Transferrin polymorphism of common carp: link with disease resistance

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General introduction

1. Introduction to the immune system

Animal health is a function of intrinsic and actual adaptation capacity determined by the intrinsic genetic background of the animal, and modified by interaction with the environment. Environmental factors constitute, among others, infections (by bacteria, viruses and parasites), management procedures (vaccination, welfare and species-specific behavior) and husbandry, food (constituents and additives) and transportation. Robust animals can adapt to various conditions while maintaining a basic level of health. Widely agreed upon, the immune system can be used as a reflection of the general health status of the individual animal. However, the selection of (immune) parameters relevant for disease protection and health are still poorly defined.

The immune systems of fish and higher vertebrates are very similar. Both have two integral components (Plouffe et al., 2005; Secombes et al., 2005). The innate, or natural defense system is formed by a series of cellular and humoral components. The adaptive, acquired or specific immune system is characterized by the humoral immune response through the production of antibodies and characterized by the cellular immune response mediated by T-lymphocytes and capable of reacting specifically with antigens (Janeway et al., 2001). Initially, most infectious agents encountered by an individual are prevented from entering the body by a variety of physical and biochemical barriers, such as the intact skin, mucus, cilia lining the trachea, acidity of the stomach, lysozyme and the presence of commensal bacteria in the gut. If an infectious agent does manage to penetrate an epithelial surface, it will meet a second set of barriers: the phagocytes and the natural killer (NK) cells (Herberman and Ortaldo, 1981). Phagocytes are able to engulf particles, including many bacteria and fungi species, and destroy them in a process called phagocytosis. The main phagocytic cells are the neutrophilic granulocytes, monocytes and macrophages. NK cells are able to recognize cell surface changes that occur in tumor cells and in virus infected cells. NK cells are then able to bind to those altered cell types and kill them by a process called cytotoxicity.

In addition to phagocytic and NK cells, soluble substances can also operate in a coordinated way to eradicate infectious agents. These substances include molecules referred to as acute-phase proteins, the complement system proteins and interferons, which increase rapidly in concentration during infection (Gabay and Kushner, 1999). Acute phase proteins encompass C-reactive protein, a protein that can bind to bacteria and promote the activation of some complement-system proteins. The

complement system is a complex of more than twenty serum proteins, whose overall functions are to facilitate phagocytosis by binding to the antigens (a process called opsonization), to control inflammation and to destroy foreign agents through lysis of these cells. The complement proteins interact with each other and with other elements of the innate and specific immune system components (Rus et al., 2005).

The innate immune system, unlike the adaptive immune system, lacks the ability to acquire memory and specific recognition based upon highly specific T- and B-cell receptors originated by rearranged gene fragments after an encounter with foreign agents. However, the innate system is highly important, especially in fish where the synthesis of antibodies is relatively slow in comparison with antibody production in warm-blooded vertebrates (Tosi, 2005). When an individual is exposed to a foreign antigen, two basic types of effector mechanisms are normally stimulated. One is mediated by specific molecules, called antibodies. Antibodies are present in the blood and various biological fluids and the antibody-mediated immunity is called humoral immunity (Pier et al., 2004). The other type of immune response is effected by cells, mainly by the so-called T lymphocytes, and confers cell-mediated immunity (Huber et al., 1976).

Most immune responses involve the activity and interplay of both the humoral and the cell-mediated immune branches of the immune system. Furthermore, the innate and the adaptive immune systems do not act in a totally independent way. This is illustrated by the formation of specific antibodies that opsonize infectious agents, so that phagocytes recognize and engulf their targets more effectively. Also, activated T lymphocytes produce certain cytokines (Smith, 1980) and some of these cytokines stimulate phagocytes to destroy infectious agents in a more efficient way, or help B lymphocytes to produce antibodies. An adaptive immune response in ectothermic vertebrates can take a considerable period of time, for example, antibody production can take 4–6 weeks to respond and is highly temperature-dependent (Rijkers et al., 1980). The much more rapidly responding innate immune system also plays a major role in antigen presentation and regulation of the functional balance of cytokines and chemokines and their receptor profiles.

