Mark O. Huising

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About the cover: The picture on the front cover represent the reading ribbon that was designed by Mr. M. B. Hoogeveen in 1910 to teach children the correct pronunciation and spelling of the different vowels that are used in Dutch.

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Het zit erop, mijn proefschrift is af. Tijd om de balans op te maken. Het boekwerk dat u nu doorbladert is het resultaat van vier jaar en een beetje noeste arbeid en ging, zoals dat kennelijk hoort, gepaard met de broodnodige frustratie en gelukkig ook een hoop lol en voldoening. En waar elke promovendus tijdens zijn onderzoek wel eens het idee heeft dat hij of zij er alleen voor staat, zijn er natuurlijk een heleboel mensen die, ieder op hun eigen manier en soms ongemerkt, een bijdrage geleverd hebben aan 'mijn' boekje. Mijn project was, dat zal niemand zijn ontgaan, een samenwerking tussen 'Wageningen' en 'Nijmegen'. Met als gevolg dat ik over het aantal begeleiders niet te klagen had, ik had er maar liefst drie. Lidy, nog voor mijn studie was afgerond vroeg je me om als aio mijn werk aan het immuunsysteem van vissen voort te zetten. Belangrijk voor mij was dat er geen dichtgetimmerd project lag, maar een paar kantjes a4 en de toezegging dat hetgeen daar op stond slechts een aanzet was tot leuk en hopelijk vernieuwend onderzoek. Je hebt het geloof ik wel eens moeilijk gehad met de vrijheid en zelfstandigheid waarmee ik probeerde mijn onderzoek naar eigen inzicht in te vullen. Toch hoop ik dat je, met het resultaat van mijn promotie-onderzoek in je handen, eveneens terugkijkt op een succesvol promotie-traject, waarop voortgeborduurd gaat worden. Huub, jij verruilde ruim vijf jaar geleden Rotterdam om ambitieus en vol plannen het waterige Wageningse onderzoek te gaan leiden. Met je aanstekelijke enthousiasme wist je al snel menig promovendus voor je te winnen, maar je ondervond ook al snel dat er wel heel veel mensen een beslag leggen op je agenda als je hoogleraar bent in Wageningen. Wat mij bij zal blijven is dat de meest geëngageerde discussies die op cbi werden gevoerd gingen over financiën. Ik hoop van harte dat je er in slaagt om die discussies binnen je staf te houden en het gesprek binnen je vakgroep weer te leiden in de richting van spannende wetenschap, want dat is toch waar juist jij het volgens mij nog steeds voor doet. Beste Gert, jij bekeek met name het begin van mijn promotietraject van een afstandje vanuit Nijmegen. Ik woonde op kruipafstand van de 'wageningse dependance' en zat daarom uit gewoonte of gemakzucht vaak daar op het lab. Jij vond dat allemaal prima, ik moest vooral doen wat voor mij het prettigst of handigst was. Dat was zeker geen desinteresse, integendeel, want wanneer ik weer een stuk had geproduceerd en je dat op de vroege avond opstuurde voor commentaren had ik het niet zelden al de volgende ochtend mèt inhoudelijke verbeteringen en geredigeerd engels retour. Daarbij maakte je je nooit zorgen over de vraag of de 'Nijmeegse' onderzoeksvragen wel voldoende voor het voetlicht kwamen, met als gevolg dat juist ook die vragen in dit boekwerk ruimschoots aan bod zijn gekomen. Mijn grote dank voor het vertrouwen en de steun die ik in de afgelopen jaren van je genoten heb. Ik hoop de komende jaren vanuit La Jolla nog een hoop leuke dingen met je te kunnen opschrijven. Dear Amanda, Wylie, and Eric, my apologies for making you go through such a thick stack of paper, your comments and suggestions are greatly appreciated.

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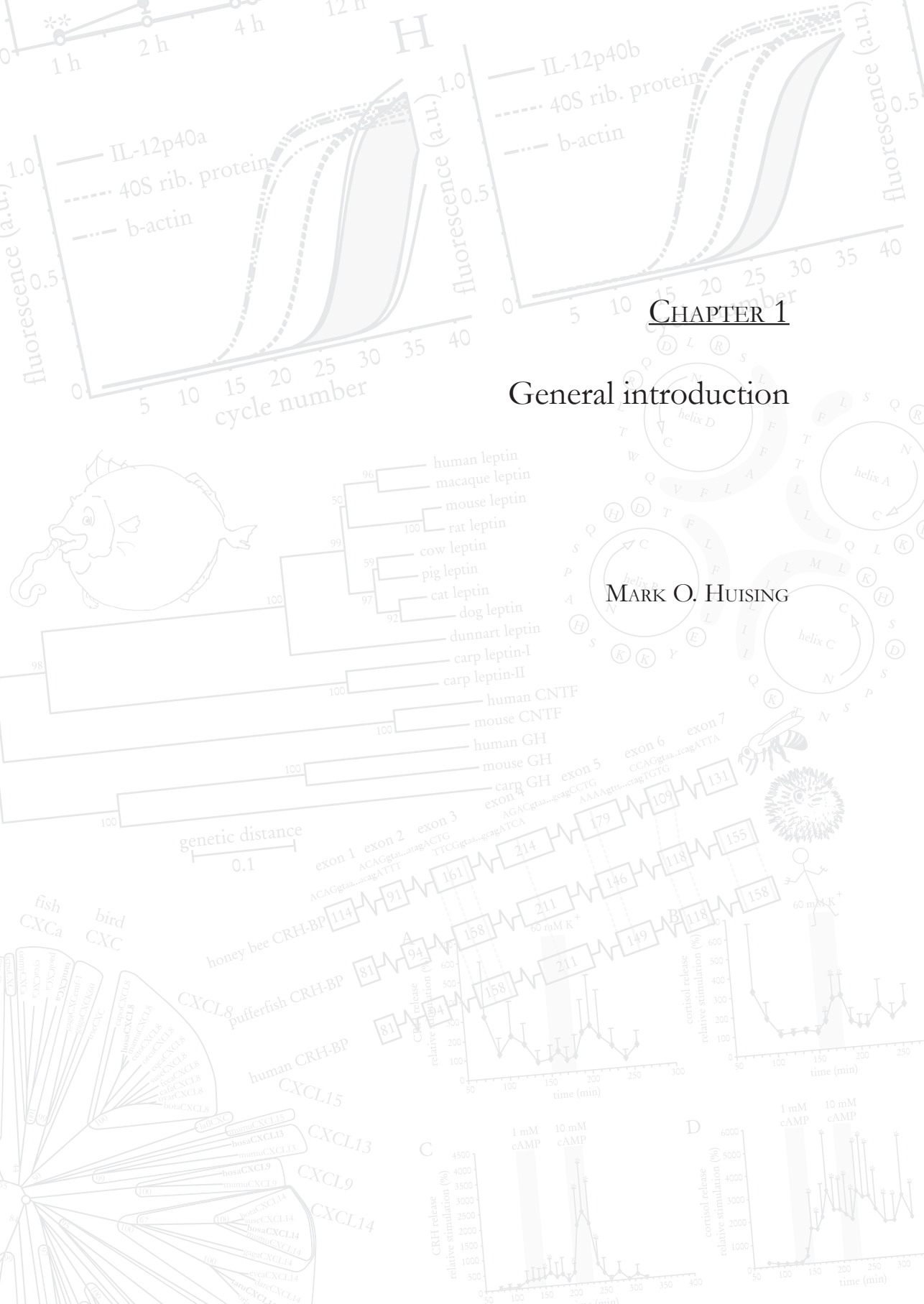

Chapter 1

General introduction

Mark O. Huising

General introduction

Multicellularity, arguably one of the greatest leaps in the evolution of life forms, offers considerable advantages over the unicellularity of *Archaea*, *Bacteria*, and early *Eukarya*. The reason is simple: two that act together stand stronger. Multicellular organisms can distribute complex tasks, such as the acquisition of energy (*e.g.* through feeding or photosynthesis), locomotion, reproduction, and host defence over different and specialised cells and organs: each specialises in one function and 'outsources' other functions. One of the first specialisations that follows from multicellularity is the formation of an integument, tissue that forms an effective demarcation between the multicellular organism and the outside world. An important consequence of this separation between in- and outside is the effective creation of an internal environment, or 'milieu intérieur' as Claude Bernard originally described it[1]. It was only a small additional step from here to control over this internal environment. This development culminated in the combined set of physiological responses that aim to maintain the 'milieu intérieur' within tight, pre-set boundaries, a conceptual framework that we know as homeostasis[2]. In recent years, the concept of homeostasis has been refined to that of allostasis, which provides some degree of flexibility to the setpoints of the physiological parameters that constitute our internal environment. Allostasis is coordinated by specific cells, which are organised in tissues and organs. The coordination of all these functions requires extensive and explicit communication and, consequently, a common language. If this communication fails, the consequences are all too well known (Fig. 1.1). The common language of the body is a biochemical one; proteins, peptides, and steroids form the words to build the sentences that direct the physiological responses of our body according to the 'grammar' dictated by the kinetics of their release. Thus, the vocabulary and syntax of this common language comprises (*i.a.*) protein and peptide signals, steroids and receptors for all of these, as well as the complex feedback and setpoint regulation in space and time. The language is perceived and processed through a variety of receptor mechanisms: the integrated result of the activation of all these receptors determines the cell's final output in response to the received signals. The actions of some of these biochemical messengers are restricted to the synaptic cleft or pericellular fluid into which they were released, others are released into the circulation and then exert their influence at greater distance peripherally. Regardless of whether the principal mechanism is endocrine, paracrine, or autocrine, these messages collectively govern the physiology of an organism: they collect and process sensory input, direct muscles via motor neurons, regulate our digestive system and energy metabolism, time the reproductive cycle, defend our body against pathogenic invasion, and mount appropriate responses to stressors. Our ability to think self-consciously, the characteristic we like to regard as setting us apart from other animals,

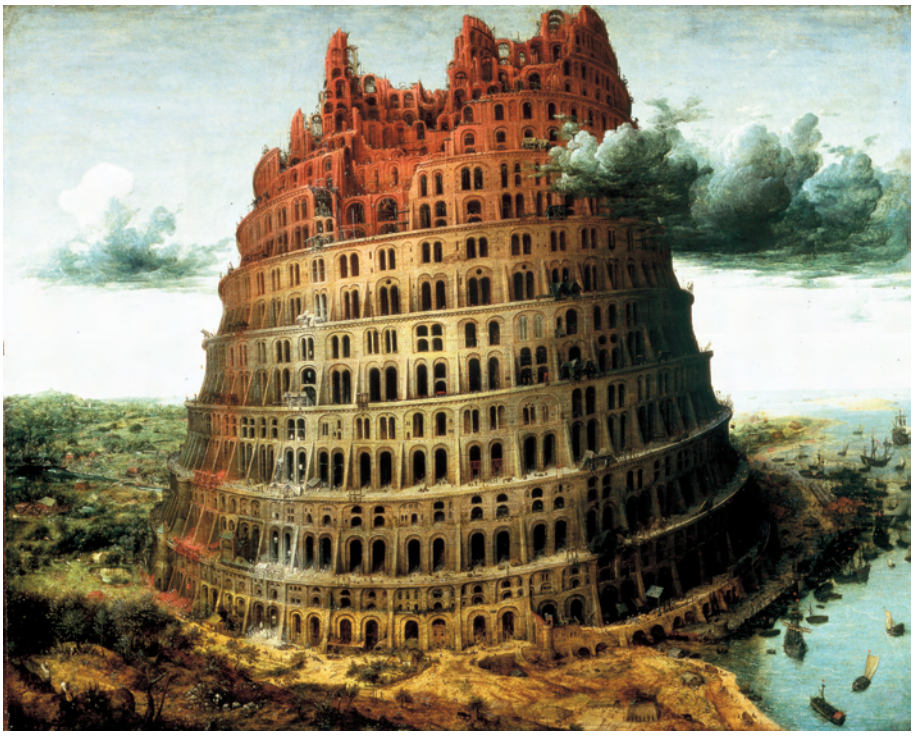

Figure 1.1: The tower of Babel, depicted in the 1563 painting by the Dutch Master painter Pieter Breughel the Elder. The painting depicts the biblical story of the people of Babylon, who set out to build a tower that would reach into the heavens. Due to Divine intervention, the tower builders adopted different languages, leading to the tower's demise as the result of miscommunication. Museum Boijmans Van Beuningen, Rotterdam.

may be nothing more than the integrated result of highly complex neural networks that fire coordinately to induce or prevent the local release of neuropeptides. And all these processes take place within the physiological environment of our body that is generally maintained at defined setpoints by potent homeostatic mechanisms.

In mammals, a central position in homeostatic control is taken by the hypothalamo-pituitary-adrenal (HPA)-axis that is activated upon imminent or ongoing disturbances of homeostasis. A threat to the homeostatic equilibrium is called the stressor, the physiological response elicited by a stressor is commonly referred to as the stress response. Once a stressor is perceived, the hypothalamus releases corticotropin-releasing hormone (CRH) into the *eminentia mediana*, which is the portal system that carries hypothalamic peptides towards the pituitary gland. This leads to release of a second neuropeptide,

adrenocorticotropic hormone (ACTH), into the general circulation[3, 4]. Subsequently, ACTH releases corticosteroids from the adrenal cortex, which is detectable in plasma within minutes following the initial perception of a threat[5]. HPA-axis activation is preceded by an adrenergic response (characterised by the release of catecholamines from the adrenal medulla) that increases heart frequency and directs blood flow to the muscle compartment at the cost of the blood volume directed e.g. at the gastrointestinal tract. The primary result of the activation of the HPA-axis is the mobilisation of blood glucose to provide readily available energy. The combined activation of the HPA-axis and the adrenergic response results in the redistribution of resources towards those organs and tissues that likely contribute to the successful handling of the stressor. Thus, the activation of the HPA-axis is ultimately aimed at preserving or restoring control of the homeostatic equilibrium through the actions of (gluco)corticosteroids directly, or indirectly via the modulation of other systems in our body.

The immune system is one of these systems under potent glucocorticoid modulation[6-9]. It is responsible for host defence in the face of continuous pathogenic exposure and is as such required to ensure homeostatic integrity. However, in contrast to the HPA-axis that involves communication between cells situated throughout the body, the immune response starts out as (and often does not exceed the stage of) a focal inflammatory response to a local infection. Neutrophilic granulocytes and macrophages, cells of the innate immune system mediate this local immune response. They attempt to neutralise, eradicate, or encapsulate the pathogenic threat by releasing cytotoxic agents, such as oxygen radicals and nitric oxide, and phagocytosis. Because the agents that are released in response to infection are cytotoxic for pathogens as well as host cells themselves[10-12], the activated innate immune response receives inhibitory feedback from the HPA-axis, via glucocorticoids[8, 13]. This mechanism prevents an overshoot of the inflammatory response that would inflict serious damage on the host and potentially disturb homeostasis.

The immune system employs a series of protein signalling molecules, collectively referred to as cytokines. Among the cytokines we distinguish interleukins, referring to the communication 'between leukocytes', and chemokines, an acronym for 'chemoattractive cytokines'. As indicated earlier, the effects of most cytokines are paracrine, but also autocrine or even intracrine actions are known[14]. The HPA-axis and endocrine systems in general, employ hormones, a group of messengers united by their common mode of action: released from effector cells and transported to their targets via the circulation. The distinction between hormones and cytokines however, is in part inspired by the historical demarcation between the fields of endocrinology and immunology and fuelled by our inherent urge to classify, in an attempt to maintain overview. Nevertheless, the current view is one of extensive and multi-directional communication between the various systems in our body and of a staggering complexity. In an attempt to unravel

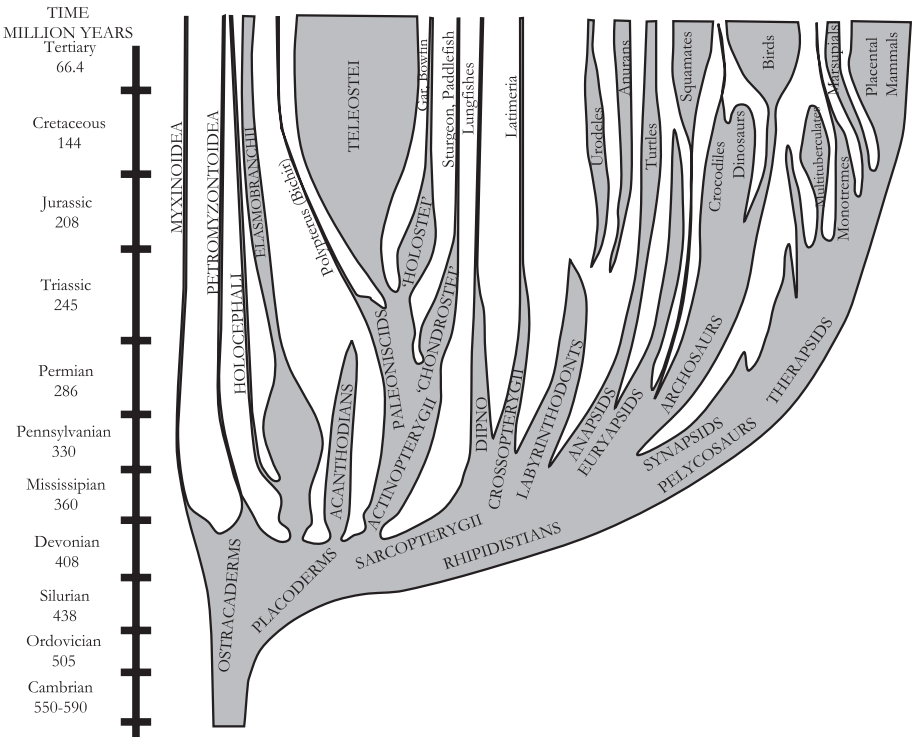

Figure 1.2: Traditional evolutionary tree of the vertebrates. Width of branches indicates the relative number of recognised genera for a given time level on the vertical axis (time in millions of years indicates the beginning of each geological period). Note that the 'fishes' do not form a monophyletic group, as they contain present-day representatives of both the actinopterygian and the sarcopterygian lineage. Reproduced from 'Vertebrate Life' by F. Harvey Pough, William N. McFarland, and John B. Heiser, third Edition, © 1989. Reprinted by permission of Pearson Education, Inc., Upper Saddle River, NJ.

the significance of some of these signalling pathways, we traced the presence of several classes of protein messengers in non-mammalian species and explored the evolutionary conservation of the physiological processes in which they are involved. Within vertebrates, actinopterygian, or ray-finned, fish occupy a unique position as they diverged from the sarcopterygians (lobe-finned fish, amphibians, reptiles, birds, and mammals) early in the vertebrate lineage, approximately 450 million years ago[15]. This landmark event is often referred to as the 'fish-tetrapod' split, although this designation ignores the phylogenetic position of the lobe-finned fishes (such as lungfishes and coelacanths) that are the earliest members of the sarcopterygians (Fig. 1.2). Besides the key phylogenetic position

that is occupied by fish, they constitute by far the most successful vertebrate class with an estimated number of 35,000 species (Dr. T. Iwamoto and Prof. dr. G. Flik, pers. comm.). The vast majority of these fish species belong to the infraclass of the '*teleostei*' and are commonly referred to as teleostean or bony fishes. Comparison of the repertoire of signalling molecules of fishes and mammals reveals the molecular signals that are evolutionarily 'old', attested by their presence in distantly related vertebrate species. On the other hand, such comparisons reveal the differences in the signalling repertoire, telltale of lineage-specific acquisition or deletion events involving the genes that encode them. Moreover, the signals that are shared by fishes and mammalian species can be compared so that their degree of similarity provides us with the means to estimate and compare their evolutionary conservation over large evolutionary distances. In some instances, the emerging picture might be one of striking similarity, whereas in other cases, comparative studies into the signalling molecules of fish might reveal the different but nonetheless equally efficacious solutions that were adopted by independent vertebrate lineages. Many of the thousands of contemporary fish species are postulated to have arisen in a relatively short period of adaptive radiation that is estimated to have occurred 300 million years ago, which is comparable to the time that has passed since the divergence of birds and mammals[15]. Consequently, many fish species differ quite extensively with regard to the genes that encode their signalling molecules and receptors. We chose common carp (*Cyprinus carpio* l.) as the subject of the majority of the studies that are presented in this thesis. Common carp is a species that is genetically very similar to the zebrafish (*Danio rerio*), the model organism of choice for vertebrate development whose genome has been sequenced. Unlike zebrafish, the carp reaches a substantial body size and thus combines the best of both worlds: extensive information on the cyprinid genetic background and a body size that enables experiments that are difficult to execute on the small zebrafish.

First, we address the cxc chemokines, a class of chemokines with a distinct signature formed by a pair of conserved cysteine residues that are separated by a single intervening amino acid (denoted by the x). In chapter 2 we characterise two cxc chemokines (cxca and cxcb) in carp that are expressed in systemic immune organs, upregulated in response to in-vitro stimulation with mitogens, and that probably mediate the chemoattraction of leukocytes. Despite their unambiguous cxc signature, neither of these chemokines proved to be orthologous to a particular mammalian cxc chemokine. In chapter 3, we describe three additional carp cxc chemokines. In contrast to the two chemokines described in chapter 2, these chemokines are clearly orthologous to mammalian cxcl12 and cxcl14 and display a much higher overall sequence identity. They are expressed very early in ontogeny and, in adult animals, at non-immune sites such as the brain. A comparison of the mammalian cxc chemokine repertoire with that of teleost fish (chapter 4) reveals that cxcl12 and cxcl14 are the only cxc chemokines with unambiguous orthologues

throughout vertebrates. Moreover, their genes are situated on separate chromosomes in human and mouse, whereas all other CXC chemokine genes are located in close proximity at a single locus. It follows that these CXC chemokine genes result from lineage-specific gene duplications that occurred after the fish-tetrapod split.

The second group of biochemical signals discussed in this thesis are the type-I cytokines. This large group of divergent proteins is united by a similar three-dimensional structure of four parallel α-helices, shared receptor chains, and related intracellular signalling pathways. CHAPTER 5 describes a carp orthologue of IL-11, one of the members of the type-I cytokine family, which substantiates the presence of IL-11 throughout vertebrates. We further show that a second, highly dissimilar IL-11 gene is present in the genomes of phylogenetically distantly related fish species such as pufferfish (*Takifugu rubripes*), spotted green pufferfish (*Tetraodon nigrovirides*), and zebrafish (*Danio rerio*). The presence of duplicate IL-11 genes is unique to the teleostean lineage. A similar situation has occurred for IL-12, a covalently linked heterodimer of a type-I cytokine (designated P35) and a truncated soluble cytokine receptor (designated P40). In CHAPTER 6, we identify the carp orthologues of IL-12P35 and IL-12P40. In contrast to mammals that so far appear to have only a single p40 gene, carp possess three very dissimilar IL-12P40 genes. These three carp P40 genes differ extensively in their constitutive expression, as well as in their in-vitro response to mitogenic stimulation. Yet, all residues that are critically required for the formation of the contact surface with the P35 subunit in human IL-12 are conserved in all three P40 genes, suggesting that they all retained the potential to engage in heterodimerisation with IL-12P35. Leptin is another member of the type-I cytokine family. Yet it fulfils all the criteria for a 'classical' hormone: in mammals it is secreted (from adipocytes) into the circulation and acts distally, in the hypothalamic *arcuate nucleus*, to regulate food intake and energy metabolism. In CHAPTER 7, we describe two carp orthologues of leptin that are predominantly expressed in the liver, and we establish their involvement in the response to short-term feeding. However, the liver expression of neither carp leptin gene changed in response to fasting for a period of six days, nor to subsequent re-feeding, which makes the involvement of leptin in the long-term regulation of energy metabolism in poikilothermic vertebrates uncertain. In CHAPTER 8, we reconstruct the phylogeny of the vertebrate type-I cytokine family, on the basis of nineteen different vertebrate type-I cytokines. Fish and mammalian type-I cytokines display relatively low overall amino acid sequence identities that often do not suffice to assign orthology. Nevertheless, the conservation of gene structure, as well as the presence and spacing of conserved cysteine residues are reliable indicators of orthology of vertebrate type-I cytokines. Reconstructing a phylogeny of the type-I cytokine family, including novel carp cytokines such as erythropoietin, reveals that many of its members were already present prior to the divergence of the fish and tetrapod lineages, a situation

that contrasts with the one described for the CXC chemokine family in CHAPTER 4.

Finally, the messengers of the teleostean stress response are investigated. The interrenal corticosteroid producing cells in fish are intermingled with the catecholamine-producing cells and embedded in the haematopoietic tissue of the head kidney. The HPA-axis of fish is therefore referred to as hypothalamus-pituitary-interrenal (HPI) axis[16]. As described earlier, the mammalian HPA-axis is activated by CRH, which evokes the release of ACTH via the CRH-receptor 1 (CRH-R1). In CHAPTER 9, we describe a CRH-binding protein (CRH-BP), along with CRH and CRH-R1, in carp, and we demonstrate their involvement in the regulation of the stress response. Collectively, this shows that the peptides and proteins of the mammalian CRH system, as well as their role in the initiation of the stress response are conserved throughout vertebrates. Besides its important role in the physiology of stress, the mammalian CRH system is involved in many more functions than those directly associated with stress[17]. A growing body of evidence in fish species points to a similarly widespread role for CRH signalling. Fish have a caudal neurosecretory system that releases CRH[18], as well as a CRH system in the gills and skin that resembles the mammalian cutaneous CRH system (Mazon *et al*., accepted). In CHAPTER 10, we describe the presence of CRH and CRH-BP within the chromaffin cells of the head kidney, and demonstrate CRH release from the head kidney following in-vitro stimulation with cyclic AMP. The presence of CRH and CRH-BP within the head kidney of teleosts resembles the endogenous CRH system of the mammalian adrenal gland. Collectively, this substantiates that CRH signalling is evolutionarily better conserved than most cytokines and chemokines. This notion is corroborated in CHAPTER 11, where we take advantage of the unique features of CRH-BP to show the presence of CRH-BP in honeybee (*Apis mellifera*) and other insect species and by doing so date the CRH system to over one billion years old. Finally, in CHAPTER 12, a synthesis of our findings is presented based on the systematic comparison of the evolutionary conservation of signalling molecules between teleostean fish and mammals. This comparison enables us to establish the type and strength of the evolutionary forces that have acted on these signalling molecules since the teleost-tetrapod split, and we discuss several explanations for the profound differences that emerge.

Chapter 2

Cxc chemokines and leukocyte chemotaxis in common carp

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Abstract

Cxc chemokines, structurally recognisable by the position of four conserved cysteine residues, are prominent mediators of chemotaxis. Here we report a novel carp cxc chemokine obtained through homology cloning and compare it with fish orthologous genes and with a second, recently elucidated, carp cxc chemokine. Phylogenetic analyses clearly show that neither cxc chemokine resembles any of the mammalian cxc chemokines in particular. However, basal expression is most prominent in immune organs like anterior kidney and spleen, suggesting involvement in the immune response. Furthermore we show that anterior kidney phagocyte-enriched leukocyte suspensions express both chemokines and that this expression is upregulated by brief (4h) stimulation with pma, but not lps. Neutrophilic granulocyte-enriched leukocytes display chemotaxis to human recombinant cxcl8 (hrcxcl8; interleukin-8), confirming cxc chemokine mediated chemotaxis of neutrophilic granulocytes in teleost fish. Factors secreted from carp phagocytes are also capable of inducing chemotaxis and secretion of these factors into culture supernatants is upregulated by pma. Finally we demonstrate involvement of both cxc chemokines as well as cxcr1 and cxcr2 in acute *Argulus japonicus* infection. Collectively the data presented implicate the involvement of cxc chemokines in chemotaxis of fish neutrophils in a fashion that shares characteristics with the mammalian situation. However, the cxc chemokines involved differ enough from those involved in neutrophil chemotaxis in mammals to warrant their own nomenclature.

Introduction

The aqueous environment that is so vital to fish survival also facilitates transmission of many water-borne pathogens. Fish rely heavily on their innate immune system to fend off these invading micro-organisms, as the specific immunoglobulin response is often not detected during the first four weeks after the first contact with a pathogen[19]. The integumental surfaces form an important first line of defence. This includes the mucus layer, as it contains many antimicrobial substances. Upon breaching of this barrier, the invading pathogen is met by a whole array of soluble factors like anti-bacterial peptides, proteases, lysozyme, complement factors and acute-phase proteins [reviewed by 20, 21]. At the same time the cellular component of innate immunity is activated upon recognition of pathogen derived pamps (pathogen associated molecular patterns)[22, 23] including lipopolysaccharide (lps) and double-stranded rna as well as by host derived cytokines. The latter group includes typical pro-inflammatory cytokines like interleukin-1β (il-1β)

and tumour necrosis factor-α (TNFα) as well as chemokines that are of pivotal importance in recruiting leukocytes to the site of inflammation.

A large array of chemokines is known in mammals, each with a spectrum of different but largely redundant properties. Chemokines are subclassified by structure and spacing of conserved cysteines into four major groups, CXC, CC, C and CX_3C[24-26], X denoting any amino acid. All chemokines possess two conserved cysteine bridges, except C chemokines that only contain one. In CXC chemokines the two N-terminal cysteines are separated by intervening amino acid, hence the name. All CXC chemokines share a tertiary structure of an α-helix overlying three β-strands. Mammalian CXC chemokines are subdivided further according to the presence of an ELR (Glu-Leu-Arg) motif directly preceding the CXC motif. Many different names have been given to the numerous members of the CXC chemokine family, we convene to the nomenclature proposed in[26], in which ligands (CXCL) and receptors (CXCR) are numbered consecutively. Functionally ELR^+ CXC chemokines (CXCL1-8 except CXCL4) are implicated in the recruitment of mammalian neutrophilic granulocytes, whereas ELR^- CXC chemokines (CXCL9-15 and CXCL4) attract lymphocytes.

CXC chemokines relay messages through the activation of specific seven-helix transmembrane G-protein coupled receptors (GPCRs). In human and mouse there are five different mammalian receptors for CXC chemokines, dubbed CXCR1-5. These receptors share 25 – 80% amino acid identity and usually possess a cysteine in each of the four extracellular loops. CXCR1 and -2 are expressed on a wide range of cell types but predominantly on neutrophilic granulocytes and monocytes/macrophages. CXCR2 is relatively non-selective for the various ELR^+ CXC chemokines, whereas CXCR1 is highly selective for CXCL8 (interleukin-8)[reviewed by 27, 28]. Besides chemotaxis, processes like degranulation and super-oxide production are also mediated through CXCRs 1 and 2 as well[29]. Receptor number and activity are tightly regulated by metalloprotease mediated receptor degradation and receptor internalisation[30, 31].

Recently we cloned a novel CXC chemokine in carp (*Cyprinus carpio* L.), which shares only little amino acid identity with a recently submitted carp CXC chemokine gene[32]. These novel sequences are discussed in the context of the growing number of CXC chemokine sequences in both fish and mammals. Based on phylogenetic analyses, a nomenclature for fish CXC chemokines is proposed, analogous to the nomenclature of mammalian CXC chemokines[26]. Furthermore we studied the regulation of expression of these two CXC chemokine genes as well as the regulation of carp CXCR1 and –2[33] *in vitro* and *in vivo*. Finally, functional evidence is provided that implies involvement of CXC chemokines in chemotaxis of neutrophilic granulocytes in fish.

Materials and methods

Animals

Common carp (*Cyprinus carpio* L.) were reared at 23°C in recirculating UV-treated tap water at the 'De Haar Vissen' facility in Wageningen. Fish were fed pelleted dry food (Provimi, Rotterdam, The Netherlands) at a daily rate of 0.7% of their estimated body weight. R3xR8 are the hybrid offspring of a cross between fish of Hungarian origin (R8 strain) and fish of Polish origin (R3 strain)[34].

Amplification, cloning and sequencing

Two oligonucleotide primers (CXCa.fw2/ CXCa.rv3; Table 2.1) were designed based on flounder[35] and trout[36] CXC chemokine sequences. Anchored PCR was performed on a λZAP cDNA library of PMA-activated anterior kidney macrophages[37] using CXCa.fw2 and CXCa.rv3 with T7 and SK anchored primers respectively (Table 2.1). The genomic sequence of carp CXCa was amplified from 200 ng genomic DNA using CXCa.fw6 and CXCa.rv5 primers (Table 2.1). Oligonucleotides were obtained from Eurogentec. (Anchored) PCR reactions were performed using 0.5 µl Taq DNA polymerase (Goldstar) supplemented with 1.5 mM $MgCl_2$, 200 µM dNTPs and 400 nM of each primer in a final volume of 25 µl. Cycling conditions were 94°C for 2 min; 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min for 30 cycles and 72°C for 10 min, using a GeneAmp PCR system 9700 (PE Applied Biosystems, Foster City, USA). Products amplified by PCR were ligated and cloned in JM-109 cells using the pGEM-T-easy kit (Promega, Leiden, The Netherlands) according to the

Table 2.1: Primer sequences and corresponding EMBL acc. numbers

gene	EMBL acc. Number	primer	sequence 5' ⇒ 3'
carp CXCa	AJ421443, AJ550164	CXCa.fw2	AIWCWGARATCATTGCCACTCTG
		CXCa.rv3	GCTGCTGGTGTTTTGTTGGC
		CXCa.fw6	TGGCAGAATGCACTTCAAAATCTT
		CXCa.rv5	GTTCAACTTCACACATGATCCAA
		qCXCa.fw1	CTGGGATTCCTGACCATTGGT
		qCXCa.rv1	GTTGGCTCTCTGTTTCAATGCA
carp CXCb	AB082985	qCXCb.fw1	GGGCAGGTGTTTTTGTGTTGA
		qCXCb.rv1	AAGAGCGACTTGCGGGTATG
carp CXCR1	AB010468	qCXCR1.fw1	GCAAATTGGTTAGCCTGGTGA
		qCXCR1.rv1	AGGCGACTCCACTGCACAA
carp CXCR2	AB010713	qCXCR2.fw1	TATGTGCAAACTGATTTCAGGCTTAC
		qCXCR2.rv1	GCACACACTATACCAACCAGATGG
β-actin	CCACTBA	qACT.fw1	CAACAGGGAAAAGATGACACAGATC
		qACT.rv1	GGGACAGCACAGCCTGGAT
40s ribosomal protein s11	AB012087	q40s.fw1	CCGTGGGTGACATCGTTACA
		q40s.rv1	TCAGGACATTGAACCTCACTGTCT
vector		T7	TAATACGACTCACTATAGGG
		SK	CGGCCGCTCTAGAACTAGTGGACT

manufacturer's protocol. Plasmid DNA was isolated using the QIAprep Spin Miniprep kit (Qiagen, Leusden, The Netherlands) following the manufacturer's protocol. Sequence reactions were carried out using the ABI Prism Bigdye Terminator Cycle Sequencing Ready Reaction kit, and analysed using an ABI 377 sequencer.

Blood collection and tissue preparation

Fish were anaesthetised with 0.2 g/l tricaine methane sulphonate (TMS) buffered with 0.4 g/l $NaHCO_3$. Fish were bled through puncture of the caudal vessel using a heparinised (Leo Pharmaceutical Products Ltd., Weesp, The Netherlands) syringe fitted with a 21 or 25 Gauge needle and processed for further analysis according to the requirements of the various techniques used. Anterior kidney was surgically removed, passed through a 50 μm nylon mesh with carp RPMI (cRPMI; RPMI 1640, Gibco; adjusted to carp osmolality (270 mOsm/kg) with 10% distilled water) and washed once. Anterior kidney cell suspension was layered on discontinuous Percoll (Amersham Pharmacia Biotech AB) gradient (1.020, 1.060, 1.070 and 1.083 g/cm^3)[38]. Following centrifugation (30 min at 2000 rpm with brake disengaged) cells at the 1.083 g/cm^3 interface (representing neutrophilic granulocyte-enriched leukocytes) or the 1.070 g/cm^3 interface (representing macrophage-enriched leukocytes) were collected and washed twice. For *in vitro* stimulation experiments, both fractions were pooled (phagocyte-enriched leukocytes).

Cell culture

For in vitro induction of gene expression 400 μl anterior kidney phagocyte-enriched cell suspension (10^7 cells/ml) was seeded in 24 well plates. Cells were stimulated for 4 h at 27°C at 5% CO_2 with LPS (10 μg/ml; *E. coli*, Sigma L2880) or PMA (phorbol 12-myristate 13-acetate; 0.1 μg/ml; P8139, Sigma). All treatments were carried out in quadruplicate. Supernatant of activated leukocytes was obtained by culturing phagocyte-enriched anterior kidney leukocytes in cRPMI (supplemented with 0.5% pooled carp serum) containing 0.1 μg/ml PMA at 27°C at 5% CO_2 for 4 h. Unstimulated controls were included. PMA was washed away thoroughly (5 times) with $cRPMI^{++}$ (cRPMI containing 200 nM 2-mercaptoethanol (Biorad), 1% L-glutamin (Merck), 1% penicillin G (Sigma) and 1% streptomycin sulphate (Sigma). Cells were cultured o.n. (overnight) in $cRPMI^{++}$ and supernatant was harvested and stored at –20°C.

Argulus japonicus infection

Fish were infected with adult (3 parasites per fish) or juvenile (10 parasites per fish) fish lice (*Argulus japonicus*)[39]. Skin samples of infected sites were collected 1 hour after infection and snap-frozen.

RNA isolation

RNA isolation from organs was conducted according to CHOMCZYNSKI and SACCHI[40]. Briefly, organs were homogenised in lysis buffer (4 M guanidium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercapthoethanol, followed by phenol/chloroform extractions. Total RNA was precipitated in ethanol, washed and dissolved in water. RNA from cultured cells was isolated using the SV Total RNA isolation system (Promega, Leiden, The Netherlands) according to the manufacturer's protocol. Concentrations were measured by spectrophotometry and integrity was ensured by analysis on a 1.5% agarose gel. RNA was stored at –80°C.

DNase treatment and first strand cDNA synthesis

For each sample a non-template (NT) control was included. One µl 10x DNase I reaction buffer and 1 µl DNase I (Invitrogen, 18068-015) was added to 2 µg total RNA and incubated for 15 min at room temperature in a total volume of 10 µl. DNase I was inactivated with 1 µl 25 mM EDTA at 65°C, 10 min. To each sample 300 ng random hexamers (Invitrogen, 48190-011), 1 µl 10 mM dNTP mix, 4 µl 5x First Strand buffer, 2 µl 0.1 M DTT and 10 U RNase inhibitor (Invitrogen, 15518-012) were added and the mix was incubated for 10 min at room temperature and for an additional 2 min at 37°C. To each positive sample (but not the NT controls) 200 U Superscript RNase H^- Reverse Transcriptase (RT; Invitrogen, 18053-017) was added and reactions were incubated for 50 min at 37°C. All reactions were filled up with demineralised water to a total volume of 1 ml and stored at –20°C until further use.

Real-time quantitative PCR

Primer Express software (Applied Biosystems) was used to design primers for use in real-time quantitative PCR (RQ-PCR; Table 2.1). For RQ-PCR 5 µl cDNA and forward and reverse primer (300 nM each) were added to 12.5 µl Sybr Green PCR Master Mix (Applied Biosystems) and filled up with demineralised water to a final volume of 25 µl. RQ-PCR (2 min 48°C, 10 min 95°C, 40 cycles of 15 sec. 95°C and 1 min 60°C) was carried out on a GeneAmp 5700 Sequence Detection System (Applied Biosystems). Data were analysed using the ΔΔCt method[41] and the relative quantitation value expressed as $2^{-\Delta\Delta Ct}$. Dual internal standards (40S and β-actin) were incorporated in all RQ-PCR experiments and results were confirmed to be very similar following standardisation to either gene.

SDS-PAGE

Culture supernatants (20 µl) were run on a 12.5% polyacrylamide gel. After electrophoresis, the gel was fixed (30' 5 g trichloro-acetic acid, 20 ml methanol, 40 ml dH_2O), washed 3x in dH_2O, reduced (20' dithiotreitol 0.5 mg/100 ml), incubated in

$AgNO_3$ (20' 1 mg/ml) and stained in 4.5 g $NaCO_3$ in 150 ml dH_2O containing 75 µl 36% formaldehyde.

Chemotaxis assay

Modified 48-well Boyden chambers (AP48 Neuroprobe, Gaithersburg, USA)[42] with 3 µm pore sized (PVP-free) polycarbonate filters were used for determination of chemotaxis. The chemoattractants were diluted in CRPMI containing 0.5% BSA (bovine serum albumin). Supernatants of stimulated and non-stimulated phagocyte-enriched anterior kidney cell suspensions were used undiluted. Human recombinant CXCL8 (hrCXCL8; 208-IL; R&D, Minneapolis, USA) was used at 0.025 - 2.5 ng/ml and FMLP (N-formyl-methionyl-leucyl-phenylalanine; F3506, Sigma)[43, 44] was used as a positive control at 10^{-7} M. Bottom wells were filled with 27 µl chemoattractant or the assay medium (CRPMI + 0.5% BSA) as a negative control. To the top wells 51.5 µl neutrophilic granulocyte-enriched (1.083 g/cm^3 Percoll) anterior kidney leukocytes (4 x 10^5 cells/ml) was added. Following 60 or 90 min incubation, the non-migrated cells (*i.e.* cells on the topside of the membrane) were washed off with PBS and wiped off. Migrated cells (*i.e.* cells on the underside of the membrane) were fixed in 4% PFA (paraformaldehyde) in PBS, stained for 5 min in Coomassie Brilliant Blue (BDH Chemicals Ltd., Poole, England) and destained for 3 min in destaining solution (30% ethanol and 10% glacial acetic acid in water). Migration was quantified by counting the number of migrated cells microscopically. All treatments were performed in quadruplet. Migration was expressed as chemotaxis index (CI), *i.e.* number of migrated cells/random migration (random migration is defined as the number of cells that migrated towards assay medium).

Phylogenetic analysis

Sequences were retrieved from the Swissprot, EMBL and Genbank databases using the SRS mirror site of the Centre of Molecular and Biomolecular Informatics (www.cmbi.kun.nl). *Takifugu rubripes* sequences were retrieved using the Ensembl Blast server. (http://ensembl.fugu-sg.org/Fugu_rubripes/blastview) Multiple sequence alignment was carried out using ClustalW on the CMBI mirror site. Calculation of pairwise amino acid and nucleotide identities was carried out using the Sim Alignment tool on the EXPASY Molecular Biology Server (http://tw.expasy.org/tools/sim-prot.html). Phylogenetic trees were constructed on the basis of amino acid difference (p-distance) by the neighbour-joining method[45] using MEGA version 2.1[46]. Reliability of the tree was assessed by bootstrapping, using 1000 bootstrap replication.

Statistics

All statistical analyses were carried out using SPSS software (version 10.1.0). Differences

were considered significant at $p<0.05$. Data were tested for normal distribution using the Shapiro-Wilk test. Homogeneity of variances was tested with the Levene test. Differences were evaluated using one-sided one factor analysis of variance (ANOVA). If ANOVA was significant, Dunnett's T-test was used to determine which means differed significantly from the control. In case of non-homogeneous variances Dunnett's C-test was used as a substitute for Dunnett's T-test. Kruskal-Wallis H-test was applied in case of non-normal distribution. If Kruskal-Wallis was significant, the Mann-Whitney U-test was used to determine which means differed significantly.

Results

Cloning and characteristics of a novel carp CXC chemokine

Homology cloning based on known flounder[35] and trout[36] CXC chemokine sequences resulted in the elucidation of a novel partial carp CXC chemokine (CXCa) sequence from a PMA-stimulated phagocyte cDNA library. The corresponding full-length (625 bp) cDNA sequence was obtained by anchored PCR and encodes a 98 aa novel carp CXC chemokine bearing considerable (57 and 73% respectively) aa identity to both previously reported genes (Table 2.2). In addition to a polyadenylation signal (bp 562-567), the 3'UTR of the mRNA contained four instability motifs (attta; bp 523-527, 529-533, 541-545, 545-549) predicted to reduce the half-life of the mRNA molecule (Fig. 2.1a). The CXC chemokine gene consisted of four exons and three introns at conserved locations but of considerably shorter length compared to human CXCL8 (Fig. 2.1b)[47], representing the most thoroughly characterised CXC chemokine. Based on similarity with human CXCL8, the first 20 aa form a signal peptide. The remaining 78 aa form a mature protein predicted to fold into an α-helix overlying three β-strands, as is the prototypical three-dimensional conformation of CXC chemokines (Fig. 2.2). Four cysteine residues were present at positions 32, 34, 58 and 75, confirming the CXC signature.

Table 2.2: Comparison of amino acid (basepair) identity in fish CXCa and CXCb chemokines and human CXCL8. Accession numbers are as in Fig. 2.3.

	carp CXCa	flounder CXCa	trout CXCa	carp CXCb	catfish CXCb	trout CXCb	human CXCL8
carp CXCa	100						
flounder CXCa	56.7 (65.3)	100					
trout CXCCa	73.3 (70.1)	57.4 (67.0)	100				
carp CXCb	33.3 (50.0)	28.4 (46.3)	33.3 (50.3)	100			
catfish CXCb	34.0 (46.9)	36.7 (45.5)	34.8 (44.4)	52.7 (62.8)	100		
trout CXCb	43.1 (48.8)	38.9 (50.5)	31.6 (54.1)	46.5 (55.1)	47.4 (59.0)	100	
human CXCL8	39.0 (49.5)	39.6 (49.3)	38.3 (49.0)	28.1 (51.4)	25.0 (45.1)	34.7 (43.7)	100

A

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attcggcacgagcagatctgatcaccatacctagcatccaaaccattgatttacttttac   60
                  ***
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                       M  H  F  K  I  F  S  V  I  V  F  L  G
ttcctgaccattggtgaaggaatgagtcttagaggtctgggtgtagatccacgctgtcgc  180
 F  L  T  I  G  E  G  M  S  L  R  G  L  G  V  D  P  R  C  R
tgcattgaaacagagagccaacgcattgggaaactcatagagagtgtggagctcttccct  240
 C  I  E  T  E  S  Q  R  I  G  K  L  I  E  S  V  E  L  F  P
ccaagcccacactgtaaagacacagagatcattgccaccctgaaggtatccagaaaagag  300
 P  S  P  H  C  K  D  T  E  I  I  A  T  L  K  V  S  R  K  E
atctgtctggaccctattgcaccctgggttaagaaagtcattgagaagatcattgccaac  360
 I  C  L  D  P  I  A  P  W  V  K  K  V  I  E  K  I  I  A  N
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 K  T  P  A  A  -
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B

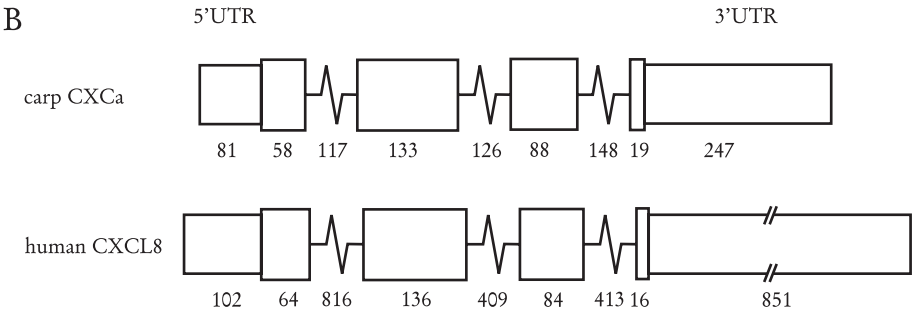

Figure 2.1: (a) CDNA and amino acid sequence of carp CXCa. Asterisks indicate the start codon. Predicted intron splice sites are shaded. Potential instability motifs are indicated in bold. A putative polyadenylation signal is underlined. (b) Genomic organisation of carp CXCa and human CXCL8. Both genes consist of four exons of similar length, except the 3'UTR that is over three times as long in the human CXCL8 gene. Intron lengths of carp CXCa are also considerably shorter. Accession numbers for the MRNA and genomic carp CXCa sequences are AJ421443 and AJ550164 respectively.

A second 97 aa carp CXC chemokine (CXCb)[32] shares the same basic features characteristic of the CXC family but bears only low (33%) aa identity to the carp CXCa sequence (Table 2.2). Moreover, comparison of aa identity of CXC chemokine sequences of several fish species suggests the presence of several CXC chemokine genes in fish. Multiple sequence alignment confirmed that the lengths of both signal peptides and mature proteins as well as the positions of the conserved cysteine residues are well conserved in fish CXC chemokines (Fig. 2.2).

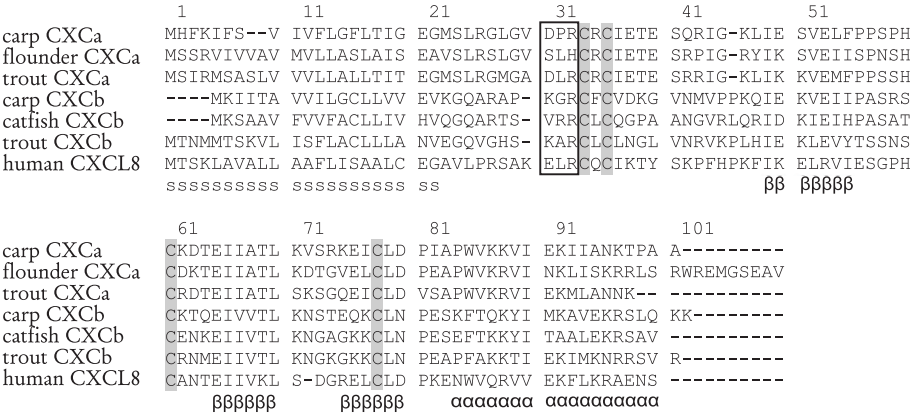

Figure 2.2: Amino acid alignment of carp CXCa and carp CXCb with their teleost fish orthologues and with human CXCL8. Conserved cysteine residues are shaded. The location of the ELR motif is boxed. Hyphens indicate gaps. The predicted signal peptide (s) and tertiary structure (α, β) are indicated below the aa residues. Abbreviations of species names are as in Fig. 2.3.

Phylogenetic analyses

The relationship of teleost fish CXC chemokines with the well-defined mammalian CXC chemokines a phylogenetic tree was made using the neighbour-joining method, comprising known vertebrate CXC chemokine aa sequences (Fig. 2.3). The overall topology of the tree shows clustering of the fish CXC chemokines in two distinct groups, supported by high bootstrap values. Mammalian CXC chemokines cluster largely according to nomenclature, some clusters comprising two (CXCL5 and CXCL6) or three (CXCL1-3) different chemokines. CXC chemokine sequences of shark (*Triakis scyllia*) and lamprey (*Lampetra fluviatilis*) cluster apart from all other sequences. Moreover, neither teleost CXC chemokine group clusters with any of the mammalian clusters. To reflect this phylogenetic segregation, we named the teleost fish CXC chemokines CXCa and CXCb respectively. Construction of a phylogenetic tree using the minimum-evolution algorithm based on aa and nucleotide sequences generated trees of very similar topology (not shown).

Regulation of CXC chemokine and CXCR expression

Carp CXCa and CXCb expression was detectable in most organs of a healthy control fish, but most notably in anterior kidney, kidney, spleen, gill, thymus and liver. Expression of each gene was expressed relative to the organ or tissue with the lowest detectable expression. Brain and pituitary displayed low expression for both chemokines. Levels

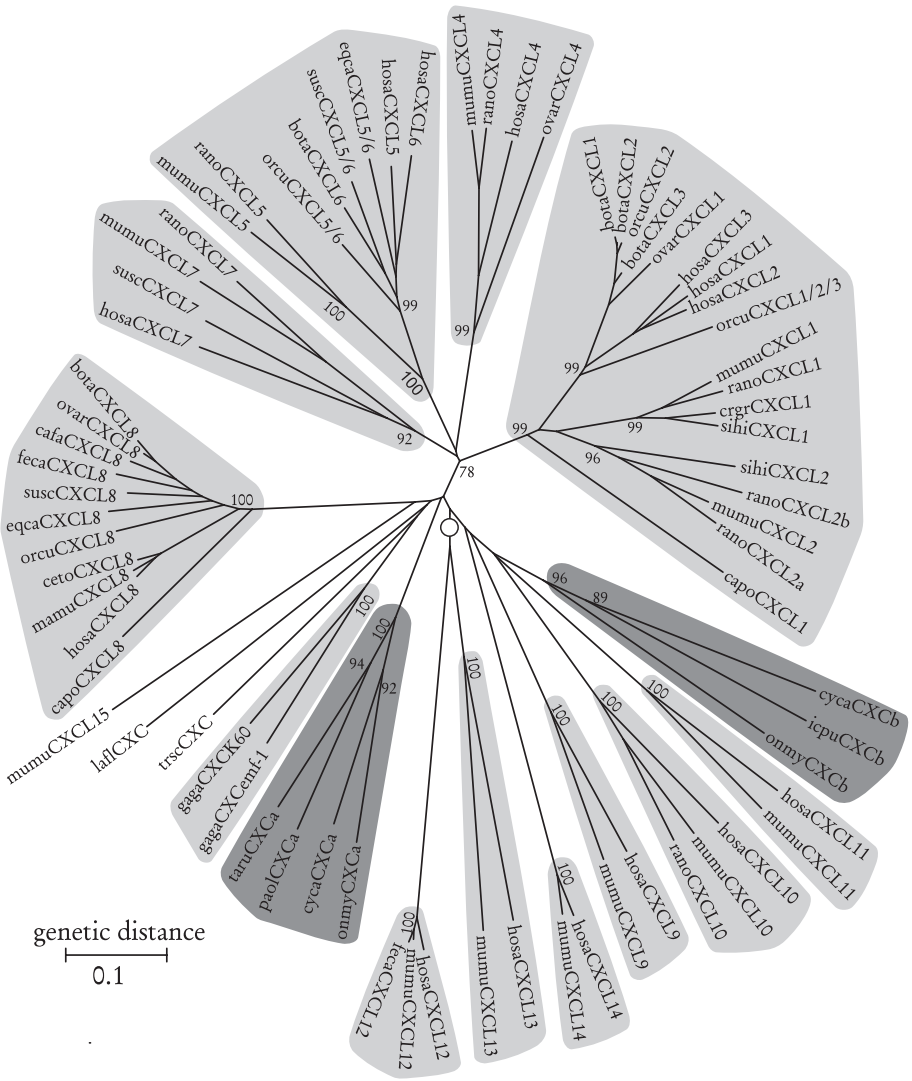

Figure 2.3: Neighbour-joining tree of mammalian and fish CXC chemokine amino acid sequences. Numbers at branch nodes represent the confidence level of 1000 bootstrap replications. Clusters are shaded for clarity, clusters of teleost fish CXC chemokines are shaded darker. An open circle indicates starting point of cluster formation. Abbreviations of species names are as follows: hosa, *Homo sapiens* (human); ceto, *Cercocebus torquatus atys* (mangabey); mamu, *Macaca mulatta* (rhesus macaque); mumu, *Mus musculus* (mouse); rano, *Rattus norvegicus* (rat); sihi, *Sigmodon hispidus* (hispid cotton rat); crgr, *Cricetulus griseus* (chinese hamster); orcu, *Oryctolagus cuniculus* (rabbit); capo, *Cavia porcellus* (guinea pig); ovar, *Ovis aries* (sheep); bota, *Bos taurus* (cow); susc, *Sus scrofa* (pig);

continued on next page

Figure 2.3, continued:
eqca, *Equus caballus* (horse); feca, *Felis cattus* (cat); cafa, *Canis familiaris* (dog); gaga, *Gallus gallus* (chicken); onmy, *Oncorhynchus mykiss* (rainbow trout); paol, *Paralichtys olivaceous* (flounder); cyca, *Cyprinus carpio* (carp); taru, *Takifugu rubripes* (pufferfish) lafl, *Lampetra fluviatilis* (lamprey); trsc, *Triakis scyllium* (shark); icpu, *Ictalurus punctatus* (channel catfish). Accession numbers are as follows: hosaCXCL1 P09341, hosaCXCL2 P19875, hosaCXCL3 P19876, hosaCXCL4 P02776, hosaCXCL5 P42830, hosaCXCL6 P80162, hosaCXCL7 P02775, hosaCXCL8 P10145, hosaCXCL9 Q07325, hosaCXCL10 P02778, hosaCXCL11 O14625, hosaCXCL12 P48061, hosaCXCL13 O43927, hosaCXCL14 O95715, mumuCXCL1 P12850, mumuCXCL2 P10889, mumuCXCL4 AB017491, mumuCXCL5 P50228, mumuCXCL9 P18340, mumuCXCL10 P17515, mumuCXCL11 Q9JHH5, mumuCXCL12 P40224, mumuCXCL13 AF044196, mumuCXCL14 Q9WUQ5, mumuCXCL15 Q9WVL7, mumuCXCL7 NP_076274, ranoCXCL1 P14095, ranoCXCL4 P06765, ranoCXCL10 P48973, ranoCXCL2a P30348, ranoCXCL2b BAB12280, ranoCXCL5 P97885, ranoCXCL7 AAK30166, orcuCXCL2 P47854, orcuCXCL8 P19874, orcuCXCL5/6 P82535, orcuCXCL1/2/3 AB93924, ovarCXCL1 O46678, ovarCXCL4 P30035, ovarCXCL8 P36925, crgrCXCL1 P09340, sihiCXCL2 AAL26705, sihiCXCL1 AAL16934, botaCXCL1 O46676, botaCXCL2 O46677, botaCXCL3 O46675, botaCXCL6 P80221, botaCXCL8 P79255, suscCXCL7 P43030, suscCXCL8 P26894, suscCXCL5/6 P22952, gagaemf-1 P08317, gagaK60 CAA75212, fecaCXCL8 Q9XSX5, fecaCXCL12 O62657, cafaCXCL8 P41324, capoCXCL1 O55235, capoCXCL8 P49113, cetoCXCL8 P46653, eqcaCXCL8 O62812, eqcaCXCL5/6 AAM76679, mamuCXCL8 P51495, onmyCXCa OMY279069, onmyCXCb AF483528, paolCXCa AF216646, cycaCXCa AJ421443, cycaCXCb AB082985, taruCXCa scaffold_215, laflCXC L231072, trscCXC AB063299, icpuCXCb BE212851.

of CXCa expression consistently exceeded those of CXCb expression in all organs with the exception of spleen, brain and pituitary. CXCR1 and CXCR2 were most abundantly expressed in anterior kidney, kidney and spleen. PBL (peripheral blood leukocytes)

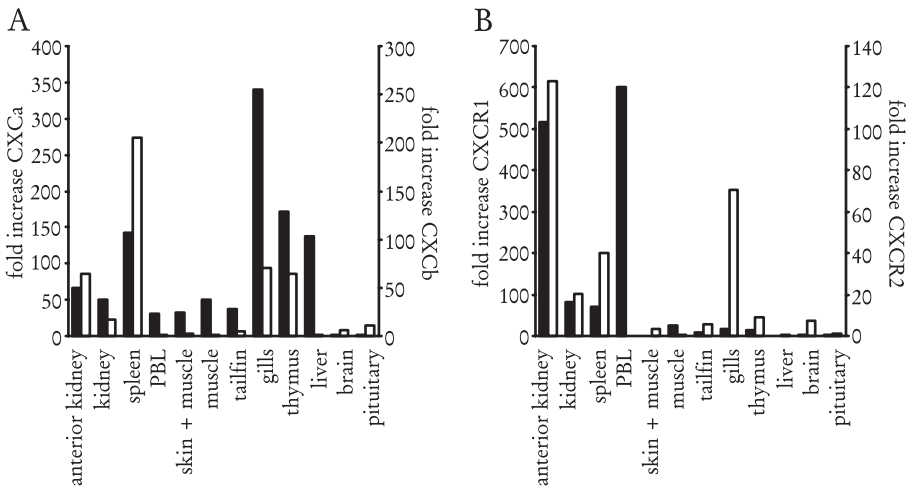

Figure 2.4: Basal expression of CXCa (filled bars) and CXCb (open bars) (a) and CXCR1 (filled bars) and CXCR2 (open bars) (b) in various organs of carp, standardised for 40s expression and relative to the organ with the lowest detectable expression, *i.e.* pituitary for CXCa and CXCR2, PBL for CXCb and muscle for CXCR1.

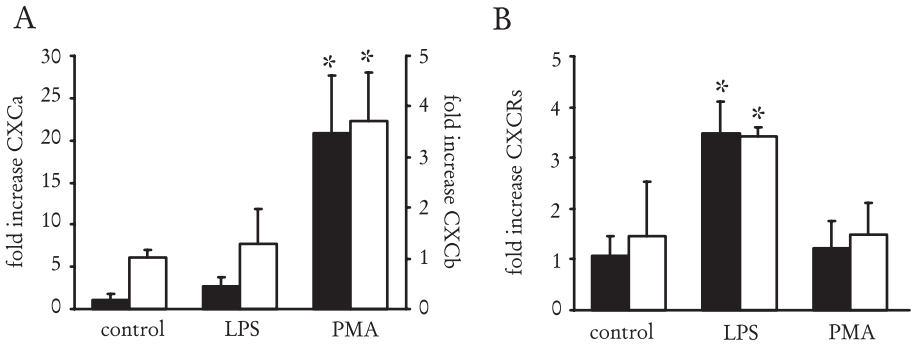

Figure 2.5: Regulation of expression of CXCa (filled bars) and CXCb (open bars) (a) and CXCR1 (filled bars) and CXCR2 (open bars) (b) by LPS (10 μg/ml) and PMA (0.1 μg/ml). Expression is standardised for β-actin expression and presented relative to control (unstimulated) cells. Error bars indicate standard deviations of four replicate measurements. Asterisks indicate significant differences from the control ($p<0.05$). Dunnett's C-test and Kruskal-Wallis H-test were used for statistic evaluation of differences in CXCa and CXCR1 expression respectively. Dunnett's T-test was applied in other cases. Representative of two experiments.

abundantly expressed CXCR1, but displayed a complete lack of CXCR2 expression, whereas in the gill the expression profile of both genes was reversed.

Regulation of expression of CXCa, CXCb as well as both CXCRs was studied in freshly isolated phagocyte-enriched anterior kidney leukocytes, stimulated *in vitro* with 10 μg/ml *E. coli* LPS or 0.1 μg/ml PMA for four hours. Expression was quantified relative to unstimulated cells and standardised to β-actin (Fig. 2.4). PMA but not LPS significantly upregulated expression of CXCa and CXCb 21- and four-fold respectively (Fig. 2.5a). Expression of both CXCR1 and CXCR2 was upregulated 3.5 fold with LPS but not PMA (Fig. 2.5b). Non-reverse transcriptase controls were negative in all experiments (not shown).

Chemotactic response of carp neutrophilic granulocytes

To ascertain if chemotaxis in carp is CXC chemokine-mediated, we assessed the chemotactic response of carp neutrophilic granulocyte-enriched leukocytes to hrCXCL8. Migrated cells adhering to the polycarbonate filter (Fig. 2.6a) were counted. A dose-dependent chemotactic response was observed towards increasing doses of hrCXCL8 (0.025 – 2.5 ng/ml) at an optimised time period of 60 min, which peaked at six-fold over random migration (Fig. 2.6b). Upon application of FMLP at 10^{-7} M as a positive control a chemotactic response of similar magnitude was observed.

Presence of a potential chemotactic property of both carp CXC chemokines was determined by measuring the response towards supernatant of o.n. leukocyte cultures.

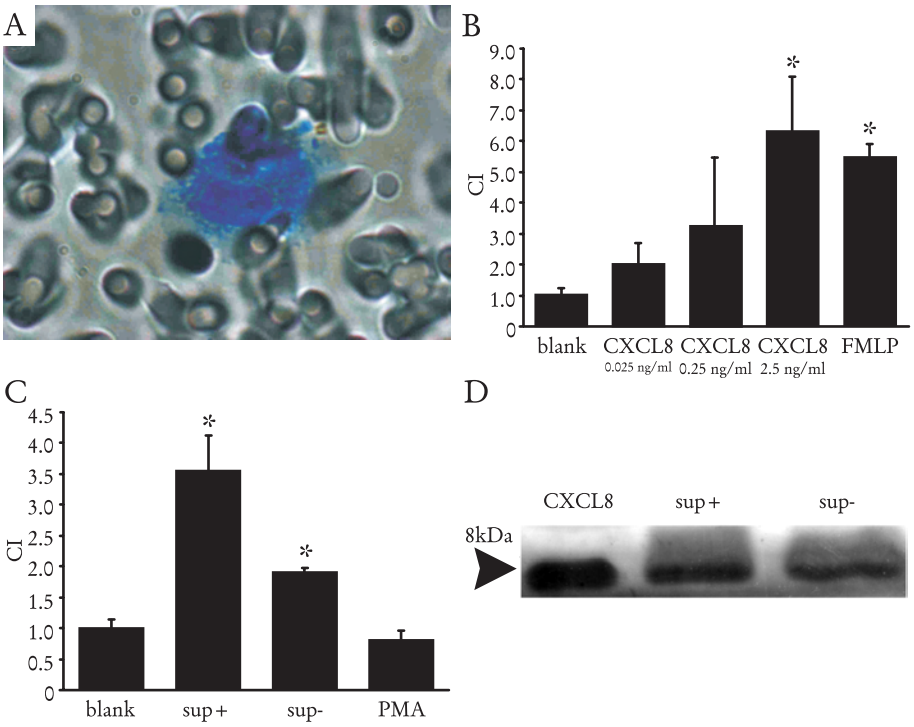

Figure 2.6: Chemotaxis of carp neutrophilic granulocyte-enriched anterior kidney leukocytes. Panel a shows a cell that has migrated through the three μm pore sized filter. Panel b shows concentration dependent chemotaxis of carp neutrophilic granulocyte-enriched leukocytes towards hrCXCL8. FMLP (10^{-7} M) is included as a positive control. Chemotactic activity of supernatant of PMA-stimulated and unstimulated phagocyte-enriched o.n. leukocyte cultures (c). Silver staining of an SDS polyacrylamide gel showing proteins of very similar size to hrCXCL8 in supernatant of both stimulated and unstimulated phagocyte-enriched anterior kidney leukocyte cultures (d). Error bars indicate standard deviations of four to eight replicate measurements. Asterisks indicate significant differences from the control ($p<0.05$). Dunnett's C-test was used for statistic evaluation of differences.

Supernatant of PMA-stimulated o.n. phagocyte-enriched anterior kidney leukocyte cultures elicited a chemotactic response of 3.5-fold over random migration at 60 min. (Fig. 2.6c). Supernatant of unstimulated phagocyte-enriched anterior leukocyte cultures also induced a significant chemotactic response of nearly two-fold over random migration, which was significantly lower compared to the chemotactic response towards supernatant of PMA stimulated o.n. cultures. Low concentrations of PMA (0.001 ng/ml) did not induce chemotaxis, ruling out that the chemotactic responses observed were

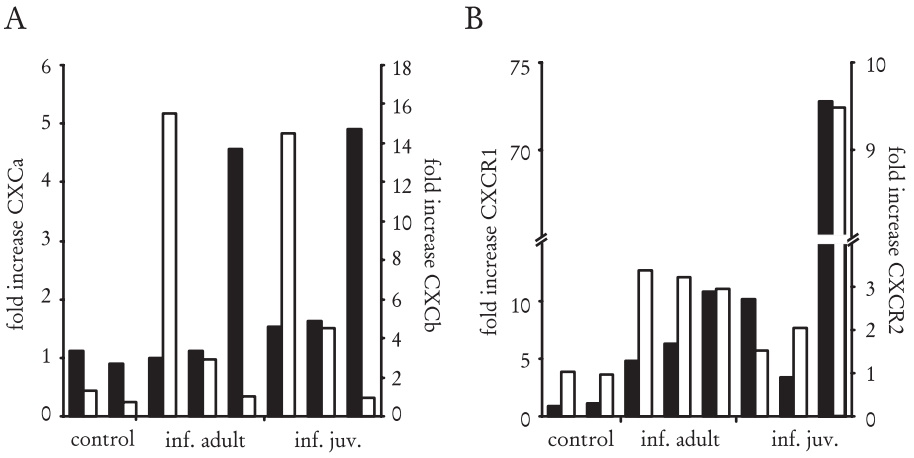

Figure 2.7: Expression of CXCa (filled bars) and CXCb (open bars) (a) and CXCR1 (filled bars) and CXCR2 (open bars) (b) in individual fish following *in vivo* infection with *A. japonicus* standardised for 40S expression and relative to control (uninfected) fish.

attributable to residual PMA. The chemotactic response to both supernatants was already clearly detectable at 30 min. (not shown). From 60 min. to 90 min. the total number of migrated cells did not increase further (not shown), indicating that a maximal chemotactic response had occurred in 60 min. Both supernatants contained a double protein band of similar size to hrCXCL8 (Fig. 2.6d) in the <10kDa range.

In vivo modulation of CXC chemokine expression

To establish involvement of CXCa and CXCb in infection, we analysed the expression of both CXC chemokines as well as their receptors in carp skin, one hour following infection with adult (n is three) or juvenile (n is three) *Argulus* at the site of infection. Expression of both CXCa and CXCb increased in some, but not all individuals following infection. However, all individuals displayed increased expression of at least one of the chemokines compared to non-infected controls (Fig. 2.7a). CXCR1 and CXCR2 expression was upregulated in all individuals 1.5 – 9.5 fold and 3.5 – 73 fold respectively compared to non-infected controls (Fig. 2.7b). Non-reverse transcriptase controls were negative (not shown).

Discussion

A cDNA molecule bearing the CXC chemokine signature ($CXCX_{23/24}CX_{14-20}C$) was sequenced by homology cloning from a PMA activated carp macrophage cDNA library. The cDNA molecule contained common features like a polyadenylation site and a poly-A tail as well as four putative instability motifs often associated with cytokine mRNAs. Both chemokine sequences described in this study encode pro-CXC molecules of 98 and 97 aa respectively, which is one aa shorter than human CXCL8 and also comparable to other teleost fish CXC chemokine sequences, which range from 95 to 109 aa. We named the CXC chemokines CXCa and CXCb, to be able to identify orthologues of different fish species and simultaneously reflect the phylogenetic distance between fish and mammalian CXC chemokines. Comparison of the percentages aa identity of carp CXCa and CXCb with previously identified fish CXC chemokine sequences and multiple alignment of these aa sequences with human CXCL8 suggests that the existence of two different CXC chemokine sequences is a common feature in teleost fish. Furthermore, none of the CXC chemokines of fish elucidated to date contains the ELR motif that is crucial for mammalian neutrophil chemotaxis[25] directly preceding the CXC signature. All fish CXC chemokine sequences do contain a positively charged aa (R or H) at the same location as the third position of the mammalian ELR motif. However, this positively charged amino acid is a common feature of all CXC chemokines except CXCL4.

Phylogenetic analyses of teleost fish CXCa and CXCb chemokines with other vertebrate CXC chemokines confirm that fish CXC chemokines cluster in two distinct clusters, supported by high bootstrapping values. Mammalian CXC chemokines cluster according to nomenclature, CXCL1-3 and CXCL5 and –6 clustering together. Neither fish CXC chemokine cluster associates with any of the mammalian CXC chemokines in particular, including CXCL8. Furthermore, ELR^+ CXCL1-7 cluster apart from the remaining CXC chemokines, supported by a relatively high bootstrap value of 78%, suggesting these chemokines originate from a common ancestor. Since this entire cluster contains only mammalian CXC chemokine sequences, it has likely arisen recently, probably after the fish-tetrapod split and possibly after the mammalian-avian split, since two CXC chemokines (gagaK60 and gagaemf-1) do not cluster with any of the mammalian sequences either. CXCL8, which is also an ELR^+ chemokine, clusters apart (99%) from all other chemokine sequences. Since three different clusters of CXC chemokines (CXCL1-7 but lost in CXCL4, CXCL8 and gagaK60 and gagaemf-1) contain an ELR motif, it has possibly arisen multiple times during evolution. The absence of the ELR motif in teleost fish CXC chemokines is therefore likely a reflection of the evolutionary novelty of this feature. Furthermore, our phylogenetic analyses clearly show a complete lack of clustering with any other group of CXC chemokine sequences. Therefore, naming teleost[35, 36] or agnathan[48] CXC chemokines

according to mammalian nomenclature, implicitly suggesting function, is premature and by no means warranted by phylogeny.

To establish function of the carp CXC chemokines reported here, we first assessed the basal expression pattern in healthy uninfected carp. Both chemokines are most notably expressed in classical fish immune organs like anterior kidney, spleen, thymus and kidney, suggesting a functional role in the immune system. The high expression of CXCa in the gill is striking, since this is generally not considered a systemic organ. However, these results are in line with those in trout[36], where gill consistently displayed the highest CXCa expression. This indicates that the gill is a more important immune compartment than generally recognised, most likely playing a significant role in innate immunity. This is supported by the gills representing by far the largest surface area of the fish, where constant activation of innate immunity is inevitable due to continuous pathogen exposure.

Expression of CXCRs in anterior kidney, spleen and kidney is likely a direct result of the sheer number of phagocytes contained in these organs in healthy, non-immune compromised individuals. However, the high expression of CXCR1 in PBL in combination with the complete absence of CXCR2 expression is striking. This suggests that lack of CXCR2 expression is a prerequisite for leukocytes to enter or remain in circulation. Assuming that the low level of CXCR1 expression in gill is attributable to circulating neutrophils, this implies that the high levels of gill CXCR2 expression is associated with resident rather than circulating leukocytes.

PMA enhanced both CXCa and CXCb expression most prominently compared to control. LPS at 10 μg/ml was unable to significantly upregulate CXC chemokine expression, although CXCa is slightly upregulated. This is in contrast to the studies of trout[36] and flounder[35] CXCa, in which an upregulation of CXCa chemokine expression by LPS is qualitatively reported. This apparent discrepancy is attributable to differences in LPS concentration used for stimulation and the source of the cells used for *in vitro* stimulation. Expression of CXCR1 and CXCR2 was induced only by LPS and not by PMA, indicating that CXC chemokine and receptor genes are regulated differently.

One of the most prominent and best-characterised functions of mammalian ELR$^+$ CXC chemokines is chemotaxis of neutrophils. Despite the lack of fish orthologues to any of the mammalian ELR$^+$ CXC chemokines, chemotaxis in fish is described[49]. We established the occurrence of chemotaxis in carp and its potential mediation through carp CXC chemokines. Carp neutrophilic granulocyte-enriched anterior kidney leukocytes displayed a chemotactic response towards hrCXCL8. This response is dose-dependent and occurred at doses (0.025 – 2.5 ng/ml) which are similar to those inducing chemotaxis of mammalian cells[50]. Also the time needed for a maximal chemotactic response to occur does not differ appreciably from normal incubation times in mammalian setups. Since PMA is the most

potent stimulator of cxc chemokine expression, we used supernatant of o.n. cultures of pma-stimulated (4h) anterior kidney phagocytes as a source of carp cxc chemokines. pma-activated, and to a lesser extent, unstimulated culture supernatant, both induced chemotaxis of carp neutrophilic granulocyte-enriched leukocytes. The chemotactic ability of supernatant of unstimulated cultures is in line with the expression of both cxca and cxcb in unstimulated anterior kidney phagocyte cultures. This corroborates the observation that unstimulated phagocytes express both cxca and cxcb. Sds-page analysis of culture supernatants confirmed the presence of proteins of similar size to hrcxcl8 in both stimulated and unstimulated supernatants. Not many proteins besides chemokines are usually contained within this low molecular weight range. Moreover, systematic analysis of the recently published pufferfish genome reveals that the number of cxc chemokines that function within the fish immune system is likely restricted to cxca and cxcb[51]. Unfortunately we do not as yet dispose of specific reagents to confirm the nature of these low molecular weight proteins.

In vivo infection of carp with *A. japonicus* indicates that cxca and cxcb can be rapidly (1h) upregulated. However it seems that some fish upregulate cxca and some cxcb compared to uninfected controls, while all infected fish upregulate expression of at least one of the chemokines. This pattern is consistent within both infection with adult and juvenile *A. japonicus*, and suggests redundancy in the limited repertoire of fish cxc chemokines alike that observed in mammalian cxc chemokines. All infected fish display an enhanced expression of both cxcr1 and cxcr2, likely due to an influx of neutrophilic granulocytes, corroborating the involvement of a cxcr mediated pathway in infection. Especially the increase of cxcr2 expression indicates the migration of phagocytes from circulation towards the infection site, since circulating leukocytes do not express cxcr2.
In conclusion we show the existence of two different clusters of cxc chemokines in teleost fish, with members of different fish species containing orthologues for both cxc chemokines. Furthermore, our phylogenetic analyses rule out a classification based on mammalian nomenclature, since neither fish cxc chemokine displays unambiguous orthology to any of the mammalian cxc chemokines in particular. However, based on *in vitro* and *in vivo* expression profiles as well as functional characterisation, a possible role of both chemokines in chemoattraction of neutrophilic granulocytes is demonstrated.

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CHAPTER 3

Three novel carp chemokines are expressed early in ontogeny and at non-immune sites

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Abstract

Three novel CXC chemokines were identified in common carp (*Cyprinus carpio* L.) through homology cloning. Phylogenetic analyses show that one of the three CXC chemokines is an unambiguous orthologue of CXCL14, whereas both others are orthologues of CXCL12, and were named CXCL12a and CXCL12b. Percentages amino acid identity between each of these carp chemokines and their human and mouse orthologues are markedly higher than those reported previously for other carp CXC chemokines, suggestive of involvement in vital processes, that have allowed for relatively few structural changes. Furthermore, all three novel carp CXC chemokines are expressed during early development, in contrast to established immune CXC chemokines. In non-infected adult carp, CXCL12b and CXCL14 are predominantly expressed in the brain. CXCL12a is highly expressed in kidney and anterior kidney, but its expression is still more abundant in brain than any other carp CXC chemokine. Clearly, these chemokines must play key roles in the patterning and maintenance of the (developing) vertebrate central nervous system.

Introduction

Chemokines are small proteins that derive their name from their chemotactic properties. Chemokine is an acronym for 'chemotactic cytokine' and reflects their discovery and characterisation as important chemoattractants in the pro-inflammatory phase of the immune response. Based on the pattern and spacing of four conserved cysteine residues, that determine tertiary structure by virtue of two disulphide bridges, chemokines are subdivided into four classes[26]. The two major chemokine classes are referred to as CXC and CC, reflecting the relative spacing of both N-terminal cysteine residues, that are separated by one amino acid residue or directly adjacent, respectively. Mammalian CXC chemokines are further subdivided based on the presence or absence of a tri-peptide ELR (glutamic acid, leucine, arginine) motif directly preceding the CXC signature. ELR^+ CXC chemokines are implicated in chemoattraction of neutrophilic granulocytes, whereas ELR^- CXC chemokines are associated with lymphocyte chemotaxis. Another useful classification depends on whether the chemokine is constitutively expressed or inducible[28]. The majority of CXC chemokines falls into the last category, but CXCL12 (SDF-1; stromal cell-derived factor-1) and CXCL13 (BCA-1; B-cell attracting chemokine-1) are examples of constitutively expressed CXC chemokines that are involved in basal leukocyte trafficking[52, 53].

Despite their initial discovery as mediators of leukocyte chemotaxis and the

ensuing attention from an immunological audience, their actions extend beyond the immune system. A large number of chemokines and chemokine receptors are expressed in the central nervous system[54-56], and whereas this expression is mostly inducible by inflammatory mediators, several chemokines, including CXCL12 and CXCL14 (BRAK; breast and kidney derived), are constitutively expressed in the (developing) central nervous system[57-60]. CXCL12 and its receptor CXCR4 play an essential role in cerebellar and neocortical neuron migration during development[57, 61-63]. Recently, both molecules were reported to be key in the migration of germ cells towards the developing reproductive organs in early development in mouse[64, 65] and zebrafish[66]. Despite its good conservation throughout vertebrate evolution[51], the number of studies addressing the *in-vivo* role(s) of CXCL14 is limited. As a consequence a lot of information, including information regarding the identity of its receptor is still unavailable.

To date a fair number of CXC chemokines has been discovered in various teleost fish species[32, 67]. For the majority of those chemokines, orthology with any particular mammalian CXC chemokine is difficult to establish as a consequence of the adaptive radiation that characterises the recent history of the mammalian CXC chemokine family[51]. In recent years common carp (*Cyprinus carpio* L.) has been established as a physiological and immunological model species, that is genetically closely related to zebrafish[68]. However, the substantially larger body size of carp allows for experimental approaches that are not feasible in the small zebrafish. To date two carp CXC chemokines (CXCa and CXCb) have been functionally characterised[32, 67]. Both chemokines are constitutively expressed in systemic immune organs, including the anterior kidney, which is considered the bone marrow equivalent of teleost fish. Moreover, their expression is upregulated in anterior kidney phagocytes upon *in-vitro* PMA (phorbol 12-myristate 13-acetate) stimulation. Although neither chemokine is orthologous to any mammalian CXC chemokine in particular, their expression patterns and *in-vitro* inducibilities are analogous to those of the majority of mammalian CXC chemokines and indicate an immune function.

Here we report the sequences and expression patterns of three novel carp CXC chemokines, orthologous to mammalian CXCL12 and CXCL14. We identified two CXCL12 genes in carp (designated CXCL12a and CXCL12b), a likely result of gen(om)e duplication, and one gene for carp CXCL14. The mRNA molecules for these three novel chemokines contain a 3'UTR (untranslated region) that is much longer compared to previously identified carp chemokine messengers. We show that in carp CXCL12a, CXCL12b and CXCL14 are expressed very early in ontogeny, in contrast to the 'immune' CXC chemokines CXCa and CXCb. In adult carp CXCL12b and CXCL14 are predominantly expressed within the central nervous system. In addition to a high central nervous system expression, CXCL12a is very highly expressed within the anterior kidney and the kidney, but, in case of the anterior kidney, this expression seems restricted to the stromal compartment. Furthermore, expression in

anterior kidney phagocytes is constitutive rather than inducible, in sharp contrast to the expression of previously characterised 'immune' CXC chemokines.

Materials and methods

Animals

Common carp (*Cyprinus carpio* L.) were reared at 23°C in recirculating UV-treated tap water at the 'De Haar Vissen' facility in Wageningen. Fish were fed pelleted dry food (Provimi, Rotterdam, The Netherlands) at a daily ration of 0.7% of their estimated body weight. R3xR8 are the offspring of a cross between fish of Hungarian origin (R8 strain) and fish of Polish origin (R3 strain)[34]. Eggs and milt were obtained by repeated injection of sexually mature female and male carp with pituitary homogenates in the days preceding spawning. Eggs and sperm were collected separately, mixed, together with some Cu^{2+}-free water and gently stirred for 30 sec. to start fertilisation.

Homology cloning, amplification and sequencing

Oligonucleotide primers were designed for CXCL12 based on a zebrafish EST (expressed

Table 3.1: Primer sequences and corresponding accession numbers

gene	acc. number	primer	sequence 5' ⇒ 3'
carp CXCL12a	AJ627274	CXCL12a.fw1	GTGCGGATCTSTTCTTCACAC
		qCXCL12a.fw1	CACCGTCACAGATATGTACCATATAGTC
		qCXCL12a.rv1	GGTGGTCTTTTGCAGAGTCATTT
carp CXCL12b	AJ536027	CXCL12.rv1	TTCTTTAGATACTGCTGAAGCCA
		CXCL12.fw3	AGGTCTGCATCAACCCCAAG
		CXCL12.fw4	GCATCAACCCCAAGACCAAATGG
		CXCL12.rv4	CGGGACGGTGTTGAGAGTGGA
		CXCL12.rv5	GAGAGTGGACCGGCACCAACA
		qCXCL12b.fw1	GAGGAGGACCACCATGCATCT
		qCXCL12b.rv1	TTGTGCAAGCAGTCCAGAAAGA
carp CXCL14	AJ536028	CXCL14.rv3	GGATGCAGGCAATACTCCTG
		CXCL14.fw5	CCATACTGCCAAGAAAAGATGAT
		qCXCL14.fw1	ACAGAGGCATACAAGTGCAGATG
		qCXCL14.rv1	TGTTTAGGCTTGATCTCCAGCTT
carp CXCa	AJ421443	qCXCa.fw1	CTGGGATTCCTGACCATTGGT
		qCXCa.rv1	GTTGGCTCTCTGTTTCAATGCA
carp CXCb	AB082985	qCXCb.fw1	GGGCAGGTGTTTTTGTGTTGA
		qCXCb.rv1	AAGAGCGACTTGCGGGTATG
carp 40s ribosomal protein S11	AB012087	q40s.fw1	CCGTGGGTGACATCGTTACA
		q40s.rv1	TCAGGACATTGAACCTCACTGTCT
carp β-actin	CCACTBA	qACT.fw1	CAACAGGGAAAAGATGACACAGATC
		qACT.rv1	GGGACAGCACAGCCTGGAT
vector		T7	TAATACGACTCACTATAGGG
		T3	CGCAATTAACCCTCACTAAAG

sequence tag) entry similar to human CXCL12 (accession number BM070896). Anchored PCR was performed on a λZAP cDNA library of carp brain[69] with T3 forward and CXCL12.rv1 reverse primers (Table 3.1). This yielded a truncated carp CXCL12 sequence (that we later named carp CXCL12b to parallel the names adopted in recent zebrafish literature[70]). The full-length CXCL12b mRNA sequence was obtained by RACE (rapid amplification of cDNA ends). We used total RNA from brain tissue of one individual adult carp for the synthesis of RACE cDNA (GeneRacer™; Invitrogen, Breda, The Netherlands), according to the manufacturer's instructions. CXCL12.fw3 and CXCL12.fw4 were used as initial and nested primer for the amplification of the 3'UTR, while CXCL12.rv4 and CXCL12.rv5 were used as initial and nested primer for the amplification of the 5'UTR. The latter combination of initial and nested primers applied on carp anterior kidney RACE cDNA resulted in the identification of a similar, but distinct sequence, encoding the 5'UTR and the N-terminal part of a second CXCL12 gene, that we named CXCL12a. The complete mRNA sequence of carp CXCL12a was amplified from a λZAP cDNA library constructed from PMA-activated anterior kidney macrophages[37]. To this end we used CXCL12a.fw1 forward primer with T7 reverse primer in an anchored, extra long PCR approach, according to the manufacturer's instructions (Expand Long Template PCR System; Roche Diagnostics, Almere, The Netherlands). Primers for carp CXCL14 were based on a zebrafish gene previously described as *scyba*[71]. Anchored PCR was performed on a λZAP cDNA library of carp brain with T3 forward and CXCL14.rv3 reverse primers yielding a 385 bp amplicon comprising the 5'UTR and the N-terminal part of an ORF (open reading frame) encoding carp CXCL14. The C-terminus and 3'UTR were amplified using CXCL14.fw5 forward and T7 reverse primers. Oligonucleotides were obtained from Eurogentec (Seraing, Belgium). Regular (anchored) PCR reactions were performed using 0.5 μl Taq DNA polymerase (Goldstar; Eurogentec, Seraing, Belgium) supplemented with 1.5 mM $MgCl_2$, 200 μM dNTPs and 400 nM of each primer in a final volume of 25 μl. Cycling conditions were 94°C for 2 min; 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min for 30-35 cycles and 72°C for 10 min, using a GeneAmp PCR system 9700 (PE Applied Biosystems, Foster City, USA). Products amplified by PCR were ligated and cloned in JM-109 cells using the pGEM-T-easy kit (Promega, Leiden, The Netherlands) according to the manufacturer's protocol. Plasmid DNA was isolated using the QIAprep Spin Miniprep kit (Qiagen, Leusden, The Netherlands) following the manufacturer's protocol. Sequences were determined from both strands using T7 and Sp6 primers and were carried out using the ABI Prism Bigdye Terminator Cycle Sequencing Ready Reaction kit, and analysed using an ABI 377 sequencer (PE Applied Biosystems, Foster City, USA.

Tissue and cell collection and preparation

Adult carp (approximately 150-200 g) were anaesthetised with 0.2 g/l TMS (tricaine

methane sulphonate) buffered with 0.4 g/l $NaHCO_3$. Fish were bled through puncture of the caudal vessels using a heparinised (Leo Pharmaceutical Products Ltd., Weesp, The Netherlands) syringe fitted with a 21 or 25 Gauge needle. Blood was mixed with an equal volume of carp RPMI (RPMI 1640, Gibco; adjusted to carp osmolality (270 mOsm kg^{-1}) with distilled water) containing 10 IU ml^{-1} heparin and centrifuged 10 min at 100 x g to remove the majority of erythrocytes. The supernatant containing PBL (peripheral blood leukocytes) was layered on a discontinuous Percoll (Amersham Pharmacia Biotech AB) gradient (1.020 and 1.083 g cm^{-3}). Following centrifugation (30 min at 800 x g with brake disengaged) cells at the 1.083 g cm^{-3} interface were collected. Anterior kidney cell suspensions were obtained by passing the tissue through a 50 μm nylon mesh with carp RPMI and washed once. The cell suspension was layered on a discontinuous Percoll gradient (1.020, 1.070, and 1.083 g cm^{-3}) and centrifuged 30 min at 800 x g with the brake disengaged. Cells at the 1.070 g cm^{-3} interface (representing predominantly macrophages) were collected, washed, and seeded at 2 x 10^6 cells per well (in a volume of 400 μl) in a 24 well cell culture plate. Following overnight culture at 27°C, 5% CO_2 in cRPMI^{++} (cRPMI supplemented with 0.5 % pooled carp serum, 1% L-glutamine (Cambrex), 200 nM β-mercaptoethanol (Biorad), 1% penicillin G (Sigma), and 1% streptomycin sulphate (Sigma)), cell cultures were stimulated for 4 h with 50 μg ml^{-1} LPS (lipopolysaccharide from *Escherichia coli*; Sigma), 20 μg ml^{-1} ConA (concanavalin A from *Canavalia ensiformes*; Sigma) or 0.1 μg ml^{-1} PMA (Sigma). A non-stimulated control group was included and all treatments were carried out in five-fold. Following stimulation cells were collected for RNA isolation. Organs and tissues for the analysis of *ex-vivo* RNA expression were carefully removed, flash-frozen in liquid nitrogen and stored at -80ºC. Carp embryos were anaesthetised with 0.2 g/l TMS buffered with 0.4 g/l $NaHCO_3$ at the indicated stages of development. Individual eggs or embryos were flashfrozen in liquid nitrogen and stored at -80ºC.

RNA isolation

RNA from PBL, anterior kidney macrophage-enriched cell cultures, and carp embryo's was isolated using the RNeasy Mini Kit (Qiagen, Leusden, The Netherlands) following the manufacturer's protocol. Final elution was done in 25 μl of nuclease-free water, to maximise concentration. RNA was isolated from tissues using Trizol reagent (Invitrogen, Breda, The Netherlands), according to the manufacturer's instructions. Total RNA was precipitated in ethanol, washed and dissolved in nuclease-free water. RNA concentrations were measured by spectrophotometry and integrity was ensured by analysis on a 1.5 % agarose gel before proceeding with cDNA synthesis.

DNase treatment and first strand cDNA synthesis

For each sample a -RT (non-reverse transcriptase) control was included. One µl 10x DNase I reaction buffer and 1 µl DNase I (Invitrogen, 18068-015) was added to 1 µg total RNA and incubated for 15 min at room temperature in a total volume of 10 µl. DNase I was inactivated with 1 µl 25 mM EDTA at 65°C, 10 min. To each sample 300 ng random hexamers (Invitrogen, 48190-011), 1 µl 10 mM dNTP mix, 4 µl 5x First Strand buffer, 2 µl 0.1 M DTT and 10 U RNase inhibitor (Invitrogen, 15518-012) were added and the mix was incubated for 10 min at room temperature and for an additional 2 min at 37°C. To each sample (but not to the -RT controls) 200 U Superscript RNase H^- Reverse Transcriptase (RT; Invitrogen, 18053-017) was added and reactions were incubated for 50 min at 37°C. All reactions were filled up with demineralised water to a total volume of 1 ml and stored at –20°C until further use.

