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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 hematopoietic 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 peptides 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.

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Introduction

Interleukin-11 (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 (Paul et al. 1990). The human and murine IL-11 genes each consist of five exons and encode a 199-amino acid peptide. 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 (Czupryn et al. 1995) that conforms to the four-helix bundle topology shared by all type I cytokines (Bazan 1990). This classification is strongly supported by the nature of the IL-11 receptor complex. IL-11 binds with low affinity to a specific IL-11Rα 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, ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM), and cardiotrophin-1 (CT-1) (Ozaki and Leonard 2002). 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 (Barton et al. 2000).

The shared signal-transducing gp130 chain as well as the similar type I cytokine four-helix bundle conformation explains, 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 (Paul et al. 1990), 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 hematopoietic progenitors (Du and Williams 1994, 1997). IL-11 contributes to the commitment of stem cells into the multilineage progenitor compartment (Du et al. 1995). Moreover, IL-11 acts in concert with IL-3, thrombopoietin (TPO), IL-6, and stem cell factor (SCF) to en-

hance megakaryocytopoiesis and increase peripheral platelet counts (Du et al. 1993a; Kobayashi et al. 1993; Neben et al. 1993; Paul et al. 1990; Teramura et al. 1992; Williams et al. 1998) and stimulates erythropoiesis alone or aided by IL-3 or erythropoietin (EPO) (Quesniaux et al. 1992). Moreover, administration of recombinant human IL-11 to sublethally irradiated mice accelerates recovery of peripheral blood platelets and neutrophils (Du et al. 1993b). 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 (Zheng et al. 2001). IL-11 also has many nonhematopoietic effects. It exerts protective effects on various mucosal epithelia of the gastrointestinal (Du et al. 1994, 1997) and respiratory systems (Redlich et al. 1996) 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 (Redlich et al. 1996; Trepicchio et al. 1996; Waxman et al. 1998, 2003; Zheng et al. 2001). Moreover, IL-11 was recently shown to mediate direct antiapoptotic effects on human colonic epithelial cells (Kiessling et al. 2004). IL-11 also prevents cartilage matrix degradation in chronically inflamed joints by inducing the expression of tissue inhibitor of metalloproteinases (TIMP) (Maier et al. 1993) and is an important regulator of bone remodeling (Elias et al. 1995; Takeuchi et al. 2002). Other physiological functions of IL-11 include the inhibition of adipogenesis (Keller et al. 1993; Takeuchi et al. 2002) and the induction of proliferation and differentiation of hippocampal neuronal cells (Du et al. 1996; Mehler et al. 1993).

IL-11 has so far been identified in only two primate and two rodent species. Very recently, an IL-11 peptide was reported in rainbow trout (*Oncorhynchus mykiss*) (Wang et al. 2005). Fish orthologues of several other mammalian four-helix bundle cytokines, including IL-12p35 (Yoshiura et al. 2003) and EPO (Chou et al. 2004), are known, and these cytokines invariably exhibited low overall amino acid similarity to their mammalian orthologues. Furthermore, a four-helix bundle cytokine designated M17 that shares similarities with OSM, LIF, and CNTF was recently described in carp (Fujiki et al. 2003). 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 (Aparicio et al. 2002). Here we report the sequence of IL-11 from common carp (*Cyprinus carpio*) and characterize 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 species 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 (Kumar and Hedges 1998), IL-11 in fish and mammals may have adopted different functions.

Experimental procedures

Animals

Common carp (*C. carpio* L.) were reared at 23°C in recirculating UV-treated tap water at the "De Haar Vissen"

Table 1 Primer sequences and corresponding accession numbers

Gene	Acc. no.	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
IL-11	AJ632159	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

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. R3×R8 are the hybrid offspring of a cross between fish of Polish origin (R3 strain) and fish of Hungarian origin (R8 strain) (Keller et al. 1993). 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 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 rapid amplification of cDNA ends (RACE). 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 1) were used as the initial and nested primers for the amplification of the 5′UTR. IL-11.fw4 and IL-11.fw3 (Table 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 anesthetized 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 heparinized syringe (Leo Pharmaceutical Products, Weesp, the Netherlands) 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 1–5×10^6 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% (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} lipopolysaccharide (LPS from *Escherichia coli*; Sigma), 20 μg ml^{-1} concanavalin A (ConA from *Canavalia ensiformes*; Sigma), or 400 ng ml^{-1} cortisol (Sigma). A nonstimulated control group was included, and all treatments were carried out in fivefold. 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 cRPMI [RPMI 1640, Gibco; adjusted to carp osmolarity (270 mOsm kg^{-1}) with distilled water] containing 0.01% (v/v) NaN_3 and 10 μU ml^{-1} heparin and centrifuged for 10 min at 100×*g* to remove the majority of erythrocytes. The supernatant containing peripheral blood leukocytes (PBL) was layered on a discontinuous Percoll (Amersham Pharmacia Biotech) 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 PBL) were collected.