2. Modulation of the immune system

Immunomodulation is the manipulation of the immune system; it may augment or decrease the magnitude of the immune responsiveness. Augmentation of the immune response is known as immunostimulation or immunopotentiation, while suppression of immune responsiveness is called immunosuppression. The necessity of suppression of the function of the immune system is well recognized in the areas of transplantation (Madsen and Allan, 2004) and immunopathological disorders like autoimmunity (Jaworski et al., 1987). Conversely, augmentation of the immune response has been a target for increasing the host's resistance to infections and diseases. Specific immunomodulation is limited to a single antigen (such as included in a vaccine) and thus immunopotentiation is used for the development of resistance against particular diseases. Non-specific immunomodulation implies a more generalized change in the immune responsiveness leading to altered host reactivity to many different antigens. Prevention and treatment of diseases in farm animals, including fish in aquaculture, can be achieved by a antibacterial, antiviral, antiparasitic, antifungal agents and vaccines. In general, the impact of chemotherapy and vaccination on many complex diseases, however, is reaching a plateau and if further progress is to be made, different strategies have to be developed. Immunomodulation is one of the most important alternatives to control local husbandry-linked diseases with the additional advantage of amplifying specific responses to vaccines, still needed to control severe, life-threatening diseases. Immunomodulation offers the additional benefit of reversing immunosuppression caused by stress, infection, food-related problems, reproductive problems, and chronic inflammatory conditions.

3. Nutritional immunity

Proper nutrition plays a critical role in maintaining normal growth and health of cultured fish. A variety of nutritional strategies may influence fish health, including adjustment of specific nutrient levels in the diet, manipulation of nutritional condition through feeding regimens, and administration of non-nutrient immunostimulants in the diet. Immunocompetence and disease resistance can be compromised by deficiencies of various nutrients, especially certain vitamins and minerals (Halver, 1954). Thus, adequate levels of these micronutrients must be supplied in diets prepared to support optimal growth and production efficiency of fish in aquaculture. Malnutrition can easily

compromise the immune function of fish. As a consequence, infectious diseases can spread easily, certainly in more intensive aquaculture systems. Moreover, poor immune function accelerates the incidence of opportunistic infections. Boosting the immune function by providing balanced nutrition will prevent infections.

A balanced diet will include many micronutrients among which metal ions such as iron, the second most abundant metal on Earth. Feeds used in intensive aquaculture often are rich in antinutrients such as phytates and polyphenols that inhibit the absorption of iron and other metal ions. The postulated mechanism for the deleterious effects of iron deficiency on infections is a reduction in immune competence, particularly in cellular immunity which is reversible by supplementation (Dhur et al., 1989). Iron plays important roles in the generation of an immune response. Iron is essential for tissues with rapid cellular differentiation and turnover – such as occur in the immune system and the gastrointestinal tract, and for various lymphocyte functions implicated in resistance to infectious disease.

During pathogen (bacteria, parasite) invasion, the body makes a considerable metabolic adjustment in order to render important nutrients unavailable to microorganisms in a process called 'nutritional immunity'. Nutritional immunity plays a key role in iron metabolism during pathogen invasion. Host defence mechanisms increase the concentration of unsaturated transferrin in plasma, reducing iron levels, while pathogens in turn, have evolved a range of mechanisms to acquire iron from the host.

4. Iron homeostasis

Iron is distinguished by its favorable oxidation-reduction characteristics (i.e., it rusts easily, thereby liberating energy) and coordination chemistry. These features have led to its incorporation as a major constituent of hemoglobin, myoglobin, cytochromes and many enzymes involved in energy transfer. Total body iron stores average about 1 gram in men and iron turnover studies using ^{59}Fe indicate that 80% of body iron is destined for bone marrow uptake (hemopoiesis). Ferric (Fe^{3+}) iron is insoluble above pH 3, allowing it to circulate as a transferrin-bound chelate, whereas dietary iron is absorbed mainly in the reduced (ferrous) Fe^{2+} state. Iron balance is maintained by the regulation of iron absorption from the intestine in response to the level of iron stores in the body and by the amount of iron needed for hemoglobin synthesis (Finch, 1994). Intestinal iron absorption increases in deficiency states and declines during iron repletion;

absorption of dietary iron from the gut lumen is mediated by the divalent metal transporter 1 protein (DMT1), an iron uptake transporter (Georgieff et al., 2000). Enterocyte export of iron to plasma is carried out in basolateral gut cell membranes by ferroportin with help from ceruloplasmin. The main proteins mediating normal iron homeostasis are: ferritin - an intracellular Fe^{3+} storage protein, and transferrin - an extracellular Fe^{3+} transport protein.