Real-time quantitative PCR

Primer Express software (Applied Biosystems) was used to design primers for use in real-time quantitative PCR (RQ-PCR; Table 3.1). For RQ-PCR 5 µl cDNA and forward and reverse primer (300 nM each, except CXCa and CXCb primer sets that were used at 250 nM each) were added to 12.5 µl Quantitect Sybr Green PCR Master Mix (Qiagen) and filled up with demineralised water to a final volume of 25 µl. RQ-PCR (15 min 95°C, 40 cycles of 15 sec 94°C, 30 sec 60°C, 30 sec 72°C followed by 1 min 60°C) was carried out on a Rotorgene 2000 real-time cycler (Corbett Research, Sydney, Australia). Following each run, melt curves were collected by detecting fluorescence from 60°C to 90°C at 1°C intervals Expression during ontogeny and in organs and tissues of adult carp was rendered as a ratio of target gene vs. reference gene and was calculated according to the following equation: ratio = $(E_{reference})^{Ct}$reference / $(E_{target})^{Ct}$target, where E is amplification efficiency and Ct is de number of PCR cycles needed for the signal to exceed a predetermined threshold value. Expression following *in-vitro* stimulation was rendered relative to the expression in non-stimulated control cells according to the following equation: ratio = $(E_{target})^{\Delta Ct}$target$^{(control - sample)}$ / $E_{reference}{}^{\Delta Ct}$reference$^{(control - sample)}$[72]. Efficiency and threshold values used for each primer set were: CXCa, 2.06, 0.0056; CXCb, 1.95, 0.0701; CXCL12a, 2.06, 0.0701; CXCL12b, 2.18, 0.0701; CXCL14, 2.14, 0.03; 40S, 2.11, 0.0077; β-actin, 2.05, 0.0513. Dual internal reference genes (40S and β-actin) were incorporated in all RQ-PCR experiments and results were confirmed to be similar following standardisation to either gene. Non-RT controls were included in all experiments and were negative.

Bioinformatics

Sequences were retrieved from the Swissprot, EMBL and Genbank databases using SRS and/or BLAST (basic local alignment search tool)[73]. Multiple sequence alignments

were carried out using Clustalw. Signal peptide predictions were carried out at using SignalP v3.0[74]. Calculation of pairwise amino acid identities was carried out using the Sim Alignment tool[75]. The organisation of zebrafish chemokine genes as well as their preliminary chromosomal location was determined at the Ensembl site (http://www.ensembl.org/). Phylogenetic trees were constructed on the basis of amino acid difference (p-distance) by the neighbour-joining method (complete deletion)[45] using MEGA version 2.1[46]. Reliability of the tree was assessed by bootstrapping, using 1000 bootstrap replications.

Statistics

Statistical analyses were carried out with SPSS software (version 11.5.0). Differences were considered significant at $p < 0.05$. Data were tested for normal distribution with the Shapiro-Wilk test. Differences were evaluated with ANOVA. If ANOVA was significant, Dunnett's T-test was used to determine which means differed significantly from the control.

Results

Cloning and characteristics of three novel carp CXC chemokines

Homology cloning based on a zebrafish EST sequence (BM070896) resembling human CXCL12 resulted in the elucidation of a partial carp CXC chemokine sequence from a cDNA library of carp brain. In obtaining the corresponding full-length sequence, we discovered a second, similar CXCL12-like sequence in RACE cDNA from the anterior kidney. Its corresponding full-length cDNA sequence was obtained from a cDNA library constructed from PMA-activated anterior kidney macrophages. We named these chemokines CXCL12b and CXCL12a, respectively, to parallel the names adopted in the recent zebrafish literature[70].

The full-length carp CXCL12a cDNA sequence (1495 bp) encodes a 99 amino acid CXC chemokine (Fig. 3.1a) bearing high (88%; Table 3.2) amino acid identity to zebrafish CXCL12a and intermediate (43%) amino acid identity to human CXCL12. In addition to a consensus polyadenylation signal (attaaa; bp 1449-1454), the 3'UTR contained six potential instability motifs (attta; bp 984-988, 1180-1184, 1219-1223, 1242-1246, 1308-1312, 1445-1449) implicated in reduction of mRNA half-life[76]. The full-length carp CXCL12b cDNA sequence (1023 bp) is shorter compared to the CXCL12a sequence and encodes a 97 amino acid CXC chemokine (Fig. 3.1b). At the amino acid level, carp CXCL12b is 91% and 44% identical to zebrafish CXCL12b and human CXCL12, respectively (Table 3.2). The

A

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                                  ***
tgcggatctgttcttcacacggcaaacatggatgtgaaactgatcgtaatattcgctctg   60
                                  M  D  V  K  L  I  V  I  F  A  L
atggcggtcgtcatgcatgcaccggttttaaatgccaagcccattagcctggtagagaga  120
 M  A  V  V  M  H  A  P  V  L  N  A  K  P  I  S  L  V  E  R
tgctggtgccgttccacagtcaacacagtcccacagagaagcattcgcgagctcaagttc  180
 C  W  C  R  S  T  V  N  T  V  P  Q  R  S  I  R  E  L  K  F
ctccacacaccaaactgccccttccaagtcattgccaaactgaagaacaacaaggaggtg  240
 L  H  T  P  N  C  P  F  Q  V  I  A  K  L  K  N  N  K  E  V
tgcattaacccagacaccaaatggctgcaacagtacctgaggagtgccatgaacaaaatg  300
 C  I  N  P  D  T  K  W  L  Q  Q  Y  L  R  S  A  M  N  K  M
aaaaaagcccaacaacagcagagctaagaagtctgccagatgacaagagaatctgacagc  360
 K  K  A  Q  Q  Q  Q  S  -
ccaccgtcacagatatgtaccatatagtcctctggcatgcttgctgacccagcacttaac  420
ctgccaaatatgcatccccagtaaatgactctgcaaaagaccaccagaccatcatcgcca  480
cccatgtagtctcctctgttagcgccaagtgcaattatatatgtatgactgtatttttt  540
acgtatgatgtatatacaatgttttttatatatcgtcttaaaacagtaaagtgatatttt  600
tggagtcgtgtttgctccttgtttgttgggttatgttgggttaggcagaacggcattgag  660
atattttggagcaaacattcaaaactctgaaatatattttaatctcagatgtaaaagaat  720
atgtggcttcactttagcaggactgaagttctgacaattcatatgcatgcaagtgctttg  780
acagaaaaatgaagggcattctatttcgttatgtattttactagttcataacttagacatc  840
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ctgttcttggaaaggcagggttactttgcaaaacaaacatgaacccaaatggttatttga  960
atgactttatgtaatagggaaggcatttaacaccaatagaaagtcttttgcaacgttctgt 1020
agataccaaaacatctgtgcttgcaaaggttaattgaccgatcagagtgtgcgtaaagag 1080
cttggtgtcacaatatactaaatattgctccaccttcagcagaatttatgtcatgaaaaga 1140
aacattagaaacgtcagttaacaaagtagaggttagtga<b>atttta</b>aagcagtgttctgtaa 1200
gtactattgagctaattc<b>atttta</b>ttcaattatgaaaactga<b>atttta</b>agcatgataatctt 1260
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ctatatataacattgcactttataacccctggtgaccattgacattgtccattacgtag 1380
tgaataggtctaggtgacctaatggtttctggtggtttagacatagttatgactggtatt 1440
gcca<b>atttat</b>taaaacatctgtatcttcagcatccaaaaaaaaaaaaaaaaaaaaa      1495
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B

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                                                        ***
actgagctctggaagctagctgtgacggacaaaagccacacacacacacttggcatggat   60
                                                          M  D
agcaaagtccttgcgctggtggccctgctgacggtcgtcatatggagcccagaggctgat  120
 S  K  V  L  A  L  V  A  L  L  T  V  V  I  W  S  P  E  A  D
gctaaacccatcagtctggtggagagatgttggtgccggtccactctcaacaccgtcccg  180
 A  K  P  I  S  L  V  E  R  C  W  C  R  S  T  L  N  T  V  P
cagaggagcatccgtgagattaagttcctccacacacccagctgccctttccaagtcatt  240
 Q  R  S  I  R  E  I  K  F  L  H  T  P  S  C  P  F  Q  V  I
gccaagctgaagaacaacagagaggtctgcatcaaccccaagaccaaatggctgcagcag  300
 A  K  L  K  N  N  R  E  V  C  I  N  P  K  T  K  W  L  Q  Q
tatctaaagaatgccattagcaaaataaagaagaagcgctcaaagtaaacacctgaggat  360
 Y  L  K  N  A  I  S  K  I  K  K  K  R  S  K  -
gtgcgaggaggaccaccatgcatctctgacctccaaagagtcgcccgtcctcttctctca  420
ctctctttgatcactcctcctctttctggactgcttgcacaaacagcacttcacctaaac  480
ctgcctctcaagcatccagacagcctacagctcaccaaaacacaagccaacacctcctc  540
ctgacgcactgctactgcggctgtgattactgactgaacgaggccaatgaatgtgaccat  600
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acatgaatgattcagcatgctaacgctagctctgttccaaaacctactgagccacctact  900
gaacaaaaatggcaattgaacacgaaatttgaagtagaaaacctgaaatagttttctct  960
tgtaaaacgtgctccaaaaaccttgaaa<u>aataat</u>ttacggtgcaaaaaaaaaaaaaaa 1020
aaa                                                          1023
```

Figure 3.1: CDNA and deduced amino acid sequences of carp CXCL12a (a) and CXCL12b (b). Asterisks indicate the start codon. Potential instability motifs are indicated in bold. The polyadenylation signal is underlined. Accession numbers for carp CXCL12a and CXCL12b are AJ627274 and AJ536027, respectively.

Table 3.2: Comparison of amino acid identity in vertebrate CXCL12 sequences. Dashed lines separate different vertebrate classes. Accession numbers are as in Fig. 3.5.

	carp CXCL12a	zebrafish CXCL12a	carp CXCL12b	zebrafish CXCL12b	*Xenopus* CXCL12	chicken CXCL12	human CXCL12b	mouse CXCL12a	cow CXCL12a	cat CXCL12b
carp CXCL12a	100									
zebrafish CXCL12a	87.8	100								
carp CXCL12b	71.7	76.3	100							
zebrafish CXCL12b	70.1	75.3	90.7	100						
Xenopus CXCL12	50.7	48.0	43.2	44.2	100					
chicken CXCL12	42.9	45.1	44.0	42.9	75.3	100				
human CXCL12b	43.2	45.7	44.0	46.2	65.2	73.0	100			
mouse CXCL12a	41.8	47.3	44.0	48.4	66.3	75.3	93.3	100		
cow CXCL12a	45.1	49.5	45.1	48.4	67.4	74.2	92.1	89.9	100	
cat CXCL12b	42.6	46.8	45.1	49.5	67.4	77.5	95.7	97.8	92.1	100

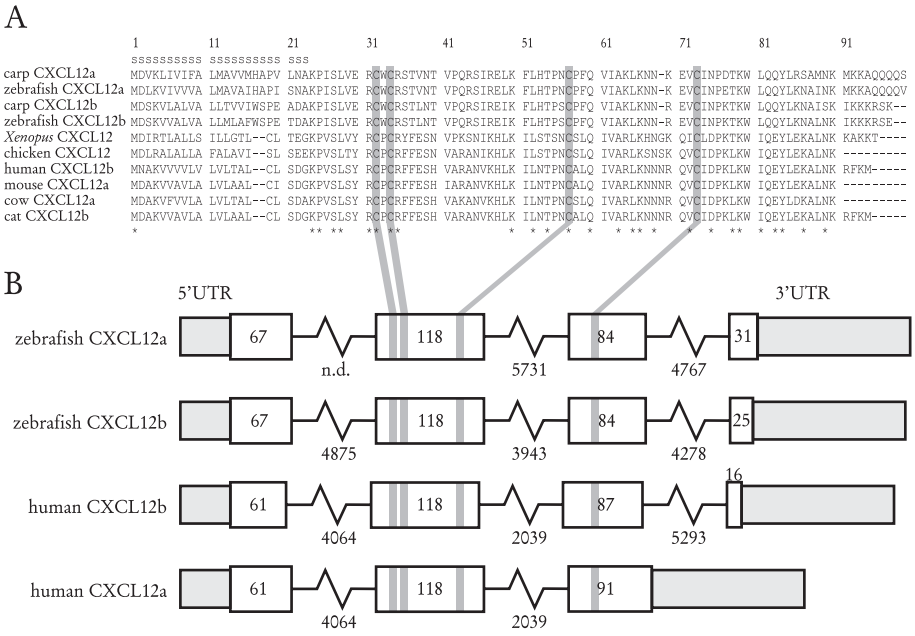

Figure 3.2: Comparison of the amino acid sequences (a) and genomic organisations (b) of cyprinid CXCL12a and CXCL12b with vertebrate orthologues. (a) Asterisks indicate amino acid residues that are conserved in all vertebrate sequences. The four conserved cysteine residues are shaded. The predicted signal peptide (s) is indicated above the alignment. Hyphens indicate gaps. Accession numbers are the same as in Fig. 3.5. (b) Genomic organisation of zebrafish CXCL12a and CXCL12b compared with human CXCL12a and CXCL12b. Exons are indicated in scale by open boxes. Grey boxes indicate the 5' UTR and 3' UTR. Note that zebrafish CXC12a and CXCL12b are duplicate genes, whereas CXCL12α and CXCL12β from mammalian species are splice variants from a single gene. Accession numbers are as follows: zebrafish CXCL12a, ENSDARG00000026725; zebrafish CXCL12b, ENSDARG00000023398; human CXCL12, NT_033985.

```
cggcacgaggggtgacgtgtcgcggactgggagcacattcagcatcacagagacagagac   60
gggttcatcatcacgctcgcgctgtttgagaatctcgattattgaatttgagtggtacaa  120
acaaaccggagactgctggagcttctcaacaaactattgggg***gaatcgctgtacggcc  180
                                          M  N  R  C  T  A
gctttacttttgctggttatcgccgtttattcactcaacacagaggcatacaagtgcaga  240
 A  L  L  L  L  V  I  A  V  Y  S  L  N  T  E  A  Y  K  C  R
tgcacgagaaaaggtccgaagatccggtacaaagatgtacaaaagctggagatcaagcct  300
 C  T  R  K  G  P  K  I  R  Y  K  D  V  Q  K  L  E  I  K  P
aaacatccatactgccaagaaaagatgatatttgtcaccatggagaatgtgtcccgtttc  360
 K  H  P  Y  C  Q  E  K  M  I  F  V  T  M  E  N  V  S  R  F
aaagggcaggagtattgcctgcaccccagactccagagcactaagaaccttgtcaagtgg  420
 K  G  Q  E  Y  C  L  H  P  R  L  Q  S  T  K  N  L  V  K  W
ttcaaaatctggaaggacaaacacagggtgtatgaggcctaaacgctcttctttatgcac  480
 F  K  I  W  K  D  K  H  R  V  Y  E  A  -
aaggctgaaaacattgcgggacgcacagcaccaccagtgaactggggaagaccagaggac  540
caatttttaaagcatgacaaagcatatacacagacaagcttataaagcacaccagacat  600
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ggtctgattcattgtgtcgtggcatataaaggggtcctgacacttatatttggataccgt  840
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acc[attta]aatcatatcatactctat[attta]cacatatacttcagtggaaatgtaccgcc 1140
acacgacgagcacatgaactttggggcctgcagtgggtcatgtgtttaaaatgaagcttt 1200
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gtaaatgaaatgtttctcatctcctcttgtgcatcagattgtatgtataagcacacagaa 1380
taggttcttgtgtacagaccacctcgtctagtctagcttattcagaactgattagtgaag 1440
cctatgtattgtatcacagaattgttgttttact[attta]aataataaatgatagaaatgt 1500
ttgtgtgtttggactgacccaccaaacacaacttaagtccaaggagtgtaatcttcaaac 1560
acagcaataaacatttaaataaaaaaaaaaaaaaaaaaaaaaaaaaaaaa           1610
```

Figure 3.3: cDNA and deduced amino acid sequence of carp CXCL14. Asterisks indicate the start codon. Potential instability motifs are indicated in bold. The polyadenylation signal is underlined. The accession number for carp CXCL14 is AJ536028.

CXCL12b 3'UTR contains a consensus polyadenylation signal (aataaa; bp 990-995) and one potential instability motif (bp 758-762). The spacing of the four cysteine residues is conserved in all vertebrate CXCL12 sequences (Fig. 3.2a). The end of the predicted signal peptide and the start of the mature protein are also conserved throughout vertebrate CXCL12 sequences. Note that both cyprinid CXCL12a sequences differ from carp and zebrafish CXCL12b throughout their amino acid sequences (70 – 75% amino acid identity; Table 3.2), but that the majority of differences are concentrated at the C- and N-terminal

Table 3.3: Comparison of amino acid identity in vertebrate CXCL14 sequences. Dashed lines separate different vertebrate classes. Accession numbers are as in Fig. 3.5.

	carp CXCL14	zebrafish CXCL14	chicken CXCL14	human CXCL14	mouse CXCL14	pig CXCL14
carp CXCL14	100					
zebrafish CXCL14	94.0	100				
chicken CXCL14	54.1	52.1	100			
human CXCL14	58.2	54.6	59.6	100		
mouse CXCL14	56.1	52.6	60.6	91.9	100	
pig CXCL14	57.1	53.6	61.6	94.9	91.9	100

ends. Both zebrafish CXCL12 genes consist of four exons of identical lengths, with the exception of exon four, that is six bp longer in CXCL12a (Fig. 3.2b), accounting for the two extra amino acid residues of CXCL12a. The introns of both genes are long (roughly 3.9 – 5.7 kb), but corresponding introns are clearly different in length in zebrafish CXCL12a and CXCL12b. The genomic organisation of both zebrafish genes is very similar to that of human CXCL12b. Human CXCL12a arises via alternative splicing from the same gene as CXCL12b and misses the fourth exon.

Carp CXCL14 was identified from a carp brain cDNA library in an homology cloning strategy based on the previously described zebrafish *scyba* gene[71]. The full-length carp CXCL14 cDNA sequence (1610 bp) encodes a 99 amino acid CXC chemokine (Fig. 3.3) that is 94% identical to zebrafish CXCL14 and 58% identical to human CXCL14 (Table 3.3). The sizeable 3'UTR of CXCL14 (1109 bp) is comparable in length to that of carp CXCL12a (1127 bp) and substantially longer than the 3'UTRs of carp CXCa and CXCb (189 and 257 bp, respectively). It contains a consensus polyadenylation signal (aataaa; bp 1566-1571) and five potential instability motifs (bp 628-632, 1084-1088, 1107-1111, 1203-1207, 1475-1479). The spacing of the four cysteine residues is conserved in all vertebrate CXCL14 sequences, as is the predicted cleavage site of the signal peptide (Fig. 3.4a). The good conservation of vertebrate CXCL14 is also reflected in its conserved genomic organisation. Like CXCL12, CXCL14 consists of four exons, although exon sizes differ substantially between CXCL12 and CXCL14. With the exception of the first exon, that is one triplet longer in zebrafish, the exons of zebrafish and human CXCL14 are identical in length (Fig. 3.4b).

Phylogenetic analyses

To compare the relationship among teleostean CXCL12 and CXCL14 sequences as well as to establish their relationship with the well-defined mammalian CXC chemokines we constructed a phylogenetic tree of vertebrate CXC chemokine amino acid sequences, using the neighbour-joining method (Fig. 3.5). The overall topology of the tree is in line with CXC chemokine nomenclature. The majority of the ELR$^+$ CXC chemokines (CXCL1 – CXCL7) form a clade, supported by a bootstrap value of 87. CXCL9, CXCL10, and CXCL11, three CXC

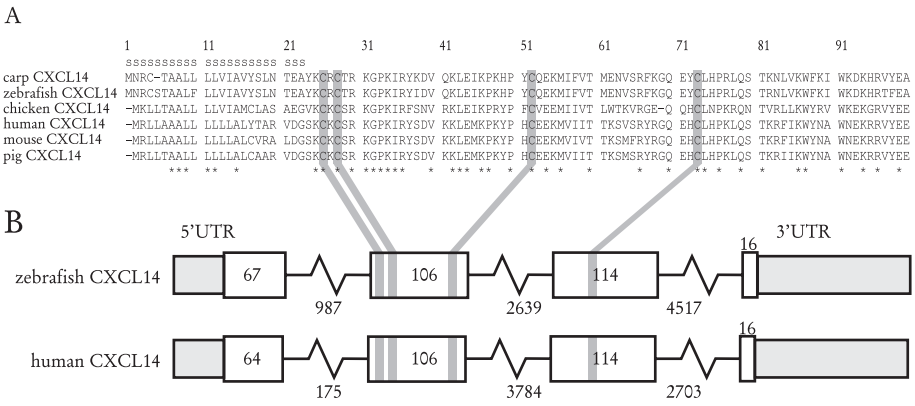

Figure 3.4: Comparison of the amino acid sequence (a) and genomic organisation (b) of cyprinid CXCL14 with vertebrate orthologues. (a) Asterisks indicate amino acid residues that are conserved in all vertebrate sequences. The four conserved cysteine residues are shaded. The predicted signal peptide (s) is indicated above the alignment. Hyphens indicate gaps. Accession numbers are the same as in Fig. 3.5. (b) Genomic organisation of zebrafish CXCL14 compared with human CXCL14. Exons are indicated in scale by open boxes. Grey boxes indicate the 5' UTR and 3' UTR. Accession numbers are as follows: zebrafish CXCL14, ENSDARG00000024941; human CXCL14, NT_034772.

chemokines that share CXCR3 as a receptor, also form a clade, supported by a bootstrap value of 94. Vertebrate CXCL12 and CXCL14 form two distinct clusters, each supported by a high bootstrap value of 99 and 100, respectively. This underscores the conservation of both chemokines throughout vertebrate evolution, as well as confirms the *bona fide* orthology of teleost CXCL12 and CXCL14 sequences to their mammalian namesakes. Note that carp and zebrafish CXCL12a sequences cluster together, as do both cyprinid CXCL12b sequences.

CXC chemokine expression during early ontogeny

We analysed the expression of carp CXCL12a, CXCL12b, and CXCL14 during the first 48 hours of development, which is well before the development of any lymphoid organs[77], and compared their expression patterns with those of two previously described carp CXC chemokines, CXCa and CXCb[32,67]. Expression of CXCL12a and CXCL14 was already detectable in substantial amounts in unfertilised eggs and this expression continued during the first 48 hours of development (Fig. 3.6). CXCL12b expression was detected from four HPF (hours post fertilisation) onwards. At this time, CXCL12a was expressed as abundantly as 40S ribosomal protein. By comparison, CXCa expression was detected only at 24 HPF and 48 HPF and only in limited amounts. CXCb expression was not detected in any of the

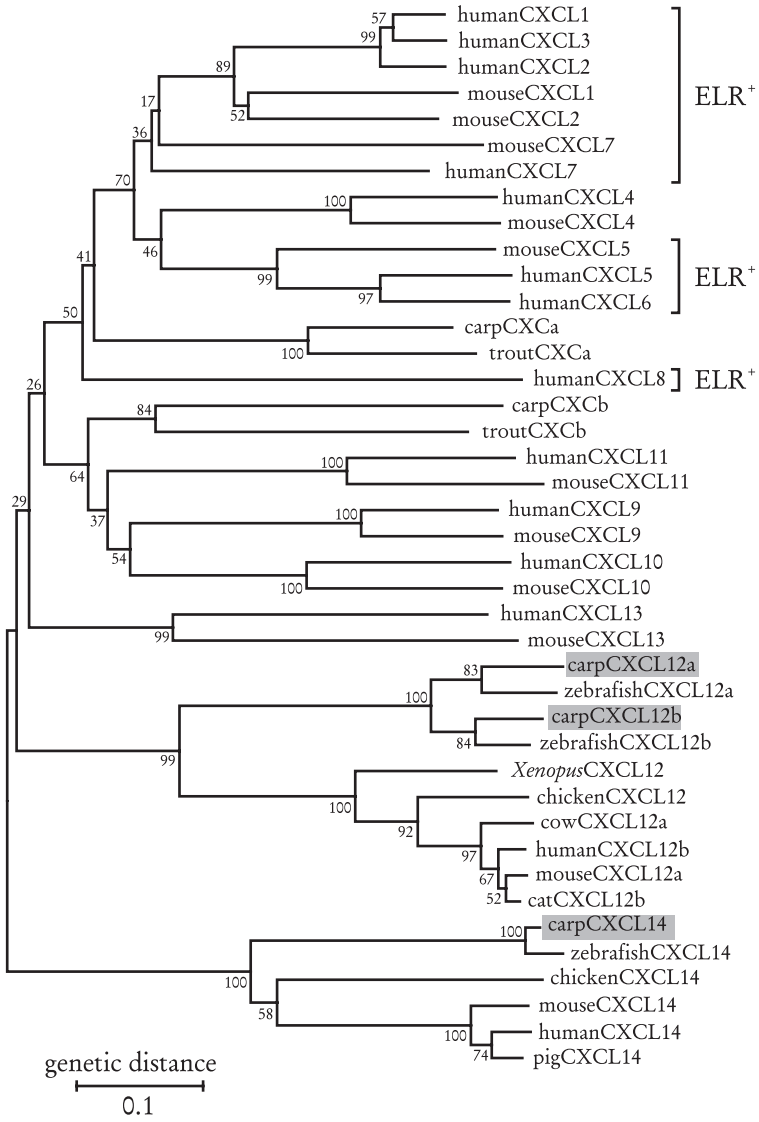

Figure 3.5: Neighbour joining tree of cyprinid CXCL12 and CXCL14 amino acid sequences with non-teleost CXC chemokines. Numbers at branch nodes represent the confidence level of 1000 bootstrap replications. Note that all vertebrate CXCL12 sequences as well as all vertebrate CXCL14 sequences form stable clusters, supported by high bootstrap values (99 and 100, respectively). Accession numbers are as follows: carp CXCL12a, AJ627274; carp CXCL12b, AJ536027; carp CXCL14, AJ536028; carp CXCa, AJ421443; carp CXCb, AB082985; cat CXCL12, O62657; chicken CXCL12, AY451855; chicken CXCL14, AF285876; cow CXCL12, BE483001; human CXCL1, P09341; human

samples (not shown). Expression of each chemokine was confirmed by sequencing the PCR amplicons from the developmental stages with the earliest detectable expression for that chemokine (not shown).

CXC chemokine expression in adult carp

The expression of CXCL12a, CXCL12b, CXCL14 was assessed in various organs and tissues of five individual adult carp and compared with the expression of CXCa and CXCb (Fig. 3.7). The expression of CXCL12a was very high in the anterior kidney and kidney (ten-fold and two-fold the expression of 40s ribosomal protein, respectively), followed by the expression in brain, gonads, and gills. CXCL12b was predominantly expressed in the brain, although expression was detectable in all organs and tissues tested, with the exception of PBL. However, expression levels of CXCL12b in the brain did not approach those of CXCL12a. CXCL14 was also predominantly expressed in the brain, expression in other organs was more restricted. In contrast, the expression of CXCa was highest in organs with mucosal surfaces, such as gills and gut, but was also high in systemic immune organs such as spleen, thymus, kidney, anterior kidney, and liver. CXCb expression was highest in spleen, and was also detectable in gills, anterior kidney, kidney, thymus and gut. Expression levels of CXCa were consistently higher than those of CXCb. Neither gene was detectable in either brain or gonads.

In-vitro CXCL12a expression in anterior kidney phagocytes

To test whether the very high CXCL12a expression observed in the intact anterior kidney is inducible or constitutive, we analysed its expression in anterior kidney phagocytes following *in-vitro* stimulation with various compounds. None of the stimuli induced any changes in CXCL12a expression (Fig. 3.8). In contrast, gene expression of CXCa showed a robust upregulation following stimulation with either ConA or PMA, but not LPS. Furthermore, the expression of CXCL12a in anterior kidney phagocytes is over 3.5 orders of magnitude lower compared to its expression in total anterior kidney. In contrast, the expression of CXCa is not significantly different in total anterior kidney compared to non-stimulated anterior kidney phagocytes.

Figure 3.5, continued,
CXCL2, P19875; human CXCL3, P19876; human CXCL4, P02776; human CXCL5, P42830; human CXCL6, P80162; human CXCL7, P02775; human CXCL8, P10145; human CXCL9, Q07325; human CXCL10, P02778; human CXCL11, O14625; human CXCL12, P48061; human CXCL13, O43927; human CXCL14, O95715; mouse CXCL1, P12850; mouse CXCL2, P10889; mouse CXCL4, AB017491; mouse CXCL5, P50228; mouse CXCL7, NP_076274; mouse CXCL9, P18340; mouse CXCL10, P17515; mouse CXCL11, Q9JHH5; mouse CXCL12, P40224; mouse CXCL13, AF044196; mouse CXCL14, Q9WUQ5; pig CXCL14, BI338800; trout CXCa, OMY279069; trout CXCb, AF483528; Xenopus CXCL12, XLA78857; zebrafish CXCL12a, AY577011; zebrafish CXCL12b, AY347314; zebrafish CXCL14, AF279919.

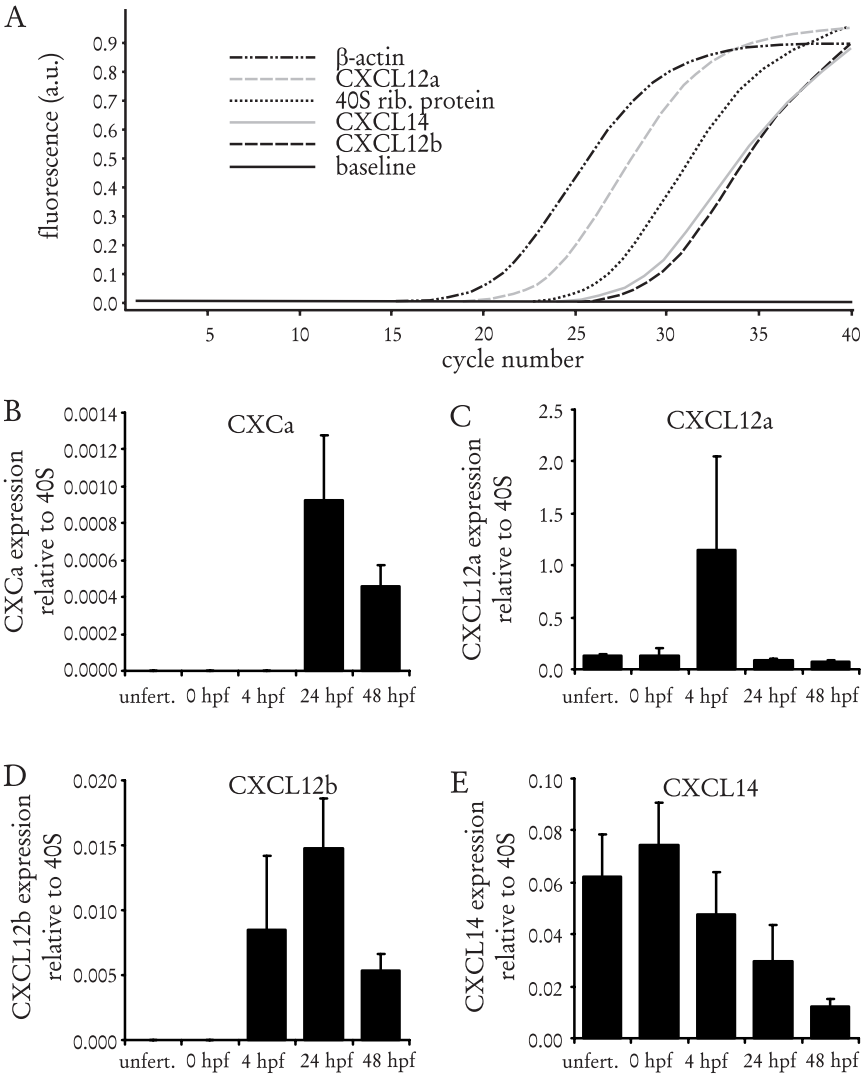

Figure 3.6: Expression of CXC chemokines during early ontogeny in carp. Panel a shows an example of typical RQ-PCR output, in this case for one of the replicates at four HPF. As the number of PCR cycles increases, fluorescence appears consecutively in the various PCR samples. Ct values are determined as the number of PCR cycles that are needed for the fluorescence to cross a predefined threshold (not shown). Note that fluorescence signal for CXCa, CXCb and –RT control does not exceed the baseline. Expression of CXCa (b), CXCL12a (c), CXCL12b (d), and CXCL14 (e) is standardised for 40S expression. Expression of CXCb was not detectable in any of the samples (not shown). Bars represent the average expression in five individual embryos. Error bars indicate standard deviations. Note the different scales of the y-axes.

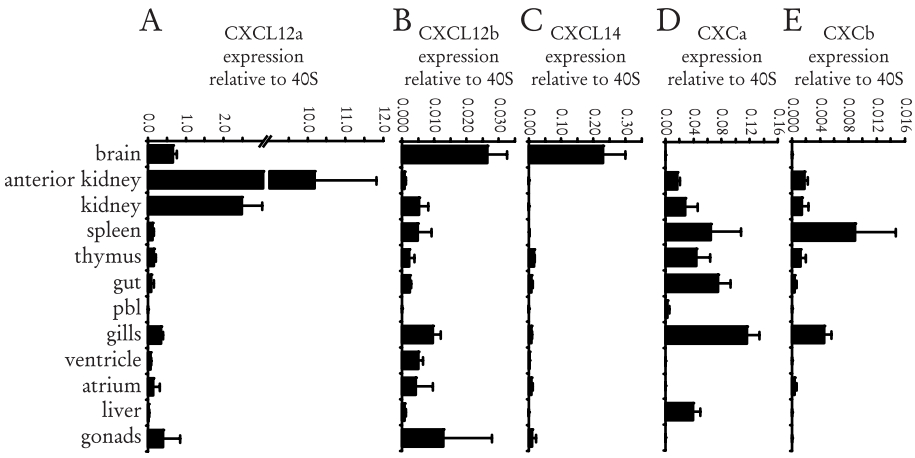

Figure 3.7: Constitutive expression patterns of CXC chemokines in various organs and tissues of carp. Expression of CXCa (a), CXCb (b), CXCL12a (c), CXCL12b (d), and CXCL14 (e) is standardised for 40S expression. Bars represent the average expression in organs or tissues obtained from five individual carp. Error bars indicate standard deviations. Note the different scales of the y-axes.

Discussion

We identified the complete cDNA sequences of three novel carp CXC chemokines by homology cloning. Based on stable clustering in phylogenetic analysis, but also on the relatively high percentages of amino acid conservation with human and mouse orthologous sequences, and the apparent conservation of genomic organisations throughout vertebrate evolution, we named them CXCL12a, CXCL12b, and CXCL14. The fact that we could unequivocally establish orthology of carp CXCL12a, CXCL12b, and CXCL14 with mammalian chemokines is in sharp contrast with both carp CXC chemokines that were earlier described. Although these chemokines also contain a consensus CXC chemokine signature and were shown to mediate chemoattraction in an immune setting, assigning orthology to any particular mammalian CXC chemokine proved impossible[32, 67]. Therefore we named these chemokines CXCa and CXCb to be able to identify orthologues within teleost fish and to simultaneously reflect their phylogenetic distance to mammalian CXC chemokines.

To better understand the relevance of the relatively good conservation of CXC12 and CXCL14 throughout vertebrates, we have to take a closer look at their functions. Despite being evolutionary ancient[51], CXCL14 was identified only recently in human and mouse[59, 78]. Somewhat surprisingly, the tissues that express CXCL14 under normal conditions

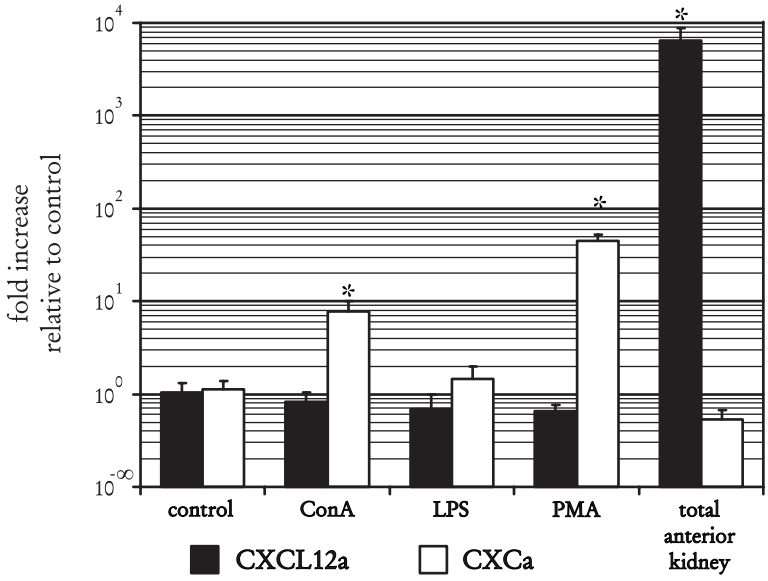

Figure 3.8: *In-vitro* regulation of CXCL12a and CXCa expression. Carp anterior kidney phagocytes were stimulated for four hours with ConA (20 μg ml^{-1}), LPS (50 μg ml^{-1}), or PMA (0.1 μg ml^{-1}). Expression of CXCL12a (black bars) and CXCa (open bars) is standardised for 40s expression and presented relative to unstimulated controls. To enable a proper comparison, the average expression of CXCL12a and CXCa in intact anterior kidneys is also presented relative to unstimulated control cells. Bars represent the average expression in five replicate measurements. Error bars indicate standard deviations. Asterisks denote significant differences from the control ($p < 0.05$). Note that the y-axis is logarithmic.

differ markedly in both species. Human CXCL14 is expressed in small intestine, kidney, spleen, liver, and to a lesser extent brain and skeletal muscle[79]. Murine CXCL14 expression predominates in brain and ovary[59], a pattern that matches the expression of carp CXCL14. The expression of zebrafish CXCL14 in the vestibulo-acoustic system and at the midbrain-hindbrain boundary at twelve HPF, and in various neural structures later in ontogeny offer strong support for a vital role of CXCL14 in central nervous system patterning. In addition, the constitutive expression of CXCL14 in adult carp and mouse brain indicates a role in normal brain physiology. These functions in patterning and maintenance of the vertebrate brain offer an explanation for its remarkable conservation. In this light it is surprising that no information on the role of CXCL14 in mammalian ontogeny, nor as to the identity of its receptor, is available.

In contrast to the paucity of information on CXCL14, far more has been reported on CXCL12. In human and mouse, CXCL12 and its exclusive receptor CXCR4 play essential roles

in bone marrow colonisation[53,80], B-cell development[61,81], and intra-thymic T-cell migration[82-84]. More importantly, CXCL12 and CXCR4 are involved in a series of non-immune functions, such as cerebellar[61, 62, 85] and neocortical[63, 86] neuron migration, astrocyte proliferation[87], germ cell migration[64, 65], angiogenesis[88-90], and cardiac development[62, 81], making CXCL12 arguably the most pleiotropic CXC chemokine. But the key to the conservation of CXCL12 is not so much the myriad of functions it is involved in, but in the critical importance of some of these functions during early development. This importance is illustrated by the perinatally lethal phenotype of CXCL12$^{-/-}$[81] and CXCR4$^{-/-}$[61, 62, 90] mice. Other chemokine and receptor knockout mice oftentimes display an immune-compromised phenotype, but are invariably viable[26].

Reverse genetics approaches, such as generation of knockouts, have not been possible in zebrafish until the entry of antisense morpholino oligos. Hence the number of traditional mutants in which a defective chemokine or chemokine receptor was shown to bring about the mutant phenotype is limited. One study describes the phenotype of the *odysseus* mutant, in which zebrafish CXCR4b is disrupted[91]. The main phenotypic effect of this mutation is the loss of directed migration of PGCs (primordial germ cells) towards their target tissue. Another, parallel study used antisense morpholinos to demonstrate the role of zebrafish CXCR4b in PGC migration[70], although both studies conflict over whether the chemotactic factor involved is CXCL12a[70] or CXCL12b[91]. The apparent phenotypic subtlety of the abrogated CXCR4b expression in zebrafish compared to the severely impaired mouse CXCR4$^{-/-}$ knockout may be explained by the presence of a second zebrafish CXCR4 receptor (CXCR4a) and that these receptors have divided the functions of mammalian CXCR4 between them[92]. Alternatively, both receptors may still display considerable redundancy that, in case of the deletion of one receptor, allows the other to take over the majority of its functions. The fact that fish have duplicate genes for both CXCL12 and CXCR4 is a likely result of a genome duplication event that has occurred in an ancestor of teleost fish, following the fish-tetrapod split[93]. This is corroborated by the preliminary assignment of zebrafish CXCL12a and CXCL12b to separate chromosomes (13 and 22, respectively). However, the conservation of both copies in the genome over extended periods of evolution, requires that each copy adopts a slightly different (set of) function(s), subject to selection[94]. To this end each copy must distinguish itself either in terms of functional properties or in spatial and/or temporal expression.

The 70 – 75% amino acid identity between both CXCL12 paralogues in carp as well as zebrafish would indeed suggest that functional differences exist between CXCL12a and CXCL12b, e.g. in receptor repertoire or affinity. Furthermore, differences in temporal and spatial expression are paramount. Both chemokines are expressed early in development, but carp CXCL12a is supplied as maternal mRNA, while carp CXCL12b expression is only detectable from four HPF onwards, which coincides with early zygotic transcription.

Moreover, expression of cxcl12a is much more abundant compared to that of cxcl12b, most notably at four hpf. Expression of both transcripts in organs and tissues of adult carp is prolific; both transcripts are detectable in the majority of organs and tissues tested. Conspicuous differences exist nonetheless with regards to the organs that contain the highest expression of each transcript. Cxcl12b is predominantly expressed in brain and gonads. Cxcl12a is also expressed in the brain and in considerably higher amounts than cxcl12b, but it is even more abundantly expressed in the anterior kidney and kidney. However, the profound reduction in cxcl12a expression in phagocytes compared to total anterior kidney expression, would suggest that anterior kidney cxcl12a expression is largely restricted to the stromal environment and serves a homeostatic purpose in retaining leukocytes within the anterior kidney. This is in agreement with the lack of upregulation of carp cxcl12a expression by conA, lps or pma. A similar role for cxcl12 has been described in mouse and gave rise to its original name: stromal cell-derived factor 1[53, 95]. Furthermore, murine cxcl12 expression is also non-inducible by pma or lps[96]. The relatively long 3'utr of carp cxcl12a is in line with the observation that the 3'utr of human cxcl12 is the longest 3'utr of all human cxc chemokines, and may be linked to its constitutive expression by containing cis-acting regulatory elements.

The appearance, early in development, of carp cxcl12a and cxcl12b expression is congruent with the presence of zebrafish cxcr4a and cxcr4b from fertilisation onwards[92] and is in line with the early and abundant expression of cxcl12 and cxcr4 during mouse ontogeny[58]. The earliest expression of cxcl12a, cxcl12b, and cxcl14 precedes the formation of the carp thymus, the systemic immune organ that appears first in embryonic development[77], by at least 48 hours. Several processes, such as pgc migration[70] and lateral line formation[97], are described as exclusively mediated by cxcl12a via cxcr4b. This delineates an engaging and straightforward scenario of a cxcl12a/cxcr4b and cxcl12b/cxcr4a as autonomous ligand/receptor pairs that each mediates an exclusive set of functions. However, as illustrated by the discrepancy over the ligand that is involved in pgc migration via cxcr4b[70, 91], it is not certain whether such a monogamous ligand receptor relationship will hold. Nevertheless, the remarkable conservation of cxcl12 and cxcl14, combined with their expression in very early ontogeny and outside established systemic immune organs throughout vertebrates indicates that the key roles these chemokines fulfil are non-immune.

Acknowledgements

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CHAPTER 4

Molecular evolution of CXC chemokines: extant CXC chemokines originate from the CNS

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Abstract

The mammalian CXC chemokine system comprises sixteen ligands and six receptors and its actions stretch well beyond the immune system. The recent elucidation of the genome of the pufferfish, a representative of an evolutionary ancient vertebrate class, enables analysis of the mammalian CXC chemokine system in a phylogenetic context. Comparing phylogenies of vertebrate CXC chemokines revealed that fish and mammals have found different solutions to similar problems, grafted on the same basic structural motif. Phylogenetic analyses showed that the large, highly redundant, CXC chemokine family is a very recent phenomenon, exclusive to higher vertebrates. Moreover, its ancestral role is found within the central nervous system and not within the immune system.

Introduction

CXC chemokines, named after a conserved pattern of cysteine residues, were initially identified as potent mediators of neutrophil chemotaxis[98, 99]. Subsequent work has revealed a much broader spectrum of functions mediated by members of the

Figure 4.1: Phylogenetic tree of CXC chemokine amino acid sequences. Sequences were retrieved by extensive systematic BLAST (Basic Local Alignment Search Tool) searching of all available databases using a multitude of different (fragments of) known CXC chemokines as reference sequences. Numbers at branch nodes represent the confidence level of 1000 bootstrap replications. Clusters of mammalian, avian, amphibian, and teleost fish sequences are shaded in red, yellow, green, and lilac, respectively. Human and pufferfish sequences are indicated in bold. Sub-clusters (statistically reliable clusters within clusters) are indicated with a dashed line. An open circle indicates starting point of branch formation. Abbreviations of species names are as follows: bota, *Bos taurus* (cow); cafa, *Canis familiaris* (dog); capo, *Cavia porcellus* (guinea pig); ceto, *Cercocebus torquatus atys* (mangabey); crgr, *Cricetulus griseus* (chinese hamster); cyca, *Cyprinus carpio* (carp); dare, *Danio rerio* (zebrafish); eqca, *Equus caballus* (horse); feca, *Felis cattus* (cat); gaga, *Gallus gallus* (chicken); hosa, *Homo sapiens* (human); icfu, *Ictalurus furcatus* (blue catfish); icpu, *Ictalurus punctatus* (channel catfish); lafl, *Lampetra fluviatilis* (lamprey); mamu, *Macaca mulatta* (rhesus macaque); mumu, *Mus musculus* (mouse); onmy, *Oncorhynchus mykiss* (rainbow trout); orcu, *Oryctolagus cuniculus* (rabbit); orla, *Oryzias latipes* (medaka); ovar, *Ovis aries* (sheep); paol, *Paralichtys olivaceous* (flounder); rano, *Rattus norvegicus* (rat); sihi, *Sigmodon hispidus* (hispid cotton rat); susc, *Sus scrofa* (pig); taru, *Takifugu rubripes* (pufferfish); trsc, *Triakis scyllium* (shark); xela, *Xenopus laevis* (African clawed toad). Accession numbers are as follows: botaCXCL1 O46676, botaCXCL2 O46677, botaCXCL3 O46675, botaCXCL6 P80221, botaCXCL8 P79255, cafaCXCL8 P41324, capoCXCL1 O55235, capoCXCL8 P49113, cetoCXCL8 P46653, crgrCXCL1 P09340, cycaCXCa AJ421443, cycaCXCb AB082985, cycaCXCL12 AJ536027, cycaCXCL14 AJ536028, dareCXC BI475704, dareCXCL12a BM070896, dareCXCL12b BM265550, dareCXCL14 AF279919, dareCXCc BM529046, eqcaCXC1/2/3 BM735057, eqcaCXCL5/6 AAM76679, eqcaCXCL8 O62812, fecaCXCL8 Q9XSX5, fecaCXCL12 O62657, gagaemf-1 P08317, gagaK60 CAA75212,

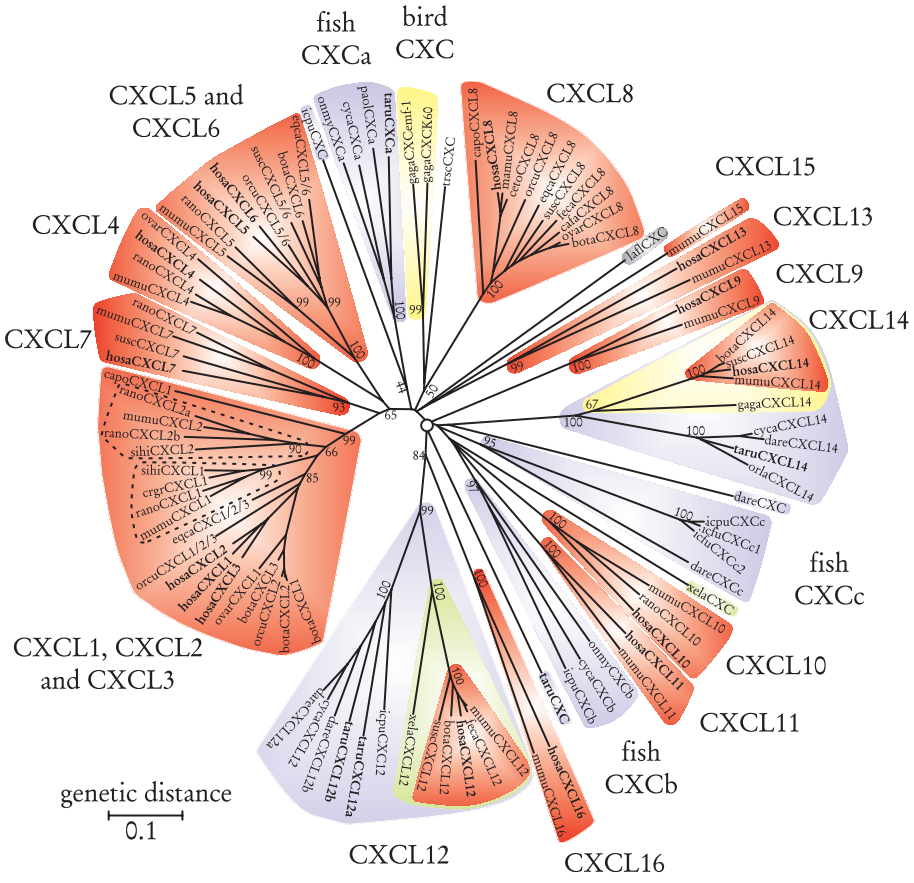


Figure 4.1, continued,

hosaCXCL1 P09341, hosaCXCL2 P19875, hosaCXCL3 P19876, hosaCXCL4 P02776, hosaCXCL5 P42830, hosaCXCL6 P80162, hosaCXCL7 P02775, hosaCXCL8 P10145, hosaCXCL9 Q07325, hosaCXCL10 P02778, hosaCXCL11 O14625, hosaCXCL12 P48061, hosaCXCL13 O43927, hosaCXCL14 O95715, hosaCXCL16 AF301016, icfuCXCc1 BQ097011, icfuCXCc2 BQ097449, icpuCXC12 BM424716, icpuCXC BE470298, icpuCXCb BE212851, icpuCXCc BM425301, laflCXC L231072, mamuCXCL8 P51495, mumuCXCL1 P12850, mumuCXCL2 P10889, mumuCXCL4 AB017491, mumuCXCL5 P50228, mumuCXCL7 NP_076274, mumuCXCL9 P18340, mumuCXCL10 P17515, mumuCXCL11 Q9JHH5, mumuCXCL12 P40224, mumuCXCL13 AF044196, mumuCXCL14 Q9WUQ5, mumuCXCL15 Q9WVL7, mumuCXCL16 AF301017, onmyCXCa OMY279069, onmyCXCb AF483528, orcuCXCL1/2/3 AB93924, orcuCXCL2 P47854, orcuCXCL5/6 P82535, orcuCXCL8 P19874, orlaCXCL14 BJ500803, ovarCXCL1 O46678, ovarCXCL4 P30035, ovarCXCL8 P36925, paolCXCa AF216646, ranoCXCL1 P14095, ranoCXCL2a P30348, ranoCXCL2b, ranoCXCL4 P06765, ranoCXCL5 P97885, ranoCXCL7 AAK30166, ranoCXCL10 P48973, BAB12280, sihiCXCL1 AAL16934, sihiCXCL2 AAL26705, suscCXCL5/6 P22952, suscCXCL7 P43030, suscCXCL8 P26894, taruCXC scaffold_3692 v6.1.1 (scrappy.fugu-sg.org), taruCXCL12a scaffold_871 v6.1.1, taruCXCL12b scaffold_1335 v6.1.1, taruCXCL14 scaffold_236 v6.1.1, taruCXCa scaffold_215 v6.1.1, trscCXC AB063299, xelaCXC BQ400268, xelaCXCL12 XLA78857.

mammalian CXC chemokine family, such as chemotaxis of monocytes and lymphocytes and angiogenesis[100, 101]. Our purpose is not to exhaustively review the plethora of CXC chemokine functions, because this is covered by a number of excellent recent reviews[25, 55, 102-104]. Instead we have traced back the evolutionary history of the CXC chemokine family. In doing so, we deduced remarkable features concerning the putative function of the ancestral CXC chemokine and the recent evolutionary history of the majority of mammalian CXC chemokines.

It takes two to make a comparison. Molecular data of several fish species, supplemented with the recent elucidation of the *Takifugu rubripes* (pufferfish) genome[105], enabled us to identify the CXC chemokine system, including their receptors, in this evolutionary ancient class of vertebrates (Box 4.1). Where appropriate we adhered to the standard nomenclature for chemokines[24, 106] and chemokine receptors[26, 107] but for clarity a table summarising the most common synonyms is included (Table 4.1).

Table 4.1: vertebrate CXC chemokines and common synonyms[a].

standard name	common synonyms	human chromosome	mouse chromosome	receptor	subclass
CXCL1	GROα/MGSAα[b]	4q21.1	5E	CXCR2>CXCR1	ELR$^+$
CXCL2	GROβ/MGSAβ/MIP-2α	4q21.1	5E	CXCR2	ELR$^+$
CXCL3	GROγ/MGSAγ/MIP-2β	4q21.1	-	CXCR2	ELR$^+$
CXCL4	PF4	4q21.1	5E	unknown	ELR$^-$
CXCL5	ENA-78	4q21.1	5E	CXCR2	ELR$^+$
CXCL6	GCP-2	4q21.1	-	CXCR1,CXCR2	ELR$^+$
CXCL7	NAP-2	4q21.1	5E	CXCR2	ELR$^+$
CXCL8	IL-8/NAP-1	4q21.1	-	CXCR1,CXCR2	ELR$^+$
CXCL9	MIG	4q21.1	5E	CXCR3	ELR$^-$
CXCL10	IP-10	4q21.1	5E	CXCR3	ELR$^-$
CXCL11	I-TAC	4q21.1	5E	CXCR3	ELR$^-$
CXCL12	SDF-1α/PBSF	10q11.21	6E	CXCR4	ELR$^-$
CXCL13	BLC/BCA-1	4q21.1	5E	CXCR5	ELR$^-$
CXCL14	BRAK/BMAC	5q31.1	13B	unknown	ELR$^-$
CXCL15	lungkine	-	5E	unknown	ELR$^+$
CXCL16	-	17p13	11B	CXCR6	ELR$^-$

[a] Partially based on[106]

[b] abbreviations: BCA-1, B-cell attracting chemokine-1; BLC, B-lymphocyte chemoattractant; BMAC, B-cell and monocyte-activating chemokine; BRAK, breast and kidney derived; ENA-78, epithelial-cell derived neutrophil activating protein-78; ELR, glutamic acid – leucine – arginine; GCP-2, granulocyte chemoattractant protein-2; GRO, growth related oncogene; IL-8, interleukin-8; IP-10, interferon-inducible protein-10 kilodaltons; I-TAC, interferon-inducible T-cell alpha chemoattractant; MGSA, melanoma growth stimulatory activity; MIG, monokine induced by interferon gamma; MIP, macrophage inflammatory protein; NAP, neutrophil activating peptide; PBSF, pre B-cell stimulating factor; PF4, platelet factor-4; SDF-1α, stromal cell-derived factor-1α.

Most CXC chemokines are evolutionary recent and exclusive to mammals

The most striking observation derived from the phylogenetic analyses of CXC chemokines is that only CXCL12 and CXCL14 have unambiguous orthologues in fish (Fig. 4.1). None of the remaining mammalian CXC chemokines has clear orthologues in any other vertebrate class, including birds. This suggests that CXCL12 and CXCL14 are phylogenetically the modern representatives of the ancestral CXC chemokine. The recently discovered membrane associated CXCL16[108] displays aberrant characteristics, which complicate assignment to a particular group of chemokines. Its pattern of conserved cysteine residues resembles that of CXC chemokines, whereas the glycosylated mucin stalk by which it is attached to the cell surface is a characteristic shared with the CX_3C class of chemokines. Human CXCL16 is located on chromosome 17, like many CC chemokines, but on opposite sides of the centromere (17p13 vs. 17q12)[25]. Since this chemokine is provisionally ranked under the CXC chemokines we have included it in our phylogenetic analyses of the multigene CXC chemokine family. CXCL16 is represented by merely two mammalian sequences. The presence of the mucin stalk and transmembrane region in combination with the aberrant CXC signature of CXCL16 made it impossible to unequivocally establish the presence or absence of fish orthologues. Phylogenetic analyses of CXC chemokine receptors (CXCRs; Fig. 4.2) provide a clue. CXCR6, the receptor to CXCL16, is only found in mammals, suggesting that orthologues of CXCL16 and CXCR6 are not present in fish. So far, sixteen different chemokines have been discovered in mammals, although the numbers vary between individual species. This makes the CXC chemokine system in mammals, much more extensive than that of fish; exhaustive sequencing and systematic database-mining yielded only five different CXC chemokine clusters in fish (CXCa, CXCb, CXCc, CXCL12, and CXCL14; Box 4.1). CXC chemokines from several mammalian species cluster largely according to nomenclature with the notable exceptions of CXCL1 to CXCL3 and CXCL5 and CXCL6 that cluster together, indicating a relatively recent common origin.

CXC chemokine receptors form an extended family

Four of the six mammalian CXCRs possess clearly distinguishable orthologues in fish (Fig. 4.2). CXCR4 is evolutionary the most conserved of all CXCRs, given the representation of mammalian, avian, amphibian, and fish orthologues as well as the relatively short branch lengths within this cluster. Moreover, CXCR4 from sea lamprey, a member of the earliest vertebrate class (agnatha), has recently been described[109]. Because CXCL12 and

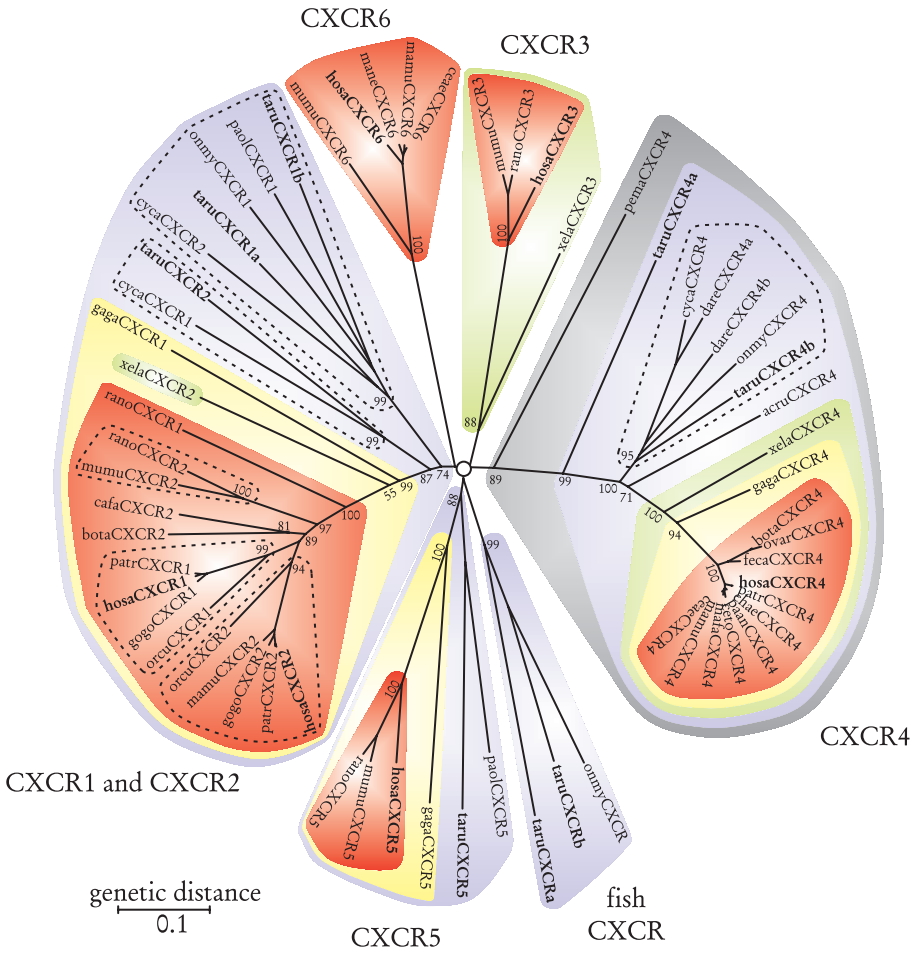


Figure 4.2: Phylogenetic tree of CXC chemokine receptor amino acid sequences. Phylogenies were constructed as in Fig. 4.1. Clusters of mammalian, avian, amphibian, teleost fish and agnathan sequences are shaded in red, yellow, green, lilac, and grey, respectively. Human and pufferfish sequences are indicated in bold. Subclusters (statistically reliable clusters within clusters) are indicated with a dashed line. An open circle indicates the starting point of branch formation. Abbreviations of species names are as follows: acru, *Acipenser ruthenus* (sterlet); bota, *Bos taurus* (cow); cafa, *Canis familiaris* (dog); ceae, *Cercopithecus aethiops* (African green monkey); ceto, *Cercocebus torquatus atys* (mangabey); chae, *Chlorocebus aethiops*; cyca, *Cyprinus carpio* (carp); dare, *Danio rerio* (zebrafish); feca, *Felis cattus* (cat); gaga, *Gallus gallus* (chicken); gogo, *Gorilla gorilla* (gorilla); hosa, *Homo sapiens* (human); mafa, *Macaca fascicularis* (crab-eating macaque); mamu, *Macaca mulatta* (rhesus macaque); mane, *Macaca nemestrina* (pig-tailed macaque); mumu, *Mus musculus* (mouse); onmy, *Oncorhynchus mykiss* (rainbow trout); orcu,

cxcr4 form an exclusive ligand-receptor pair, this underpins the conserved nature of cxcl12. Collectively, the conservation of both ligand and receptor strongly argues in favour of cxcl12 and cxcr4 structurally resembling the ancestral cxc chemokine-receptor pair. The receptor for cxcl14 is as yet unknown but because conserved ligands tend to bind to conserved receptors[110], the receptor for cxcl14 will probably be conserved as well.

Cxcr1 and cxcr2 cluster together and thus apparently are the product of several independent gene duplication events. This is corroborated by the fact that both genes are, in mammals, found in the same chromosomal region[26]. Both pufferfish and common carp (*Cyprinus carpio*) possess multiple cxcr1 and cxcr2 genes but because fish cxcr sequences branch off separately in the phylogenetic tree, abiding by standard nomenclature rules is complicated. Cxcr5 is also represented in birds and fish, in spite of the absence of non-mammalian orthologues to cxcl13, the unique ligand to cxcr5. *Bona fide* fish orthologues of mammalian cxcr3 and cxcr6 lack altogether, although a pufferfish cxcr sequence is located near the cxcr3 cluster. Representation of the majority of cxc chemokine receptors in teleost fish, in conjunction with the strikingly low number of fish cxc chemokines compared to mammals indicates that redundancy of the cxc chemokine system has mainly been generated by expansion of ligands rather than of the receptors.

Figure 4.2, continued,

Oryctolagus cuniculus (rabbit); ovar, *Ovis aries* (sheep); paan, *Papio anubis* (olive baboon); paol, *Paralichtys olivaceous* (flounder); patr, *Pan troglodytes* (chimpanzee); pema, *Petromyzon marinus* (sea lamprey); rano, *Rattus norvegicus* (rat); taru, *Takifugu rubripes* (pufferfish); xela, *Xenopus laevis* (African clawed toad). Accession numbers are as follows: acrucxcr4 aj249438, botacxcr2 q28003, botacxcr4 af399642, cafacxcr2 o97571, ceaecxcr4 af019378, ceaecxcr6 o18983, cetocxcr4 af051906, chaecxcr4 ab015943, cycacxcr1 ab010713, cycacxcr2 ab010468, cycacxcr4 ab012310, darecxcr4a ay057094, darecxcr4b ay057095, fecacxcr4 aj009816, gagacxcr af029369, gagacxcr1 af227961, gagacxcr4 af294794, gogocxcr1 p55919, gogocxcr2 q28422, hosacxcr1 p25024, hosacxcr2 p25025, hosacxcr3 p49682, hosacxcr4 af025375, hosacxcr5 p32302, hosacxcr6 o00574, mafacxcr4 af291672, mamucxcr2 q28519, mamucxcr4 af001928, mamucxcr6 q9xt45, manecxcr6 o19024, mumucxcr2 p35343, mumucxcr3 o88410, mumucxcr5 q04683, mumucxcr6 af301018, onmycxcr ca352557, onmycxcr1 aak48496, onmycxcr4 aj001039, orcucxcr1 p21109, orcucxcr2 p35344, ovarcxcr4 q28553, paancxcr4 af031089, paolcxcr1 bab97378, paolcxcr5 au091121, patrcxcr1 p55920, patrcxcr2 q28807, patrcxcr4 u89798, pemacxcr4 ay178969, ranocxcr1 p70612, ranocxcr2 p35407, ranocxcr3 af452185, ranocxcr5 p34997, tarucxcr1a scaffold_1375 v6.1.1, tarucxcr1b scaffold_2158 v6.1.1, tarucxcr2 scaffold_2580 v6.1.1, tarucxcr4a scaffold_302, tarucxcr4b contig_45848 v6.1.1, tarucxcr5 scaffold_127 v6.1.1, tarucxcra scaffold_2667 v6.1.1, tarucxcrb scaffold_2667 v6.1.1, xelacxcr2 cac85089, xelacxcr3 bg407320, xelacxcr4 y17894.

Box 4.1: Reconstruction of the phylogeny of CXC chemokines

The recent completion of the *Takifugu rubripes* (pufferfish) genome enables us to identify and compare complete multigene families between mammals and teleost fish, a large and relatively early vertebrate class. Gene families arise through and are shaped by processes like gene duplication, loss of genes or even (partial) genome duplications[124]. Speciation is another process that profoundly influences the way in which gene families are shaped. Speciation is a process by which genomes become reproductively isolated; mutational changes or duplications occurring within one genome are no longer transferred to the other via gene flow. Throughout the text, we refer to genes as 'orthologous' or 'paralogous' depending on their relationship. Orthologous is used in those cases where a common ancestor of two genes from different species is apparent, hence speciation is the event that accounts for the presence of two genes[131] (e.g. human and pufferfish CXCL12; Fig. 4.1). Paralogous refers to two genes within the same species that are the product of gene duplication[131] (e.g. human CXCL5 and human CXCL6; Fig. 4.1). Genes, like any part of the genome are also subject to mutational changes. The accumulation of these mutations was used for the phylogenetic reconstruction of the CXC chemokine family. In doing so we assumed that vertebrate CXC chemokines stem from one common ancestral gene. In the resulting phylogenetic tree, a node indicates a common ancestor of the sequences, whereas the length of a branch represents the extent of sequence differences. Our model for the evolution of the CXC chemokine multigene family (Fig. 4.3) is based on a much broader combination of data, such as: chromosomal location, the occurrence of common features (e.g. the ELR motif, glutamic acid (E), leucine (L), arginine (R)), clusters of CXC chemokines that are supported by statistics in phylogenetic analyses (e.g. CXCL5 and CXCL6), protein function and sharing of receptors. It is the combination of all these features that allows for the construction of a model for the evolution of CXC chemokines.

Brief methodology: All available CXC chemokine sequences were retrieved from various sequence databases by extensive systematic BLAST (Basic Local Alignment Search Tool)[73] searches. The alignment of these sequences was performed using the ClustalW algorithm (www.cmbi.kun.nl) and uploaded into MEGA (Molecular Evolutionary Genetics Analysis) version 2.1[46]. We compared several different algorithms for the construction of phylogenetic trees (neighbour-joining (NJ) and minimum evolution (ME) each based on the uncorrected (p) or Poisson corrected proportion of amino acid differences) and all generated trees with a very similar topology. Only trees obtained by the NJ method based on p-distances are presented. The numbers at nodes giving rise to separate branches or clusters indicate their statistical reliability (in percent) of their position within the tree. Reliable clusters of fish CXC chemokines that were not clearly orthologous to any of the systematically defined mammalian CXC chemokines were assigned a letter in addition to the classification CXC, e.g. CXCa for fish.

Why the CXC chemokine system lacks redundancy in fish

Redundancy is one of the key features of mammalian CXC chemokines and is believed to ensure a robust operational system relatively insensitive to polymorphisms[111]. The redundancy of the mammalian CXC chemokine system is to a large extent inferred from research on the inducible and/or inflammatory CXC chemokines bearing the tri-peptide ELR (glutamic acid (E), leucine (L), arginine (R)) motif directly preceding the first conserved cysteine residue. This group consists of CXCL1 to CXCL8 with the exception of CXCL4 and is collectively implicated in chemotaxis of neutrophils, mediated through CXCR1 and CXCR2. Neutrophil chemotaxis in fish too is mediated by CXC chemokines. Carp neutrophils display chemotaxis towards human CXCL8 [67]. Carp CXCa and CXCb are probably the endogenous mediators of this process. The question that remains is what has been the evolutionary force driving the dramatic adaptive radiation of mammalian ELR$^+$ CXC chemokines compared to the modest expansion of fish CXC chemokines. One intriguing hypothesis is that pirating of chemokines and chemokine receptors by viral pathogens forced the rapid expansion of the repertoire of inducible CXC chemokines, to continuously cope with the threat of viral infections[112].

Teleost fish represent by far the greatest number of extant vertebrate species. An elaborate CXC chemokine system was not an absolute prerequisite for successful evolutionary radiation. Apparently a much simpler solution sufficed for adequate immunological protection. The strikingly lower number of CXC chemokines and receptors in fish is in line with the apparent simplicity of especially the adaptive immune system. One of the hallmarks of the mammalian adaptive immune system is the distinct architecture of lymphoid organs, which is instrumental in fine-tuning key processes, such as affinity maturation and memory formation. The compartmentalisation of secondary lymphoid organs, such as spleen and lymph nodes, is maintained by a number of chemokines. CXCL13 interacting with CXCR5 is instrumental in the homing of B-cells towards the B-cell follicle[113-115]. During a primary immune response antigen-stimulated T-cells upregulate CXCR5, thereby becoming sensitive to CXCL13 in the B-cell areas[116]. Together this illustrates a dual key role for CXCL13 and CXCR5. Fish lack orthologues of CXCL13 as well as the lymphoid tissue architecture[117] that is so paramount in mammals. Furthermore, the presence of an orthologue to CXCR5, despite the absence of CXCL13, also suggests a different pivotal function for CXCR5.

Another classic phenomenon associated with mammalian adaptive immunity is the elaborate thymic architecture that is instrumental in positive and negative selection of thymocytes. A number of chemokines are implicated in the sequential migration from cortex to medulla, including CXCL10 to CXCL12 and their receptors CXCR3 and CXCR4[82, 118]. The limited repertoire of fish chemokines fits with the less intricately organised fish

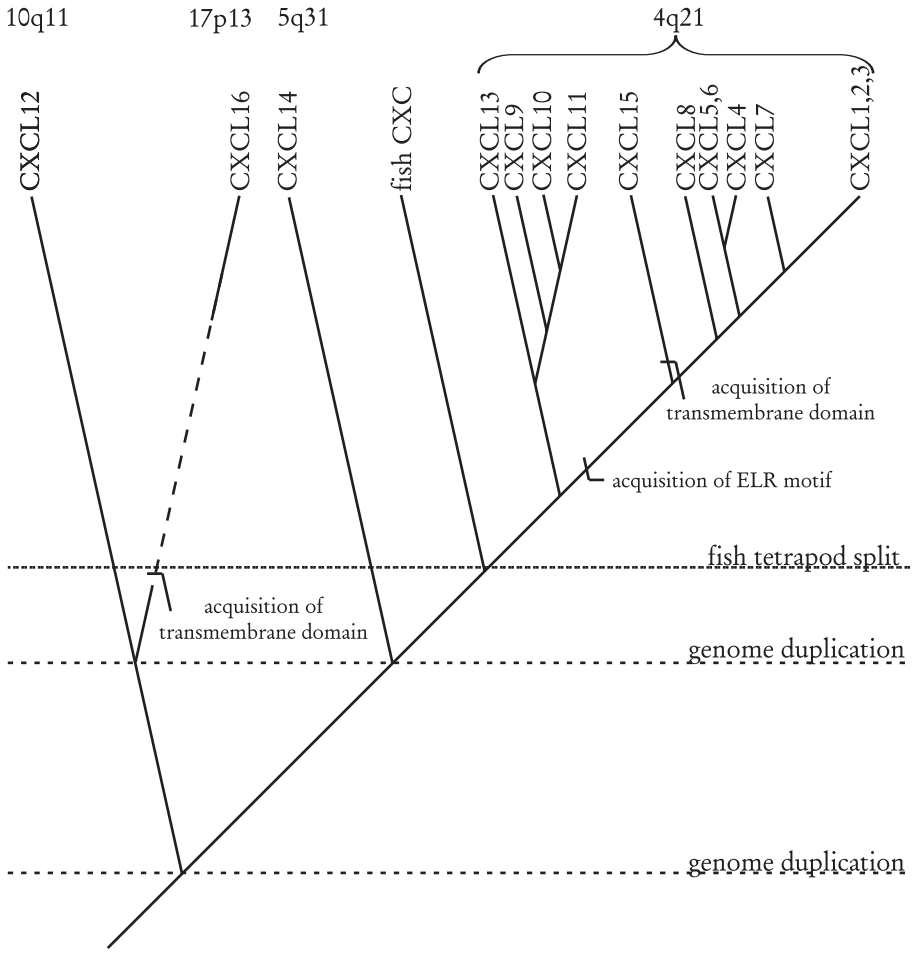


Figure 4.3: Cladogram depicting the individual events shaping the CXC chemokine family as inferred from structural and functional features. Genome duplications may account for the distribution of CXC chemokines over four paralogous regions. Not until much later, well after the fish-tetrapod split did the ancestral CXC chemokine encoded on one of the paralogous regions display a profound radiation by gene duplication to form the extensive CXC chemokine family we know to date. Chromosomal locations of the human CXC chemokines are indicated above the sequences.

thymus[119]. Furthermore, the skewing of helper T-cells on antigen stimulation towards a TH1 phenotype is accompanied by selective expression of chemokine receptors, including CXCR3[120, 121]. Although research on TH1 and TH2 immune responses in fish is scant to say the least, so far no evidence has been found that warrants the distinction between TH1 and TH2, in line with the absence of CXCR3 and its ligands CXCL9 to CXCL11 in fish.

Cxc chemokines originate from the CNS

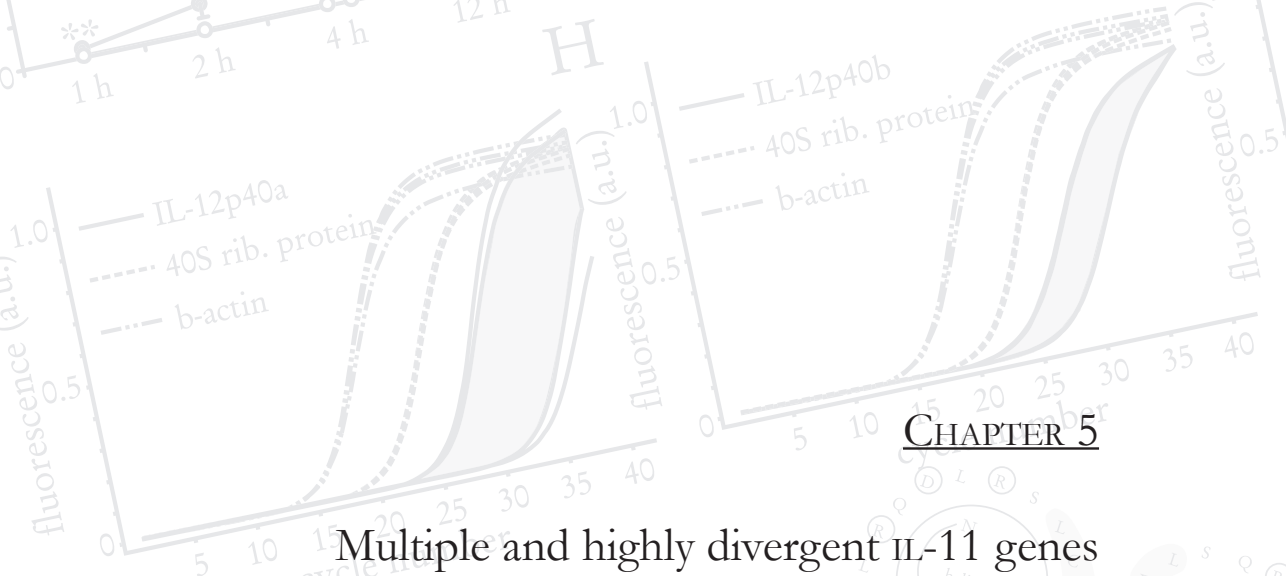
The limited structural alterations of CXCL12, CXCL14 and CXCR4 predict a vital function. Although CXC chemokines are classically associated with the immune system, CXCL12 and CXCL14 perform pleiotropic functions. CXCL12 and its exclusive receptor CXCR4 are crucially required for normal cerebellar development[61, 62], vascularisation of the gastrointestinal tract[90] and cardiac development[62, 81] in mammals, besides mediating normal B-cell lymphopoiesis and bone-marrow myelopoiesis[61, 81]. The importance of this chemokine receptor pair in early central nervous system (CNS) development is explicitly illustrated by the perinatally lethal phenotype of CXCL12$^{-/-}$ and CXCR4$^{-/-}$ mice. The cellular basis for this developmental defect is the premature migration of a subset of cerebellar neurones[61]. This makes CXCL12 and CXCR4 the only members of the CXC chemokine and receptor family essential in the early life history stages[26]. Other CXC chemokine and receptor knockout mice give rise to oftentimes immunodeficient but invariably viable offspring[26, 113]. Less is known of CXCL14. In zebrafish it is expressed in selected brain regions during early development and is implicated in development of the acousto-lateralis system[71]. Adult murine and human brain also abundantly express CXCL14[59]. Although embryonic expression of mammalian CXCL14 has yet to be reported, it is tempting to speculate that the phenotype of CXCL14$^{-/-}$ mice might be severely disrupted, analogous to CXCL12$^{-/-}$ mice, owing to defective CNS development.

The CNS is evolutionary older[122, 123] compared to a highly specialised adaptive immune system, such as that of vertebrates. Therefore, we conclude that CXC chemokines and their receptors predate the vertebrate immune system. Originally the primordial CXC chemokine and receptor mediated functions within the developing CNS, such as chemotaxis of neurones. Genome duplications in protovertebrates[124, 125] could account for the distribution of CXC chemokines on four paralogous regions in mammals (Fig. 4.3). At some point during the emergence of the immune system the ancestral CXC chemokine system, successfully mediating chemotaxis in the CNS, was recruited by the immune system. Effectively, this implicated the transfer of a successful old function to a new setting. Not until much later, well after the fish-tetrapod split, did the CXC chemokine

genes in the mammalian ancestor display a dramatic adaptive radiation towards the highly redundant CXC chemokine family we know to date. CXC chemokines that have a role within the CNS do not merely constitute an added function to the myriad of chemokine functions; this might well reflect their ancestral role.

Concluding remarks

CXC chemokines and their receptors predate the specialised vertebrate immune system as we know it. We postulate that they were initially involved in processes concerning CNS development. Not until the vertebrate immune system began to take shape as a separate and specialised organised system was the CXC chemokine family, already mediating chemotaxis of neurones, recruited to perform similar functions in an ectopic setting (at that time). All mammalian CXC chemokines except CXCL12, CXCL14 and CXCL16, map to the same chromosomal region[126], serving as a silent witness to the recent expansion of the majority of CXC chemokines. Chemokines are not unique with respect to the dual role they have adopted in the CNS and the immune system. Evidence of shared signalling pathways is accumulating; either originally identified by virtue of their CNS function[127, 128], or initially characterised within the immune system[129, 130]. Given their shared requirements for the generation of complexity, flexibility and specificity[129] the brain and immune systems might have more in common than first meets the eye.



CHAPTER 5

Multiple and highly divergent IL-11 genes in teleost fish

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Abstract

Interleukin-11 (IL-11) is a key cytokine in the regulation of proliferation and differentiation of haematopoietic progenitors and is also involved in bone formation, adipogenesis, and protection of mucosal epithelia. Despite this prominent role in diverse physiological processes, IL-11 has been described in only four mammalian species and, recently in rainbow trout (*Oncorhynchus mykiss*). Here we report the presence of IL-11 in common carp (*Cyprinus carpio*), a bony fish species related to zebrafish. IL-11 is expressed in most carp organs and tissues. In-vitro expression of IL-11 in cultured macrophages is enhanced by stimulation with lipopolysaccharide and is markedly inhibited by cortisol. A detailed and systematic scan of several fish genome databases confirms that IL-11 is present in all fish, but also reveals the presence of a second, substantially different IL-11 gene in the genomes of phylogenetically distant fish species. We designated both fish paralogues IL-11a and IL-11b. Although sequence identity between fish IL-11a and IL-11b proteins is low, the conservation of their gene structures supplemented by phylogenetic analyses clearly illustrate the orthology of both IL-11a and IL-11b genes of fish with mammalian IL-11. The presence of IL-11 genes in fish demonstrates its importance throughout vertebrate evolution, although the presence of duplicate and divergent IL-11 genes differs from the single IL-11 gene that exists in mammals.

Introduction

IL-11 was discovered in the early 1990s as a molecule distinct from IL-6 that was able to drive the proliferation of an 'IL-6 dependent' plasmacytoma cell line[132]. The human and murine IL-11 genes each consist of five exons and encode a 199 amino acid protein. Although IL-11 shares limited primary sequence identity with IL-6 and its related cytokines, it is classified as a member of the type-I cytokine family based on the predicted high helical content of the molecule[133] that conforms to the four-helix bundle topology shared by all type-I cytokines[134]. This classification is strongly supported by the nature of the IL-11 receptor complex. IL-11 binds with low affinity to a specific IL-11R chain, that is incapable of eliciting an intracellular signal. To that end the promiscuous signal-transducing GP130 β-chain is recruited, which also participates in the receptor complex for other type-I cytokines including IL-6, CNTF (ciliary neurotrophic factor), LIF (leukemia inhibitory factor), OSM (oncostatin M), and CT-1 (cardiotrophin-1)[135]. The stoichiometry of the functional IL-11 receptor complex is a hexamer that consists of two molecules each of IL-11, IL-11Rα, and GP130[136].

The shared signal-transducing GP130 chain as well as the similar type-I cytokine four-helix bundle conformation explain, at least in part, the redundancy between IL-11 and other type-I cytokines. IL-11 overlaps in function with IL-6, as illustrated by its discovery in an 'IL-6 dependent' bioassay[132], and with other type I cytokines. Nonetheless, over the last decade IL-11 has emerged as pleiotropic cytokine that mediates a unique spectrum of different functions at various sites in the body. IL-11, either by itself or in synergy with various other growth factors, stimulates proliferation and differentiation of both early and late haematopoietic progenitors[137, 138]. IL-11 contributes to the commitment of stem cells into the multilineage progenitor compartment[139]. Moreover, IL-11 acts in concert with IL-3, TPO (thrombopoietin), IL-6, and SCF (stem cell factor) to enhance megakaryocytopoiesis and increase peripheral platelet counts[132, 140-144] and stimulates erythropoiesis alone or aided by IL-3 or EPO (erythropoietin)[145]. Moreover, administration of recombinant human IL-11 to sublethally irradiated mice accelerates recovery of peripheral blood platelets and neutrophils[146]. The prominent effects of IL-11 on thrombopoiesis in particular have led to its clinical use as a therapeutic agent following bone marrow ablative chemotherapy[147]. IL-11 also has many non-hematopoietic effects. It exerts protective effects on various mucosal epithelia of the gastrointestinal[148, 149] and respiratory systems[150] in response to injury. The underlying mechanism is considered to relate to the dampening effects of IL-11 on the inflammatory response by reducing macrophage cytokine and reactive oxygen species production[147, 150-153]. Moreover, IL-11 was recently shown to mediate direct anti-apoptotic effects on human colonic epithelial cells[154]. IL-11 also prevents cartilage matrix degradation in chronically inflamed joints by inducing the expression of TIMP (tissue inhibitor of metalloproteinases)[155] and is an important regulator of bone remodeling[156, 157]. Other physiological functions of IL-11 include the inhibition of adipogenesis[156, 158] and the induction of proliferation and differentiation of hippocampal neuronal cells[159, 160].

So far IL-11 has only been identified in two primate and two rodent species. Very recently, an IL-11 protein was reported in rainbow trout (*Oncorhynchus mykiss*)[161]. Fish orthologues of several other mammalian four-helix bundle cytokines, including IL-12P35[162] and EPO[163], are known and these cytokines invariably exhibited low overall amino acid similarity to their mammalian orthologues. Furthermore, a four-helix bundel cytokine designated M17 that shares similarities with OSM, LIF, and CNTF was recently described in carp[164]. The inability to find fish orthologues to many mammalian cytokines following the completion of the first draft fish genome has initially led to speculation on their absence from the fish lineage[105]. Here we report the sequence of IL-11 from common carp (*Cyprinus carpio*) and characterise its expression in-vivo and in-vitro in a carp primary macrophage cell culture system. Surprisingly, a systematic and detailed search of the available fish genomes not only confirmed the presence IL-11, but also revealed the presence of a second, IL-11 gene in the genomes of such distantly related families

such as pufferfishes (pufferfish, *Takifugu rubripes* and spotted green pufferfish, *Tetraodon nigroviridis*), and cyprinids (zebrafish, *Danio rerio*). In these species we designated both paralogues as IL-11a and IL-11b. Despite their obvious sequence dissimilarity, both teleost IL-11a and IL-11b genes share their intron-exon organization with mammalian IL-11 genes. Furthermore, all vertebrate IL-11 genes form a stable cluster in phylogenetic analysis, apart from other type-I cytokines, which confirms their orthology with mammalian IL-11. The presence of IL-11 in teleost fishes is testimony of its importance throughout vertebrate evolution. Nonetheless, the presence of duplicate and substantially different IL-11 genes in fish indicates that, during the 450 million years since the bony fish-tetrapod split[165], IL-11 in fish and mammals may have adopted different functions.

Materials and methods

Animals

Common carp (*Cyprinus carpio* L.) were reared at 23 ºC in recirculating UV-treated tap water at the 'De Haar Vissen' facility in Wageningen. Fish were fed pelleted dry food (Provimi, Rotterdam, The Netherlands) at a daily rate of 0.7% of their estimated body weight. R3xR8 are the hybrid offspring of a cross between fish of Polish origin (R3 strain) and fish of Hungarian origin (R8 strain)[34]. All experiments were performed according to national legislation and approved by the institutional animal experiments committee.

Homology cloning, amplification and sequencing

Oligonucleotide primers (IL-11.fw1 and IL-11.rv1; Table 5.1) were designed for carp IL-11 based on a partial zebrafish IL-11 sequence retrieved from the Ensembl zebrafish genome in a BLAST search. The corresponding full-length carp IL-11 sequence was obtained by RACE (rapid amplification of cDNA ends). We used total RNA from the head kidney of one individual adult carp for the synthesis of RACE cDNA (GeneRacer; Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions. IL-11.rv3 and IL-11.rv4 (Table 5.1) were used as the initial and nested primers for the amplification of the 5'UTR. IL-11.fw4 and IL-11.fw3 (Table 5.1) were used as initial and nested primers for the amplification of the 3'UTR.

Tissue and cell collection and preparation

Adult carp (±150-200 g) were anesthetised with 0.2 g l^{-1} tricaine methane sulfonate buffered with 0.4 g l^{-1} $NaHCO_3$. Blood was collected by puncture of the caudal vessels using a heparinised syringe (Leo Pharmaceutical Products Ltd, Weesp, the Netherlands)

Table 5.1: Primer sequences and corresponding accession numbers

gene	acc. number	primer	sequence 5' ⇒ 3'
IL-11	AJ632159	IL-11.fw1	CAGCACCATTTTGAGTGGCT
		IL-11.rv1	IACTCGAGCGGCCCAGTC
		IL-11.fw3	CTCAGGAACTCCTGCAGCAG
		IL-11.rv3	TAATGTGGGAGATCATGTCTGT
		IL-11.fw4	CCTAATCCCGCTTTTCATTGG
		IL-11.rv4	GCTGCTGCTTGCGTGTCGT
		qIL-11.fw1	GCTGTCACGTCATGAACGAGAT
		qIL-11.rv1	CCCGCTTGAGATCCTGAAATAT
IL-10	AB110780	qIL-10.fw1	GCTGTCACGTCATGAACGAGAT
		qIL-10.rv1	CCCGCTTGAGATCCTGAAATAT
IL-1β	CCA245635	qIL-1β.fw1	CTGGAGCAATGCAATACAAAGTTC
		qIL-1β.rv1	CAAGGTAGAGGTTGCTGTTGGAA
40s ribosomal protein s11	AB012087	q40s.fw1	CCGTGGGTGACATCGTTACA
		q40s.rv1	TCAGGACATTGAACCTCACTGTCT
β-actin	CCACTBA	qACT.fw1	GCTATGTGGCTCTTGACTTCGA
		qACT.rv1	CCGTCAGGCAGCTCATAGCT

fitted with a 21 or 25 Gauge needle. Anterior kidney cell suspensions were obtained by passing the tissue through a 50 μm nylon mesh with carp RPMI [cRPMI; RPMI 1640, Gibco, adjusted to carp osmolarity (270 mOsm kg^{-1}) with distilled water] and washed once. The cell suspension was layered on a discontinuous Percoll gradient (1.020, 1.070, and 1.083 g cm^{-3}) and centrifuged for 30 min at 800 g with the brake disengaged. Cells at the 1.070 g cm^{-3} interface (representing predominantly macrophages) were collected, washed, and seeded at 1-5x10^6 cells per well (in a volume of 400 μl) in a 24-well cell culture plate. Following overnight culture at 27 ºC, 5% CO_2 in $cRPMI^{++}$ [cRPMI supplemented with 0.5% (v/v) pooled carp serum, 1% (v/v) L-glutamine (Cambrex), 200 nM β-mercaptoethanol (Biorad), 1% (v/v) penicillin G (Sigma), and 1% (v/v) streptomycin sulfate (Sigma)], cell cultures were stimulated for 4 h with 50 μg ml^{-1} LPS (lipopolysaccharide from *Escherichia coli*; Sigma), 20 μg ml^{-1} ConA (concanavalin A from *Canavalia ensiformes*; Sigma) or 400 ng ml^{-1} cortisol (Sigma). A non-stimulated control group was included and all treatments were carried out in five-fold. Following stimulation cells were collected for RNA isolation. Organs and tissues for the analysis of in-vivo RNA expression were carefully removed, flash-frozen in liquid nitrogen and stored at -80 °C. Blood was mixed with an equal volume of carp RPMI (RPMI 1640, Gibco; adjusted to carp osmolarity (270 mOsm kg^{-1}) with distilled water) containing 0.01% (v/v) NaN_3 and 10 U ml^{-1} heparin and centrifuged for 10 min at 100 g to remove the majority of erythrocytes. The supernatant containing PBL (peripheral blood leukocytes) was layered on a discontinuous Percoll (Amersham Pharmacia Biotech AB) gradient (1.020 and 1.083 g cm^{-3}). Following centrifugation (30 min at 800 g with brake disengaged) cells at the 1.083 g cm^{-3} interface (representing total peripheral blood leukocytes) were collected.

RNA isolation

RNA from PBL and from anterior kidney macrophage-enriched cell cultures was isolated using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. Final elution was carried out in 25 µl of nuclease-free water, to maximise concentration. RNA was isolated from tissues using Trizol reagent (Invitrogen), according to the manufacturer's instructions. Total RNA was precipitated in ethanol, washed and dissolved in nuclease-free water. RNA concentrations were measured by spectrophotometry and integrity was ensured by analysis on a 1.5% agarose gel before proceding with cDNA synthesis.

DNase treatment and first strand cDNA synthesis

For each sample a non-RT (non-reverse transcriptase) control was included. One µl of 10x DNase I reaction buffer and 1 µl DNase I (Invitrogen, 18068-015) was added to 1 µg total RNA and incubated for 15 min at room temperature in a total volume of 10 µl. DNase I was inactivated with 1µl 25 mM EDTA at 65 ºC for 10 min. To each sample, 300 ng random hexamers (Invitrogen, 48190-011), 1 µl 10 mM dNTP mix, 4 µl 5x First Strand buffer, 2 µl 0.1 M dithiothreitol and 10 U RNase inhibitor (Invitrogen, 15518-012) were added and the mix was incubated for 10 min at room temperature and for an additional 2 min at 37 ºC. To each sample (but not to the non-RT controls) 200 U Superscript RNase H⁻ Reverse Transcriptase (RT; Invitrogen, 18053-017) was added and reactions were incubated for 50 min at 37 ºC. All reactions were filled up with demineralised water to a total volume of 1 ml and stored at -20 ºC until further use.

Real-time quantitative PCR

Primer express software (Applied Biosystems) was used to design primers for use in real-time quantitative PCR. For RQ-PCR 5 µl cDNA and forward and reverse primer at 5 µM each (Table 5.1) were added to 12.5 µl Quantitect Sybr Green PCR Master Mix (Qiagen) and filled up with demineralised water to a final volume of 25 µl. RQ-PCR (15 min at 95 ºC, 40 cycles of 15s at 94 ºC, 30 s at 60 ºC, and 30 s at 72 ºC followed by 1 min at 60 ºC) was carried out on a Rotorgene 2000 real time cycler (Corbett Research, Sydney, Australia). Following each run, melt curves were collected by detecting fluorescence from 60 to 90 ºC at 1 ºC intervals. Expression in organs and tissues of adult carp was rendered as a ratio of target gene vs. reference gene and was calculated as previously described[166]. Expression following in vitro stimulation was corrected for reference gene expression and rendered relative to the expression in non-stimulated control cells as previously described[72, 166]. Efficiency and threshold values used for each primer set were: IL-11, 2.09, 0.0170; 40S, 2.11, 0.0970; β-actin, 2.05, 0.0513. Dual internal reference genes (40S and β-actin) were incorporated in all RQ-PCR experiments and results were confirmed to be similar following standardisation to either gene. Non-RT controls were included in all

```
tgacaacatctggagcttcatttatttattaatttctacgcttttgtttgtttgtttatt    60
tatttctctgcgtatttatttgcaacgtatctcctttataaaataaggacctggacaaat   120
atgaaattgctgggtgactcctcctcatcgctgctcctctcgctgctgctggctcaactt   180
 M  K  L  L  G  D  S  S  S  S  L  L  L  S  L  L  L  A  Q  L        20
catctgttagcatctgccttccctgctcaccccaggcggatccagactgactttgacaag   240
 H  L  L  A  S  A  F  P  A  H  P  R  R  I  Q  T  D  F  D  K        40
ctgagcaatcagaccagacaccttctgaagctgactcaggatctactgaaaaacccagtg   300
 L  S  N  Q  T  R  H  L  L  K  L  T  Q  D  L  L  K  N  P  V        60
tttgccacagagattgatcaccaaaggttcaagtctcttccagcgatcagcagcagagtc   360
 F  A  T  E  I  D  H  Q  R  F  K  S  L  P  A  I  S  S  R  V        80
agtgacctcaccactctggagttcaagcctacactttctcagctctatgcagacctaaag   420
 S  D  L  T  T  L  E  F  K  P  T  L  S  Q  L  Y  A  D  L  K       100
tcctttgagcaccactttgagtggctgaacagaacgacacgcaagcagcagcacagctca   480
 S  F  E  H  H  F  E  W  L  N  R  T  T  R  K  Q  Q  H  S  S       120
gtaccaaagctgacagacatgatctcccacattaaaagcctcataaactccttacagcgt   540
 V  P  K  L  T  D  M  I  S  H  I  K  S  L  I  N  S  L  Q  R       140
cagatgacccgagcagaggctccacggatccccgttccctctccctcactcccacctaat   600
 Q  M  T  R  A  E  A  P  R  I  P  V  P  S  P  S  L  P  P  N       160
cccgcttttcattgggaggtggttcaatcctctcaggaactcctgcagcagttcaggctc   660
 P  A  F  H  W  E  V  V  Q  S  S  Q  E  L  L  Q  Q  F  R  L       180
ttctgtgactgggcctcacgagtgttccttaccctcaaatccaaattaccagcatgatga   720
 F  C  D  W  A  S  R  V  F  L  T  L  K  S  K  L  P  A  -         199
acctcagaggcatcacacaaaaaacattgggccaggactcaatcattggccaaatggact   780
ggacatagggagcaccgggactttccccagtgggtagggtcaatgtatgggataatagtt   840
ttgatattgtcaggccggaacatctgagtaacaggatggtagctgagtatttcaacttgt   900
tctctcgtacccattcacaacctactcttgttcagtctcgttccaaccgccactgctttg   960
aactcccaaatcataagaatgagagtaaatctgtgcattattaagacatgcataaatata  1020
aatattgcatatggaaccaggaagacgatgactgatttgatcaactgaaatatagtgcta  1080
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Figure 5.1: Nucleotide and deduced amino acid sequence of carp IL-11. The accession number for carp IL-11 is AJ632159. Nucleotide and amino acid numbers are given at the end of each line. A potential N-glycosylation site (NQT) is indicated in bold and italics, the predicted signal peptide is underlined.

experiments and were negative.