RNA isolation

RNA was isolated from PBL, and from anterior kidney, macrophage-enriched cell cultures were 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 maximize 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 proceeding with cDNA synthesis.

DNase treatment and first strand cDNA synthesis

For each sample a non-RT (nonreverse transcriptase) control was included. One microliter of 10× DNase I reaction buffer and 1 μl DNase I (Invitrogen, 18068-015) were 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 for 10 min. To each sample, 300 ng random hexamers (Invitrogen, 48190-011), 1 μl of 10 mM dNTP mix, 4 μl of 5× First Strand buffer, 2 μl of 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°C. 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°C. All reactions were filled up with demineralized 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. For RQ-PCR, 5 μl of cDNA and forward and reverse primer at 5 μM each (Table 1) were added to 12.5 μl of Quantitect Sybr Green PCR Master Mix (Qiagen) and filled up with demineralized 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 1 min at 60°C)

Fig. 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

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°C at 1°C 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 (Huising et al. 2004b). Expression following in vitro stimulation was corrected for reference gene expression and rendered relative to the expression in nonstimulated control cells as previously described (Huising et al. 2004b; Pfaffl 2001). 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 standardization 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 the SRS mirror site of the European Bioinformatics Institute (http://www.ebi.ac.uk/services). *D. rerio*, *T. rubripes*, and *T. nigroviridis* sequences were retrieved from the Ensembl web site (http://www.ensembl.org) (Hubbard et al. 2005). 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/ (Bendtsen et al. 2004). 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 (Pearson and Lipman 1988). 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 neighbor-joining method (Saitou and Nei 1987) using MEGA version 2.1 (Kumar et al. 2001). Reliability of the tree was assessed by bootstrapping, using 1,000 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 (*D. 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 complete coding strands was obtained by RACE and encodes a 198-amino acid novel carp interleukin (Fig. 1). Protein–protein BLAST against the Swissprot database revealed significant hits with rat, mouse, macaque, and human IL-11 amino acid sequences only (Table 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, as well as an IL-11 sequence from rainbow trout (*O.*

Table 2 List of BLAST hits following comparison of carp IL-11 to the Swissprot database

Acc. no.	Species	Description	*E* value
P20808	Macaque	IL-11 precursor	2×10^{-4}
Q99MF5	Rat	IL-11 precursor	2×10^{-4}
P47873	Mouse	IL-11 precursor	3×10^{-4}
P20809	Human	IL-11 precursor	2×10^{-3}
Q8R6G8	*Fusobacterium nucleatum*	Bifunctional glycosyltransferase/methyltransferase	1.6
P19137	Mouse	Laminin alpha-1 chain precursor	2.3