Iron is fundamental to the biology of eukaryotic cells since it plays a key role in metabolic functions including oxygen transport, electron transfer and catalysis (Frausto da Silva and Williams, 1991). Iron concentrations in body tissues are tightly regulated because excessive iron leads to tissue damage as a result of the formation of highly reactive hydroxyl radicals (·OH) or oxidants of similar reactivity from hydrogen peroxide via the Fenton reaction: $H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + \cdot OH$. These hydroxyl radicals are responsible for toxic processes including peroxidation of biological membranes and DNA damage (Miller and Britigan, 1997). Ferritin is important to control the amount of iron available to the body. In conditions of iron deficiency, ferritin can release Fe and in conditions of iron overload, this protein can help to store the excess ions. Ferritin is an acute phase protein and it is often elevated in the course of disease (Wooldridge and Williams, 1993; Jurando, 1997), sequestering iron from microorganisms.

Iron cannot cross cellular membranes directly and requires mechanisms to facilitate its transport. In plasma, iron is transported by transferrin (Tf), a 80 kDa glycoprotein with high affinity for ferric iron and which can transport in a human body 4 mg of body iron at any one time. Although Tf has the highest affinity to Fe molecules, it is also responsible for binding and transporting of other heavy metals such as zinc or cadmium (Trisak et al., 1990). In common carp, Tf has been identified as the main protein transporting cadmium (De Smet et al., 2001). Alterations in Tf expression can occur quickly. These alterations are accompanied by changes in ferritin and Tf mRNA availability (see below). Transferrin exists as a mixture of iron-free (apo-Tf), one iron (monomeric) and two iron (diferric, holo-Tf) forms. Under normal conditions most of the iron molecules are bound to Tf (Lieu et al., 2001). In mammalian serum, Tf is 25 – 30% saturated (De Silva, 1996). Transferrin has also been identified for common carp (*Cyprinus carpio* L.), where Tf is recognized to be highly polymorphic with more than 7 different alleles identified according to differences in electrophoretic mobility.

Between the alleles, no differences in iron binding capacity were detected (Valenta et al., 1976). Functional studies into the allelic differences have not been reported for the Tf alleles of common carp.