Bioinformatics

Sequences were retrieved from the Swissprot, EMBL and Genbank databases using the SRS mirror site of the European Bioinformatics Institute (www.ebi.ac.uk/services). *Danio rerio*, *Takifugu rubripes*, and *Tetraodon nigroviridis* sequences were retrieved from the Ensembl website (http://www.ensembl.org)[167]. Isoelectric point prediction was carried out at http://www.iut-arles.up.univ-mrs.fr/w3bb/d_abim/compo-p.html. The gene structure of carp IL-11a was predicted by comparison with the zebrafish IL-11a gene. Signal peptides were predicted by SignalP 3.0 at http://www.cbs.dtu.dk/services/SignalP/[74]. Multiple sequence alignment was conducted with ClustalW on the EBI mirror site. Calculation of multiple pairwise amino acid identities was carried out using FASTA version 3.4t10[168]. Helical wheels were predicted at http://www.site.uottawa.ca/~turcotte/

resources/HelixWheel. Phylogenetic trees were constructed on the basis of amino acid difference (p-distance) by the neighbour-joining method[45] using MEGA version 2.1[46]. Reliability of the tree was assessed by bootstrapping, using 1000 bootstrap replications. Trees with very similar topologies to the one shown were obtained by the application of either Minimum Evolution or Maximum Parsimony algorithms.

Statistics

Statistical analysis was carried out with SPSS software (version 12.0.1). Differences were evaluated with a Mann-Whitney U-test, $p<0.05$ was accepted as significant.

Results

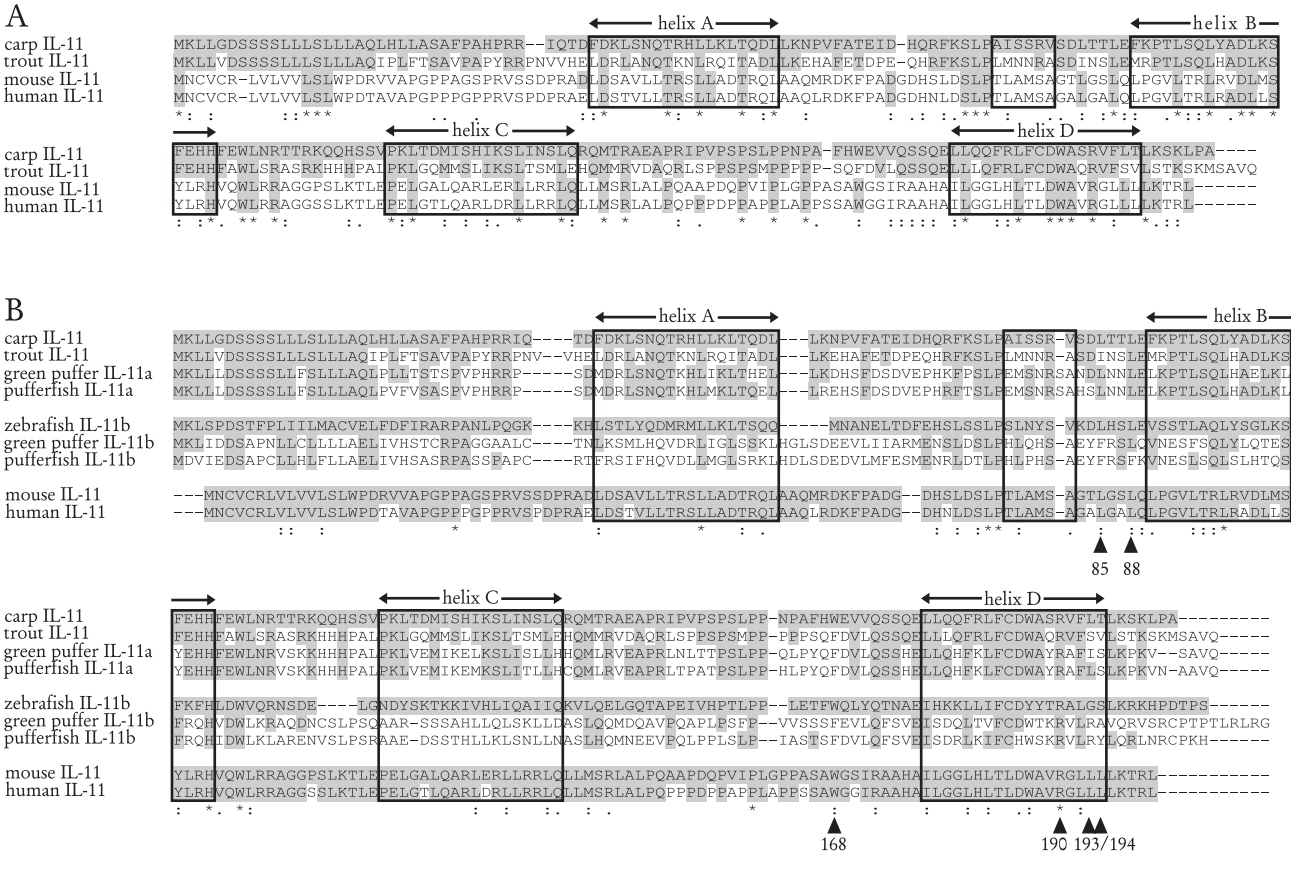
Cloning and characteristics of carp IL-11

A systematic BLAST search of the Ensembl zebrafish (*Danio rerio*) genome database with mammalian IL-11 sequences revealed a partial zebrafish IL-11(a) sequence. This partial zebrafish sequence was used in a homology cloning approach to identify a partial carp IL-11 sequence. The corresponding full-length cDNA sequence containing the

Table 5.2: List of BLAST hits following comparison of carp IL-11 to the Swissprot database.

acc. number	species	description	E-value
P20808	macaque	Interleukin-11 precursor	2×10^{-4}
Q99MF5	rat	Interleukin-11 precursor	2×10^{-4}
P47873	mouse	Interleukin-11 precursor	3×10^{-4}
P20809	human	Interleukin-11 precursor	2×10^{-3}
Q8R6G8	*Fusobacterium nucleatum*	bifunctional glycosyltransferase/methyltransferase	1.6
P19137	mouse	Laminin alpha-1 chain precursor	2.3

Figure 5.2: Multiple amino acid sequence alignment of vertebrate IL-11 sequences. (a) The alignment of carp IL-11 with selected vertebrate IL-11 sequences illustrates the presence of four potential α-helices (boxed), despite the relatively low overall amino acid identity. A fifth, short α-helix that is characteristic for many four-helix bundle cytokines is present in the amino acid loop that connects helices A and B. (b) Inclusion of additional bony fish IL-11a and IL-11b sequences illustrates their extensive dissimilarity. Asterisks indicate amino acids that are identical throughout the alignment. Colons and dots indicate decreasing degrees of conservative substitutions. Residues important for receptor binding or activation are indicated with their position number and an arrowhead. Shading reflects amino acid identity throughout the alignment (a) or within clusters of fish IL-11a, fish IL-11b, and mammalian IL-11 amino acid sequences (b). Green puffer and pufferfish refer to *Tetraodon nigroviridis* and *Takifugu rubripes*, respectively. Accession numbers are: carp IL-11, AJ632159; trout IL-11, AJ535687; green puffer IL-11a, BN000715/CAAE01014543.1; pufferfish IL-11a, BN000713/CAAB01000705.1; zebrafish IL-11b, BN000718/CAAK01009193.1; green puffer IL-11b, AY374508; pufferfish IL-11b, BN000714/CAABB01000522.1; mouse IL-11, P47873; human IL-11, P20809.


A
helix A
helix B
helix C
helix D
carp IL-11
trout IL-11
mouse IL-11
human IL-11
B
helix A
helix B
helix C
helix D
carp IL-11
trout IL-11
green puffer IL-11a
pufferfish IL-11a
zebrafish IL-11b
green puffer IL-11b
pufferfish IL-11b
mouse IL-11
human IL-11
85
88
168
190
193/194

complete coding strands was obtained by RACE (rapid amplification of cDNA ends) and encodes a 198 amino acid novel carp interleukin (Fig. 5.1). Protein-protein BLAST against the Swissprot database revealed significant hits with rat, mouse, macaque, and human IL-11 amino acid sequences only (Table 5.2), which supports the identity of the carp interleukin sequence as IL-11. Alignment of carp IL-11 with IL-11 of human and mouse, and well as an IL-11 sequence from rainbow trout (*Oncorhynchus mykiss*), reveals that their overall amino acid identity is modest (Fig. 5.2a). Nonetheless, several short stretches of amino acid residues throughout the alignment are identical in all four IL-11 sequences. Many of these conserved amino acids are leucine residues that converge at the center face of each of the four α-helices when subjected to a helical wheel projection (Fig. 5.3). In contrast, the solvent exposed exterior surface of the predicted carp IL-11 molecule contains many charged amino acid residues, predominantly arginines (R), that account for its relatively high predicted isoelectric point (pI; Table 5.3), a feature shared by bony fish and mammalian IL-11 proteins.

Bony fish possess duplicate and highly divergent IL-11 genes

We retrieved another bony fish sequence from the nucleotide databases that was annotated as IL-11 (AY374508)[169]. This sequence, from the spotted green pufferfish (*Tetraodon nigroviridis*), differs from the carp and rainbow trout IL-11 sequences more than is expected based on the evolutionary distance between the spotted green pufferfish and both other bony fish species. Therefore we revisited the zebrafish genome database, as well as the publicly available genomes of the spotted green pufferfish and the pufferfish species 'torafugu' (*Takifugu rubripes*), for a careful, detailed, and systematic search for teleostean IL-11 genes. In each of these species we identified duplicate and highly divergent IL-11 genes. In all three species, one of these genes (designated IL-11a) is clearly similar to carp IL-11 (52 – 76 % amino acid identity), whereas in each species the other IL-11 gene,

Table 5.3: Predicted isoelectric point (pI) of IL-11 sequences

species	sequence	predicted pI
human	IL-11	11.55
macaque	IL-11	11.12
mouse	IL-11	11.70
rat	IL-11	11.70
carp	IL-11	10.50
zebrafish	IL-11a	10.77
trout	IL-11	10.34
green puffer	IL-11a	9.67
pufferfish	IL-11a	9.71
zebrafish	IL-11b	9.42
green puffer	IL-11b	6.66
pufferfish	IL-11b	6.63

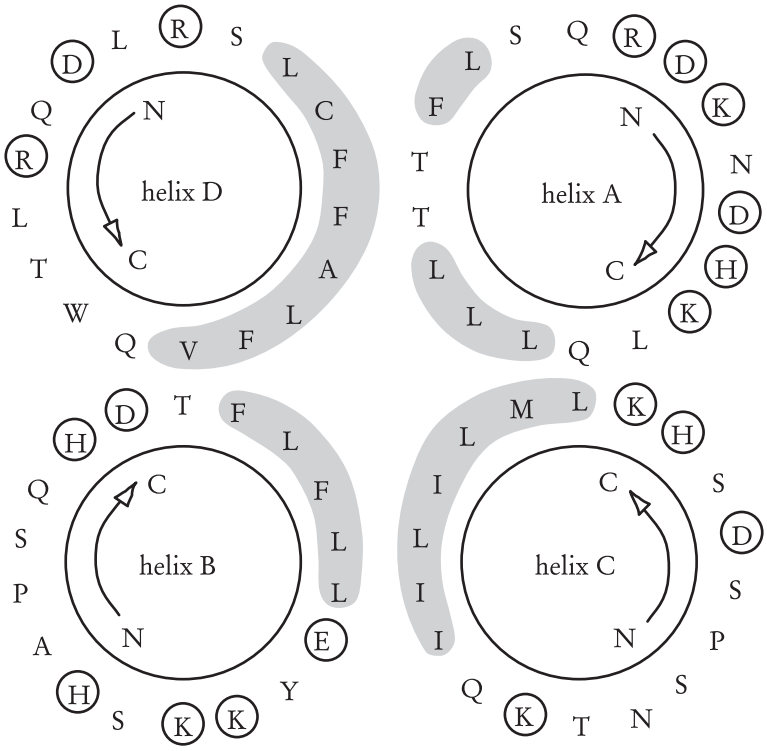


Figure 5.3: Helical wheel projection of each of the four α-helices of carp IL-11 suggests a hydrophobic protein core (hydrophobic faces are shaded) and a hydrophilic, solvent exposed exterior surface that contains many charged residues (circled).

that we designated IL-11b, bears much lower amino acid identity to mammalian IL-11 as well as to bony fish IL-11a (Table 5.4). Expansion of the multiple sequence alignment with the newly discovered bony fish IL-11a and IL-11b proteins also illustrates their low overall amino acid similarity (Fig. 5.2b). As bony fish IL-11a and IL-11b paralogues share relatively low amino acid identity with each other and with mammalian IL-11, only a few amino acid residues are identical throughout the alignment, although identities within the blocks of fish IL-11a, fish IL-11b, and mammalian IL-11 are markedly higher.

Carp IL-11 contains a conserved arginine (corresponding to human R190) in helix D and a tryptophan (corresponding to human W168) in the CD loop that were identified as crucial for receptor binding and signalling, respectively, in mouse[170]. The authors also identified four leucine residues (corresponding to L85, L88, L193, and L194) that

Table 5.4: Percentages of amino acid sequence identity for vertebrate IL-11 sequences

	carp IL-11	zebrafish IL-11a	trout IL-11	green puffer IL-11a	pufferfish IL-11a	zebrafish IL-11b	green puffer IL-11b	pufferfish IL-11b	halibut IL-11	human IL-11	macaque IL-11	mouse IL-11	rat IL-11
carp IL-11	100												
zebrafish IL-11a	76.0	100											
trout IL-11	58.4	53.0	100										
green puffer IL-11a	57.3	54.2	63.0	100									
pufferfish IL-11a	57.8	52.6	56.2	88.9	100								
zebrafish IL-11b	31.5	27.1	29.4	30.5	30.2	100							
green puffer IL-11b	30.0	29.6	28.9	28.5	26.8	29.6	100						
pufferfish IL-11b	27.4	30.6	27.6	29.1	28.9	25.0	65.5	100					
halibut IL-11	28.3	28.9	27.4	30.2	29.6	27.5	54.8	57.8	100				
human IL-11	30.6	28.7	31.6	29.1	30.3	25.6	30.3	25.8	27.6	100			
macaque IL-11	31.1	29.9	30.9	29.8	30.3	26.2	30.7	25.1	26.9	94.5	100		
mouse IL-11	28.5	27.4	27.7	28.0	28.8	27.7	28.8	24.3	24.6	87.9	83.9	100	
rat IL-11	28.5	27.4	27.7	28.0	28.8	27.0	28.8	24.3	31.1	87.9	84.4	97.5	100

are important for the binding of IL-11 to its receptor complex. Three of these four leucines are conserved in carp IL-11 and only the carboxy-terminal leucine in helix D was substituted by a threonine (T). Also in other bony fish IL-11a and IL-11b sequences these amino acids are conserved or conservatively substituted, with the exception of both carboxy-terminal leucines of helix D.

Regardless of their considerable amino acid sequence dissimilarity, the predicted pI of fish IL-11a proteins approached the cationic values of mammalian IL-11, whereas the pI of IL-11b proteins, especially those of both puffer species, were more neutral (Table 5.3). Despite the moderate similarities at the amino acid level and their different pI values, the bony fish IL-11a and IL-11b gene structures are very similar to those of human and mouse IL-11 (Fig. 5.4). All IL-11 genes consist of five exons of identical or similar length and differences in exon size are small and restricted to one or several triplets.

In phylogenetic analyses that include other four-helix bundle cytokine sequences, bony fish IL-11 sequences form two clades that cluster together with mammalian IL-11, supported by a high bootstrap value (Fig. 5.5). Carp IL-11 clusters within the teleost IL-11a clade, which also contains the trout IL-11 sequence. The fish IL-11b clade contains a halibut IL-11 sequence in addition to the IL-11b paralogues of pufferfish, spotted green pufferfish, and zebrafish. This pattern of clustering, separate from IL-6, CNTF, OSM, LIF, and M17, supports the ancestral relationship between mammalian IL-11 and the proteins in both fish IL-11 clades. The fish IL-11a clade is notably more compact compared to IL-11b clade, although both clusters consist of sequences from cyprinid (*Cyprinidae*, zebrafish)

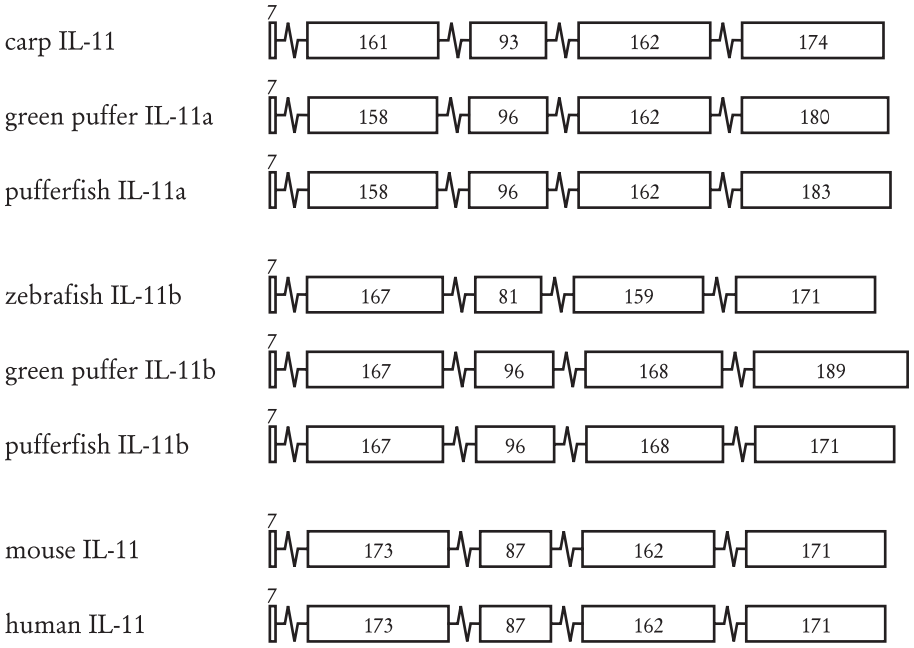


Figure 5.4: The gene structures of bony fish IL-11 sequences closely resembles those of mammalian IL-11 genes. Boxes represent coding exons and are drawn to scale. Numbers indicate exon sizes in nucleotides. The gene structure of carp IL-11 is inferred by the comparison of its CDNA sequence with the zebrafish IL-11a gene. Accession numbers are: carp IL-11, AJ632159; green puffer IL-11a, BN000715/CAAE01014543.1; pufferfish IL-11a, BN000713/CAAB01000705.1; zebrafish IL-11b, BN000718/CAAK01009193.1; green puffer IL-11b, AY374508; pufferfish IL-11b, BN000714/CAAB01000522.1.

and puffer (*Tetraodontidae*, pufferfish and spotted green pufferfish) species. This likely reflects the observation that the amino acid differences in the IL-11b clade are larger than within the IL-11a clade (Table 5.4).

Constitutive expression of carp IL-11

The expression of IL-11 was determined in various organs and tissues of five individual adult carp, relative to the expression of 40S ribosomal protein S11. IL-11 was constitutively expressed in all systemic immune organs of carp (Fig. 5.6a), including thymus, spleen, kidney, and head kidney (the anatomical equivalent of the mammalian adrenal gland, that in fish is a major systemic immune organ). The gills, that constitute a major mucosal surface of the fish, also express IL-11, as does the carp brain. IL-11 expression was not detectable in PBL (peripheral blood leukocytes) of four of the five fish

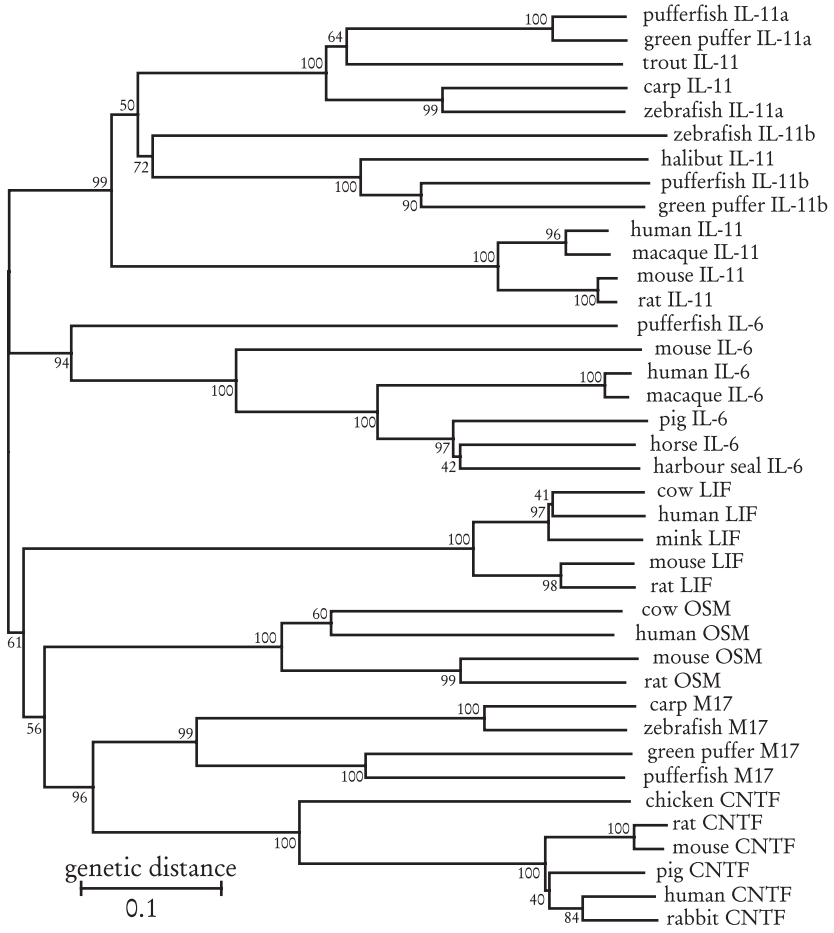


Figure 5.5: Phylogenetic tree of vertebrate IL-11 sequences. Numbers at branch nodes represent the confidence level of 1000 bootstrap replications. The related type I cytokines IL-6, CNTF, OSM and LIF are included as outgroup. human IL-11, P20809; macaque IL-11, P20808; rat IL-11, AAK29623; mouse IL-11, P47873; green puffer IL-11a, BN000715/CAAE01014543.1; pufferfish IL-11a, BN000713/CAAB01000705.1; trout IL-11, AJ535687; zebrafish IL-11a, BN000717/CAAK01001584.1; carp IL-11, AJ632159; zebrafish IL-11b, BN000718/CAAK01009193.1; green puffer IL-11b, AY374508; pufferfish IL-11b, BN000714/CAAB01000522.1; halibut IL-11, AU090873; pufferfish IL-6, AJ544721; human IL-6, P05231; macaque IL-6, P79341; horse IL-6, Q95181; pig IL-6, P26893; harbour seal IL-6, Q28819; mouse IL-6, P08505; pig CNTF, O02732; human CNTF, P26441; rabbit CNTF, P14188; rat CNTF, P20294; mouse CNTF, P51642; chicken CNTF, Q02011; human OSM, P13725; cow OSM, P53346; rat OSM, NP_001006962; mouse OSM, P53347; human LIF, P15018; mink LIF, O62728; cow LIF, Q27956; rat LIF, P17777; mouse LIF, P09056; carp M17, AY102632; pufferfish M17, SINFRUP00000170397;green puffer M17, GSTENP00017261001; zebrafish M17, ENSDARP00000047694.

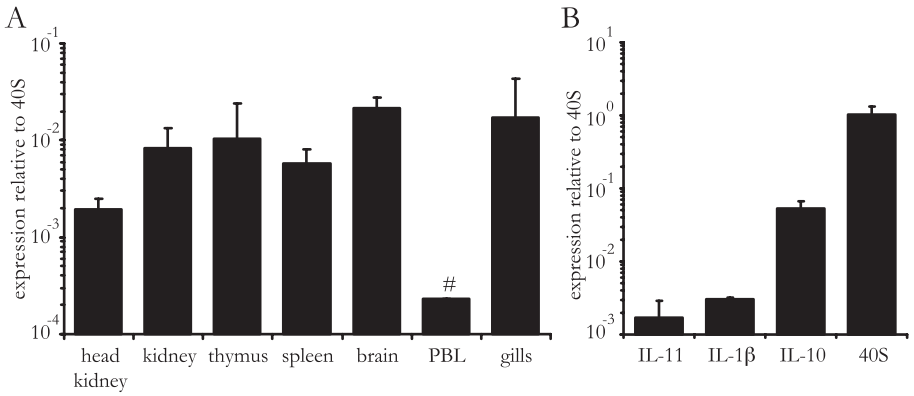


Figure 5.6: Constitutive expression of carp IL-11. Carp IL-11 is constitutively expressed in the organs investigated, with the exception of PBL, where IL-11 was not detectably expressed in four out of five replicates (indicated by #) (a). The constitutive expression of IL-11 in carp head kidney macrophages is lower than that of IL-1β and IL-10 (b). Expression is determined by RQ-PCR and expressed relative to 40S ribosomal protein S11. Gene expression of the different cytokines in panel b is determined at a threshold value of 0.05 for all genes and corrected for primer efficiency. Error bars indicate the standard deviation of five replicate samples. Note the logarithmic scale of the y-axis in both panels.

and the expression in the fifth individual was low compared to that of the other organs and tissues. To compare the constitutive levels of IL-11 expression with those of other cytokine genes, we reverted to carp head kidney macrophage cultures that we routinely use for our in-vitro analysis. Constitutive expression of IL-11 in these cultures is slightly lower than the constitutive expression of IL-1β. Both cytokines are modestly expressed compared to IL-10 that is expressed only one order of magnitude less than 40S ribosomal protein S11 (Fig. 5.6b).

In-vitro regulation of IL-11 expression in carp macrophages

We first tested the inducibility of carp IL-11 expression by in-vitro stimulation of carp head kidney macrophages with ConA and LPS. Following four hours of stimulation, both agents had significantly enhanced the expression of IL-11a, with LPS being a slightly more potent stimulator (Fig. 5.7a, b). We next studied the kinetics of IL-11 upregulation in response to LPS in two separate experiments. IL-11 expression was modestly increased following in-vitro LPS stimulation for two and four hours whereas a period of one hour stimulation was too short to elicit increased IL-11 expression (Fig. 5.7c). Continuous stimulation with LPS for an extended period of twelve or 24 hours resulted in a further increase in the IL-11 expression, although at 24 hours, cells that received no exogenous

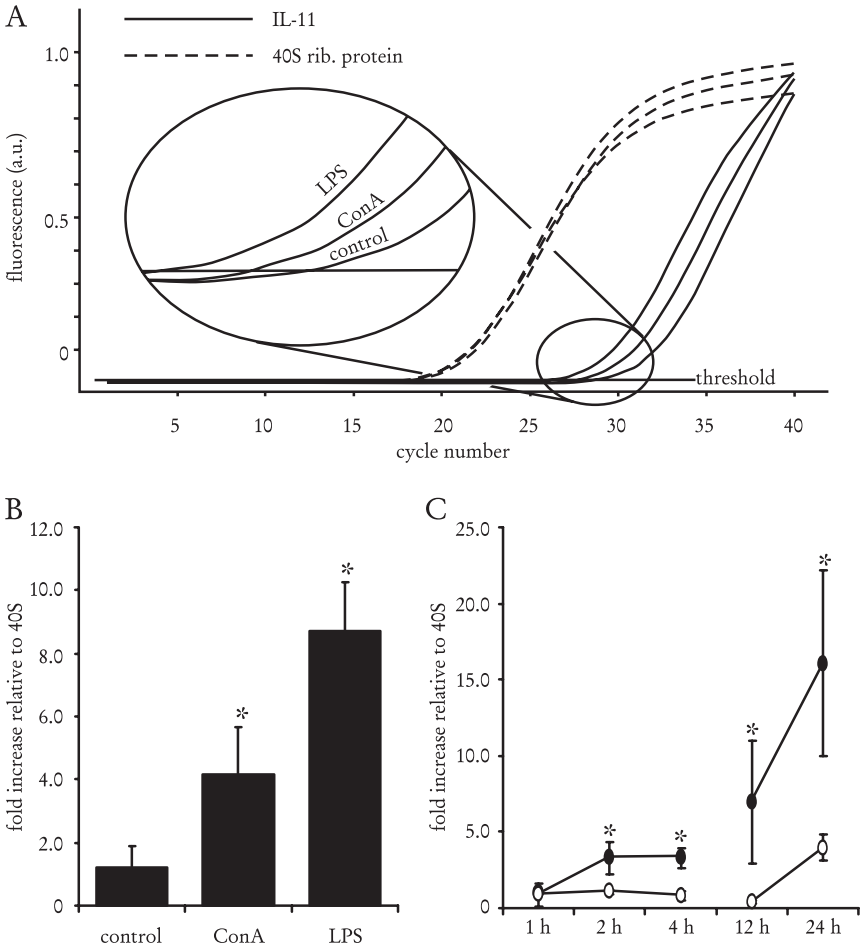


Figure 5.7: IL-11 expression in carp head kidney macrophages following in-vitro stimulation for four h with ConA (20 μg ml^{-1}) or LPS (50 μg ml^{-1}), determined with RQ-PCR. In panel a the amplification curves of IL-11a and 40S ribosomal protein S11 are given for one replicate of each group. Note that the IL-11 curve, but not the 40S curve, of the ConA and LPS stimulated sample crosses the threshold earlier compared to the control sample, indicative of increased expression. Panel b depicts the quantified differences in expression of five replicate samples of each group, corrected for 40S expression and plotted relative to unstimulated controls. The kinetics of IL-11 expression (c) in carp head kidney macrophages following continued in vitro stimulation with LPS (50 μg ml^{-1}) (filled symbols). Results are obtained by RQ-PCR from two consecutive experiments. IL-11 expression in non-stimulated controls cultured for the same amount of time are included (open symbols). Results are corrected for 40S expression and plotted relative to the control group at one h. Asterisks indicate a significant difference ($p<0.05$) with the corresponding control group. Error bars indicate the standard deviation of five replicate samples, a.u., arbitrary units.

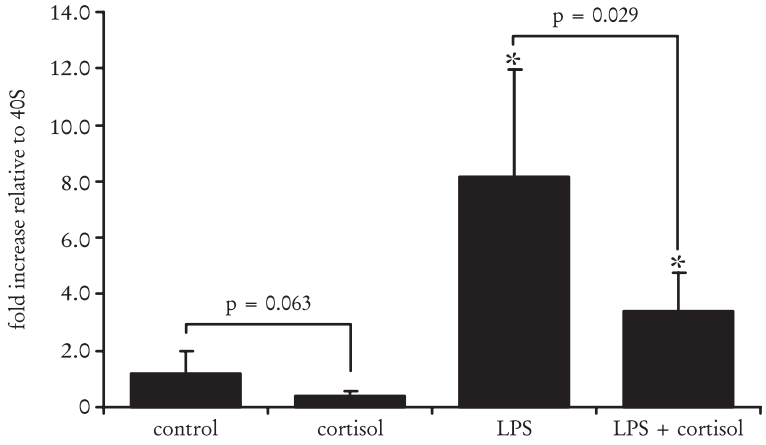


Figure 5.8: IL-11 expression is inhibited by cortisol. IL-11 expression in carp head kidney macrophages following four h in-vitro stimulation with cortisol (400 ng ml^{-1}), LPS (50 µg ml^{-1}), or a combination of LPS and cortisol. Expression is corrected for 40S expression and plotted relative to unstimulated controls. Asterisks indicate a significant difference ($p<0.05$) from the control group. Error bars indicate the standard deviation of four to five replicate samples.

stimulus also start to display elevated levels of IL-11 expression compared to previous timepoints.

We next investigated the inhibitibility of constitutive as well as LPS-induced macrophage IL-11 expression by the glucocorticoid hormone cortisol. We used 400 ng ml^{-1} cortisol, which corresponds to circulating plasma cortisol levels during the peak of the acute stress response[69]. LPS stimulation for a period of four hours reproduced a significantly increased IL-11 expression. Administration of cortisol, either by itself or simultaneously with LPS, resulted in a marked decrease of IL-11 expression compared to control, or LPS stimulated cells respectively (Fig. 5.8).

Discussion

Here we characterise IL-11 of common carp. Several lines of evidence substantiate the orthology of carp IL-11 to known mammalian IL-11 genes and to the recently reported trout IL-11 gene. Protein-protein BLAST of the deduced amino acid sequence encoded by the carp IL-11 gene against the Swissprot database revealed significant hits

with rat, mouse, macaque, and human IL-11 amino acid sequences only. Unlike most type-I cytokines that utilise one or several intrachain disulphide bridges to stabilise their four-helix bundle topology, IL-11 lacks conserved cysteine pairs. Instead, it relies on hydrophobic interactions for stability[133]. And although the amino acid identity between carp IL-11 and mammalian IL-11 proteins is modest, the predicted tertiary structure of carp IL-11 is supportive of a topology of four α-helices that are stabilised by the interplay of hydrophobic internal surfaces and hydrophilic solvent-exposed surfaces. Moreover, the stable clustering of bony fish IL-11 with mammalian IL-11 proteins as well as their highly similar gene organisations confirm the unambiguous orthology of fish and mammalian IL-11 genes.

A systematic search of the genome databases of other bony fish species confirms that an IL-11 gene is present throughout bony fish, and that the similarity between IL-11 proteins of different fish species decreases with increasing phylogenetic distance. Remarkably, our in-silico analyses also suggest the presence of second IL-11 gene in the genomes of zebrafish, pufferfish, and spotted green pufferfish. We designated the fish paralogues IL-11a and IL-11b. The fish IL-11b proteins not only bear low amino acid identity with mammalian IL-11 proteins, but also share limited sequence identity with their fish paralogues. This is illustrated by the recent description of trout IL-11, where the recently deposited green pufferfish IL-11b sequence was inadvertently regarded as the immediate orthologue of trout IL-11[161]. As a consequence it was concluded that the identity between IL-11 proteins of both bony fish species is approximately 30%. In reality, the identity between the actual green pufferfish IL-11a and trout IL-11 is markedly higher at 63%. Nevertheless, the fish IL-11a and IL-11b, like mammalian IL-11, are encoded by genes that consist of five exons. Furthermore, any differences in exon sizes between the various IL-11 genes are small and restricted to one or several triplets. Moreover, many of the other type-I cytokines are encoded by fewer (CNTF, LIF, OSM, M17) or more (IL-12P35) than five exons[162, 164]. IL-6, which like IL-11 has five exons, is distinctly different with regard to the sizes of each of these exons. Moreover, unlike most four-helix bundle cytokines, all vertebrate IL-11 proteins lack conserved cysteine pairs, a property that sets them apart from all other type-I cytokines except CNTF.

Despite the relatively high dissimilarity between fish IL-11a, fish IL-11b and mammalian IL-11 proteins, several residues that are key for the interaction between IL-11 and its receptor complex in mammalian species[170] are conserved in fish IL-11a and IL-11b proteins. Most of the residues that are critical (the arginine (R) residue in helix D) or important for receptor binding are conserved or conservatively substituted. The same is true for the tryptophan (W) in the CD loop that, although dispensible for receptor binding, is essential for receptor activation[170]. This trypthophan is conserved in cyprinid IL-11a and IL-11b proteins and is substituted for a likewise aromatic phenylalanine (F) in the other fish

sequences. Although IL-11 and CNTF are the only type-I cytokine family members that lack intrachain disulphide bridges, a single conserved cysteine residue is present in helix D of all teleostean IL-11a and IL-11b proteins, but not in mammalian IL-11. Although a single cysteine residue is insufficient to form an intrachain disulphide bridge, it may participate in the formation of a disulphide-linked dimer, similar to IL-12, that is composed of a cytokine (P35) covalently linked to a soluble receptor (P40). The position of this cysteine however, central in the helix and facing inwards, seems to preclude its participation in a disulphide bridge.

The stable clustering in phylogenetic analysis, close similarity in gene structure, conservation of key amino acids as well as the absence of conserved cysteine pairs that are characteristic other type-I cytokines, collectively confirm the unambiguous orthology of both fish IL-11 genes with mammalian IL-11. Nevertheless, the relatively low overall amino acid identity that is shared between teleost and mammalian IL-11, indicates that mammalian and fish IL-11 proteins, although structurally orthologous, are not necessarily functionally analogous. Clearly, the duplication of fish IL-11 adds to the complexity of this issue. The presence of duplicate IL-11 genes in representatives of the evolutionarily distantly related families of *Cyprinidae* and *Tetraodontidae*, complemented by their high dissimilarity, pinpoints the origin of both IL-11 paralogues to early in the teleostean lineage. Therefore, the genome duplication that occurred early in teleost fish, following their divergence from tetrapods[93], offers a plausible explanation for the observed duplication. Nevertheless, the estimated gene numbers of teleostean genomes are similar to those of tetrapod species, indicating that many of the duplicated genes have been lost secondary to this genome duplication event[169]. The conservation of duplicate genes in a genome over extended periods of evolution requires that each of the copies adopts a (slightly) different function that is subject to selection[94]. In other words, each copy must distinguish itself either in its spatial or temporal expression, or in the functional characteristics of the protein it encodes. Currently we have expression data of only carp (this paper) and trout[161] IL-11, genes that both cluster within the fish IL-11a clade, yet, based on their extensive protein dissimilarity alone, it is obvious that IL-11a and IL-11b proteins differ profoundly. These differences may become manifest in different receptor affinities, or potentially even different receptor repertoires. Regardless of the precise nature and extent of the differences between both teleost IL-11 genes, apparently there has been an evolutionary advantage to the possession of duplicate IL-11 genes in fish. As IL-11 is a pleiotropic cytokine, it is possible that the many functions it fulfills in mammals have been divided between two genes in bony fish, as has been suggested for other duplicate genes in fish[92, 166].

Obviously, many differences exist in the physiology of fish and terrestrial animals. As most terrestrial animals have developed a keratinous skin to prevent excessive

dehydration, the external surfaces of fish are covered by a mucus-coated epithelium. Moreover, a large part of the total external surface area of fish is made up by the gas exchange epithelial layer of the gills. As mucosal epithelia in general, and the delicate epithelia of the gills in particular are easily damaged by external mechanical, pathogenic, and chemical influences that abound in the fishes' aqueous surroundings[171], a properly balanced and timely responding protective system is an absolute necessity to ensure lasting epithelial integrity. Therefore, the expression of IL-11 in carp gills as well as trout gills and intestine may reminisce the protective effects IL-11 exerts on the mucosal surfaces of the mammalian respiratory and digestive systems. The constitutive expression of IL-11 in most carp organs tested resembles the constitutive expression of trout IL-11, that was detectable in all organs tested, although differences in the level of expression between different organs appear larger in trout[161] than in carp.

In-vitro, carp IL-11 expression is upregulated several-fold in a carp primary culture of head kidney macrophages within two hours following stimulation with LPS, a key component of the cell wall of gram$^-$ bacteria. This expression steadily increases to approximately fifteen-fold after 24 hours of LPS stimulation. This is comparable to the effects of LPS on IL-11 expression in a trout RTS-11 cell line, where LPS-stimulation for the duration of three h, seven h, and 24h all increased IL-11 expression relative to non-stimulated controls[161]. Cortisol, the main glucocorticoid released upon activation of the teleost fish stress axis, is capable of inhibiting both the constitutive as well as LPS-induced IL-11 expression. Similar inhibitory effects of cortisol on fish cytokine expression have been reported for carp IL-1β and TNFα following co-stimulation of cortisol with LPS or a fish tryponosome lysate[172, 173]. Furthermore, the inhibitibility of constitutive as well as LPS-induced IL-11 expression by glucocorticoids in carp macrophages is in line with reports on the regulation of mammalian IL-11 gene expression and protein release in-vitro[157, 174, 175].

In conclusion, the presence of IL-11 in early vertebrates illustrates its importance throughout vertebrate evolution. And although the duplication of IL-11 in teleostean fish is interesting in its own right, it is the sustained presence of both duplicated genes in several, phylogenetically distant fish species that is truly remarkable, as it suggests that each copy has taken on its own distinct role.

Acknowledgements

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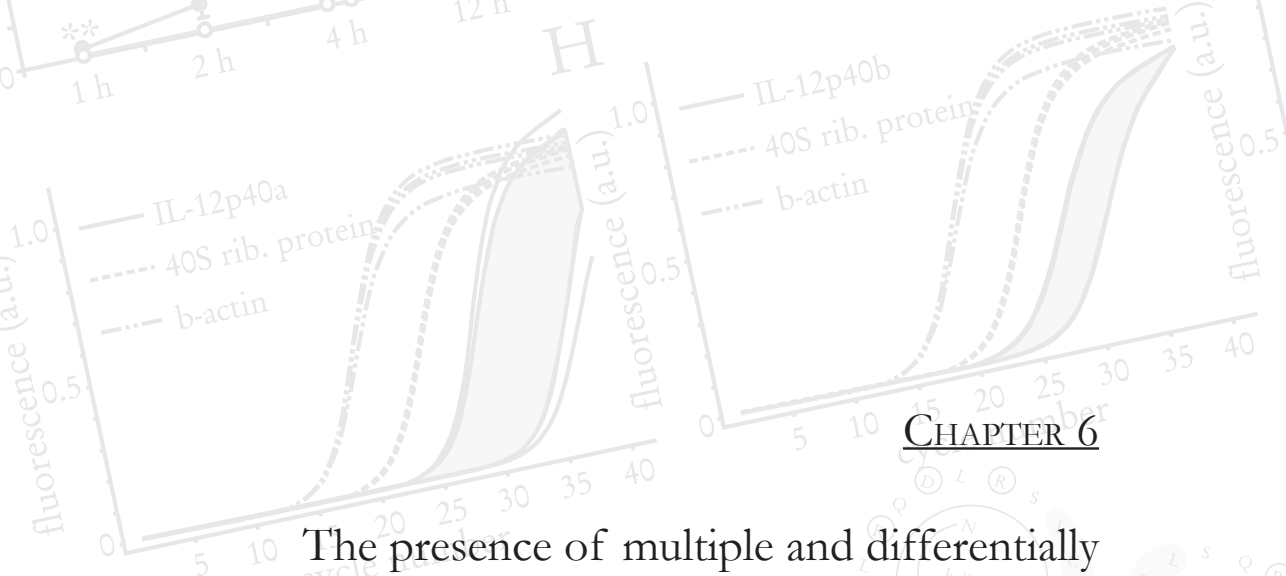

Chapter 6

The presence of multiple and differentially regulated interleukin-12p40 genes in bony fishes signifies an expansion of the vertebrate heterodimeric cytokine family

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Abstract

IL-12 is the founding member of a rapidly growing family of heterodimeric cytokines. It consists of two subunits, designated P35 and P40 that together constitute a disulphide-linked heterodimeric cytokine. IL-12 is well-known for its prominent role in both the early innate immune response and the skewing of the ensuing acquired immune response towards TH1. Here we report the presence of IL-12P35 and three highly distinct IL-12P40 genes in common carp (*Cyprinus carpio*). The carp is a bony fish species genetically similar to the zebrafish, but its substantially larger body size facilitates immunological studies. A comparison of IL-12P35 genes of mammalian and non-mammalian species reveals the presence of a duplicated exon that is unique to the mammalian lineage. The organisation of the three carp IL-12P40 genes is similar to that of higher vertebrates. Phylogenetic analyses that include the P40-related subunits of other composite cytokines confirm the presence of three genuine IL-12P40 genes in carp and indicate that they are evolutionary ancient and possibly not restricted to bony fishes. The orthology of the different carp P40 subunits to mammalian IL-12P40 is further evident from the conservation of key residues involved in the formation of intra- and interchain disulphide bridges and the tight interlocking topology between P35 and P40. The expression of each of the carp IL-12P40 genes differs profoundly, constitutively as well as in response to in-vitro stimulation of carp macrophages. Collectively, the presence of multiple and substantially different IL-12 genes signifies a considerable expansion of the vertebrate heterodimeric cytokine family.

Introduction

IL-12 is a key cytokine in the immune response to intracellular pathogens and parasites[176]. It is produced early in the immune response by monocytes, macrophages, dendritic cells or neutrophils in response to Toll-like receptor signaling induced by various microbial agents, such as LPS (lipopolysaccharide), LTA (lipoteichoic acid), and prokaryote (CpG) DNA[177-179]. IL-12 induces the production of other cytokines, including the TH1 signature cytokine IFNγ (interferon-γ) in NK- and T-cells and it does so either alone or in synergy with IL-18[178, 180]. Thus IL-12 is a major determining factor in the skewing of naïve $CD4^+$ T-cells towards a TH1 phenotype, by its ability to potently induce IFNγ[181] and by its capacity to inhibit transcription of the key TH2 cytokines IL-4, IL-5, and IL-13 in concert with IFNγ[180].

IL-12 is a heterodimer, composed of two disulphide-linked subunits, P35 and P40.

The P35 subunit is a member of the IL-6 family and has a four-helix bundle topology, whereas the P40 subunit resembles a soluble cytokine receptor. Both subunits have to be coexpressed in the same cell in order to yield the functional IL-12P70 heterodimer[182]. IL-12P70 binds to a receptor complex that consists of two subunits (IL-12Rβ1 and IL-12Rβ2) that resemble the common IL-6 family receptor signaling chain GP130[183]. Production of heterodimeric IL-12P70 is accompanied by the release of a large excess of P40, either in a monomeric form or as a disulphide linked homodimer, $(P40)_2$[184]. IL-12P40, and especially the homodimeric form, is a potent antagonist of IL-12P70 bioactivity *in vitro*[185-187]. *In vivo*, pretreatment with $(P40)_2$ lowers serum levels of IFNγ and protects mice from LPS induced shock[188, 189]. Mechanistically, this antagonism is explained by the ability of $(P40)_2$ to bind to the IL-12Rβ1 chain of the IL-12 receptor complex[186, 187]. Whereas $(P40)_2$ indeed inhibits IFNγ release from alloreactive $CD4^+$ T-cells, IFNγ release from alloreactive $CD8^+$ T-cells is enhanced, indicating that $(P40)_2$ under certain conditions augments rather than antagonises the actions of IL-12[190]. Later studies established IL-12P40 as a macrophage chemoattractant[191] and as an initiator of iNOS (inducible NO synthase) expression in microglial cells[192], thereby enhancing the spectrum of immunostimulatory actions of IL-12P40.

IL-12 is the founding member of a larger family of composite cytokines[reviewed by 193, 194, 195]. The P40 subunit of IL-12 participates in the formation of another heterodimeric cytokine, IL-23. This novel cytokine consists of the P35-related P19 subunit, disulphide-linked to P40[196]. Another composite cytokine, IL-27, is formed by the combination of the cytokine-like P28 subunit with the P40-like EBI3 (EBV-induced protein-3) protein[197]. Both IL-23 and IL-27 functionally resemble IL-12 in their involvement in TH1 responses, although their functions are distinct from those of IL-12. IL-27 is produced early by activated APCs (antigen-presenting cells), and synergises with IL-12 in the initiation of TH1 response[197-199]. In contrast, IL-23 induces strong proliferation of memory T-cells, but not of naïve T-cells[196, 200, 201].

Despite the rapid succession of reports on novel heterodimeric cytokine chains in mammalian species, the presence of heterodimeric cytokines in non-mammalian species remains elusive. A single IL-12P35 and IL-12P40 gene have recently been reported in chicken[202] and pufferfish[162]. The latter is a bony fish species that is only distantly related to common carp[68]. Here we describe the presence of three separate and substantially different, yet *bona fide* P40 genes in common carp (*Cyprinus carpio*), in addition to the carp orthologue of IL12P35. The overall amino acid identity between the three different carp P40 genes is low. Moreover, the topology of our phylogenetic analyses substantiates that the presence of three separate and functionally distinct IL-12P40 genes is not specific for cyprinids and may not be restricted to bony fishes.

Materials and methods

Identification of carp p35 and p40 genes

We systematically screened the Ensembl zebrafish genome database with sequences of mammalian p35 and p40 genes, using the BLAST (basic local alignment search tool) algorithm[73]. This initial screen revealed several short hits, which were used as a basis for an homology cloning approach to pick up orthologous partial cDNA sequences from common carp, a species genetically similar to the zebrafish[68]. We obtained partial cDNA sequences from a cDNA library that was constructed from PMA-activated head kidney macrophages. The corresponding full-length cDNA sequences were obtained from head kidney RNA via RACE (rapid amplification of cDNA ends; Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The primers used for RACE PCR are given in Table 6.1. PCR was carried out as previously described[166] and sequences were determined from both strands. This approach yielded one carp IL-12p35 cDNA sequence, and three distinct carp IL-12p40 sequences, denoted as IL-12p40a, -p40b, and -p40c.

Phylogenetic analyses

Multiple sequence alignments were constructed with Clustalw. Phylogenetic trees were constructed on the basis of amino acid differences (p-distance) using the neighbor-joining algorithm (complete deletion) in MEGA (molecular evolutionary genetics analysis) version 2.1[46]. Reliability of the trees was assessed by bootstrapping, using 1000 bootstrap replications. Amino acid sequences of the CNTFR (ciliary neurotrophic factor receptor) were truncated to their predicted soluble form (sCNTFR), corresponding to human CNTFR1-316[203] before inclusion in phylogenetic analyses.

Modeling of carp IL-12

The structure of the human IL-12p70 complex (PDB entry 1f45), which was solved at 2.8 Å resolution[204], was used as a template to build models of carp IL-12p70 complexes. Initial alignments of both the carp p35 and p40 sequences to the template structure were obtained from the PSIPRED fold recognition server[205] using GenTHREADER[206]. The amino acid sidechains in the models were positioned by SCWRL3.0[207]. Subsequently the model was refined in YASARA to improve model accuracy[208]. Coordinate files are available from the authors upon request.

Animals

Common carp (*Cyprinus carpio* L.) were reared at 23°C in recirculating UV-treated water at the 'De Haar Vissen' facility in Wageningen, The Netherlands. Fish were fed dry food pellets (Provimi, Rotterdam, The Netherlands) at a daily ration of 0.7 % of

Table 6.1: Primers used in RACE PCR.

gene	acc. number	primer	sequence 5' ⇒ 3'
IL-12P35	AJ580354	IL-12P35.fw4	TCACAGCAAAACGCAGAGGT
		IL-12P35.fw2 (nested)	GCCTACAGAGAATTCTAGAAGA
		IL-12P35.rv3	ACACGATTTATTGTTATTGTTCG
		IL-12P35.rv4 (nested)	ACTGCACTGTGAGCGCAGTT
IL-12P40a	AJ621425	IL-12P40a.fw5	TGACCATATACTTCAGAAGCAG
		IL-12P40a.fw6 (nested)	CATGGATATTGTGAGGCCTGA
		IL-12P40a.rv5	GGTGCGTTCCAGGATCGTG
		IL-12P40a.rv6 (nested)	TCGTGGATATGTGACCTCTAC
IL-12P40b	AJ628699	IL-12P40b.fw1	TTCCTCATCCGAGACATTGTTAA
		IL-12P40b.fw2 (nested)	CGAGACATTGTTAAGCCAGA
		IL-12P40b.rv3	CACTCAAATACATCGTCTTGGAT
		IL-12P40b.rv4 (nested)	CTTGGATCTTGGTGATGCTAAC
IL-12P40c	AJ628700	IL-12P40c.fw3	TGATTGGCCTGCAGGAAGAC
		IL-12P40c.fw4 (nested)	CAGGAAGACCTAACAGGGAAT
		IL-12P40c.rv4	GTCTTCCTGCAGGCCAATCA
		IL-12P40c.rv3 (nested)	ATTCCCTGTTAGGTCTTCCTG

their estimated body weight. R3xR8 are the 8th generation inbred offspring of a cross between fish of Polish (R3 strain) and Hungarian (R8 strain) origin. The institutional Animal Experiments Committee approved of the experiments in this study.

Cell culture

Adult carp were anesthetised with 0.2 g l^{-1} tricaine methane sulfonate buffered with 0.4 g l^{-1} $NaHCO_3$. Fish were bled through puncture of the caudal vessels using a heparinised syringe. Head kidney cell suspensions were obtained by passing the tissue through a 50 μm nylon mesh with carp RPMI (RPMI 1640, Invitrogen, Carlsbad, CA; adjusted to carp osmolarity (270 mOsm kg^{-1}) and containing 10 IU ml^{-1} heparin (Leo Pharmaceutical Products Ltd, Weesp, The Netherlands) and washed once. This cell suspension was layered on a discontinuous Percoll (Amersham Biosciences, Piscataway, NJ) gradient (1.020, 1.070, and 1.083 g cm^{-3}) and centrifuged for 30 min at 800 g with the brake disengaged. Cells at the 1.070 g cm^{-3} were collected, washed, and seeded at 1-5 x 10^6 cells per well (in a volume of 400 μl) in a 24-well cell culture plate. Following overnight culture at 27°C, 5% CO_2 in carp $RPMI^{++}$ (carp RPMI supplemented with 0.5 % (v/v) pooled carp serum, 1% L-glutamine (Merck, Whitehouse Station, NJ) 200 nM 2-mercaptoethanol (Bio-Rad, Hercules, CA), 1% (v/v) penicillin G (Sigma-Aldrich, St. Louis, MO), and 1% (v/v) streptomycin sulfate (Sigma), cell cultures were stimulated with 50 μg ml^{-1} LPS (from *E. coli*), 20 μg ml^{-1} ConA (from *Canavalia ensiformes*), 0.1 μg ml^{-1} PMA, or 400 ng ml^{-1} cortisol (all Sigma-Aldrich, St. Louis, MO).

RNA isolation and cDNA synthesis

RNA from cell cultures and PBL was isolated with the RNeasy Mini Kit (Qiagen,

Table 6.2: Primers used for gene expression studies. Numbers following each primer indicate their position relative to the translation start site.

gene	acc. number	primer	sequence 5' ⇒ 3'	amplicon size
IL-12P35	AJ580354	qP35.fw1	TGCTTCTCTGTCTCTGTGATGGA (424-446)	86
		qP35.rv1	CACAGCTGCAGTCGTTCTTGA (489-509)	
IL-12P40a	AJ621425	qP40a.fw1	GAGCGCATCAACCTGACCAT (565-584)	151
		qP40a.rv1	AGGATCGTGGATATGTGACCTCTAC (691-715)	
IL-12P40b	AJ628699	qP40b.fw2	TCTTGCACCGCAAGAAACTATG (358-379)	121
		qP40b.rv2	TGCAGTTGATGAGACTAGAGTTTCG (454-478)	
IL-12P40c	AJ628700	qP40c.fw1	TGGTTGATAAGGTTCACCCTTCTC (7-30)	127
		qP40c.rv1	TATCTGTTCTACAGGTCAGGGTAACG (108-133)	
IL-10	AB110780	qIL-10.fw1	GCTGTCACGTCATGAACGAGAT (236-257)	132
		qIL-10.rv1	CCCGCTTGAGATCCTGAAATAT (346-367)	
IL-1β	CCA245635	qIL-1β.fw1	CTGGAGCAATGCAATACAAAGTTC (452-475)	114
		qIL-1β.rv1	CAAGGTAGAGGTTGCTGTTGGAA (543-565)	
40s ribosomal protein S11	AB012087	q40s.fw1	CCGTGGGTGACATCGTTACA (365-384)	69
		q40s.rv1	TCAGGACATTGAACCTCACTGTCT (410-433)	
β-actin	M24113	qACT.fw1	GCTATGTGGCTCTTGACTTCGA (650-671)	85
		qACT.rv1	CCGTCAGGCAGCTCATAGCT (715-734)	

Valencia, CA) following the manufacturer's instructions. Final elution was carried out in 25 µl nuclease free water, to maximise concentration. RNA from whole organs was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). RNA concentrations were measured by spectrophotometer and integrity was ensured by analysis on a 1.5% agarose gel. First strand cDNA synthesis was carried out as previously described[166]. Briefly, total RNA was DNAse treated, followed by random hexamer primed cDNA synthesis (Invitrogen, Carlsbad, CA). A non-reverse transcriptase control was included for each sample.

Expression analysis

Gene expression was assessed by RQ-PCR (real-time quantitative PCR). Five µl cDNA and forward and reverse primers (300 nM each) were added to 12.5 µl Quantitect Sybr Green PCR Master Mix (Qiagen, Valencia, CA) and filled up with demineralised water to a final volume of 25 µl. RQ-PCR (15 min at 95°C, 40 cycles of 15 s at 94°C, 30 s at 60°C, and 30 s at 72°C, followed by a final extension of 1 min at 60°C) was carried out on a Rotorgene 2000 real-time cycler (Corbett Research, Sydney, Australia). Following each run, melt curves were collected by detecting fluorescence from 60 - 90°C at 1°C intervals. Constitutive expression of all genes in organs and tissues of carp was determined at a threshold of 0.05, corrected for primer efficiency and plotted as a ratio of target gene vs. reference gene[166]. Expression following in-vitro stimulation was determined at optimal threshold values determined for each primer set (always below 0.10), corrected for primer efficiency and reference gene expression and plotted relative to non-stimulated control cells[72, 166]. Efficiency and threshold values for each primer set were: IL-12P35 2.04, 0.0990;

IL-12P40a 2.11, 0.0970; IL-12P40b 1.79, 0.0106; IL-12P40c 1.99, 0.0134; IL-10 2.02, 0.0439; IL-1β 1.94, 0.0048; 40S 2.11, 0.0077; β-actin 2.05, 0.0513. Primers used are displayed in Table 6.2. Dual internal reference genes (40S ribosomal protein S11 and β-actin) were incorporated in all experiments and results were confirmed to be similar following standardisation to either gene. Non-reverse transcriptase controls were consistently negative.

Statistics

Statistical analyses were carried out with SPSS software (version 12.0.1). Differences were evaluated with a Student's T-test. Homogeneity of variances was tested with Levene's test and we corrected the Student's T-test for unequal variances when necessary.