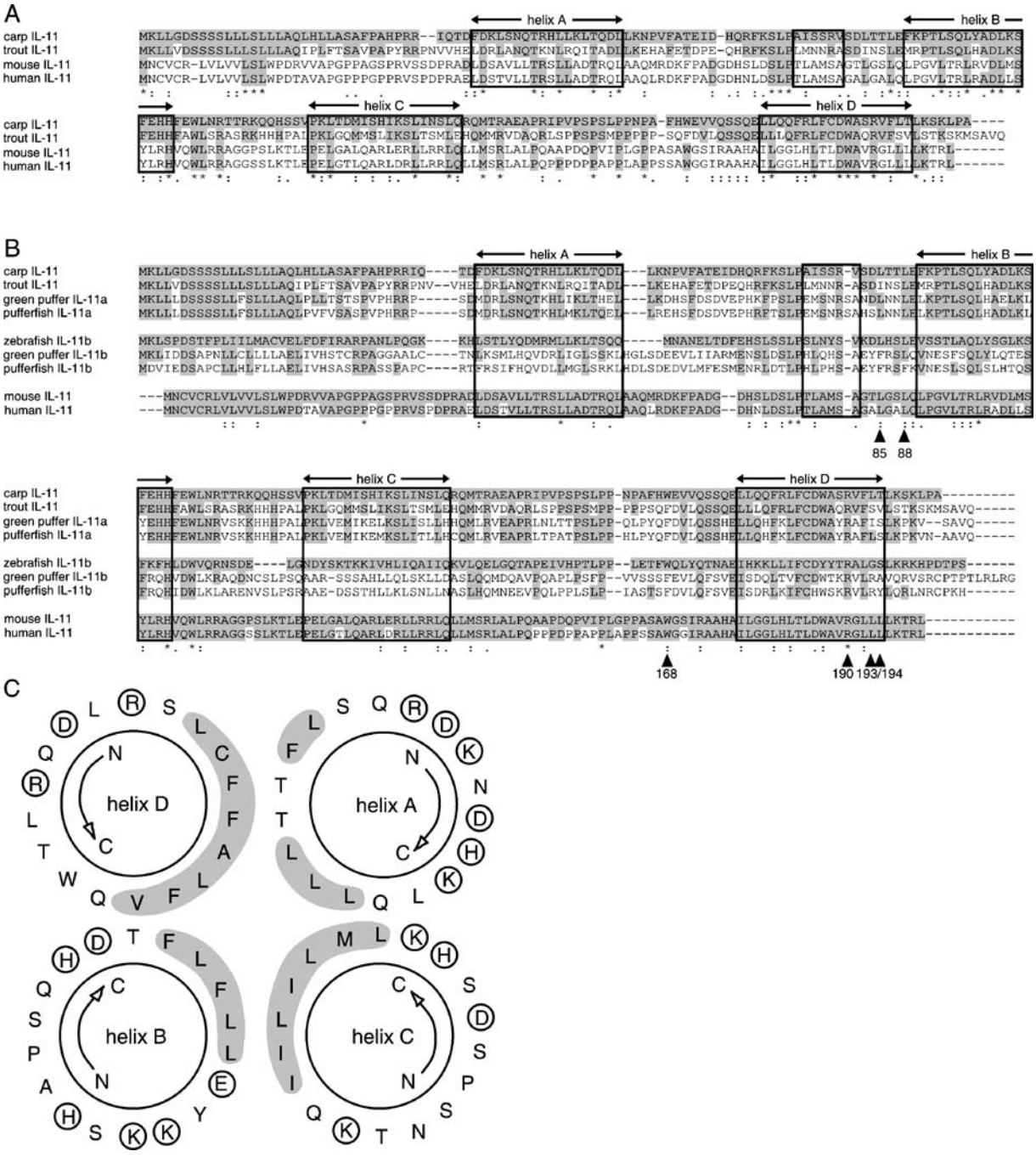

Fig. 2 Multiple amino acid sequence alignment of vertebrate IL-11 sequences. **a** The alignment of carp IL-11 with selected vertebrate IL-11 sequences illustrates the presence of four potential α-helices (*boxed*), despite the relatively low overall amino acid identity. A fifth, short α-helix that is 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. Amino acids conserved throughout the alignments are indicated by asterisks. 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/CAAB01000522.1; mouse IL-11, P47873; human IL-11, P20809. **c** Helical wheel projection of each of the four α-helices suggests a hydrophobic protein core (hydrophobic faces are shaded) and a hydrophilic, solvent-exposed exterior surface that contains many charged residues (*circled*)

Table 3 Predicted isoelectric point 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

mykiss), reveals that their overall amino acid identity is modest (Fig. 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. 2c). 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 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) (Jaillon et al. 2004). This sequence, from the spotted green pufferfish (*T. 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" (*T. 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 that we designated IL-11b bears much lower amino acid identity to mammalian IL-11 as well as to bony fish IL-11a (Table 4). Expansion of the multiple sequence alignment with the newly discovered bony fish IL-11a and IL-11b peptides also illustrates their low overall amino acid similarity (Fig. 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 tryptophane (corresponding to human W168) in the CD loop that were identified as crucial for receptor binding and signaling, respectively, in mouse (Barton et al. 1999). The authors also identified four leucine residues (corresponding to L85, L88, L193, and L194) that 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.

Table 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

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, was more neutral (Table 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. 3). 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. 4). 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 peptides 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) 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 4).