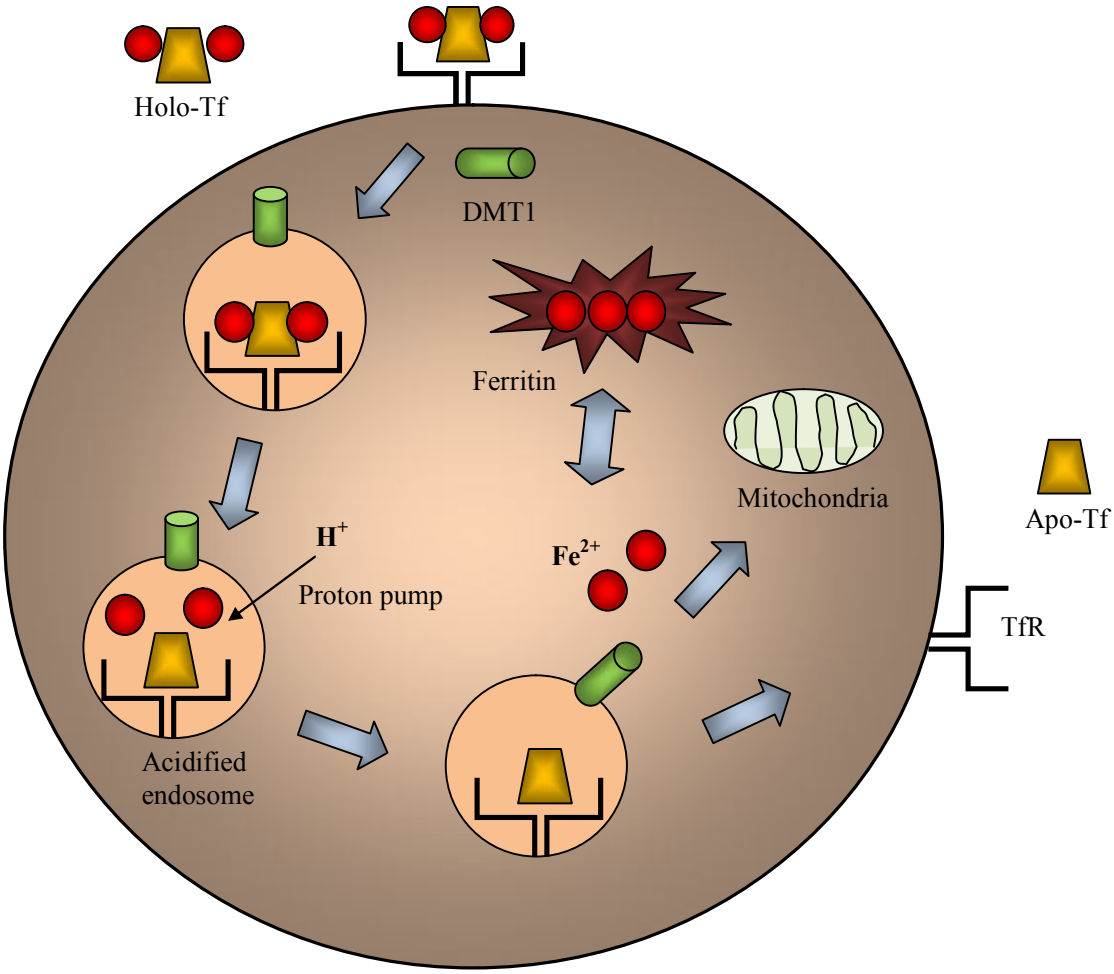

Fig. 1.1. The transferrin cycle. Holo-transferrin (Holo-Tf) binds to transferrin receptors (TFR) on the cell surface. The complex is internalized and transported to endosomes where at acidic pH iron is released from the Tf molecule. Specialized endosomes form, and become acidified through the action of a proton pump. Acidification leads to protein conformational changes that release iron from transferrin. Acidification also enables proton-coupled iron transport out of the endosomes through the activity of the divalent metal transporter 1 protein (DMT1). Subsequently, apo-transferrin (Apo-Tf) and the transferrin receptor both return to the cell surface, where they dissociate at neutral pH. Both proteins participate in further rounds of iron delivery. Iron is transported directly to mitochondria or stored as ferritin in non-erythroid cells.

Most cells acquire iron from Tf first by binding of holo-Tf to Tf receptors. Following binding, the Tf-TfR complex is internalised via a classic receptor-mediated endocytic

pathway (Fig. 1.1). For zebrafish (*Danio rerio*), a close relative of the common carp, two TfR1-like genes and a single TfR2 ortholog have been reported (Wingert et al., 2004). Iron is released from Tf within acidic endosomal compartments and apo-Tf and the TfR both return to the cell surface, where they dissociate at neutral pH (Dautry-Varsat et al., 1983). Iron is transported across the endosomal membrane into the cytoplasm. Acidification of the endosome also enables proton-coupled iron transport out of the endosomes through the activity of DMT1, a protein reported to also exist in common carp (Saeij et al., 1999).

5. Translational regulation of iron metabolism

Iron metabolism can have clear effects of translational control on gene function. Ferritin and the Tf receptor are functional opposites, with one acting as a storage protein and the other as a distributor. Changes in microenvironmental iron balance lead to reciprocal effects on the availability of ferritin and TfR mRNAs. Iron abundance activates a stem-loop iron-response element (IRE, (Rouault and Klausner, 1997)) in the 5' untranslated region of the ferritin mRNA; this IRE sequence is a potent translational repressor. Increases in extracellular iron concentration interact with the IRE to cause translational derepression of ferritin mRNA, resulting in a 50-fold enhancement of ferritin synthesis. Like other heavy metal response elements, IREs are not activated by iron per se, but by a regulatory protein (the IRE-binding protein, IRE-BP) which again may be inactivated by inflammatory mediators such as nitric oxide. This protein does not bind iron, but rather recognizes the mRNA secondary structure of both the ferritin and TfR transcripts (i.e., the stem-loop IRE). A molecule playing a reciprocal role in ferritin mRNA translation is ferritin repressor protein, which is degraded in the presence of iron (see Fig. 1.2).