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                    ***
acgaggttgaacgcaacaatgctgtccagggtttgtaataacttggtcgtcagcattgcg
                     M  L  S  R  V  C  N  N  L  V  V  S  I  A
tgcttagctttgctgtggagatgtagtatcggcggtccggtcggagcacgtgaatcacca
 C  L  A  L  L  W  R  C  S  I  G  G  P  V  G  A  R  E  S  P
ttgaacctcactggaaagtgcaacgagcttgcgcgctcactgctctggaacgtgtcagcg
 L  N  L  T  G  K  C  N  E  L  A  R  S  L  L  W  N  V  S  A
gtgctcgagatggaccacctgttcagcgggtttgactgctcacagcaaaacgcagaggtg
 V  L  E  M  D  H  L  F  S  G  F  D  C  S  Q  Q  N  A  E  V
catctcaggaggcaaacagtgtctgcttgcacaccacagaactctaactgcgctcacagt
 H  L  R  R  Q  T  V  S  A  C  T  P  Q  N  S  N  C  A  H  S
gcagttctaaatattgatgagaatgaatgcctacagagaattctagaagatctccactac
 A  V  L  N  I  D  E  N  E  C  L  Q  R  I  L  E  D  L  H  Y
tatcgggagacattgagagcttactctaacccagagctcaccaatagtgtagtttggagc
 Y  R  E  T  L  R  A  Y  S  N  P  E  L  T  N  S  V  V  W  S
attgatgacctcctgcagaactgcttctctgtctctgtgatggacatctctccagccaag
 I  D  D  L  L  Q  N  C  F  S  V  S  V  M  D  I  S  P  A  K
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 V  S  V  D  H  Q  K  S  F  Q  E  R  L  Q  L  C  K  V  L  K
ggttttaaccttcgaacaataacaataaatcgtgttttcaactacattttgtcaaaatag
 G  F  N  L  R  T  I  T  I  N  R  V  F  N  Y  I  L  S  K  -
caaactagcaattgctacaggccaaacacctgttagtttactgttacaaaaagcaatgat
tatttattagttaattattgttgtattttaacattatttatttatgcagtaatgtatgc
tattttatttttttgtgaacttctgatttgtttcttgttgttttattaaaaggtggccatg
cacttcagcaaaaaaaaaaaaaaaa
```

Figure 6.1: Nucleotide and deduced amino acid sequence of carp IL-12P35. Asterisks indicate the start codon. Potential instability motifs are indicated in bold. The polyadenylation signal is underlined. Accession number for carp IL-12P35 is AJ580354.

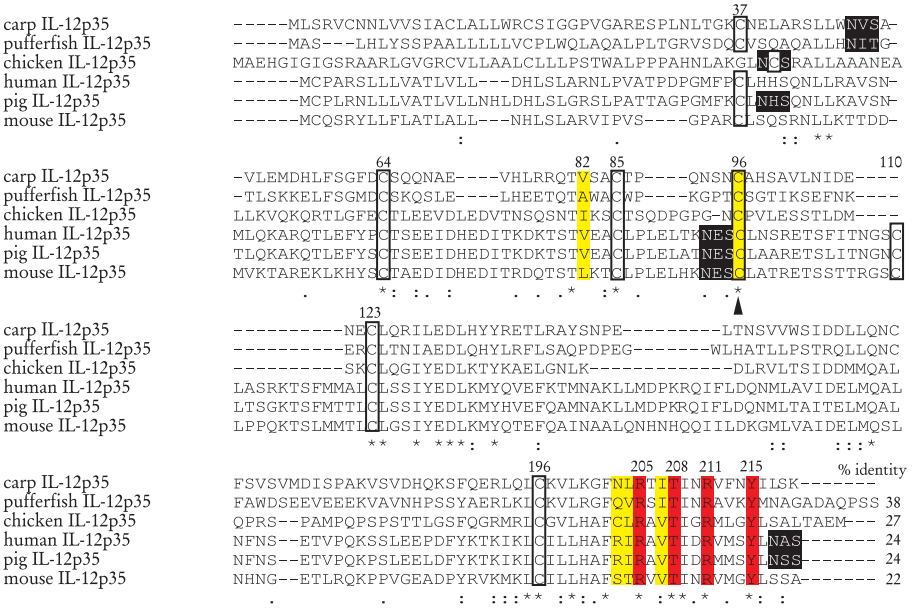


Figure 6.2: Multiple amino acid sequence alignment of vertebrate IL-12P35. Asterisks indicate amino acids that are conserved in all sequences. Colons and dots indicate decreasing degrees of conservative substitutions. Conserved cyteine residues are boxed. The arrowhead indicates the cysteine residue that forms an interchain disulphide bridge with IL-12P40. Residues that are critical or important for IL-12P70 formation are indicated in red and yellow, respectively. Conserved N-glycosylation motifs NXT/S (as determined by their presence in at least two aa sequences) are boxed in black. The precentage aa identity of carp IL-12P35 with each of the other IL-12P35 proteins is indicated at the end of the alignment.

Results

Characteristics of carp IL-12P35

The full-length carp IL-12P35 cDNA sequence (806 bp) displays a consensus polyadenylation signal and five potential mRNA instability motifs (auuua) in its 3'untranslated region (Fig. 6.1). The cDNA encodes a 193 aa protein with several potential N-glycosylation sites that displays intermediate amino acid identity (38 %) to the P35 sequence of the phylogenetically distant pufferfish, and shares lower amino acid identity (22 – 27 %) with other, non-piscine, IL-12P35 sequences (Fig. 6.2). Despite these intermediate to low amino acid identities, most of the key residues involved in intrachain disulphide binding (corresponding to human C64-C196, and C85-C123) and heterodimerisation with P40 (corresponding to human C96, R205, T208, and R211)[204] are

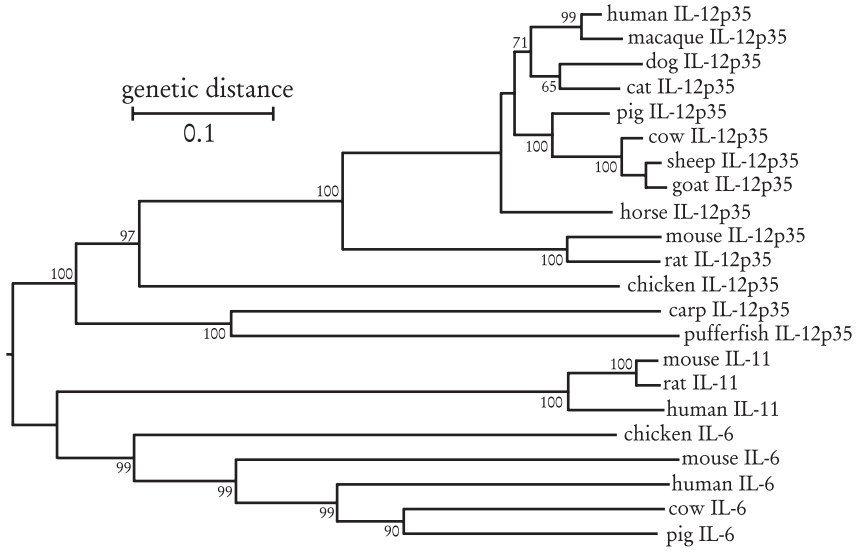


Figure 6.3: Phylogenetic tree of full-length vertebrate IL-12P35 amino acid sequences. Numbers at branch nodes represent the confidence level of 1000 bootstrap replications. IL-6 and IL-11 are included as outgroup. Accession numbers are as follows: human IL-12P35, P29459; macaque IL-12P35, P48091; dog IL-12P35, Q28267; cat IL12-P35, O02743; pig IL-12P35, Q29053; cow IL-12P35, P54349; sheep IL-12P35, Q9TU27; goat IL-12P35, O02814; horse IL-12P35, Q9XSQ6; mouse IL-12P35, P43431; rat IL-12P35, AAD51364; chicken IL-12P35, AY262751; carp IL-12P35, AJ580354; pufferfish IL-12P35, AB096265; mouse IL-11, P47873; rat IL-11, AAK29623; human IL-11, P20809; chicken IL-6, CAC40812; mouse IL-6, P08505; human IL-6, P05231; cow IL-6, P26892; pig IL-6, P26893.

conserved (Fig. 6.2). The only key residue that is absent upon inspection of the alignment is the cysteine residue (corresponding to human C110) that has been suggested to form a third intrachain disulphide bridge together with human C37. Instead, a gap is introduced in both carp and chicken P35 in this region of our alignment (see below). Nonetheless, in phylogenetic analysis carp IL-12P35 clusters with other vertebrate IL-12P35 amino acid sequences, supported by high bootstrap values that confirm the orthology of carp IL-12P35 with P35 of mammalian species (Fig. 6.3).

Recent exon duplication in the IL-12P35 gene in the mammalian lineage

Inspection of the genomic organization of non-mammalian IL-12P35 genes reveals that mammalian P35 genes possess one extra exon compared to pufferfish P35 (and two compared to chicken IL-12P35 which lacks intron one-two) (Fig. 6.4a). More precisely, mammalian P35 genes possess two exons (exon four and five) of 42 bp that contain a cysteine-encoding triplet that starts exactly nine bp (*i.e.* three aa) following the initiation

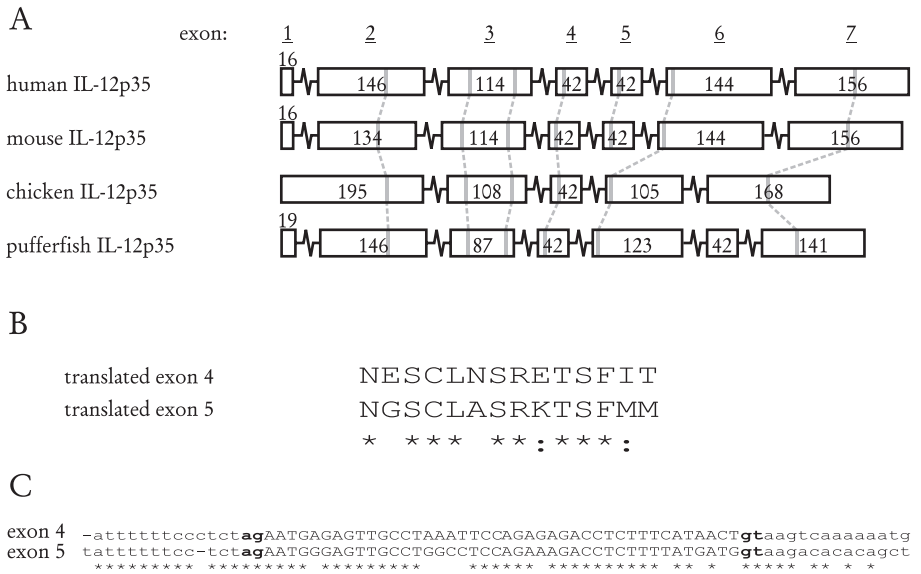


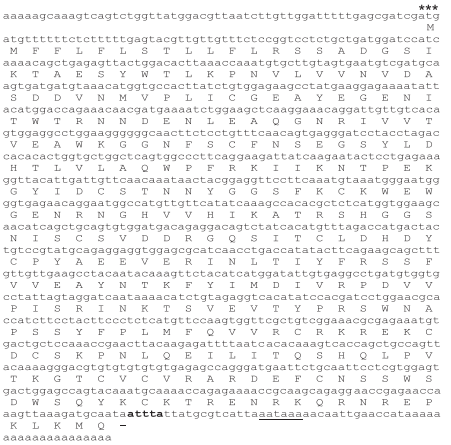
Figure 6.4: The gene structure of non-mammalian IL-12p35 reveals a recent exon duplication in the p35 gene of the mammalian lineage. (a) Gene structure of IL-12p35 of human, mouse, chicken and pufferfish IL-12p35. Boxes represent coding exons and are drawn to scale, conserved cysteine residues are shaded. Numbers indicate exon sizes in nucleotides. Note that mammalian exon four and five are identical in length and both encode a cysteine residue at the exact same position (b). This exon is present only once in the IL-12p35 genes of chicken and pufferfish. A nucleotide alignment of exons four and five of human IL-12p35 (c) reveals that their high similarity extends to the non-coding sequences of the flanking introns. Coding nucleotides are presented as capitals. Donor and acceptor splice sites (gt…ag) are indicated in bold. Asterisks indicate identical residues.

of the exon (Fig. 6.4b). This exon is present only once in the p35 genes of chicken and pufferfish, which suggests a recent exon duplication in the mammalian lineage, following its divergence from birds. The high similarity between human exon four and five, which extends to their flanking intron sequences, is consistent with such an event (Fig 6.4c). The presence of this duplicated exon in mammalian p35 genes is responsible for the gap in the multiple sequence alignment of IL-12p35 (Fig. 6.2).

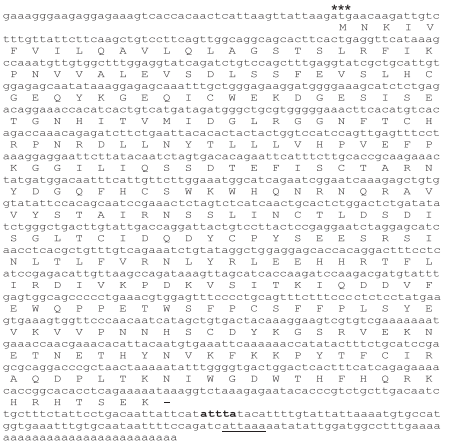
Three distinct IL-12p40 genes in bony fishes

Our initial screen for zebrafish orthologues of IL-12 yielded several short fragments that resembled the mammalian p40 subunit. Their corresponding full-length cDNA sequences were obtained in common carp, which resulted in the identification of three distinct carp p40 genes that were designated p40a, p40b, and p40c. The 3'untranslated

A



B



C

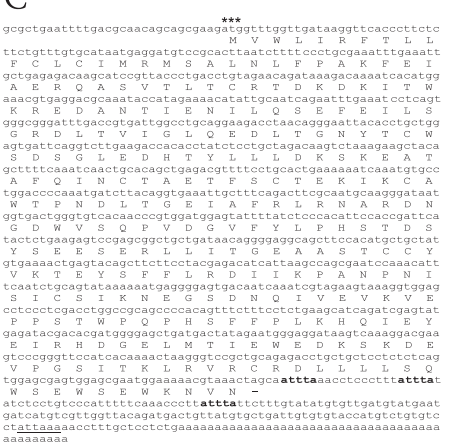


Figure 6.5: Nucleotide and deduced amino acid sequence of three distinct carp IL-12P40 genes, IL-12P40a (a), IL-12P40b (b), and IL-12P40c (c). The start codon is indicated by asterisks. Potential instability motifs are indicated in bold. The polyadenylation signal is underlined. Accession numbers are: carp IL-12P40a, AJ621425; carp IL-12P40b, AJ628699; carp IL-12P40c, AJ628700.

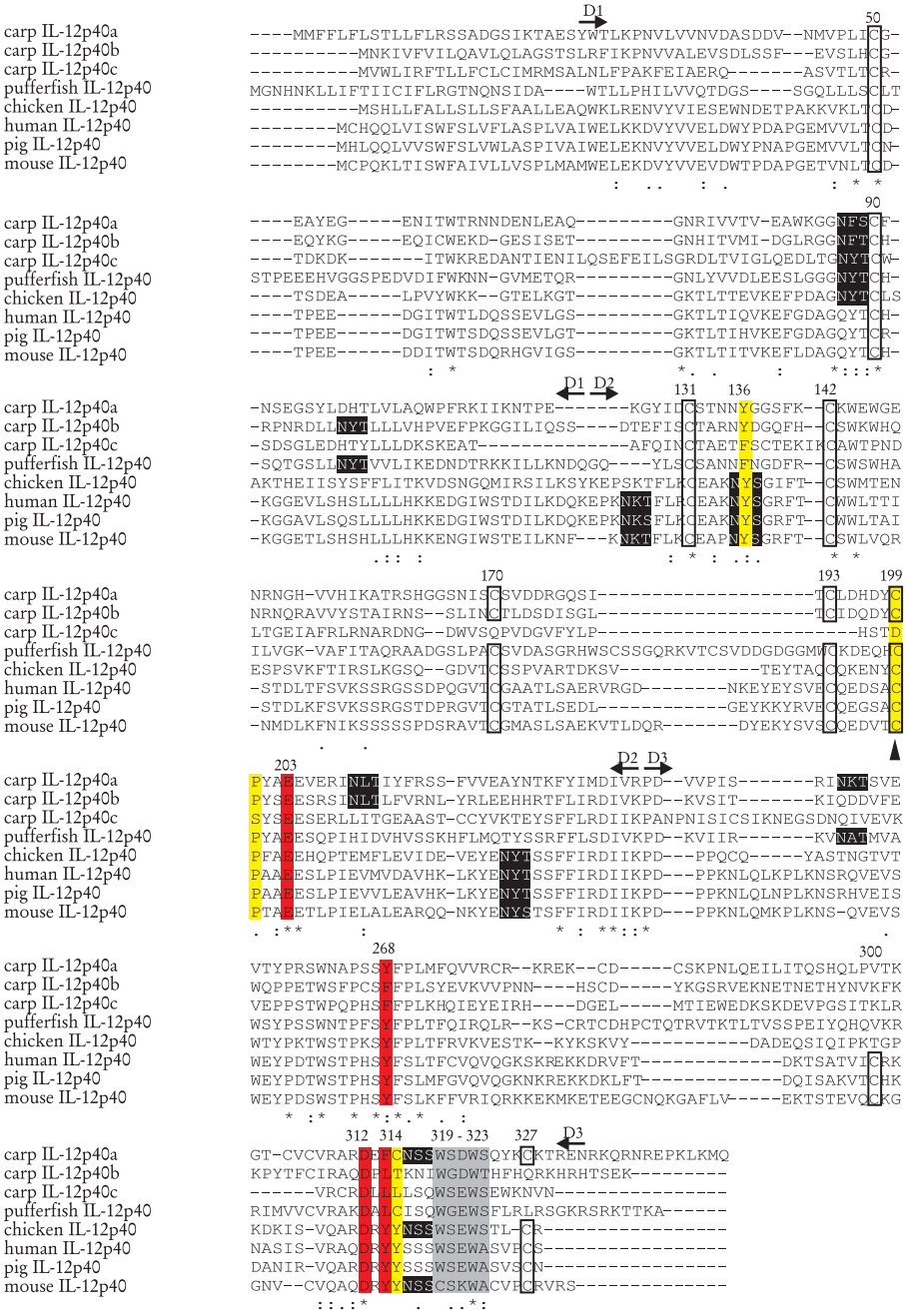
region of each of these full-length sequences included a consensus polyadenylation signal, as well as one or several potential MRNA instability motifs (Fig. 6.5). The proteins encoded by each of these three CDNA sequences were similar in length (326, 312, and 301 aa, respectively) but were only 32.4 – 23.9% identical (Table 6.3). The amino acid similarities between the three carp IL-12P40 proteins is higher and ranges from 71.5 – 50.3%. The amino acid identities of each of the carp IL-12P40 proteins to mammalian P40 amino

Table 6.3: Amino acid identity between vertebrate IL-12P40 sequences

	carp P40a	carp P40b	carp P40c	pufferfish	chicken	human	pig	mouse
carp P40a	100							
carp P40b	32.4	100						
carp P40c	25.7	23.9	100					
pufferfish	33.6	30.0	25.1	100				
chicken	29.4	26.1	25.9	28.8	100			
human	29.0	28.1	26.9	27.8	45.4	100		
pig	29.6	27.3	26.2	27.9	45.3	82.6	100	
mouse	30.6	25.2	27.2	28.5	41.6	66.7	62.8	100

acid sequences are comparable to those of carp and mammalian IL-12P35, with IL-12P40a consistently displaying slightly higher identity to mammalian P40 proteins than -P40b, and -P40c. Inspection of a multiple amino acid sequence alignment with pufferfish, chicken, and mammalian P40 sequences, confirms that carp IL-12P40a is most similar to mammalian IL-12P40 (Fig. 6.6). Each of the three carp P40 proteins contains one or several conserved N-glycosylation motifs (NXT/S). The human P40 protein consists of three domains (D1 – D3) that are stabilised by one (D1 and D3) or two (D2) intrachain disulphide bridges[204]. The cysteine residues that form the disulphide bridges of the D1 (corresponding to human C50-C90) and D2 (human C131-C142, C170-C193) domains are conserved in presence and spacing in all three carp P40 proteins, with the exception of P40c that lacks the second cysteine pair of the D2 domain. However, the cysteine pair that forms a disulphide bridge within the D3 domain of human P40 is absent from all fish P40 proteins that are identified to date. Carp P40a does possess a single cysteine that aligns with the C-terminal cysteine residue (human C327) of mammalian P40s. The cysteine that engages in an interchain disulphide bridge that stabilises the IL-12P70 heterodimer (human C199) is conserved in all P40 sequences except carp IL-12P40c. All three carp P40 proteins contain the C-terminal WSXWS consensus motif that is characteristic for type-I cytokine receptors[209].

Figure 6.6: Multiple amino acid sequence alignment of the three novel carp IL-12P40 sequences with the single IL-12P40 sequence from pufferfish, chicken, and mammalian species. Asterisks indicate amino acids that are conserved in all sequences. Colons and dots indicate decreasing degrees of conservative substitutions. Conserved cysteine residues are boxed. The arrowhead indicates the cysteine residue that forms an interchain disulphide bridge with IL-12P35. Residues that are critical or important for IL-12P70 formation are indicated in red and yellow, respectively. Conserved N-glycosylation motifs NXT/S (as determined by their presence in at least two aa sequences) are boxed in black. The consensus WSXWS motif is shaded. Arrows above the alignment mark the borders of each of the three domains (D1 - D3) of human IL-12P40.


D1
50
carp IL-12p40a
carp IL-12p40b
carp IL-12p40c
pufferfish IL-12p40
chicken IL-12p40
human IL-12p40
pig IL-12p40
mouse IL-12p40
90
D1 D2
131
136
142
170
193
199
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D2 D3
268
300
312 314
319 - 323
327
D3

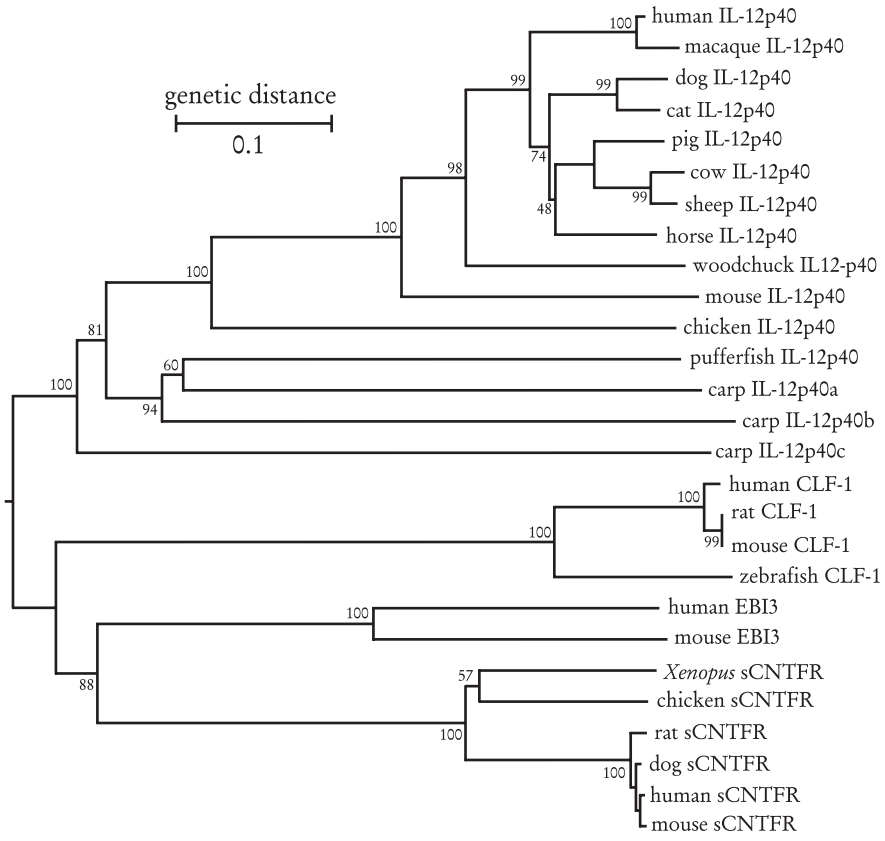

Figure 6.7: Phylogenetic analysis of receptor-like subunits of heterodimeric cytokines confirms the presence of three bona fide IL-12p40 genes in carp. Full-length amino acid sequences were used with the exception of sCNTFR sequences that were truncated to their predicted soluble form (corresponding to human CNTFR1-316). Numbers at branch nodes represent the confidence level of 1000 bootstrap replications. CLF-1 (cytokine-like factor-1), EBI3 (EBV-induced protein-3), and sCNTFR (soluble ciliary neurotrophic factor receptor) are included as outgroup. Accession numbers are as follows: human IL-12p40, P29460; macaque IL-12p40, P48095; dog IL-12p40, Q28268; cat IL-12p40, O02744; pig IL-12p40, Q28938; cow IL-12p40, P46282; sheep IL-12p40, O02815; horse IL-12p40, Q9XSQ5; woodchuck IL12-p40, Q61729; mouse IL-12p40, P43432; chicken IL-12p40, AY262752; pufferfish IL-12p40, AB096266; carp IL-12p40a, AJ621425; carp IL-12p40b, AJ628699; carpIL-12p40c, AJ628700; human CLF-1, O75462; rat CLF-1, XP_214312; mouse CLF-1, Q9JM58; zebrafish CLF-1, NM_001002650; human EBI3, Q14213; mouse EBI3, O35228; *Xenopus* sCNTFR, AAH43961; chicken sCNTFR, P51641; rat sCNTFR, Q08406; dog sCNTFR, AAQ09101; human sCNTFR, P26992; mouse sCNTFR, AF068615.

The presence of multiple IL-12p40 genes might not be exclusive for bony fishes

In phylogenetic analyses that include the receptor-like subunits of other heterodimeric cytokines, all vertebrate p40 sequences including the carp p40 proteins, cluster together (Fig. 6.7). The high bootstrap values that support the clustering of vertebrate p40 sequences, separate from CLF-1 (cytokine-like factor-1), EBI3, and SCNTFR, corroborate the *bona fide* identity of the carp genes as IL-12p40. Nonetheless, the branch lengths that separate the three carp p40 sequences are long, reminiscent of their relatively low overall amino acid identity. Furthermore, carp p40a clusters with the pufferfish IL-12p40 sequence (although supported by an intermediate bootstrap value of 60), before clustering with the carp p40b sequence. As carp and pufferfish are two of the most distantly related bony fish species[210], this pattern of clustering would suggest that p40a and p40b arose very early in the bony fish evolution, before the separation of the carp and pufferfish lineages. The topology of the carp p40c branch indicates that this sequence arose even earlier in evolution. As a consequence the presence of multiple IL-12p40 genes is likely not restricted to cyprinid fishes, and might extend to higher vertebrate phyla.

Protein modeling of carp IL-12p70

We investigated the possibility of heterodimerisation of carp p35 with each of its three p40 subunits. To that end we constructed protein models of carp IL-12 heterodimers of p35 with p40a, p40b, and p40c, based on the human IL-12p70 crystal structure[204]. Based on these models, each of the three carp p40 subunits was capable of heterodimer formation with carp p35 (Fig. 6.8, only heterodimer of p35 with p40a is shown). The p35-p40 interface is characterised by a unique interlocking topology in which charged residues of the p35 and the p40 subunits interact to form a stable heterodimer, described in detail by Yoon and colleagues[204]. A positively charged arginine residue of p35 (corresponding to human R211) interacts with a negatively charged aspartic acid of p40 (human D312) that is situated at the bottom of an otherwise hydrophobic pocket formed by several aromatic residues (human Y136, Y268, Y314, and Y315). Additional charged interactions, most notably between a second arginine in p35 (human R205) and a glutamic acid in p40 (human E203) strengthen the interaction between p35 and p40, which is further stabilised by an interchain disulphide bond. Based on mutagenesis studies, Yoon and colleagues[204] identified several residues in the p35-p40 interface that are critical or important for heterodimer formation. A detailed inspection of the carp IL-12p70 models revealed that both critical arginines of p35 (corresponding to human R205 and R211) as well as the critical aspartic acid and glutamic acid of p40 (human D312 and E203) are conserved in all four carp IL-12 subunits (Fig. 6.8). Moreover, the hydrophobic residues that constitute the pocket of p40 are identical or conservatively substituted by residues with similar biochemical properties (e.g. Y136F in p40c). Based on our models, it is plausible that all three carp p40 subunits have retained the capability of heterodimerisation with p35.

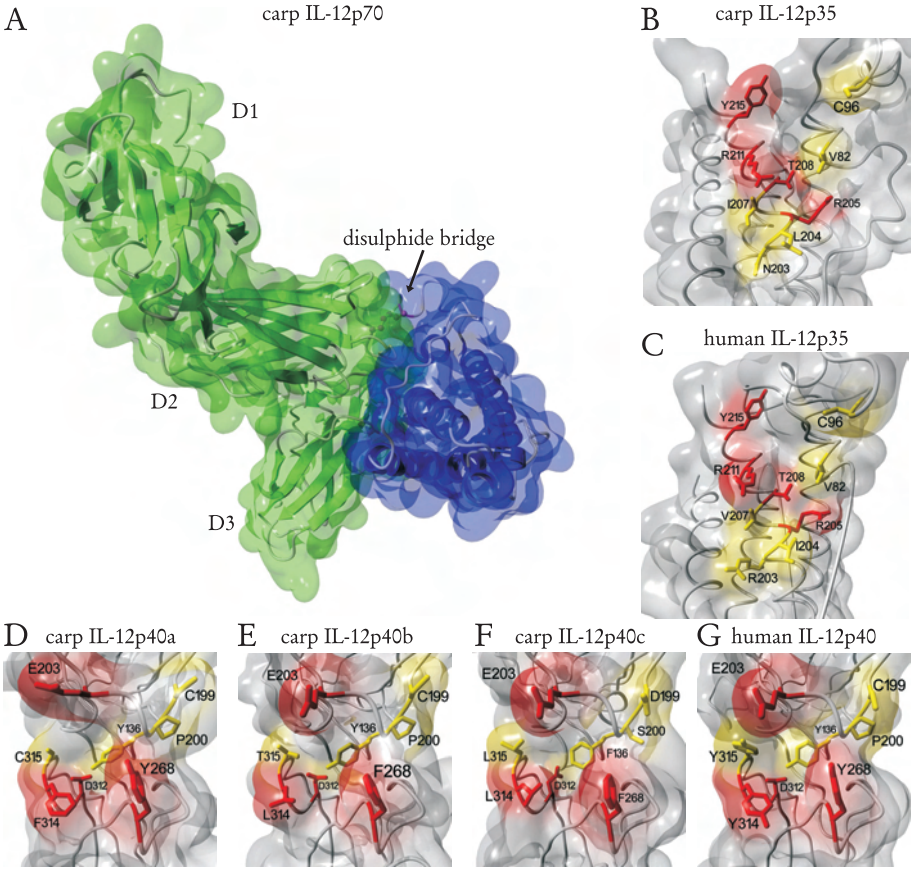

Figure 6.8: Protein modeling of carp IL-12 heterodimers. The heterodimer of carp P35 and P40a modeled over the human IL-12P70 crystal structure (PDB accession number 1F45) illustrates the heterodimeric cytokine conformation adopted by carp P35 (blue) and carp P40a (green), stabilised by an interchain disulhpide bridge (magenta) (a). The P35 binding interface of carp P35 (b) contains the residues that are critical (red) or important (yellow) for IL-12P70 formation in human (c), as described by [204]. Also both critical (red), negatively charged residues of human P40 (E203 and D312) and most of the critical (red) and important (yellow) residues that participate in the formation of the hydrophobic pocket of P40 are identical or conservatively substituted in carp P40a (d), P40b (e), and P40c (f). Note that carp P40c lacks the important (yellow) cysteine residue that forms the stabilising interchain disulphide bridge with P35 (human C199).

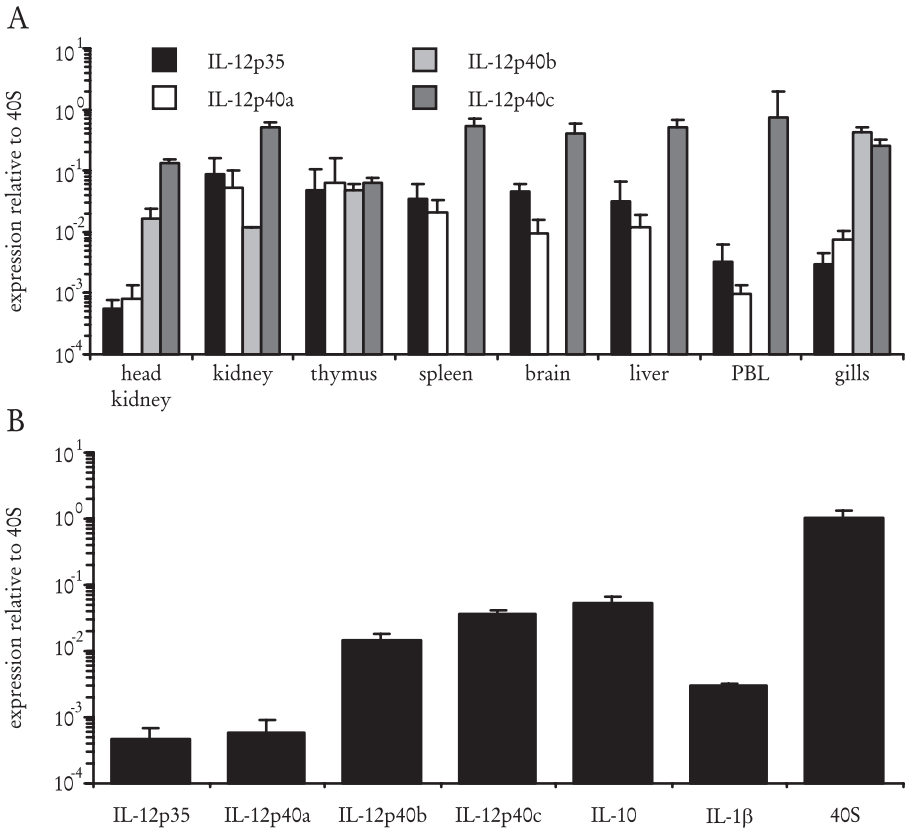

Figure 6.9: Constitutive expression of carp IL-12P35, IL-12P40a, IL-12P40b, and IL-12P40c in various carp organs (a) and in carp head kidney macrophages (b). Expression is determined by RQ-PCR and expressed relative to 40S ribosomal protein S11. Expression is determined at a threshold value of 0.05 for all genes and corrected for primer efficiency. Error bars indicate the standard deviation of five replicate samples. The experiment in panel (b) is representative of two experiments performed. Note the logarithmic scale of the y-axes.

Differences in the constitutive expression of carp IL-12 genes

The expression of IL-12 genes was assessed in various organs of five individual carp, relative to the expression of 40S ribosomal protein S11. These organs included the head kidney (the anatomical equivalent of the mammalian adrenal gland, which in fish is a major systemic immune organ), the kidney (which is also a systemic immune organ), thymus, and spleen, as well as liver, brain, PBL, and gills (that in fish constitute a major

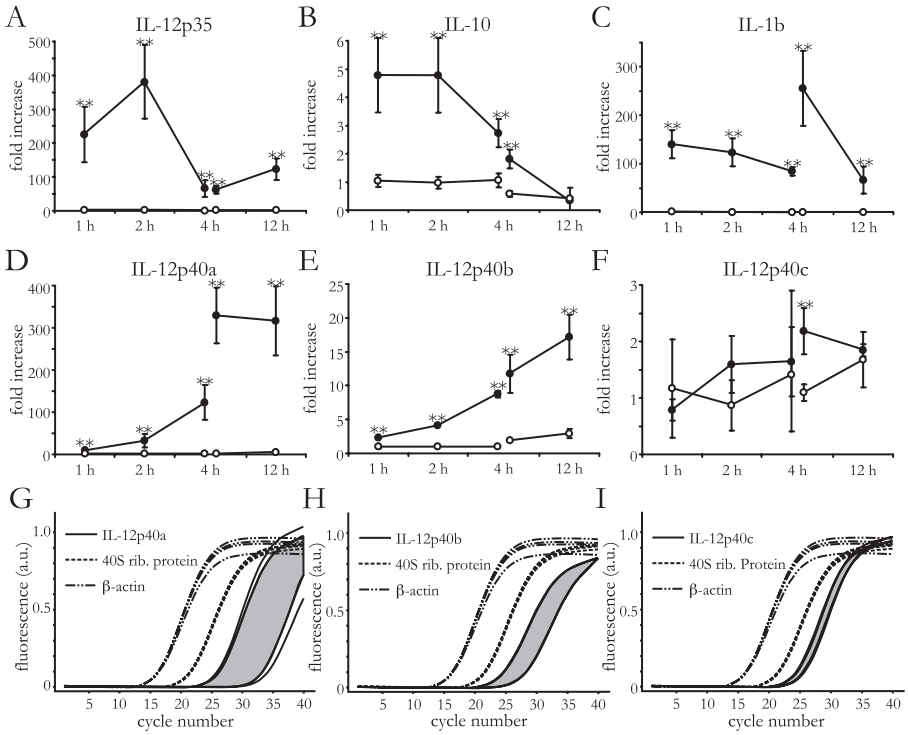

Figure 6.10: Kinetics of cytokine expression in carp head kidney macrophages following stimulation with LPS (50 μg ml-1) (filled symbols). Kinetics of non-stimulated controls are included (open symbols). Results are obtained by RQ-PCR from two consecutive experiments and are presented relative to the control group at one h. Asterisks indicate a statistically significant difference ($p<0.01$) from the corresponding control group. Error bars indicate the standard deviation of five replicate samples. This experiment is representative of two experiments performed. Note the different scales of the y-axes. Representative RQ-PCR graphs that display the expression of 40S ribosomal protein S11, β-actin, and IL-12P40a (g), IL-12P40b (h), or IL-12P40c (i) in two LPS-stimulated and two control samples illustrate the marked differences in inducibility and constitutive expression between the different P40 genes.

mucosal surface area). In most organs, expression of all four IL-12 genes was detectable, although obvious differences existed. The expression of IL-12P40a closely paralleled that of IL-12P35 in all organs, but ranged from barely detectable in the head kidney to about two orders of magnitude higher in the kidney (Fig. 6.9a). In contrast, the expression of IL-12P40c was invariably high in all organs tested. The expression of IL-12P40b was intermediate to that of P40a and P40c in the head kidney, equal to P40a and P40c in the thymus, and most abundant in the gills. In spleen, brain, PBL, and liver, P40b expression

could not be detected. The constitutive expression pattern of IL-12 genes in isolated head kidney macrophages matches that of the in-toto organ, which establishes these primary macrophage cultures as a suitable in-vitro system to study the regulation of gene expression (Fig. 6.9b). Expression of IL-12P35 and IL-12P40a is hardly detectable, whereas P40c is abundantly expressed and P40b expression is intermediate to the former two. Interestingly, the anti-inflammatory cytokine IL-10 is abundantly and constitutively expressed in non-stimulated head kidney macrophages, as well as in the complete head kidney and other organs (not shown). Constitutive expression of the pro-inflammatory cytokine IL-1β is low, but nonetheless an order of magnitude higher than the expression of IL-12P35 and IL-12P40a.

Carp IL-12P40 genes differ profoundly in their in-vitro inducibility

We tested the inducibility and kinetics of carp IL-12 gene expression by in-vitro stimulation of head kidney macrophages with LPS. For reasons not entirely understood, fish macrophages are relatively insensitive to *E. coli* LPS, therefore we used a concentration of 50 μg ml^{-1} LPS, as similar concentrations are commonly used for in-vitro stimulation of fish leukocytes[211, 212]. The amplitude of the response of both the IL-12P35 and IL-12P40a genes was similarly large following in-vitro LPS stimulation, although their kinetics differed. IL-12P35 expression peaked within the first two hours following in-vitro stimulation, but remained elevated roughly 100-fold until the end of the experiment (Fig. 6.10a), whereas IL-12P40a expression gradually increased to over 300-fold during the course of a twelve h stimulation (Fig. 6.10d). The expression of IL-12P40b was similar in kinetics, but was maximally upregulated only about fifteen-fold (Fig. 6.10e). In contrast, IL-12P40c expression barely responded to LPS stimulation, although a minimal two-fold increase in expression was observed following LPS stimulation in one of the groups at four h (Fig. 6.10f). Nevertheless, analysis of the relative increases in gene expression following in-vitro stimulation form merely one side of the story. Inspection of the RQ-PCR graphs of two control and two LPS-stimulated replicates further illustrates the large differences in in-vitro inducibility between the expression of the three P40 genes, whereas the expression of both reference genes remains constant (Fig. 6.10g-i). Moreover, these panels illustrate the large differences in constitutive expression between IL-12P40a (low), IL-12P40b (intermediate), and IL-12P40c (high). Somewhat surprising is the finding that the expression of the anti-inflammatory cytokine IL-10 increases in the initial hours following LPS stimulation, albeit only modestly (Fig. 6.10b). As expected and reported earlier[213], stimulation with LPS rapidly enhanced the expression of the pro-inflammatory cytokine IL-1β several hundred-fold (Fig. 6.10c).

We next tested the in-vitro response to stimulation with ConA (20 μg ml^{-1}), PMA (100 ng ml^{-1}), or cortisol (400 ng ml^{-1}), in addition to LPS (50 μg ml^{-1}). As the actions of ConA

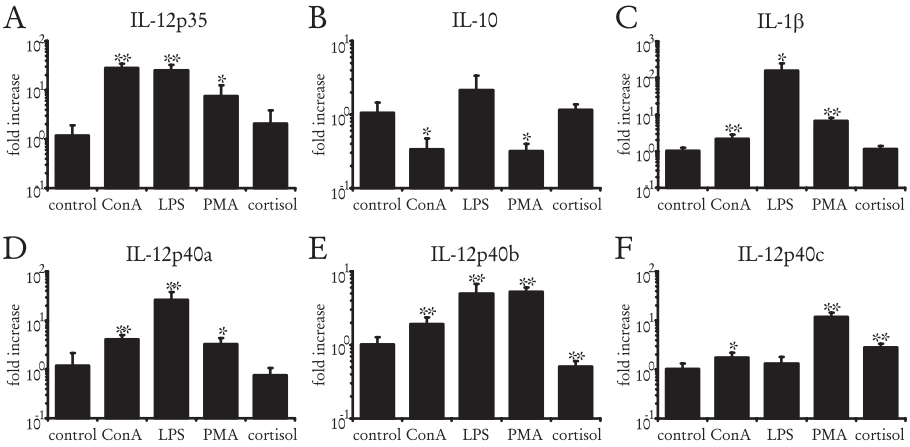

Figure 6.11: Cytokine expression in carp head kidney macrophages following four h stimulation with ConA (20 μg ml^{-1}), LPS (50 μg ml^{-1}), PMA (100 ng ml^{-1}), or cortisol (400 ng ml^{-1}). Expression is plotted relative to unstimulated controls. Asterisks indicate a significant difference (*<0.05, **<0.01) from the control group. Error bars indicate the standard deviation of five replicate samples. This experiment is representative of two experiments performed. Note the logarithmic scale of the y-axes.

on teleostean leukocytes are incompletely understood, we investigated its effect on the gene expression of carp macrophages, despite the fact that ConA is generally considered a stimulator of mammalian T-cells. Again, IL-12p35 and IL-12p40a expression is upregulated more profoundly, compared to that of IL-12p40b and IL-12p40c (Fig. 6.11). IL-12p35 is equally responsive to ConA and LPS stimulation, whereas PMA is a less potent stimulator of p35 expression. IL-12p40a is most responsive to LPS (Fig. 6.11d), whereas LPS and PMA induce equal, but modest, expression of IL-12p40b (Fig. 6.11e). Expression of IL-12p40c is again not enhanced by LPS stimulation, but is upregulated more profoundly by PMA than any other cytokine tested (Fig. 6.11f). Furthermore, expression of IL-12p40c is increased several-fold by stimulation with 400 ng ml^{-1} cortisol, which reflects peak plasma cortisol levels during acute stress[69]. In contrast, expression of IL-12p40b is markedly inhibited by cortisol. The expression of IL-12p35 and IL-12p40a is not altered appreciably, which should be interpreted in the light of their barely detectable constitutive expression. IL-10 expression was insensitive to cortisol, despite the stimulatory effect of glucocorticoids on mammalian TH2 cytokine expression[214, 215]. ConA and PMA inhibit expression of IL-10 (Fig. 6.11b). LPS and to a lesser extent PMA and ConA enhance the expression of IL-1β (Fig. 6.11c).

Discussion

We describe the presence of three separate and functionally distinct IL-12p40 genes in bony fishes, that were designated IL-12p40a, -p40b, and –p40c, in decreasing order of similarity to human IL-12p40. All three carp p40 proteins share relatively low amino acid identity, although the residues critical for disulphide bridge formation and heterodimerisation with the p35 subunit in human IL-12[204] are conserved in all three carp IL-12p40 proteins. The relatively low overall amino acid identities between the different carp IL-12p40 genes are reflected in the topology of the phylogenetic tree, where the long branch lengths pinpoint their origin to early in the bony fish lineage, at the latest. The major differences between the three carp p40 genes in both constitutive expression as well as in-vitro inducibility indicate pronounced differences in functionality. In addition to the identification of three distinct IL-12p40 genes, we identified the carp orthologue of IL-12p35. Inspection of non-mammalian IL-12p35 genes reveals the recent occurrence of an exon duplication in the mammalian p35 lineage, following its divergence from birds.

The identification of multiple IL-12p40 genes in teleost fish is congruent with the occurrence of a genome duplication event that has occurred early in the bony fish lineage, following the fish-tetrapod split[93, 216]. However, as such an event would result in the presence of duplicate genes, at least one other gene duplication is accountable for the presence of three highly distinct p40 genes in bony fishes. The topology of our phylogenetic tree indicates that p40a and p40b share a common ancestor following their divergence from p40c, but before the split between the carp and pufferfish lineages. Therefore, the early teleost genome duplication is a likely cause for the presence of p40a and p40b. As a consequence, p40c arose before this genome duplication and possibly before the fish-tetrapod split. Indeed, partial sequences that represent orthologues of carp p40b as well as p40c can be retrieved from the pufferfish genome (not shown), in addition to the published pufferfish IL-12p40a sequence. Whether orthologues of p40c are still present in higher vertebrates is currently not known. The gene duplications that gave rise to multiple IL-12p40 genes are not unique within the vertebrate heterodimeric cytokine family. The IL-23R chain that forms the receptor complex for IL-23 in concert with IL-12Rβ1 is the likely product of a gene duplication event, as it is situated on the same chromosome and in close proximity to the IL-12Rβ2 gene, the receptor chain it substitutes in the IL-23 receptor complex[217]. Also the IL-12Rβ1 chain and the IL-27R chain WSX-1/TCCR (T-cell cytokine receptor)[218] are situated in proximity on chromosomes 19 and 8 in human and mouse, respectively. Collectively this indicates that the family of heterodimeric cytokines has certainly not been static during recent vertebrate evolution. Of the three carp IL-12p40 genes, IL-12p40a is most similar to mammalian p40 proteins, with respect to overall amino acid identity as well as the conservation of key amino acid residues

that are involved in the interlocking topology with the p35 subunit. Its close interaction with p35 is further illustrated by their closely linked patterns of constitutive expression, and their similar response to in-vitro induction of gene expression. The similarities in the magnitude of constitutive expression and the in-vitro responses of the il-12p35 and il-12p40a genes slightly contrast the situation described in human and mouse, where the secretion of a large excess of p40 monomer and homodimer accompanies the release of il-12p70[184, 219]. Unfortunately, we are currently unable to assess how the expression of fish il-12 genes translates into protein release. Despite their decreasing overall amino acid identity with mammalian il-12p40, carp p40b and p40c still contain the four key charged residues and most hydrophobic residues that participate in the formation of the tight interlocking heterodimerisation with the p35 subunit[204]. This suggests that all three fish p40 subunits have retained the capacity to form a heterodimer with p35. As a comparison, ebi3 lacks two of these four key residues[220], but is still capable of heterodimerisation with the p35 subunit[221], although a biological function for this heterodimer has not been demonstrated. Nonetheless, whether all three carp p40 genes heterodimerise with the p35 subunit *in vivo*, will for a large part depend on their simultaneous production with p35 in one cell-type, as this is a prerequisite not only for the efficient secretion of bioactive il-12p70, but also for il-23 and il-27[182, 196, 197]. Carp il-12p40c has lost the cysteine residue that forms an interchain disulphide bridge, but this bridge is merely stabilising and not essential for heterodimer formation proper[204]. In fact, the loss of this stabilising cysteine residue might hold a clue to the function of this aberrant il-12p40 subunit. It is possible that any of the p40 subunits in fish acts as a chaperone for the secretion of p35 or other, as yet unidentified cytokine subunits. Alternatively, they may act as carrier molecules that affect the bioavailability and circulating half-life of the subunits they bind, as has been described for other members of the il-6 family[222]. Thirdly, p40 homodimers may interact directly with the il-12r complex, either supplementary or antagonistically to il-12p70 as has been well-documented[185-189, 191, 192]. The differential effects of the anti-inflammatory steroid cortisol on the expression of the various p40 subunits *in vitro* is consistent with this scenario. Cortisol considerably enhances the expression of p40c, which is already highly and constitutively expressed in many organs, suggestive of a dampening effect on the immunostimulatory actions of il-12p70. Taken together, the major differences in constitutive expression and in-vitro responses to various mitogens between carp il-12p40a, -40b, and -40c clearly indicate differences in functionality.

Obviously, the il-12 family in bony fish may well turn out to be far more complex compared to what has been documented in mammalian species, as the potential for the formation of heterodimers, not only between p35 and any of the three carp p40 subunits, but also directly between the different p40 subunits is great. In fact, the presence of such a number of substantially different il-12 genes in fish constitutes an unprecedented

example of combinatorial immunology[193].

Ever since its initial characterization in the late 1980s and early 1990s[182, 223, 224], IL-12 has been in the spotlights for its early and determining role in the development of TH1 responses. In this respect, its signature function is the induction of IFNγ by lymphoid cells, either alone or in synergy with IL-18. The presence of IL-12 in fish substantiates that it has been an important cytokine in the regulation of immune responses throughout vertebrates. Nonetheless, whether it has adopted a similarly central role in the differential regulation of the bony fish immune response is not yet clear. In fact we are just starting to identify the orthologues to some of the major mammalian cytokines in bony fishes, including IL-10[225], IL-18[226], IL-12[162 and this chapter], IL-6, and IL-11[227]. But overall amino acid identities between mammalian and non-mammalian cytokines are generally intermediate to low. Furthermore, for most cytokines that are identified in fish, conspicuous differences emerge with their mammalian orthologues, that prevent an indiscriminate extrapolation of the mammalian dogma of immune regulation to lower vertebrates[51 and this chapter]. Although it is evident that pathogen-dependent skewing of acquired immune responses is a capacity shared by vertebrates from fish to mammals, it is currently unclear whether these differential responses in lower vertebrates respond to descriptives such as TH1 and TH2. Therefore, further study of the regulation of immunity in early vertebrates may assist us in appreciating the evolutionary significance of the paradigms that shape the field of immunology.

Acknowledgements

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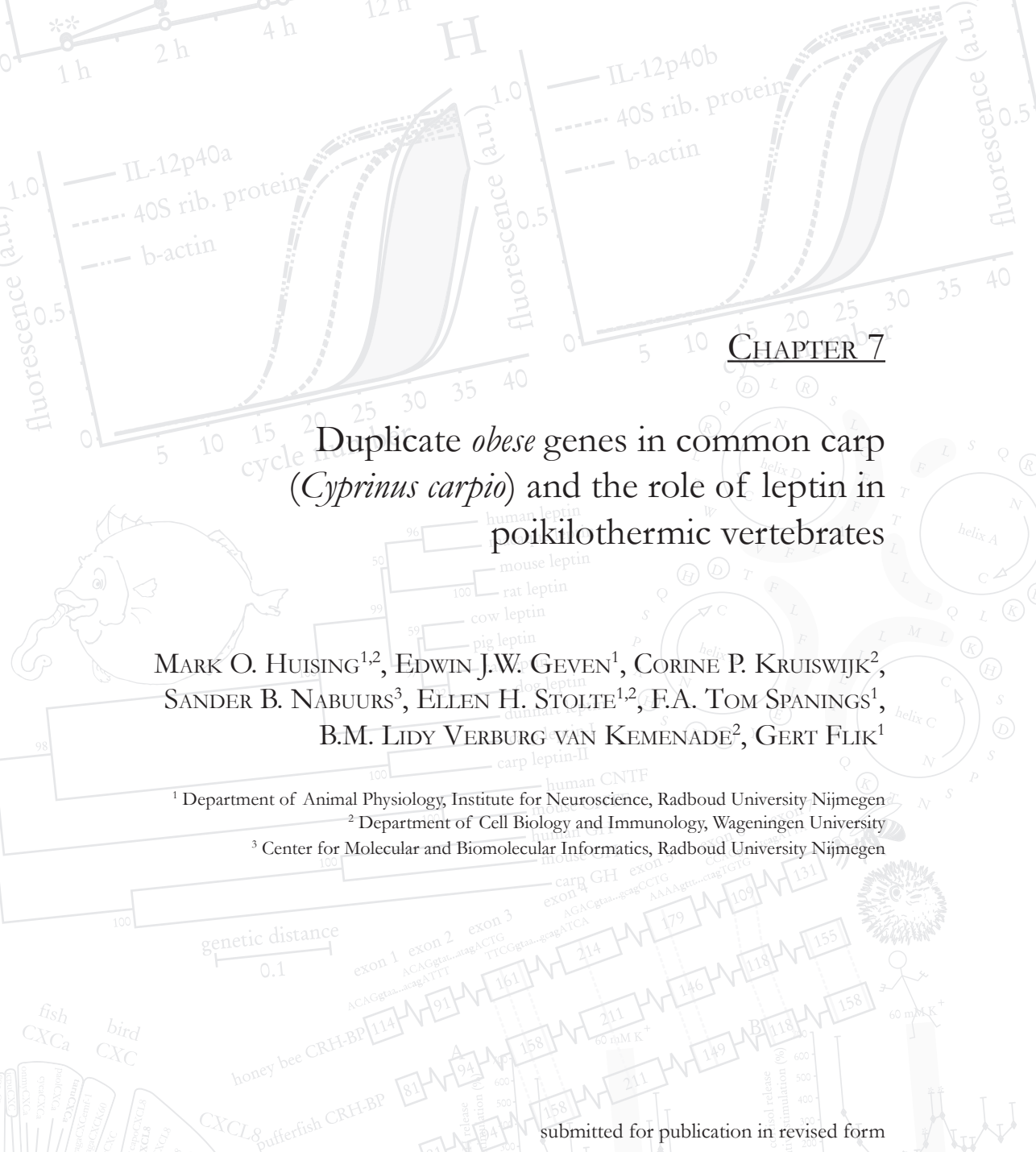

CHAPTER 7

Duplicate *obese* genes in common carp (*Cyprinus carpio*) and the role of leptin in poikilothermic vertebrates

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submitted for publication in revised form

Abstract

Obesity is taking on epidemic proportions in the Western world. Leptin, the product of the *obese* (*ob*) gene, is a key factor in the etiology of obesity and has consequently received wide interest. It is mainly produced by adipocytes and circulates in proportion to body fat mass. Via actions on the hypothalamus, leptin inhibits food intake and regulates energy metabolism. Given its important role in these vital processes, leptin is widely regarded as evolutionarily conserved. However, in the decade that passed since the discovery of human and mouse leptin, information regarding leptin in poikilothermic vertebrates has been scant. We describe two *obese* genes, encoding duplicate leptin proteins, in common carp (*Cyprinus carpio* L.). Their conserved and unique gene structure, the conservation in presence and spacing of both cysteines that form leptin's single disulphide bridge, and stable clustering in phylogenetic analyses substantiate the unambiguous orthology of mammalian and carp leptins, despite low amino acid identity. The liver is the major site of carp leptin expression. Moreover, liver leptin expression is characterised by an acute, transient postprandial increase that follows the postprandial plasma glucose peak. However, neither six days of fasting, nor subsequent re-feeding, changed leptin expression, although carp responded predictably to the altered feeding regime by shifting between carbohydrate and lipid metabolism. Thus, in carp leptin appears to be involved in the acute regulation of food intake and energy metabolism, whereas the involvement of leptin in the long-term regulation of energy balance in poikilothermic vertebrates remains uncertain.

Introduction

The positional cloning of the *obese* (*ob*) gene in mouse, over a decade ago[228], identified the factor responsible for the profound obesity and type II diabetes associated with the *obese* mouse mutant[229]. The *ob* gene encodes a 167 amino acid soluble protein that was named leptin after the Greek root *leptos*, meaning 'lean'. The absence of a soluble factor as underlying cause for the obese phenotype was already established by parabiosis: a soluble factor in the blood of wild-type mice reduced food intake and weight gain of the obese parabiont and partially reverted the profoundly obese, diabetic and hyperphagic phenotype[230].

Leptin is a member of the type-I cytokine family, which includes IL-6 and growth hormone; the protein is characterised by a typical four-helix bundle conformation[231]. In mammals, it is secreted by adipocytes in response to feeding, and reaches the brain to

evoke satiety and terminate food intake[232-234]. Leptin informs the brain about the energy status of the body and is considered a major factor in appetite control and body weight regulation[235,236]. Mutations in the human *OB* gene are very rare[232], which explains why leptin therapy does not provide an effective solution for the treatment of obesity. Moreover, mean body fat and serum leptin levels are positively correlated in mice and men, which indicates that in the obese the peripheral leptin signal is inadequately perceived by the brain, a condition referred to as leptin resistance. Shuttling of leptin across the blood-brain barrier, mediated by a short non-signalling splice variant of the leptin receptor[237], is considered the critical step in leptin signalling that underlies leptin resistance[238]. Within the brain, leptin conveys its signal via the long form of its receptor that is found in several hypothalamic nuclei[239, 240]. In mammals, the hypothalamic arcuate nucleus plays a central role in the control of energy metabolism. The nucleus contains two distinct and antagonistic sets of leptin-responsive neurons[241, 242]. One set of neuropeptide Y (NPY) positive neurons is inhibited by leptin. A high percentage of these NPY^+ neurons co-expresses agouti gene-related protein (AgRP)[243], which is an α-melanocyte stimulating hormone (α-MSH) antagonist at the level of the melanocortin-receptor 4 (MC4R) and MC3R[244]. The other set of leptin-responsive neurons co-expresses the anorexigens proopiomelanocortin (POMC; *i.a.* the precursor for α-MSH) and cocaine and amphetamine regulated transcript (CART)[245]. Thus, within the arcuate nucleus leptin targets antagonistic populations of anabolic (NPY^+, $AgRP^+$) and catabolic ($POMC^+$, $CART^+$) neurons[241, 246]. More neuropeptides, including the anorexigen CRH, function in the complex neural network that controls food intake and energy balance[242].

ZHANG and colleagues (1994) addressed the evolutionary conservation of the *OB* gene by hybridizing genomic DNA of representative vertebrate species with a murine *OB* probe. Positive signals from genomic DNA of species that are evolutionarily distantly related to mammals, such as chicken and eel led them to conclude that the *OB* gene is highly conserved among vertebrates[228]. Indeed, leptin-like immuno-crossreactivity was reported in blood, brain, and liver of several fish species[247] and intracerebroventricular injection of human recombinant leptin in goldfish inhibits food intake[248], suggesting the presence of a leptin-like molecule in bony fish. Only very recently, the sequence of a leptin-like molecule in teleostean fish has been reported in pufferfish (*Takifugu rubripes*)[249], but any information regarding the possible role(s) of leptin in the regulation of food intake and energy metabolism in poikilothermic vertebrates lacks altogether at this point.

We here report the presence of duplicate *OB* genes in common carp (*Cyprinus carpio*). In sharp contrast to the evolutionarily well-conserved hypothalamic factors involved in the regulation of energy metabolism, both carp leptin proteins display surprisingly low amino acid identity to their mammalian orthologues. Nonetheless, the conservation of both the *OB* gene structure and the characteristic four-helix bundle conformation

adopted by leptin, supplemented by stable clustering in phylogenetic analyses, support unambiguous orthology of carp leptin with mammalian counterparts. The liver is the main site of leptin expression in carp and this expression is acutely upregulated following ingestion of a meal. Fasting for six days followed by re-feeding did not lead to any changes in leptin gene expression, despite altered nutrient status, indicated by several plasma nutrient parameters.

Materials and methods

Animals

Common carp (*Cyprinus carpio*) of the R3xR8 line were reared at 23°C in recirculating UV-treated water. Carp were fed dry food pellets (LDX Filia slow sinking, Trouw Nutrition International, Putten, The Netherlands) at a daily ration of 1.2 or 2.0 % of their estimated body weight, once daily. R3xR8 are the inbred offspring of a cross between fish of Polish (R3 strain) and Hungarian (R8 strain) origin. Fish were irreversibly anaesthetised with 0.1 % 2-phenoxyethanol before the collection of plasma and tissue samples. All animal experiments were performed in accordance with national legislation and institutional guidelines regarding the treatment of experimental animals.

Identification of carp OB genes

We screened the Ensembl zebrafish genome database with mammalian leptin sequences, using the BLAST algorithm[73]. This initial screen revealed a partial zebrafish leptin-like sequence. Using leptin.fw3 and leptin.rv2 (Table 7.1) primers based on this

Table 7.1: Primer sequences and corresponding accession numbers

gene	accession number	primer	sequence 5' ⇒ 3'
leptin	AJ830744,	leptin.fw3	AAACTGCAGGCAAAGACCATCAT
	AJ830744	leptin.rv2	ATCAGCTTTTGCATAAACTGTTT
		leptin.fw4	GCAAAGACCATCATCGTCAGAAT
		leptin.rv5	CTCCTTGGATGTGCAATGCAT
		qleptin-I.fw1	AGATACGCAACGATTTGTTCACA
		qleptin-I.rv1	GCGTTGTTCTCCAAGAAAGCA
		qleptin-II.fw1	CATATTGATTTGTCCACCCTTCTG
		qleptin-II.rv1	CCATTAGCTGGCTCCTTGGAT
40s ribosomal protein S11	AB012087	q40s.fw1	CCGTGGGTGACATCGTTACA
		q40s.rv1	TCAGGACATTGAACCTCACTGTCT
β-actin	CCACTBA	qACT.fw1	CAACAGGGAAAAGATGACACAGATC
		qACT.rv1	GGGACAGCACAGCCTGGAT

partial zebrafish leptin-like sequence, two similar and partial cDNA sequences were obtained from the liver of common carp, which is genetically similar to zebrafish[166]. The corresponding full-length cDNA sequences were obtained from carp liver by nested RACE (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions with leptin.fw3, leptin.fw4, leptin.rv2, and leptin.rv5 primers. This approach led to the identification of duplicate carp *ob* genes, designated *ob1* (encoding leptin-I) and *ob2* (encoding leptin-II). The gene structure of both carp *ob* genes was determined by amplification of the coding sequences from genomic DNA. Cloning and sequencing was carried out as previously described[166]. Briefly, PCR products were ligated and cloned into JM-109 cells using the pGEM-T-easy kit (Promega, Leiden, The Netherlands). Plasmid DNA was isolated with the QIAprep Spin Miniprep kit (Qiagen, Leusden, The Netherlands) and sequences were determined from both strands using the ABI Prism Bidye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems).

Phylogenetic analysis

Multiple sequence alignments were carried out with ClustalW. A phylogenetic tree was constructed on the basis of amino acid differences (p-distance) with the neighbour-joining algorithm (complete deletion) in MEGA version 3.0[250]. Reliability of the tree was assessed by bootstrapping, using 1000 bootstrap replications.

Modelling of carp leptin

The structure of human leptin (PDB entry 1AX8) that was solved at 2.4 Å resolution[231], was used as a template to build models of carp leptin-I and leptin-II. Initial alignments were obtained from the PSIPRED fold recognition server[251]. Side chain rotamers were modelled using SCWRL3.0[207]. Both models were refined in YASARA using the YAMBER2 forcefield[252]. Coordinate files are available from the authors upon request.

RNA isolation and cDNA synthesis

RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA), following the manufacturer's instructions; RNA concentrations were measured by spectrophotometry. First strand cDNA synthesis was carried out as previously described[166]. Briefly, total RNA was DNase treated, followed by random hexamer primed cDNA synthesis (Invitrogen, Carlsbad, CA). A non-reverse transcriptase control was included for each sample.

Real-time quantitative PCR

Primer Express software (Applied Biosystems) was used to design primers for analysis of gene expression by real-time quantitative PCR (Table 7.1). Five µl cDNA and forward and reverse primers (300 nM each) were added to 12.5 µl Sybr Green PCR Master

Mix (Applied Biosystems) and the volume adjusted to 25 μl with demineralised water. RQ-PCR (2 min at 48°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C) was carried out on a GeneAmp 5700 sequence detection system (Applied Biosystems). Data were analysed with the ΔΔCt method. Dual internal standards (40S ribosomal protein S11 and β-actin) were incorporated in all RQ-PCR experiments and results were confirmed to be similar following standardization to either gene. Results standardised for 40S expression are shown.

Plasma parameters

Plasma glucose concentration was determined with a Stat Profile pHOx Plus analyser with automated two-point calibration and equipped with an enzymatic glucose electrode (Nova Biomedical, Waltham, MA, USA)[3]. Plasma non-esterified fatty acids (NEFA) were measured via to the NEFA-C method (Wako Chemicals, Neuss, Germany), according to the manufacturer's instructions.

Statistics

Statistical analyses were carried out with SPSS software (version 12.0.1). The level of significance of differences assessed by ANOVA was evaluated with a two-sided Student's T-test. Homogeneity of variances was tested with Levene's test and corrected for where necessary.

Results

Characteristics of carp leptin

Both carp *OB* genes encode 171 amino acid leptin proteins that share 82% amino acid identity (Fig. 7.1; Table 7.2). Both carp leptins are equally similar (62 and 61 %, respectively) to the single leptin of zebrafish. Amino acid identity to mammalian leptins is markedly lower at between 20 and 25%. The identity of the carp leptins with leptin proteins of *Xenopus laevis* and the Eastern tiger salamander (*Ambystoma tigrinum tigrinum*), which were retrieved from the primary sequence databases, are only marginally higher. Identical amino acid residues of carp and mammalian leptins are distributed evenly throughout the leptin protein and include both cysteine residues that together form a disulphide bridge connecting the carboxy-terminal ends of α-helices C and D (Fig. 7.2). The gene structure of the carp *OB* genes is identical and consists of two exons, separated by a short intron with consensus 5' donor (gt) and 3' acceptor (ag) splice sites. Both exons of the carp *OB* genes are very similar in length to those of the human and mouse

Table 7.2: Percentage amino acid identity between vertebrate leptin amino acid sequences

	carp leptin-I	carp leptin-II	zebrafish	*Xenopus*	salamander	fat-tailed dunnart	mouse	rat	pig	macaque	human
carp leptin-I	100										
carp leptin-II	82	100									
zebrafish	62	61	100								
Xenopus	27	27	28	100							
salamander	23	24	28	60	100						
fat-tailed dunnart	20	22	21	33	36	100					
mouse	24	24	23	34	31	67	100				
rat	24	23	23	34	32	67	96	100			
pig	24	24	23	34	35	71	82	83	100		
macaque	23	24	22	34	33	69	81	80	84	100	
human	24	25	22	36	34	68	83	82	85	90	100

OB genes, and differ only one and three codons, respectively (Fig. 7.3). Furthermore, although information on the genomic context of the carp leptin genes is unavailable, the *OB* gene of the closely related zebrafish is positioned in the opposite orientation and in close proximity (less than three kb) to the gene encoding RNA binding motif protein 28 (RBM28) (not shown). RBM28 is also present in juxtaposition of and directly adjacent to the *OB* gene in the genomes of both human and mouse, which further strengthens the orthology of fish and mammalian leptins.

In phylogenetic analyses that include other members of the type-I cytokine family, all vertebrate leptins cluster together, supported by a high bootstrap value (Fig. 7.4). The high bootstrap value that anchors the vertebrate leptin cluster corroborates the *bona fide* identity of the carp leptins, although the branch lengths that separate mammalian and fish leptins are long, reflecting their considerable sequence dissimilarity. Furthermore, the branching pattern with the leptin cluster is in accordance with the established patterns of vertebrate evolution, as the teleostean leptins branch of prior to the separation of the amphibian and mammalian leptin cluster. Within the mammalian leptin cluster, the sequence of the fat-tailed dunnart (*Sminthopsis crassicaudata*), the only marsupial leptin that is known, branches outside the leptin sequences of placental mammals.

Human leptin consists of four α-helices, arranged in a bundle with an up-up-down-down topology [231]. Despite the relatively low overall amino acid identity with their mammalian orthologues, our models of carp leptin, based on the crystal structure of human leptin, indicate that both carp leptins conform well to the four-helix bundle topology characteristic of leptin (Fig. 7.5).

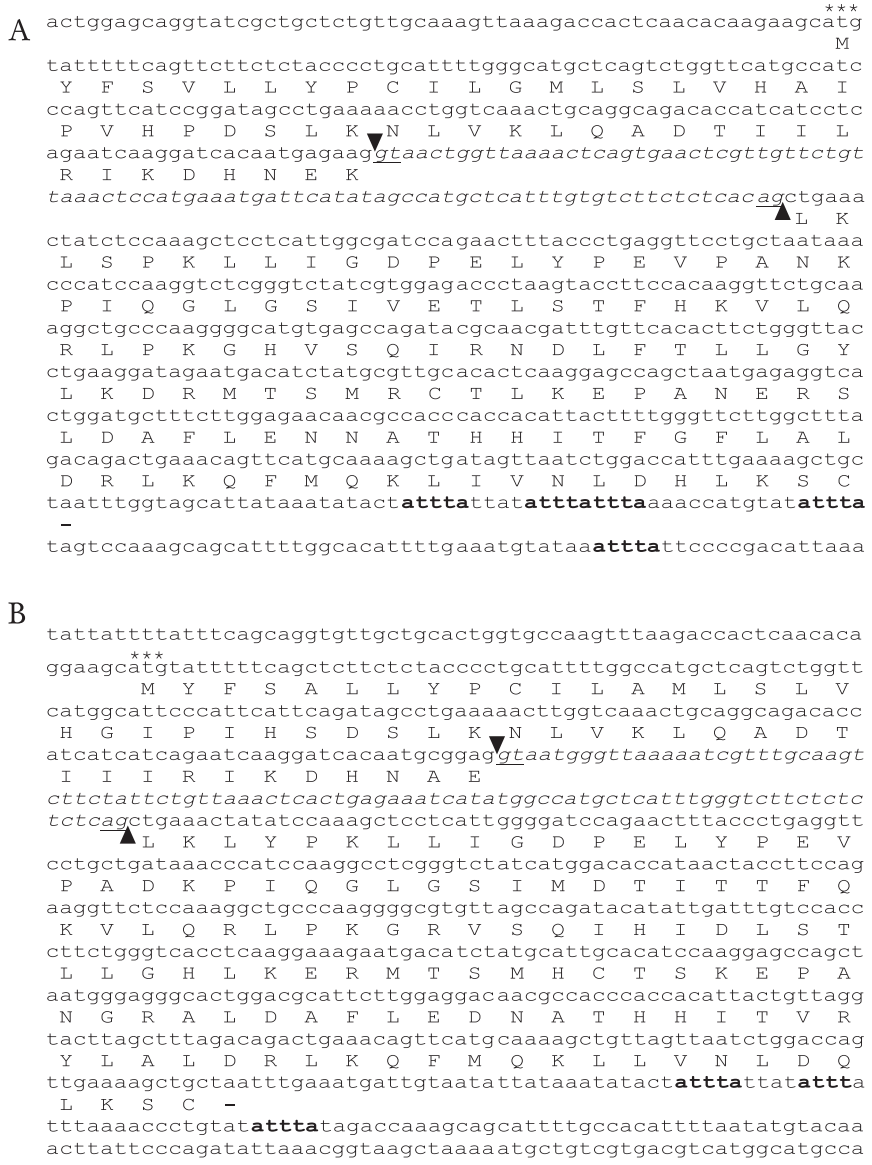

Figure 7.1: DNA and deduced amino acid sequences of carp *OB1* (encoding leptin-I; a) and *OB2* (encoding leptin-II; b). Asterisks indicate the start codon. Potential instability motifs are in bold. Introns are in italics, 5' donor (gt) and 3' acceptor (ag) splice sites are underlined. Accession numbers for are AJ868357 and AJ868356, respectively.

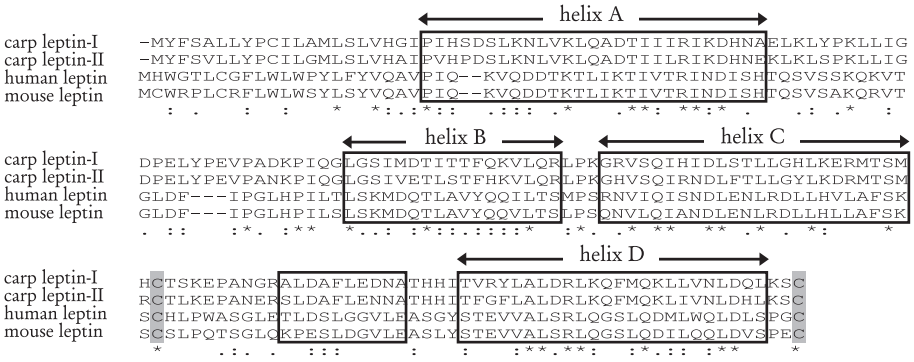

Figure 7.2: Multiple sequence alignment of carp and mammalian leptins. Asterisks indicate amino acids that are conserved in all sequences, while colons and dots reflect decreasing degrees of similarity. The cysteine residues involved in disulphide bridge formation are shaded, the α-helices, inferred from human leptin, are boxed.

Constitutive expression of carp leptins

We investigated the expression of both carp *ob* genes in a panel of peripheral organs. Carp leptin-I is predominantly and almost exclusively expressed in the carp liver, but faint expression was also detectable within the combined hypothalamic and preoptic area of the brain (Fig. 7.6). In addition to prominent liver expression of leptin-II,

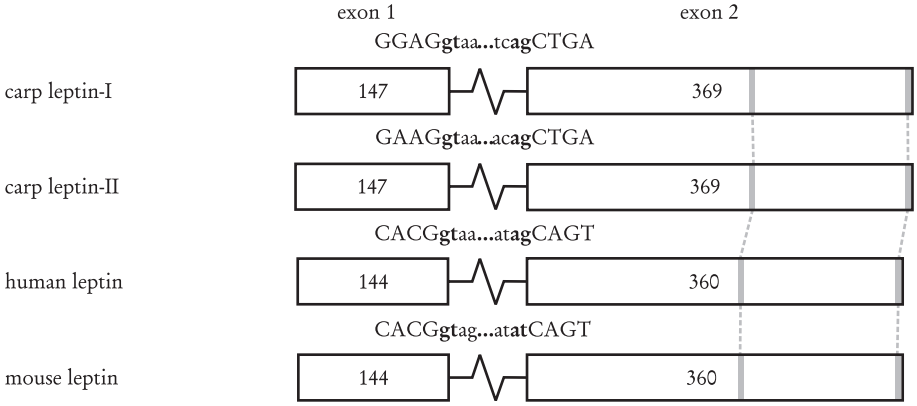

Figure 7.3: The gene structure of vertebrate *ob* genes is conserved. Boxes represent coding exons and are drawn to scale, numbers indicate exon sizes in nucleotides. Conserved cysteine residues are shaded. The residues surrounding each splice site are given, coding residues are represented as capitals. The 5' donor (gt) and 3' acceptor (ag) splice sites are in bold.

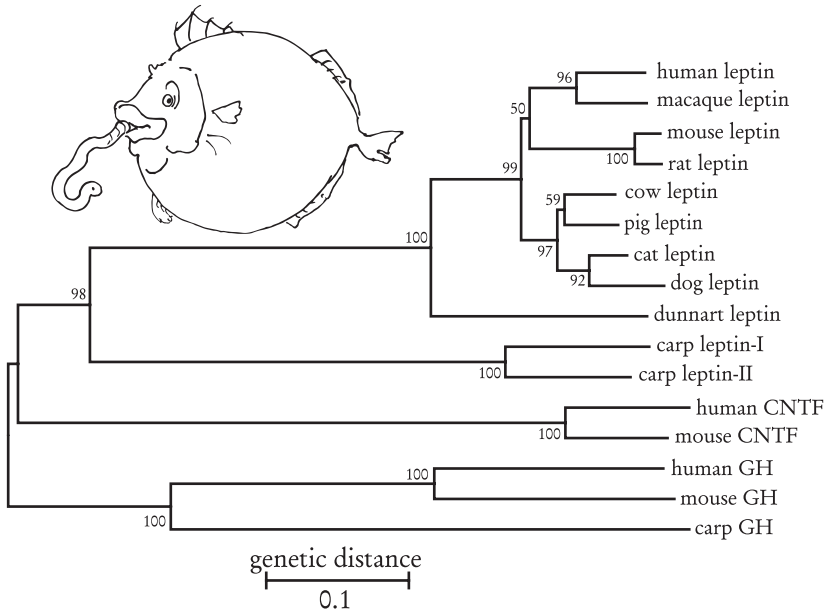

Figure 7.4: Phylogenetic tree of vertebrate leptin amino acid sequences. Numbers at branch nodes represent the confidence level of 1000 bootstrap replications. Growth hormone and CNTF are included as outgroup. Accession numbers are as follows: human leptin, P41159; rhesus macaque leptin, Q28504; mouse leptin, P41160; rat leptin, P50596; cow leptin, P50595; pig leptin, Q29406; dog leptin, O02720; cat leptin, AB041360; fat-tailed dunnart leptin, AF159713; *Xenopus* leptin, AY884210; Eastern tiger salamander leptin, CN054256; zebrafish leptin, GENSCAN00000029170; carp leptin-I, AJ830745; carp leptin-II, AJ830744, human CNTF, P26441; mouse CNTF, P51642; human GH, P01241; mouse GH, P06880; carp GH, P10298.

constitutive leptin-II expression was observed in several other organs, including gonads, hypothalamus/preoptic area, and thymus. Low leptin-II expression is also detectable in adipose tissue, muscle, heart, spleen, and head kidney of carp.

Postprandial changes in leptin expression

Following the establishment of the liver as the primary site of leptin-I expression and a major site of leptin-II expression, we investigated the expression of leptin-I and leptin-II in the liver in response to a single meal. The liver expression of both leptins displayed a marked postprandial rise at six hours and three hours post-feeding, respectively (Fig. 7.7a). Interestingly, in the initial hours following feeding, prior to its postprandial peak, liver leptin-I expression slightly but significantly dropped below the pre-feeding expression values. The postprandial peak in leptin-I and leptin-II expression was preceded

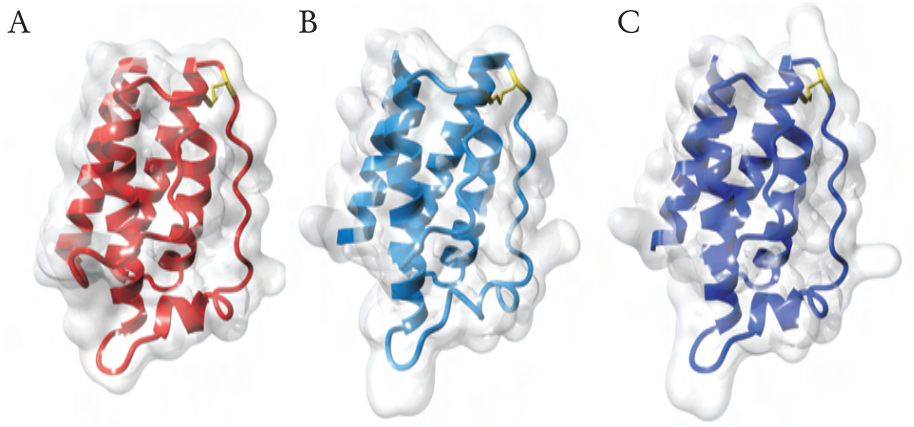

Figure 7.5: Protein models of carp leptin. Carp leptin-I and -II, modelled over the human leptin crystal structure (a) illustrate the four-helix bundle conformation adopted by carp leptin-I (b) and leptin-II (c), stabilised by a single disulphide bridge (yellow).

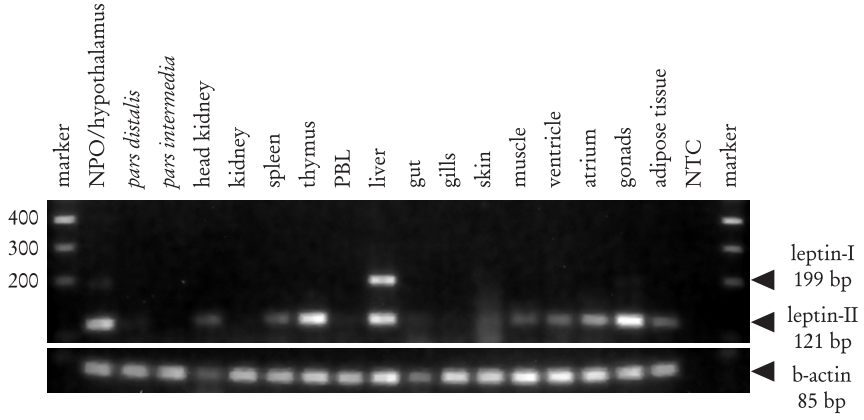

Figure 7.6: Constitutive expression of carp leptin-I and -II in carp organs. Carp leptin-I is predominantly expressed in liver, with faint expression observed in the combined hypothalamus and preoptic area. Constitutive expression of leptin-II is more widespread. Liver, hypothalamus/NPO, gonads, and thymus are sites of dominant leptin-II expression, with modest expression detected in several other organs and tissues, including adipose tissue. PCR reactions for leptin-I and leptin-II were carried out separately (40 cycles each) and PCR products were pooled prior to loading on the gel. Carp β-actin (30 cycles) was included as a positive control.

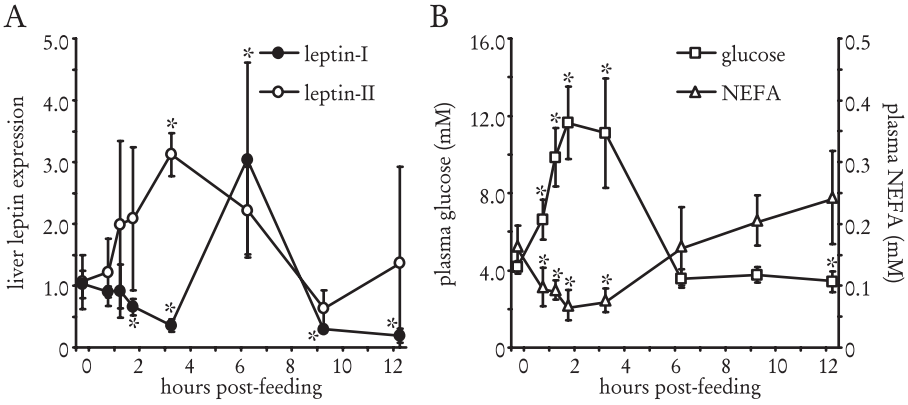

Figure 7.7: The liver expression of leptin-I and leptin-II displays a marked and significant postprandial peak in expression, at six hours and three hours post-feeding, respectively (a). During the course of a single day, fish from the same tank were sampled prior to, and at various times following feeding. Directly after feeding 2.0 % of their estimated body weight, leptin-I expression is slightly reduced, whereas leptin-II expression at that time inclines towards its postprandial peak. The peak in leptin expression is preceded by a postprandial rise in plasma glucose that is accompanied by a concomitant drop in plasma NEFA values (b). Leptin expression is standardised to expression of 40S ribosomal protein S11 and expressed relative to pre-feeding controls. Error bars indicated the standard deviation of four to five replicates, asterisks indicate significant differences with the pre-feeding controls, $p<0.05$ was accepted as fiducial limit.

by a postprandial increase in plasma glucose values and a concomitant drop in plasma NEFA values (Fig. 7.7b).

Leptin expression in response to fasting and re-feeding

We next investigated liver leptin expression in response to prolonged (six days) fasting, followed by re-feeding. Surprisingly, food deprivation over a six day period did not result in any changes in leptin-I expression (Fig. 7.8a). The expression of leptin-II was also not affected by fasting, with the exception of the fourth day of fasting, where leptin-II expression values significantly dropped below control values (Fig. 7.8b). Furthermore, the expression of neither leptin was changed in response to re-feeding. Plasma glucose and NEFA values did clearly differ between fed and fasted animals. Plasma glucose values in the control animals consistently ranged from eight - eleven mM. In contrast, plasma glucose values in the fasted animals were maintained at 2.5 – 3.0 mM (Fig. 7.8c). Since these values were determined at one hour following the scheduled feeding time, they likely reflect the absence of a postprandial peak in plasma glucose in the fasted animals, rather than a drop in baseline glucose values. In contrast to glucose, plasma NEFA values

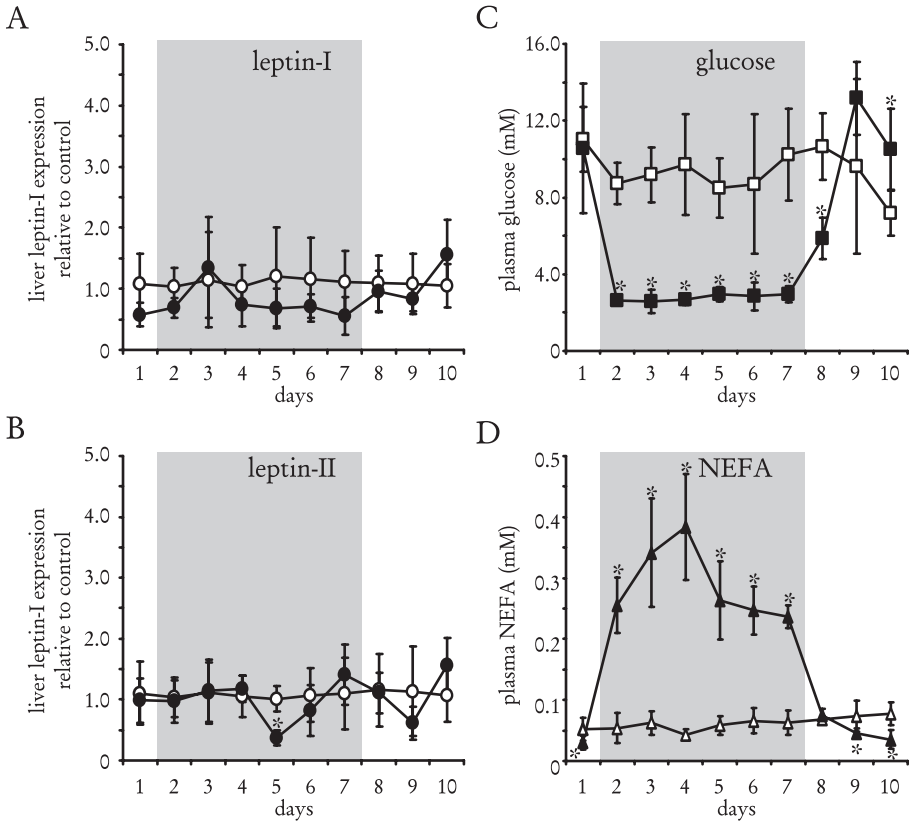

Figure 7.8: Liver leptin expression in response to fasting and re-feeding. Two groups of animals reared from the same offspring were acclimatised to a daily ration of 1.2 % of their estimated body weight, once daily at 09.00 am. The control animals (open symbols) were maintained on this feeding regime throughout the ten-day experiment. The experimental animals (closed symbols) were fasted for six days (day two to seven, indicated by the grey background), followed by re-feeding in the final three days of the experiment (*ad lib.* at day eight, 1.2 % at days nine and ten). Each day fish were samples at one hour following the (scheduled) feeding time. Liver expression of leptin-I (a) and leptin-II (b) does not respond to fasting or to subsequent re-feeding. Plasma glucose values in the experimental animals are maintained at 2.5- 3.0 mM (c), whereas plasma NEFA values are significantly elevated in the experimental animals throughout the fasting period (d). Leptin expression is standardised to expression of 40s ribosomal protein s11 and expressed relative to their corresponding fed controls. Error bars indicate the standard deviation of four to five replicates, asterisks indicate significant differences with the corresponding control group, $p<0.05$ was accepted as fiducial limit.

rapidly rose in response to food deprivation from approximately 0.05 mM to 0.25 – 0.35 mM (Fig. 7.8d). Upon resumption of the normal feeding regime, plasma glucose and NEFA rapidly normalised, although glucose and NEFA did exhibit a slight over- and undershoot, respectively, in the days following re-feeding.

Discussion

Carp, an early vertebrate, expresses duplicate *ob* genes that encode two similar leptins, designated leptin-I and leptin-II. Given their similarity, both carp leptins are the likely result of a recent duplication event, possibly the duplication of the carp genome, that occurred less than sixteen million years ago[253]. Contrary to the prevailing opinion that leptin is evolutionarily conserved, both carp leptins share low amino acid sequence identity of maximally 25% with mammalian leptins. Despite this relatively poor sequence identity, we are confident in the assignment of orthology to carp and mammalian leptins based on several key observations. Firstly, the carp leptins are encoded by two exons that differ merely one and three triplets in length, respectively, from the human and mouse *ob* genes. Importantly, this composition of only two exons offers strong support to the orthology of carp and human leptins, as vertebrate type-I cytokines are almost invariably encoded by three or more (usually five) exons. The only other type-I cytokine gene composed of two exons is ciliary neurotrophic factor (CNTF), but its exons clearly differ in size from those of leptin. Secondly, the spacing of the cysteines that constitute the single disulphide bridge of leptin, which are conserved in both carp leptins, is unique among type-I cytokines. Finally, the stable clustering in phylogenetic analyses, the predicted conformation of both carp leptins to the characteristic four helix bundle topology of type-I cytokines, and the conservation of synteny between the zebrafish and human *ob* genes further support the unambiguous orthology of fish and mammalian leptins. Nevertheless, the unexpectedly high sequence dissimilarity between mammalian and fish leptins has likely contributed to the difficulties in retrieving *ob* orthologues in lower vertebrates.

Based on the original Southern blot experiment by ZHANG and colleagues, where a murine *ob* probe was hybridised successfully to genomic DNA of chicken and eel, and on the sequence identity between human and mouse leptin, leptin has been widely regarded as evolutionarily conserved[228, 232]. A comprehensible view, since leptin plays a key role in the regulation of food intake and energy metabolism, physiological processes that are vital for survival, and thus evolutionary fitness. Although the mere presence of leptin in bony fish could be regarded as testimonial to this widespread supposition, it is unlikely

that an identity of less than 25% between leptins of different vertebrate species was what investigators had in mind when referring to leptin as 'evolutionarily conserved'.

The duplicate carp *ob* genes are not the first published non-mammalian leptins. Chicken leptin was reported to be 83% and 97% identical to human and mouse leptin, respectively[254, 255]. Clearly, this exquisite conservation between avian and mammalian leptins fitted the prevailing supposition that leptin is evolutionarily conserved. As mouse and human leptin are also 83% identical (Table 7.2), these percentages indicate that chicken and human leptin are equally similar to mouse leptin, despite the fact that the avian and mammalian lineages diverged approximately 219 million years prior to the split between human and mouse[15]. The reconciliation of these observations would involve a remarkable and unprecedented example of evolutionary convergence. The inability of several groups to repeat the amplification of the chicken *ob* gene[256, 257], added to the absence of chicken leptin as it was originally reported from the recently published chicken genome indicates that this topic needs to be readdressed. Very recently, a leptin sequence from pufferfish (*Takifugu rubripes*) was reported. In slight contrast to the leptin proteins of cyprinids, which are only slightly longer than their mammalian orthologues, the pufferfish leptin sequence is fifteen amino acids shorter than mammalian leptins. Moreover, pufferfish and human leptin share only 13.2 % amino acid identity[249], which is very low even compared to the modest amino acid identity shared by carp and human leptins.

In mammals leptin exerts its central effects via two antagonistic populations of neurons within the arcuate nucleus. Both populations share the long signalling form of the leptin receptor, but their response to leptin is opposite: the 'anabolic' NPY$^+$/AgRP$^+$ neurons are inhibited, while the 'catabolic' POMC$^+$/CART$^+$ neurons are activated by leptin. In recent years, most key factors involved in the hypothalamic regulation of food intake and energy metabolism have been identified in non-mammalian vertebrates[258]. The orthologues of NPY[259], AgRP[260], POMC[261], and CART[262] are evolutionarily conserved in bony fish species and their expression is localised to the *nucleus lateralis tuberis* (NLT)[260, 263, 264], the teleostean equivalent of the arcuate nucleus[265]. In fact the amino acid identities between these (mature) hypothalamic peptides range from 50 to 100%, when human and cyprinid fish are compared[259-262]. Furthermore, the hypothalamic expression of NPY and AgRP is upregulated in response to food deprivation in goldfish[260, 266], whereas CART expression is downregulated[262], comparable to the situation in mammals. In sharp contrast to the hypothalamic peptide hormones, leptin shows only 25% amino acid identity over a similar evolutionary distance. The good conservation of these neuropeptides, both in sequence and hypothalamic organization, and the poor sequence conservation of the peripheral ligand that operates this system in mammals is paradoxal. It raises the question whether leptin is the principle peripheral indicator of nutrient status in fish.

Both antagonistic populations of leptin-responsive neurons within the mammalian arcuate nucleus also express receptors for the other major adiposity signal, insulin[267-269]. Besides the well-known peripheral effects of insulin in the promotion of energy storage in the form of carbohydrate, fat, and protein, insulin and leptin act complementary and partially redundantly on the hypothalamic systems that control energy metabolism and food intake[241, 267, 268]. Moreover, hypothalamic leptin and insulin signals converge intracellularly at several shared signalling pathways[234, 268, 270]. In contrast to the 25 % amino acid identity between human and carp leptins, the amino acid identities between the A and B chains of the mature bioactive insulin peptides of both species are much higher at 67 and 76 %, respectively[271], which is more in line with the degree of conservation of the hypothalamic peptides controlling food intake. Moreover, the insulin signalling pathway is highly conserved during evolution, as it is present in evolutionarily distant species such as *Drosophila melanogaster* and *Caenorhabditis elegans*, where it plays a key role in the regulation of metabolism, reproduction, and longevity[267, 272, 273]. Thus, it appears that insulin signalling outdates leptin in the central regulation of energy metabolism.

Energy metabolism revolves around the maintenance of balance between energy intake and expenditure. And whereas energy intake is largely in the form of food consumption, expenditure is the sum of basal metabolism, physical activity, and thermogenesis[269]. During times of limited nutrient access, when peripheral energy stores become depleted, plasma levels of leptin and insulin drop as they circulate in proportion to body fat mass[267]. Centrally, a lowered tonic inhibition of leptin and insulin will stimulate food intake and lower metabolic rate to preserve adipose stores[274]. This physiological response to starvation has an adaptive advantage, as it allows animals to cope with periods of famine. But, although periods of prolonged caloric restriction are of all times, the need to inhibit metabolism during fasting may have become acutely necessary with the onset of homoiothermic animals, in which thermoregulation accounts for a large proportion of resting energy expenditure. In contrast, poikilotherms are much more flexible in the regulation of their energy metabolism as they do not require thermoregulation. And as the transition from poikilothermia to homeothermia was accompanied by the development of an insulating layer of adipose tissue, it may explain why the subcutaneous adipose compartment is the main source of circulating leptin in mammals[275, 276]. Therefore, the modest expression of leptin-II, and the lack of expression of leptin-I in the visceral adipose compartment of carp are in line with the notion that the contribution of visceral adipose tissue to circulating leptin levels in mammals is limited[275]. Instead, the liver is the main source of leptin-I, and one of the major sources of leptin-II in carp. These observations are in line with previous studies that suggest that the liver, rather than adipose tissue, is one of the main sites of immunoreactive leptin in fish[247] and supported by the recent demonstration of leptin expression exclusively in the

liver of pufferfish[249]. Proceding from a role of leptin in short-term regulation of energy metabolism, as evidenced by the upregulation of leptin-I and -II expression within hours following feeding, the liver would be a logical location as it directly receives the blood from the gastro-intestinal tract through the hepatic portal vessel system. Leptin-I expression is restricted to the liver, but leptin-II expression is more ubiquitous. Although we have not investigated extrahepatic leptin expression in detail, several of the carp tissues that express leptin-II have precedent with regard to leptin expression in mammalian species[277-279].

So what is the role of leptin in teleost fish? The marked postprandial rise in the expression of both carp *ob* genes in the liver is similar to the postprandial rise in leptin mRNA in the hours following eating in mice[233]. This links liver leptin expression to short term regulation of food intake in carp. Furthermore, the peak in postprandial liver leptin mRNA expression follows the postprandial peak of plasma glucose and drop in plasma NEFA. Whether the upregulation of leptin expression is brought about directly via these indicators of nutrient status, or indirectly via the actions of other hormones such as insulin or cholecystokinin is presently unclear. Nonetheless, the rapid and transient changes in *ob* gene expression in response to a single meal are in line with work of the group of Peter, who observed a rapid inhibitory effect of recombinant murine leptin on goldfish food intake following direct injection into the third ventricle[248].

An involvement of leptin in the acute regulation of food intake in carp does not preclude a role for leptin in the regulation of food intake and metabolism on the longer term. Following food deprivation, carp rapidly switch to lipid metabolism, as indicated by the rapid and profound increase in plasma NEFA levels, which is characteristic of starvation[280]. Nevertheless, the obvious changes in metabolic status are reflected by neither a decrease in leptin expression in response to starvation, nor an increase in response to re-feeding. Rodents, in contrast, respond to fasting for several days with a drop in leptin expression that is rapidly restored by re-feeding. Nonetheless, as eluded to earlier, fasting over a period of six days in the homeothermic rat results in a considerable 36 % weight loss[280]. In contrast, our carp subjected to six days of fasting, lost only 10.7 ± 2.2 % of body weight (data not shown). The differences in the rate of body weight loss following six days fasting in carp and rats are likely attributable to the energy costs involved in the maintenance of body temperature in the latter species. Therefore, if leptin plays a role in the long-term regulation of energy metabolism, it may require considerably longer periods of food deprivation before appreciable changes in *ob* gene expression may become detectable. After all, many fish species routinely encounter, and cope with, periods of starvation of up to several months as a result of reproductive strategy or seasonal variation in food availability.

In summary, duplicate *ob* genes encode two leptins in common carp. The low

amino acid identity with their mammalian orthologues illustrates leptins relatively poor evolutionary conservation, despite the weight attributed to leptin in the regulation of food intake and energy metabolism in mammals. Furthermore, our initial characterisation of leptin gene expression in response to ingestion of a single meal establishes a role for leptin in the acute regulation of food intake and energy metabolism. Whether leptin in fish is also involved in long-term energy metabolism is at present unclear. No changes in liver leptin gene expression were observed in response to fasting or subsequent re-feeding in carp, but this apparent unresponsiveness of leptin gene expression may reflect the less stringent need for regular food intake that characterises poikilothermic animals. The discovery of leptin in carp opens up the possibility to investigate the role of leptin in early vertebrates, as this may ultimately assist us in the elucidation of the mechanisms that are responsible for the ongoing obesity epidemic.

Acknowledgements

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CHAPTER 8

Phylogeny and evolution of vertebrate type-I cytokines

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Introduction

The type-I cytokines constitute a large group of signaling molecules that play key roles in a plethora of physiological processes including as host defense, immune regulation, somatic growth, reproduction, food intake and energy metabolism, regulation of neural growth and many more. Type-I cytokines are considered a monophyletic group, although they share little primary sequence identity[134]. This implies that a single ancestral gene that expanded by successive gene duplication events (largely in the vertebrate lineage) is at the basis of the contemporary multigene cytokine family. All type-I cytokines fold into a bundle of four α-helices and signal via related receptors that share molecular signatures[281]; moreover, following ligand binding, these receptors activate similar intracellular signaling pathways[209, 282-284]. Virtually all of our knowledge on type-I cytokine signaling derives from research on primate and rodent species[285]. Information on the presence, structure, and function of type-I cytokines in non-mammalian vertebrates and non-vertebrates is fragmentary. Consequently, our ideas about the evolution of this versatile multigene family are often based on a limited comparison of human and mouse orthologues. In the last five years, whole genome sequencing projects yielded draft genomes of the early vertebrates pufferfish (*Takifugu rubripes*), spotted green pufferfish (*Tetraodon nigroviridis*), and zebrafish (*Danio rerio*)[105, 169]. Fueled by this development, fish orthologues of a number of mammalian type-I cytokines have recently been discovered. In this review we characterise the mammalian type-I cytokine family and compare it with the emerging type-I cytokine repertoire of teleost fish. This approach offers important insights into cytokine evolution as it identifies the cytokines shared by fish and mammals that, consequently, existed before the divergence of teleosts and tetrapods. A 'fish-mammalian' comparison will identify the cytokines that still await discovery in fish, or, alternatively may have been evolutionarily recent additions to the mammalian cytokine repertoire. All fish type-I cytokines described to date share limited primary sequence identity with their mammalian orthologues. In contrast, the gene structure of cytokines is conserved throughout vertebrates, a characteristic that is instrumental in the identification of orthologous relationships within the multigene cytokine family that is characterised by poor primary sequence conservation. Even though the fish cytokine repertoire is as yet not completely known, fish cytokines characterised to date reveal several examples of conspicuous differences between fish and mammalian orthologues that illustrate the eventful history of vertebrate type-I cytokines. Following an introduction on the principles of type-I cytokine receptors and their intracellular signaling that is based on mammalian studies, we separately introduce each mammalian type-I cytokine and discuss the evidence for their presence in early vertebrates.

List of abbreviations

ACTH	adrenocorticotrophic hormone
CIS	cytokine-inducible SH2-containing domain
CLC	cardiotrophin-like cytokine
CLF	cytokine-like factor
CNTF	ciliary neutrotrophic factor
CNTFR	ciliary neutrotrophic factor receptor
ConA	concanavalin A
CT-1	cardiotrophin-1
EBI3	Epstein Barr Virus-induced protein-3
EPO	erythropoietin
FDA	Food and Drug Administration
G-CSF	granulocyte-colony stimulating factor
GH	growth hormone
GHR	growth hormone receptor
GM-CSF	granulocyte-macrophage colony stimulating factor
GPI	glycosyl-phosphatidyl-inositol
HIF-1	hypoxia-inducible factor-1
HOP	hopscotch
IFN	interferon
IL	interleukin
JAK	Janus kinase
LIF	leukemia inhibitory factor
LIFR	leukemia inhibitory factor receptor
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MGF	myelomonocytic growth factor
NK	natural killer
NP	neuropoietin
OSM	oncostatin M
OSMR	oncostatin M receptor
PBL	peripheral blood leukocytes
PHA	phytohaemagglutinin
PIAS	protein inhibitors of activated STAT
PMA	phorbol myristate acetate
POMC	pro-opiomelanocortin
PRF	plethodontid receptivity factor
PRL	prolactin
TGF	tissue growth factor
TNF	tumor necrosis factor
TPO	thrombopoietin
SH2 domain	SRC homology-2 domain
SHP	SH2 domain-containing phosphatase
SL	somatolactin
SOCS	suppressor of cytokine signaling
STAT	signal transducer and activator of transcription
UPD	unpaired

Type-I cytokines share a common fold

The tertiary structure of type-I cytokines is characterised by a bundle of four tightly packed α-helices, designated helix A – D (Fig. 8.1); their primary amino acid sequences share little to no sequence similarity. Their common fold separates type-I cytokines from others. For example, type-II cytokines (interferons, IL-10, and IL-20) are homodimers of two cytokine molecules that contain six α-helices each[286, 287]. The IL-1 family, which includes IL-1β and IL-18, is characterised by a fold rich in β-strands[288]. A unique aspect of the type-I cytokine fold is that the four α-helices are arranged in an 'up-up-down-down' fashion as a result of the anti-parallel orientation of two consecutive pairs of helices. In most type-I cytokines this four-helix bundle fold is stabilised by up to three disulphide bridges. In interleukin-11 (IL-11), that lacks conserved cysteine residues, the four-helix bundle is stabilised solely by hydrophobic interactions that result from buried hydrophobic and exposed charged residues[133].

On the basis of the length of their α-helices, type-I cytokines are subdivided into either 'long-chain' or 'short-chain'[285]. The long-chain cytokines such as growth hormone

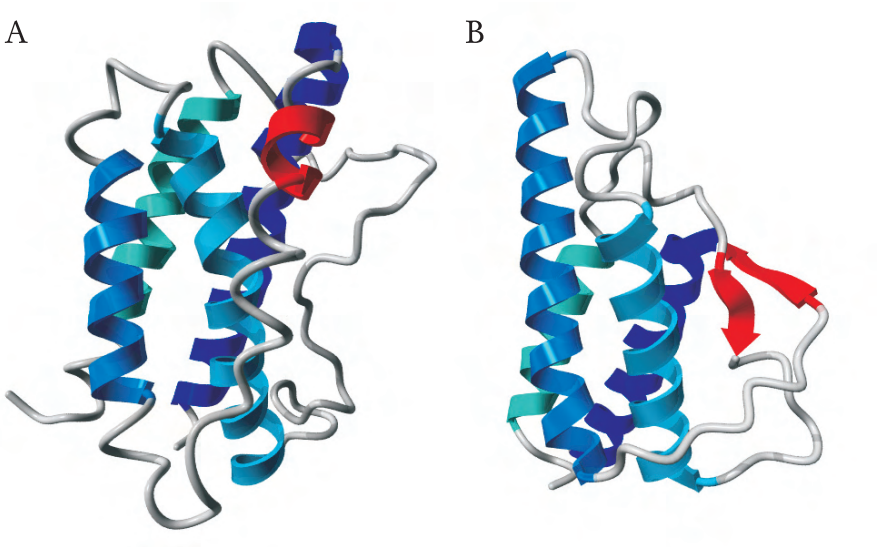

Figure 8.1: Type-I cytokines fold into a bundle of four tightly packed α-helices. On the basis of their helix length, type-I cytokines are characterised as long-chain, such as IL-6 (a), or short-chain, such as IL-4 (b). Many long-chain type-I cytokines are also recognisable by an additional short α-helix in the AB or CD loop, indicated in red in panel a. In some short-chain type-I cytokines, these loops both contain a stretch of β-strand that connect to form a β-sheet (b).

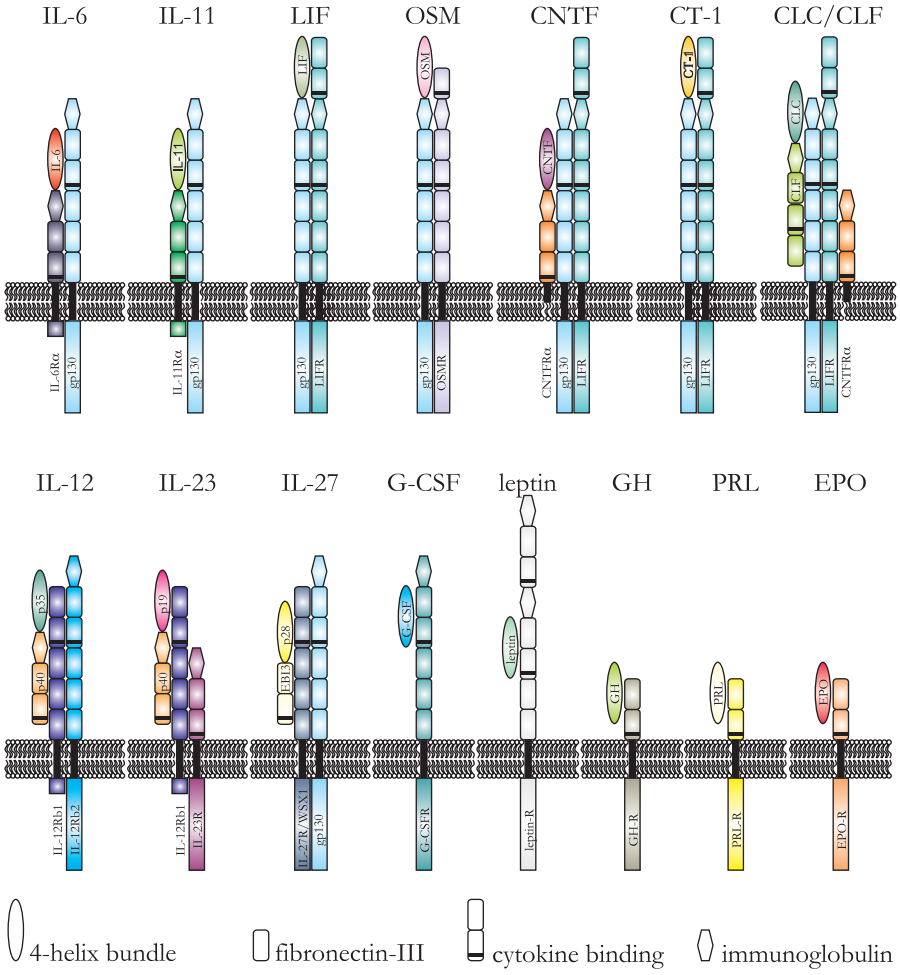

Figure 8.2: Cytokine receptors share a modular make-up. Type-I cytokines signal via a multimeric receptor complex that usually consists of several cytokine receptor chains. All receptor chains contain at least one cytokine-binding domain, formed by two fibronectin type-III domains, of which the membrane-proximal contains a consensus WSXWS motif. Many cytokine receptor chains contain an immunoglobulin domain as well as several fibronectin type-III domains in addition to the pair that forms the cytokine-binding domain. The typical cytokine receptor complex is composed of a ligand-specific α-chain that lacks intracellular signaling capacity and a signaling β-chain that is shared by multiple receptor complexes.

(GH), prolactin (PRL), leptin, erythropoietin (EPO), and the GP130 cytokines (see below) are made up of 170 – 250 amino acid residues, the of 'short-chain' cytokines (*i.a.* IL-2, IL-3, IL-4, IL-13) typically do not exceed 160 amino acids. An additional difference between the long- and short-chain type-I cytokines resides in the loops that connect α-helices A, B, C, and D. Given the anti-parallel orientation of the A-B and C-D helix pair, the loop connecting helix B and C (the BC-loop) is invariably short. In contrast, the AB- and CD-loops are much longer, as they span the entire length of the helix bundle. In short-chain cytokines, the AB- and the CD-loops often connect to form a small section of β-sheet (Fig. 8.1). In contrast, the AB- or CD-loop in several long-chain type-I cytokines contains an additional short α-helix.

The modular make-up of cytokine receptors

Cytokines exert their actions via cell surface receptors. Extracellularly, type-I cytokine receptors share a modular make-up of several fibronectin type III domains and usually a single immunoglobulin domain (Fig. 8.2). Furthermore, all receptor chains contain one or two cytokine binding domains, formed by a pair of fibronectin type-III domains, of which the membrane proximal domain contains a characteristic WSXWS signature[281,282]. The WSXWS motif is required for proper receptor folding, but is not directly involved in ligand binding[282,289]. Whereas some type-I cytokine receptors have long intracellular domains, the cytoplasmatic tails of others are short, and incapable of intracellular signaling. Functional receptor complexes are formed by the combination of long, signaling (or β-) chains and short, non-signaling (or α-) chains (Fig. 8.2) that determine the ligand-specificity of the receptor complex[209,290]. The interleukin-6 (IL-6) receptor complex provides a prototypical example: IL-6 binds with high affinity to its specific α chain (IL-6Rα) and the IL-6/IL-6Rα complex subsequently recruits GP130 as the signaling β-chain[291]. The stoichiometry of this receptor complex indicates a hexamer consisting of doublets of the cytokine, the α-chain, and the β-chain[292]. The GP130 signaling chain is shared by several cytokine receptor complexes[293]. For that reason, the matching cytokines are collectively referred to as 'GP130 cytokines'. This group includes IL-6, IL-11, ciliary neutrotrophic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM), and cardiotrophin-1 (CT-1). The IL-11Rα and CNTFRα resemble the IL-6Rα in that they are short and cannot signal intracellularly. A characteristic feature of the CNTFRα is the lack of a transmembrane domain and its anchoring via a glycosylphosphatidylinositol (GPI)-anchor. The IL-11 and CNTF receptor complexes are hexamer, similar in conformation to the IL-6 receptor[136, 209, 294]. The CNTF receptor complex contains a single GP130 chain instead, the second GP130

chain is replaced by the LIFR (Fig. 8.2), a signaling chain similar to GP130. The other GP130 cytokines, OSM, LIF, and CT-1, bind directly and with high affinity to signaling β-chains. Their receptor complexes lack α-chains and consist of a combination of GP130 and LIFR (LIF and CT-1), or GP130 and OSMR (OSM) (Fig. 8.2)[209].

Some members of the type-I cytokine receptor family lack a transmembrane domain, effectively making them soluble rather than membrane-bound. Interleukin-12 (IL-12) provides the prototypical example of such a soluble receptor. IL-12 is a heterodimeric cytokine that consists of two disulphide-linked subunits, designated P35 and P40. Whereas P35 is a typical four-helix bundle type-I cytokine, P40 is effectively a short soluble type-I receptor with a cytokine binding domain and an immunoglobulin domain (Fig. 8.2). The receptor for IL-12 is a heterodimer that consists of IL-12Rβ1 and IL-12Rβ2, receptor chains that both resemble GP130[193]. IL-12 is usually not listed as a GP130 cytokine, yet the recent characterization of several novel heterodimeric cytokines, including interleukin-23 (IL-23) and interleukin-27 (IL-27), and their receptors illustrates that the emerging IL-12 family of heterodimeric cytokines is a mere variation on the common GP130 theme. IL-23 is a disulphide-linked heterodimer of the P40 subunit and the newly identified P19 type-I cytokine subunit[196]. Its receptor complex is a combination of the IL-12Rβ1 chain with the newly identified IL-23R[217]. Another novel heterodimeric cytokine, IL-27, is a covalently linked combination of the soluble cytokine receptor Epstein Barr virus-induced protein-3 (EBI3) and the type-I cytokine P28[197]. The IL-27 receptor complex consists of the IL-27R (also known as WSX1) and GP130[195, 197]. The shared use of the latter receptor chain illustrates the close parallels between the original GP130 cytokines and the growing family of heterodimeric cytokines. These parallels are further evidenced by the type-I cytokine cardiotrophin-like cytokine (CLC) that circulates as a dimer with the soluble receptor molecule cytokine-like factor (CLF). The CLC/CLF complex signals via the CNTF receptor complex (Fig. 8.2). The heterodimer conformation of CLC/CLF is similar to that of IL-12, although both subunits are not linked via a disulphide bridge[295]. The non-covalently linked heterodimer conformation of CLC/CLF is not unique among the GP130 cytokines. Similar heterodimers of IL-6, IL-11, and CNTF with truncated, soluble versions of each of their specific α-chains occur[209]. This adds an extra level of complexity to GP130 signaling, since these circulating heterodimers convey signals to cells that express only the GP130 chain and lack the endogenous specific receptor α-chain[282].

The remaining long-chain type-I cytokines all signal via homodimers of receptor chains that combine high affinity ligand-binding with intracellular signaling. The receptors for granulocyte-colony stimulating factor (G-CSF) and leptin are similar to GP130 in size and extracellular composition. In contrast, the receptors for GH, PRL, EPO, and thrombopoietin (TPO) are notably shorter and consist of a single cytokine binding domain connected to a long cytoplasmatic tail (Fig. 8.2). Despite these differences, the

early studies that addressed the complex of GH and its receptor are at the basis of our knowledge of type-I cytokine receptor complex formation and stoichiometry[290, 291], as the crystal structure of the GH-GHR complex was the first cytokine/receptor complex that was solved[296].

Intracellular signaling pathways, phosphorylation is the message

Intracellularly, the recruitment of the multimeric cytokine receptor complex upon cytokine binding brings together the signaling domains of two β-chains, leading to the activation of several distinct intracellular signaling cascades. Each cytoplasmatic domain of GP130 and related signaling chains is constitutively associated with a tyrosine kinase of the JAK family (Janus kinase; after the two-faced, Roman god Janus, referring to JAKs dual (pseudo)-catalytic domains). Dimerisation of two β-chains leads to JAK activation via the transphosphorylation of both JAKs[297]. Phosphorylated JAKs, in turn, phosphorylate several intracellular membrane-distal tyrosine residues in the cytoplasmatic domain of the receptor chain. The phosphorylated tyrosines serve as docking site for members of the STAT (signal transducer and activator of transcription) family that bind to phosphorylated tyrosine through their SRC homology-2 (SH2) domain[283]. The mammalian STAT family consists of seven members (STAT1, -2, -3, -4, -5a, -5b, and 6) that are phosphorylated upon docking to a phosphorylated tyrosine. Phosphorylated STATs dissociate from the receptor chain and form STAT homo- or heterodimers that are translocated into the nucleus to initiate transcription[298-300]. Which genes are transcribed depends on the composition of the STAT dimer. As untimely or prolonged JAK/STAT signaling can shift the balance from adequate immune regulation and host defense to a derailed immune response with potentially serious consequences, multiple inhibitory mechanisms control JAK/STAT activity.

One of these inhibitory mechanisms is formed by SH2 domain containing tyrosine phosphatases (SHP), such as SHP1 and SHP2. These phosphatases dephosphorylate tyrosines of key signaling components such as JAKs, STATs, and cytokine receptors, thereby abrogating cytokine signaling[209, 297, 301-303].

The protein inhibitors of activated STAT (PIAS) constitute a second class of JAK/STAT signaling inhibitors. Currently five PIAS members have been identified (PIAS1, PIAS3, PIASxα, PIASxβ, and PIASy) . By binding to activated STAT dimers, PIAS1 and PIAS3 directly prevent association with DNA, whereas PIASx or PIASy prevent activated STAT from transcription without directly interfering with DNA-binding[297].

Among the many target genes that are transcribed in response to JAK/STAT activation

is one particular group of genes that encodes members of the SOCS (suppressor of cytokine signaling) family. Currently the SOCS family consists of eight members (CIS and SOCS1-7); the role of SOCS4-7 in cytokine signaling has received limited attention. The SOCS proteins exert a classical negative feedback. Like STATs, SOCS proteins contain a central SH2 domain that allows them to bind phosphorylated tyrosine residues[209]. SOCS proteins suppress in several ways: SOCS1 directly binds to phosphorylated JAKs and inhibits JAK catalytic activity. CIS, SOCS2, and SOCS3 bind directly to phosphorylated cytokine receptor tyrosines, effectively blocking STAT binding[297, 303-305].

Redundancy and specificity in cytokine signaling

Many type-I cytokines, such as IL-6 and IL-11, and LIF and OSM, display considerable overlap in their spectrum of biological actions. This redundancy is explained by the shared use of the GP130 signaling chain that funnels the intracellular response that follows receptor activation along the same signaling cascade. The cell's response is eventually determined by integration of a plethora of biological signals that may differ among cell type. The redundancy that stems from the shared use of GP130 is illustrated by the embryonically lethal phenotype of GP130 knockout mice; which is considered to result from the cumulative effects of the loss of signaling capacity for a large group of cytokines[306].

Besides the overlap in cytokine receptor conformation, redundancy in cytokine signaling is achieved by promiscuity of components of the JAK/STAT signaling pathway. Four JAKs (JAK1-3 and TYK2) and seven STATs have been identified in mammals. Together they constitute the intracellular mechanism that is responsible for signaling in response to at least 30 different type-I cytokines. This can only be achieved when different cytokines share an intracellular signaling cascade. The involvement of the various JAKs and STATs in signaling in response to specific (groups of) type-I cytokines has been defined by assessing the phenotypes of mice lacking specific components of JAK/STAT signaling. The absence of JAK1 leads to a complex perinatally lethal phenotype that is characterised by neurological as well as lymphoid defects[297]. JAK1$^{-/-}$ mice fail to respond to interferons and lack signaling capacity via the γc-receptor chain, which is the receptor chain responsible for intracellular signal transduction in the receptor complex of many short-chain type-I cytokines[283]. The phenotype of JAK3 knockout mice in part resembles that of the JAK1 knockout, but is less severe. JAK3$^{-/-}$ mice are viable, although they suffer from severe combined immunodeficiency (SCID)[297, 302]. This immunological defect is caused by abrogated γc-receptor signaling and since the γc-receptor$^{-/-}$ and JAK3$^{-/-}$ phenotypes are

identical, it follows that JAK3 is only employed by the short-chain type-I cytokines that signal via this common receptor chain[307]. JAK2 is employed by all type-I cytokines that signal via a homodimeric receptor. This group includes leptin, PRL, GH, EPO, TPO, and G-CSF[283, 284]. Phenotypically, JAK2$^{-/-}$ mice are characterised by defective erythropoiesis as a consequence of silenced EPO signaling[297, 302]. This renders JAK2 knockouts embryonically lethal, before a phenotype of defective signaling by any of the other cytokines that depend on JAK2 becomes manifest. The TYK2 knockout is viable, which is a mild phenotype compared to the phenotypes described earlier for JAK1-3. TYK2 can be activated in response to interferon-α/β, IL-10 and various GP130 cytokines, but appears only critically required for IL-12 signaling[283, 284, 302]. Due to this defective IL-12 signaling and possibly diminished interferon signaling capacity, TYK2$^{-/-}$ mice are susceptible to (viral) pathogen infection, but appear otherwise normal[308, 309].

STAT3 is the principle signal transducer that is activated in response to GP130-cytokines as well as leptin and G-CSF. The type-I cytokines that bind receptors with short extracellular domains (GH, PRL, EPO, and TPO) all engage STAT5[283, 284]. Duplicated and highly similar STAT5 genes, designated STAT5a and STAT5b, are encoded in both the human (locus 17q11.2) and mouse (locus 11D) genome[310]. Although both are activated by the cytokines that depend on STAT5 *in vitro*, STAT5a$^{-/-}$ and STAT5b$^{-/-}$ mice each have a specific phenotype. Female STAT5a knock-outs have impaired mammary gland development and fail to lactate as a consequence of abrogated PRL signaling[311]. In contrast STAT5b$^{-/-}$ mice display growth defects due to defective GH signaling[312].

Despite the general redundancy of intracellular signaling pathways, the use of some STATs is highly restricted to a single cytokine, or a small group of related type-I cytokines. These restrictions are so specific, that activation of these STATs has become synonymous for the activation of distinct immunological pathways. This is pre-eminently illustrated by the specific role of STATs in the determination of TH1 and TH2 immune responses. The TH1/TH2 dogma states that the modality of an immune response depends on and is tailored for the effective eradication of the eliciting pathogen. TH1 and TH2 refer to the extremes of a continuous spectrum of immune responses and are, as a rule of thumb, associated with intracellular and extracellular pathogens, respectively. Both TH1 and TH2 responses are associated with a panel of 'signature' cytokines[313, 314]. IL-12 is a key cytokine in the determination of TH1 responses that, when released from activated macrophages, induces IFNγ production in T-cells[178, 180]. A TH2 response, in contrast, is associated with the release of IL-4, IL-5, and IL-13, a group of related short-chain type-I cytokines that uses similar receptors[315, 316]. TH1 cytokines inhibit the expression of TH2 cytokines and *vice versa*, thus the balance between TH1 and TH2 cytokines determines the outcome of the overall immune response. And whereas most STATs are promiscuous to some degree, TH1 and TH2 signalling each involve a unique STAT: IL-4 and IL-13 activate STAT6 whereas IL-12

relies solely on STAT4[283, 284, 298]. Consequently, the activation of STAT4 or STAT6 is telltale of the type of immune response unfolding[178].

Type-I cytokines in teleosts

In the next paragraphs we separately introduce the long-chain type-I cytokines and discuss evidence for orthologous non-mammalian cytokines. Cytokines that are found on the same locus in mammals (e.g. LIF and OSM) are discussed together, as are cytokines that share distinct structural characteristics and/or functional properties such as the heterodimeric cytokines. Also the order in which the various (pairs of) cytokines are covered is loosely determined by shared features such as gene structure and shared cysteine residues.

Interleukin-6

The pleiotropic nature of interleukin-6 (IL-6) is aptly illustrated by the history of its discovery. The cloning and characterisation of IL-6 by four separate groups[317-320] revealed that a variety of factors known as B-cell stimulatory factor 2, B-cell differentiation factor, hybridoma-plasmacytoma growth factor, interferon-β2, hepatocyte-stimulating factor, T-cell replacing factor-like factor, and monocyte-granulocyte inducer type 2 were in fact one and the same. IL-6 is an important inducer of antibody production, although the finding that IL-6 deficient mice display reduced IgG, but normal early IgM responses to *Vaccinia* virus infection indicates that IL-6 is not equally important for the production of all Ig isotypes[321]. IL-6 also has the capacity to stimulate T-cells, and participates in haematopoiesis by inducing the proliferation of pluripotent progenitor cells. In addition, IL-6 stimulates the differentiation of myeloid progenitors into granulocytes and macrophages and promotes megakaryocyte maturation[282]. In the liver, IL-6 is one of the major inducers of the acute phase reaction, in concert with IL-1β and TNFα[322]. An interesting property of IL-6 is that it is one of several cytokines that can modulate the hypothalamic-pituitary-adrenal axis, thus directly influencing the stress response[323]. The IL-6 gene in human and mouse consists of five exons[324] and the four-helix bundle topology of IL-6 is stabilised by two internal disulphide bridges. The elucidation of the pufferfish genome has revealed the existence of IL-6 in fish (Fig. 8.3). The pufferfish IL-6 gene is similar in organization to that of mammalian IL-6 and G-CSF genes. Pufferfish IL-6 shares two conserved cysteine residues with mammalian IL-6 and G-CSF sequences but lacks their N-terminal cysteine pair (Fig. 8.4). In-vitro stimulation of pufferfish head kidney cells with the T-cell activator phytohaemagglutinin (PHA) rapidly upregulates IL-6 expression,

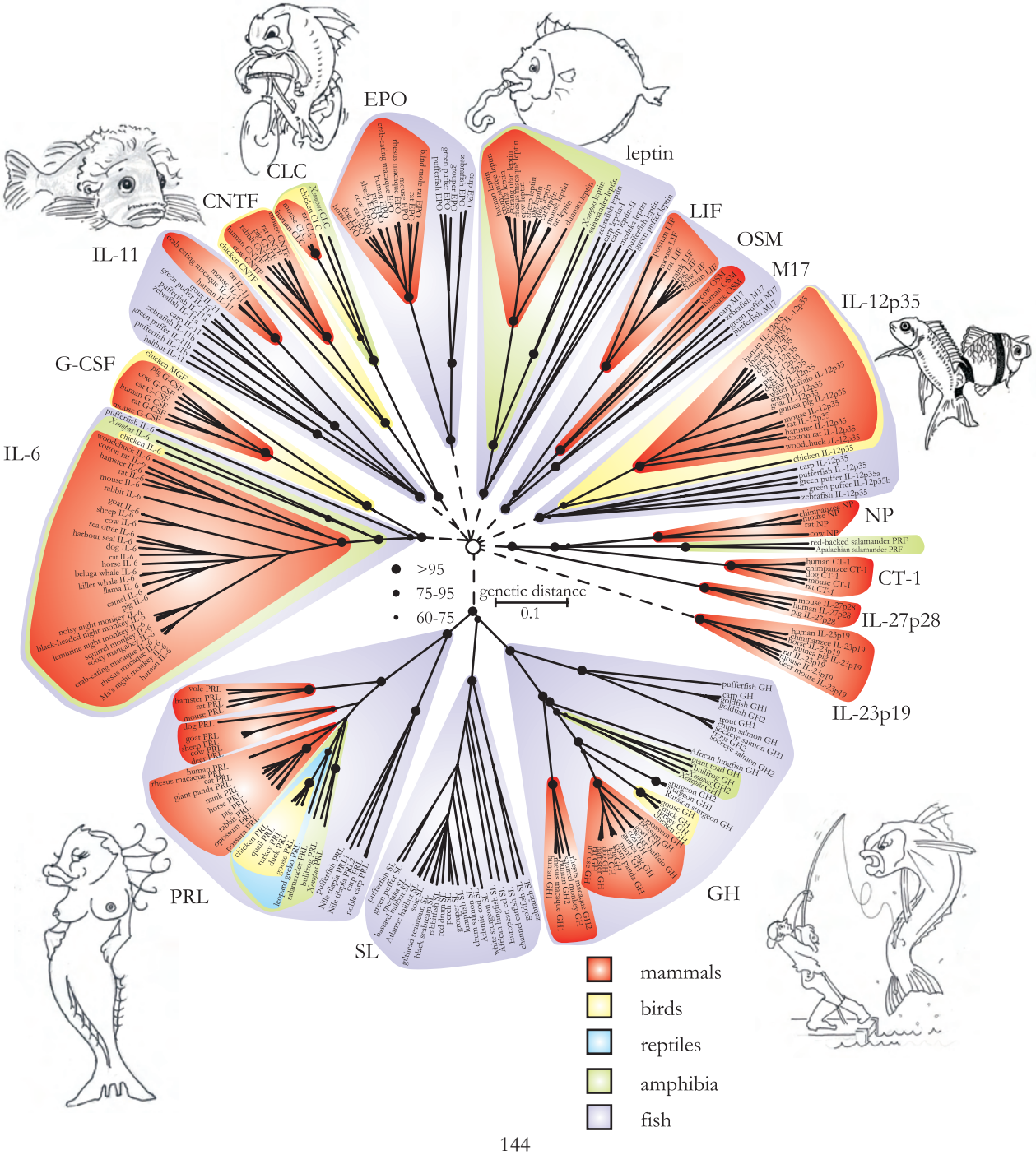
EPO
leptin
CLC
CNTF
LIF
OSM
M17
IL-11
IL-12p35
G-CSF
IL-6
NP
CT-1
IL-27p28
IL-23p19
PRL
GH
SL
>95
75-95
60-75
genetic distance
0.1
mammals
birds
reptiles
amphibia
fish

Figure 8.3: Phylogeny of the vertebrate type-I cytokines. For most mammalian type-I cytokines non-mammalian orthologues have been identified to date. The amino acid sequence alignment obtained by T-COFFEE[411], was refined by hand to correct for overt alignment mismatches. Phylogenetic analysis of such a large number of amino acid sequences (>250 sequences), which are only weakly similar both within a single species (paralogues) as well as between distantly related species (orthologues), is sensitive to artefactual topologies. To prevent this from occurring, constraints (indicated by dashed lines) were introduced for the deep topology of the tree based on the information regarding gene organisation, chromosomal location, and conserved cysteine pairs, which is summarised in Fig. 8.4. The phylogeny was reconstructed on the basis of a amino acid differences (p-distance) in MEGA 3.0[250] using the neighbor-joining algorithm. The tree obtained via the minimum evolution algorithm had an essentially identical topology (not shown). Branch lengths reflect the extent of the genetic distance between sequences. The confidence level of 1000 bootstrap replications is indicated by the size of the dots at the branch nodes. Clusters of mammalian, avian, reptilian, amphibian, and teleostean cytokines are indicated in red, yellow, blue, green, and lilac, respectively. Note that some clusters are notably more compact than others, reflecting a higher degree of primary sequence conservation. Accession numbers are as follows: human IL-6, P05231; rhesus macaque IL-6, P51494; crab-eating macaque IL-6, P79341; sooty mangabey IL-6, P46650; Ma's night monkey IL-6, AF014510; squirrel monkey IL-6, AF294757; lemurine night monkey IL-6, AF097323; black-headed night monkey IL-6, AF097322; noisy night monkey IL-6, AF014505; mouse IL-6, P08505; rat IL-6, P20607; rabbit IL-6, AAF86660; hamster IL-6, BAA78766; cotton rat IL-6, AF421389; woodchuck IL-6, O35736; cow IL-6, P26892; pig IL-6, P26893; sheep IL-6, P29455; horse IL-6, Q95181; camel IL-6, AB107656; llama IL-6, AB107647; goat IL-6, Q28319; cat IL-6, P41683; dog IL-6, P41323; killer whale IL-6, Q28747; beluga whale IL-6, AAD42929; harbour seal IL-6, Q28819; sea otter IL-6, AAB01428; chicken IL-6, CAC40812; *Xenopus* IL-6, AW63707; pufferfish IL-6, NM_001032722; mouse G-CSF, P09920; rat G-CSF U37101; human G-CSF, P09919; cat G-CSF, NM_001009227; cow G-CSF, AF092533; pig G-CSF, U68481; chicken MGF, NM_205279; human IL-11, P20809; crab-eating macaque IL-11, P20808; mouse IL-11, P47873; rat IL-11, AAK29623; carp IL-11, AJ632159; halibut IL-11, AU090873; pufferfish IL-11a, BN000713; pufferfish IL-11b, BN000714; green puffer IL-11a, BN000715; green puffer IL-11b, AY374508; zebrafish IL-11a, BN000717; zebrafish IL-11b, BN000718; trout IL-11, AJ535687; horse EPO, AB100030; cow EPO, P48617; dog EPO, P33707; cat EPO, P33708; human EPO, P01588; crab-eating macaque EPO, P07865; rhesus macaque EPO, Q28513; mouse EPO; P07321; rat EPO, P29676; sheep EPO, P33709; blind mole rat EPO, AJ715792; pig EPO, NM_214134; pufferfish EPO, AY303753; zebrafish EPO, GENSCAN00000009653; carp EPO, AJ831393; green puffer EPO, AY374507; grouper EPO, AY735012; human IL-12P35, P29459; rhesus macaque IL-12P35, P48091; mouse IL-12P35, P43431; rat IL-12P35, AAD51364; hamster IL-12P35, AB085791; woodchuck IL-12P35, X97018; guinea pig IL-12P35, AB025723; cotton rat IL-12P35, AF421396; cowIL-12P35, P54349; water buffalo IL-12P35, AY232819; deer IL-12P35, U57751; pig IL-12P35, Q29053; horse IL-12P35, Q9XSQ6; sheep IL-12P35, Q9TU27; goat IL-12P35, O02814; dog IL-12P35, Q28267; cat IL-12P35, O02743; carp IL-12P35, AJ580354; zebrafish IL-12P35, AB183001; chicken IL-12P35, AY262751; pufferfish IL-12P35, AB096265; green puffer IL-12P35a, AY374509; green puffer IL-12P35b, AY374510; human IL-23P19, AF301620; chimpanzee IL-23P19, AY412450; mouse IL-23P19, AF301619; rat IL-23P19, NM_130410; deer mouse IL-23P19, AY259629; guinea pig IL-23P19, AB058509; horse IL-23P19, AY704416; mouse IL-27P28, AY099297; human IL-27P28, AY099296; pig IL-27P28, AY788913; human CT-1, Q16619; chimpanzee CT-1, XM_523348; mouse CT-1, Q60753; rat CT-1, Q63086; dog CT-1, XM_547034; chimpanzee NP, Q6R2R2; mouse NP, P83714; rat NP, AY518205; cow NP, XM_609151; red-backed salamander PRF, AY926884; Appalachian salamander PRF, AY926937; human leptin, P41159; chimpanzee leptin, O02750; gorilla leptin, Q95189; orang utan leptin, Q95234; rhesus macaque leptin, Q28504; mouse leptin, P41160; rat leptin, P50596; cow leptin, P50595; sheep leptin, Q28603; pig leptin, Q29406; dog leptin, O02720; cat leptin, AB041360; dunnart leptin, AF159713; *Xenopus* leptin, AY884210; salamander leptin, CN054256; medaka leptin, AB193548; zebrafish leptin, GENSCAN00000007598; pufferfish leptin, AB193547; green puffer leptin, AB193549; carp leptin-I, AJ830744; carp leptin-II, AJ830745; human CLC, AF176912; mouse CLC, Q9QZM3; rat CLC, NM_207615; chicken CLC, XM_427323; *Xenopus* CLC, CR762259; human CNTF, P26441; rat CNTF, P20294; mouse CNTF, P51642; pig CNTF,

continued on next page

Fig. 8.3, continued,
O02732; cow CNTF, XM_607445; rabbit CNTF, P14188; chicken CNTF, Q02011; carp M17, AY102632; green puffer M17, GSTENP00017261001; zebrafish M17, ENSDARP00000047694; pufferfish M17, SINFRUP00000170397; cow LIF, Q27956; human LIF, P15018; mouse LIF, P09056; rat LIF, P17777; mink LIF, O62728; pig LIF, Q9GKZ8; hamster LIF, AY171245; possum LIF, AF303448; cow OSM, P53346; human OSM, P13725; mouse OSM, P53347; human GH1, P01241; human GH2, P01242; mouse GH, P06880; rat GH, P01244; rhesus macaque GH1, P33093; rhesus macaque GH2, Q07370; squirrel monkey GH, AF339060; cow GH, V00111; dog GH, Z23067; cat GH, U13390; water buffalo GH, X72947; mink GH, X56120; giant panda GH, AF540936; guinea pig GH, AF233853; hamster GH, S66299; possum GH, AF052192; opossum GH, AF312023; pig GH, AY536527; goat GH, Y00767; chicken GH, P08998; turkey GH, M33697; duck GH, X07079; goose GH, AY149895; *Xenopus* GH1, P12855; *Xenopus* GH2, P12856; bullfrog GH, AY251538; giant toad GH, AF062746; carp GH, P10298; pufferfish GH, O12980; goldfish GH1, O93359; goldfish GH2, O93360; trout GH1, P09538; trout GH2, P20332; sockeye salmon GH1, Q91222; sockeye salmon GH2, Q91221; chum salmon GH, P07064; Russian sturgeon GH, AY941176; sturgeon GH1, P26773; sturgeon GH2, P26774; African lungfish GH, AF062745; human PRL, P01236; rhesus macaque PRL, U09018; mouse PRL, P06879; rat PRL, P01237; hamster PRL, S66296; vole PRL, AF178933; cat PRL, U25974; rabbit PRL, Q28632; deer PRL, AY373035; sheep PRL, X13483; goat PRL, X76049; cow PRL, V00112; horse PRL, AY373339; dog PRL, AY741405; mink PRL, P29234; giant panda PRL, AY161285; pig PRL, NM_213926; opossum PRL, AF067726; possum PRL, AF054634; chicken PRL, P14676; quail PRL, AB162003; duck PRL, AB158610; turkey PRL, U05952; goose PRL, AY993962; leopard gecko PRL, AB182277; bullfrog PRL, X16063; salamander PRL, AY332494; *Xenopus* PRL, BC075216; pufferfish PRL, SINFRUP00000132442; carp PRL, X12543; Nile tilapia PRL1, A07820; Nile tilapia PRL2, A07824; noble carp PRL, X61049; zebrafish SL, AJ867249/ ENSDARP00000023510; goldfish SL, P79697; green puffer SL, AY374504; pufferfish SL, SINFRUP00000163505; red drum SL, AF062520; rabbitfish SL, AB026186; black seabream, SLAY714370; gilthead seabream SL, L49205; sole SL, U06753; medaka SL, AY530202; Atlantic cod SL, D10639; European eel SL, U63884; channel catfish SL, AF267991; lumpfish SL, L02118; grouper SL, AY129310; perch SL, AY332490; chum salmon SL, D10640; Atlantic halibut SL, L02117; bastard halibut SL, M33696; African lungfish SL, O73847; white sturgeon SL, AB017200.

demonstrating its expression and upregulation within the leukocyte compartment[325].

Granulocyte-colony stimulating factor

Circulating neutrophilic granulocytes constitute an essential component of innate immunity and respond rapidly to local pathogenic insults by local margination. The circulating neutrophil population is short-lived and is continuously replenished from the bone marrow haematopoietic precursor population. Granulocyte-colony stimulating factor (G-CSF) is one of the principle regulators of neutrophil differentiation and activation. In the bone marrow, stromal cell-derived G-CSF stimulates the proliferation and maturation of bone marrow granulocytic progenitors and promotes the release of neutrophils into the circulation[326]. Moreover, peripherally administered G-CSF induces a rapid (minutes) margination of circulating neutrophils, resulting in acute neutropenia that is gradually (hours) resolved by the release of neutrophils from the bone marrow[327]. The main phenotypic aberrations of G-CSF$^{-/-}$ mice are a reduced number of bone-marrow neutrophil progenitor cells accompanied by moderate neutropenia, which is in line with the actions of G-CSF described above[328]. Although G-CSF is often not included in the list of GP130 cytokines (since its receptor complex lacks the GP130 chain), the organisation of

the G-CSF gene is similar to that of IL-6[291]. Not only do both genes consists of five exons of similar length, the four conserved cysteine residues that make up the two disulphide bridges of IL-6 are identically spaced in G-CSF (Fig. 8.4). This suggests that IL-6 and G-CSF are related within the large type-I cytokine family. Chicken myelomonocytic growth factor (MGF) represents an avian G-CSF orthologue that shares limited similarity with mammalian IL-6 and G-CSF sequences (Fig. 8.3)[329]. Its gene structure is also reminiscent of that of G-CSF, and cMGF expression is upregulated in response to LPS in vitro and in vivo[330, 331]. To date, no G-CSF-like molecule has been described outside mammalian and avian species.

Interleukin-11

The discovery of interleukin-11 (IL-11) as a growth factor that drives the proliferation of an 'IL-6 dependent' plasmacytoma is illustrative of the redundancy within the type-I cytokine family, but also indicates the close relation between IL-6 and IL-11[132]. Like IL-6, IL-11 is encoded by five exons, although it lacks the conserved cysteine pairs that stabilise the four-helix bundle conformation of other cytokines, including IL-6. Instead, IL-11 is stabilised solely by the hydrophobic interactions that result from the convergence of hydrophobic residues at the buried side of each α-helix, whereas charged residues abound at the solvent exposed surface of each helix[133]. IL-11 is no exception with regard to the pleiotropy characteristic of type-I cytokines. It is an important growth factor of haematopoietic lineages that give rise to neutrophilic granulocytes, erythrocytes, and megakaryocytes[143, 145, 146]. The stimulatory effects on the megakaryocytic lineage in particular, have led to the FDA (Food and Drug Administration) approval of IL-11 as a therapeutic agent in the prevention of thrombocytopenia resulting from severe bone-marrow ablative chemotherapy[147]. IL-11 also exerts protective effects on mucosa of respiratory organs and the gastro-intestinal tract. The inhibitory actions of IL-11 on macrophage reactive oxygen species production and pro-inflammatory cytokine release are considered to be the underlying mechanism of the protective effects on mucosa[150-152].

IL-11 has been considered as 'evolutionary conserved' based on the primary sequence conservation of a limited number of primate and rodent orthologues. IL-11 orthologues have been characterised recently in carp[227] and rainbow trout[161] that share only limited primary sequence identity (maximally 31%) with mammalian IL-11. In fish, IL-11 is ubiquitously, albeit not very abundantly, expressed in most organs and tissues examined[161, 227]. Differences in the IL-11 expression between organs are less pronounced in carp than in trout, where gills and intestine express more IL-11 than any other organ. This may reflect protective effects of IL-11 at mucosal surfaces throughout vertebrates. IL-11 expression in carp head kidney primary macrophages or a trout RTS-11 monocyte/macrophage cell line is reproducibly upregulated in response to various pro-inflammatory stimuli such as LPS, ConA, polyI:C, and recombinant IL-1β[161, 227], establishing

the involvement of IL-11 in the immune response from fish to mammals.

In addition to the orthologues of carp and trout IL-11 that are discussed above, a second IL-11 gene is present in the genome of evolutionarily distantly related fish species such as zebrafish (*Danio rerio*), spotted green pufferfish (*Tetraodon nigroviridis*), and pufferfish (*Takifugu rubripes*) (Fig. 8.3)[227]. Both fish IL-11 paralogues, referred to as IL-11a (which includes carp and trout IL-11) and IL-11b, share limited amino acid identity (27 – 29%), but have similar gene structures that closely resemble those of mammalian IL-11 genes (Fig. 8.4). In line with mammalian IL-11, neither fish IL-11 paralogue contains a pair of conserved cysteines, although all fish IL-11 proteins do contain a single conserved C-terminal cysteine of unknown significance (Fig. 8.4). The extensive sequence dissimilarity of both paralogous fish IL-11 proteins, coupled to their presence in phylogenetically disparate species pinpoints their origin to early in the teleostean lineage[227]. The genome duplication that has occurred around this time[93] provides a plausible explanation for the fish specific duplication of IL-11 genes.

Leukemia inhibitory factor and oncostatin M

The genes encoding leukemia inhibitory factor (LIF) and oncostatin M (OSM) are located in close proximity on the same locus in human and mouse[332]. The same is true for the LIFR and OSMR, suggesting that LIF and OSM as well as the genes encoding their receptors arose through gene duplication (Fig. 8.4). Both cytokines were initially identified as potent regulators of tumor cell proliferation, but have since been implicated in an impressive number of diverse and partially redundant functions[333]. OSM was originally identified as a product of PMA-stimulated macrophage-like cells that inhibited the proliferation of a human melanoma cell line[334]. LIF was initially discovered for its potential to limit self-renewal and induce macrophage maturation of an undifferentiated murine myeloid cell line[335]. Subsequently LIF and OSM were both found to be essential for the maintenance of embryonic stem cell pluripotency[336-338]. Although these observations are intuitively opposite from a functional perspective, they do illustrate that LIF and OSM are important factors in the regulation of proliferation and differentiation of cells of diverse origins. Additional actions of OSM and LIF include the induction of hepatocyte acute phase

Figure 8.4: The gene structure of orthologous cytokines is conserved throughout vertebrates, a feature that is instrumental in the identification of orthologous relationships within the multigene cytokine family, which is characterised by limited primary sequence conservation. Boxes represent exons and are drawn to scale; numbers indicate their size in nucleotides. Grey bars indicate conserved cysteine residues, which are generally conserved in their presence and spacing between orthologous cytokines. The numbers beneath the human and mouse cytokine names reflect their chromosomal locations. The carp and pufferfish icons reflect the gene structure of cyprinid (*Cyprinidae*) and puffer (*Tetraodontidae*) species, respectively. Note that the human NP gene is a pseudogene due to an eight-nucleotide deletion in its third exon, indicated by the gap.

IL-6
IL-6
7p21
IL-6
3B1
G-CSF
17q11.2-q12
G-CSF
11D
IL-11a
IL-11a
IL-11b
IL-11b
IL-11
19q13.3-
IL-11
7A1
IL-12p35
IL-12p35
3p12-q3.2
IL-12p35
3D
CT-1
16p11.2-
CT-1
7F3
NP
NP
7F3
IL-23p19
12q13.3
IL-23p19
10D3
IL-27p28
16p11
IL-27p28
7F3
leptin
leptin
7q31.3
leptin
6A3.2
M17
LIF
22q12.2
LIF
11A1
OSM
22q12.2
OSM
11A1
CNTF
11q12.2
CNTF
19A
CLC
11q13.3
CLC
19A
EPO
EPO
EPO
7q22
EPO
5G
PRL
PRL
PRL
6p22.2-
PRL
13A3.1
SL
SL
GH
GH
17q24.2
GH
11D

protein production[339, 340] and the stimulation of various haematopoietic lineages, mostly in concert with other growth factors[333, 341]. LIF and OSM also stimulate ACTH production in the pituitary[323, 342, 343] and affect the secretion of PRL and GH[344]. Moreover, LIF is required for blastocyst implantation[345, 346] and exerts a multitude of proliferative and differentiating effects on various neuronal cell populations[333]. The extensive redundancy between LIF and OSM is at least in part explained by the fact that human (but not mouse) OSM can signal via the LIF receptor complex in addition to its own receptor[341].

LIF and OSM genes both have three exons and share four conserved cysteine residues that form a pair of intra-chain disulphide bridges (Fig. 8.4)[347]. LIF has an additional third disulphide bridge, but the most conspicuous structural difference between LIF and OSM is the observation that the latter cytokine is synthesised as a pre-pro-protein: in addition to an N-terminal signal peptide (pre) OSM possesses a C-terminal (pro) region of 31 amino acids (57 in mouse) that is removed to yield a mature OSM with increased biological activity[348].

M17

In 2003, a novel cytokine was reported in common carp that shares features with several different mammalian type-I cytokines. As this resemblance to multiple mammalian cytokines precludes the assignment of one-to-one orthology, this carp cytokine was designated M17 after the original clone number assigned to different clones of a carp leukocyte cDNA library[164]. The M17 protein shares little yet highest sequence identity with chicken CNTF (25%), and its amino acid identity with human CT-1, IL-11, OSM, and LIF ranges from 19 – 15%. M17 is predominantly expressed in brain and to some extent in PBL, a pattern that resembles the expression of CNTF. Nevertheless, in contrast to CNTF, M17 has a predicted signal peptide and is encoded by three exons of comparable size to the exons of OSM and LIF[164]. Moreover, M17 contains five cysteine residues that are similarly spaced compared to the conserved cysteine residues of LIF and OSM (Fig. 8.4). Taken together, these observations suggest that M17 is closely related to the mammalian LIF/OSM locus and likely represents the teleost orthologue of LIF and OSM. Since its discovery in carp, genes that encode M17 have been found in the genomes of several phylogenetically distantly related fish species[227] (Fig. 8.3), indicating that M17 is a type-I cytokine common to most teleost fish. The recent discovery of a single LIFR-like gene in goldfish that clusters ancestral to the bifurcation of mammalian LIFR and OSMR sequences[349] (that are in human both located at chromosome 5p13) suggests that LIFR and OSMR may have originated from a duplication event specific to the tetrapod lineage.

Ciliary neurotrophic factor and cardiotrophin-like cytokine/cytokine-like factor

The genes that encode ciliary neurotrophic factor (CNTF) and cardiotrophin-like cytokine (CLC), which is the type-I cytokine component of the heterodimeric CLC/CLF complex, are located in proximity on the same locus (Fig. 8.4)[350, 351], suggestive of a common origin through gene duplication. And although the CNTF gene is shorter than the CLC gene and lacks a signal peptide (as well as the short first exon that partially encodes it in most other type-I cytokines), the second exon of CNTF and the third of CLC closely resemble in length (Fig. 8.4). Unlike most type-I cytokines, the activity of CNTF was first described in a non-mammalian vertebrate prior to its cloning in rabbit[352]. It was initially characterised for its ability to support the survival of chicken neurons that were isolated from embryonic ciliary and sensory ganglia[353]. Since then CNTF has emerged as a pleiotropic neurotrophic factor that supports survival and growth of diverse cells of neuronal origin[354]. CNTF is also expressed in a variety of mammalian peripheral tissues, but is in those cases usually associated with peripheral nerves. Its non-neuronal effects include the induction of hepatocyte acute phase protein expression[355], a property shared with several other type-I cytokines. $CNTF^{-/-}$ mice display only a mild loss of motor neuron numbers, which is accompanied by increased muscle atrophy later in adult life, despite its prominent effects on neuron growth and survival[356]. Also in the human population, CNTF null mutations occur rather frequently (2.5% of the Japanese population) without overt consequences for the bearer[357]. In contrast, mice lacking the CNTFRα die perinatally due to severe motor neuron loss that prevents jaw movement resulting in a failure to suckle[356]. The phenotypic discrepancy between $CNTF^{-/-}$ and $CNTFR\alpha^{-/-}$ mice is explained by the observation that CNTFRα is also an essential component of the receptor complex for several other type-I cytokines, including CLC/CLF (Fig. 8.2)[358, 359]. Heterodimerisation of CLC with the truncated cytokine receptor cytokine-like factor (CLF) is required for the secretion of this non-covalently coupled soluble complex from the cell[295]. The requirement of the CNTFRα for CLC/CLF signaling is supported by the observation that $CLF^{-/-}$ mice, like $CNTFR\alpha^{-/-}$ mice, die shortly after birth due to a similar suckling defect[360]. CLC/CLF acts as another neurotrophic factor for motor neurons[351, 361], but also initiates POMC expression and ACTH release from murine pituitary ATT20 cells[343] and potently stimulates B-cells[351].

Despite its early discovery in chicken, no orthologues for CNTF have yet been described in poikilothermic vertebrates to date. The same is true for CLC, although a zebrafish orthologue of its obligatory binding partner CLF has been identified (acc. number BE016629). Moreover, this zebrafish CLF is considerably better conserved compared to related soluble type-I cytokine receptors such as the carp IL-12P40 (CHAPTER 6, 12).

Cardiotrophin-1 and neuropoietin

Cardiotrophin-1 (CT-1) and neuropoietin (NP) provide yet another example of a pair of mammalian type-I cytokines that is located on a single locus (Fig. 8.4). CT-1, like

CNTF, lacks an N-terminal signal peptide, but is nevertheless readily secreted by an as yet unknown mechanism. As its name suggests, CT-1 promotes cardiac myocyte survival and is expressed only in heart cells during murine embryonic development[362]. Additionally CT-1 shares several functions with other type-I cytokines, such as the induction of hepatocyte acute phase proteins production[340] and the release of ACTH[343]. The CT-1 receptor complex contains GP130 as well as LIFR, and there is evidence to suggest that a third, GPI-anchored receptor chain distinct from the CNTFRα participates in the CT-1 receptor complex[363, 364]. To date, no orthologues for CT-1 have been described in non-mammalian vertebrates.

NP, also known as cardiotrophin-2, was discovered in 2004 is the latest addition to the type-I cytokine family. It is the third cytokine, in addition to CNTF and CLC/CLF, which is known to use the tripartite CNTF receptor complex that consists of CNTFRα, LIFR, and GP130. In mouse, NP is expressed only during embryonic development in the brain. Moreover, the embryonic expression of NP and CNTFRα, but not CLC or CNTF, overlap and this indicates that defective NP signaling may partially contribute to the perinatally lethal phenotype observed in CNTFRα$^{-/-}$ mice[365]. Curiously, the human NP gene contains an eight nucleotide deletion within the third exon that causes a frame-shift, which renders human NP a pseudogene[365]. This observation seems to preclude a critical role for NP signaling during early mammalian ontogeny.

NO NP has been described in non-mammalian species to date. Interestingly, in certain species of terrestrial salamander that belong to the *Plethodontidae* family, the males produce a proteinaceous pheromone substance in a specialised gland under the chin that, when applied directly to the female nostrils, enhances female receptivity. This factor is called plethodontid receptivity factor (PRF) and displays modest amino acid identity to human CT-1 and CNTF (20 and 16 %, respectively)[366]. However, comparison of PRF with the newly reported mammalian NP gene reveals a 28% overall amino acid identity, which is considerably higher than the identity between PRF and any other mammalian type-I cytokine, suggesting that PRF may represent the amphibian orthologue of the mammalian NP gene.

Interleukin-12 and other heterodimeric cytokines

Interleukin-12 (IL-12) is composed of the type-I cytokine subunit P35, covalently linked to the soluble cytokine receptor P40. Together, they constitute a 70 kDa bioactive heterodimeric cytokine, IL-12P70, that is usually plainly referred to as IL-12. It is secreted from antigen-presenting cells in response to stimulation by microbial agents such as LPS, LTA, or prokaryotic DNA via Toll-like receptors. IL-12 induces the release of other cytokines, in particular IFNγ, from T-cells and NK-cells and by doing so is an early and key factor that drives an immune response towards a TH1 phenotype. In recent years IL-12 has emerged as the founding member of a larger family of composite cytokines that share

involvement in the TH1 response[193, 195]. Interleukin-23 (IL-23) is formed by the covalent association of the P40 subunit with the novel type-I cytokine subunit P19[196]. Besides the P40 subunit, IL-23 shares the IL-12Rβ1 chain with IL-12 (Fig. 8.2). Furthermore, the IL-23R lies in close proximity to IL-12Rβ2, the receptor chain it substitutes in the IL-23 receptor complex, on human chromosome one[217]. The third composite cytokine, interleukin-27 (IL-27), is constituted by the non-covalently associated type-I cytokine P28 with Epstein Barr Virus-induced protein-3 (EBI3), that has all the characteristics of a soluble cytokine receptor[197, 220]. IL-23 and IL-27, like IL-12, are involved in the TH1 response and their roles differ qualitatively[194]. IL-27 precedes the early actions of IL-12 in the initiation of a TH1 response and consequently acts on naïve $CD4^+$ T-cells[218]. IL-23 on the other hand acts much later in the TH1 response and predominantly activates memory T-cells[200, 201]. Although, in contrast to IL-12 and IL-23, the IL-27 subunits are not linked by an interchain disulphide bridge, co-expression of both subunits by the same cell is required for the secretion of all three composite cytokines[182, 196, 197].

The P35 and P40 subunits of IL-12 have recently been identified in chicken[202, 367], pufferfish[162], and carp (CHAPTER 6) (Fig. 8.3), but the amino acid identity of these non-mammalian IL-12 proteins compared to mammalian orthologues is invariable low at around 20 – 25%. Interestingly, the resolution of the gene structure of non-mammalian IL-12P35 genes[162,202] revealed that, although the P35 gene structure is conserved throughout vertebrates, mammalian IL-12P35 genes possess an extra exon (Fig. 8.4). This additional exon may have resulted from an exon duplication specific to the mammalian lineage: in mammals exons four and five both consist of 42 nucleotides, contain an identically spaced conserved cysteine residue, and are similar throughout their coding as well as their flanking non-coding regions (CHAPTER 6).

Pufferfish IL-12P35 was induced in the head kidney as well as spleen in response to i.p. injection of polyI:C but not LPS. In contrast to IL-12P35, pufferfish IL-12P40 is constitutively expressed in both organs, but was not detectably upregulated in response to injection of either stimulus. This suggests that IL-12P35 expression is the determining step in the production of pufferfish IL-12P70[162], a situation that is comparable to the regulation of IL-12 release from human monocytes[368]. We investigated the expression of carp IL-12P35 and P40 expression in head kidney macrophages *in vitro* and found that their constitutive expression was barely detectable. Following LPS stimulation however, the expression of both subunits was upregulated several 100-fold within hours (CHAPTER 6).

To our surprise we identified two more paralogous carp IL-12P40 genes, which we designated IL-12P40b and -40c, in addition to the P40a subunit discussed above. These three carp P40 paralogues are maximally 32% identical at the amino acid level, but all residues that are critically required for the formation of the interlocking topology between P35 and P40 in human IL-12[204] are conserved in all three carp P40 subunits

(chapter 6). Moreover, their identification as il-12p40 is supported by phylogenetic analyses, where all three carp p40 genes cluster within the vertebrate il-12p40 cluster and separate from other soluble type-i cytokine receptors such as clf, ebi3, and scntfrα (chapter 6). The constitutive expression of each carp p40 paralogue in head kidney macrophages varies profoundly from barely detectable (p40a) to abundant (p40c) and their inducibility by in-vitro lps stimulation is inversely proportional to the level of their constitutive expression (chapter 6). Although no non-mammalian orthologues of any of the subunits that constitute il-23 and il-27 (other than il-12p40) have been identified to date, the discovery of multiple, substantially different p40 subunits in carp, suggests that there are considerable differences between the subunit repertoires involved in the formation of composite cytokines in mammals and bony fish. This notion may extend to p35 as dual il-12p35 subunits are present in spotted green pufferfish (Fig. 8.3).

Leptin