Fig. 3 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

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 con-

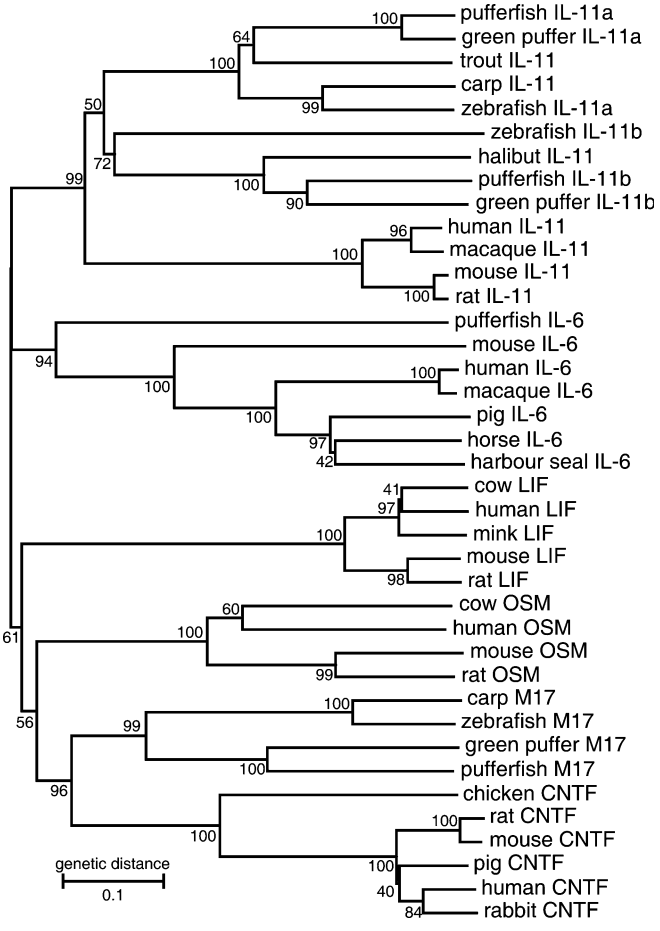

Fig. 4 Phylogenetic tree of vertebrate IL-11 sequences. Numbers at branch nodes represent the confidence level of 1,000 bootstrap replications. The related type I cytokines IL-6, CNTF, OSM, and LIF are included as out-group. Human IL-11, P20809; macaque IL-11, P20808; rat IL-11, AAK29623; mouse IL-11, 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; harbor 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

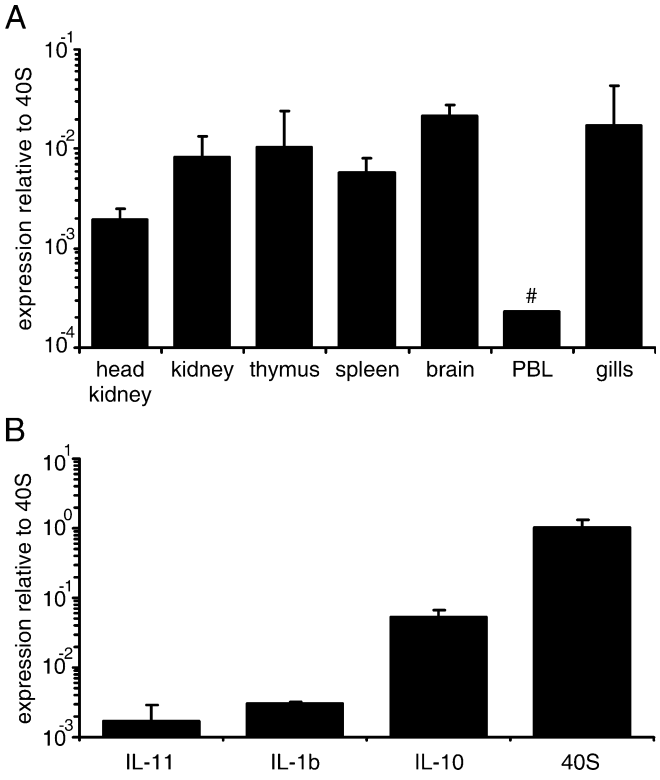

Fig. 5 Constitutive expression of carp IL-11. Carp IL-11 is constitutively expressed in the organs investigated, with the exception of PBL, where IL-11 was not detectably expressed in four out of five replicates (indicated by #) (**a**). The constitutive expression of IL-11 in carp head kidney macrophages 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

stitutively expressed in all systemic immune organs of carp (Fig. 5a), including thymus, spleen, kidney, and head kidney (the anatomical equivalent of the mammalian adrenal gland, that is 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 of four of the five fish, 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