Iron deficiency converts the IRE-binding protein to a high-affinity RNA binding state, leading to the repression of ferritin translation. However, absolute concentrations of ferritin mRNA may remain high, despite iron flux. The transferrin receptor mRNA also contains IREs - five in all - which are positioned in the 3' untranslated region of the message. Iron deficiency has no effect on transferrin receptor translation, but potently inhibits receptor mRNA degradation. Hence, unlike ferritin, iron deficiency increases transferrin receptor mRNA concentrations, whereas iron excess reduces mRNA levels.

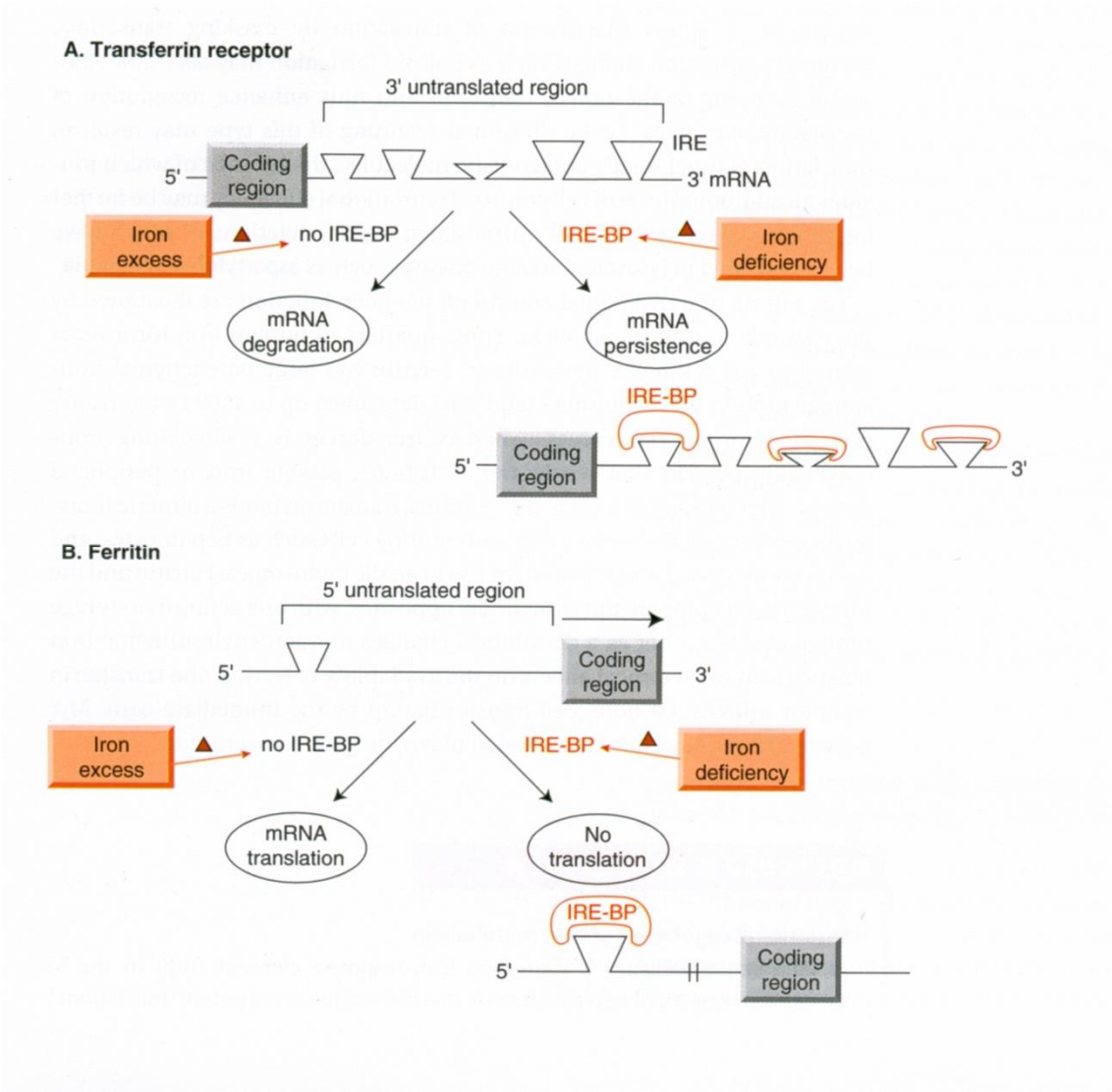

Fig. 1.2. Regulation of transferrin receptor and ferritin mRNA translation by iron availability. (**A**) In the transferrin receptor mRNA, binding of iron-responsive elements (IRE) by binding proteins (IRE-BP) in the 3'untranslated region leads to increased abundance, reflecting inhibition of mRNA degradation. (**B**) In contrast, binding of an IRE-BP to the single IRE in the 5'untranslated region of ferritin mRNA prevents translation of the coding region. Hence, iron availability differentially modulates the effects of these iron-regulatory molecules on iron availability. *(After R.J. Epstein, Human Molecular Biology, Cambridge University Press, 2003).*