Leptin is a soluble factor that, in mammals, circulates in proportion to body fat mass. It traverses the blood-brain barrier to reach the hypothalamus, where it evokes satiety and regulates energy metabolism[241]. The involvement of a soluble, circulating factor in the morbidly obese, diabetic and hyperphagic phenotype of the obese mouse strain was already established in the 1970s by parabiosis. A circulating factor from a wild-type mouse was capable of suppressing food intake and weight gain in the obese parabiont[230]. Nevertheless, this factor was not identified until 1994 with the cloning of leptin as the product of the obese gene[228]. Since leptin does not share considerable sequence identity with other proteins, it took another three years (until the crystal structure of leptin was resolved) before leptin was recognised as a member of the type-i cytokine family[231]. Leptin is a 167 amino acid protein encoded by two exons. Its four helix bundle conformation is stabilised by a single conserved disulphide bridge that connects the c-terminal ends of helix c and d. Consequently, the final amino acid before the stop codon is a cysteine residue (Fig. 8.4), a distinctive feature among type-i cytokines. Leptin's important role in food intake, a process that is considered key in the etiology of obesity, has spurred an enormous boost in leptin research. Despite the immense attention for leptin, no *bona fide* leptin orthologue from any non-mammalian species had been reported in the decade that followed leptin's discovery in mouse. A leptin molecule from chicken was reported by two groups in the late 1990s[254, 255]. This chicken leptin sequence was 97% identical to murine leptin at the amino acid level, which would signify an incredible evolutionary conservation. Since its discovery, several research groups have reported their inability to repeat the amplification of chicken leptin[256, 257]. The chicken leptin receptor has been cloned, but displays only modest amino acid identity of around 50% to its mammalian orthologues[369, 370]. The difference between the degree of conservation of chicken

leptin and its cognate receptor is counterintuitive, since ligands and the ligand-binding domain of their receptors tend to co-evolve. Also, the recently published draft chicken genome does not contain a gene that encodes chicken leptin as it was originally reported. Collectively, these considerations raise doubts on the validity of the published chicken leptin sequence and warrant a careful re-evaluation of chicken leptin.

Very recently, we reported the presence of two obese genes that encode duplicate leptins in common carp (CHAPTER 7). Both carp leptins are 83% identical and are likely the result of a recent duplication event. In line with our prediction that cytokines are generally poorly conserved from fish to mammals, we found that the amino acid identity of the carp leptins with their mammalian orthologues is relative low at 22 - 25%. Recently, the leptin sequences of two species of pufferfish have also become available[249]. Among the amino acid residues that are conserved between fish and mammalian leptins are the two characteristically spaced cysteines that make up leptins single disulphide bridge. Leptin's gene structure is also conserved, as the first and second exon of both carp leptin genes extend merely one and three triplets, respectively, compared to those of human and mouse leptins (Fig. 8.4). Their leptin identity is further supported by phylogenetic analyses that place the carp leptins in a single cluster with mammalian orthologues, separate from other type-I cytokines (Fig. 8.3). In contrast to mammalian species, where leptin is predominantly expressed in the subcutaneous adipose compartment, the liver is the major site for leptin expression in carp (CHAPTER 7) and pufferfish[249]. Moreover, the liver mRNA expression of both carp leptins displays a postprandial peak in response to a single meal at scheduled feeding time (CHAPTER 7). Prolonged (six days) fasting followed by refeeding for three days did not affect liver leptin expression. This contrasts with what has been reported for rodents, where food deprivation for a short period results in a more considerable weight loss and a drop in leptin expression[280, 371]. The divergent responses of leptin to fasting in fish and mammals may well relate to the overt physiological differences that are associated with homoiothermic and poikilothermic vertebrates, and further experiments are ongoing to further unravel the role of leptin in early vertebrates and the primary site of its production.

Erythropoietin

Erythropoietin (EPO) is the principle regulator of the proliferation and differentiation of progenitors of the erythroid lineage. EPO is mainly produced in the fetal liver and adult kidney in response to hypoxia and stimulates bone-marrow erythropoiesis[372]. Central to the oxygen-sensing mechanism that is responsible for the transcription of EPO is the transcription factor complex HIF-1 (hypoxia-inducible factor-1), which is activated in response to hypoxia[373]. The human EPO gene consists of five exons and contains two conserved cysteine bridges[372, 374]. One of these disulphide bridges is lost in the murine

EPO protein[375] (Fig. 8.4). The EPO gene of several fish species has recently been resolved (Fig. 8.3). The genes encoding carp and pufferfish EPO contain five exons of comparable size to those of human EPO. Moreover, both fish EPO proteins share four conserved cysteine residues with human EPO (Fig. 8.4). The amino acid identity of EPO of either fish species is higher than 30%, which ranks EPO among the best conserved vertebrate type-I cytokines (Fig. 8.5). The heart is the site of major EPO expression in the pufferfish, where modest expression is also observed in liver and brain[163]. In contrast to mammalian species, the pufferfish (head) kidney does not express EPO, which may relate to the fact that in fish the head kidney and kidney are major haematopoietic sites, analogous to mammalian bone marrow. Transfection of the pufferfish EPO gene into a fish hepatoma cell line, shows that the pufferfish EPO gene is upregulated under hypoxic conditions[163], which would suggest that EPO is involved in hypoxia induced erythropoiesis throughout vertebrates.

Prolactin and growth hormone

In contrast to most other type-I cytokines, prolactin (PRL) and growth hormone (GH) both are secreted as classical hormones: they are released into the circulation from the anterior pituitary gland and act peripherally in an endocrine fashion. GH is released in response to hypothalamic stimulation via GH-releasing hormone and exerts negative feedback onto the hypothalamus. Its most prominent and best-known effect throughout vertebrates is the stimulation of postnatal growth by inducing the growth and differentiation of bone, cartilage and muscle. Many of the growth-promoting effects of GH are mediated via insulin-like growth factors produced in the liver[376]. In addition to the effects of GH on somatic growth, GH and GH receptors are expressed in many peripheral sites, including the immune system, where GH promotes cellular growth and survival[377].

The actions of PRL were already recognised in the 1920s as an anterior pituitary hormone capable of inducing milk secretion in female rabbits[378]. Not much later PRL was shown to induce the growth of the pigeon crop sac, an organ that is used by these birds to feed their chicks with crop milk released under PRL control[379]. The similarity of the actions of PRL in birds and mammals alike demonstrates the involvement of PRL in reproduction in different vertebrate classes. And also in early vertebrates such as fish, PRL induces parental behavior[380]. However, in the decades that have passed since PRL's discovery as an inducer of lactation, over 300 distinct functions for PRL have been described, indicating that PRL is extremely pleiotropic and arguable the most versatile type-I cytokine of all[381, 382]. PRL is also the only type-I cytokine to date where orthologues have been identified in fish, amphibia, reptiles, birds, and mammals (Fig. 8.3). Its actions have been classified into six major categories: osmoregulation, growth and development, endocrinology and metabolism,

brain and behavior, reproduction, and immune regulation[381]. Osmoregulatory actions of PRL are particularly important for (although not exclusive to) fish. Fish are mostly exposed to an aqueous environment of different osmolarity compared to the body fluids (not in some brackish waters) without the protective barrier of a water-impermeable epidermis. Especially euryhaline fish (fish that migrate from salt- to fresh water or *v.v.*) rely on PRL for osmoregulation in fresh water. When seawater adapted fish migrate to fresh water, their pituitary and plasma PRL levels rise. PRL decreases permeability of the integumental surfaces to water and ions and increased ion retention, properties that both favor life in a fresh water environment[383]. This principle is aptly demonstrated by the discovery that hypophysectomised killifish (*Fundulus heteroclitus*) are only able to survive in fresh water following PRL replacement[384, 385]. The important osmoregulatory properties of PRL in fish as well as more recent vertebrates have led to the postulation that osmoregulation may reflect PRL's original function; the vertebrate lineage stems from an aquatic environment[383]. The water-land transition was followed by many physiological changes, including *i.a.* the development of a keratinous skin, decreased litter size and increased parental care, and PRL might have expanded its repertoire by taking on these additional roles.

PRL and GH are generally considered to share a direct common ancestor[381, 383], as they are both encoded by five exons of comparable size and signal via related receptors (Fig. 8.2). Moreover, the four conserved cysteine residues in vertebrate GH proteins are also present and identically spaced in PRLs (Fig. 8.4). Mammalian PRLs have an additional N-terminal cysteine pair that is lost in actinopterygian fish due to a partial deletion of exon two[383]. In addition to PRL and GH that are present throughout vertebrates, several lineage-specific members of the PRL/GH subfamily cytokines exist. Somatolactin (SL) is a cytokine that is considered specific to the fish lineage with approximately equal amino acid identity to PRL and GH. SL is secreted from the *pars intermedia* of the pituitary gland and has been implicated in a variety of functions such as background adaptation, stress responses, energy metabolism, acid-base regulation, and the control of reproduction[386, 387]. Interestingly and in contrast to teleostean PRLs, SLs share all three conserved cysteine residues with mammalian PRLs (Fig. 8.4). No SL orthologue has been discovered in a non-piscine vertebrate to date. However, the presence of SL in the sarcopterygian lungfish indicates that the SL gene has been present in the early tetrapod ancestor and may have been lost prior to the origin of most classes of present-day vertebrates[386, 388]. Another example of a lineage-specific cytokine within the GH/PRL subfamily is provided by the placental lactogens (PL) that are the result of gene duplications that are specific to the mammalian lineage. Mammalian PLs do not form a monophyletic group. Instead, they independently arose by duplication of the PRL gene in rodents and ruminants and by a duplication of the GH gene in primates[388]. PLs are expressed mainly in the placenta and serve as a luteotrophic factor and regulate fetal growth[389]. Collectively this indicates that,

although GH and PRL are present throughout the vertebrate lineage, their subfamily of type-I cytokines underwent lineage-specific duplication and deletion in different classes of vertebrates. In contrast to other type-I cytokines that cluster according to the accepted patterns of vertebrate evolution, mammalian GH and PRL sequences form respectively two and three separate clusters (Fig. 8.3). This slightly erratic branching pattern of GH and PRL has been observed previously and has been attributed to variability in the evolutionary rate of GH and PRL between different mammalian lineages[388].

'Short-chain' type-I cytokines: the TH2 example

Type-I cytokine genes are scattered over the genome of mice and men, where they are found mostly isolated. Some occur in small aggregations of two or sometimes three genes. Examples include cytokine pairs, such as LIF/OSM, and CNTF/CLC, and the threesome of CT-1, NP, and IL-27P28 (Fig. 8.4). Although these clusters of cytokine genes residing on a single locus may reflect recent gene duplication events, it is likely that the distribution of type-I cytokine genes over multiple loci occurred earlier in the vertebrate lineage. A similar situation is encountered within the CXC chemokine family. This subclass of the chemokines currently has sixteen mammalian representatives, of which thirteen reside on a single locus (human 4q21), the remaining three each occupy a separate locus. Representatives of at least three of these loci are found fish, indicating that the distribution over multiple loci occurred prior to the fish-tetrapod divergence[51]. The total repertoire of fish CXC chemokines differs profoundly from that of mammalian species, and even primate and rodent species differ substantially with regard to their total number of chemokine genes. Collectively this indicates that the expansion of the number of chemokine genes on a single locus occurred much later in evolution and in a lineage specific way[51].

In contrast to the long-chain type-I cytokines that in majority occur isolated in the genome, many of the genes for short-chain four-helix bundle cytokines are concentrated in a cluster on human chromosome 5q31.1 (mouse 11B1). This gene cluster is also referred to as the TH2 cluster, as it contains the genes encoding for IL-4, IL-5, and IL-13, which are important TH2 cytokines. In addition to these TH2 cytokines, this cluster also contains the genes that encode IL-3 and GM-CSF. Their close proximity indicates that the genes that form the TH2 cluster expanded from a single gene by gene duplication, similar to the CXC chemokines on human locus 4q21. The next question is when these gene duplications occurred, or, in other words, is the TH2 cluster restricted to the mammalian lineage, or a feature shared by mammals and earlier vertebrates alike? Recently, the TH2 cluster of

chicken was characterised and found to contain six cytokine genes. Five of these chicken genes were designated as orthologous to the five cytokines of the mammalian TH2 cluster[390]. Since the sequence identity between the chicken and mammalian cytokines is limited, and the gene structure of all genes in the TH2 cluster is similar this designation of orthology depends in part on the conservation of synteny. The human, mouse, and chicken TH2 cluster are situated at syntenic regions of chromosomes 5, 11, and 13, respectively (Fig. 8.5). Although the synteny of the chicken TH2 cluster is conserved with regard to gene order and orientation[390] (Fig. 8.5), the genes for chicken IL-3 and GM-CSF are reversed in the recently completed chicken genome. This difference may be attributable to a strain effect, as a BAC library of the White Leghorn strain was used in the characterisation of the chicken TH2 cluster, whereas the chicken genome is assembled from the genetic material of red jungle fowl, the ancestor of domesticated chicken. Alternatively, the reversed gene order of IL-3 and GM-CSF may represent an artefact of the chicken genome assembly. Several differences exist between the chicken and mammalian TH2 cluster. Chicken IL-5 lacks a promoter region and is not expressed, effectively demoting it to a pseudo-gene in both the White Leghorn strain[390] and the red jungle fowl (DR. P. KAISER, pers. comm.). Furthermore, the chicken TH2 cluster contains a sixth cytokine gene, designated KK34[391], that is probably unique to the avian lineage. Collectively, this indicates that, although the TH2 cytokine locus predates the avian-mammalian split estimated to have occurred at around 310 million year ago[15], several differences exist between the TH2 clusters of birds and mammalian species.

Of the three fish genomes currently available, the genome assembly of *Tetraodon nigroviridis* is most complete in the region that is syntenic to the human, mouse, and chicken TH2 cluster. This *Tetraodon* region is located on chromosome seven and contains orthologues to many of the genes that flank the cytokine genes of the TH2 cluster in mammalian and avian genomes (Fig. 8.5). The synteny of these genes is not completely conserved. The genes for the *Tetraodon* orthologues of P4HA2, PDLIM4, SLC22A5, and IRF1 are inversed in gene order and orientation, indicative of a block inversion event (Fig. 8.5). An artefactual *Tetraodon* genome assembly, which could provide an alternative explanation for the inversed gene order, is unlikely in this particular instance (DR. ROEST CROLLIUS, pers. comm.). Although we identified a *Tetraodon* locus that is syntenic to the TH2 locus of human, mouse, and chicken, there is as yet no evidence for any cytokine gene within this approximately 700 Mb region of the *Tetraodon* genome. Also the genomes of zebrafish and *Takifugu rubripes* do not reveal any of the cytokine genes of the TH2 cluster, although orthologues of several non-cytokine genes within the mammalian TH2 cluster are readily identifiable in both these fish species, as they were in *Tetraodon* (not shown). There are two explanations for the lack of identifiable fish orthologues of the mammalian TH2 cluster cytokines. The first explanation is that one or several orthologues of IL-3, -4,

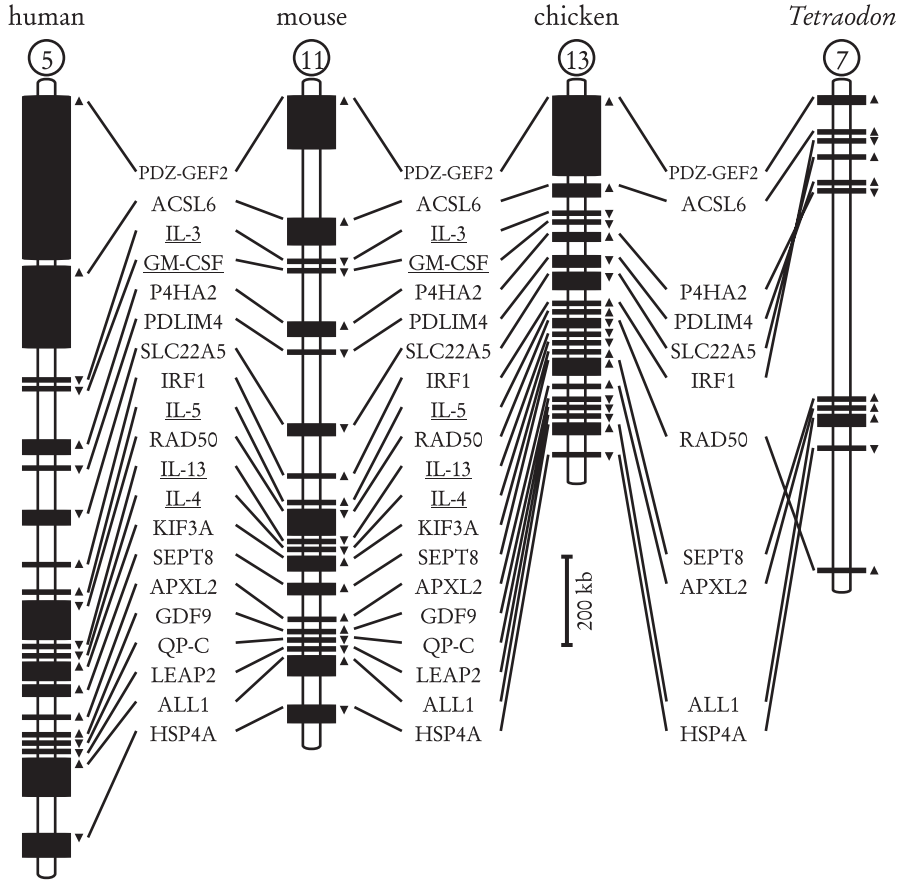

Figure 8.5: The synteny of the TH2 cluster is conserved in human, mouse, and chicken. A comparison of the human TH2 cluster (chromosome 5q31) with syntenic regions of mouse (11B1) and chicken (13 15.5 – 16.2 Mb) reveals that synteny is conserved with regard to gene order and orientation. The region of *Tetraodon* chromosome seven that is syntenic to human 5q31 contains orthologues to eleven genes that flank the cytokine genes in human and mouse, but appears to lack cytokine genes. Genes (black boxes) and the distance between them are drawn to scale. The arrowheads indicate the gene orientation. Cytokine genes are underlined. Abbreviations: PDZ-GEF2, PDZ-domain containing-guanine nucleotide exchange factor-2; ACLS6, long-chain fatty acid CoA ligase 6; P4HA2, prolyl 4-hydroxylase α2 subunit precursor; PDLIM4, PDZ and LIM domain protein-4; SCL22A5, solute carrier family 22 member 5; IRF1, interferon regulatory factor-1; RAD50, DNA repair protein RAD50; KIF3A, kinesin-like protein KIF3A; SEPT8, septin-8; APXL2, apical protein-2; GDF9, growth/differentiation factor-9 precursor; QP-C, low molecular mass ubiquinone-binding protein; LEAP2, liver-expressed antimicrobial peptide-2 precursor; ALL1, ALL1 fused gene; HSP4A, heat shock 70 kDa protein-4.

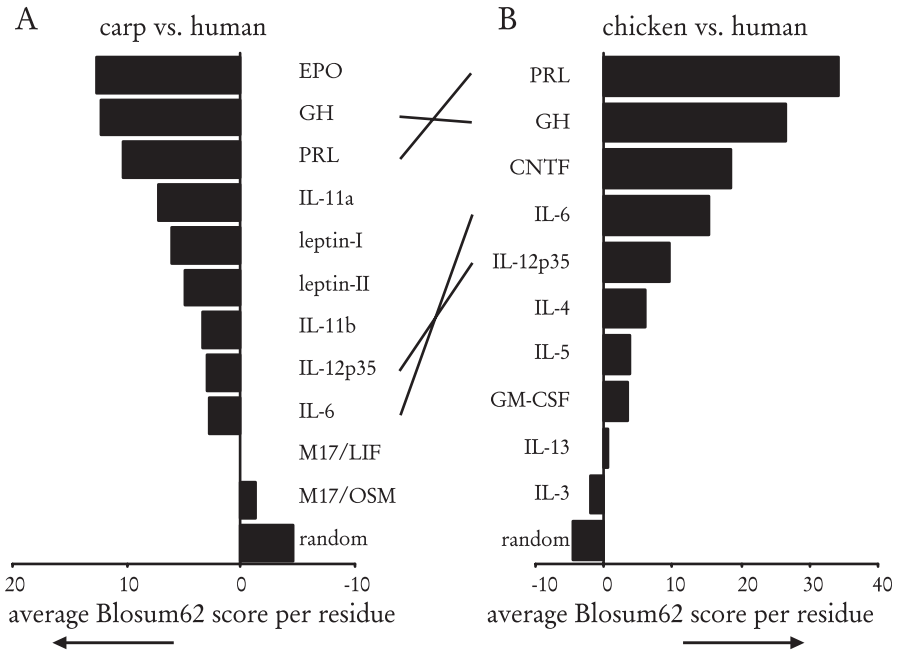

Figure 8.6: Comparison of the degree of evolutionary conservation of various type-I cytokines. The score assigned to a pairwise amino acid alignment (via the T-COFFEE algorithm) of orthologous cytokines on the basis of the Blosum62 matrix, divided by the length of the alignment to correct for the differences in sequence length, is used to quantify their degree of conservation (identity and similarity). The average score of pairwise alignments of three randomly generated amino acid sequences of 200 residues is included to provide a baseline value. The conservation scores of all known carp type-I cytokines are ranked from high (EPO) to low (M17/OSM) to provide a direct comparison of their evolutionary conservation (a). Since the IL-11b and IL-6 from carp have not yet been identified, their conservation scores are the result of the comparison of human with zebrafish (IL-11b) and pufferfish (IL-6), respectively. In panel b, the conservation scores of all known chicken type-I cytokines compared to their human orthologues are ranked. Note that the five members of the TH2 cytokine cluster are the five most poorly conserved chicken cytokines. In carp as well as chicken, the sequences of the pituitary hormones GH and PRL rank among the best conserved type-I cytokines. Pairwise comparison of carp or chicken with mouse instead of human results in very similar rankings (not shown).

-5, -13, and GM-CSF are present in the fish genome, but are not retrieved in searches with the usual computational algorithms. The other explanation is that these genes are absent in fish altogether. This is in line with the lack of TH2 cytokines in the long list of cytokine and chemokine genes that were identified via the sequencing of numerous fish EST libraries, constructed via subtractive hybridization to enrich for genes that are upregulated in leukocytes in response to stimulation. It is also striking that the five

members of the chicken TH2 cluster are also the five cytokines that display the poorest sequence conservation of all chicken type-I cytokines described to date when compared with human orthologues (Fig. 8.6). This indicates that TH2 cytokines, although present in chicken, are poorly conserved and raises the possibility that fish orthologues to TH2 cytokines may lack altogether. However, the arguments above are merely circumstantial; it is much more difficult to definitively prove the absence instead of the presence of a gene. Nevertheless, the close association of all major TH2 cytokine genes on a single locus suggests a scenario where the appearance of the TH2 response depended on the expansion of a single locus by gene duplication. From the presence of the TH2 cluster in chicken it follows that this expansion had already occurred prior to the avian-mammalian split. The question remains whether an ancestor of the TH2 cytokine cluster exists in the teleostean lineage. The identification of teleostean orthologues of the receptors of TH2 cytokines may provide us with an answer to this question. Although the extracellular domain of the TH2 cytokine receptors is not necessarily better conserved than their ligands, their overall sequences are longer and will contain a conserved intracellular domain; both characteristics that facilitate their discovery compared to the discovery of their ligands. Through the identification of their receptors we may find valuable clues regarding the presence and number of TH2 cytokines, and consequently a TH2-like response, in early vertebrates. Orthologues of IL-12, IL-18, and IFNγ, the main initiators of the mammalian TH1 response, have been identified in fish[162, 226, 392-394] (chapter 6), suggestive of the existence of a fish TH1-like immune response. However, although TH1 and TH2 are considered the Yin and Yang of mammalian immunology, they may not necessarily have developed simultaneously. A fish TH1-like response may function autonomously, without the cross-regulatory activity of TH2 cytokines. Th2 responses in mammalian species are associated with a predominantly humoral immune response elicited by extracellular pathogens. And although fish can mount an antigen-specific antibody response, evidence for refined properties such as isotype switching and affinity maturation is limited, in line with the absence of lymph nodes and histologically identifiable germinal centers[117]. Recently, fish orthologues of several short-chain type-I cytokines other than those of the TH2 cluster, such as IL-2, IL-15, and IL-21[169, 395], have been described, indicating that short-chain type-I cytokines are represented within the fish genome. From the above, it is clear that the presence or absence of the TH2 cytokine cluster in lower vertebrates needs to be addressed, as it is the last major group of cytokines that lacks representatives in lower vertebrate species.

Vertebrates outdated: insect cytokines

Most long-chain type-I cytokines are situated at a distinct locus, either alone or in pairs (Fig. 8.4). For many of these (pairs of) cytokines unambiguous orthologues have recently been identified in bony fish species and more are likely to follow in the near future. Under the plausible assumption that type-I cytokines constitute a monophyletic group, this implies that the origin of cytokines predates vertebrates. The draft genome of the invertebrate chordate *Ciona intestinalis* contains orthologues of JAK, STAT, SOCS, and PIAS[396], but ascidian cytokine or cytokine receptor genes have not been described to date.

In *Drosophila melanogaster*, compelling evidence has emerged for the involvement of cytokine signaling in various physiological processes. A *Drosophila* JAK/STAT pathway was first identified through its role in embryonic segmentation. The *Drosophila* JAK is named hopscotch (HOP) and shares 27% overall amino acid identity with human JAK2[397]. The single *Drosophila* STAT identified to date is named STAT92E (or marelle) and shares 37% amino acid identity with human STAT5[398, 399]. Fly embryos deficient in either HOP or STAT92E gene activity are characterised by the stripe-specific loss of the expression of several pair-rule genes, leading to the selective loss of a specific subset of body segments[397-399]. The same phenotype was observed in the absence of gene activity of a third gene, unpaired (UPD). UPD is a secreted glycoprotein of over 400 amino acids, that associated with the extracellular matrix through association with glycosaminoglycans, effectively confining its range of actions[400]. The complete overlap in phenotypes displayed upon the absence of either UPD, HOP, or STAT92E suggests that they are all components in the same pathway. Indeed, UPD is capable of inducing HOP phosphorylation, reminiscent of the JAK phosphorylation that follows cytokine activation in vertebrates[400]. The receptor that mediates the UPD-induced phosphorylation of HOP was later identified as domeless (DOME) and belongs to the type-I cytokine receptor family. Extracellularly, DOME has five fibronectin type-III domains, two of which form a cytokine binding domain with an incomplete WSXWS motif that is characteristic of vertebrate type-I cytokine receptors[401-403]. DOME also has a single intracellular consensus STAT binding motif (YXXQ). Since its discovery as important regulators of embryonic segmentation, involvement of the *Drosophila* DOME/HOP/STAT92E pathway has been shown in oogenesis[404], eye development[403, 405, 406], hindgut epithelial cell rearrangement[407], trachea formation[400, 403], and the fly immune response[408]. Recently, two additional *Drosophila* unpaired proteins, designated UPD2 and UPD3, have been discovered at the UPD locus[402]. The three *Drosophila* UPD proteins differ, at least to some extent, in their involvement in the physiological processes that require DOME/HOP/STAT92E activation. For example, UPD3, but not UPD, is upregulated in fly hemocytes in response to septic injury[408].

Given the similarity of their downstream signaling components with the vertebrate type-I cytokine family, the obvious question is whether *Drosophila* UPD is a cytokine. The UPD amino acid sequence is longer than that of any mammalian cytokine and although predicted to be of high α-helical content[400] it is not known whether these α-helices fold into a four-helix bundle motif. The predicted pI of UPD is extremely basic at almost twelve, a characteristic that is generally considered to be unlike any vertebrate cytokine[400, 403], although e.g. vertebrate IL-11 proteins have a similarly high predicted pI[147, 227]. Although the similarities between UPD signaling and the vertebrate JAK/STAT pathway are compelling, the definitive classification of *Drosophila* UPD proteins as members of the cytokine family may require the resolution of their crystal structures.

Summary and perspectives

Although the list of known fish cytokines is certainly incomplete at this stage, the picture that emerges from the description of the individual members of the type-I cytokine family is that many mammalian cytokines have orthologues in fish. It follows that these cytokines already existed before the fish-tetrapod divergence that occurred approximately 450 million years ago[15]. Following this split, the type-I cytokine family witnessed several lineage-specific expansions by gene duplications. Examples include the duplication of IL-11 and IL-12P40 genes and the presence of SL that are all specific to the teleost fish lineage. On the other hand, PLS are restricted to placental mammals and the gene duplications that have led to the occurrence of pairs or triplets of type-I cytokines at a single locus (LIF/OSM, 22q12.2; CNTF/CLC, 11q12-13; CT-1/NP/IL-27P28, 16p11) may also be unique to the tetrapod lineage. However, these examples of (potential) lineage-specific gene duplications do not change the view that the type-I cytokine family was already largely established and distributed over many loci early in the vertebrate lineage. And since the three unpaired genes, which are the only *Drosophila* cytokine-like proteins discovered to date, all reside on a single locus, the resolution of the genome of species that occupy an evolutionarily intermediate position between vertebrates and insects, such as *Ciona intestinalis*, may reveal important insights into the evolution of the type-I cytokine family.

A number of cytokines that belong to the other cytokine families have been discovered in the last decade in teleost fish. These include pro-inflammatory cytokines such as IL-1β and TNFα as well as anti-inflammatory cytokines such as TGFβ[409] and IL-10[225]. Representatives of several classes of chemokines are also present in fish[67, 166, 410], although there is compelling evidence that the chemokine repertoire of fish and mammals differs

extensively due to lineage-specific gene duplications[51]. Also IFNγ and IL-18, key cytokines that drive the immune response towards TH1 in concert with IL-12, are present in fish[226, 393, 394]. Although the status of many individual cytokines is still uncertain, the only major group of cytokines for which not a single fish orthologue has been reported to date is the TH2 cluster. Clearly, the question whether orthologues of mammalian TH2 cytokines are present in early vertebrates needs to be addressed in the near future, as the answer to this question will assist us in appreciating the evolutionary significance of one of the major paradigms of the mammalian immune system. A common feature of all fish cytokines discovered to date is the low degree of primary sequence conservation they share with their mammalian orthologues. This relatively poor sequence conservation has complicated (and still complicates) the discovery of non-mammalian orthologues of cytokine genes. This extensive sequence dissimilarity also serves as a reminder that the functions of orthologous cytokines in different vertebrate classes may differ considerably. For only a very select number of fish cytokines has their discovery been followed up by a functional characterisation that exceeds the level of gene expression. Nevertheless, a better understanding of fish immunity as well as a more refined comparison between immune systems of different vertebrate classes will eventually require such an approach.

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CHAPTER 9

Structural characterisation of a cyprinid CRH, CRH-BP, and CRH-R1 and the role of these proteins in the acute stress response

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Abstract

We elucidated the structure of the principle factors regulating the initiation of the acute stress response in common carp: CRH (corticotropin releasing hormone), CRH-R1 (CRH-receptor 1) and CRH-BP (CRH-binding protein). Phylogenetic analyses reveal that these proteins are evolutionary well conserved in vertebrates. CRH and CRH-BP expression are not co-localised in the same hypothalamic perikarya. On the contrary, CRH-BP expression is limited to the perimeter of the NPO (*nucleus preopticus*), but is abundant in other regions, including an area directly rostral from, and in close proximity to the NPO. Despite the lack of co-expression, the nerve fibres projecting onto both the rPD (*rostral pars distalis*) as well as the large fibre bundles projecting onto the PI (*pars intermedia*) contain CRH as well as CRH-BP, suggesting that both ACTH (adrenocorticotropic hormone) release from the rPD as well as the release of PI melanotrope content is regulated via CRH and CRH-BP. Finally, we show via real time quantitative PCR that expression of hypothalamic CRH and CRH-BP following a 24 h restraint significantly increases, whereas PD CRH-R1 expression decreases, and this reflects desensitisation of the PD for hypothalamic CRH output. We conclude that these factors are actively involved in the regulation of acute stress responses in the teleost fish.

List of abbreviations

ACTH	adrenocorticotropic hormone	PI	*pars intermedia*
AVT	arginine vasotocin	PN	*pars nervosa*
CRH	corticotropin releasing hormone	pPD	*proximal pars distalis*
CRH-BP	corticotropin releasing hormone-binding protein	rPD	*rostral pars distalis*
		TRH	thyrotropin releasing hormone
CRH-R*i*	corticotropin releasing hormone-receptor (*i = 1, 2, 3*)	TSH	thyrotropin
		UCN1	urocortin-1
NPO	*nucleus preopticus*	UCN2	urocortin-2
NPOpmc	*nucleus preopticus pars magnocellularis*	UCN3	urocortin-3
NPOppc	*nucleus preopticus pars parvocellularis*	UI	urotensin-I
NO	*nervus opticus*	UII	urotensin-II
PD	*pars distalis*		

Introduction

In fish, as in other vertebrates, corticotropin releasing hormone (CRH) is the dominant hypothalamic hormone controlling the stress axis[16, 412]. Upon central registration of an imminent or ongoing disturbance of homeostasis, CRH is released from the *nucleus preopticus* (NPO). This evokes the sequential secretion into the circulation of adrenocorticotropic hormone (ACTH) from the pituitary *pars distalis* (PD) and cortisol from the interrenal cells of the head kidney. The stress axis of teleost fish is generally similar to that of mammals, although some striking differences exist. Firstly, the cortisol secreting cells are found around the blood vessels of the head kidney, an organ unique to fish and an important site of haematopoiesis[413]. Secondly, cortisol in fish combines glucocorticoid and mineralocorticoid functions[16]. It is thus not only involved in recruitment of energy to cope with stressors, but also acts to restore and maintain the hydromineral balance in concert with prolactin (fresh water fish) and growth hormone (sea water fish). Despite these differences, the stress-axis is conserved throughout vertebrate evolution: several 'classical' peptide hormones and receptors, including CRH have been identified in teleost fish[414, 415].

CRH is derived from a 160 amino acid prepro-hormone, that is cleaved into a mature 41 amino acid bioactive peptide[416]. With the discovery of urotensin-I (UI) in teleost fish[417, 418] followed by the identification of its mammalian orthologue urocortin-1 (UCN1)[419, 420] it became apparent that a family of CRH-like factors exists. Aided by recent whole genome sequencing efforts, two more CRH-like family members have been identified to date, named urocortin-2 and -3 (UCN2 and UCN3)[421, 422]. These peptides signal through seven-helix G-protein coupled receptors. The two receptors for CRH and UCN1 (CRH-R1 and CRH-R2) display overlapping ligand specificities. CRH-R1 has equal affinity for both CRH and UCN1/UI in catfish[423] and mammals[424], while CRH-R2 has higher affinity for UCN over CRH[425, 426]. Recently a third CRH receptor (CRH-R3) has been identified in catfish (*Ameiurus nebulosus*) with five-fold higher affinity for CRH over UI [423].

Levels of bioactive CRH in circulation are influenced by CRH-binding protein (CRH-BP)[427], a 322 amino acid soluble protein structurally unrelated to the CRH-receptors. The affinity of CRH-BP for CRH is higher than that of the CRH-R1[427, 428]. CRH-BP is generally considered to be an antagonist of CRH[429].

Here we report on the characterisation of the hormones and receptors of the CRH system involved in the stress axis in common carp (*Cyprinus carpio*). Our data include extensive sequence and expression analyses of CRH-BP in a teleost fish. From a phylogenetic perspective it follows that the novel CRH, CRH-R1 and CRH-BP sequences described here are highly conserved in structure and are orthologous to CRH, CRH-R1 and CRH-BP of other non-teleostean vertebrates. Furthermore we assess the expression of

CRH and CRH-BP at the mRNA as well as the protein level. Finally we describe the integrated regulation of hypothalamic and pituitary expression of these genes in an acute restraint stress paradigm.

Materials and methods

Animals

Common carp (*Cyprinus carpio* L.) were reared at 23°C in recirculating UV-treated tap water at the 'De Haar Vissen' facility in Wageningen, at a photoregimen of 12 h light, 12 h dark. Fish were fed pelleted dry food (Provimi, Rotterdam, The Netherlands) at a daily ration of 0.7% of their estimated body weight. R3xR8 are the hybrid offspring of a cross between fish of Hungarian (R8 strain) and Polish origin (R3 strain)[34]. Carp of the same strain housed under identical conditions at the fish facilities of the Department of Animal Physiology at the University of Nijmegen were used for analyses of in-vivo stress responses. Fish were anaesthetised with 0.2 g l^{-1} tricaine methane sulphonate (TMS) buffered with 0.4 g l^{-1} $NaHCO_3$ or with 0.1 % 2-phenoxyethanol.

Cloning and sequencing

For CRH, PCR was carried out with CRH16sense and CRH11antisense (Table 9.1) primers on carp hypothalamic cDNA. The 5' and 3' UTR were amplified from a PMA-stimulated phagocyte cDNA library[37] by the use of SK and T7 anchored primers, respectively. For CRH-R1, degenerate oligonucleotide primers (CRH-R.fw3 and CRH-R1.rv1) were designed based on mammalian, *Xenopus* and catfish CRH-R1 sequences, which yielded a 0.4 kb partial CRH-R1 sequence. The majority of remaining coding sequence was amplified by the use of a second set of primers (CRH-R1.fw4 and CRH-R1.rv2). CRH-BP was amplified by the use of degenerate oligonucleotide primers (CRH-BP.fw2 and CRH-BP.rv1) based on conserved regions from mammalian and *Xenopus* sequences, which yielded a partial sequence of 0.55 kb. The complete coding sequence and the 5' and 3' UTR were amplified from on a λZAP cDNA library of total carp brain without pituitary gland, constructed from pooled total RNA of 10 adult carp, of which five individuals were restrained for 90 minutes. T3 and T7 anchored primers in combination with two new sequence specific primers (CRH-BP.fw4 and CRH-BP.rv4) were used in anchored PCR. Oligonucleotides were obtained from Eurogentec. PCR reactions were performed with 0.5 µl Taq DNA polymerase (Goldstar) supplemented with 1.5 mM $MgCl_2$, 200 µM dNTPs and 400 nM of each primer in a final volume of 25 µl. Cycling conditions were 94°C for 2 min, 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min for 30 cycles and 72°C for 10 min, by the use of a

Table 9.1: Primer sequences and corresponding accession numbers

gene	acc. number	primer	sequence 5' ⇒ 3'
CRH	AJ317955, AJ576243	CRH16sense	GGRGAGGARRAYTWCATCCG
		CRH11antisense	CATTTGTTCGGCTCTGGCCAT
		qCRH.fw2	CATCCGGCTCGGTAACAGAA
		qCRH.rv2	CCAACAGACGCTGCGTTAACT
CRH-R1	AJ576244	CRH-R.fw3	GAYSHATYGGIACSTGYTGGCC
		CRH-R1.rv1	CCAAACCAGCACTTYTCATTG
		CRH-R1.fw4	CATGAGAGCAATGTGATCTGGTG
		CRH-R1.rv2	CCACICGIGCICGGATKGARTGC
		qCRH-R1.fw2	CCCTGCTGATCGCCTTCAT
		qCRH-R1.rv2	GCAGGATAAATGCTGTAATCAGGTT
CRH-BP	AJ490880, AJ490881	CRH-BP.fw2	GGCTGGATGATGAAGGGAGAGAA
		CRH-BP.rv1	CACCATTCTGACCACAGTGTTATC
		CRH-BP.fw4	AATGAAGGTTGGCTGTGATAACACT
		CRH-BP.rv4	CGCAGTAATCAGAGTAACGCTGTAC
		qCRH-BP.fw1	ACAATGATCTCAAGCGGTCCAT
		qCRH-BP.rv1	CCACCCAGAAGCTCGACAAA
UI	P01146	qUI.fw1	GCACCTGTGTCCAGCATGAA
		qUI.rv1	GGTGCTCAGCGGGATGTG
40S ribosomal protein S11	AB012087	q40s.fw1	CCGTGGGTGACATCGTTACA
		q40s.rv1	TCAGGACATTGAACCTCACTGTCT
β-actin	CCACTBA	qACT.fw1	CAACAGGGAAAAGATGACACAGATC
		qACT.rv1	GGGACAGCACAGCCTGGAT
vector		SK	CGGCCGCTCTAGAACTAGTGGACT
		T7	TAATACGACTCACTATAGGG
		T3	CGCAATTAACCCTCACTAAAG

GeneAmp PCR system 9700 (PE Applied Biosystems, Foster City, USA). Products amplified by PCR were ligated and cloned in JM-109 cells with the pGEM-T-easy kit (Promega, Leiden, The Netherlands) according to the manufacturer's protocol. Plasmid DNA was isolated from the cloned cells by the use of the QIAprep Spin miniprep kit (Qiagen, Leusden, The Netherlands). Sequence reactions were carried out by the use of the ABI Prism Bigdye terminator cycle sequencing ready reaction kit according to the manufacturers protocol and analysed with an ABI 377 sequencer. Signal peptide predictions were done by the use of the SignalP program v2.0[430] at http://www.cbs.dtu.dk/services/SignalP-2.0/. Prediction of the pufferfish CRH-BP gene organisation was done with GENSCAN at http://genes.mit.edu/ GENSCAN.html.

Restraint stress paradigm

We used an acute (30 min.) and a prolonged (24h) restraint model to study the stress response. Fish (n = 8) were housed in identical tanks (0.6 x 0.3 x 0.3 m (30 min.) or 0.9 x 0.5 x 0.4 m (24 h)). At t = 0 the fish were restrained for 30 min or 24 h, by netting. Fish were sampled at the indicated times following the initiation of stressor by rapid anaesthesia applied to their tank, followed by sampling of the anaesthetised animal. Blood was collected by puncture of the caudal vessels using a heparinised (Leo Pharmaceuticals

Products, Ltd.) 25 Gauge needle. This method was previously successfully employed for 'stress-free' sampling of fish[171]. For each time point a non-stressed control group was included in the experimental design to rule out circadian or sampling effects.

Plasma hormone determination

Freshly collected heparinised blood was spun down in a cooled microcentrifuge (10 min at 10,000 rpm). Plasma was taken off and stored at –20°C until use. Cortisol was measured by radioimmunoassay[261], by the use of a commercial antiserum (Bioclinical Services Ltd., Cardiff, UK). All constituents were in phosphate-EDTA buffer (0.05 M Na_2HPO_4, 0.01 M Na_2EDTA, 0.003 M NaN_3, pH 7.4). Ten-µl samples or standards in RIA buffer (phosphate-EDTA buffer containing 0.1 % 8-anilio-1-naphtalene sulfonic acid and 0.1 % w/v bovine γ-globulin) were incubated with 100 µl antiserum (in RIA buffer containing 0.2 % normal rabbit serum) for 4 h. Samples were incubated overnight with 100 µl iodinated cortisol (approx. 1700 cpm/tube; ^{125}I-cortisol, Amersham Nederland BV, 's Hertogenbosch, The Netherlands) and 100 µl goat anti-rabbit γ-globulin (in RIA buffer). Bound and free cortisol were separated by the addition of 1 ml of ice-cold precipitation buffer (phosphate-EDTA buffer containing 2 % w/v bovine serum albumin and 5 % w/v polyethylene glycol). The tubes were centrifuged at 4°C (20 min at 2,000g), the supernatant aspirated and counted in a gamma counter (1272 clinigamma, LKB Wallac, Turku, Finland).

RNA isolation

Organs for RNA isolation were harvested and flash-frozen on dry ice. RNA isolation was conducted by the use of Trizol (Invitrogen) according to the manufacturers protocol. Total RNA was precipitated in ethanol, washed and dissolved in water. Concentrations were measured by spectrophotometry and integrity was ensured by analysis on a 1.5 % agarose gel. RNA was stored at –80°C until use.

DNAse treatment and first strand cDNA synthesis

For each sample a non-template control was included. One µl 10x DNASE I reaction buffer and 1 µl DNASE I (Invitrogen, 18068-015) was added to 1 µg total RNA and incubated at room temperature, 15 min in a total volume of 10 µl. DNASE I was inactivated by the addition of 1 µl 25 mM EDTA and incubation at 65°C, 10 min. To each sample 300 ng random hexamers, 1 µl 10 mM dNTP mix, 4 µl 5x First Strand buffer, 2 µl 0.1 M DTT and 10 U RNASE inhibitor (Invitrogen, 15518-012) were added and the mix was incubated 10 min at room temperature and an additional 2 min at 37°C. To each positive sample (but not the NT controls) 200 U Superscript RNASE H$^-$ Reverse Transcriptase (RT; Invitrogen, 18053-017) was added and reactions were incubated 50 min at 37°C. All reactions were

filled up with demineralised water to a total volume of 1 ml and stored at –20°C until further use.

Real-time quantitative PCR

Primer Express software (Applied Biosystems) was used to design primers for use in real-time quantitative PCR (RQ-PCR; Table 9.1). For RQ-PCR 5 µl cDNA and forward and reverse primer (300 nM each) were added to 12.5 µl Sybr Green PCR Master Mix (Applied Biosystems) and filled up with demineralised water to a volume of 25 µl. RQ-PCR (2 min 48°C, 10 min 95°C, 40 cycles of 15 sec. 95°C and 1 min 60°C) was carried out on a GeneAmp 5700 Sequence Detection System (Applied Biosystems). Data were analysed with the ΔΔCt method[41]. Dual internal standards (40s and β-actin) were incorporated in all RQ-PCR experiments and results were confirmed to be very similar following standardisation to either gene. Only results standardised for 40s expression are shown.

Western blotting

Fresh aqueous homogenates of hypothalamus and pituitary gland of carp were prepared by the use of a Potter glass-to-glass homogenisation device. The water insoluble moiety was removed by centrifugation (10 min. 15,800g) and the water-soluble moiety was run under denaturing conditions on a 12.5% SDS gel. Human recombinant CRH-BP (hrCRH-BP 254-299; a generous gift of Prof. dr. Wylie Vale) was loaded as size marker. After running, the gel was blotted on a nitro-cellulose filter and blocked with PBS containing 1% casein. CRH-BP was detected by the use of a rabbit anti-human CRH-BP antiserum (#5144; a generous gift of Prof. dr. Wylie W. Vale)[431] at a 1:2000 dilution during 1h followed by goat anti-rabbit Ig-HRP (1:1500, 1h; Bio-rad). Signal was detected with a chemoluminescence kit (Amersham) according to the manufacturers protocol and visualised by the use of Lumni-film chemiluminescent Detection Film (Roche). Controls without primary antibody were negative.

Immunohistochemistry

Tissue was fixed in Bouin (15 ml picric acid, 5 ml formol, 1 ml glacial acetic acid), dehydrated and embedded in paraffin. Sections of 5 µm were used for immunohistochemistry. CRH was detected by the use of a rabbit anti-sheep CRH antiserum (1:1000; Incstar)[432]. CRH-BP was detected with a rabbit anti-human CRH-BP antiserum (#5144) at a dilution of 1:1000. Arginine vasotocin (AVT) was detected with a rabbit anti-AVT antiserum at a dilution of 1:4000[433]. Primary antibodies were incubated overnight. Goat anti-rabbit IgG-biotin (1:200, 1h; Bio-rad) was used as secondary antibody followed by amplification via the vectastain® ABC amplification kit (Vector laboratories) according to the manufacturer's protocol. Signal was visualised with AEC (3-amino-6-ethylcarbazole,

Sigma) as a substrate. Controls for cross-reactivity of the secondary reagents and for endogenous enzyme activity were included in all experiments and were negative.

Phylogenetic analyses

Sequences were retrieved from the Swissprot, EMBL and Genbank databases at the SRS mirror site of the Centre of Molecular and Biomolecular Informatics (www.cmbi.kun.nl). Pufferfish (*Takifugu rubripes*) sequences were retrieved from http://www.ensembl.org/Fugu_rubripes/. Zebrafish sequences were retrieved from http://www.ensembl.org/Danio_rerio/. Multiple sequence alignment was carried out with ClustalW at the CMBI mirror site. Phylogenetic trees were constructed on the basis of amino acid difference (p-distance) by the neighbour-joining method[45] with MEGA version 2.1[46]. Reliability of the tree was assessed by bootstrapping, using 1000 bootstrap replications.

Statistics

All statistical analyses were carried out with SPSS software (version 10.1.0). Data were tested for normal distribution with the Shapiro-Wilk test. Homogeneity of variances was tested with the Levene test. Differences were evaluated with one-sided one factor analysis of variance (ANOVA). Kruskal-Wallis H-test was applied in case of non-normal distribution. If Kruskal-Wallis was significant, the Mann-Whitney U-test was used to determine which means differed significantly from the control.

Results

Cloning and characteristics of two carp CRH genes

A partial sequence of carp CRH was obtained in a homology cloning approach from carp hypothalamic cDNA. In search for the corresponding full-length sequence, two highly similar sequences were found (Fig. 9.1). They were designated CRH1 and CRH2. The 3' UTR contains a polyadenylation site (bp 918-923) and four potential instability motifs (attta; bp 615-619, 670-674, 683-687, 687-691). Both genes consist of an open reading frame encoding a 162 amino acid protein, which encompasses a signal peptide (M^{1}-A^{24}), a conserved region within the cryptic peptide (R^{48}-R^{60}), and the mature peptide (S^{120}-F^{160}) based on similarity with mammalian CRH sequences. The mature peptide is flanked by a dibasic cleavage site and a potential C-terminal amidation site. The coding regions of both genes differs in thirteen nucleotides, that result in eight amino acid substitutions. One of these substitutions (L^{12} to P^{12}) is within the predicted signal peptide and one (A^{123} to P^{123}) is within the mature peptide. The remaining substitutions are situated within the

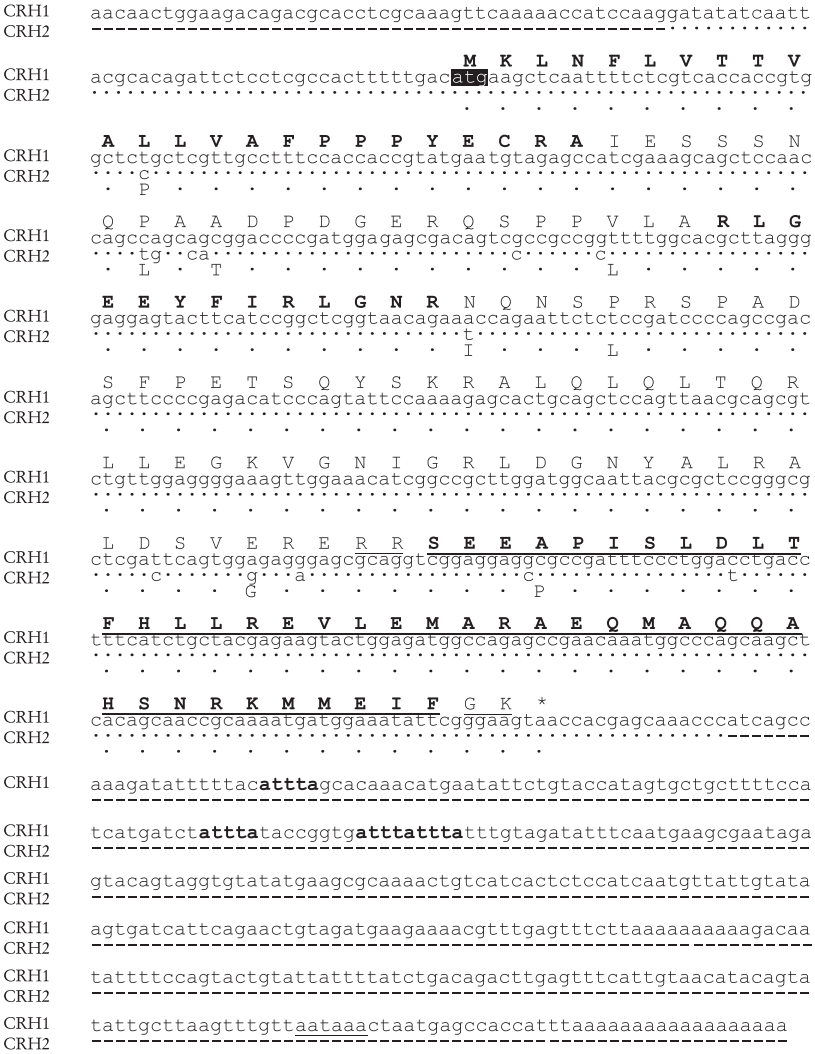


Figure 9.1: Nucleotide and deduced amino acid sequences of two carp CRH cDNAs. The start codon is boxed, the stop codon is indicated by the asterisk. Dots indicate identical residues, hyphens indicate gaps. Potential instability motifs are indicated in bold. A putative polyadenylation signal is underlined. The deduced amino acid sequences of CRH1 and CRH2 are displayed above and underneath the nucleotide sequences respectively. The predicted signal peptide (M^{1}-A^{24}) and the conserved cryptic motif within the cryptic peptide (R^{48}-R^{60}) are presented in bold capitals. The sequence of the mature peptide (S^{120}-F^{160}) is underlined and presented in bold capital. The predicted cleavage site and the C-terminal amidation site are underlined. Accession numbers: CRH1, AJ317955; CRH2, AJ576243.

Table 9.2: Percentages amino acid sequence identity of CRH sequences of various vertebrate species. The identity is given for the complete sequence and for the mature peptide (in brackets)

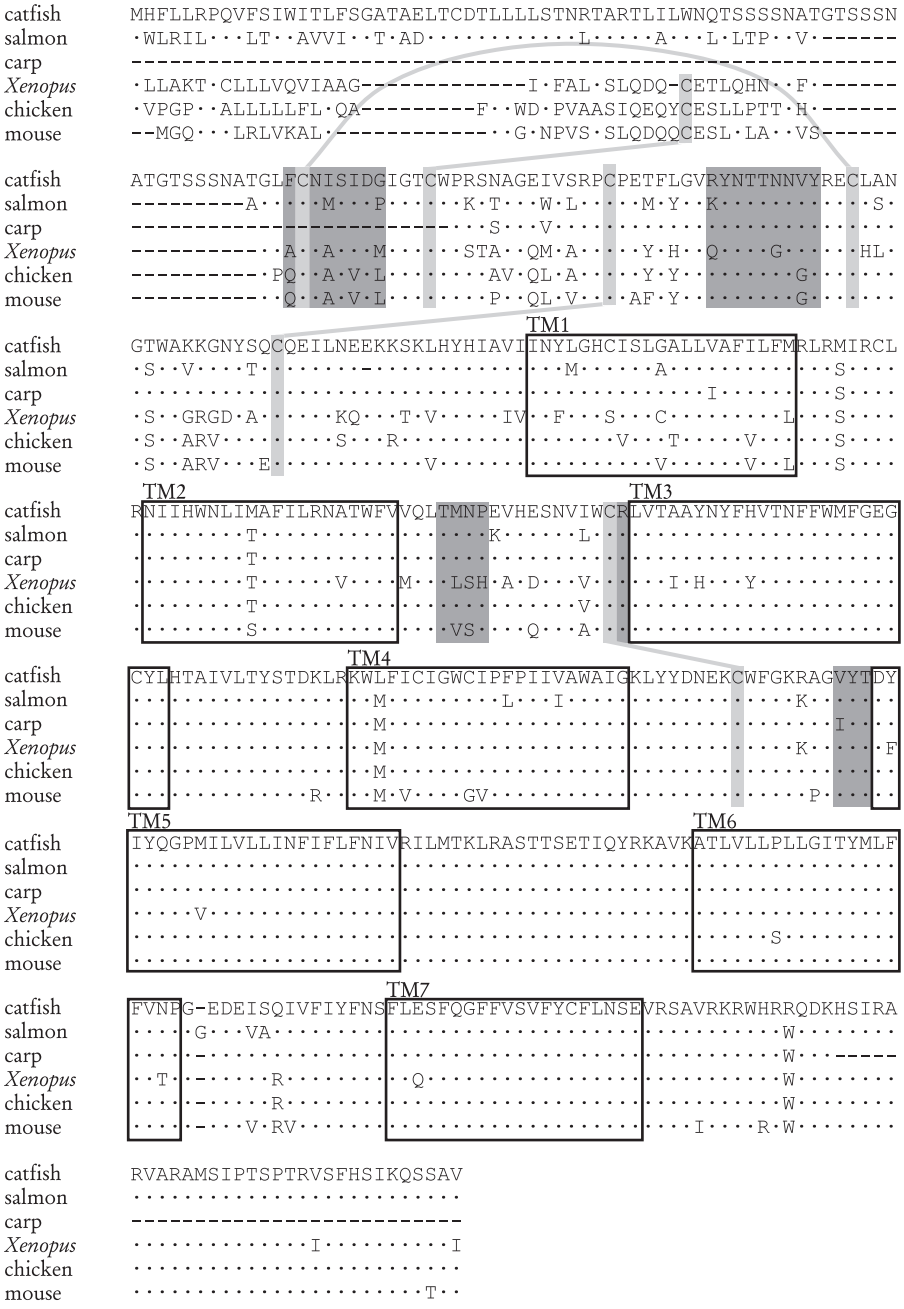
	carp 1	carp 2	white sucker 1	white sucker 2	tilapia	*Xenopus*	sheep	rat	human
carp 1	100								
carp 2	96 (98)	100							
white sucker 1	91 (95)	90 (98)	100						
white sucker 2	88 (93)	85 (95)	91 (98)	100					
tilapia	62 (76)	61 (78)	62 (83)	59 (78)	100				
Xenopus	51 (85)	49 (88)	48 (88)	49 (85)	49 (68)	100			
sheep	48 (76)	46 (78)	48 (80)	51 (78)	46 (37)	49 (80)	100		
rat	54 (90)	53 (93)	54 (95)	56 (93)	51 (76)	58 (93)	67 (83)	100	
human	56 (90)	54 (93)	55 (95)	56 (93)	50 (76)	56 (93)	77 (83)	82 (100)	100

pro-hormone. Both carp CRH sequences bear high (96%) to intermediate (60%) sequence identity to other teleost fish CRH sequences (Table 9.2). Sequence identity to mammalian CRH sequences is considerably lower (around 45%). However, sequence identity within the various conserved regions, most notably the mature peptide, is considerably higher (up to 90% identity between carp and human).

Cloning and characteristics of carp CRH-R1

Two highly similar partial cDNA sequences encoding the carp CRH-R1 were obtained from brain cDNA using a homology cloning approach. Both sequences differ at seventeen nucleotide positions, but none of these result in an amino acid substitution (not shown). Overall, the sequences of teleost fish and mammals are highly similar at the amino acid level (Fig 9.2). The N-terminal part of the protein, which contains the signal peptide is the most variable part of the protein. The seven predicted transmembrane regions are well conserved (Fig. 9.2). The C-terminal region of the receptor is extremely well conserved; the third intracellular loop, presumably involved in G-protein binding, is identical in amino acid sequence between fish, amphibian, avian and mammalian sequences.

Figure 9.2: Amino acid alignment of CRH-R1 sequences from various vertebrate species, including carp. Dots indicate identical residues, hyphens indicate gaps. Cysteine residues involved in the formation of disulphide bonds (mouse C^{30}-C^{54}, C^{44}-C^{87}, C^{68}-C^{102}) are shaded light. The residues implicated in ligand binding in the human CRH-R1 are shaded dark. The seven predicted transmembrane regions are boxed. Note the exceptional conservation of the C-terminal part of the protein. Accession numbers: catfish (*Ameiurus nebulosus*), AF229359; salmon (*Oncorhynchus keta*), AJ277157; carp (*Cyprinus carpio*), AJ576244; *Xenopus* (*Xenopus laevis*), O42602; chicken (*Gallus gallus*), Q90812; mouse (*Mus musculus*), P35347.


catfish
salmon
carp
Xenopus
chicken
mouse
TM1
TM2
TM3
TM4
TM5
TM6
TM7

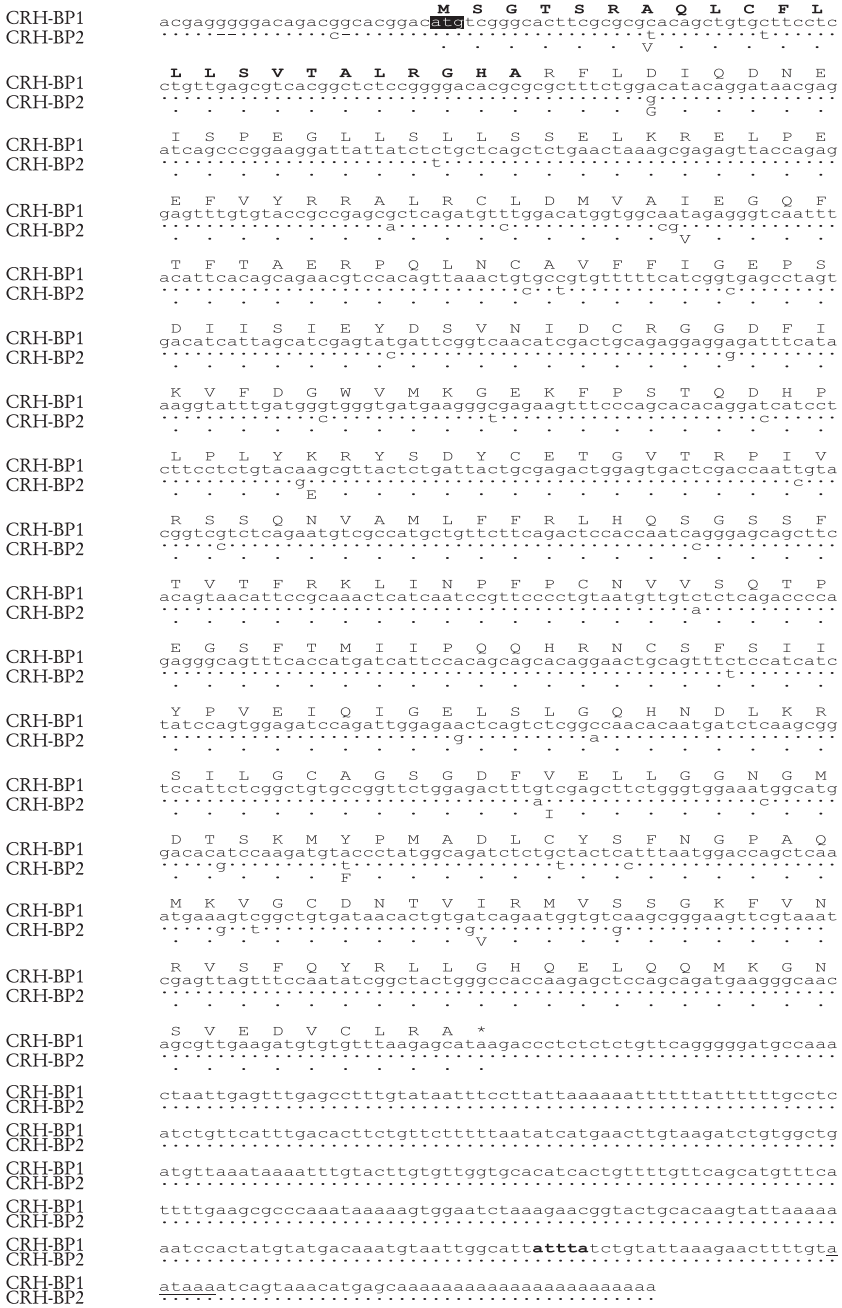

CRH-BP1
CRH-BP2
M S G T S R A Q L C F L
L L S V T A L R G H A R F L D I Q D N E
I S P E G L L S L L S S E L K R E L P E
E F V Y R R A L R C L D M V A I E G Q F
T F T A E R P Q L N C A V F F I G E P S
D I I S I E Y D S V N I D C R G G D F I
K V F D G W V M K G E K F P S T Q D H P
L P L Y K R Y S D Y C E T G V T R P I V
R S S Q N V A M L F F R L H Q S G S S F
T V T F R K L I N P F P C N V V S Q T P
E G S F T M I I P Q Q H R N C S F S I I
Y P V E I Q I G E L S L G Q H N D L K R
S I L G C A G S G D F V E L L G G N G M
D T S K M Y P M A D L C Y S F N G P A Q
M K V G C D N T V I R M V S S G K F V N
R V S F Q Y R L L G H Q E L Q Q M K G N
S V E D V C L R A *
ctaattgagtttgagccttttgtataatttccttattaaaaaattttttattttttgcctc
atctgttcatttgacacttctgttctttttaatatcatgaacttgtaagatctgtggctg
atgttaaataaaatttgtacttgtgttggtgcacatcactgttttgttcagcatgtttca
ttttgaagcgcccaaataaaaagtggaatctaaagaacggtactgcacaagtattaaaaa
aatccactatgtatgacaaatgtaattggcatt**atttta**tctgtattaaagaacttttgta
ataaaatcagtaaacatgagcaaaaaaaaaaaaaaaaaaaaaa

Table 9.3: Percentages amino acid sequence identity of CRH-BP sequences of various vertebrate species

	carp 1	carp 2	pufferfish	*Xenopus*	chicken	sheep	rat	human
carp 1	100							
carp 2	97.8	100						
pufferfish	68.2	68.7	100					
Xenopus	58.9	59.2	55.3	100				
chicken	59.8	59.5	57.9	72.0	100			
sheep	57.0	57.0	55.3	62.6	70.8	100		
rat	58.3	58.6	55.7	63.9	71.1	77.3	100	
human	61.7	62.0	58.5	68.2	73.9	85.1	84.5	100

Cloning and characteristics of two carp CRH-BP genes

Two highly similar genes encoding the carp orthologues of mammalian CRH-BP were amplified from a total brain cDNA library. They were designated CRH-BP1 and CRH-BP2 and both genes contain an open reading frame encoding a 321 protein (Fig. 9.3). The 3' UTR contains one potential instability motif (bp 1294-1298) and a polyadenylation signal (bp 1320-1325). In the 5' UTR both genes differ in two small insertions and/or deletions (indels) and a nucleotide substitution. The coding regions of both genes differ at 34 additional nucleotide positions, resulting in seven amino acid substitutions. The predicted signal peptide (M^{1}-R^{24}) is the most variable part of the protein, whereas the rest of the protein is better conserved (Table 9.3). The ten cystein residues implicated in the formation of five disulphide bonds (carp C^{62}-C^{83}, C^{106}-C^{143}, C^{185}-C^{207}, C^{239}-C^{266}, C^{279}-C^{320}) are conserved between fish, amphibian, avian and mammalian sequences (Fig. 9.4). Using the complete carp CRH-BP sequences we retrieved the pufferfish CRH-BP gene from its genome database, to study the CRH-BP gene organisation in fish. Comparison of the genomic organisation of the pufferfish CRH-BP gene with that of human revealed a well-conserved gene, consisting of seven exons (Fig. 9.5). The exons comprising the pufferfish CRH-BP gene are of identical length as those of human CRH-BP, with the exception of exons five and seven, that both extend one triplet in the human gene. All pufferfish introns contain well-recognisable 5' donor (gt) and 3' splice acceptor (ag) sites.

Phylogenetic analyses

To address the relationship between carp CRH, CRH-R1 and CRH-BP and their mammalian and non-mammalian orthologues, we constructed phylogenetic trees using the neighbour-joining method. The overall topology of the CRH-family tree (Fig. 9.6a) shows clustering

Figure 9.3: Nucleotide and deduced amino acid sequences of two carp CRH-BP cDNAs. The start codon is boxed, the stop codon is indicated by the asterisk. Dots indicate identical residues, hyphens indicate gaps. Potential instability motifs are indicated in bold. A putative polyadenylation signal is underlined. The deduced amino acid sequences of CRH-BP1 and CRH-BP2 are displayed above and underneath the nucleotide sequences respectively. The predicted signal peptide is indicated in bold. Accession numbers: CRH-BP1, AJ490880; CRH-BP2, AJ490881.

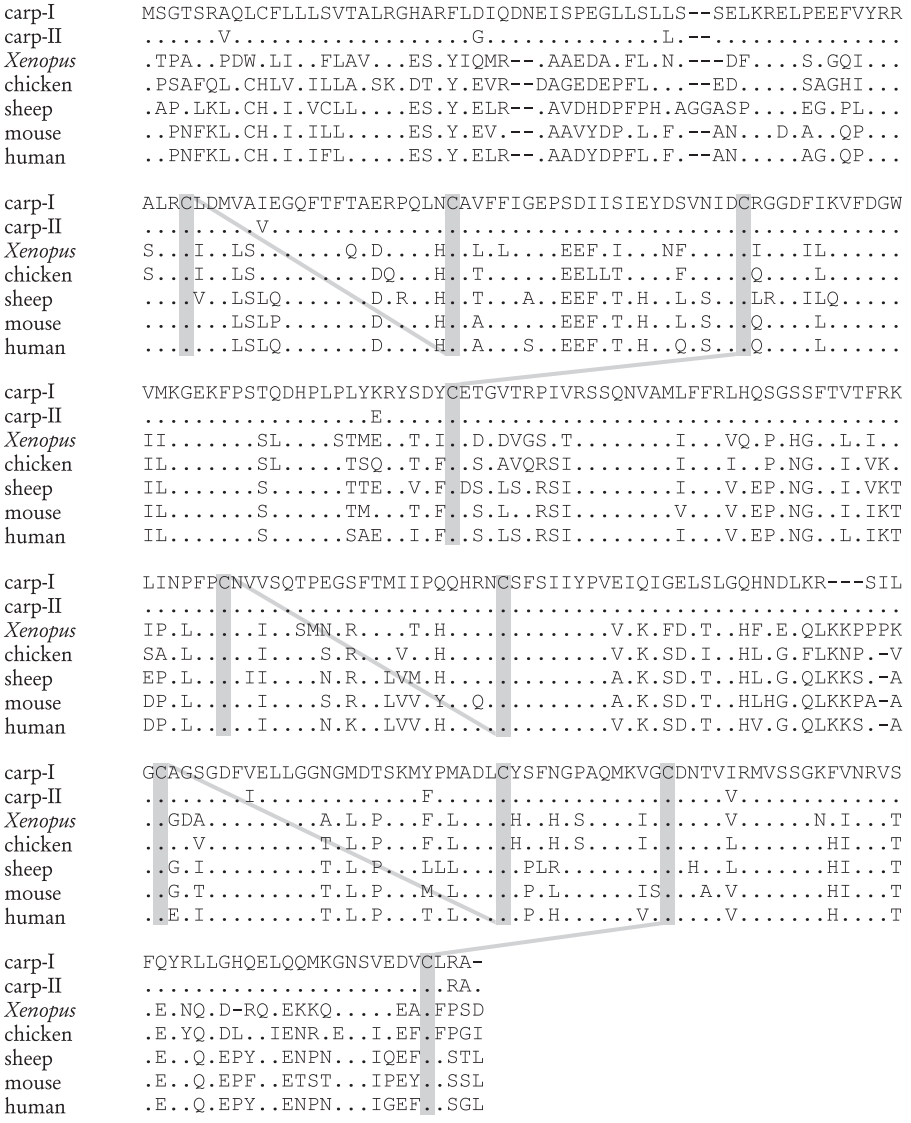


Figure 9.4: Amino acid alignment of CRH-BP sequences from various vertebrate species, including carp. Dots indicate identical residues, hyphens indicate gaps. Cysteine residues involved in the formation of disulphide bonds (carp C^{62}-C^{83}, C^{106}-C^{143}, C^{185}-C^{207}, C^{239}-C^{266}, C^{279}-C^{320}) are shaded. Accession numbers: carp (*Cyprinus carpio*) CRH-BP1, AJ490880; carp CRH-BP2, AJ490881; Xenopus (*Xenopus laevis*), Q91653; chicken (*Gallus gallus*), predicted from BU358572 and BU367671; sheep (*Ovis aries*), Q28557; mouse (*Mus musculus*), Q60571; human (*Homo sapiens*), P24387.

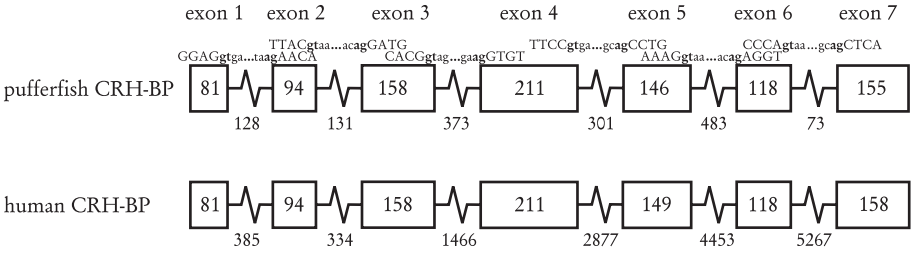


Figure 9.5: Comparison of the genomic organisation of the pufferfish and human CRH-BP genes. Boxes represent exons. Exon sizes are indicated within the boxes, intron sizes are given underneath the introns. The four nucleotide residues surrounding each splice site are given for the pufferfish gene, coding residues are represented by capitals. The actual splice donor and acceptor residues are indicated in bold. Accession number for the pufferfish CRH-BP gene is BN000457.

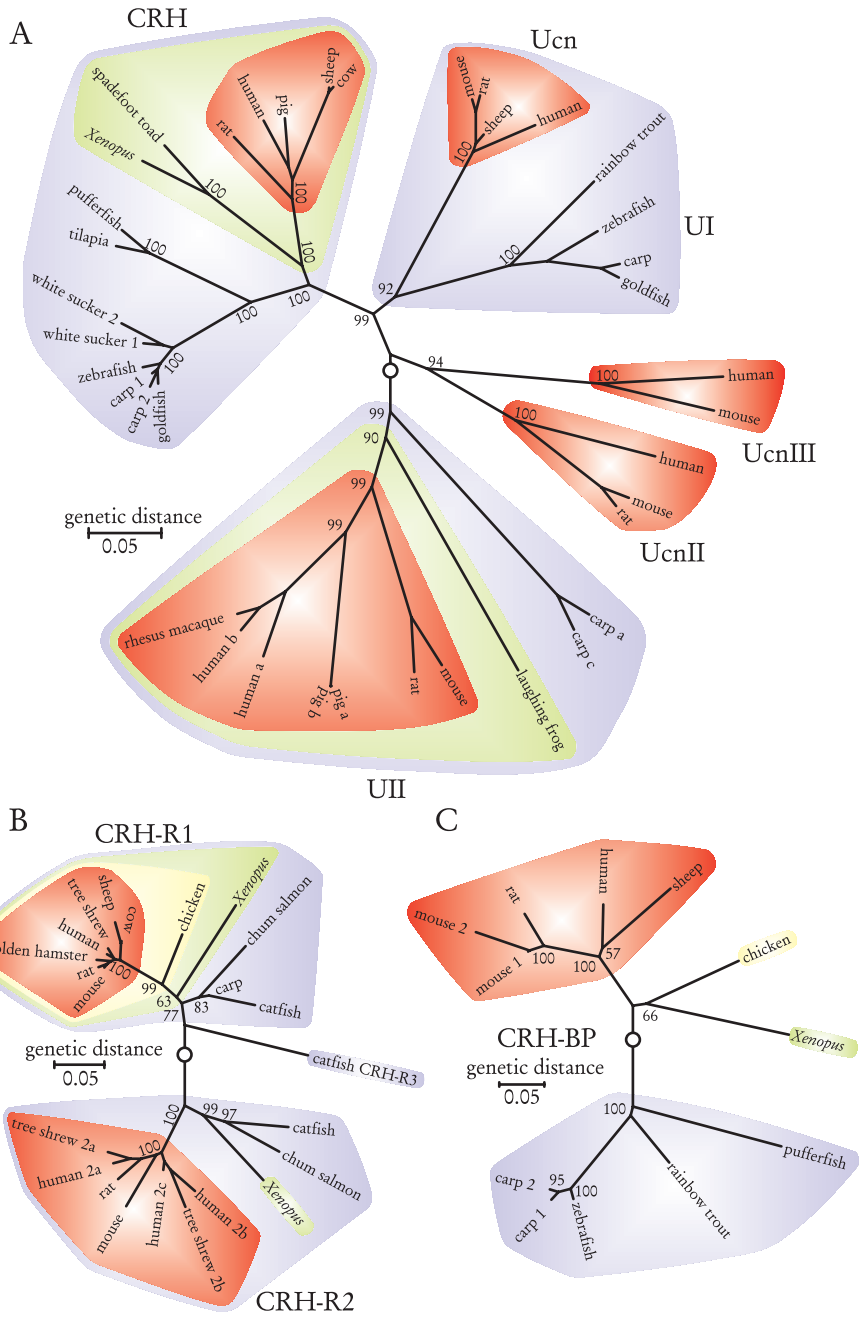
of the fish CRH sequences, with sequences from cyprinid species clustering together apart from CRH sequences from the more recent teleost species tilapia and pufferfish. The cluster of mammalian CRH sequences is separated from the fish sequences by *Xenopus* CRH. Mammalian UCN and teleost fish UI sequences cluster together, reflecting their proposed orthology. The vertebrate CRH and UI/UCN clusters together form one clade, supported by a bootstrap value of 99.

All vertebrate CRH-R1 sequences cluster separately from CRH-R2 (Fig. 9.6b). The topology of each cluster reflects the evolutionary relationship between the vertebrate classes. The carp CRH-R1 sequence clusters well within the teleost fish CRH-R1 cluster. The catfish CRH-R3[423] clusters separately from both the CRH-R1 and the CRH-R2 cluster.

Avian and amphibian CRH-BP cluster close to the mammalian CRH-BP sequences, whereas the evolutionary more distantly related teleost fish sequences also cluster more distantly (Fig 9.6c). The very similar carp CRH-BP sequences cluster in very close proximity, as do both murine CRH-BP sequences, reflecting a recent common origin.

Expression of the CRH system

The expression of CRH, CRH-R1 and CRH-BP as well as UI was studied in the hypothalamus and pituitary gland of healthy non-stressed carp. Expression was plotted relative to the expression of ribosomal protein 40S. CRH and UI expression was detected in the hypothalamus only (Fig. 9.7a). CRH-R1 was expressed abundantly in the hypothalamus (Fig. 9.7b). Within the pituitary gland, CRH-R1 was expressed more abundantly (approximately four-fold) in the PD than in the PI. Hypothalamic CRH-BP expression was very high, with CRH-BP messengers approaching the abundance of 40S mRNA. Pituitary gene expression of CRH-BP was relatively low, with most CRH-BP expressed in the PI. None


A
CRH
Ucn
spadefoot toad
Xenopus
pufferfish
tilapia
white sucker 2
white sucker 1
zebrafish
carp 1
carp 2
goldfish
human
pig
sheep
cow
rat
mouse
sheep
human
rainbow trout
zebrafish
carp
goldfish
UI
UcnIII
UcnII
UII
genetic distance
0.05
rhesus macaque
human b
human a
pig b
pig a
rat
mouse
laughing frog
carp a
carp c
B
CRH-R1
golden hamster
tree shrew
sheep
cow
human
rat
mouse
chicken
Xenopus
chum salmon
carp
catfish
catfish CRH-R3
genetic distance
0.05
tree shrew 2a
human 2a
rat
mouse
human 2c
human 2b
tree shrew 2b
catfish
chum salmon
Xenopus
CRH-R2
C
mouse 2
mouse 1
rat
human
sheep
chicken
Xenopus
CRH-BP
genetic distance
0.05
carp 2
carp 1
zebrafish
rainbow trout
pufferfish

of these four genes were significantly expressed within either muscle or liver tissue (not shown). Non-reverse transcriptase controls were negative (not shown).

To study the expression of CRH-BP in conjunction with CRH at the protein level we performed immunohistochemistry. The anti-CRH-BP antiserum detected a protein of very similar size to the 37 kDa human recombinant CRH-BP (hrCRH-BP) in the water-soluble fraction of homogenates from non-stressed carp hypothalamus and pituitary gland (Fig. 9.8). Although we cannot compare total avidity and affinity of the antiserum to hrCRH-BP and carp CRH-BP, this suggests that at least some of the epitopes recognised by the antiserum are formed by sufficiently conserved parts of the carp CRH-BP sequence. In sections of the hypothalamus of non-stressed carp, the NPO shows prominent CRH positive neurones. A dorsally situated magnocellular area (*pars magnocellularis*; NPOpmc) was clearly distinguishable from a ventral parvocellular area (*pars parvocellularis*; NPOppc) (Fig. 9.9a). Higher magnification of the NPOpmc on serial sections stained with haemaluin/eosin (Fig. 9.9b), CRH (Fig. 9.9c) and arginine vasotocin (AVT; Fig. 9.9d) showed that a considerable number of the magnocellular perikarya contained either CRH or AVT

Figure 9.6: Neighbour joining trees of vertebrate CRH-like amino acid sequences (a), CRH-R sequences (b) and CRH-BP sequences (c). Numbers at branch nodes represent the confidence level of 1000 bootstrap replications. Clusters of sequences are shaded in red (mammals), yellow (birds), green (amphibia), and lilac (teleostean fishes). An open circle indicates the starting point for cluster formation. Scientific names are as follows: carp, *Cyprinus carpio*; catfish, *Ameiurus nebulosus*; cow, *Bos taurus*; chicken, *Gallus gallus*; chum salmon, *Oncorhynchus keta*; golden hamster, *Mesocricetus auratus*; goldfish, *Carassius auratus*; human, *Homo sapiens*; laughing frog, *Rana ridibunda*; mouse, *Mus musculus*; pig, *Sus scrofa*; pufferfish, *Takifugu rubripes*; rainbow trout, *Oncorhynchus mykiss*; rat, *Rattus norvegicus*; rhesus macaque, *Macaca mulatta*; sheep, *Ovis aries*; spadefoot toad, *Spea hammondii*; tilapia, *Oreochromis mossambicus*; tree shrew, *Tupaia belangeri*; white sucker, *Catostomus commersoni*; *Xenopus*, *Xenopus laevis*; zebrafish, *danio rerio*. Accession numbers are as follows: carp CRH1, AJ317955; carp CRH2, AJ576243; carp CRH-BP1, AJ490880; carp CRH-BP2, AJ490881; carp CRH-R1, AJ576244; carp UI, P01146; carp UIIa, P04560; carp UIIg, P06580; catfish CRH-R1, AF229359; catfish CRH-R2, AF229360; catfish CRH-R3, AF229361; chicken CRH-BP, BU358572/BU367671; chicken CRH-R1, Q90812; cow CRH, AAK83231; cow CRH-R1, BAB21864; chum salmon CRH-R1, AJ277157; chum salmon CRH-R2, CAC81754; golden hamster CRH-R1, AAK59707; goldfish CRH, Q9PTS1; goldfish UI, Q9PTQ4; human CRH, P06850; human CRH-BP, P24387; human CRH-R1, P34998; human CRH-R2α, Q13324; human CRH-R2β, AAB94503; human CRH-R2γ, AAC71654; human UCN, NP_003344; human UCN2, Q96RP3; human UCN3, Q969E3; human UIIa, NP_068835; human UIIb, O95399; laughing frog UII, P33715; mouse CRH-BP1, Q60571; mouse CRH-BP2, XP_138793; mouse CRH-R1, P35347; mouse CRH-R2, Q60748; mouse UCN, P81615; mouse UCN2, Q99ML8; mouse UCN3, Q924A4; mouse UII, Q9QZQ3; pig CRH, P06296; pig UIIa, Q95J46; pig UIIb, Q95K72; pufferfish CRH, SINFRUP00000155211 (http://www.ensembl.org/Fugu_rubripes/); pufferfish CRH-BP, BN000457; rainbow trout CRH-BP, BX076908; rainbow trout UI, O93448; rat CRH, P01143; rat CRH-BP, P24388; rat CRH-R1, P35353; rat CRH-R2, P47866; rat UCN, P55090; rat UCN2, Q91WW1; rat UII, Q9QZQ4; rhesus macaque UII, AAL55429; sheep CRH, P01142; sheep CRH-BP, Q28557; sheep CRH-R1, O62772; sheep UCN, AAC35555; tilapia CRH, Q9I9D0; spadefoot toad CRH, AY262255; tree shrew CRH-R1, CAD19577; tree shrew CRH-R2α, CAD19578; tree shrew CRH-R2β, CAD19579; white sucker CRH1, P13241; white sucker CRH2, P25308; *Xenopus* CRH, P49188; *Xenopus* CRH-BP, Q91653; *Xenopus* CRH-R1, O42602; *Xenopus*, CRH-R2, O42603; zebrafish CRH, ENSDARP00000003130 (http://www.ensembl.org/Danio_rerio/); zebrafish UI, BI844591; zebrafish CRH-BP, BQ450925/BQ615049.

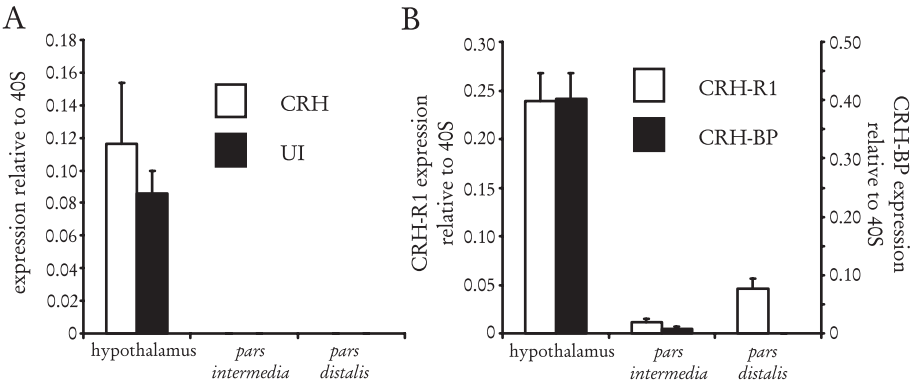


Figure 9.7: Basal expression of CRH (open bars), UI (filled bars) (a) CRH-R1 (open bars) and CRH-BP (filled bars) (b) in various organs of carp. CRH and UI expression within the *pars intermedia* and *pars distalis* was non-detectable. Expression was assessed by RQ-PCR and is plotted relative to the expression of 40S. Error bars indicate the SD of five replicate measurements.

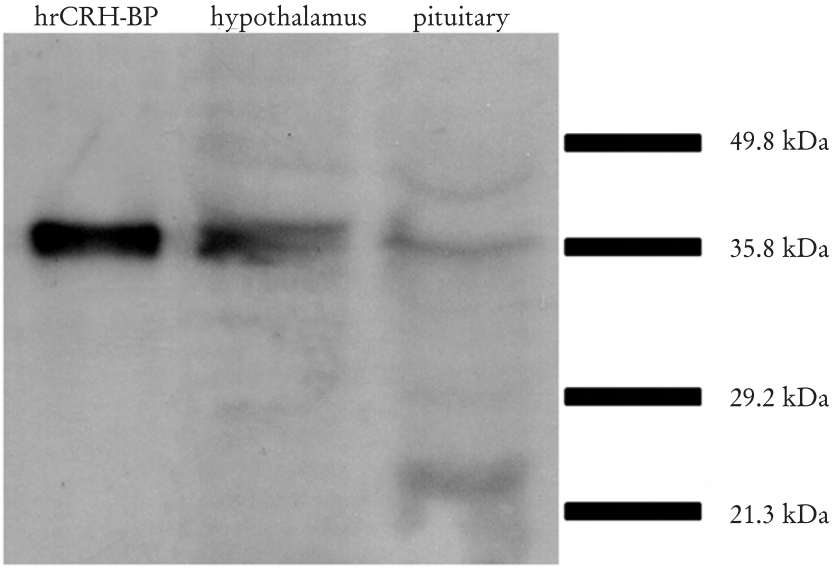


Figure 9.8: Western blot showing proteins of very similar size to human recombinant CRH-BP in the water soluble moiety of crude lysates of carp hypothalamus and pituitary gland.

and that some perikarya were positive for both hormones. Within the NPOppc a similar pattern was observed with regard to the presence of CRH and AVT (not shown). When the same area was stained on serial sections for the presence of CRH-BP, a limited number of relatively small CRH-BP$^+$ perikarya was observed at the caudal perimeter of the NPO (Fig. 9.9e). These CRH-BP$^+$ perikarya did not contain CRH or AVT. Abundant CRH-BP staining was observed in a cluster of cells and nerve fibres located at the floor of the hypothalamus 600 μm rostrally of the NPOppc (Fig. 9.9f). This area contained neither CRH nor AVT (not shown).

In the pituitary gland CRH was most conspicuously present in the nerve fibre bundles of the *pars nervosa* (PN) that project to the PI melanotropes. Also, a relatively small set of nerve fibres of the rostral *pars distalis* (rPD) stained clearly for the presence of CRH (Fig. 9.10a). The proximal *pars distalis* (pPD) did not contain CRH immunoreactivity. A higher magnification of the rPD showed that the ACTH cells were aligned in a sheet around these fibres (Fig. 9.10b). The nerve fibres proper contained immunoreactive CRH in a distinct granular pattern (Fig. 9.10c) as well as immunoreactive CRH-BP that was distributed more evenly within the fibre (Fig. 9.10d). The PN also contained considerable CRH-BP immunoreactivity (see below). Controls for cross-reactivity of the secondary reagents or endogenous enzyme activity were consistently and completely negative.

Regulation of the CRH system during acute restraint stress

To asses the regulation of CRH, CRH-R1 and CRH-BP during stress, we subjected carp to either a 30 min or a 24 h period of restraint stress. In the 30 min restraint stress paradigm two groups of fish were followed up to 90 and 240 min, respectively, to allow potential changes in gene transcription to establish. After 30 min restraint, plasma cortisol values had risen to 100 ng/ml (Fig 9.11a). Within the next 90 min they had returned to baseline. The 30 min restraint period did not have a detectable effect on hypothalamic expression of CRH or CRH-BP as expression levels of both genes remained constant throughout the experiment (Fig. 9.11c). Following 24 h of restraint, plasma cortisol levels had reached nearly 500 ng/ml (Fig. 9.11b). Following 24 h restraint hypothalamic CRH as well as CRH-BP expression had increased significantly, whereas UI expression remained unaltered (Fig. 9.11d). The expression of CRH-R1 within the PD showed a concomitant and significant decrease following 24 h restraint (Fig. 9.11e). CRH-BP expression within the PD did not change significantly.

Upon histological examination of the hypothalamic area following 24 h restraint no differences were observed with regard to CRH, AVT or CRH-BP content (not shown). Within the PN, considerable differences were observed between non-stressed and 24 h restrained fish. The PN fibre bundles of non-stressed carp contained considerable amounts of CRH (Fig. 9.12a) as well as CRH-BP (Fig. 9.12b). CRH and CRH-BP were co-localised as visualised

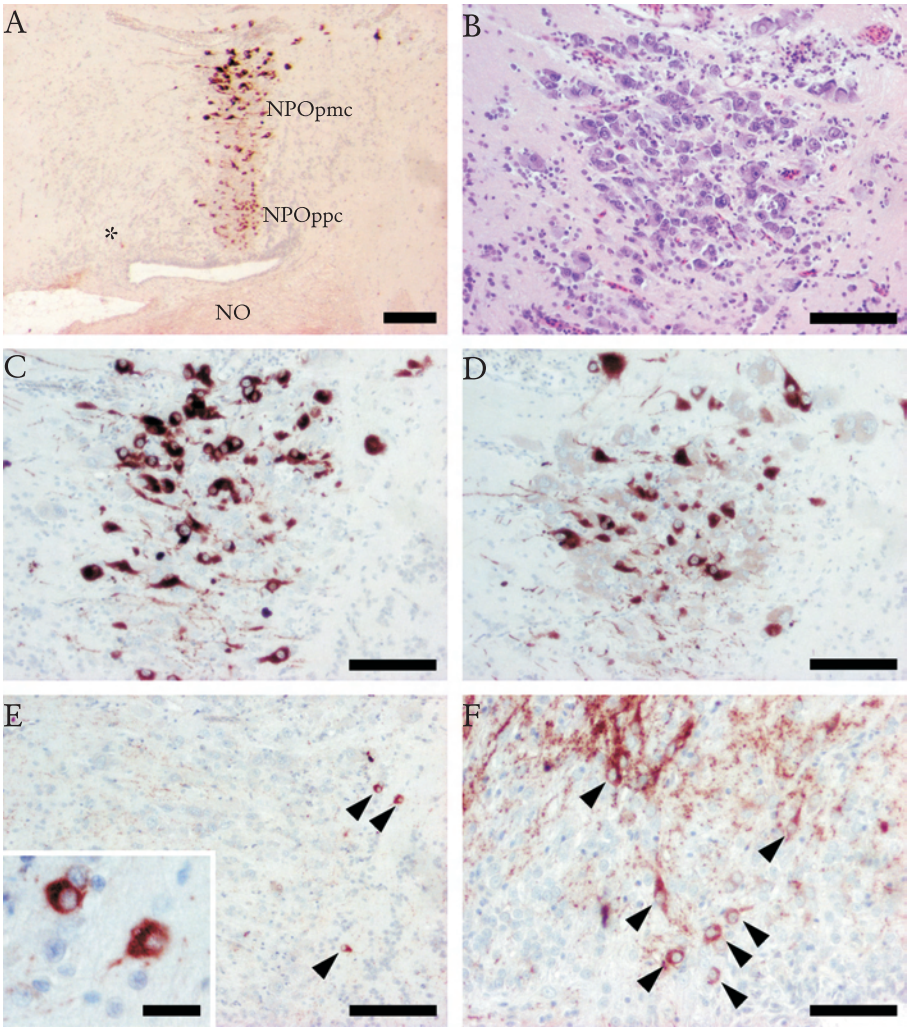


Figure 9.9: Serial immunohistochemistry on paramedian sagittal sections of the NPO. Panel a shows an overview of the preoptic area, stained with an anti-CRH antiserum. Panel b shows the NPOpmc enlarged with an haemaluin-eosin stain. Panels c and d show the same area on serial sections stained for CRH and AVT respectively. Panel e shows in a serial section the presence of several small CRH-BP positive perikarya on the caudal perimeter of the NPO, at the region marked by the asterisks in panel a. The same cells are shown in enlargement in the inset. Panel f shows abundant CRH-BP staining in perikarya as well as neurones in the recessus opticus, just rostral from the NPO. NPOpmc, *nucleus preopticus pars magnocellularis*; NPOppc, *nucleus preopticus pars parvocellularis*; NO, *nervus opticus*. Arrowheads mark CRH-BP positive perikarya. Scale bars: a 200 μm, b-e 100 μm, e (inset) 20 μm, f 50 μm.

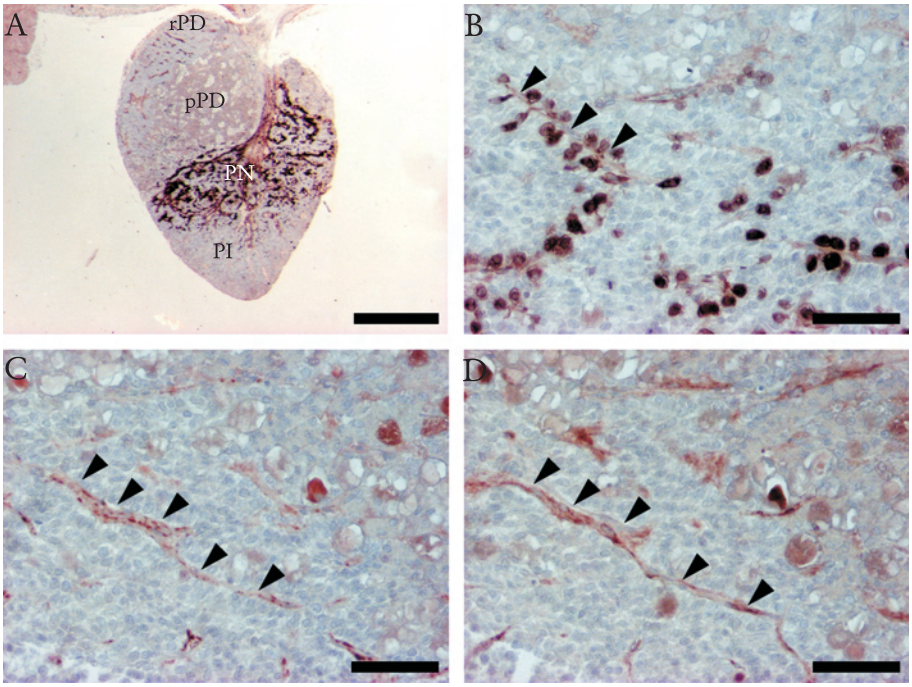


Figure 9.10: Serial immunohistochemistry on sagittal sections of the pituitary gland. Panel a shows an overview of the pituitary gland, stained with an anti-CRH antiserum. Note the profound staining of the PN fibres projecting on the PI as well as the staining of the fibres within the rPD but not the pPD. Panel b-d show details of the rPD of serial sections stained for ACTH, CRH and CRH-BP respectively. Note the alignment of ACTH positive cells in a double row (b) surrounding a nerve fibre that is positive for both CRH (c) and CRH-BP (d). Arrowheads mark a distinct CRH/CRH-BP double positive nerve fibre. PI, *pars intermedia*; PN, *pars nervosa*; pPD, *proximal pars distalis*; rPD, *rostral pars distalis*; NO, *nervus opticus*. Scale bars: a 500 μm, b-d 50 μm.

in serial sections. Following 24 h restraint the amount of CRH contained within the PN fibres was unaltered or slightly diminished (Fig. 9.12c). However, the amount of immunoreactive CRH-BP contained within the PN fibre bundles was markedly reduced as shown by serial sections (Fig. 9.12d).

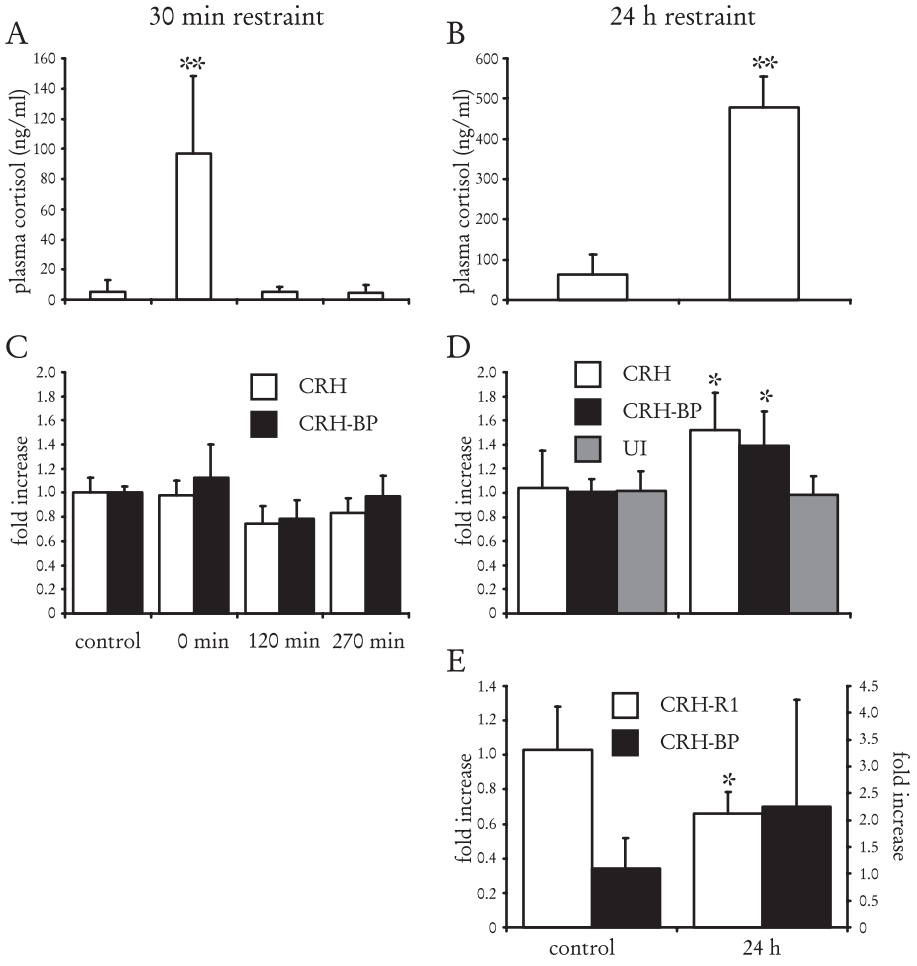


Figure 9.11: Changes in plasma cortisol values (a, b) and in hypothalamic and PD expression levels (c-e) during 30 min (a, c) and 24 h (b, d, e) restraint. The indicated times reflect the time passed since the initiation of restraint stress. Note the different scales of the y-axes in panels a and b. Error bars indicate the SD of eight (cortisol) or five (expression) replicate measurements. Asterisks indicate significant differences from the control (** $p>0.01$; * $p>0.05$). Expression is standardised for 40s and the relative quantitation value expressed as $2^{-\Delta\Delta C_T}$.

Discussion

CRH, CRH-R1 and CRH-BP, some of the principal molecules that regulate the initiation of the vertebrate stress response were sequenced in common carp. The overall amino acid identity of these proteins between fish and mammalian species is relatively high, generally around 55 – 60%, confirming that the signal system regulating the stress response is conserved throughout vertebrate evolution. The high degree of conservation of the CRH family of peptides earlier facilitated the discovery of several of the mammalian family members following the elucidation of their teleost fish orthologues[419, 434]. The elucidation of the complete set of CRH, CRH-R1 and CRH-BP in one teleost fish species allowed the study of the expression of CRH in relation to the expression of CRH-BP and CRH-R1, the principle pituitary gland receptor for CRH. The involvement of these molecules in the regulation of the teleost fish stress response is demonstrated in a restraint stress paradigm.

Protein characteristics

For each of the three proteins described, two highly similar (>95% amino acid identity) genes were found; a likely result of the teleost fish tetraploid genome. This is in agreement with previous findings for many other genes, including β-actin, growth hormone, prolactin and pro-opiomelanocortin that are present in duplicate form in the carp[253, 261]. Nonetheless, any amino acid substitution observed potentially alters protein structure and consequently function. Most noteworthy is the substitution of amino acid residue P[123] of carp CRH2 for A[123] in CRH1, which is a unique substitution among all known vertebrate CRH sequences. This amino acid is not essential for binding to CRH-R1[435] or CRH-BP[436] but may yet have implications for protein structure, as proline residues are known to bend the protein backbone.

Several features of the carp sequences described here are more conserved than the overall sequence between representatives of an early (teleost fish) and a more recent (mammals) group of vertebrates. These include the mature CRH peptide as well as the majority of the extracellular CRH-R1 residues that have been implicated in CRH agonist binding[437, 438]. The small sequence dissimilarities between the proposed peptide-binding region of mammals and fish should be viewed in the light of the highly conserved, but nevertheless slightly different fish CRH sequences. Besides the conserved peptide binding regions, the carp CRH-R1 sequence contains several cystein residues that have been suggested to form extracellular disulphide bridges[439, 440]. Finally, the complete absence of amino acid substitutions in the third intracellular loop, which is involved in G-protein coupling[441], between carp and mammalian CRH-R1 sequences is additional proof of orthology of the carp CRH-R1 sequence to other vertebrate CRH-R1 sequences.

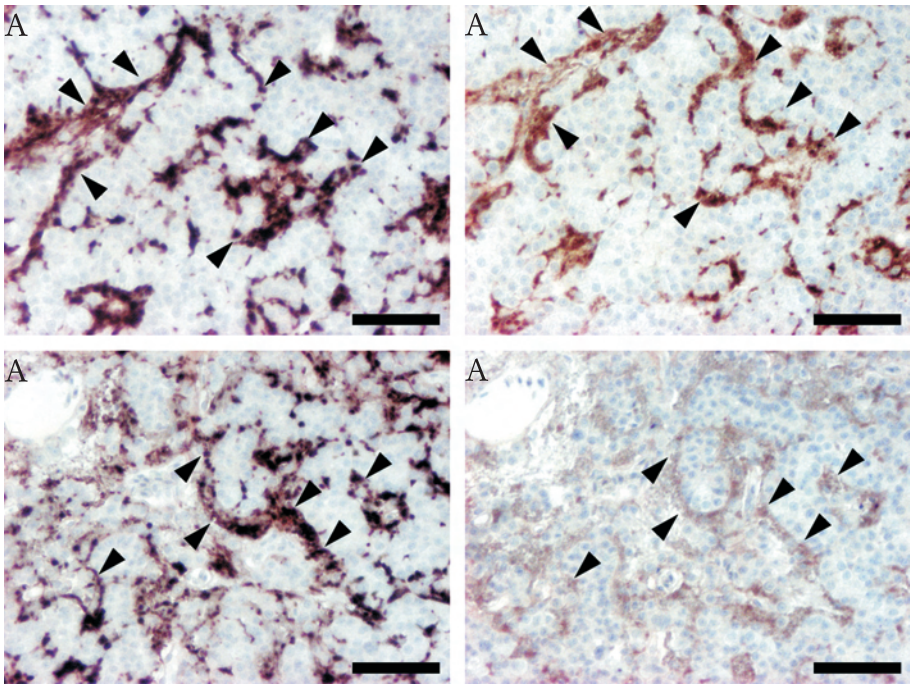


Figure 9.12: Serial immunohistochemistry on saggital sections of the PI. In control carp, the nerve fibres projecting on the PI contain both CRH (a) and CRH-BP (b). Following 24 h restraint, the amount of immunoreactive CRH (c) remained unaltered or was slightly diminished, whereas immunoreactivity to CRH-BP (d) was sharply reduced. Arrowheads mark the same features within panels a and b, and c and d respectively. Immunoreactivity was assessed on serial sections of two fish per group. Scale bars: 50 μm.

Also CRH-BP is evolutionary well conserved. In our in-silico searches we reconstituted the chicken CRH-BP sequence from two EST sequences and we retrieved CRH-BP sequences of several teleost fish species in addition to the two carp genes described here. Both the presence and spacing of the ten cysteines forming the five consecutive disulphide bonds that determine tertiary structure were conserved in all sequences found. The orthology of fish CRH-BP to their non-teleostean vertebrate orthologues was confirmed in phylogenetic analyses, that established the CRH-BP origin before the fish-tetrapod split in vertebrate evolution. This is confirmed by recent work of SEASHOLTZ and colleagues, who showed that a CRH binding factor of similar size to human CRH-BP was present in brain extracts of representatives of many vertebrate classes, including jawless fish[442].

Whereas CRH-BP is evolutionary well-conserved, it bears no considerable sequence

similarity to any known group of proteins and might constitute an autonomous family of proteins. This implies that, unlike many soluble ligand-binding factors that structurally resemble the ligand receptor, CRH-BP is unrelated to the receptors for any of the CRH family of ligands. This partially relates to the heptahelical nature of the CRH receptors that would radically alter their folding in solution (if they were soluble at all). Nonetheless this implies a compelling scenario of two different, structurally non-related proteins with high affinity for the CRH family of ligands that arose independently in evolution.

Innervation of the teleost pituitary gland

In contrast to mammals, most teleost fish species lack a hypothalamo-pituitary portal vessel system[443]. Consequently, neurosecretory fibres emanating from the NPO project directly to the pituitary, and this enables an histological assessment of their target cells. Within the NPO, CRH co-localises with AVT, which has been reported earlier[444]. Both hormones are present in nerve fibers that project onto the pituitary and have been suggested to exert either additive or synergistic effects on ACTH secretion[445, 446]. Consistent with the original studies on the distribution of CRH in the teleost pituitary gland[415], we find CRH positive fibres within the rPD. These fibres are surrounded by ACTH secreting cells, in line with the well-established role for CRH as an ACTH releasing factor[416]. We also observed prominent CRH immunoreactivity within the PN fibre bundles that project onto the PI. Although many previous investigations have focussed at CRH immunoreactivity within the teleost rPD, also considerable CRH immunoreactivity has been reported within the PI of several species[444, 447, 448]. The large majority of the PI melanotropes innervated by these CRH positive fibre bundles secrete α-MSH and contain various acetylated forms of β-endorphin[432]. In some species of fish CRH is capable of stimulating the secretory activity of PI melanotropes *in vitro*[449-451], which concurs with early reports on the α-MSH or β-endorphin releasing capability of CRH in higher vertebrates[416, 452, 453]. Notably, transcription levels of CRH-R1 are seven-fold higher in the PD compared to the PI, while ACTH-releasing cells constitute merely an estimated 10% of the PD cell mass. Given the abundance of PI melanotropes and the paucity of CRH-R1 in the PI, the regulation of PI output by CRH is likely mediated by another receptor with considerable affinity for CRH. Candidates are CRH-R2 and CRH-R3, although the latter has so far been described in only one fish species[423].

In teleosts, β-endorphin and α-MSH alone or in concert display corticotropic activity in some species[454, 455]. Recently, in carp, the presence of a corticotropic factor in the pituitary PI was confirmed, but this factor was neither α-MSH nor β-endorphin, alone or in concert[456]. Nevertheless, the vast quantities of CRH observed within the PN projecting onto the PI melanotropes substantiate a likely role for CRH in the regulation of the release of one or several PI corticotropic factors *in vivo*. Alternatively, CRH (and CRH-BP) could be

released to the circulation.

In Chinook salmon (*Oncorhynchus tshawytscha*), co-localisation of CRH$^+$ fibres and thyrotropin (TSH) secreting cells was reported[447]. However, in carp no CRH immunoreactivity was observed in nerve fibres projecting onto the PPD, that contains the TSH releasing cells. This is corroborated by previous reports in several species[444, 448]. The absence of direct innervation of the PPD TSH cells by CRH is remarkable and in paradox with the potent in-vitro and in-vivo TSH releasing capacity of CRH in fish[457, 458] as well as in amphibians and birds[459, 460]. In chicken, CRH is a potent TSH releasing factor, although the pituitary thyrotropes do not express CRH-R1[461]. Possibly the regulation of the thyroid axis is a secondary effect of CRH, potentially mediated in paracrine way via one of the pituitary hormones that is under direct CRH control.

CRH-BP mode of action

In mammals the pituitary corticotropes constitute a prominent site of CRH-BP mRNA expression[462]. This is suggestive of a role for CRH-BP as a negative regulator of CRH activity during acute stress[463], a function that is corroborated by the in-vitro attenuation of CRH-induced ACTH release from pituitary cells[427] or ATT-20 cells[428]. Contrary to this notion, CRH-BP immunoreactivity within the rat anterior pituitary is largely associated with secondary lysozomes and multivesicular bodies, suggesting a role for CRH-BP in the processing and degradation of CRH and/or ligand receptor complexes[464].

The co-localisation of CRH-BP with CRH in both the rPD as well as the PI strongly suggests that it is a key regulator of CRH activity in both pituitary parts. Furthermore, the localisation within fibre bundles is suggestive of an in-vivo modulatory role in the regulation of the levels of 'free' CRH, rather than a role in protein degradation. This is substantiated by our observation of the decrease in PD CRH-R1 expression and the concomitant increase in PD CRH-BP expression following a 24 h restraint period, as both effects would reflect the desensitisation of the PD for the ACTH releasing effects of hypothalamic CRH. Substantial CRH-BP gene expression is observed in both parts of the carp pituitary gland, despite the lack of significant CRH-BP immunoreactivity associated with cell bodies. This suggests tight regulation at the translational level. Thus, although we observe substantial CRH-BP gene expression at the pituitary level, we are currently unable to attribute this gene expression to a particular pituitary cell type.

Although localised in the same pituitary fibre bundles, CRH-BP is not co-expressed by the CRH$^+$ neurones of the NPO, that project onto the pituitary[415], but must originate from other cells. Indeed, a limited number of relatively small perikarya at the caudal perimeter of the NPO express CRH-BP and may be the source of this CRH-BP. Another hypothalamic site of considerable CRH-BP expression in many fibres as well as perikarya is found ventro-rostrally of and in close proximity to the NPO. Also the fibres emanating from

this area potentially project onto the pituitary, although we can, as yet, not conclusively demonstrate this.

Regulation of restraint stress

In our in-vivo experiments we could not detect altered gene expression following a 30 min restraint, but following a prolonged (24) restraint period, considerable differences in hypothalamic as well as PD gene expression were observed. This is not entirely surprising for two reasons. Firstly, the moderate peak plasma cortisol values that had dissipated within 120 min following the onset of the stress response are indicative of a mild and transient stress response. On the contrary, a 24 h restraint period resulted in very high plasma cortisol values of nearly 500 ng/ml at the termination of restraint that had likely persisted for the major part of the restraint period. Secondly, ACTH release by CRH is initiated within approximately minutes following the application of a stressor[3], which implies that the initiation of the stress response depends on stored signal protein. The amounts of peptide released following a typical acute and transient stressor (*e.g.* 30 min restraint) are so small in comparison to the amount of stored protein, that any additional gene transcription that is initiated on top of the constitutive gene expression will remain undetectable. However, the persistent stimulation of the stress axis during the 24 h restraint period may have depleted CRH and CRH-BP protein stores, necessitating the observed enhancement of hypothalamic CRH and CRH-BP gene expression to guarantee homeostasis. At the same time this observation illustrates that hypothalamic CRH-BP is directly involved in the regulation of the stress axis, despite the virtual absence of CRH-BP immunoreactivity from the NPO. Hypothalamic UI expression remained unaltered throughout both experiments, confirming that the increases in CRH and CRH-BP expression are specific.

Despite the increased hypothalamic CRH-BP gene expression, PN CRH-BP protein content had markedly dropped following 24 h of restraint. This suggests that some regulation of PI output had occurred, consistent with a role for PI corticotrope output under longer term stress conditions[451]. A 24 h restraint period might have crossed the 'border' between acute stress, regulated by PD output, and chronic stress, where the PI output takes over the chronic regulation of plasma cortisol levels[451].

Perspectives

The observations above indicate that the regulation of pituitary gland output by CRH extends well beyond the release of ACTH from the rPD. The co-localisation of CRH-BP with CRH throughout the pituitary gland indicates a profound regulatory capacity for CRH-BP in the regulation of rPD as well as PI output. CRH and ACTH are generally regarded as the principle initiators of the acute stress response in fish, whereas thyrotropin releasing hormone

(TRH) and α-MSH have been suggested to regulate the stress response during exposure to chronic stressors. This study indicates that, although acute and chronic stress responses are regulated by rPD and PI output respectively, CRH and CRH-BP potentially contribute to the regulation of both phases of the stress response by virtue of their presence in both parts of the pituitary gland. Now that we know the major regulatory molecules in teleost fish, we can further unravel the intricate regulation of the pituitary gland together with the ensuing stress response. To this end, teleost fish form a convenient model system, since they lack an eminentia mediana and consequently reveal many discrete regulatory pathways via the direct hypothalamic innervation of pituitary target cell populations.

Acknowledgements

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Chapter 10

CRH and CRH-BP expression in and release from the head kidney of common carp: evolutionary conservation of the adrenal CRH system

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in preparation

Abstract

Corticotropin-releasing hormone (CRH), along with CRH-binding protein (CRH-BP) and the CRH-receptors plays a central role in the regulation of the stress axis. In mammals, this CRH system is expressed in variety of organs and tissues outside the central nervous system, where it is mostly involved in local physiological processes. One of the sites of extra-hypothalamic CRH expression is the adrenal gland, where the paracrine actions of adrenal CRH influence cortical steroidogenesis and adrenal blood flow. Although the central role of CRH-signaling in the initiation and regulation of the stress response has now been established throughout vertebrates, information about possible peripheral functions in lower vertebrates is scant. We established the expression of CRH, CRH-BP, and the CRH-receptor 1 (CRH-R1) in a panel of peripheral organs of common carp (*Cyprinus carpio*). CRH and CRH-BP are most abundantly expressed in the carp head kidney out of all the peripheral organs tested. This expression pattern is confirmed by immunohistochemistry and localised to the chromaffin cells of the endocrine compartment. Furthermore, CRH is released in detectable quantities from the intact head kidney following in-vitro stimulation with 8-bromo-cyclic AMP (cAMP) in an superfusion setup, demonstrating involvement of protein kinase A. The presence of CRH and CRH-BP within the chromaffin compartment of the head kidney suggests that a signaling pathway homologous to the intra-adrenal CRH system is present in the head kidney of fish. It follows that a system to locally fine-tune the outcome of the centrally activated stress response has been present since the common ancestor of teleostean fishes and mammals.

Introduction

Corticotropin-releasing hormone (CRH) was initially identified and is still best-known as the principle hypothalamic initiator of the stress response. Via the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland CRH induces glucocorticoid secretion from the adrenal cortex. This axis is appropriately named the hypothalamus-pituitary-adrenal (HPA) -axis. Nevertheless, the expression of CRH has since its discovery in 1981[416] been reported in many cells and tissues other than the hypothalamus[424, 465, 466]. One of the sites where extra-hypothalamic CRH has clear ties to the regulation of the stress response is the mammalian adrenal gland[467, 468]. The adrenal cortex is the site of synthesis and release of the glucocorticoid hormones, which are directly responsible for many of the downstream effects of stress-axis activation, whereas the medulla of the adrenal gland is the principle site of catecholamine secretion

(adrenalin and noradrenalin). The adrenal medulla in particular produces and contains many neurotransmitters and peptide hormones other than CRH, such as neuropeptide Y (NPY), serotonin (5-HT), and vasoactive intestinal peptide (VIP); presence has been established, but functions are incompletely understood[467, 468]. CRH is found exclusively in a subpopulation of medullary chromaffin cells[469-473]. Moreover, direct effects of CRH, independently of HPA-axis activation, have been demonstrated on adrenocortical steroid release. This is demonstrated by experiments in hypophysectomised rats, where externally administered CRH exerts a trophic effect on the adrenal cortex, as it reversed the cortical atrophy that normally follows this procedure[474]. Moreover, in calves that were functionally hypophysectomised, CRH induced considerable glucocorticoid release, which demonstrates that CRH can exert effects on the adrenal gland that do not require pituitary-derived ACTH[475]. The enhanced release of glucocorticoids induced by CRH is partially attributable to the stimulation of adrenal blood flow *in vivo*, or perfusion rate *in vitro*[467, 475, 476]. These direct effects of CRH on the adrenal gland require the local presence of CRH-receptors. Indeed CRH-receptors, predominantly CRH-R1, are found within the adrenal gland[477-481]. In addition, CRH-binding protein (CRH-BP), an important modulator of the concentration of free, bioavailable CRH, has also been demonstrated in chromaffin cells of rat adrenal gland[482]. The absence of CRH-receptors in adrenal cortex in all species investigated to date[478, 480, 481] (with the exception of mouse[479]) seems to preclude a direct effect of CRH on cortical cells. Indeed, the effects of CRH on the adrenal cortex require the presence of medullary tissue, as CRH has no effect on the steroid release from isolated adrenocortical cells *in vitro*[476] or from autotransplants of cortical cells deprived of chromaffin tissue[483]. This implies that the actions of CRH on cortical steroidogenesis are indirect, as they apparently require an intermediate adrenomedullary component[483, 484]. There is good evidence to suggest that the intermediate medullary component responsible for the indirect effects of CRH on steroidogenesis is ACTH, which is also found in the adrenal medulla[473, 485, 486]. Indeed, the adrenal effects of CRH are inhibited not only by the specific CRH-antagonist α-helical CRH (α-CRH; CRH_{9-41})[487], but also by the ACTH-antagonist corticotropin-inhibiting peptide (CIP; $ACTH_{7-38}$)[484, 487]. Collectively this indicates the presence of a local, paracrine CRH-system within the adrenal gland that is capable of fine-tuning adrenal output via the modulation of either cortical steroidogenesis or adrenal blood flow.

The presence of an adrenal CRH-system that modulates the output of the activated HPA-axis has now been firmly established in mammals. We know virtually nothing about the evolutionary origins of this modulatory CRH system. The central initiation of the stress response in fish, as in mammals, is controlled by CRH, CRH-R1 and CRH-BP[69]. The stress-axis of teleostean fish differs anatomically from that of mammals. One of these differences is the location of the catecholamine producing cells and the interrenal cells

(which release cortisol as main glucocorticoid) that are located within the paired head kidneys, the fish homolog of the mammalian adrenal gland. Therefore, the stress-axis of fish is referred to as the hypothalamus-pituitary-interrenal (HPI) -axis. The fish head kidney however, lacks the clear cortex-medulla architecture that is characteristic of the mammalian adrenal gland. Instead, the interrenal and chromaffin cells are intermingled and lie around the cardinal veins of the head kidney. Here we report the first indications of the presence of a local CRH-system in the carp head kidney that is similar to that of the mammalian adrenal gland.

Materials and methods

Animals

Common carp (*Cyprinus carpio* L.) of the R3xR8 strain were obtained from the 'De Haar Vissen' facility of Wageningen University (The Netherlands). R3xR8 are the hybrid offspring of a cross between fish of Polish origin (R3 strain) and Hungarian origin (R8 strain)[34]. Carp were maintained at 23 ºC in recirculating UV-treated tap water at our fish facilities and were fed pelleted dry food (Provimi, Rotterdam, The Netherlands) at a daily ration of 0.7 % of their estimated body weight. Fish were killed by anesthesia with 0.1 % 2-phenoxyethanol before the collection of plasma and tissue samples. All animal experiments were carried out in accordance with national legislation.

RNA isolation and gene expression analysis

RNA from carp tissues was isolated according to CHOMCZYNSKI and SACCHI[40]. Briefly, organs were homogenised in lysis buffer (4 M guanidium thiocyanate; 25 mM sodium citrate, pH 7.0; 0.5 % sarcosyl; 0.1 M 2β-mercaptho-ethanol), followed by phenol/chloroform extractions. Total RNA was precipitated in ethanol, washed and dissolved in water. Concentrations were measured by spectrophotometry and integrity was ensured by analysis on a 1.0 % (w/v) agarose gel. Gene expression was assessed by RT-PCR with the Superscript One-Step RT-PCR system (GibcoBRL, Breda, The Netherlands). Briefly, 1 μg total RNA and forward and reverse primer (400 nM each; Table 10.1) were added to 12.5 μl 2x reaction mix, 0.2 μl RNase inhibitor, and 1 μl Platinum Superscript II RT/Taq mix and filled up with diethyl pyrocarbonate-treated water to a total volume of 25 μl. Reverse transcription was performed at 50 ºC for 30 minutes. The reaction was subsequently denatured at 94 ºC for 4 minutes and subjected to 30 – 40 cycles of 94 ºC for 30 s, 55 ºC for 30 s, 72 ºC for 60 s, followed by a final extension step of 10 min at 72 ºC. RT-PCR reactions were analysed on a 1.0 % agarose gel.

Table 10.1: Primer sequences and corresponding accession numbers and amplicon lengths

gene	accession number	amplicon length	primer	sequence 5' ⇒ 3'
CRH	AJ317955,	549 bp	CRH.fw2	GATATATCAATTACGCACAGATT
	AJ576243		CRH.rv1	TGATGGGTTTGCTCGTGGTTA
CRH-BP	AJ490880,	507 bp	CRH-BP.fw2	GGGTGGGTGATGAAGGGCGAGAA
	AJ490881		CRH-BP.rv1	CACCATTCTGATCACAGTGTTATC
CRH-R1	AJ576244	409 bp	CRH-R1.fw3	GACGTGTTGGGCCCAGGAGCAG
			CRH-R1.rv4	CACCAGATCACATTGCTCTCAT
β-actin	CCACTBA	708 bp	β-actin.fw1	AGACATCAGGGTGTCATGGTTGGT
			β-actin.rv1	GATACCGCAAGACTCCATACCCA

Immunohistochemistry

Tissue was fixed in Bouin (15 ml picric acid, 5 ml formol, 1 ml glacial acetic acid), dehydrated, embedded in paraffin and sectioned in 5 μm sections. CRH was detected with a rabbit anti-sheep CRH antiserum (Biotrend, Cologne, Germany) at a dilution of 1:50. CRH-BP was detected with a rabbit anti-human CRH-BP antiserum (a generous gift of PROF. DR. WYLIE W. VALE) at a dilution of 1:1000. Primary antibodies were incubated overnight. Goat anti-rabbit IgG-biotin (1:200, 1h; Vector Laboratories, Burlingame, CA, USA) was used as second antibody, followed by amplification with the Vectastain ABC amplification kit (Vector Laboratories,) according to the manufacturer's protocol. The signal was visualised with 3-amino-6-ethylcarbazole (AEC; Sigma) as the substrate. Controls for the cross-reactivity of the secondary reagents and for endogenous enzyme activity were included in all experiments and were negative. Nuclei were counterstained with haematoxilin before embedding in Kaiser's gelatin.

Confocal laser scanning microscopy

In a two-color immunofluorescence approach, interrenal cells were visualised either via their higher autofluorescence (in double staining with CRH), or (in double staining with CRH-BP) by staining for cortisol with an anti-cortisol antibody (1:150; Campro Scientific, Veenendaal, The Netherlands). Goat anti-rabbit IgG-HRP (Biorad) was used as the second antibody at 1:200 and the signal was visualised with tyramide-FITC (1:50 for 30 min; NEN Life Science Products, Boston, MA, USA). For the detection of CRH and CRH-BP, the same primary and secondary antibodies as before were used at the same dilutions. Signal was detected by incubating with avidin-Texas Red (Vector Laboratories) for 10 min. Sections were embedded in Vectashield (Vector Laboratories) and examined with a Zeiss LSM-510 laser scanning microscope. Fluorescein signal was excited with a 488 nm argon laser and detected using a band-pass filter (505-530 nm) and Texas Red was excited with a 543 nm helium-neon laser and detected with a long-pass filter (585 nm).

Radioimmunoassays

Cortisol was measured by RIA, using a commercial antiserum (Campro Scientific, Veenendaal, The Netherlands) as previously described[69]. For the detection of carp CRH we developed a RIA on the basis of a rabbit antiserum directed at human/rat CRH_{24-41} (C5348; Sigma, St. Louis, USA). According to the manufacturer, the antibody exhibits less than 0.01 % cross-reactivity with rat urocortin-I, sauvagin (*Phylomedusa sauvagei*), and human ACTH and also did not cross-react with flounder (*Platichtys flesus*) CRH or urotensin-I (kindly donated by PROF. DR. RICHARD BALMENT). The optimal antibody dilution was experimentally established at 1:10,000. Human/rat Tyr-CRH (H-2455l; Bachem, Bubendorf, Switzerland) was used as standard. The standard was also used as tracer following labeling with ^{125}I (ICN, Costa Mesa, CA, USA) by the iodogen method[488] and purified through solid-phase extraction (octadecyl Bakerbond column). All constituents were in phosphate-EDTA RIA buffer of pH 7.4 (63 mM Na_2HPO_4, 13 mM Na_2EDTA, 0.02 % (w/v) NaN_3, 0.1 % (v/v) Triton X-100, 0.25 % (w/v) bovine serum albumin (Sigma) and 2.5 % (v/v) aprotinin (Trasylol; Bayer, Leverkusen, Germany)). Samples and standards of 25 µl were preincubated in duplicate or triplicate, respectively, with 100 µl primary antibody (1:10,000) for 96 h at 4 ºC. Then, tracer was added at a volume of 100 µl (approximately 4000 cmp) and incubated for 24 h at 4 ºC. A volume of 100 µl secondary antibody solution (goat anti-rabbit IgG; Biogenesis, Ede, The Netherlands diluted 1:16 v/v in RIA buffer containing 0.007 % (w/v) rabbit IgG; Sigma) was added and incubated for 30 min at room temperature. Immune complexes were precipitated by adding 1 ml ice-cold polyethylene glycol 6000 (PEG) and centrifuged (10 min, 2000 g, 4 ºC). Supernatants were aspirated and the pellets were counted in a gamma counter (1272 Clinigamma, LKB Wallac, Turku, Finland). The RIA has a sensitivity of 2.5 – 5.0 pg/tube (0.5 – 1.0 fmol/tube). Inter-assay variation was 5.97 ± 2.05 % (n=6) and the intra-assay variation was 1.90 ± 1.63 % (n=5).

In vitro superfusion

To asses CRH and cortisol release *in vitro*, freshly collected head kidneys were placed on a cheesecloth filter in a superfusion chamber and superfused with 0.015 M HEPES/Tris-buffered medium (pH 7.4) containing 128 mM NaCl, 2 mM KCl, 2 mM $CaCl_2 \cdot 2H_2O$, 0.25 % (w/v) glucose, 0.03 % (w/v) bovine serum albumin (BSA; Sigma) and 0.1 mM ascorbic acid. Medium was saturated with carbogen (95% o2/5% co2) and pumped through the superfusion chambers at 20 µl/min with a multichannel peristaltic pump (Watson-Marlow, Falmouth, UK). Medium and tissues were maintained at 23 ºC throughout the experiment. At the indicated times, head kidneys were stimulated by a pulse of 60 mM KCl or 8-br-cAMP (8-bromoadenosine 3'-5'-cyclic monophosphate; B-7880; Sigma) dissolved in superfusion medium. Fractions were collected every 10 or 15 minutes, stored on ice for the immediate determination of CRH content and stored at -20 ºC for the determination

of cortisol content at a later time. Basal, unstimulated release was calculated based on the three values preceding the (first) pulse and designated at 100 %. Stimulation is expressed as a percentage of basal release.

Statistics

Statistical analyses were carried out with SPSS software (version 11.5.0). Differences were evaluated with the non-parametric Kruskal-Wallis H-test. When this test indicated significant differences in the dataset, the Mann-Whitney U-test was used to determine which samples differed significantly from controls. Differences were considered significant at $p<0.05$ (one-sided).

Results

To investigate the presence of CRH, CRH-R1, and CRH-BP, outside the central nervous system, we assessed gene expression in brain and a panel of peripheral organs. In the periphery, CRH as well as CRH-BP are expressed most abundantly in the head kidney (Fig. 10.1).

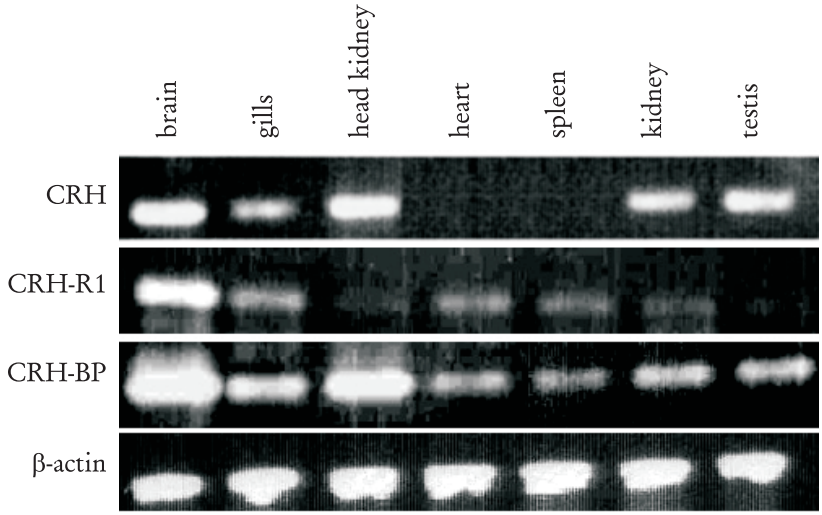


Figure 10.1: Expression of CRH, CRH-R1, and CRH-BP in brain and peripheral organs of carp. Note that the most prominent gene expression of CRH and CRH-BP outside the central nervous system is observed in the head kidney. Weak expression of CRH-R1 is detectable in gills, head kidney, heart, spleen, and kidney, but not in testis. The β-actin gene was included as a housekeeping gene. PCR reactions for CRH, CRH-R1, CRH-BP, and β-actin were performed for 40, 30, 35, and 30 cycles, respectively.

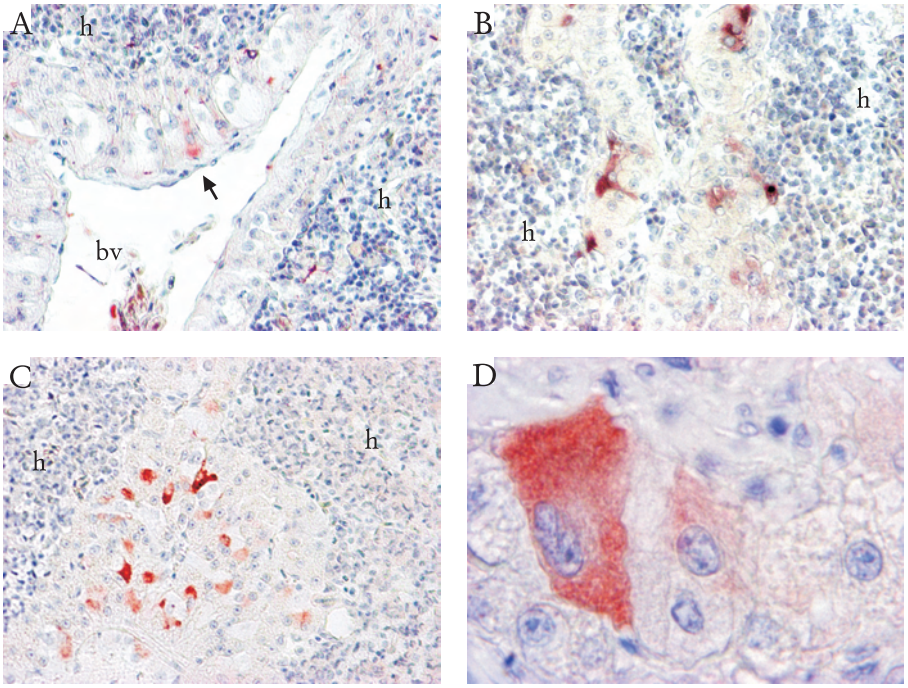


Figure 10.2: CRH and CRH-BP immunoreactivity in the islands of endocrine tissue that surround the blood vessels of the head kidney. Panels a and b show the presence of CRH immunoreactivity (red) in a subset of endocrine cells at a magnification of 20x. The endocrine tissue of the teleostean head kidney is organised around blood vessels (bv) and surrounded by densely-packed haematopoietic tissue (h). Panels c and d show the presence of CRH-BP immunoreactivity at a magnification of 20x and 100x, respectively. Nuclei were counterstained with haematoxilin.

CRH expression was also present in gills, kidney, and testis, but was undetectable in heart and spleen. CRH-BP expression was detected throughout the panel of peripheral organs. CRH-R1 is expressed to some extent in all organs except testis and is weakest in the head kidney.

To investigate the cellular location of CRH and CRH-BP within the head kidney, we investigated their distribution via immunohistochemistry. This approach revealed that CRH and CRH-BP immunoreactivities are present in the endocrine compartment of the carp head kidney. The interrenal and chromaffin cells are organised in patches of endocrine tissue around the blood vessels of the head kidney, embedded in densely packed haematopoietic tissue. A small proportion of these endocrine cells is positive for CRH or CRH-BP (Fig. 10.2). To establish whether CRH and CRH-BP are expressed in the interrenal or

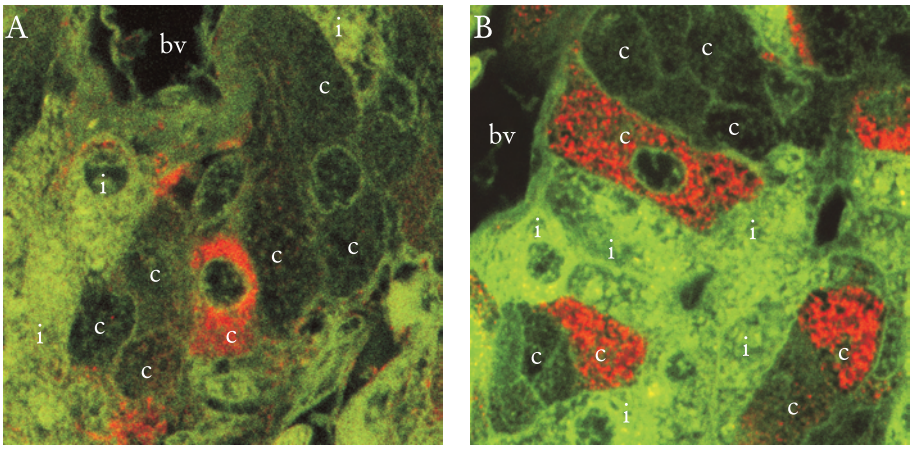


Figure 10.3: CRH and CRH-BP immunoreactivity is localised to a subset of the chromaffin cells. Panel a shows a confocal micrograph of a detail of the head kidney endocrine tissue that features a single cell stained for CRH (red). The slide's autofluorescence is recorded in the green channel of the confocal microscope and highlights the interrenal cells (i), which have stronger autofluorescence that the chromaffin cells (c) that remain dark. Panel b shows a few chromaffin cells that contain CRH-BP immunoreactivity (red) amidst a group of interrenal cells that are stained with an antibody against cortisol (green). Note that most chromaffin cells do not contain CRH-BP and that 'bv' indicates the lumen of the blood vessel. Magnification is 189x for both panels.

the chromaffin cell compartment, we further investigated their expression via confocal laser scanning microscopy. The autofluorescence of Bouin-fixed interrenal cells is more intense than that of the chromaffin cell compartment. We exploited this characteristic to establish that CRH immunoreactivity is absent from the interrenal compartment but localises to a subset of chromaffin cells (Fig. 10.3a). In a similar approach, CRH-BP immunoreactivity was also demonstrated in a subpopulation of chromaffin cells and not in the interrenal cell compartment (Fig. 10.3b).

The release of CRH from the head kidney was studied in an in-vitro superfusion assay. For the detection of carp CRH we developed and validated a radioimmunoassay (RIA). The carp CRH released from head kidney displaces radiolabeled human CRH in parallel with the standard curve (Fig. 10.4), which validates the RIA for the detection and relative quantitation of carp CRH. Depolarising concentrations of K^+ (60 mM) did not induce the release of CRH (Fig. 10.5a), but did induce a modest and transient increase in cortisol secretion (Fig. 10.5b), demonstrating that depolarisation did occur. Stimulation of protein kinase C (PKC) via phorbol 12-myristate 13-actetate (PMA) did not result in a reproducible release of CRH (not shown). Direct activation of protein kinase A (PKA) by one mM 8-bromo-cAMP resulted in a marginal CRH release, ten mM resulted in a rapid and

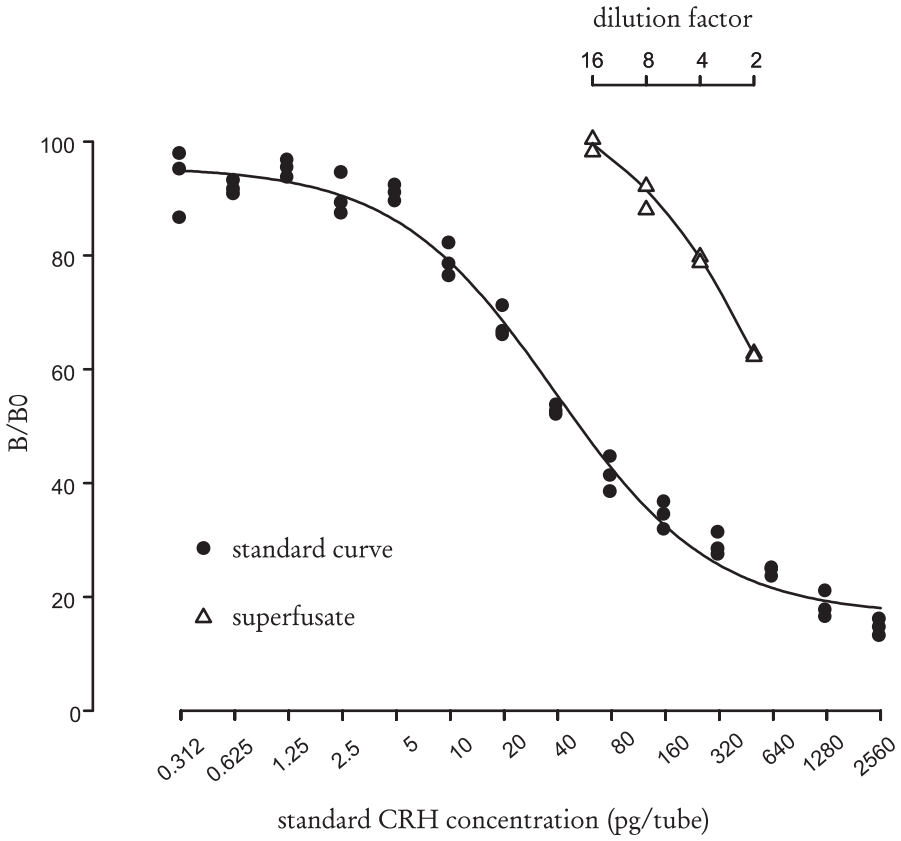
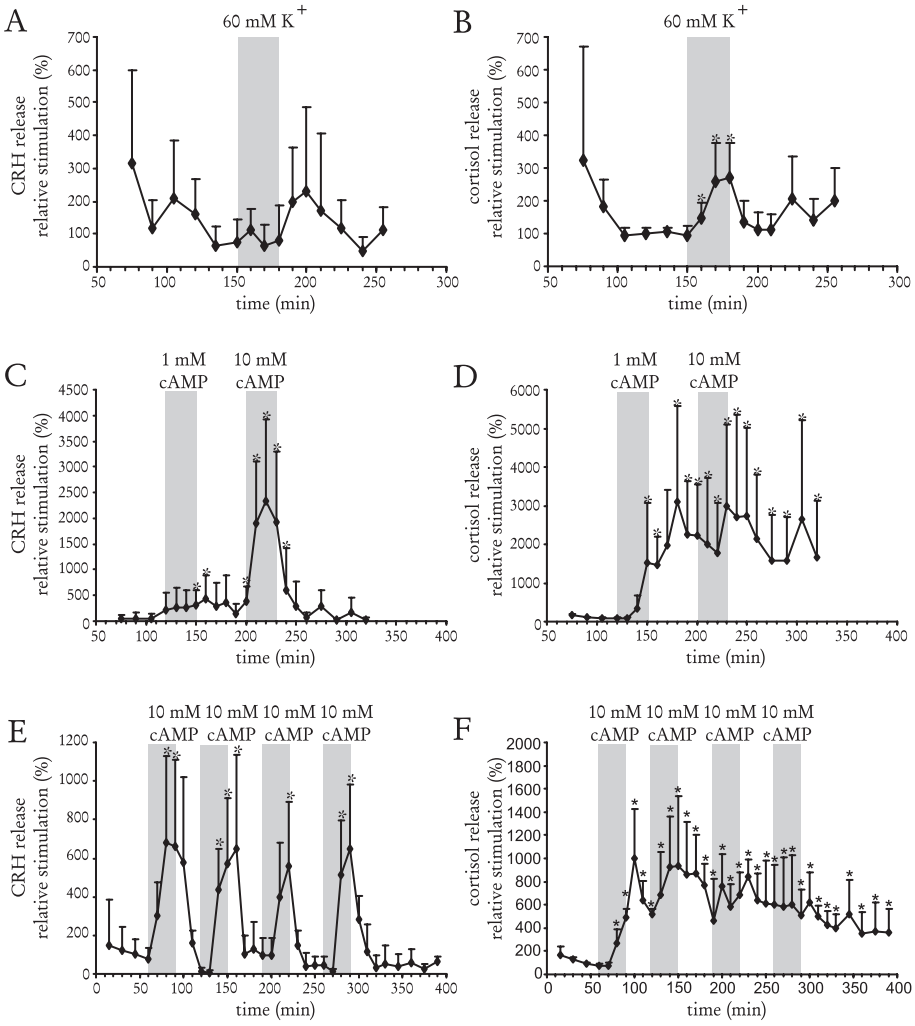


Figure 10.4: The CRH radioimmunoassay binding curve for standard (human) CRH and the dilution curve of carp CRH run in parallel. Carp CRH was obtained from pooled and concentrated head kidney superfusion fractions.

pronounced release of CRH from the head kidney (Fig. 10.5c). CRH concentrations rapidly returned to baseline values upon termination of the stimulus. Simultaneously, cortisol release is induced by one mM 8-bromo-cAMP, although the secretion starts only towards the end of the 30-minute pulse and persists until the subsequent stimulation with ten

Figure 10.5: The effects of K^+ and 8-bromo-cAMP on the release of CRH and cortisol from superfused carp head kidneys. Depolarisation induced by 60 mM K^+ failed to induce CRH release (a), but did induce a modest release of cortisol (b), n is five. Direct stimulation of PKA via 8-bromo-cAMP induced a marginal CRH release when applied at one mM but resulted in the rapid and pronounced release of CRH at ten mM (c). Stimulation via 8-bromo-cAMP also induced the release of cortisol, albeit with a considerable delay (d), n is six. Four consecutive 30 minute pulses with ten mM 8-bromo-cAMP result in four distinct peaks of CRH release that closely follow



the application of 8-bromo-cAMP (e). The same stimulation also induced the profound release of cortisol (f), n is three. The cortisol response to 8-bromo-cAMP displays a delay in both its initiation and termination, which leads to the continued secretion of cortisol throughout the duration of the superfusion experiment. Note that within each of the three experiments that are displayed, CRH and cortisol content is measured within the same superfusion samples. Stimulation is expressed as a percentage of basal release, which was calculated based on the three values preceding the (first) pulse. Basal CRH release release corresponds to 4.1 pg min^{-1}, 1.3 pg min^{-1}, and 1.4 pg min^{-1} in panels a, c, and e, resepctively. Basal cortisol release corresponds to 199.6 pg min^{-1}, 97.8 pg min^{-1}, and 256.2 pg min^{-1} in panels b, d, and f, respectively. Asterisks denote a significant increase from basal release ($p < 0.05$).

mM 8-bromo-cAMP (Fig. 10.5d). To demonstrate the responsiveness of head kidney in vitro for the duration of the experiment, we subjected head kidneys to four subsequent 30-minute pulses with ten mM 8-bromo-cAMP. Each of the four stimulations is closely followed by a rapid, profound, and transient release of CRH (Fig. 10.5e). Moreover, the magnitude of the CRH responses is constant throughout the experiment, which indicates that the CRH content of the head kidney suffices to sustain repeated episodes of in-vitro stimulation. As predicted, the repetitive stimulation with ten mM 8-bromo-cAMP initiated a profound increase in cortisol secretion that is delayed compared to the simultaneous release of CRH (Fig. 10.5f). Because of the lag in the termination of the cortisol release, its enhanced secretion is maintained throughout the experiment.

Discussion

Here we presented the fist indication of the presence of local CRH-system in the head kidney of a teleostean fish, in analogy to the intra-adrenal CRH-system of mammals. Of the peripheral tissues examined, CRH and CRH-BP were expressed most prominently in the head kidney, which also contained low level expression of CRH-R1. Within the head kidney, CRH and CRH-BP immunoreactivity is present in a subset of chromaffin cells and not detected in the interrenal compartment. This is a similar situation as that of the CRH system in the medulla (but not the cortex) of the mammalian adrenal gland[467, 468]. The distribution of CRH in the chromaffin but not the glucocorticoid compartment of vertebrates is in agreement with their ontogenic roots: interrenal or adrenocortical cells are mesodermally derived, whereas the chromaffin cells originate from neural crest[489]. Apparently, the expression of neuropeptides or peptide hormones in the fish head kidney and its homologs of more recent vertebrates is largely restricted to the chromaffin cells of neurectodermal origins[467, 490].

The release of CRH from intact head kidneys was detectable *in vitro* and is most potently induced by 8-bromo-cAMP, which directly activates a PKA pathway. This is similar to studies on the regulation of hypothalamic and amygdalar CRH in mammals that report an increase of gene expression and peptide release following stimulation with the PKA activator forskolin[491, 492]. The rapid response of the carp head kidney following stimulation by 8-bromo-cAMP is too fast (minutes) to involve *de novo* peptide synthesis and indicates that 8-bromo-cAMP induces the direct release of stored CRH. This is supported by the presence of CRH-immunoreactivity in the cytoplasm of chromaffin cells. The kinetics of CRH and cortisol secretion following repeated stimulation with ten mM 8-bromo-cAMP indicate that CRH is released faster than cortisol and thus independently of the latter. The differences

in response time between CRH and cortisol likely stem from the different mechanisms that are responsible for their release: CRH is stored cytoplasmatically and can be release rapidly via exocytosis, whereas cortisol is the end-product of an enzymatic cascade that requires more time to become maximally activated. The magnitude of 8-bromo-cAMP-induced CRH release is not diminished by a prolonged (hours) simultaneous secretion of cortisol, which indicates that direct activation of the PKA pathway overrules any potential negative feedback mechanism of glucocorticoids on the secretion of CRH.

The mammalian intra-adrenal CRH-system is considered to exert local, paracrine effects that modulate the overall adrenal glucocorticoid response. A similar paracrine function seems likely now too for the teleostean head kidney CRH as the number of CRH and CRH-BP positive cells is relative small compared to the bulk of head kidney endocrine cells. And although CRH was clearly detectable at the peak of its release in an in-vitro superfusion setup, where release is measured immediately downstream of the source, it is likely that head kidney CRH will be diluted beyond detection in the general circulation before it can induce systemic effects. We detected only a relatively modest amount of CRH-R1 expression in the head kidney. This level of expression (the result of only 30 cycles of amplification) apparently suffices for the mediation of paracrine effects under circumstances where the concentration of CRH locally within the head kidney may reach significant values. Alternatively, direct effects of CRH may be mediated by CRH-R2 or a third, as yet unidentified CRH receptor in carp[423]. Nevertheless, the direct corticotropic effect of CRH on co-cultures of human glucocorticoid and chromaffin cells is completely inhibited by the specific CRH-R1 antagonist antalarmin, suggesting that the CRH-R1 is the most important CRH receptor in the adrenal CRH-system[477].

Whether the local presence of CRH-BP in the carp head kidney serves the sole purpose of modulating the paracrine response to local CRH is presently unclear. It is conceivable that the local presence of CRH-BP is intended for the modulation of the head kidney response to CRH that is derived from sources outside the head kidney such as the hypothalamus or the pituitary *pars intermedia* that in fish contains many CRH-positive nerve fiber bundles[69,444]. In tilapia (*Oreochromis mossambicus*) high concentrations of CRH are detected in circulation following acute stress[493]. It is also conceivable that CRH-BP modulates the response of the head kidney to urotensin-I (UI), which is a member of the CRH family of peptide hormones. The major source of UI in fish is the caudal neurosecretory system (CNSS) that in flounder (*Platichthys flesus*) also contains CRH[18]. Indeed, UI enhances the steroidogenic actions of ACTH on the head kidney of flounder[494], although UI, in contrast to CRH, is not expressed in the flounder head kidney[18].

The head kidney of fish is unique among vertebrates as it combines key endocrine functions with important haematopoietic properties. This provides the opportunity for paracrine modulation of the outcome of HPI-axis activation by signals from the immune

system, which include cytokines but also CRH. The presence of CRH in the mammalian immune system is well-documented[466] and recently, CRH and CRH-BP immunoreactivity have also been established in macrophage-like cells of the gills and skin in carp (De Mazon *et al.*, accepted).

Based on *i)* the presence of CRH as well as its modulator CRH-BP in a subset of chromaffin cells and *ii)* the demonstration of PKA-dependent CRH release from the head kidney *in vitro*, we conclude that a local CRH-system is present in the head kidney of teleostean fish. The intra-adrenal CRH system of mammals is implicated in the modulation of glucocorticoid release by effects on glucocorticoid release as well as adrenal blood flow. Our in-vitro superfusion setup will allow us to further investigate the effects of CRH on the modulation of cortisol release from the carp head kidney, independently of any potential modulatory effects of CRH on blood flow. The presence of a local CRH system in the head kidney of fish indicates that the capacity to locally modulate the output of systemic stress-axis activation at the level of glucocorticoid release has apparently provided an adaptive advantage to the early vertebrate ancestor.

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Chapter 11

The remarkable conservation of corticotropin-releasing hormone (CRH)-binding protein in the honeybee (*Apis mellifera*) dates the CRH system to a common ancestor of insects and vertebrates

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Abstract

Corticotropin-releasing hormone-binding protein (CRH-BP) is a key factor in the regulation of CRH-signaling: it modulates the bioactivity and bioavailability of CRH and its related peptides. The conservation of CRH-BP throughout vertebrates was only recently demonstrated. Here we report the presence of CRH-BP in the honeybee *(Apis mellifera*) and other insects. Honeybee CRH-BP resembles previously characterised vertebrate CRH-BP sequences with respect to conserved cysteine residues, gene organization, and overall sequence identity. Phylogenetic analyses confirm the unambiguous orthology of insect and vertebrate CRH-BP sequences. Soon following their discovery, it was noted that insect diuretic hormone-1 (DH-1) and its receptor share similarities with the vertebrate CRH-family and their receptors. Despite these similarities, demonstration of common ancestry of DH-1 and the vertebrate CRH-family is still speculative: the mature neuropeptides are short, and their genes differ substantially with regards to the number of coding exons. Moreover, DH- and CRH-receptors belong to the much larger family of G-protein coupled receptors. In contrast, the unique and conspicuous features of CRH-BP greatly facilitate the establishment of orthology over much larger evolutionary distances. The identification of CRH-BP in insects clearly indicates that this gene predates vertebrates by at least several hundred million years. Moreover, our findings imply that a CRH system is shared by insects and vertebrates alike, and consequently, that it has been present at least since the common ancestor to both phylogenetic lines of proto- and deuterostomians.

Introduction

Corticotropin-releasing hormone-binding protein (CRH-BP) is a 322-amino acid soluble protein that is structurally unrelated to the CRH-receptors. It is unique with respect to its ten cysteine residues that form five consecutive disulphide bonds[495]. CRH-BP was initially discovered in late gestational maternal plasma[496], where it prevents hypothalamus-pituitary-adrenal axis (HPA-axis) activation by the high concentrations of placenta-derived CRH that circulate around parturition[497]. Human CRH binds to CRH-BP with a considerably higher affinity than to either CRH-R1 or CRH-R2[419, 436]. It has been suggested that the binding of CRH to CRH-BP protects the former from degradation, and by doing so acts as a delivery system in a fashion similar to that described for the various insulin growth factor-binding proteins (IGF-BP)[498]. However, upon bolus injection of CRH, CRH-BP/CRH complexes are rapidly cleared from circulation[499], indicating an antagonistic role of CRH-BP in CRH signaling. Indeed, CRH-BP abrogates CRH induced adrenocorticotropic hormone

(ACTH) release *in vitro*[427, 428]. Besides its well-documented role in HPA-axis regulation, CRH-BP recently received interest as a potential therapeutic target in the treatment of *anorexia nervosa*, obesity, depression, and Alzheimers disease, disorders that are associated with dysregulated brain CRH signaling[500-503].

Only recently, the CRH-BP gene was cloned in several non-mammalian vertebrates including bony fish[69, 504], confirming that the CRH-BP gene is conserved throughout vertebrate evolution. Moreover, in common carp (*Cyprinus carpio*) CRH-BP and CRH positive nerve fibers project onto the *pars distalis* and prominently onto the *pars intermedia* of the pituitary gland, and the hypothalamic expression of both corresponding genes is subject to regulation during acute restraint stress[69]. Collectively, it seems that the CRH system (comprising CRH, CRH-BP, and CRH-R1) is involved in the regulation of the stress response throughout the vertebrate lineage.

Insects too have a neuropeptide that complies with the CRH family motif (PS00511): diuretic hormone-1 (DH-1)[505]. Insect DH-1 is released from a pair of endocrine glands, the *corpora cardiaca*, that receive input from the insect brain. The *corpus cardiacum* is a neurohemal organ where the products of neurosecretory cells from the *pars intercerebralis* (PI) are released, and the insect PI-CC axis is regarded as an analog of the vertebrate hypothalamus-pituitary axis[506]. The effect of DHs is mediated via diuretic hormone receptors that belong to the seven-helix transmembrane G-protein coupled receptor (GPCR) superfamily[507]. Insect DH-1 and its cognate receptor have been hypothesised to share a common ancestor with the vertebrate CRH system[508, 509]. Despite the general similarities of insect DH-1 and DH-receptors with the vertebrate CRH-family and CRH-receptors, establishing orthology is complicated for three major reasons. First, the mature neuropeptides are short in length; second, DH-1 and the vertebrate CRH-family members differ vastly in gene structure; third, the DH- and CRH-receptors are only a few members of the much larger GPCR family. In contrast, the evolutionary well-conserved CRH-BP does not bear appreciable sequence similarity to any other protein and appears to constitute an autonomous protein family. Therefore, CRH-BP is far better suited to establish the age of the CRH system.

We here report the presence of CRH-BP in insects. We cloned the complete coding sequence of the CRH-BP gene from the honeybee (*Apis mellifera*). Inspection of both the honeybee gene, as well as the corresponding protein, reveals striking similarities to vertebrate CRH-BP sequences. For completeness we confirm that the honeybee, like many other insect species, possesses a DH-1 sequence. Collectively our findings strongly support the notion that the endocrine CRH signaling system, including its binding protein, is shared by insects and vertebrates and has been present since the common ancestor to both phyla.

Materials and Methods

Animals

Honeybees (*Apis mellifera*) were obtained from the Dutch beekeeping expertise centre 'Het Bijenhuis' in Wageningen where they were housed according to standard beekeeping practice. Animals were rapidly sedated on ice, preceding dissection.

RNA isolation and first strand cDNA synthesis

Organs for RNA isolation were flash-frozen in liquid nitrogen. RNA isolation was conducted with Trizol (Invitrogen) according to the manufacturer's protocol. Total RNA was precipitated in ethanol, washed, and dissolved in water. All reagents for cDNA synthesis were obtained from Invitrogen and cDNA synthesis was carried out as previously described[69].

Cloning and sequencing

PCR was carried out with bBP.fw1 (AATGACAATGAGGAGGTCTGT) and bBP.rv1 (TT CATACCGATATTTTTACCACA) primers based on a honeybee EST sequence (BI514351). The sequence encoding the remaining N-terminal part of the sequence as well as a short stretch of 5'UTR (untranslated region) was obtained by PCR on cDNA from the head of a single bee with bBP.fw4 (GGATTCTTGAGGTTTCATTAGAA) and bBP.rv2 primers. Similarly, the C-terminal part of the sequence as well as a partial 3'UTR were obtained by PCR with bBP.fw2 (TCAACTTCATTACTTTTGATATACC) and bBP.rv7 (GATAAATTTATGAAAGACATCTAG) primers. For the assessment of CRH-BP gene expression we used the following primers bBP.fw3 (CTGGAGATCGTTTCTCAAAGG) and bBP.rv3 (GAGCGCGACATAAGTGCAATT). Honeybee actin (XM_393368) and 40S ribosomal protein S11 (XM_394541; not shown) were used as internal reference genes and results were very similar following comparison to either gene. Reference gene primers were actin.fw1 (CCTAGCACCATCCACCATGAA), actin.rv1 (GAAG CAAGAATTGACCCACCAA), 40S.fw1 (CCCAAAAGACGGAAGCCTATG), and 40S.rv1 (AAGAATGCGTCC TCTAATAGAAATGTT). The mature honeybee DH-1 sequence was amplified with bDH-1.fw2 (GAAACGTCTTGAATCAAAACGTATC) and bDH-1.rv2 (CTTTTTCCAATCGTCTCCAAAAG) primers based on a honeybee genomic sequence retrieved from the Baylor College of Medicine honeybee genome project (assembly Amel 1.2). All oligonucleotides were obtained from Eurogentec, Seraing, Belgium. PCR reactions were performed with 0.2 μl Taq DNA polymerase (Goldstar, Eurogentec) supplemented with 1.5 mM $MgCl_2$, 200 μM dNTPs and 400 nM of each primer in a final volume of 25 μl. Cycling conditions were 94 °C for 2 min, 30 – 35 cycles of 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C, followed by 10 min at 72 °C and PCRs were carried out on a GeneAmp PCR System 9700 (PE Applied Biosystems, Foster

City, USA). PCR products were ligated in the pGEM-T-easy vector (Promega) and cloned in JM-109 cells according to the manufacturer's protocol. Plasmid DNA was isolated with the Qiaprep Spin miniprep kit (Qiagen) and sequence reactions were carried out with the ABI Prism Bigdye terminator cycle sequencing ready reaction kit according to the manufacturer's protocol and analysed with an ABI 377 sequencer.

Bioinformatics

Multiple sequence alignments were carried out using Clustalw 1.82. The organization of the honeybee CRH-BP gene structure was carried out by comparison of the complete cDNA sequence with the honeybee genome sequence at the Ensembl site (http://www.ensembl.org/). Other non-vertebrate CRH-BP sequences were retrieved via BLAST searches. Phylogenetic trees were constructed on the basis of amino acid differences (p-distance) by the neighbor-joining method using MEGA version 3.0[250]. Reliability of the tree was assessed by bootstrapping, using 1000 bootstrap replications.

Table 11.1: Percentages of amino acid sequence identity between CRH-BP sequences of various animal species

	honeybee	malaria mosquito	fruit fly	sea squirt	carp 1	carp 2	pufferfish	*Xenopus*	chicken	sheep	rat	mouse	human
honeybee	100												
malaria mosquito	33.1	100											
fruit fly	29.8	46.3	100										
sea squirt*	26.8	28.0	28.6	100									
carp 1	29.0	22.1	24.0	33.9	100								
carp 2	28.3	23.3	24.6	33.9	97.8	100							
pufferfish	26.6	23.7	23.3	33.3	67.8	68.4	100						
Xenopus	24.9	21.5	25.6	31.6	58.9	59.2	54.7	100					
chicken	26.1	21.5	24.3	34.5	59.8	59.5	57.2	72.0	100				
sheep	26.5	24.3	23.6	33.9	57.0	57.0	55.3	64.5	70.8	100			
rat	26.7	21.5	23.6	33.9	58.3	58.6	55.0	63.9	71.1	77.3	100		
mouse	26.7	21.8	24.3	32.1	60.8	60.4	55.9	67.6	73.0	79.8	93.8	100	
human	28.9	24.3	24.6	31.6	61.7	62.0	58.1	68.2	73.9	85.1	84.5	87.3	100

*partial sequence only, potentially influencing the percentages amino acid identity with other sequences