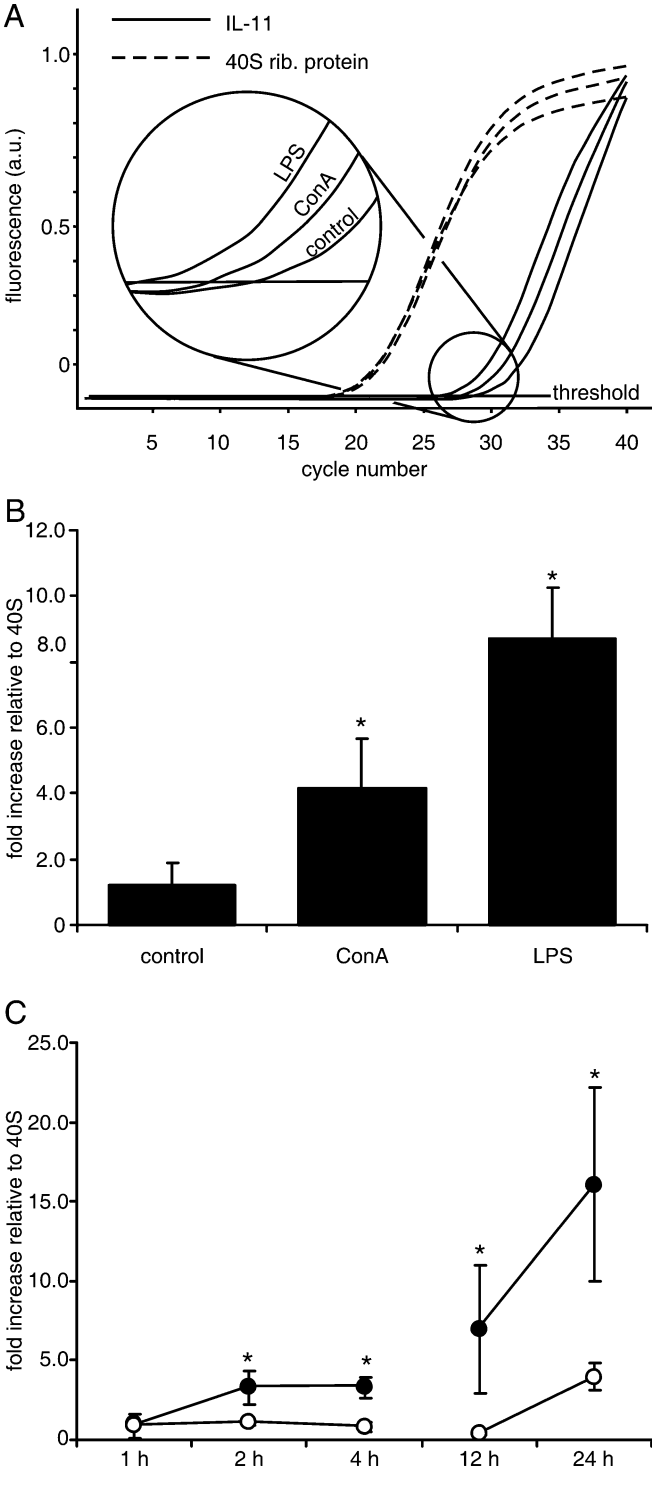

Fig. 6 IL-11 expression in carp head kidney macrophages following in vitro stimulation for 4 h with ConA (20 μg ml^{-1}) or LPS (50 μg ml^{-1}), determined with RQ-PCR. In *panel a*, the amplification curves of IL-11a and 40S ribosomal protein S11 are given for one replicate of each group. Note that 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 (*panel 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 nonstimulated controls cultured for the same amount of time is included (*open symbols*). Results are corrected for 40S expression and plotted relative to the control group at 1 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

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 expressed only one order of magnitude less than 40S ribosomal protein S11 (Fig. 5b).