In summary: ferritin and transferrin receptor are functionally opposed molecules which both contain IREs in their untranslated mRNA sequences. The ferritin IRE lies in the 5'untranslated sequence, whereas the transferrin receptor IRE lies in the 3' untranslated sequence. Activation of the 5' (ferritin) IRE by iron deficiency causes translational repression; ferritin synthesis thus depends on ferritin mRNA translational initiation. Finally, activation of the 3' (transferrin receptor) IREs by iron deficiency leads to

mRNA stabilization. The net rate of transferrin receptor synthesis therefore depends primarily on the transferrin receptor mRNA half-life. Hence, common regulatory sequences in the 5' untranslated region of ferritin mRNA and the 3' untranslated region of transferrin receptor MRNA coordinately regulate opposite net effects on ferritin and transferrin receptor biosynthesis (Fig. 1.2 (Epstein, 2003)).

6. Role of macrophages in iron metabolism

Macrophages are a major cell population of the innate immune system and also play a critical role in iron metabolism. Iron participates in many cellular functions, including the regulation of the function of different enzymes including metalloproteases, superoxide dismutase (SOD) and inducible nitric oxide synthase (iNOS). Also in common carp, macrophages are considered a prime source of hydroxyl radicals (Verburg van Kemenade et al., 1994) and nitric oxide (NO) (Saeij et al., 2000). NO is involved in the pathogenesis and control of many infectious diseases and can react with DNA, proteins, thiols, prosthetic groups and reactive oxygen intermediates (ROIs), among others. Many of the targets of NO are transcription factors. The control of iron uptake and storage is regulated by the feedback system of IRE-containing gene products and regulatory proteins that modulate the expression levels of the genes involved in iron metabolism (Lieu et al., 2001). The regulatory proteins, as mentioned above, may again be inactivated by inflammatory mediators such as nitric oxide.

Transferrin itself, however, may also exert effects that are not directly linked with maintaining iron levels. In goldfish, Tf cleavage products have been shown to act as macrophage activating factor (MAF) by stimulating macrophages to produce large amounts of nitric oxide (Stafford and Belosevic 2003; Stafford et al. 2001). Possibly, neutrophilic granulocytes, that are generally the first immune cells recruited to the site of inflammation, initiate the cleavage of transferrin via the production of neutrophil-derived proteases (Miller et al. 1996). The recognition of endogenous ‘danger’ signals (*e.g.* molecules produced by stressed cells, or products that are usually found inside a healthy cell) by the immune system is the basis of the "Danger model" (Matzinger 1994). The innate immune system is now considered the real gatekeeper of whether the immune system needs to respond or not. The danger hypothesis states that antigen-presenting cells respond to "danger signals" - most notably from cells undergoing injury, or stress or necrosis (mechanical cell death as opposed to apoptosis, which

genetically controlled cell death). The alarm signals released by these cells instruct the immune to mount an appropriate immune response. It would be intriguing to investigate if Tf polymorphic differences, as observed for the common carp, would relate to differences in the production of endogeneous danger signals, rather than to differences in iron-binding capacity.