```
honeybee CRH-BP  MFLNGSLYCFARLFFIIGFFNAISGAIKGNDYQRYHQQISGDRFSKDFYRQDTKNNLLNA
carp CRH-BP      ----MSGTSRAQLCFLLLSVTALRGHAR---FLDIQDNEISPEGLLSLLSSELKRELPEE
Xenopus CRH-BP   ----MTPASRPDWCLILLFLAVLRGESR---YIQMR--EAAEDALFLLN-SDFKRELSEG
chicken CRH-BP   ----MPSAFQLQCHLVLILLAASKGDTR---YLEVR--DAGEDEPFLLLSEDLKRELSAG
mouse CRH-BP     ----MSPNFKLQCHFILILLTALRGESR---YLEVQ--EAAVYDPLLLFSANLKRDLAEE
human CRH-BP     ----MSPNFKLQCHFILIFLTALRGESR---YLELR--EAADYDPFLLFSANLKRELAGE
                      .        :::  . .  *  :   :   :    .      :   : *.:*

honeybee CRH-BP  YVRFKLVTDCIFVTSEPGYFLYTSKNDNEEVCGIYFLAEPDQKIEINFITFDIPCEHRGL
carp CRH-BP      FV-YRRALRCLDMVAIEGQFTFTAERP-QLNCAVFFIGEPSDIISIEYDSVNIDCRGGDF
Xenopus CRH-BP   QI-YRRSLRCIDMLSIEGQFTFQADRP-QLHCALFLIGEPEEFIIIEYNFVNIDCIGGDI
chicken CRH-BP   HI-YRRSLRCIDMLSIEGQFTFTADQP-QLHCATFFIGEPEELLTIEYDFVNIDCQGGDF
mouse CRH-BP     QP-YRRALRCLDMLSLPGQFTFTADRP-QLHCAAFFIGEPEEFITIHYDLVSIDCQGGDF
human CRH-BP     QP-YRRALRCLDMLSLQGQFTFTADRP-QLHCAAFFISEPEEFITIHYDQVSIDCQGGDF
                    ::    *: : :  * * : :..  :  *. :::.**.: : *.:  ..* *   .:

honeybee CRH-BP  VSIIDGWELNGEVFPSKMDHQLPLKQRSSEFCGKNIGMKRIFTSSQNIAVIEYRIPKSGK
carp CRH-BP      IKVFDGWVMKGEKFPSTQDHPLPLYKRYSDYCETGV-TRPIVRSSQNVAMLFFRLHQSGS
Xenopus CRH-BP   LKVFDGWIIKGEKFPSSLDHPLSTMERYTDICEDGD-VGSITRSSQNVAMIFFRVQQPGH
chicken CRH-BP   LKVFDGWILKGEKFPSSLDHPLPTSQRYTDFCESGA-VQRSIRSSQNVAMIFFRIHQPGN
mouse CRH-BP     LKVFDGWILKGEKFPSSQDHPLPTMKRYTDFCESGL-TRRSIRSSQNVAMVFFRVHEPGN
human CRH-BP     LKVFDGWILKGEKFPSSQDHPLPSAERYIDFCESGL-SRRSIRSSQNVAMIFFRVHEPGN
                 :.::*** ::** ***. ** *.  :*  : *  .        ****:*:: :*: :.*

honeybee CRH-BP  GFSLFARFLKNPRPCNVLATSLTEPYTLRNYGRRINCTYVALYPSSVQVIALGVGVSNFL
carp CRH-BP      SFTVTFRKLINPFPCNVVSQTPEGSFTMIIPQQHRNCSFSIIYPVEIQIGELSLGQHNDL
Xenopus CRH-BP   GFTLTIRKIPNLFPCNVISQSMNGRFTMITPHQHRNCSFSIIYPVVIKIFDLTLGHFNEL
chicken CRH-BP   GFTITVKKSANLFPCNVISQTPSGRFTMVIPHQHRNCSFSIIYPVVIKISDLILGHLNGL
mouse CRH-BP     GFTITIKTDPNLFPCNVISQTPSGRFTLVVPYQHQNCSFSIIYPVAIKISDLTLGHLHGL
human CRH-BP     GFTLTIKTDPNLFPCNVISQTPNGKFTLVVPHQHRNCSFSIIYPVVIKISDLTLGHVNGL
                 .*::  :   *  ****:: :    :*:    :: **::  :**  :::  * :*  : *

honeybee CRH-BP  SSTRTAETGTIRKCDESSPHDQVIIGGSNGLDTSKVHIIDSICGIDSKPDYRELTEYSVT
carp CRH-BP      KR---SILG----CAGS--GDFVELLGGNGMDTSKMYPMADLCYSFNGP-AQMKVGCDNT
Xenopus CRH-BP   QLKKPPPKG----CGDA--GDFVELLGGAGLDPSKMFPLADLCHSFHGS-AQMKIGCDNT
chicken CRH-BP   FLKNPS-VG----CAGV--GDFVELLGGTGLDPSKMFPLADLCHSFHGS-AQMKIGCDNT
mouse CRH-BP     QLKKPA-AG----CGGT--GDFVELLGGTGLDPSKMMPLADLCYPFLGP-AQMKISCDNA
human CRH-BP     QLKKSS-AG----CEGI--GDFVELLGGTGLDPSKMTPLADLCYPFHGP-AQMKVGCDNT
                      .  *    *      * * : *. *:*.**:  : .:*     .  :     . :

honeybee CRH-BP  SVRLISSGFFDNFVTVQIQPLKNELFNANIGI----------
carp CRH-BP      VIRMVSSGKFVNRVSFQYRLLGHQELQQMKGNSVEDVCLRA-
Xenopus CRH-BP   VVRMVSSGNFINRVTFEYNQLD-RQLEKKQGNSVEEACFPSD
chicken CRH-BP   VLRMVSSGKHINRVTFEYYQLDLQEIENRKENSIEEFCFPGI
mouse CRH-BP     VVRMVSSGKHINRVTFEYRQLEPFELETSTGNSIPEYCLSSL
human CRH-BP     VVRMVSSGKHVNRVTFEYRQLEPYELENPNGNSIGEFCLSGL
                  :*::*** . * *:.:   *    ::
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Figure 11.1: Amino acid alignment of honeybee CRH-BP with CRH-BP sequences of various vertebrate species. Cysteine residues that are involved in the formation of disulphide bonds are shaded. Asterisks indicate amino acid identity, whereas colons and dots indicate decreasing degrees of amino acid similarity. Accession numbers: honeybee (*Apis mellifera*), AJ780964; carp (*Cyprinus carpio*), AJ490880; *Xenopus* (*Xenopus laevis*), Q91653; Chicken (*Gallus gallus*), BU358572 and BU367671; mouse (*Mus musculus*), Q60571; human (*Homo sapiens*), P24387.

Table 11.2: List of BLAST hits following comparison of honeybee CRH-BP to the Swissprot database.

acc. number	species	description	E-value
P24388	rat	corticotropin-releasing factor-binding protein	8×10^{-29}
Q28557	sheep	corticotropin-releasing factor-binding protein	4×10^{-27}
Q60571	mouse	corticotropin-releasing factor-binding protein	3×10^{-26}
P24387	human	corticotropin-releasing factor-binding protein	1×10^{-25}
Q91653	*Xenopus*	corticotropin-releasing factor-binding protein	2×10^{-25}
P22482	*Bacillus pseudofirmus*	ATP synthase gamma chain	1.2
Q9Y6W3	Human	Calpain 7	6.4
P43153	*Clostridium perfringens*	Microbial collagenase precursor	8.1

Results

The honeybee orthologue of vertebrate CRH-BP sequences was amplified from cDNA of the head of a single worker bee. The complete coding sequence measures 999 nucleotides and encodes a 332 amino acid protein, which is ten amino acids longer than most vertebrate CRH-BPs. Honeybee CRH-BP shares highest identity (up to 33%) with two automatically annotated dipteran CRH-BP sequences of the malaria mosquito (*Anopheles gambiae*) and the fruitfly (*Drosophila melanogaster*) (Table 11.1). Amino acid identity with the more distantly related CRH-BP sequences of various vertebrate species is slightly lower at 25 - 29%. Eight of the ten cysteine residues that characterise vertebrate CRH-BP sequences are conserved and identically spaced in honeybee CRH-BP, but the final pair of cysteines is absent (Fig. 11.1). Furthermore, throughout the alignment several short stretches of amino acids are identical in all CRH-BP sequences. When honeybee CRH-BP is subjected to a BLAST search to identify the sequences in the Swissprot database that are most similar, the only significant hits are other CRH-BP sequences (Table 11.2). This once again illustrates

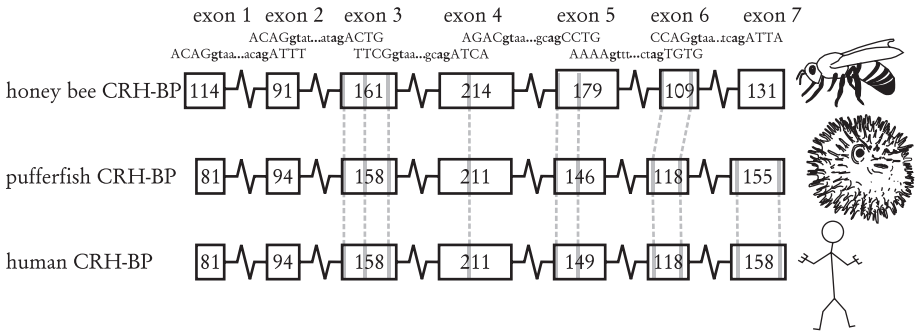

Figure 11.2: Comparison of the honeybee, pufferfish, and human CRH-BP genes. Boxes represent exons and are drawn to scale. Exon lengths are indicated in nucleotides. The nucleotide residues surrounding each splice site are displayed, coding residues are represented by capitals.