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 4 h of stimulation, both agents had significantly enhanced the expression of IL-11a, with LPS being a slightly more potent stimulator (Fig. 6a,b). We next studied the kinetics of IL-11 up-regulation in response to LPS in two separate experiments. IL-11 expression was modestly increased following in vitro LPS stimulation for 2 and 4 h, whereas a period of 1-h stimulation was too short to elicit increased IL-11 expression (Fig. 6c). Continuous stimulation with LPS for an extended period of 12 or 24 h resulted in a further increase in the IL-11 expression, although at 24 h, cells that received no exogenous stimulus start to display elevated levels of IL-11 expression compared to previous time.

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 (Huising et al. 2004a). LPS stimulation for a period of 4 h 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. 7).

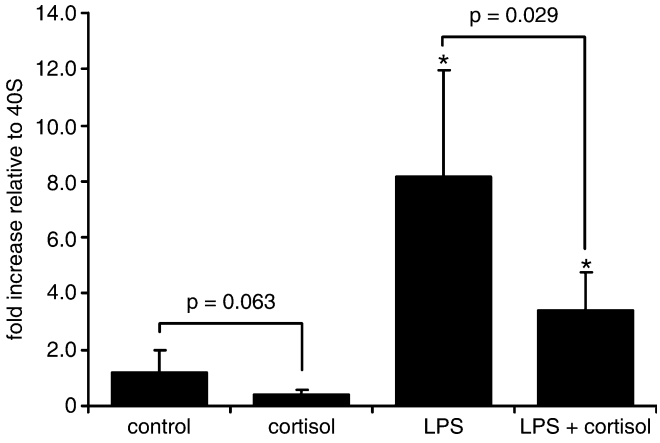

Fig. 7 IL-11 expression is inhibited by cortisol. IL-11 expression in carp head kidney macrophages following 4 h in vitro stimulation with cortisol (400 ng ml^{-1}), LPS (50 μg ml^{-1}), or a combination of LPS and cortisol. Expression is corrected for 40S expression and plotted relative to unstimulated 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

Discussion

Here we characterize 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 utilize one or several intrachain disulfide bridges to stabilize their four-helix bundle topology, IL-11 lacks conserved cysteine pairs. Instead, it relies on hydrophobic interactions for stability (Czupryn et al. 1995). And although the amino acid identity between carp IL-11 and mammalian IL-11 peptides is modest, the predicted tertiary structure of carp IL-11 is supportive of a topology of four α-helices that are stabilized 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 peptides as well as their highly similar gene organizations 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 peptides 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 peptides not only bear low amino acid identity with mammalian IL-11 peptides, 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 (Wang et al. 2005). As a consequence, it was concluded that the identity between IL-11 peptides 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 (Fujiki et al. 2003; Yoshiura et al. 2003). 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 peptides, several residues that are key for the interaction between IL-11 and

its receptor complex in mammalian species (Barton et al. 1999) are conserved in fish IL-11a and IL-11b peptides. 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 dispensable for receptor binding, is essential for receptor activation (Barton et al. 1999). This tryptophan is conserved in cyprinid IL-11a and IL-11b peptides 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 disulfide bridges, a single conserved cysteine residue is present in helix D of all teleostean IL-11a and IL-11b peptides, but not in mammalian IL-11. Although a single cysteine residue is insufficient to form an intrachain disulfide bridge, it may participate in the formation of a disulfide-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 inward, seems to preclude its participation in a disulfide 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 characteristics of 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 peptides, 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 (Taylor et al. 2003), 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 (Jaillon et al. 2004). 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 (Force et al. 1999). 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 (Wang et al. 2005) 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 (Chong et al. 2001; Huising et al. 2004b).

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 (Huising et al. 2003), 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, which was detectable in all organs tested, although differences in the level of expression between different organs appear larger in trout (Wang et al. 2005) than in carp.

In vitro, carp IL-11 expression is up-regulated several-fold in a carp primary culture of head kidney macrophages within 2 h following stimulation with LPS, a key component of the cell wall of gram-negative bacteria. This expression steadily increases to approximately 15-fold after 24 h 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 3, 7, and 24 h all increased IL-11 expression relative to nonstimulated controls (Wang et al. 2005). Cortisol, the main glucocorticoid released upon activation of the teleost fish stress axis, is capable of inhibiting both the constitutive as well as the LPS-induced IL-11 expression. Similar inhibitory effects of cortisol on fish cytokine expression have been reported for carp IL-1β and TNF-α following costimulation of cortisol with LPS or a fish trypanosome lysate (Engelsma et al. 2003; Saeij et al. 2003). 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 (Angeli et al. 2002; Elias et al. 1995; Kim et al. 1999).

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.

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