7. Competition for iron by pathogens

During nutritional immunity the body reacts with a metabolic adjustment in order to render important nutrients unavailable to invading microorganisms. However, pathogens also have evolved a range of mechanisms to acquire iron from the host. Most bacteria posses surface receptors binding Tf or lactoferrin. Iron acquisition can also include bacterial proteases that cleave host iron binding proteins and release iron for microbial use (Wilson and Britigan, 1998). Also parasitic protozoa have evolved mechanisms for iron uptake from the host. Studies on *Trypanosoma cruzi* showed binding and uptake of Tf by endocytosis via vesicles that appear uncoated and showed delivery to membrane-bound acidic organelles, called reservosomes (Soares et al., 1992). *Trichomonas vaginalis*, a protozoan found in the female vaginal tract, harbors specific receptors for host lactoferrin (Peterson and Alderete, 1984), but can also take up iron from hemoglobin by a receptor-mediated fashion (Lehker et al., 1990). The malaria parasite *Plasmodium* spp. do not acquire iron via degradating hemoglobin, but heme-bound iron is sequestered as an insoluble pigment called hemozin (Salas et al., 1995). *Leishmania* sp. promastigotes can be cultured in medium containing serum (as a source of Tf), blood, hemin or hemoglobin (Chang and Hendricks, 1985). The protozoan *Trypanosoma brucei* has up to 20 expression sites in its genome that encode similar but not identical transferrin receptors, one being active at a given time. During natural transmission from one host species to another, trypanosomes have to deal with the diversity of mammalian transferrins. By switching between different expression sites, the parasite can express different host - specific transferrin receptors (Bitter et al, 1998; Steverding, 2000). Transferrin uptake by *Trypanosoma brucei* involves Tf binding to a receptor encoded by two homologous expression site-associated genes (ESAG6 and ESAG7; Steverding et al., 1994; Steverding and Overath, 1996). The TfR-TF complex is internalised and transported to lysosomes, where Tf is proteolytically degraded. The resulting large peptide fragments are released from the trypanosomes

while iron remains cell-associated. The receptor is probably recycled to the membrane of the flagellar pocket (Wilson and Britigan, 1998; Steverding, 2000) (Fig. 1.3).

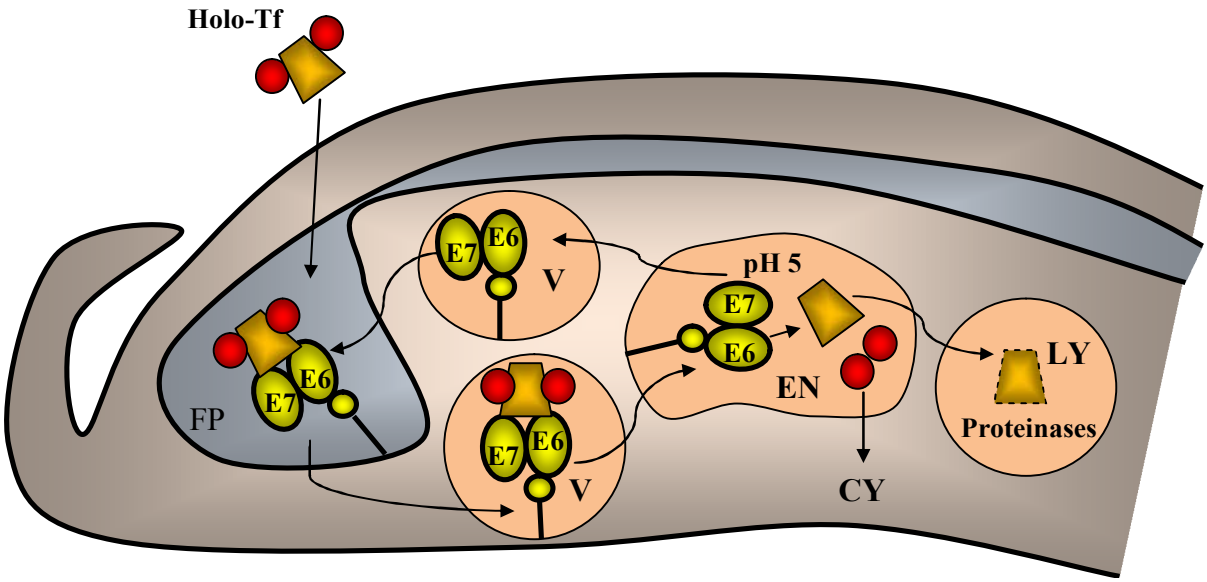

Fig. 1.3. Transferrin uptake by *Trypanosoma brucei*. Holo-transferrin (Holo-Tf) binds to a heterodimeric Tf receptor (E6/E7) situated in flagellar pocket (FP). The complex is internalized by endo- and exocytotic vesicles (V) and delivered into the endosome (EN) where at acidic pH, iron is released from the Tf molecule. Apo-transferrin is delivered into lysosomes (LY) where it is proteolytically degraded. Iron remains cell-associated. The transferrin receptor returns to the membrane of flagellar pocket.