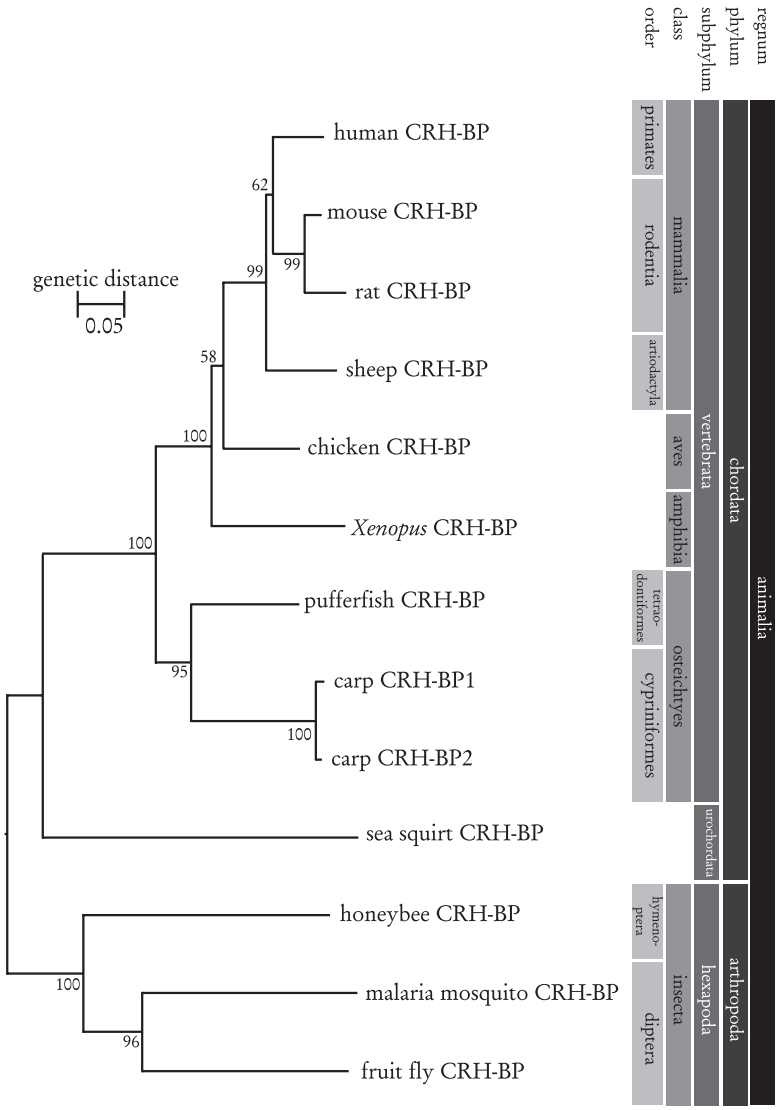

Figure 11.3: Neighbor-joining tree of CRH-BP amino acid sequences. Numbers at branch nodes represent the confidence level of 1000 bootstrap replications. Accession numbers are as follows: human (*Homo sapiens*), P24387; mouse (*Mus musculus*), Q60571; rat (*Rattus norvegicus*), P24388; sheep (*Ovis aries*), Q28557; chicken (*Gallus gallus*), BU358572/BU367671; *Xenopus* (*Xenopus laevis*), Q91653; pufferfish (*Takifugu rubripes*), BN000457; carp (*Cyprinus carpio*), CRH-BP1 AJ490880, CRH-BP2 AJ490881; sea squirt (*Ciona intestinalis*), AABS01000063; honeybee (*Apis mellifera*), AJ780964; malaria mosquito (*Anopheles gambiae*), XP_309147; fruitfly (*Drosophila melanogaster*), NM_143536.

```
honeybee DH-I       --RIGSLSIVNSMDVLRQRVLLELARRKALQDQAQIDANRRLLETI
house fly DH-I      --NKPSLSIVNPLDVLRQRLLLEIARRQMKENTRQVELNRAILKNV
cockroach DH-I      TGTGPSLSIVNPLDVLRQRLLLEIARRRMRQTQNMIQANRDFLESI
                      ******.:******:***:***:   :     ::  **  :*:.:
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Figure 11.4: Amino acid alignment of the mature honeybee DH-1 peptide with DH-1 peptides of other insects. Residues that conform to the prosite CRH motif (PS00511) are shaded. Asterisks indicate amino acid identity, whereas colons and dots indicate decreasing degrees of amino acid similarity. Accession numbers: honeybee (*Apis mellifera*), AJ876408; house fly (*Musca domestica*), P41537; Pacific beetle cockroach (*Diploptera punctata*), P82373.

the uniqueness of CRH-BP and provides further testimony of the unambiguous orthology of honeybee and vertebrate CRH-BP sequences.

The honeybee CRH-BP gene consists of seven exons, as is the case for all vertebrate CRH-BP genes that have been characterised (Fig. 11.2). Furthermore, exon sizes correspond well to the lengths of each of the seven vertebrate exons, with honeybee exons two, three, and four each differing merely one triplet over the sizes of their corresponding vertebrate exons. Also the distribution of the conserved cysteine residues over the exons is highly similar in honeybee and vertebrates and all honeybee introns contain well-recognisable 5' donor (gt) and 3' acceptor (ag) splice sites.

Phylogenetic analyses corroborate the notion that all CRH-BP sequences conform to

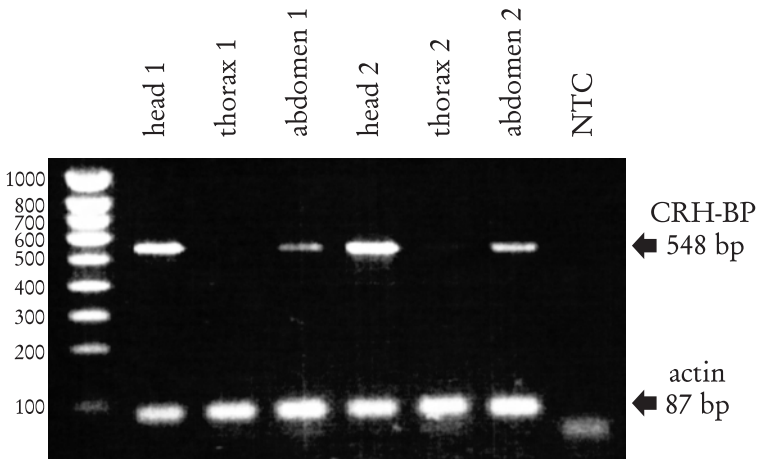

Figure 11.5: Expression of CRH-BP mRNA in the head, thorax, and abdomen of two individual female worker bees. Expression of CRH-BP (35 cycles) and actin (30 cycles) was assessed by two-step RT-PCR. Reactions were carried out in separate vials and corresponding reactions (housekeeping gene and gene of interest) were loaded in the same slots of a 1.5% agarose gel. NTC, non-template control. The slight primer-dimer formation in the NTC lane is fully attributable to the CRH-BP primers.

the accepted patterns of animal evolution, with sequences from more distantly related species clustering less proximate (Fig. 11.3). Inclusion of the human IGF-BP family as an outgroup results in a phylogenetic tree where all CRH-BP sequences form a stable cluster, separate from the IGF-BP sequences (not shown).

Because of its CRH motif, insect DH-1 would be a likely candidate to bind to insect CRH-BP. Despite its identification in many different insect species, DH-1 has not yet been reported in the honeybee. To confirm the presence of DH-1 in the honeybee, we amplified the DNA sequence encoding the honeybee mature DH-1 peptide. This peptide resembles previously identified DH-1 peptides from other insect species well (Fig. 11.4). Moreover, a honeybee DH-receptor sequence has been automatically annotated (XP_397268) from the honeybee genome.

To learn whether insect CRH-BP, like its vertebrate orthologues, is largely centrally expressed, we established the expression pattern of CRH-BP mRNA in the three major body regions of the honeybee. CRH-BP gene expression occurs in the head of honeybee (Fig. 11.5), and to a lesser extent in the abdomen, but is absent from the thorax.

Discussion

Here we describe the identification of the complete cDNA sequence of CRH-BP from the honeybee. The conservation of unique features such as key cysteine residues and gene structure, provide testimony to its *bona fide* orthology with vertebrate CRH-BP sequences. The discovery of CRH-BP in the honeybee substantiates that the CRH system predates vertebrates and is likely to share ancestry with insect DH-1 and its receptor. Overall amino acid identity of honey bee CRH-BP with various vertebrate CRH-BP sequences is moderate at around 25 – 29%, which is not surprising as the evolutionary distance between insects and vertebrates is estimated at between 700 and 993 million years[510-512]. Nonetheless, the high similarity in gene structure, stable clustering in phylogenetic analyses, as well as the conservation in presence and spacing of the first eight cysteine residues all point clearly to the unambiguous orthology of vertebrate and insect CRH-BPs. The final two C-terminal cysteine residues are missing from the honeybee CRH-BP sequence. The simultaneous disappearance of this pair of cysteines is in line with the observation that they form an intrachain disulphide bridge[495]. Furthermore, the obvious sequence identity in-between the location of both missing cysteine residues, complemented by the presence of this C-terminal cysteine pair in the predicted CRH-BP sequences of *Drosophila melanogaster* and *Anopheles gambiae*, indicates a loss of these two cysteines in the honeybee following its divergence from both dipteran species. Other than the cysteine residues, several short

amino acid stretches are identical in all sequences, which suggests that these residues are structurally imperative or indispensable for ligand binding.

Establishment of orthology for the vertebrate CRH-family members with insect DH-1 is not straightforward, as the CRH motif is not very stringent and the mature neuropeptides are short (46 amino acids or less), which impairs phylogenetic analyses. Furthermore, the genes encoding all four vertebrate CRH family members (CRH, urotensin-I/urocortin-I, urocortin-II, and urocortin-III) possess two exons, and are encoded completely by the second exon, whereas the coding region of tobacco hornworm (*Manduca sexta*) DH-1 is divided over four exons[513]. And although insect diuretic receptors and vertebrate CRH-receptors both belong to the class B (secretin-like) family of GPCRs, this family also includes receptors for secretin, vasoactive intestinal peptide, parathyroid hormone and its related peptide, growth hormone releasing hormone, calcitonin, and others[514], which complicates establishment of one-to-one orthology. In contrast, the uniqueness of CRH-BP greatly facilitates establishment of orthology over large evolutionary distances.

CRH-BP takes its name from the modulation of CRH bioactivity, either antagonistically via abrogated CRH signaling[427, 428], or agonistically via extension of protein half-life[498]. But CRH-BP also has the potential to bind to and modulate signaling of other members of the CRH-family. In fact, several reports indicate that CRH-BP has a similar or higher affinity for urocortin-I and urotensin-I compared to CRH[436, 515]. Insect DH-1, with its CRH family motif, is the most likely candidate to bind to CRH-BP. Given the colocalisation of CRH and CRH-BP in the *pars intermedia* of carp, it is obvious to assume that CRH-BP colocalises with DH-1 in the insect *corpora cardiaca*. Although we were unable to demonstrate so in the honeybee, this is indeed the case in the locust *Schistocerca gregaria* (DE LOOF and HUISING, unpublished observation). Furthermore, the gene expression pattern of honeybee CRH-BP is consistent with these findings.

While the PI-CC axis is considered the analog of the vertebrate hypothalamus-pituitary axis in general, the insect *corpora cardiaca* bear a structural resemblance to the pituitary gland of fish in particular. As a consequence of a lacking median eminence, the fish pituitary gland is directly innervated by hypothalamic neurons. Furthermore, the nerve terminals in the pituitary *pars intermedia* of fish contain such an abundance of several neuropeptides, including CRH and CRH-BP[69], that it is considered a neurohemal site, analogous to the insect *corpora cardiaca*. A second neurohemal organ in fish, the caudal neurosecretory system (CNSS), also releases CRH as well as urotensin-I[18]. Interestingly, the latter peptide has a role in osmoregulation[516], analogous to insect DH-1 that acts distally on the Malpighian tubules within the insect abdomen to promote active cation transport, thereby increasing primary urine production[517].

In summary, we have demonstrated that CRH-BP is well conserved and is clearly identifiable in insect species. It follows that CRH-BP has been present since the common

ancestor to insects and vertebrates. More importantly, the unequivocal orthology of insect and vertebrate CRH-BPs adds substantial weight to the supposition that the vertebrate CRH system and the insect DH system stem from a common ancestor.

Acknowledgements

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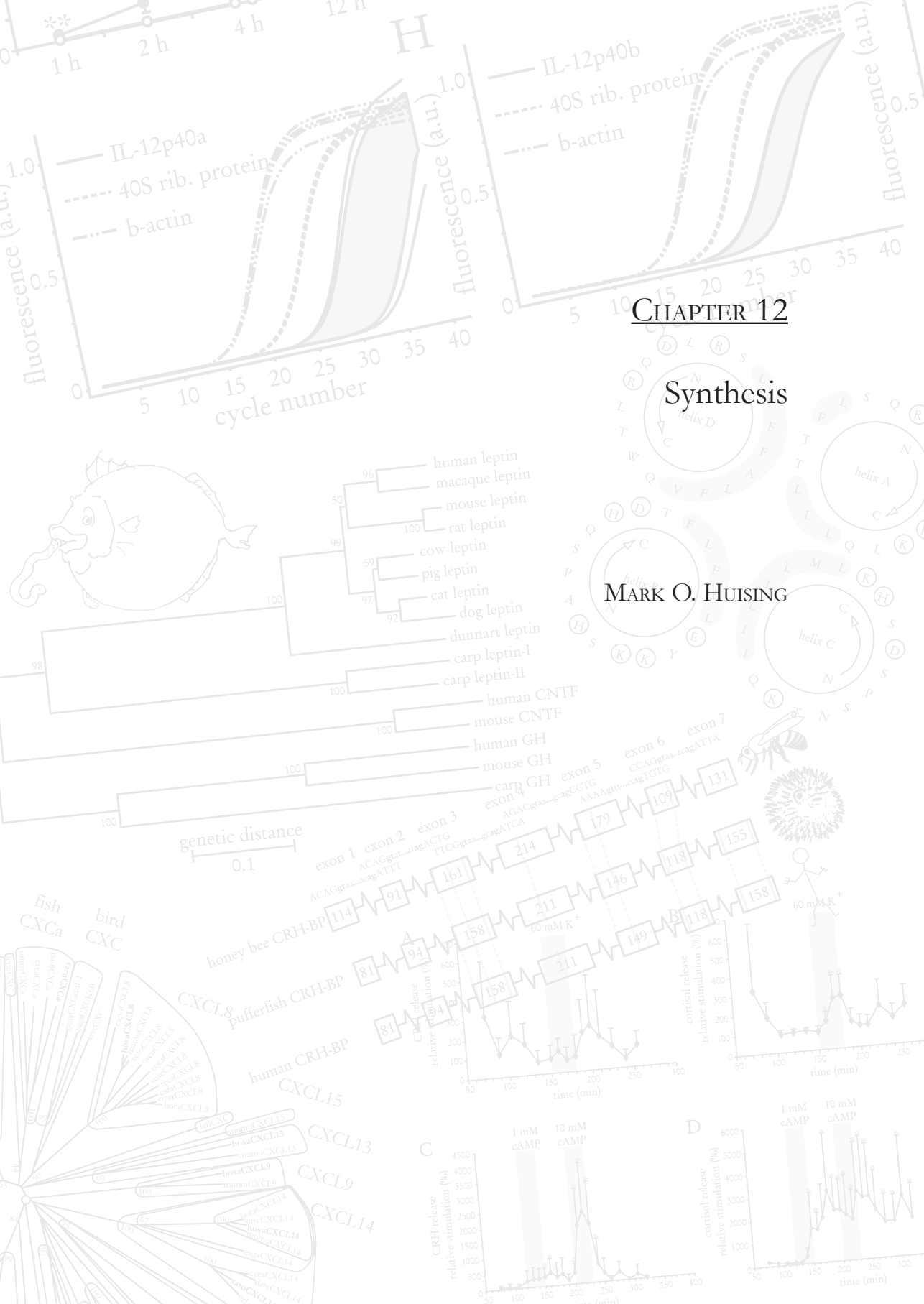


Chapter 12

Synthesis

Mark O. Huising

Purpose of review

Evolutionary theory is – almost 150 years following the first edition of Darwin's 'On the Origin of Species' – beyond discussion still the most thought- and debate- provoking concept in biology. Comparative studies, e.g. on early vertebrates such as teleostean fishes, by definition embrace the evolutionary concept, as they parallel the situation in the organism of interest with the *status quo* that follows from the mammalian literature, thereby implicitly assuming common descent. In comparative studies, conspicuous differences in the degree of evolutionary conservation between different proteins and peptides quickly emerge. Here, we compare teleostean extracellular signaling proteins and peptides (hormones, cytokines, and growth factors) with their human orthologues. We anticipate that such systematic comparison will provide us with a better understanding as well as an objective perspective on 'what' is evolutionary conserved. To obtain an answer to the question of 'how' these differences in the degree of evolutionary conservation are achieved, we calculate the type and strength of the average selective forces that have acted during 450 million years that have passed since the fish-tetrapod split. Finally, we discuss several possibilities (the 'why') that collectively may offer an explanation for the differences in the degree of conservation that are observed between the molecular signals of the endocrine and the immune system.

Introduction

The signals that coordinate the actions of our endocrine and immune system belong to several structurally unrelated peptide families, yet they all share the property that they convey messages from one cell of the body to another. Our body consists of hundreds of billions of cells that engage in a far-reaching form of cooperation, as they cannot survive independently. This cooperation requires tight coordination, achieved through extensive communication. Much of this communication is ligand-receptor mediated. Our body has an impressive array of protein and peptide messenger molecules that is employed by virtually every cell of the body. Communication has two sides: successful communication depends on both the transmission and reception of a message. When we consider the synthesis and secretion of protein and peptide messengers as the transmission of a message, its reception is taken care of by cell surface receptors specific to a particular soluble peptide. Upon their release, these proteins and peptides reach their cellular targets, either by passive diffusion or facilitated by the circulation. Neural synaptic communication could be regarded as a variation on the common theme of

communication via soluble messengers and their cell surface receptors, with the proviso that the messengers are released within the narrow confinement of the synaptic cleft and diffuse only a minimal distance to reach their target cell. Thereby, this mode of communication combines two advantages: unprecedented high speed and great resolution with regard to the receiving cell. Most communication in the vertebrate body however, would not benefit from the speed and precision with regard to the receiver so characteristic of neuronal communication. On the contrary, in many instances it is quite useful that the message transmitted from a single cell persists for a period of several minutes (instead of milliseconds) and is received by a wider audience.

In the early 1900s Bayliss and Starling, the founders of Endocrinology, first discovered that messages are transmitted from one site in the body to another, without interference of the nervous system. Working with anaesthetised dogs, they observed that contact of the duodenal mucosa with a dilute acid solution (mimicking stomach acid) evoked the release of pancreatic juices, even when the duodenum was completely freed of its neural in- and outputs[518]. They concluded that the only possible explanation for the transmission of a signal from the duodenum to the pancreas would be a soluble factor that is released by the duodenum and reaches the pancreas via the circulation. This putative factor was aptly named secretin and was much later shown to be a 27 amino acid peptide[519, 520]. In 1905, a few years following their first (indirect) demonstration of the presence of soluble factors in the blood that relay messages between distal sites in our body, Starling proposed the term 'hormone' as the name that covered all messenger substances of the body that are released into circulation from an endocrine gland[521]. Now, in the year that we celebrate the centennial anniversary of the term 'hormone', the list of hormones is long and still growing, as new hormones are discovered on a regular basis.

In their field, immunologists realised that sera and media of leukocyte cultures contained signaling substances and growth factors that induce or inhibit cell proliferation. These signaling molecules were collectively referred to as 'cytokines', referring to the Greek verb κινειν (kinein), which means 'to move'. In the 1980s the number of newly discovered cytokines rapidly expanded and were subdivided into several groups, including chemokines (an acronym of chemotactic cytokines) and interleukins, a name referring to a role in communication 'between leukocytes'. In contrast to hormones that are secreted into the circulation to act distally in the body, most cytokines act locally in an autocrine or paracrine fashion, although this distinction should be regarded as no more than a rule of thumb. In reality, the distinction between hormones and cytokines is for a large part historical and determined by whether they were originally discovered as important signals of either the endocrine or the immune system. In the last two decades, we are increasingly aware of the extensive cross-communication between the endocrine and the

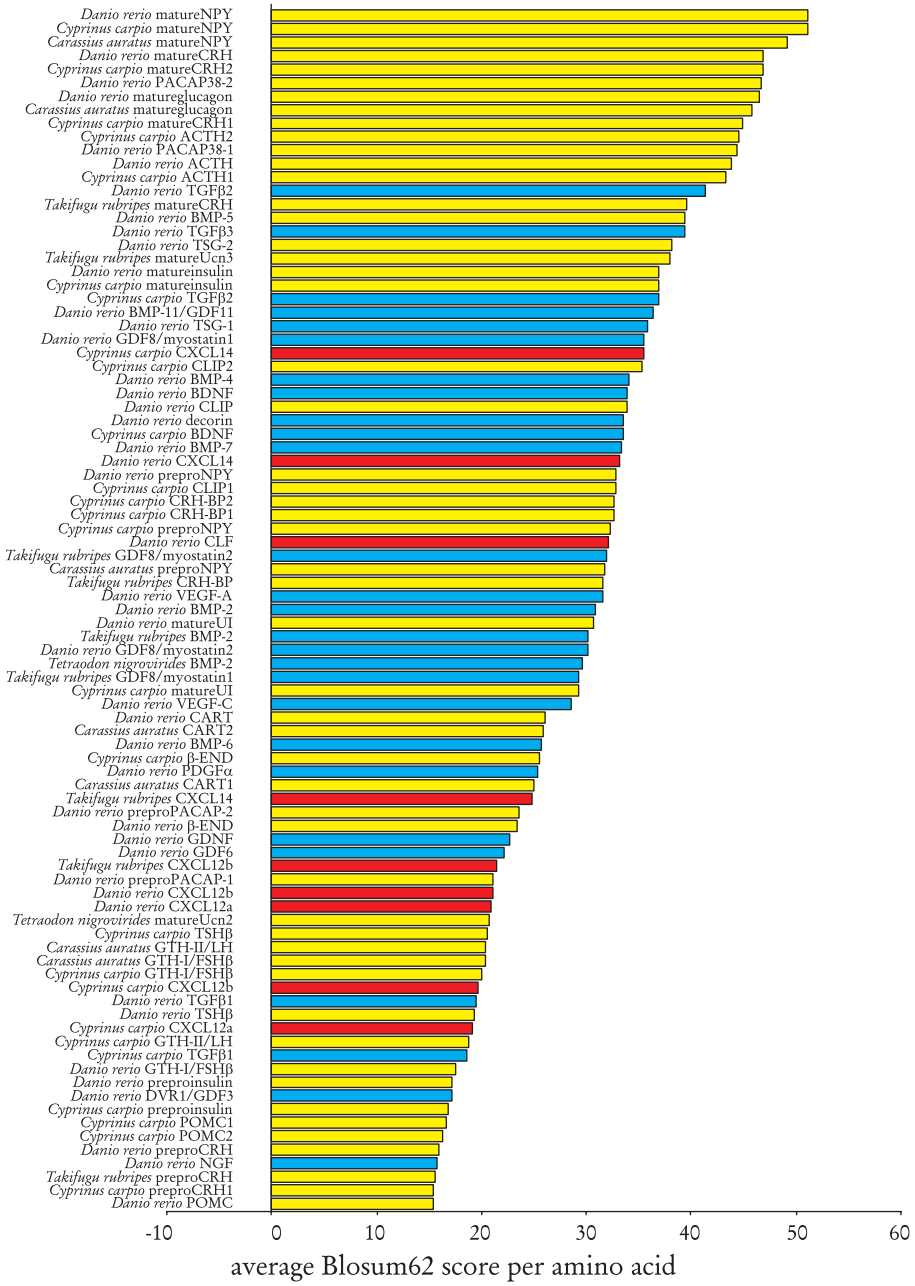
immune system, which is mediated by hormones and cytokines alike. The acceptance of this notion of bidirectional communication meant that the borders that separated the immune and endocrine system have gradually faded. Nevertheless, the fields of immunology and endocrinology are still largely regarded as separate disciplines, fueled by our own drive to classify and maintain overview.

Instigated by the incentive to investigate our evolutionary origins, or perhaps by scientific biological curiosity, researchers have addressed the signaling pathways of the immune and endocrine system in non-mammalian vertebrates and even invertebrates. This has lead to the discovery of orthologues of many mammalian cytokines and hormones as well as their receptors in early vertebrates such as bony fish. In a few instances, the discovery of a fish messenger molecule was instrumental in the discovery of its mammalian orthologue. This was the case for the peptide hormone urotensin-I (UI) as well as for the hypocalcemic hormone stanniocalcin, that were both identified in fish years prior to their discovery in mammalian species[417, 419, 522-524]. In the majority of cases, however, the identification of cytokines and hormones in non-mammalian vertebrates followed their discovery in rodent or primate species. Nevertheless, the identification of hormones and cytokines in fish, *Xenopus*, or chicken is not merely of interest to comparative endocrinologists and immunologists. Study of the communication systems of early vertebrates reveals valuable insights into which signaling pathways are evolutionarily old and which messengers are recent additions or deletions to the signal vocabulary of selected vertebrate species. Comparative studies that involve species of different classes of vertebrates can reveal the extent of evolutionary conservation of orthologous signaling molecules over greater evolutionary distances. Many hormones and cytokines are regarded as 'evolutionarily conserved', oftentimes solely based on the primary sequence identity between two mammalian species. The opposite statement, namely that a particular peptide is evolutionarily poorly conserved, is far less commonly encountered. This is undoubtedly caused by the aura of importance that is conveyed by emphasising the evolutionary conservation of one's hormone or cytokine of interest. Surprisingly, the degree of evolutionary conservation is rarely put into perspective.

The most comprehensive way to achieve such a perspective is to compare the degree of sequence conservation of orthologous sequences over a particular evolutionary distance. Although this approach may sound straightforward, it requires further refinement on three issues: 1) the most appropriate way to quantify the degree of sequence conservation, 2) the concept of orthology, and 3) the notion of evolutionary distance.

Quantification of the degree of sequence conservation

To determine the degree of sequence conservation, one can look at nucleotide or amino acid sequences. When comparing amino acid sequences, one can look at sequence identity only, or also include the degree of sequence similarity. The latter case requires the assignment of scores to amino acid substitutions, a high score to a substitution of an amino acid by a residue with similar characteristics (e.g. arginine for lysine, both of which have a positively charged side-chain), a low score to amino acid pairs with little overlap in biochemical properties. Since a comparison of amino acid similarities provides maximal resolution, particularly when a substantial number of sequences is compared over a large evolutionary distance (see below), we used this as a measure of evolutionary conservation. For all orthologous sequence pairs (fish vs. human), we constructed a pairwise amino acid alignment with the T-Coffee algorithm[411] and scored the degree of sequence similarity according the Blosum62 amino acid substitution matrix[525]. As this similarity score is cumulative and would therefore impose a positive bias on longer sequences, we divided the similarity score by the length of the pairwise alignment. This yields an average Blosum62 score per amino acid that is suitable for a direct comparison of sequence conservation between unrelated sequences. Figure 12.1 is the result of such a comparison between signaling substances of teleost fish species of the cyprinid (*Cyprinidae*) and puffer (*Tetraodontidae*) families with their human orthologues. Here, the sequences are ranked from well conserved (high Blosum62 score) to poorly conserved (low Blosum62 score), revealing that considerable differences exist in evolutionary conservation between vertebrate signaling substances. Classification of these signaling molecules according to a 'historical' perspective as cytokine, hormone, or growth factor, simply reflecting their discovery as signals of the immune or endocrine system, or as regulators of ontogeny or tissue growth and repair, demonstrates the differences in conservation that exist between these three groups of signaling molecules. Of the 50 sequences that display the poorest sequence conservation, the majority (38) are cytokines, whereas hormones and growth factors are generally much better conserved (Fig. 12.1). A complicating factor in the comparison of sequence conservation of some hormones in particular, is that they are secreted as prepro-peptides. The pre-sequence of the hormones equals the signal peptide, which is required for efficient secretion and is consequently shared by the vast majority of messenger proteins. The pro-part of many hormones, such as corticotropin-releasing hormone (CRH) and glucagon, is not involved in the actual signaling and is consequently in many cases less conserved than the mature bioactive peptide. For these hormones we therefore calculated the degree of sequence similarity for the prepro-hormone as well as the mature peptide. Indeed, many of these mature peptides rank among the best-conserved sequences in our analyses, whereas their corresponding prepro-hormones score considerably lower.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Cyprinus carpio matureNPY
Carassius auratus matureNPY
Danio rerio matureCRH
Cyprinus carpio matureCRH2
Danio rerio PACAP38-2
Danio rerio matureglucagon
Carassius auratus matureglucagon
Cyprinus carpio matureCRH1
Cyprinus carpio ACTH2
Danio rerio PACAP38-1
Danio rerio ACTH
Cyprinus carpio ACTH1
Danio rerio TGFβ2
Takifugu rubripes matureCRH
Danio rerio BMP-5
Danio rerio TGFβ3
Danio rerio TSG-2
Takifugu rubripes matureUcn3
Danio rerio matureinsulin
Cyprinus carpio matureinsulin
Cyprinus carpio TGFβ2
Danio rerio BMP-11/GDF11
Danio rerio TSG-1
Danio rerio GDF8/myostatin1
Cyprinus carpio CXCL14
Cyprinus carpio CLIP2
Danio rerio BMP-4
Danio rerio BDNF
Danio rerio CLIP
Danio rerio decorin
Cyprinus carpio BDNF
Danio rerio BMP-7
Danio rerio CXCL14
Danio rerio preproNPY
Cyprinus carpio CLIP1
Cyprinius carpio CRH-BP2
Cyprinus carpio CRH-BP1
Cyprinus carpio preproNPY
Danio rerio CLF
Takifugu rubripes GDF8/myostatin2
Carassius auratus preproNPY
Takifugu rubripes CRH-BP
Danio rerio VEGF-A
Danio rerio BMP-2
Danio rerio matureUI
Takifugu rubripes BMP-2
Danio rerio GDF8/myostatin2
Tetraodon nigrovirides BMP-2
Takifugu rubripes GDF8/myostatin1
Cyprinus carpio matureUI
Danio rerio VEGF-C
Danio rerio CART
Carassius auratus CART2
Danio rerio BMP-6
Cyprinus carpio β-END
Danio rerio PDGFα
Carassius auratus CART1
Takifugu rubripes CXCL14
Danio rerio preproPACAP-2
Danio rerio β-END
Danio rerio GDNF
Danio rerio GDF6
Takifugu rubripes CXCL12b
Danio rerio preproPACAP-1
Danio rerio CXCL12b
Danio rerio CXCL12a
Tetraodon nigrovirides matureUcn2
Cyprinus carpio TSHβ
Carassius auratus GTH-II/LH
Carassius auratus GTH-I/FSHβ
Cyprinus carpio GTH-I/FSHβ
Cyprinus carpio CXCL12b
Danio rerio TGFβ1
Danio rerio TSHβ
Cyprinus carpio CXCL12a
Cyprinus carpio GTH-II/LH
Cyprinus carpio TGFβ1
Danio rerio GTH-I/FSHβ
Danio rerio preproinsulin
Danio rerio DVR1/GDF3
Cyprinus carpio preproinsulin
Cyprinus carpio POMC1
Cyprinus carpio POMC2
Danio rerio preproCRH
Danio rerio NGF
Takifugu rubripes preproCRH
Cyprinus carpio preproCRH1
Danio rerio POMC
-10
0
10
20
30
40
50
60
average Blosum62 score per amino acid

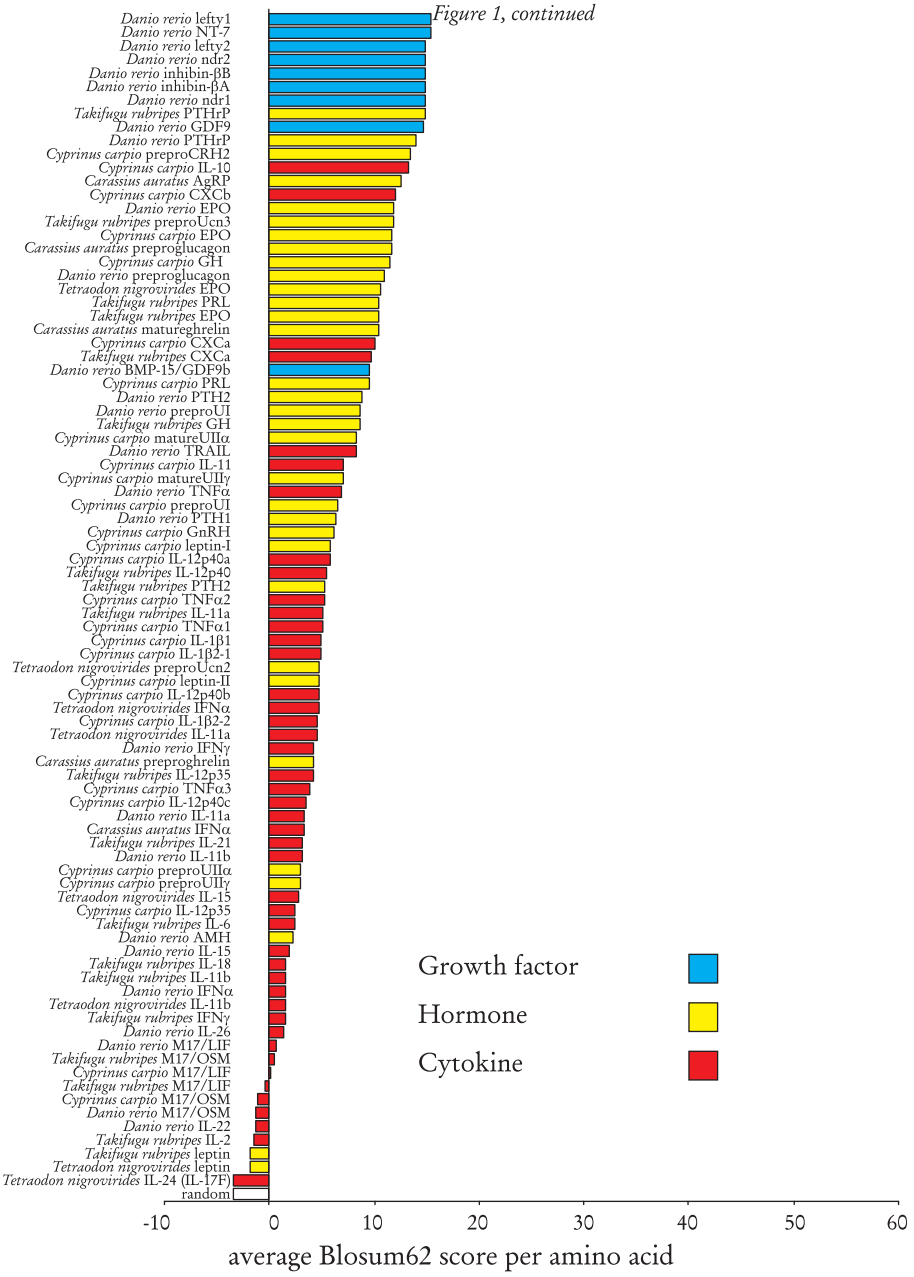


Figure 1, continued
Danio rerio lefty1
Danio rerio NT-7
Danio rerio lefty2
Danio rerio ndr2
Danio rerio inhibin-βB
Danio rerio inhibin-βA
Danio rerio ndr1
Takifugu rubripes PTHrP
Danio rerio GDF9
Danio rerio PTHrP
Cyprinus carpio preproCRH2
Cyprinus carpio IL-10
Carassius auratus AgRP
Cyprinus carpio CXCb
Danio rerio EPO
Takifugu rubripes preproUcn3
Cyprinus carpio EPO
Carassius auratus preproglucagon
Cyprinus carpio GH
Danio rerio preproglucagon
Tetraodon nigrovirides EPO
Takifugu rubripes PRL
Takifugu rubripes EPO
Carassius auratus matureghrelin
Cyprinus carpio CXCa
Takifugu rubripes CXCa
Danio rerio BMP-15/GDF9b
Cyprinus carpio PRL
Danio rerio PTH2
Danio rerio preproUI
Takifugu rubripes GH
Cyprinus carpio matureUIIα
Danio rerio TRAIL
Cyprinus carpio IL-11
Cyprinus carpio matureUIIγ
Danio rerio TNFα
Cyprinus carpio preproUI
Danio rerio PTH1
Cyprinus carpio GnRH
Cyprinus carpio leptin-I
Cyprinus carpio IL-12p40a
Takifugu rubripes IL-12p40
Takifugu rubripes PTH2
Cyprinus carpio TNFα2
Takifugu rubripes IL-11a
Cyprinus carpio TNFα1
Cyprinus carpio IL-1β1
Cyprinus carpio IL-1β2-1
Tetraodon nigrovirides preproUcn2
Cyprinus carpio leptin-II
Cyprinus carpio IL-12p40b
Tetraodon nigrovirides IFNα
Cyprinus carpio IL-1β2-2
Tetraodon nigrovirides IL-11a
Danio rerio IFNγ
Carassius auratus preproghrelin
Takifugu rubripes IL-12p35
Cyprinus carpio TNFα3
Cyprinus carpio IL-12p40c
Danio rerio IL-11a
Carassius auratus IFNα
Takifugu rubripes IL-21
Danio rerio IL-11b
Cyprinus carpio preproUIIα
Cyprinus carpio preproUIIγ
Tetraodon nigrovirides IL-15
Cyprinus carpio IL-12p35
Takifugu rubripes IL-6
Danio rerio AMH
Danio rerio IL-15
Takifugu rubripes IL-18
Takifugu rubripes IL-11b
Danio rerio IFNα
Tetraodon nigrovirides IL-11b
Takifugu rubripes IFNγ
Danio rerio IL-26
Danio rerio M17/LIF
Takifugu rubripes M17/OSM
Cyprinus carpio M17/LIF
Takifugu rubripes M17/LIF
Cyprinus carpio M17/OSM
Danio rerio M17/OSM
Danio rerio IL-22
Takifugu rubripes IL-2
Takifugu rubripes leptin
Tetraodon nigrovirides leptin
Tetraodon nigrovirides IL-24 (IL-17F)
random
Growth factor
Hormone
Cytokine
-10
0
10
20
30
40
50
60
average Blosum62 score per amino acid

Figure 12.1: Evolutionary conservation of signaling molecules that are classically associated with the immune system, the endocrine system, or regulation of tissue growth and repair. Protein and peptide messengers of five different fish species, belonging to the distantly related teleostean families of cyprinids (*Cyprinidae*) and puffers (*Tetraodontidae*) were compared with their human orthologues. Each fish messenger molecule is aligned pairwise with its human orthologue, using the T-Coffee algorithm[411]. Their overall amino acid similarity is scored via the Blosum62 substitution matrix[525]. For better comparison the overall Blosum62 score is divided by the alignment length to obtain an 'average score' per amino acid. All messenger molecules were ranked on the basis of their average Blosum62 score. Three randomly generated amino acid sequences of 200 residues each, which are aligned pairwise, yield an average Blosum62 score per amino acid of approximately -4.5 and this reflects the minimally achievable score. The bars that belong to each of the fish messenger molecules are colored to reflect their classification as a growth factor, hormone, or cytokine.

The concept of orthology

The second issue concerns the concept of orthology. In this thesis, the designation of orthology is reserved for pairs of sequences from different species that share sufficient structural features (such as gene organisation, conserved cysteine residues, and overall sequence identity) to safely assume that they share a direct common ancestor. Thus, the designation of orthology is done strictly from a structural point of view and without implying functional analogy, although orthologous genes will in many instances serve similar functions in different species. As illustrated in several chapters of this thesis, a one-to-one orthologous relationship cannot always be established, which complicates pairwise sequence alignments. Lineage-specific gene duplications are a common factor that complicates the assignment of orthology. When comparing fish and mammalian sequences, lineage-specific gene duplication can have occurred in either lineage. Gene duplications in the fish lineage are common and are in many cases attributed to genome duplication events. One round of genome duplication is estimated to have occurred early in the fish lineage (approximately 300-450 million year ago), directly following the fish-tetrapod divergence[93, 526, 527], and is likely responsible for the presence of duplicate genes for CXCL12 (chapter 3), IL-11 (chapter 5), and IL-12p40 (a and b, chapter 6). Several teleostean lineages, e.g. that of the salmonids and the catostomids (suckers), witnessed a second, more recent genome duplication[527, 528]. In common carp (*Cyprinus carpio*), a second genome duplication occurred a mere twelve million years ago[253, 529]. It is this genome duplication event that is likely responsible for the presence of duplicate yet highly similar set of genes for leptin (chapter 7), pro-opiomelanocortin (POMC)[261], corticotropin-releasing hormone (CRH) (chapter 9), and CRH-binding protein (CRH-BP) (chapter 9) in carp. Similar duplications are not indicated to date in related cyprinid species such as zebrafish (*Danio rerio*) since it diverged from the carp ancestor approximately 50 million years ago, prior to the duplication of the carp genome. Regardless of the cause

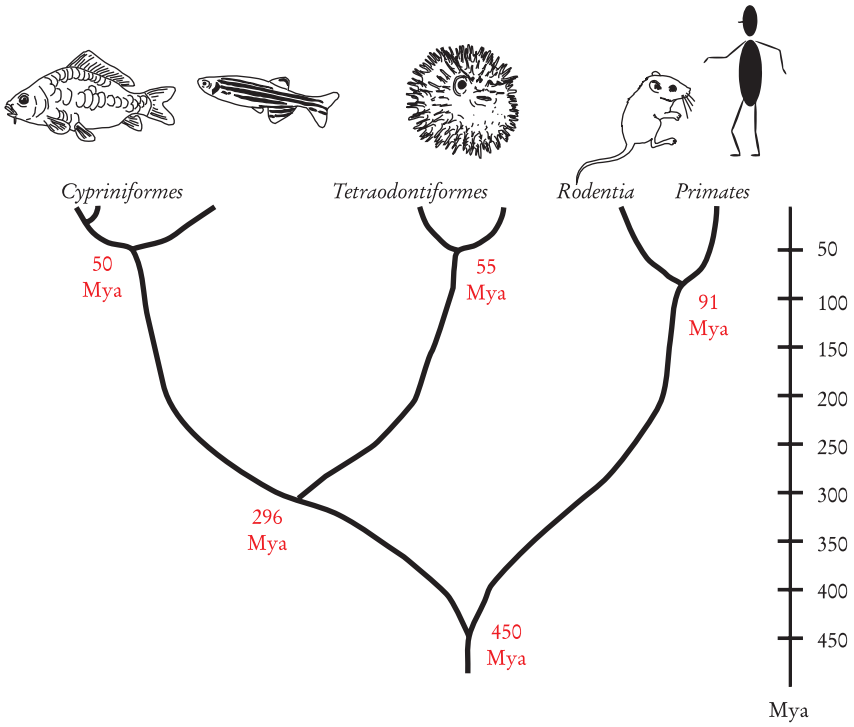
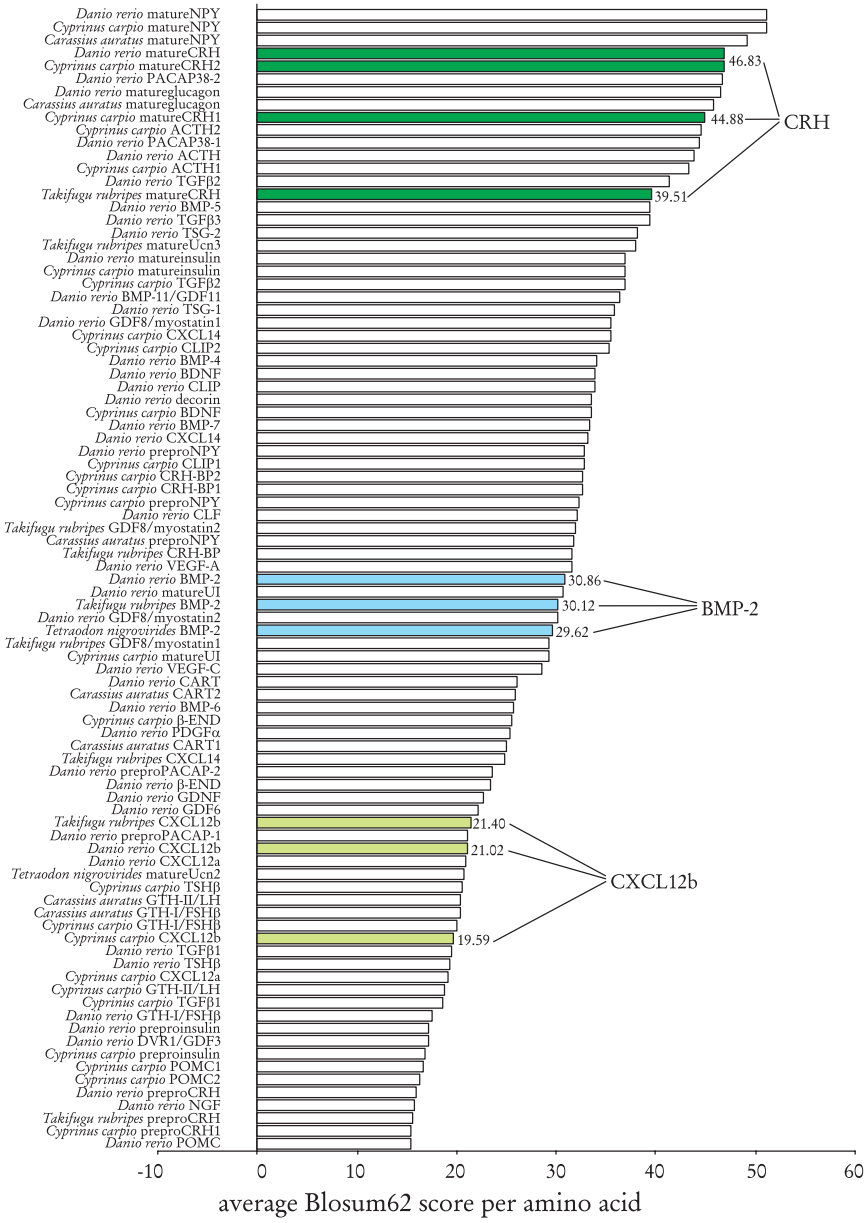


Figure 12.2: The evolutionary distance that separates contemporary fish and mammalian species is identical. The fish and tetrapod lineages separated approximately 450 million years ago. This event defines the evolutionary distance that separates each of the teleostean species with any other, more recent vertebrate, although the speciation events that have led to each of the contemporary fish and mammalian species occurred much later. Divergence estimates are based on refs[15, 527, 543, 544].

of duplication, in case of duplicate fish genes, one can compare each fish paralogue separately with its mammalian orthologue.

The opposite situation, gene duplications that are specific to the mammalian lineage, also occurs. As explained in CHAPTERS 2 and 4, the majority of mammalian CXC chemokines arose by gene duplication only after the fish-tetrapod divergence. Most of these gene duplications, particularly those that have led to the cluster of CXC chemokines at human chromosome 4q21, are not found in the chicken and may be specific to the mammalian CXC chemokine family[530]. In these situations, the average score is presented of the comparisons of a single fish cytokine with each of the members of its mammalian orthologous cluster. The potentially adaptive value of lineage-specific paralogues is addressed later in this discussion.


Danio rerio matureNPY
Cyprinus carpio matureNPY
Carassius auratus matureNPY
Danio rerio matureCRH
Cyprinus carpio matureCRH2
Danio rerio PACAP38-2
Danio rerio matureglucagon
Carassius auratus matureglucagon
Cyprinus carpio matureCRH1
Cyprinus carpio ACTH2
Danio rerio PACAP38-1
Danio rerio ACTH
Cyprinus carpio ACTH1
Danio rerio TGFβ2
Takifugu rubripes matureCRH
Danio rerio BMP-5
Danio rerio TGFβ3
Danio rerio TSG-2
Takifugu rubripes matureUcn3
Danio rerio matureinsulin
Cyprinus carpio matureinsulin
Cyprinus carpio TGFβ2
Danio rerio BMP-11/GDF11
Danio rerio TSG-1
Danio rerio GDF8/myostatin1
Cyprinus carpio CXCL14
Cyprinus carpio CLIP2
Danio rerio BMP-4
Danio rerio BDNF
Danio rerio CLIP
Danio rerio decorin
Cyprinus carpio BDNF
Danio rerio BMP-7
Danio rerio CXCL14
Danio rerio preproNPY
Cyprinus carpio CLIP1
Cyprinus carpio CRH-BP2
Cyprinus carpio CRH-BP1
Cyprinus carpio preproNPY
Danio rerio CLF
Takifugu rubripes GDF8/myostatin2
Carassius auratus preproNPY
Takifugu rubripes CRH-BP
Danio rerio VEGF-A
Danio rerio BMP-2
Danio rerio matureUI
Takifugu rubripes BMP-2
Danio rerio GDF8/myostatin2
Tetraodon nigrovirides BMP-2
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Cyprinus carpio matureUI
Danio rerio VEGF-C
Danio rerio CART
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Danio rerio BMP-6
Cyprinus carpio β-END
Danio rerio PDGFα
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Tetraodon nigrovirides matureUcn2
Cyprinus carpio TSHβ
Carassius auratus GTH-II/LH
Carassius auratus GTH-I/FSHβ
Cyprinus carpio GTH-I/FSHβ
Cyprinus carpio CXCL12b
Danio rerio TGFβ1
Danio rerio TSHβ
Cyprinus carpio CXCL12a
Cyprinus carpio GTH-II/LH
Cyprinus carpio TGFβ1
Danio rerio GTH-I/FSHβ
Danio rerio preproinsulin
Danio rerio DVR1/GDF3
Cyprinus carpio preproinsulin
Cyprinus carpio POMC1
Cyprinus carpio POMC2
Danio rerio preproCRH
Danio rerio NGF
Takifugu rubripes preproCRH
Cyprinus carpio preproCRH1
Danio rerio POMC
46.83
44.88
39.51
CRH
30.86
30.12
29.62
BMP-2
21.40
21.02
19.59
CXCL12b
-10
0
10
20
30
40
50
60
average Blosum62 score per amino acid

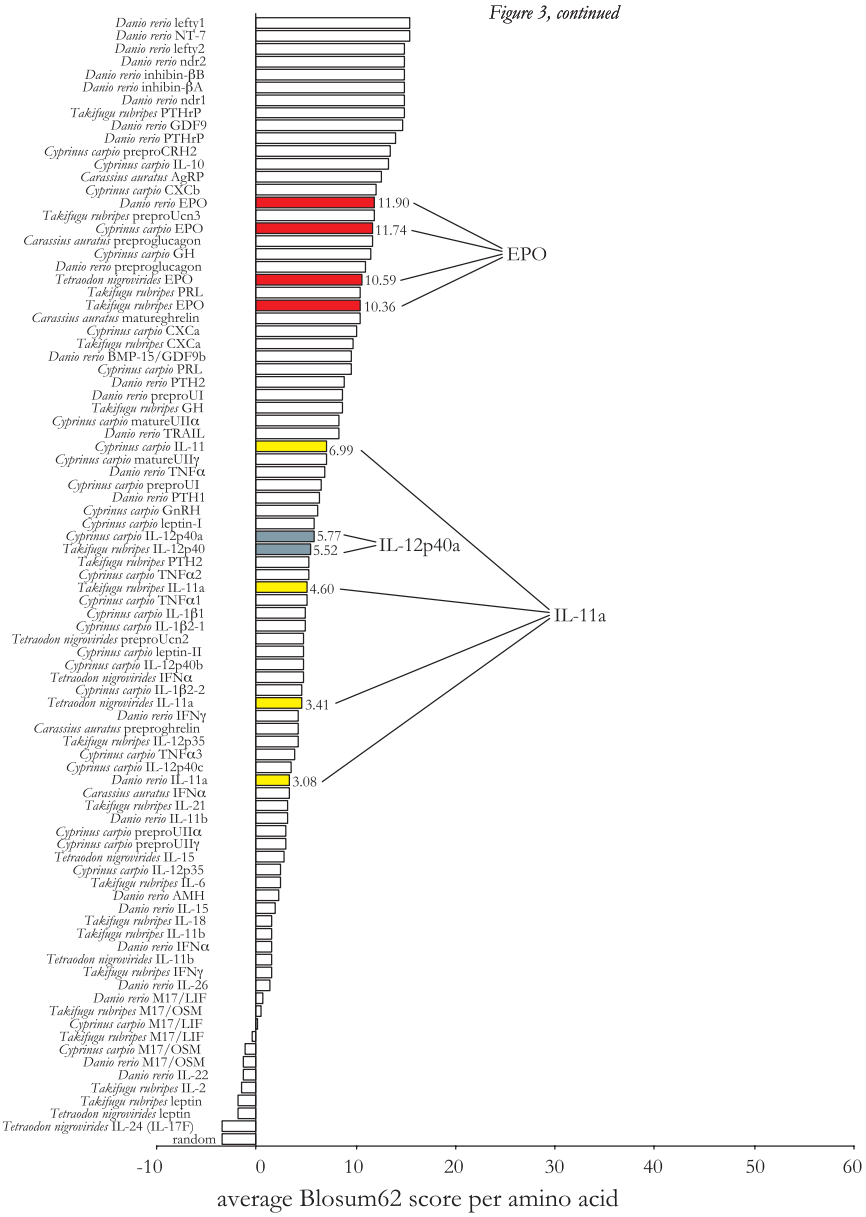


Figure 12.3: Under the assumption of equal rates of random mutations in all teleostean lineages, orthologous fish proteins are predicted to reach similar Blosum62 scores when compared to their human orthologue. Consequently, orthologues of different fish species rank in proximity.

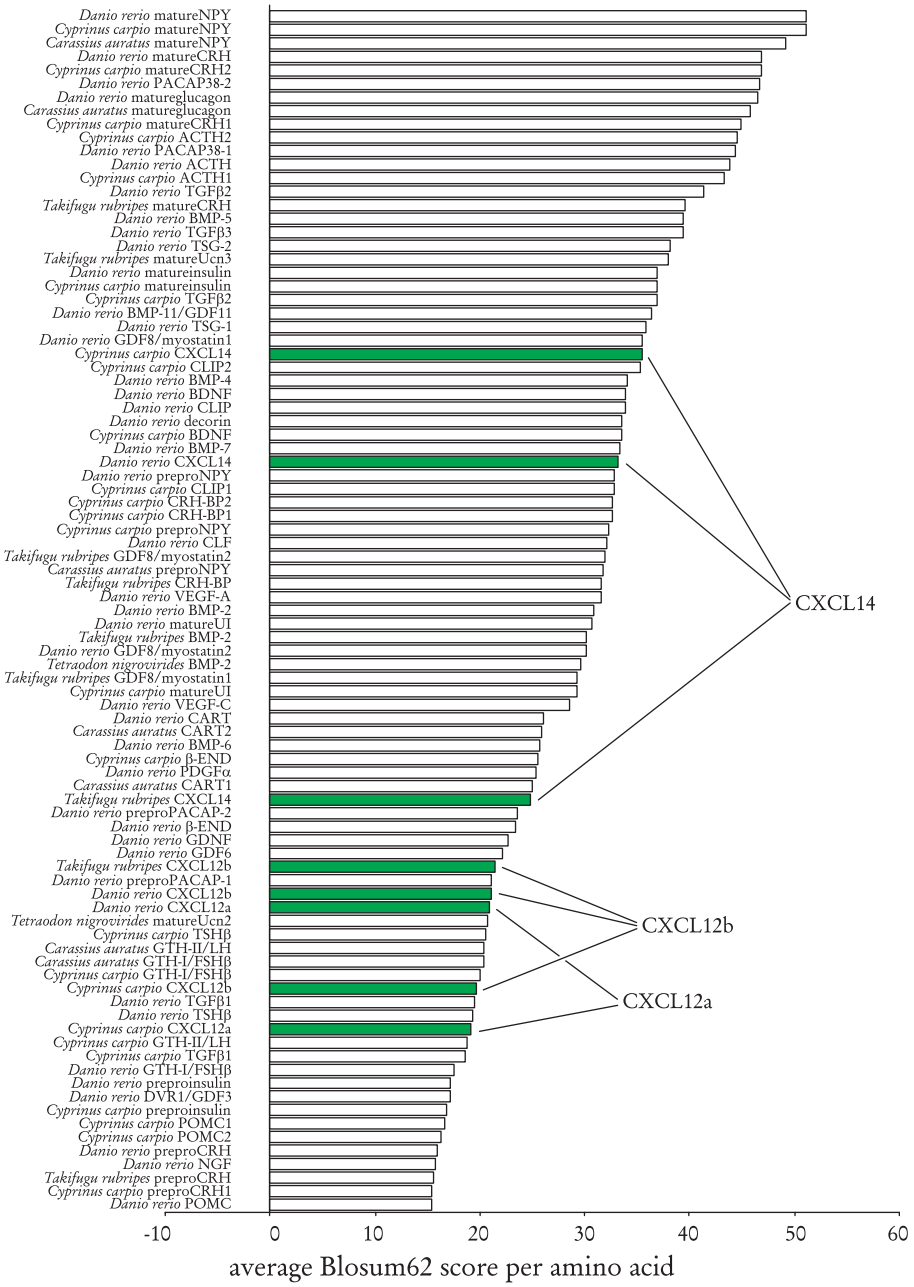
Evolutionary distance

The degree of divergence between two orthologous sequences is dependent on the time that has passed since the separation of both species. Therefore, a meaningful comparison of the evolutionary conservation between different signaling molecules can only be achieved when comparing pairs of orthologous sequences that have been separated for the same amount of time. Ideally this would mean a comparison between orthologous sequences of two species, e.g. common carp and human. But as the number of carp genes that encodes signaling peptides identified to date is relatively small, this would result in a fairly limited comparison. One alternative is to resort to another fish species, such as zebrafish, where many of the growth factors that play key roles in early development have been identified. Unfortunately, many cytokine genes have not yet been identified in zebrafish. In the spotted green pufferfish (*Tetraodon nigrovirides*) the opposite situation is true: relatively few signaling molecules have been identified in this species to date, but many of these are cytokine genes that still await discovery in other fish species. Therefore, to maximise the number of signaling sequences in our comparison, we included all signaling molecules that are identified in two of the most distantly related families of teleost fish: cyprinids (*Cyprinidae*) and puffer (*Tetraodontidae*) species. The cyprinid family is represented by common carp, zebrafish, and goldfish (*Carassius auratus*), whereas the puffer family is represented by two species: *Takifugu rubripes* and *Tetraodon nigrovirides*. Each of these five species is separated from human, mouse (or any other mammalian species) by the same amount of evolutionary time (*i.e.* approximately 450 million years[15, 527], which is the time that has passed since the divergence of actinoptergyian fish and tetrapods (Fig. 12.2). Therefore, orthologous sequences of distantly related fish species are expected to be equally (dis)similar to their human or mouse orthologues, under the assumption of equal evolutionary rates among the different lineages. Figure 12.3 shows that this assumption appears justified when comparing fish and human signaling molecules. Orthologous fish sequences generally achieve similar Blosum62 scores when compared with their human orthologues, although fish orthologues of some sequences (IL-12p40, BMP-2) achieve more similar scores than others (IL-11, leptin). Whether these minor differences in Blosum62 score are caused by mere stochastic events or lineage-specific selective forces is not known, but the extent of the variation in Blosum62 score between fish orthologues is sufficiently small to state that once the rank of any fish signaling molecule is known, their orthologues from other fish species will rank in their proximity. Thus the approximate position of a particular signaling molecule in this ranking is independent of the fish species chosen here. The same is true for the mammalian species involved, as a ranking that is constructed by comparing fish sequences with their murine instead of human orthologues is largely similar (not shown).

Cxc chemokines

Let us now have a closer look at the groups of sequences that feature this thesis and their rankings in the list of evolutionary conservation, starting with the CXC chemokines. CXC chemokines typically measure approximately 90-100 amino acids (including their signal peptide) and contain two highly conserved disulphide bridges. The N-terminal cysteines of each pair are separated by a single intervening amino acid (denoted by the X), which explains their name. The relative abundance of cysteines in CXC chemokines (four conserved cysteines in a total of approximately 100 residues) also illustrates an inherent flaw that is encountered when one weighs similarities, which is that the amino acid composition of a signaling molecule will influence its Blosum62 score. A cytokine or hormone with many conserved residues that are attributed high scores in similarity matrices (such as cysteine and tryptophan) will be positively biased compared to sequences that display a relative paucity in those conspicuous features. As structurally related sequences, such as CXC chemokines, generally share their most conspicuous amino acids, this will not influence the position of a CXC chemokine relative to other CXC chemokines, but it may impose a slight bias on the ranking of CXC chemokines compared to other, structurally unrelated classes of messenger molecules.

Many will associate chemokines with the acute inflammatory response that follows local injury or infection. Indeed, expression of the majority of mammalian CXC chemokines is inducible in response to stimulation with a variety of microbial and non-microbial agents such as lipopolysaccharide (LPS), polyI:C (combination of polyinosinic acid and polycytidylic acid), or phorbol-myristate acetate (PMA). The expression of CXCa and CXCb, fish chemokines that are likely orthologous to the mammalian clusters of CXCL1-8 and CXCL9-11, respectively, is also induced by in-vitro stimulation with PMA or concanavalin A (ConA). Despite these similarities in in-vitro induction, CXCa and CXCb are relatively poorly conserved compared to CXCL12a, CXCL12b, and CXCL14 (Fig. 12.4), as shown in CHAPTER 3. In contrast to the majority of mammalian CXC chemokines, CXCL12 and CXCL14 are constitutively expressed[57, 59, 531] and CXCL12 does not respond to in-vitro stimulation with microbial agents[96], a situation that is also true for carp CXCL12a. Involvement of CXCL12 in a plethora of functions has been established, many of which are non-immune. CXCL12 is involved in several key processes of early ontogeny, such as the development of the central nervous system (CNS), as well as cardiac development in mice[61, 62, 81, 90]. Moreover, CXCL12 and its cognate receptor CXCR4 are involved in the migration of primordial germ cells in both mouse and zebrafish[64, 65, 70, 91, 532]. As suggested in CHAPTERS 3 and 4, the relatively high degree of sequence conservation of CXCL12 (and CXCR4; CHAPTER 4) compared to other CXC chemokines probably relates to its pivotal role in key developmental processes throughout vertebrates.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Carassius auratus matureglucagon
Cyprinus carpio matureCRH1
Cyprinus carpio ACTH2
Danio rerio PACAP38-1
Danio rerio ACTH
Cyprinus carpio ACTH1
Danio rerio TGFβ2
Takifugu rubripes matureCRH
Danio rerio BMP-5
Danio rerio TGFβ3
Danio rerio TSG-2
Takifugu rubripes matureUcn3
Danio rerio matureinsulin
Cyprinus carpio matureinsulin
Cyprinus carpio TGFβ2
Danio rerio BMP-11/GDF11
Danio rerio TSG-1
Danio rerio GDF8/myostatin1
Cyprinus carpio CXCL14
Cyprinus carpio CLIP2
Danio rerio BMP-4
Danio rerio BDNF
Danio rerio CLIP
Danio rerio decorin
Cyprinus carpio BDNF
Danio rerio BMP-7
Danio rerio CXCL14
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Cyprinus carpio CLIP1
Cyprinus carpio CRH-BP2
Cyprinus carpio CRH-BP1
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Danio rerio BMP-2
Danio rerio matureUI
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Danio rerio GDF8/myostatin2
Tetraodon nigrovirides BMP-2
Takifugu rubripes GDF8/myostatin1
Cyprinus carpio matureUI
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Danio rerio CART
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Danio rerio BMP-6
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Danio rerio preproPACAP-2
Danio rerio β-END
Danio rerio GDNF
Danio rerio GDF6
Takifugu rubripes CXCL12b
Danio rerio preproPACAP-1
Danio rerio CXCL12b
Danio rerio CXCL12a
Tetraodon nigrovirides matureUcn2
Cyprinus carpio TSHβ
Carassius auratus GTH-II/LH
Carassius auratus GTH-I/FSHβ
Cyprinus carpio GTH-I/FSHβ
Cyprinus carpio CXCL12b
Danio rerio TGFβ1
Danio rerio TSHβ
Cyprinus carpio CXCL12a
Cyprinus carpio GTH-II/LH
Cyprinus carpio TGFβ1
Danio rerio GTH-I/FSHβ
Danio rerio preproinsulin
Danio rerio DVR1/GDF3
Cyprinus carpio preproinsulin
Cyprinus carpio POMC1
Cyprinus carpio POMC2
Danio rerio preproCRH
Danio rerio NGF
Takifugu rubripes preproCRH
Cyprinus carpio preproCRH1
Danio rerio POMC
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CXCL12b
CXCL12a
-10
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average Blosum62 score per amino acid

Figure 4, continued

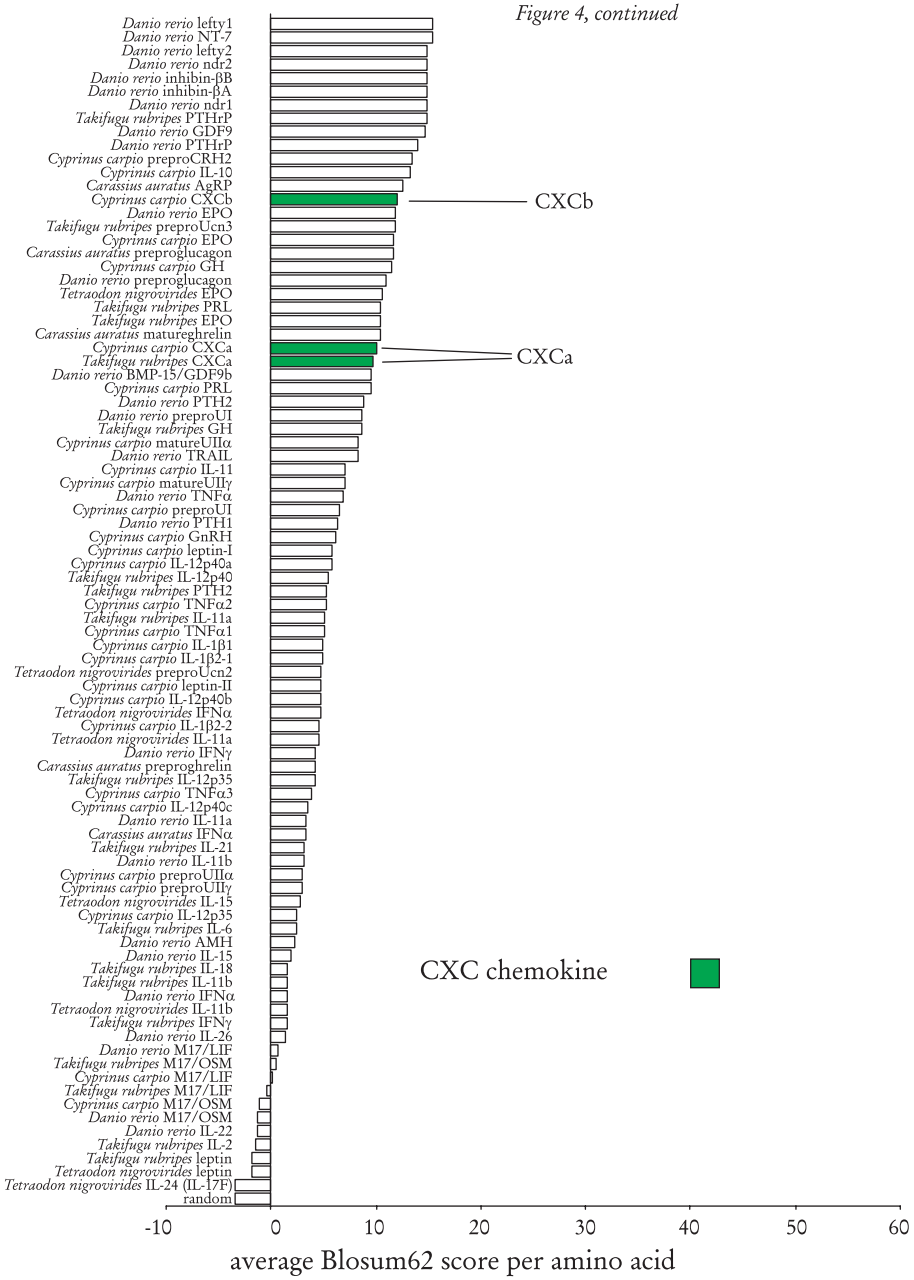


Figure 12.4: The ranking of fish CXC chemokines. CXCL14 displays by far the best evolutionary conservation of all CXC chemokines, followed by CXCL12a and CXCL12b. CXCa and CXCb rank much lower compared to other fish CXC chemokines. CXCL12 has been duplicated in the teleostean lineage. Therefore, each of the teleostean CXCL12 paralogues were aligned individually with human CXCL12. CXCa and CXCb were regarded as the fish orthologues to the mammalian clusters of CXCL1-8 and CXCL9-11, respectively. Their ranking is determined by the average score of the pairwise comparisons with each of the human members of these clusters.

CXCL14 represents something of an enigma. It was discovered as recently as 1999 as 'breast and kidney derived' (BRAK)[78] and has received only limited attention since, possibly since an appealing immunological role was not demonstrated. Yet, CXCL14 is by far the best-conserved vertebrate CXC chemokine (Fig. 12.4). The little information we have regarding its function suggests involvement in the development of the CNS of mice[59], zebrafish[71], and carp (CHAPTER 3). Nevertheless, much information, including information regarding the identity of its receptor, which would greatly assist the appreciation of the role of CXCL14, is still missing. A knock-out model for CXCL14 has also not been constructed, although this experiment could potentially confirm the important role of CXCL14 that is suggested by its extraordinary sequence conservation. After all, knockouts for CXCL12 (the second best conserved chemokine) and its cognate receptor CXCR4 are the only chemokine (receptor) knockouts to date that yield non-viable phenotypes[26].

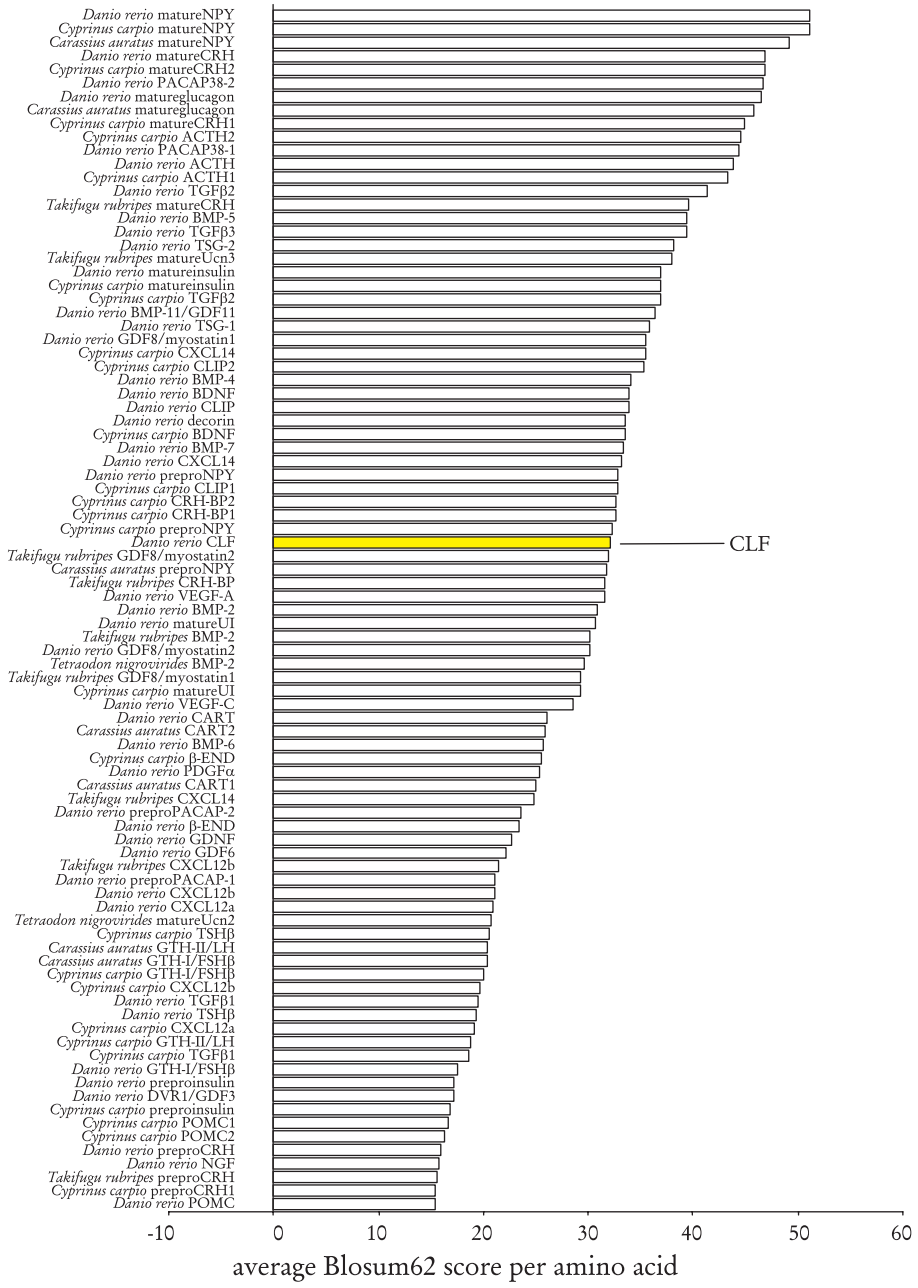
Type-I cytokines

Many of the messenger molecules that are classified as 'cytokines' according to the historical perspective that is presented in figure 12.1, belong to families of structurally related proteins. The largest of these families is constituted by the type-I cytokines. Type-I cytokines share a common fold of four α-helices in a characteristic up-up-down-down topology, as explained in CHAPTER 8. Furthermore, type-I cytokine receptors also form a monophyletic group[134] that relay the cytokine signal through to an intracellular JAK/STAT signaling mechanism[302]. All teleost members of the type-I cytokine family that have been identified to date, rank in the lower half of the panel of comparisons between orthologous fish and human messenger molecules (Fig. 12.5). This indicates that the amino acid sequences of most type-I cytokines are similar only to a limited degree between fish and mammals, although the mere presence in fish does indicate that the majority of type-I cytokines originated before the fish-tetrapod split. Nevertheless, conspicuous differences exist between different type-I cytokines regarding their degree of sequence conservation. Erythropoietin (EPO), growth hormone (GH), and prolactin (PRL) are the best-conserved type-I cytokines that have been discovered in fish to date. EPO is, in mammalian species,

released from the kidney in response to a drop in blood oxygen levels to enhance bone marrow erythropoiesis[373], and GH and PRL are both released from the pituitary gland to exert effects at distant sites. Therefore, the three best-conserved type-I cytokines act as typical hormones, whereas the majority of other type-I cytokines are players of the immune response that, in many instances signal in a paracrine or autocrine fashion. Also among these 'immune' type-I cytokines, differences exist with regard to their degree of evolutionary conservation. Duplicate IL-11 genes occur in several, evolutionarily distantly related fish species (CHAPTER 5), which suggests a gene duplication event that occurred early in the teleostean lineage. Of these paralogous fish IL-11 genes, IL-11a consistently displays a higher Blosum62 score than IL-11b, which is in line with the slightly higher amino acid identity of IL-11a to its mammalian orthologues reported in CHAPTER 5.

In mammals, leptin is secreted from adipocytes and circulates in proportion to body fat mass. Since its principle site of action is the *arcuate nucleus* of the hypothalamus (*i.e.* distant from its site of release), leptin is considered a hormone. Yet, in contrast to EPO, GH, and PRL, the other type-I cytokines that act in an endocrine fashion, the degree of conservation of the teleostean leptin proteins is much poorer. The recently published leptin sequences of *Takifugu rubripes* and *Tetraodon nigroviridis*[249] even score the lowest Blosum62 score of all protein and peptide messengers in our analysis, with the sole exception of IL-24 (Fig. 12.5). This difference is partially explained by differences in sequence length (the Blosum62 score is divided by the alignment length and not the sequence length), as both puffer leptins (152 and 160 amino acids, respectively) are considerably shorter than the leptins of carp (171 amino acids) and mammalian species (167 amino acids). Regardless of these differences between leptins of different fish species, a comparison of the degree of amino acid sequence conservation of leptin with that of other vertebrate messenger molecules reveals that leptin displays the poorest evolutionary conservation of all protein hormones. In fact, leptin is as poorly conserved as any typically 'immune' type-I cytokine. This is intuitively at odds with the weight that has been attributed to leptin in the regulation of food intake and energy metabolism. Moreover, the first experiments aimed at the resolving the physiological role of leptin in fish reveal a postprandial peak in the hours following food intake, but fail to establish a link between leptin expression and prolonged (six days) fasting or subsequent refeeding. As discussed in CHAPTER 7, the relatively poor evolutionary conservation of leptin complemented by the differences in its gene expression in response to fasting between fish and mammals, raise the question whether the role of leptin differs between homoiothermic vertebrates, which maintain their body temperature at a constant level, and poikilotherms that allow their body temperature to fluctuate with the temperature of their environment.

Type-I cytokines signal via receptor complexes that are composed of related receptor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Danio rerio matureglucagon
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Danio rerio TSG-2
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Danio rerio TSG-1
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Cyprinus carpio CXCL14
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Danio rerio CLIP
Danio rerio decorin
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Danio rerio BMP-7
Danio rerio CXCL14
Danio rerio preproNPY
Cyprinus carpio CLIP1
Cyprinus carpio CRH-BP2
Cyprinus carpio CRH-BP1
Cyprinus carpio preproNPY
Danio rerio CLF
CLF
Takifugu rubripes GDF8/myostatin2
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Cyprinus carpio CXCL12b
Danio rerio TGFβ1
Danio rerio TSHβ
Cyprinus carpio CXCL12a
Cyprinus carpio GTH-II/LH
Cyprinus carpio TGFβ1
Danio rerio GTH-I/FSHβ
Danio rerio preproinsulin
Danio rerio DVR1/GDF3
Cyprinus carpio preproinsulin
Cyprinus carpio POMC1
Cyprinus carpio POMC2
Danio rerio preproCRH
Danio rerio NGF
Takifugu rubripes preproCRH
Cyprinus carpio preproCRH1
Danio rerio POMC
-10
0
10
20
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60
average Blosum62 score per amino acid

Figure 5, continued

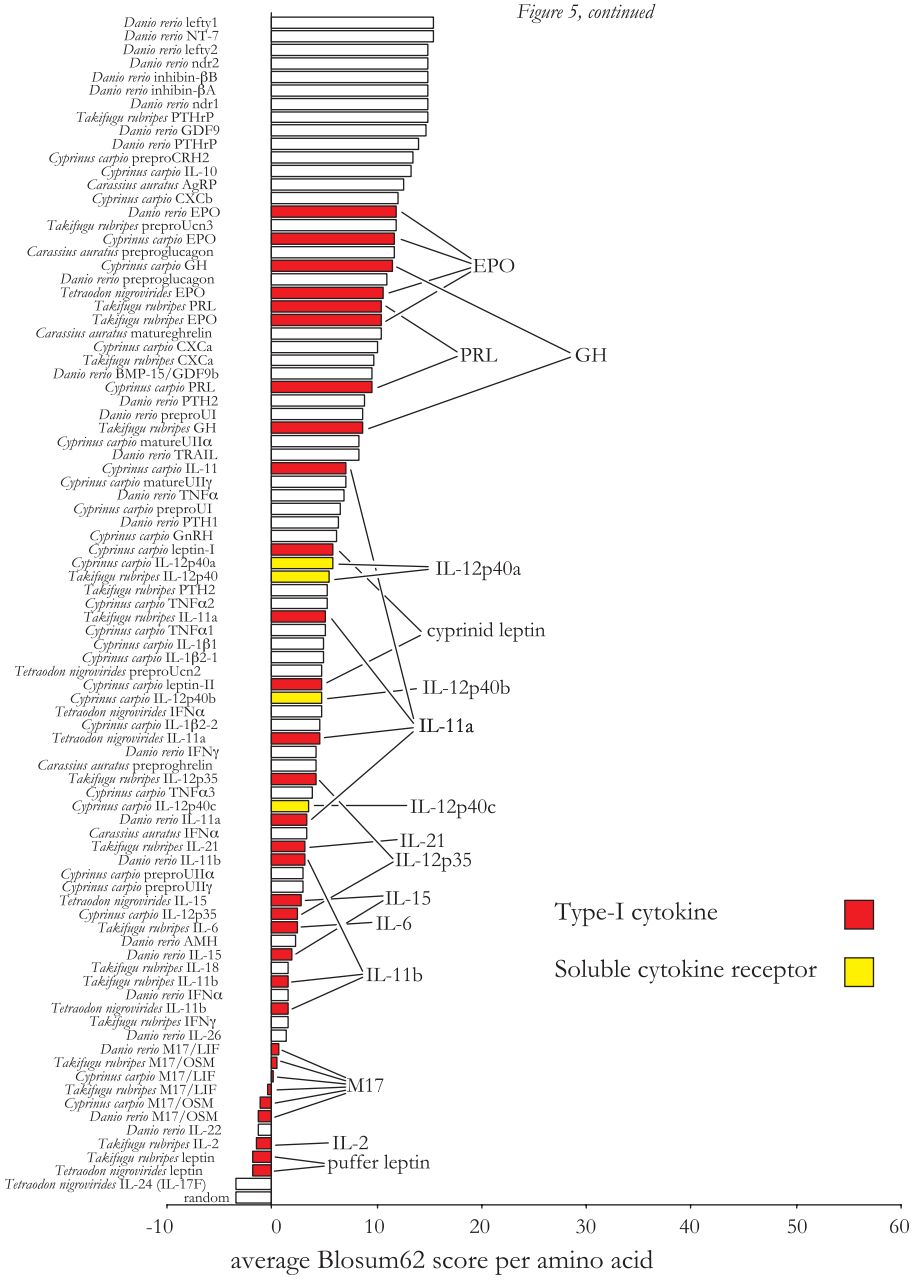


Figure 12.5: The ranking of teleostean type-I cytokines and their soluble receptors. Type-I cytokines in general display a poor overall amino acid conservation compared to other messenger molecules. Within the type-I cytokine family, EPO, GH, and PRL are the best-conserved family members. The three teleostean IL-12P40 molecules achieve similarly low rankings compared to teleostean IL-12P35 molecules. Interestingly, the zebrafish orthologue of CLF, a recently identified type-I cytokine receptor family member, displays a much higher degree of amino acid conservation that is unique among type-I cytokines and their receptors.

chains. The basic conformation of such a receptor complex is a heterodimer, which is composed of a ligand-specific α-chain without intracellular signaling capability and a β-chain that forms the signaling component in the receptor complexes of several different type-I cytokines[209,290]. As reviewed in CHAPTER 8, several variations on this common theme exist. One of these variations is the existence of soluble type-I cytokine receptors that resemble a ligand-specific α-chain, but lack a transmembrane and intracellular domain. IL-12 is the best-known example of a soluble cytokine receptor. It is a heterodimer that consists of a type-I cytokine (the P35 subunit) and a soluble cytokine receptor (the P40 subunit). In the case of IL-12, both subunits are covalently associated via an interchain disulphide bridge, although this is not necessarily true for all other heterodimeric cytokine complexes. In contrast to what has been described to date for mammalian species[224], chicken[202], and pufferfish[162], carp has three different IL-12P40 genes that encode three highly dissimilar IL-12P40 subunits (CHAPTER 6). The extent of their sequence dissimilarity suggests that the three carp P40 genes arose by gene duplication events that occurred early in the teleostean lineage at the latest, but we do not rule out the possibility that the presence of multiple IL-12P40 genes antedates the teleostean lineage (CHAPTER 6). The three carp P40 genes were designated P40a, P40b, and P40c, in reflection of their decreasing sequence identity with human IL-12P40, and a comparison on the basis of amino acid similarity results in a similar ranking (Fig. 12.5).

One other soluble type-I cytokine receptor of teleostean fish has been deposited in the nucleotide databases. It is the zebrafish orthologue of cytokine-like factor (CLF; accession number BE016629). The most interesting aspect about this particular soluble receptor chain is its unusual degree of evolutionary conservation: it ranks within the top twentyfive percent of best-conserved teleostean protein and peptide messengers that has been identified to date, in sharp contrast to all other type-I cytokines and soluble receptors (Fig. 12.5). The obligate binding partner of the soluble cytokine receptor CLF is the type-I cytokine cardiotrophin-like cytokine (CLC). The CLC/CLF complex is an important stimulator of motor neuron growth, which is illustrated by the observation that mice pups that lack a functional CLF gene die of a failure to suckle shortly after birth because they cannot move their lower jaw muscles[360]. Although a teleostean CLC molecule has not been discovered to date, the excellent evolutionary conservation of its cognate

soluble receptor CLF justifies the prediction that a CLC molecule of teleosts will display a degree of amino acid sequence similarity with its mammalian orthologue that is much larger than that observed for other type-I cytokines. Indeed, partial CLC-like sequences that display high sequence identity (up to 54% amino acid identity within the aligned region) to the third exon of the mammalian CLC gene, which encodes the largest part of the CLC protein, are readily retrieved from the genome databases of several fish species (not shown).

CRH-family peptides

Corticotropin-releasing hormone (CRH) was discovered in 1981 as the principle activator of the mammalian stress response[416]. Urotensin-I (UI) is a related protein that was purified not much later from the caudal neurosecretory system (CNSS), a neuroendocrine organ specific to fish, of white sucker (*Catastomus commersonii*)[417]. As mentioned earlier, urocortin-1 (UCN1), the mammalian orthologue of UI, was identified years later by screening a rat midbrain cDNA library with a (fish) UI probe[419]. With the subsequent discovery of urocortin-2 (UCN2) and urocortin-3 (UCN3) in human and mouse (also known as stresscopin-related peptide and stresscopin, respectively), the family of CRH-like peptides now totals four mammalian family members[421, 422, 425]. The number of four CRH-family members has led to the hypothesis that they may have originated from a single ancestral gene that was distributed over four paralogous loci in the proto-vertebrate lineage by two consecutive rounds of genome duplication[508, 509]. According to this hypothesis, orthologues to each of the four mammalian family members should be present in fish. The presence of CRH and UI in fish was already established in the 1980s[414, 417] and the hypothalamic expression of carp CRH and UI following restraint stress is discussed in CHAPTER 9. The publications that reported the presence of UCN2 and UCN3 in mammalian species also reported the presence of UCN2 and UCN3 orthologues in *Takifugu rubripes* and *Tetraodon nigroviridis*[421, 425, 533]. In addition to *Xenopus* orthologues of UCN1 and UCN3, the group of DENVER recently retrieved from the *Tetraodon* genome database another *Tetraodon* UCN3 sequence that is more similar to mammalian UCN3[534]. The UCN3 that was originally reported along with the mammalian UCN3 sequences[425] is now considered to be the *Tetraodon* orthologue of UCN2[534].

With fish orthologues of all four mammalian CRH-family members identified, we can assess and compare their evolutionary conservation. As already mentioned, CRH-like peptides are encoded as a prepro-hormone. Consequently, conserved amino acids will not be distributed evenly across the coding region, but will instead be concentrated in

the mature peptides. To prevent bias either way, we assessed the degree of similarity of the mature as well as the prepro-hormone sequences of each fish CRH-family member that is included in our analysis. For the reasons explained above, the mature CRH-family peptides rank higher than their corresponding prepro-peptides (Fig. 12.6). Regardless of whether one considers the mature peptides or the prepro-hormones, the differences in evolutionary conservation between the four CRH-family members is substantial. CRH is the best-conserved family member throughout vertebrates, followed by UCN3. UI (compared to UCN1) ranks lower, whereas UCN2 is the least conserved of all four paralogous members of the CRH-family.

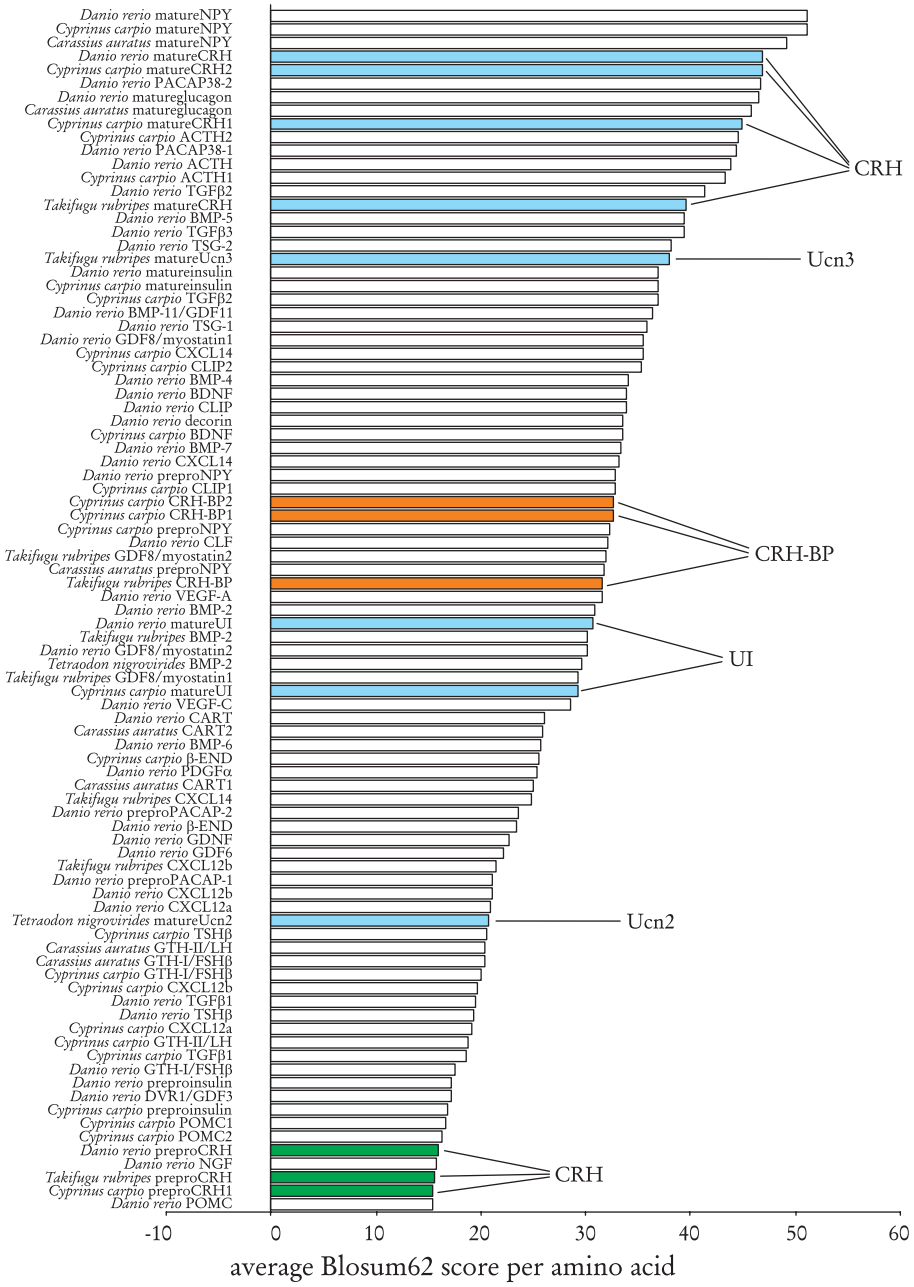
Following secretion in the circulation or extracellular fluid, the bioavailability of CRH and related peptides can be modulated via a 320 amino acid CRH-binding protein (CRH-BP). The presence of CRH-BP in teleostean fish is established in CHAPTER 9. Its involvement in the central regulation of the stress response and the local expression of CRH-BP within the chromaffin cells of the head kidney are discussed in CHAPTERS 9 and 10, respectively. CRH-BP is unrelated to any other protein and is conserved throughout vertebrate evolution (Fig. 12.6). Taking advantage of its unique and conspicuous features, which include five characteristically spaced disulphide bridges, the presence of CRH-BP could also be demonstrated in several insect species, including the honeybee (*Apis mellifera*) (CHAPTER 11). This observation offers strong support to the notion that CRH-like signals have been present since the ancestor of chordates and arthropods.

Selection

By determining the degree of evolutionary conservation of the various protein and peptide messengers that feature in this thesis, we have demonstrated the profound differences in evolutionary conservation that exist between as well as within families of structurally related messenger molecules. Moreover, the comparison of amino acid similarity of a large number of proteins that are compared over the same evolutionary distance offers a more objective perspective to compare the evolutionary conservation of a particular protein with others. Now that we have answered the question 'What is evolutionary conserved?', let us try to also address the questions of 'how' and 'why'.

When seeking answers to the question how evolutionary conservation is achieved, we have to consider the different types of selective forces that can act on a particular sequence. The first of these forces, purifying selection, is aimed at maintaining a particular amino acid sequence constant. Under purifying selection, amino acid substitutions that

occur via random nucleotide mutations will likely not be fixed in the population. Positive Darwinian selection, or simply positive selection, is the exact opposite of purifying selection. Random nucleotide acid substitutions that lead to changes in amino acids will, under positive selection, preferentially be maintained in the population instead of discarded. The third selective force is called neutral selection and refers to the situation that occurs when neither purifying nor positive selection acts on a particular sequence. To determine which selective force has acted on a particular sequence we have to consider the coding regions of our genes of interest. Random mutations will occur throughout all nucleotide sequences (we assume that this has occurred at a constant rate throughout vertebrate evolution). Since all amino acids, except methionine and tryptophan are encoded by multiple codons, some of these random nucleotide mutations are silent, *i.e.* they will not result in an amino acid substitution. Such nucleotide mutations are commonly referred to as synonymous substitutions. Nucleotide substitutions that do result in an amino acid substitution are referred to as nonsynonymous. The selective forces acting on a particular sequence do not influence the rate at which nonsynonymous substitutions occur, but they do determine the likelihood at which these substitutions are maintained or discarded. Therefore, a comparison of the number of nonsynonymous and synonymous substitutions that has occurred in a particular sequence (in comparison with a second, orthologous sequence) will reveal information with regard to the selective forces that have acted on this sequence. In most nucleotide sequences there are more positions that potentially produce nonsynonymous substitutions than sites that produce synonymous substitutions[131]. Therefore, the number of nonsynonymous and synonymous substitutions is corrected for the total number of nonsynonymous and synonymous sites. Thus, the proportion of nonsynonymous (p_N) and synonymous (p_S) substitutions is estimated by dividing the number of nonsynomous (N_d) and synonymous (S_d) differences by the total number of nonsynonymous (N) and synonymous (S) sites, respectively. When p_N and p_S are large (*i.e.* when the divergence time between two sequences is large) they underestimate the actual proportion of nonsynonymous (d_N) and synonymous (d_S) substitutions as they do not account for backward or double mutations[131]. Therefore, it is customary to correct p_N and p_S for this phenomenon to obtain a better estimate of the actual proportion of nonsynonymous (d_N) and synonymous (d_S) substitutions. A comparison of the d_N and d_S ratio reveals the type of selective force that has acted on a particular sequence. A d_N that is smaller than d_S indicates purifying selection, whereas positive selection is assumed when d_N is larger than d_S[131]. The comparison of d_N and d_S is usually expressed either as their ratio (d_N/d_S) or as the difference between both proportions (d_N-d_S). Under neutral selection, d_N and d_S are the same and therefore, d_N/d_S will equal 1 and d_N-d_S will equal 0. By definition, purifying selection is assumed when $d_N/d_S < 1$ and d_N-$d_S < 0$. Under positive selection, $d_N/d_S > 1$ and d_N-$d_S > 0$.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Cyprinus carpio CLIP1
Cyprinus carpio CRH-BP2
Cyprinus carpio CRH-BP1
Cyprinus carpio preproNPY
Danio rerio CLF
Takifugu rubripes GDF8/myostatin2
Carassius auratus preproNPY
Takifugu rubripes CRH-BP
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Danio rerio VEGF-C
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Danio rerio GDF6
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Danio rerio preproPACAP-1
Danio rerio CXCL12b
Danio rerio CXCL12a
Tetraodon nigrovirides matureUcn2
Cyprinus carpio TSHβ
Carassius auratus GTH-II/LH
Carassius auratus GTH-I/FSHβ
Cyprinus carpio GTH-I/FSHβ
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Danio rerio TGFβ1
Danio rerio TSHβ
Cyprinus carpio CXCL12a
Cyprinus carpio GTH-II/LH
Cyprinus carpio TGFβ1
Danio rerio GTH-I/FSHβ
Danio rerio preproinsulin
Danio rerio DVR1/GDF3
Cyprinus carpio preproinsulin
Cyprinus carpio POMC1
Cyprinus carpio POMC2
Danio rerio preproCRH
Danio rerio NGF
Takifugu rubripes preproCRH
Cyprinus carpio preproCRH1
Danio rerio POMC
CRH
Ucn3
CRH-BP
UI
Ucn2
CRH
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average Blosum62 score per amino acid

Figure 6, continued

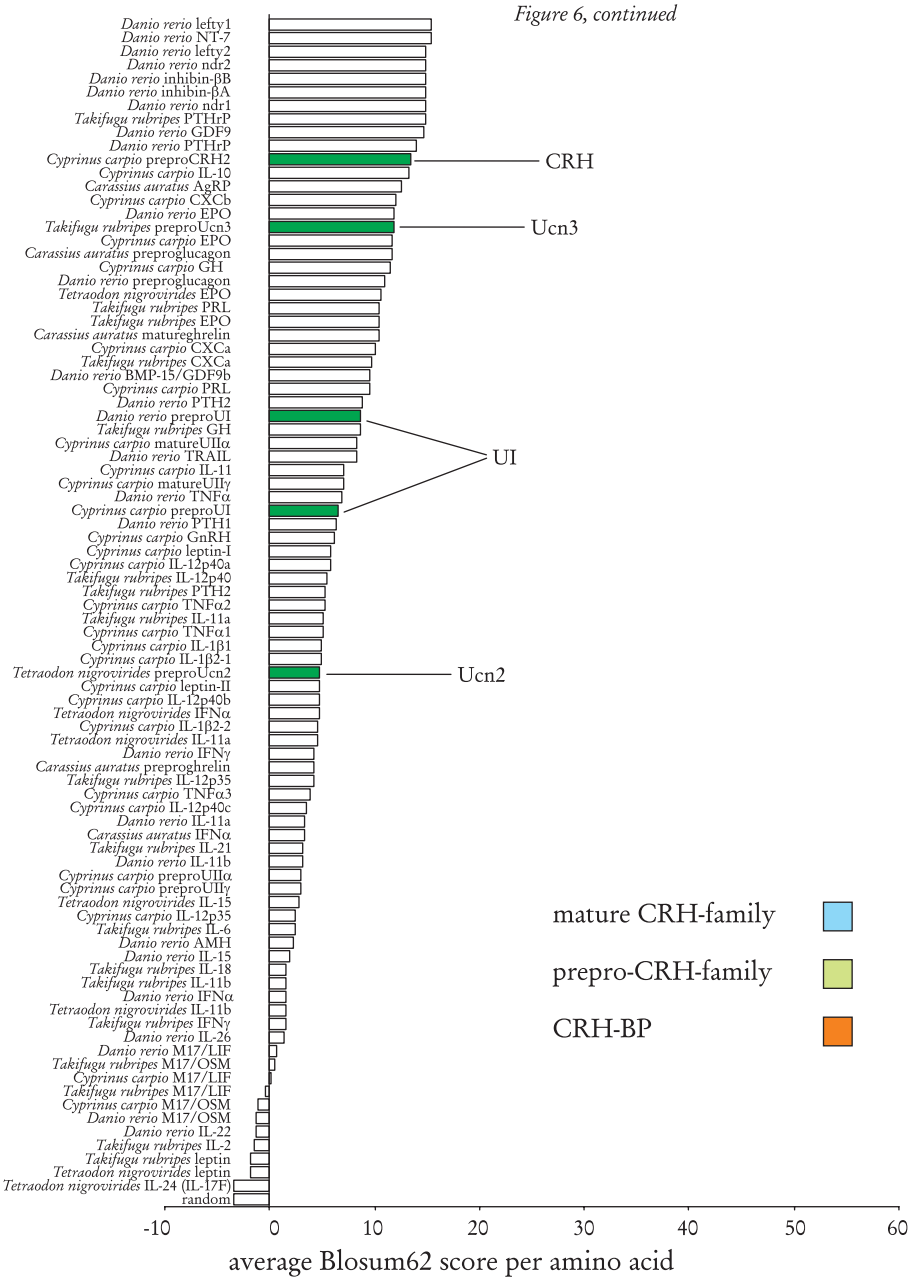


Figure 12.6: The ranking of the CRH-family members and of CRH-BP. Since the CRH-family members are encoded by a prepro-protein, we assessed the evolutionary conservation of complete proteins as well as the mature peptides. CRH is best conserved among the complete as well as the mature proteins, followed by UCN3, UI, and UCN2.

To assess the selective forces that have acted on our panel of messenger molecules throughout vertebrate evolution, in other words to determine how rather profound differences in amino acid conservation are achieved, we calculated the d_N and d_S values separately for each teleostean sequence in an alignment with its human orthologue. The amino acid sequences with the highest Blosum62 score per amino acid residue have the lowest p_N/p_S and d_N/d_S ratios (Fig. 12.7), which is hardly surprising as it indicates that these sequences have been subject to the strongest purifying selection. The same conclusion is indicated by their negative d_N-d_S values. The d_N/d_S ratios of the teleostean messenger molecules that are poorly conserved are much more interesting, as they reveal whether their poorer evolutionary conservation is the result of weaker purifying selection, or perhaps neutral or even positive selection? Inspection of the d_N/d_S ratios of the messenger sequences with low Blosum62 scores reveals that they are smaller than one (suggestive of purifying selection), with the exception of leptin and M17 of *Takifugu rubripes*. Despite the fact that the d_N/d_S ratios consistently point at purifying selection, the hypothesis of neutral selection cannot be discarded for many of the poorly conserved signaling molecules individually (Fig. 12.7). The trend that is present throughout our dataset, however, is clearly that of purifying selection. This is best illustrated by plotting the average Blosum62 score per amino acid against its corresponding d_N/d_S ratio. The trendline that describes the correlation between both characteristics does not intercept with the line $y = 1$ (neutral selection) within the range of the dataset (Fig. 12.8).

Why do endocrine and immune signals differ in their evolutionary conservation?

Whereas the mechanism of selection can be determined by calculus and statistics, reasons for the observed differences in strength of purifying selection are not as straightforward to ascertain. The easiest conclusion to draw from the differences in the strength of purifying selection that has acted on the messengers of the endocrine and the immune system throughout vertebrate evolution, is that the latter group apparently has been 'less important'. Stated differently: the vertebrate endocrine system has been

more crucial for the survival and reproductive success of vertebrates than their immune system. This statement offers the simplest explanation for the observed differences in the strength of purifying selection and simple explanations tend to be right ones according to Ockham's razor principle. Perhaps to the relief of immunologists, several alternative explanations exist.

Arguably the largest qualitative difference between the endocrine and the immune system is the difference in the degree to which external influences dictate their responses. The overall function of the endocrine system may be summarised as the regulation of a plethora of different physiological processes, within the constant 'milieu intérieur'[1], as explained in chapter 1. This constancy is key to understand the reasons for a stronger purifying selection that has acted on the messengers of the endocrine system and refers to two aspects: the function carried out by a particular endocrine protein or peptide has generally remained constant and most aspects of the internal environment in which endocrine processes take place have been maintained at similar values throughout vertebrate evolution. One of the exceptions to the latter rule is formed by regulation of body temperature, which differs fundamentally between homoiothermic and poikilothermic vertebrates. As suggested in chapter 7, this fundamental difference may pertain to the poor overall evolutionary conservation of leptin, which is unusually low for a protein hormone. Nevertheless, the constancy in the environment of and the demands to most endocrine processes has allowed their messengers to adapt (near) optimally to their function. The motto for endocrine messengers seems to have been 'not to change a winning team'. This is comparable to the example of the body shapes of many species of sharks and fishes, which have remained relative unchanged over hundreds of million years of evolution. Their body has adopted the most optimal shape for swimming in an aqueous environment that has remained constant indeed over a long period.

The main function of our immune system is to prevent us from being infested, either by tumor cells (self) or pathogens (non-self). The variation among these threats, especially in pathogens, is large: we find ourselves amidst the constant threat of invasion by a range of different pathogens including rna and dna viruses, gram$^-$ and gram$^+$ bacteria, trypanosomes, schistosomes, cestodes, trematodes, roundworms, and more. Moreover, the array of pathogens is also largely dependent on factors such as environment (*e.g.* aqueous or terrestrial) and lifestyle (*e.g.* carnivorous vs. herbivorous). The limited ability of an individual to anticipate infection is an extra, complicating factor in host defense that usually deprives the host of initiative. Thus, the inseparable involvement of an external component (pathogenic pressure) in immunity represents a fundamental difference between the immune and the endocrine system. Therefore, the motto 'not to change a winning team' is not applicable when the rules of the game are subject to constant change.

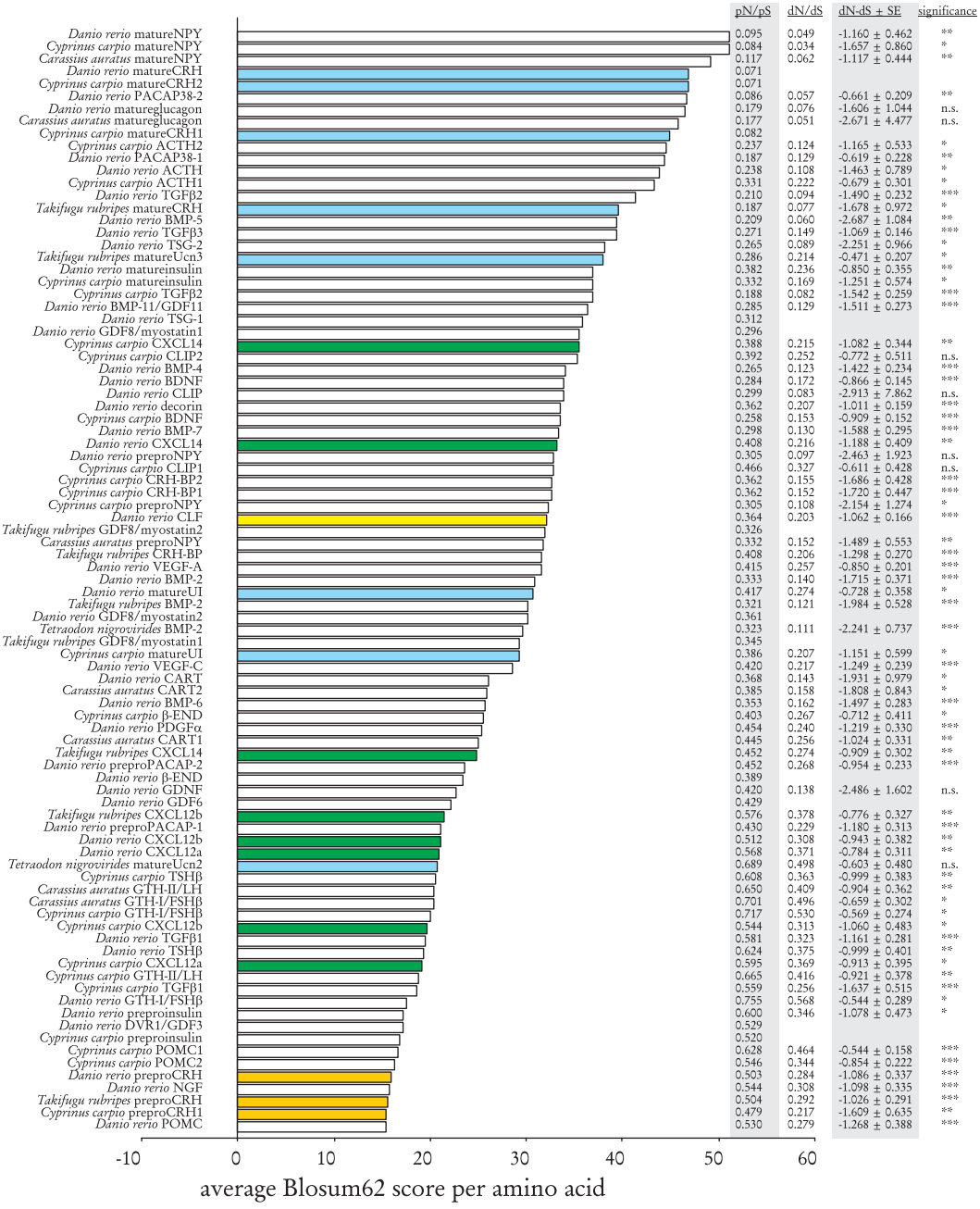
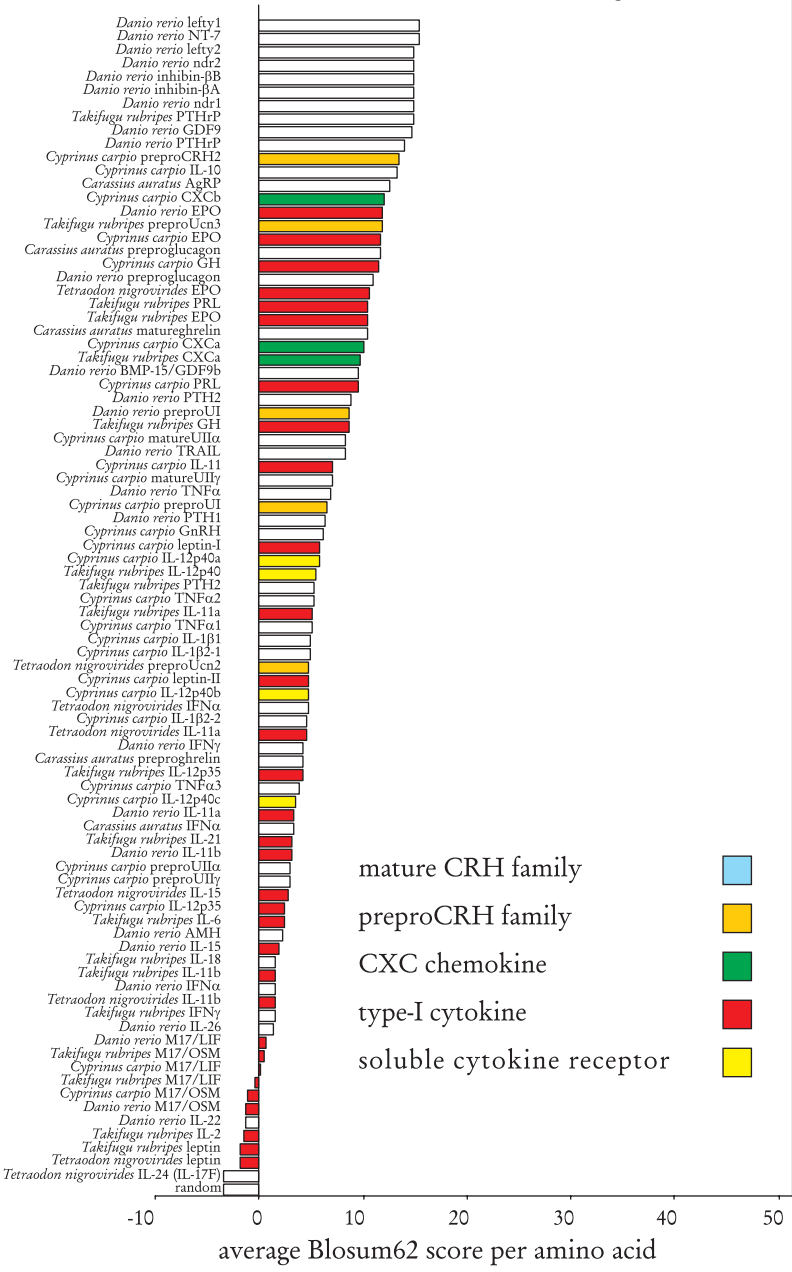

pN/pS
dN/dS
dN-dS ± SE
significance
Danio rerio matureNPY 0.095 0.049 -1.160 ± 0.462 **
Cyprinus carpio matureNPY 0.084 0.034 -1.657 ± 0.860 *
Carassius auratus matureNPY 0.117 0.062 -1.117 ± 0.444 **
Danio rerio matureCRH 0.071
Cyprinus carpio matureCRH2 0.071
Danio rerio PACAP38-2 0.086 0.057 -0.661 ± 0.209 **
Danio rerio matureglucagon 0.179 0.076 -1.606 ± 1.044 n.s.
Carassius auratus matureglucagon 0.177 0.051 -2.671 ± 4.477 n.s.
Cyprinus carpio matureCRH1 0.082
Cyprinus carpio ACTH2 0.237 0.124 -1.165 ± 0.533 *
Danio rerio PACAP38-1 0.187 0.129 -0.619 ± 0.228 **
Danio rerio ACTH 0.238 0.108 -1.463 ± 0.789 *
Cyprinus carpio ACTH1 0.331 0.222 -0.679 ± 0.301 *
Danio rerio TGFβ2 0.210 0.094 -1.490 ± 0.232 ***
Takifugu rubripes matureCRH 0.187 0.077 -1.678 ± 0.972 *
Danio rerio BMP-5 0.209 0.060 -2.687 ± 1.084 **
Danio rerio TGFβ3 0.271 0.149 -1.069 ± 0.146 ***
Danio rerio TSG-2 0.265 0.089 -2.251 ± 0.966 *
Takifugu rubripes matureUcn3 0.286 0.214 -0.471 ± 0.207 *
Danio rerio matureinsulin 0.382 0.236 -0.850 ± 0.355 **
Cyprinus carpio matureinsulin 0.332 0.169 -1.251 ± 0.574 *
Cyprinus carpio TGFβ2 0.188 0.082 -1.542 ± 0.259 ***
Danio rerio BMP-11/GDF11 0.285 0.129 -1.511 ± 0.273 ***
Danio rerio TSG-1 0.312
Danio rerio GDF8/myostatin1 0.296
Cyprinus carpio CXCL14 0.388 0.215 -1.082 ± 0.344 **
Cyprinus carpio CLIP2 0.392 0.252 -0.772 ± 0.511 n.s.
Danio rerio BMP-4 0.265 0.123 -1.422 ± 0.234 ***
Danio rerio BDNF 0.284 0.172 -0.866 ± 0.145 ***
Danio rerio CLIP 0.299 0.083 -2.913 ± 7.862 n.s.
Danio rerio decorin 0.362 0.207 -1.011 ± 0.159 ***
Cyprinus carpio BDNF 0.258 0.153 -0.909 ± 0.152 ***
Danio rerio BMP-7 0.298 0.130 -1.588 ± 0.295 ***
Danio rerio CXCL14 0.408 0.216 -1.188 ± 0.409 **
Danio rerio preproNPY 0.305 0.097 -2.463 ± 1.923 n.s.
Cyprinus carpio CLIP1 0.466 0.327 -0.611 ± 0.428 n.s.
Cyprinus carpio CRH-BP2 0.362 0.155 -1.686 ± 0.428 ***
Cyprinus carpio CRH-BP1 0.362 0.152 -1.720 ± 0.447 ***
Cyprinus carpio preproNPY 0.305 0.108 -2.154 ± 1.274 *
Danio rerio CLF 0.364 0.203 -1.062 ± 0.166 ***
Takifugu rubripes GDF8/myostatin2 0.326
Carassius auratus preproNPY 0.332 0.152 -1.489 ± 0.553 **
Takifugu rubripes CRH-BP 0.408 0.206 -1.298 ± 0.270 ***
Danio rerio VEGF-A 0.415 0.257 -0.850 ± 0.201 ***
Danio rerio BMP-2 0.333 0.140 -1.715 ± 0.371 ***
Danio rerio matureUI 0.417 0.274 -0.728 ± 0.358 *
Takifugu rubripes BMP-2 0.321 0.121 -1.984 ± 0.528 ***
Danio rerio GDF8/myostatin2 0.361
Tetraodon nigroviridis BMP-2 0.323 0.111 -2.241 ± 0.737 ***
Takifugu rubripes GDF8/myostatin1 0.345
Cyprinus carpio matureUI 0.386 0.207 -1.151 ± 0.599 *
Danio rerio VEGF-C 0.420 0.217 -1.249 ± 0.239 ***
Danio rerio CART 0.368 0.143 -1.931 ± 0.979 *
Carassius auratus CART2 0.385 0.158 -1.808 ± 0.843 *
Danio rerio BMP-6 0.353 0.162 -1.497 ± 0.283 ***
Cyprinus carpio β-END 0.403 0.267 -0.712 ± 0.411 *
Danio rerio PDGFα 0.454 0.240 -1.219 ± 0.330 ***
Carassius auratus CART1 0.445 0.256 -1.024 ± 0.331 **
Takifugu rubripes CXCL14 0.452 0.274 -0.909 ± 0.302 **
Danio rerio preproPACAP-2 0.452 0.268 -0.954 ± 0.233 ***
Danio rerio β-END 0.389
Danio rerio GDNF 0.420 0.138 -2.486 ± 1.602 n.s.
Danio rerio GDF6 0.429
Takifugu rubripes CXCL12b 0.576 0.378 -0.776 ± 0.327 **
Danio rerio preproPACAP-1 0.430 0.229 -1.180 ± 0.313 ***
Danio rerio CXCL12b 0.512 0.308 -0.943 ± 0.382 **
Danio rerio CXCL12a 0.568 0.371 -0.784 ± 0.311 **
Tetraodon nigrovirides matureUcn2 0.689 0.498 -0.603 ± 0.480 n.s.
Cyprinus carpio TSHβ 0.608 0.363 -0.999 ± 0.383 **
Carassius auratus GTH-II/LHβ 0.650 0.409 -0.904 ± 0.362 **
Carassius auratus GTH-I/FSHβ 0.701 0.496 -0.659 ± 0.302 *
Cyprinus carpio GTH-I/FSHβ 0.717 0.530 -0.569 ± 0.274 *
Cyprinus carpio CXCL12b 0.544 0.313 -1.060 ± 0.483 *
Danio rerio TGFβ1 0.581 0.323 -1.161 ± 0.281 ***
Danio rerio TSHβ 0.624 0.375 -0.999 ± 0.401 **
Cyprinus carpio CXCL12a 0.595 0.369 -0.913 ± 0.395 *
Cyprinus carpio GTH-II/LHβ 0.665 0.416 -0.921 ± 0.378 **
Cyprinus carpio TGFβ1 0.559 0.256 -1.637 ± 0.515 ***
Danio rerio GTH-I/FSHβ 0.755 0.568 -0.544 ± 0.289 *
Danio rerio preproinsulin 0.600 0.346 -1.078 ± 0.473 *
Danio rerio DVR1/GDF3 0.529
Cyprinus carpio preproinsulin 0.520
Cyprinus carpio POMC1 0.628 0.464 -0.544 ± 0.158 ***
Cyprinus carpio POMC2 0.546 0.344 -0.854 ± 0.222 ***
Danio rerio preproCRH 0.503 0.284 -1.086 ± 0.337 ***
Danio rerio NGF 0.544 0.308 -1.098 ± 0.335 ***
Takifugu rubripes preproCRH 0.504 0.292 -1.026 ± 0.291 ***
Cyprinus carpio preproCRH1 0.479 0.217 -1.609 ± 0.635 **
Danio rerio POMC 0.530 0.279 -1.268 ± 0.388 ***
-10
0
10
20
30
40
50
60
average Blosum62 score per amino acid

Figure 7, continued



pN/pS	dN/dS	dN-dS ± SE	significance
0.654	0.404	-0.951 ± 0.252	***
0.580	0.341	-1.024 ± 0.302	***
0.683	0.461	-0.754 ± 0.199	***
0.710	0.457	-0.887 ± 0.268	***
0.315	0.159	-1.262 ± 0.207	***
0.427	0.227	-1.186 ± 0.216	***
0.730	0.489	-0.800 ± 0.246	***
0.502	0.241	-1.470 ± 0.588	**
0.564			
0.453			
0.520	0.274	-1.249 ± 0.417	**
0.700	0.442	-0.926 ± 0.389	**
0.676	0.480	-0.640 ± 0.275	*
0.631	0.302	-1.593	
0.780	0.570	-0.622 ± 0.286	*
0.867	0.789	-0.167 ± 0.148	n.s.
0.791	0.609	-0.505 ± 0.327	*
0.540	0.320	-0.986 ± 0.363	**
0.733	0.514	-0.693 ± 0.268	**
0.522	0.231	-1.706 ± 0.926	*
0.816	0.671	-0.369 ± 0.216	*
0.688	0.347	-1.505 ± 0.786	*
0.798	0.629	-0.456 ± 0.289	*
0.650	0.385	-1.052 ± 1.154	n.s.
0.722	0.406	-1.244	
0.780	0.557	-0.677	
0.707	0.319	-1.865 ± 1.020	*
0.661	0.254	-2.310 ± 2.187	n.s.
0.841	0.686	-0.396 ± 0.336	n.s.
0.662	0.428	-0.849 ± 0.374	*
0.678	0.294	-1.950 ± 1.469	n.s.
0.680	0.431	-0.903 ± 0.777	n.s.
0.684			
0.816	0.615	-0.569 ± 0.283	*
0.674	0.409	-1.006 ± 0.879	n.s.
0.712	0.405	-1.200 ± 0.530	*
0.623	0.309	-1.484 ± 0.826	*
0.719	0.437	-1.031 ± 0.679	n.s.
0.758	0.512	-0.794 ± 0.533	n.s.
0.786	0.522	-0.861 ± 0.475	*
0.747	0.331	-2.000 ± 1.664	n.s.
0.734	0.347	-1.767 ± 1.168	n.s.
0.650			
0.853	0.697	-0.400 ± 0.235	*
0.849	0.675	-0.461 ± 0.270	*
0.799	0.592	-0.598 ± 0.274	*
0.729			
0.736	0.354	-1.718 ± 1.162	n.s.
0.799	0.549	-0.786 ± 0.550	n.s.
0.777	0.467	-1.120 ± 0.692	*
0.778	0.400	-1.584 ± 1.071	n.s.
0.825	0.649	-0.472	
0.740	0.350	-1.776 ± 1.279	n.s.
0.896	0.772	-0.298 ± 0.241	n.s.
0.667			
0.608			
0.779	0.416	-1.466 ± 1.083	n.s..
0.851	0.664	-0.509 ± 0.300	*
0.761			
0.890	0.765	-0.297 ± 0.227	n.s.
0.894	0.728	-0.436	
0.754			
0.886	0.629	-0.797 ± 0.672	n.s.
0.885	0.771	-0.268 ± 0.264	n.s.
0.822	0.639	-0.500 ± 0.337	n.s.
0.663			
0.737			
0.831	0.527	-1.039 ± 0.485	n.s.
0.830	0.513	-1.112 ± 0.472	*
0.716			
0.683			
0.837	0.478	-1.363 ± 1.171	n.s.
0.846	0.569	-0.894	
0.854	0.633	-0.639 ± 0.400	n.s.
0.865	0.650	-0.615 ± 0.492	n.s.
0.694			
0.922	0.783	-0.349 ± 0.369	n.s.
1.011	1.028	0.034 ± 0.246	n.s.
0.911	0.722	-0.524 ± 0.511	n.s.
1.053	1.144	0.170 ± 0.256	n.s.
0.852	0.580	-0.869 ± 0.609	n.s.
0.844	0.569	-0.885 ± 0.594	n.s.
0.863	0.608	-0.788 ± 0.644	n.s.
0.757			
1.008	1.025	0.036 ± 0.371	n.s.
0.951	0.847	-0.257 ± 0.454	n.s.
0.878	0.665	-0.607 ± 0.535	n.s.

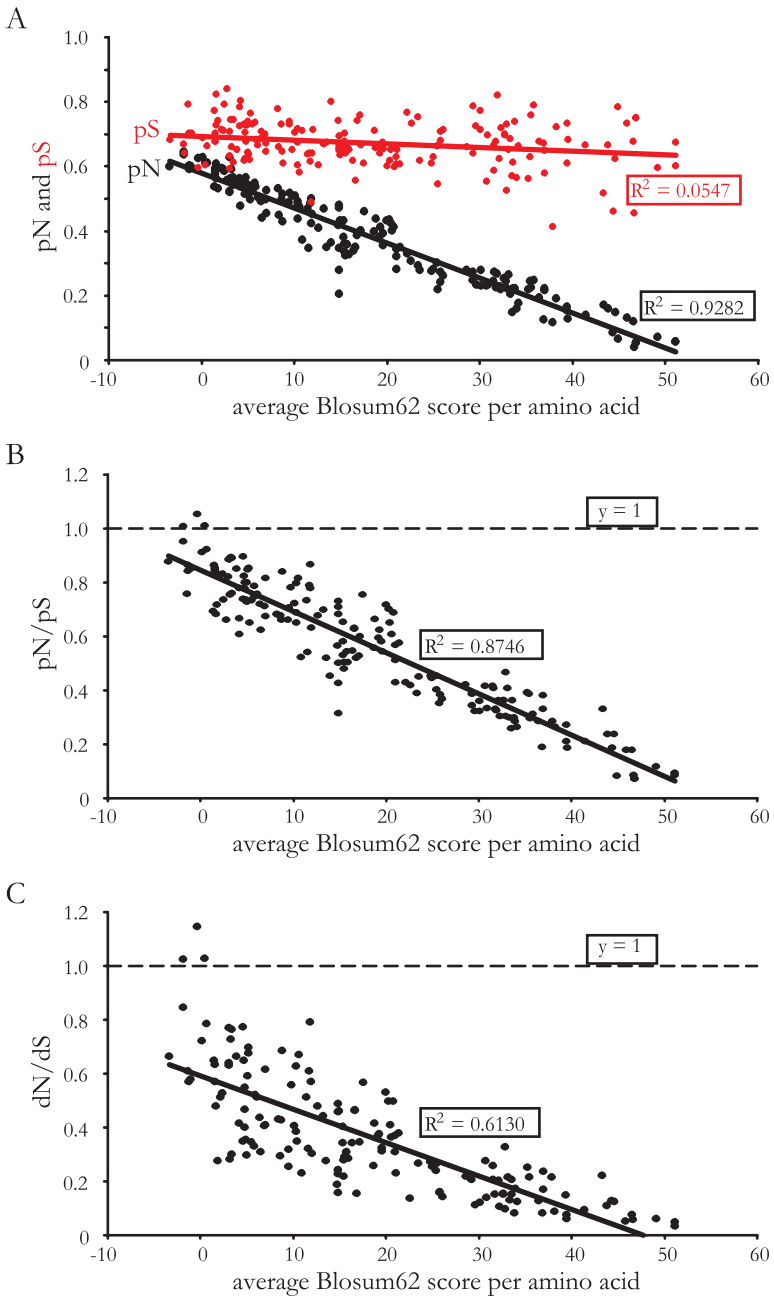
Figure 12.7: The predominant selective force on vertebrate messenger molecules has been purifying. The ratio between the proportion of nonsynonymous substitutions per nonsynonymous site (p_N) and the proportion of synonymous substitutions per synonymous site (p_S) was calculated for all messenger molecules. To this end, the coding region of each teleostean sequence was aligned pairwise with its mammalian orthologue and manually corrected to match the corresponding amino acid alignment where necessary. The number of (non)synonymous sites and (non)synonymous differences were determined according to the method of NEI and GOJOBORI[545], modified for the actual transition/transversion ratio (R) for each sequence pair. To correct for backward or double nucleotide mutations, we corrected p_N and p_S with the JUKES-CANTOR model[546] to obtain a better estimate of the actual proportion of nonsynonymous (d_N) and synonymous (d_S) substitutions. All determinations were carried out with MEGA 3.0[250]. The ratio as well as the difference between d_N and d_S are indicated for each sequence. Neutral selection is indicated when $d_N/d_S = 1$ and $d_N\text{-}d_S = 0$. The direction of the net evolutionary force that has acted on a particular sequence is considered purifying when $d_N/d_S < 1$ and $d_N\text{-}d_S < 0$. In case of positive selection, $d_N/d_S > 1$ and $d_N\text{-}d_S > 0$. Inspection of the d_N/d_S and $d_N\text{-}d_S$ values reveals the direction of the net selective force on our panel of messengers has been purifying. Note that the JUKES-CANTOR correction is inapplicable when $p_S > 0.75$. To test if the level of purifying selection is statistically different from neutral selection (in other words if the H_0 hypothesis that $d_N\text{-}d_S = 0$ can be rejected in favour of H_1: $d_N\text{-}d_S < 0$), we employed a Z-test on the basis of $d_N\text{-}d_S$ values. The level of significance is indicated by the asterisks: ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$; n.s., not significant. Since the level of significance that is achieved by a Z-test (or many other statistical tests) is dependent on the sample size, which is determined by the sequence length in this case, for some short sequences with low d_N/d_S ratios, purifying selection cannot be proven with statistical significance. For other sequences that have considerably higher d_N/d_S ratios (e.g. many members of the TGFβ superfamily), the alternative hypothesis of purifying selection is accepted with very high significance, given their much longer sequence lengths.

The continuous interplay between a host and the pathogens it has to fend off suggests a picture of a perpetual 'race of arms'. One the best examples of this arms race is provided by the major histocompatibility complex (MHC) genes and their role in host defense. MHC proteins are present on the cell surfaces of virtually all cells of our body. Their main task is the presentation of fragments of degraded pathogenic proteins, viral as well as bacterial, to T-cells that recognise the specific combination of a 'self' MHC molecule with a 'non-self' pathogenic peptide. MHC molecules display a high degree of specificity with regard to the antigenic peptide they present and this specificity is largely determined by the so-called peptide-binding residues (PBRs). An individual that has the ability to present many different antigenic peptides, in other words, an individual with a large MHC repertoire has an evolutionary advantage. Therefore, these PBRs of an MHC molecule are often subject to pathogen-driven positive selection[535].

Is this picture of an arms race between host and pathogen also applicable to the messengers of the immune system? Several examples have been documented where pathogens, usually viral, have 'hijacked' copies of host signaling molecules to enable immune modulation. Among the host molecules that are adopted by viral pathogens are viral homologs of cytokines and chemokines, soluble cytokine receptors, or cytokine binding-proteins. Some of these 'virokines' contribute to immune evasion, but others

actually promote leukocyte migration to assist in virus dissemination, which is the case for the human cytomegalovirus (CMV)-encoded CXC chemokine agonist vCXC-1[536, 537]. Collectively, this depicts a compelling scenario of an arms race between host and pathogen for the control of the host immune system, leading to positive selection on the cytokine involved. There are, however, several reasons that argue against the notion that a race of arms, driven by pathogenic molecular mimicry has been the major contributing factor to the relatively poor evolutionary conservation of immune messengers throughout vertebrates. The first is that viral copies of cytokines and their receptors and binding proteins are known for only a few cytokines, most notably IL-10, IL-6, and (inducible) chemokines and their receptors[536-540]. If pathogen-driven positive selection has had a major influence on the evolution of these cytokines, one would predict them to rank among those immune messengers with the poorest evolutionary conservation. In reality, our comparison shows that this is not the case. In fact, for IL-10 in particular, the opposite appears to be true, as it ranks among the best-conserved cytokines (Fig. 12.7). A variation on the theme of viral exploitation of host immune defense proteins is formed by the viral abuse of host cell surface receptors to infect cells. A well-known example is that of human immunodeficiency virus (HIV), which uses the chemokine receptors CCR5 and CXCR4 (in addition to CD4) to gain entry into a target cell. In case of lasting pathogen-driven positive selection that would be associated with a race of arms, one would predict these chemokine receptors to be relatively poorly conserved, whereas for CXCR4 the opposite is true (CHAPTER 4). This paradoxical situation is placed in perspective by the fact that the vast majority of pathogens is infectious only to a single, or at best a handful, of related host species. Additionally, the majority of virally encoded cytokines, cytokine receptors, and cytokine binding proteins identified to date, has been found in strains that belong to only two classes of large DNA viruses: the pox-viruses and the herpes-viruses[538]. Thus, whereas under specific circumstances an arms race between host and pathogen is certainly imaginable, the positive selection it may have enforced on the cytokine involved will be restricted to a specific host lineage. Given the scope of our analyses, 450 million years, a relative short period of lineage specific positive selection would probably have been lost in the purifying selection that has been the prevalent selective force throughout the evolution of vertebrate messengers. After all, the determination of d_N and d_S only reveals the net evolutionary force that has acted on a particular sequence over the time that has passed since the divergence of both sequences. Thus, although the scenario of a 'race of arms' between pathogen and host is compelling, and certainly imaginable between certain viral pathogens and specific vertebrate species, the restrictions that are discussed above seem to preclude a dominant role for pathogen-driven positive selection in the poor evolutionary conservation that is observed for cytokines in general.

There is an alternative explanation for the relatively poor conservation of immune


A
pS
pN
R^2 = 0.0547
R^2 = 0.9282
pN and pS
average Blosum62 score per amino acid
B
y = 1
R^2 = 0.8746
pN/pS
average Blosum62 score per amino acid
C
y = 1
R^2 = 0.6130
dN/dS
average Blosum62 score per amino acid

messengers. Many mammalian cytokines display considerable overlap in their functions (reviewed for the type-I cytokine family in chapter 8). Effectively this means that rather than acting independently, most mammalian cytokines participate in a redundant network of immune signaling. Redundancy has a negative connotation as it seems inefficient: 'a waste of resources to do the same thing twice'. In case of host defense however, the redundancy in the cytokine repertoire clearly could serve a purpose. Redundancy may be the host's answer to the inherent lack of initiative and predictability that is associated with defense against unpredictable pathogens. Rather than selecting for communicative pathways that are optimally adopted for the eradication of a single pathogen, redundancy offers several alternatives that, alone or in concert, will suffice for an effective coordination of the eradication of almost any of the many potential pathogens we may encounter. Gene duplication events are key for the development of redundancy, as they provide organisms with an extra copy of a gene that is dispensable (at first), but offers the opportunity to adopt a (slightly) new role that may provide adaptive advantage. In fact, genome duplications in particular are not just instrumental in the development of redundancy, but are considered a driving mechanism of speciation, as the massive expansion of genetic information facilitates the occurrence of major evolutionary changes[541]. Indeed, the aforementioned duplication of the early teleostean genome coincides with a period of adaptive radiation that is at the basis of the amazing diversity in contemporary species of fish[93, 527, 542]. A useful redundant network, however, requires that its different components are no longer exact copies. If this were the case, any pathogen that would succeed to corrupt the signaling of a single cytokine would effectively have succeeded in circumventing them all. Thus, redundancy requires dissimilarity and this requirement may provide an explanation for the relatively poor conservation of immune messengers. Apparently the adaptive value of the robustness that is provided by redundancy[111] has been a driving force behind a lesser degree of purifying selection. It is under these conditions of continuous exposure to a plethora of constantly changing micro-organisms, that fish and mammals apparently have adopted different solutions for the same problem of host defense. Fish rely heavily on innate mechanisms to fend of pathogenic invaders, while the focus in the

Figure 12.8: The net selective force throughout the evolution of messenger molecules is purifying. Panel a indicates a clear correlation between the proportion of nonsynonymous differences per nonsynonymous site (p_N) and the average Blosum62 score per amino acid. This correlation does not exist between the average Blosum62 score and the proportion of synonymous substitutions per synonymous site (p_S), which illustrates the validity of the general assumption that the rate of synonymous substitutions is independent of the degree of amino acid conservation. Panels b and c present the correlation between the average Blosum62 score per amino acid with the p_N/p_S ratio and the d_N/d_S ratio, respectively. The trendline that describes these correlations does not intercept the line y = 1 (indicating neutral selection) within the range of the Blosum62 scores in the dataset (-3.40 to 51.11), as these intercepts are -9.97 and -32.97 in panel b and c, respectively.

homoiothermic mammals (or rather the focus of investigators in this field) seems to have shifted towards adaptive immunity.

Summary and conclusions

Substantial differences exist in the degree of evolutionary conservation between the many different signaling molecules of vertebrates. The direction of the net selective evolutionary force over the 450 million years that separate teleostean fish from mammals has been purifying, although this purifying selection was not shown to be statistically significant for each individual messenger molecule. For some this conclusion may hardly come as a surprise. For others, the analyses in this chapter will hopefully have provided a useful demonstration of the profound differences in the evolutionary conservation of the messenger molecules of the immune and endocrine system as well as the selective forces that have shaped them. Key to this differential degree of conservation are the constantly changing circumstances under which adequate host defense had to be realised, although other factors, such as the adaptive value of redundancy, will have contributed to the relatively poor conservation of immune messengers.

Acknowledgements

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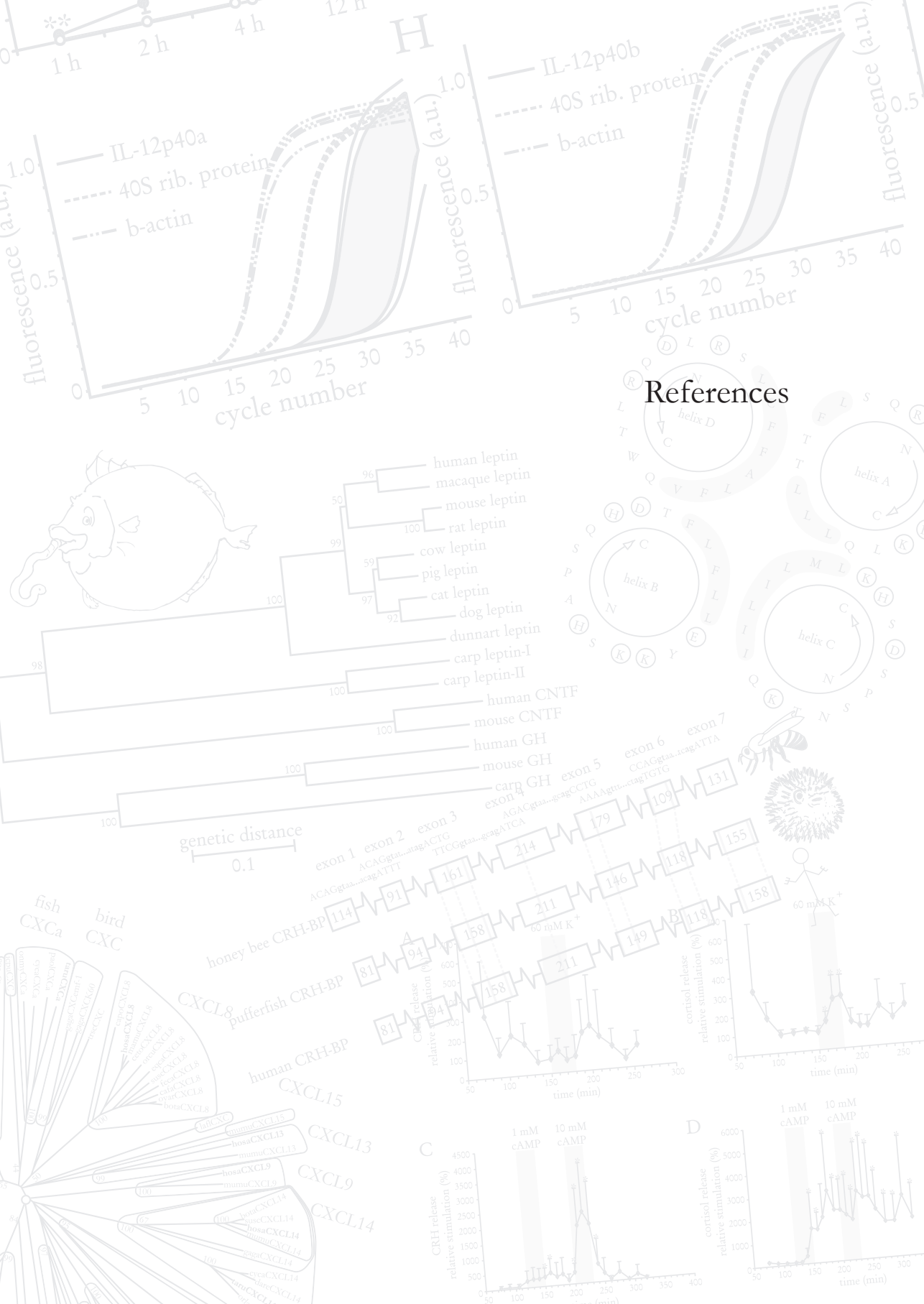
References


IL-12p40a
40S rib. protein
b-actin
fluorescence (a.u.)
cycle number
IL-12p40b
human leptin
macaque leptin
mouse leptin
rat leptin
cow leptin
pig leptin
cat leptin
dog leptin
dunnart leptin
carp leptin-I
carp leptin-II
human CNTF
mouse CNTF
human GH
mouse GH
carp GH
genetic distance
honey bee CRH-BP
pufferfish CRH-BP
human CRH-BP
fish CXCa
bird CXC
CXCL8
CXCL15
CXCL13
CXCL9
CXCL14
helix A
helix B
helix C
helix D
CRH release relative stimulation (%)
cortisol release relative stimulation (%)
time (min)
60 mM K+
1 mM cAMP
10 mM cAMP

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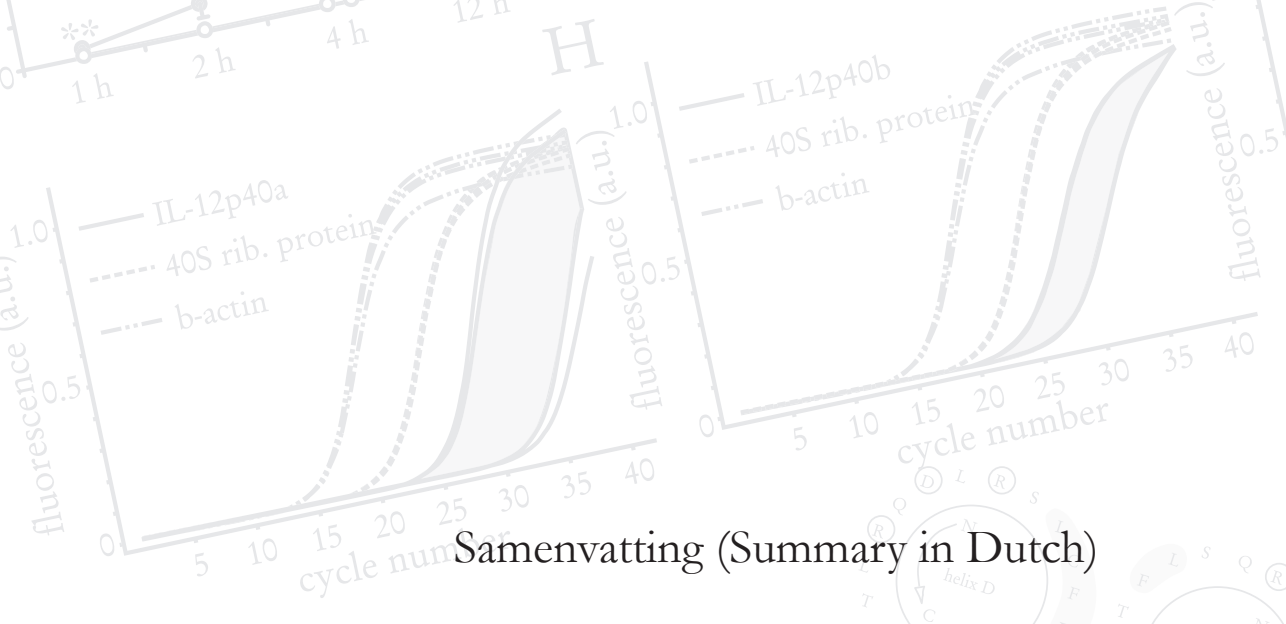

Samenvatting (Summary in Dutch)

Mark O. Huising

Samenvatting (Summary in Dutch)

Het ontstaan van meercellig leven is misschien wel één van de grootste doorbraken in de evolutionaire geschiedenis. Meercelligheid biedt aanzienlijke voordelen ten opzichte van het solitaire bestaan van bacteriën en andere eencellige organismen. Het universele principe dat ten grondslag ligt aan de opkomst en het succes van meercelligheid is eenvoudig: 'samen sta je sterker dan alleen'. Meercelligheid stelt een organisme in staat om complexe taken, zoals het verkrijgen van energie (bijv. door voedselinname of fotosynthese), voortbeweging, voortplanting, en afweer tegen ziekteverwekkers over verschillende, gespecialiseerde cellen en weefsels te verdelen. Cellen van een meercellig organisme die zich toeleggen op één functie kunnen voor andere functies vertrouwen op andere cellen in het lichaam. De coördinatie van al deze verschillende functies vereist uitgebreide en heldere communicatie en dus een universele biochemische taal. Deze communicatie verloopt deels snel en via zenuwcellen met hun soms lange uitlopers en deels wat langzamer en via biochemische signaalstoffen die aan het bloed of de extracellulaire vloeistof worden afgegeven. Deze signaalstoffen zijn veelal kleine of grotere eiwitten of cholesterol-derivaten; ze sturen de vele verschillende fysiologische processen in het lichaam. De signaalstoffen worden herkend door specifieke receptoren die zich op het oppervlak of in het cytoplasma van cellen bevinden die de boodschap ontvangen. Sommige biochemische signaalstoffen sorteren een effect in de nabije omgeving van de plek waar ze worden afgegeven, terwijl de afgifte van andere een fysiologische verandering ver(der) weg, elders in het lichaam tot gevolg heeft. Ongeacht het type of de afstand waarover zij gestuurd worden, signaalstoffen regelen alle fysiologische processen in ons lichaam: ze geven informatie door die binnenkomt via de zintuigen, sturen spieren aan via zenuwbanen, regelen de spijsvertering en energiehuishouding, zetten de klok van de voortplantingscyclus, regelen de voortplanting, verdedigen het lichaam tegen een duizelingwekkend groot aantal ziekteverwekkers en stellen het organisme in staat om te gaan met stress. Ook ons vermogen tot zelfbewust denken, een eigenschap waarvan we graag denken dat die ons 'boven' de dieren plaatst, is waarschijnlijk het geïntegreerde resultaat van een groot aantal uiterst complexe neurale netwerken en signaalroutes, waarvan de activatie met uiterste precisie wordt gecontroleerd.

Veel van onze kennis over communicatie tussen de cellen en weefsels van het lichaam is gebaseerd op studies aan zoogdieren. Om de geldigheid van deze inzichten te verbreden en te toetsen heb ik in dit proefschrift gekeken naar het voorkomen van een groot aantal signaalmoleculen in niet-zoogdieren. Ook werd de functie van deze signaalstoffen en de fysiologisch processen die ze reguleren vergeleken tussen zoogdieren en andere gewervelde dieren. Binnen de evolutie van gewervelde dieren nemen vissen een unieke plaats in. Ze hebben zich zo'n 450 miljoen jaar geleden afgesplitst van andere gewervelde

dieren (amfibieën, reptielen, vogels en zoogdieren). Bovendien zijn vissen, gemeten naar het aantal soorten dat naar schatting 35.000 bedraagt, evolutionair gezien een groot succes. Een vergelijking van het repertoire aan signalen tussen vissen en zoogdieren (dus tussen vroege en meer recente gewervelde dieren) leert ons welke signaalstoffen aanwezig zijn in alle gewervelde dieren en dus evolutionair 'oud' zijn. Omgekeerd komen we zo ook te weten welke signaalstoffen alleen voorkomen in bepaalde groepen gewervelde dieren en dus recenter of voor heel specifieke functies zijn ontstaan. Dergelijke vergelijkingen leveren nu eens opvallende overeenkomsten op tussen signaalstoffen die vele miljoenen jaren vrijwel ongewijzigd zijn gebleven, dan weer onthullen ze dat verschillende groepen gewervelde dieren hele andere oplossingen (signaalstoffen) hebben ontwikkeld voor een vergelijkbaar fysiologisch proces. In dit proefschrift staat de karper (*Cyprinus carpio*) centraal, omdat deze vissoort verschillende voordelen heeft. Hij is zeer nauw verwant aan de zebravis (*Danio rerio*), een kleine, populaire tropische aquariumvis. Veel van de informatie die in de afgelopen jaren is verzameld over de basenpaarvolgorde van het genetische materiaal van de zebravis is dan ook relatief eenvoudig 'over te zetten' naar de karper. Omdat de karper aanzienlijk groter wordt dan de zebravis, is het een veel geschikter model voor fysiologisch onderzoek dat zich richt op de werking van het endocriene systeem of de afweer van vissen tegen ziekteverwekkers.

In dit proefschrift heb ik gekeken naar drie verschillende groepen signaalstoffen. De geselecteerde vertegenwoordigers van die groepen vervullen steeds cruciale functies binnen het endocriene systeem dan wel binnen het immuunsysteem. De signaalmoleculen van het endocriene systeem worden hormonen genoemd. De vele signaalstoffen die gezamenlijk de afweer tegen ziekteverwekkers reguleren worden 'cytokines' genoemd. Tot de cytokines rekenen we ook de 'interleukines', een naam die verwijst naar de communicatie *tussen leukocyten* (witte bloedcellen), en 'chemokines'. De chemokines zijn verantwoordelijk voor het aantrekken van witte bloedcellen naar de plaats van een ontsteking of infectie. In de eerste sectie van het proefschrift hebben we gekeken naar de zogenaamde 'CXC' chemokines. Dit is een klasse van chemokines met een karakteristiek patroon van cysteine-residuen (aangeduid met de letter 'C'). In HOOFDSTUK 2 hebben we gekeken naar twee CXC chemokines van de karper (CXCa en CXCb) die tot expressie komen in immuunorganen. CXC-expressie kan in kweek worden gestimuleerd door toevoeging van verschillende stoffen die van ziekteverwekkers afkomstig zijn, zoals lipopolysaccharide (LPS). Gezamenlijk wijst dit erop dat deze CXC chemokines waarschijnlijk betrokken zijn bij het aantrekken van leukocyten. Hoewel het onmiskenbaar CXC chemokines zijn, blijkt het onmogelijk om vast te stellen op welk type CXC van zoogdieren de karper chemokines CXCa en CXCb het meeste lijken. In HOOFDSTUK 3 hebben we nog eens drie karper CXC chemokines beschreven. In tegenstelling tot de beide CXC chemokines uit HOOFDSTUK 2, zijn de aminozuursequenties van deze drie CXC chemokines veel beter geconserveerd,

waardoor we kunnen concluderen dat ze het nauwst verwant zijn aan de chemokines CXCL12 en CXCL14 van zoogdieren. Opmerkelijk is dat CXCL12a, CXCL12b en CXCL14 van de karper al heel vroeg in de ontwikkeling (al vanaf de bevruchting van het ei) tot expressie komen. In de volwassen karper komen ze voornamelijk nog in de hersenen tot expressie en veel minder in immuunorganen. Een vergelijking van het repertoire aan CXC chemokines van zoogdieren en vissen (HOOFDSTUK 4) maakt duidelijk dat CXCL12 en CXCL14 de enige twee CXC chemokines zijn die in alle klassen gewervelde dieren zijn terug te vinden. Dit klopt met de waarneming dat de genen die deze CXC chemokines coderen ieder op een apart chromosoom liggen, terwijl alle andere CXC chemokines van zoogdieren zich in elkaars nabijheid op een en hetzelfde chomosoom bevinden. Deze distributie duidt erop dat deze groep chemokines door genduplicaties is ontstaan. Dat er niet voor elk van deze chemokines een tegenhanger in vissen werd gevonden, betekent dat deze genduplicaties pas recent zijn opgetreden, nadat de vissen en overige gewervelde dieren van elkaar zijn afgesplitst.

In de tweede sectie van dit proefschrift is gekeken naar de zogenaamde 'type-I' cytokines. Dit is een grote groep eiwitsignaalstoffen met een gemeenschappelijke driedimensionale structuur. Ook maken deze signaalstoffen gebruik van dezelfde of verwante receptoren en worden er in de cel vergelijkbare reacties in gang gezet na activatie van deze receptoren. In HOOFDSTUK 5 beschrijven we interleukine-11 (IL-11), een klassiek type-I cytokine, in karper. Het is gebleken dat IL-11 al vroeg aanwezig is geweest in de evolutie van gewervelde dieren. Verschillende vissoorten die slechts in de verte aan elkaar verwant zijn, zoals de kogelvissen en zebravis, beschikken echter ook over een gen dat codeert voor een tweede, radicaal verschillend IL-11 molecuul. Het bezit van twee verschillende IL-11 genen is uniek voor de vissen. Iets soortgelijks hebben we gevonden voor interleukine-12 (IL-12). Dit cytokine is samengesteld uit twee verschillende eiwitten (P35 en P40) die met een zwavelbrug aan elkaar zijn gekoppeld. In HOOFDSTUK 6 beschrijven we de genen die coderen voor karper P35 en P40. In tegenstelling tot zoogdieren, die slechts één P35- en één P40-gen bezitten, beschikt de karper over drie heel verschillende P40-genen. Deze drie genen verschillen in de mate waarin ze normaal tot expressie komen en ook in de mate waarin zij in kweek gestimuleerd kunnen worden. Hoewel ze slechts beperkt op elkaar lijken, delen ze wel vrijwel alle aminozuren waarvan bekend is dat ze essentieel zijn om een stabiele verbinding aan te gaan met P35 om zo IL-12 te vormen. Een derde lid van de type-I cytokinefamilie dat geanalyseerd werd is leptine. Leptine wordt algemeen beschouwd als één van de belangrijkste hormonen in de regulatie van voedselinname en energiehuishouding. In zoogdieren wordt leptine afgegeven door de onderhuids vetweefsel en bereikt het de hersenen via de bloedsomloop. In HOOFDSTUK 7 beschrijven we twee vergelijkbare leptine moleculen in de karper die, in tegenstelling tot leptine in zoogdieren, vooral tot expressie komen in de lever. Deze genexpressie verandert na de

maaltijd, maar blijft verrassenderwijs onveranderd na een periode van zes dagen vasten. Ratten die gedurende een vergelijkbare periode geen voer krijgen vertonen aanzienlijke veranderingen in de expressie van leptine. Deze verschillen tussen karper en rat moeten worden geïnterpreteerd in het licht van de fundamenteel verschillende manieren waarop de warmbloedige zoogdieren en de koudbloedige vissen hun metabolisme reguleren. Tot slot van deze sectie reconstrueren we in HOOFDSTUK 8 de evolutionaire verwantschappen tussen een groot aantal verschillende type-I cytokines van gewervelde dieren. De aminozuur sequenties van deze cytokines verschillen vaak te veel om op zichzelf te dienen als basis voor een evolutionaire reconstructie. De structuur van de bijbehorende genen en ook de positie van cysteine-residuen binnen deze genen zijn echter voldoende geconserveerd om te dienen als goede indicatoren voor een betrouwbare reconstructie van de evolutionaire verwantschappen tussen type-I cytokines. Deze reconstructie laat zien dat een groot deel van de type-I cytokines al aanwezig was voordat de vissen en viervoetigen zich afsplitsten; voor de type-I cytokines is de situatie dus wezenlijk anders dan die in HOOFDSTUK 4 is beschreven voor de CXC chemokines.

In de derde en laatste sectie van het proefschrift nemen we de signaalmoleculen van de 'stress respons' onder de loep. De stress respons wordt gegeven wanneer de integriteit van een dier in het geding is, of in het geding dreigt te komen. In zo'n geval wordt er vanuit de hersenen het hormoon CRH afgegeven. Dit CRH zorgt voor de afgifte van een tweede hormoon, ACTH, vanuit de hypofyse. ACTH op zijn beurt induceert de afgifte van het 'stress-hormoon' cortisol, dat in zoogdieren vanuit de bijnierschors wordt afgegeven aan het bloed. In vissen verloopt de stressrespons in grote lijnen vergelijkbaar. Het grootste verschil is dat de cortisol-producerende cellen in vissen samen met de (nor)adrenaline-producerende cellen ingebed liggen in het immuunweefsel van de kopnier. Gedacht wordt dat deze anatomische organisatie mogelijkheden schept voor subtiele en directe communicatie tussen cellen van het endocriene en het immuunsysteem. In HOOFDSTUK 9 karakteriseren we CRH, één van de receptoren voor CRH, en 'CRH-binding protein' (CRH-BP) in de karper. Dit CRH-BP is in staat om CRH te binden en zo de activiteit van CRH te moduleren. We laten zien dat de genexpressie van deze factoren verandert tijdens stress. Hieruit volgt dat de rol van CRH in de regulatie van de stressrespons in alle gewervelde dieren vergelijkbaar is. Hoewel CRH is ontdekt als de 'startmotor' van de stressrespons, weten we inmiddels dat CRH betrokken is bij veel meer fysiologische processen. In hoofdstuk 10 laten we zien dat CRH en CRH-BP eiwitten aanwezig zijn buiten de hersenen in de (nor)adrenaline producerende cellen van de kopnier. De afgifte van CRH vanuit de kopnier kan bovendien worden gestimuleerd in kweek. Een soortgelijk CRH-systeem is beschreven in de bijnier van zoogdieren. Dit suggereert dat communicatie via CRH evolutionair beter is geconserveerd dan communicatie via de meeste chemokines en cytokines. Deze stelling wordt nog eens onderstreept door de waarneming dat CRH-BP

ook buiten gewervelde dieren voorkomt, zelfs in de honingbij (*Apis mellifera*) en andere insecten (hoofdstuk 11). Dit betekent dat crh-bp, en waarschijnlijk het gehele crh systeem, meer dan een miljard jaar oud is. Tot slot gaan we in hoofdstuk 12 dieper in op de grote verschillen die tussen signaalmoleculen bestaan in de mate van hun evolutionaire conservering. Dit doen we door een antwoord te zoeken op drie vragen. In de eerste plaats 'Wat is geconserveerd?'. Voor een betekenisvolle discussie over evolutionaire conservering is het noodzakelijk om eerst een perspectief vast te stellen waaraan de mate van conservering van individuele signaalmoleculen objectief kan worden gekwantificeerd. Vervolgens kunnen we proberen uit te rekenen welke selectiekrachten er hebben gewerkt op de basenpaar-sequenties die coderen voor signaaleiwitten. Ofwel: 'Hoe zijn verschillen in conservering tot stand gebracht'. Tot slot bediscussiëren we in dit hoofdstuk een aantal overwegingen die van cruciaal belang zijn bij het verkrijgen van een antwoord op de vraag 'waarom' sommige signaalstoffen de evolutionaire tand des tijds veel beter doorstaan hebben dan anderen.

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Mark (Olaf) Huising werd geboren op 10 mei 1977 in Hengelo (O) en groeide op in Hoogeveen. Daar heeft hij in 1995 zijn vwo diploma behaald aan het Menso-Altingh College. Datzelfde jaar begon hij aan de studie Biologie aan de Wageningen Universiteit. Tijdens de doctoraalfase heeft hij tijdens twee afstudeervakken onderzoek verricht bij de vakgroepen Celbiologie en Immunologie en Experimentele Zoölogie (sectie ontwikkelingsbiologie), beide aan de Wageningen Universiteit. Een derde afstudeervak werd uitgevoerd in de Verenigde Staten, aan de Oregon Health Sciences University in Portland, or. Daarnaast is Mark tijdens zijn studie opgetreden als student-assistent voor verschillende biologische practica. Naast studeren vond hij tijd voor volleybal en was hij gedurende ruim drie jaar actief lid van Stichting Flat Overleg, een organisatie die de belangen van huurders bij de Stichting Sociale Huisvesting Wageningen behartigt. In September 2000 studeerde hij *cum laude* af om een maand later te beginnen aan zijn promotie onderzoek dat voortvloeide uit een samenwerking tussen de leerstoelgroepen Organismale Dierfysiologie (Radboud Universiteit Nijmegen) en Celbiologie en Immunologie (Wageningen Universiteit). De resultaten van dit promotie onderzoek staan in dit proefschrift beschreven en werden daarnaast gepresenteerd op meer dan een dozijn nationale en internationale wetenschappelijke bijeenkomsten. Naast zijn promotie onderzoek was Mark van 2000 tot 2003 lid van de Raad van Commissarissen van de Stichting Sociale Huisvesting Wageningen, pakte hij duiken op als hobby en bleef hij met onverminderd enthousiasme volleyballen. Na zijn promotie zal Mark als postdoc aan de slag gaan aan het Salk instituut in La Jolla, ca, waar hij in samenwerking met de leerstoelgroep Organismale Dierfysiologie zijn onderzoek verder zal verdiepen.