8. Genetic selection for transferrin genes in common carp

Freshwater cyprinids (e.g. *Cyprinus carpio* L.) in Central and Eastern Europe are cultivated in natural ponds on a semi-intensive scale. But carp fry and young-of-the-year fishes are highly vulnerable to diseases caused by a wide range of pathogenic organisms (viruses, bacteria and parasites). One of the key issues improving the immune capacity of fish is genetic selection for innate disease resistance. The Institute of Ichthyobiology and Aquaculture of the Polish Academy of Sciences (IIA-PAS) possesses a carp live gene bank that contains 19 breeding lines with considerable genetic variation in survival under pond conditions (9-68 % survival at one year of age). To detect correlations between these differences in survival rate and differences in resistance to a specific pathogen under laboratory circumstances, we will use infection with *Trypanoplasma borreli*, a well-characterized and widely used parasite model. Gene of prime interest will be transferrin (Tf) The host's iron reserve bound by transferrin is a critical factor for parasite growth. Genes encoding for Tf are polymorphic in carp.

Impact of fish diseases on carp aquaculture

One of the key issues facing aquaculture development in Europe is the semi-intensive and extensive production of freshwater finfish such as cyprinids in Central and Eastern European countries, targeted toward the domestic market. Freshwater cyprinids are the second major group of finfish cultivated in Europe (105,000 mt), with common carp *(Cyprinus carpio L.)* accounting for 83,000 mt (FAO, 1995). Aquaculture production in Central and Eastern European countries will increase with increasing economic stability and the continued privatization of the sector. Greater production will be achieved, in part through further intensification of existing traditional extensive farming practices. Carp in Central Europe are cultivated on a semi-intensive scale in natural ponds that hold fishes from one-year class only. In these systems, carp fry and young-of-the-year fishes are highly vulnerable to diseases, like spring viraemia, *Aeromonas salmonicida, Ichthyophthiritis multifilliis, Trypanoplasma borreli,* and farmers accept "unavoidable" losses caused by predators, handling and pathogens of 30-90 % during the first year of life (Proske, 1998). Thus, heavy losses during the first year impose a serious constraint on the profitability of carp farming. Diminishing the impact of fish diseases is an important issue in sustaining and developing an economic aquaculture industry. Antibiotics and other chemotherapeutants are generally cost-effective in controlling some of the diseases and have consequently become an integral part of modem aquaculture. Chemotherapeutants are currently used to treat bacterial and parasite infections in fish and have become an integral part of modern aquaculture, but their use will increasingly be restricted by the European Drug Agency. The recognition of farming ponds as valued ecosystems (Szarowski, 1997) further argues against pharmacological interventions and strongly in favor of sustainable approaches, i.e. genetic selection for increased resistance to disease. Important alternatives and certainly more sustainable approaches are vaccination, immunostimulation by feed additives and/or genetic selection for inherited immune capacity. Effective vaccines are only cost-effective in intensive culture systems. Genetic selection will therefore be a potential and sustainable approach to disease control in semi-intensive carp pond farming.

Genetic selection and disease resistance

A long-term aim of the IIA-PAS is to prevent diseases by improving the immune capacity of fish by genetic selection, increasing production in a sustainable manner.

Notable success has been reported for genetic selection for disease resistance in fish (Chevassus and Dorson, 1990; Wiegertjes et al, 1996), and two major approaches can be discerned: (1) direct breeding for families with higher resistance and (2) indirect breeding based upon markers potentially associated with an improved health status. Probably the best example of direct breeding of fish for resistance to infectious disease is the selection of carp resistant to "infectious dropsy" (Kirpichnikov et al, 1993). However, difficulties associated with direct breeding for genetic disease resistance of carp, such as the long generation intervals (slow genetic progress), the need to apply mass or family selection (labor intensive), and the need to use large pond surfaces (high costs) strongly limit the possibility of direct selection. Indirect breeding is based upon markers potentially associated with an improved health status. Such markers can range from phenotypic features, such as scale patterns, to immune parameters and gene polymorphisms (Teale, 1994). To date, the number of potential markers for disease resistance in carp is rapidly increasing and includes polymorphic sequences for i.e. interleukins, complement, transferrin and alpha-2-macroglobulin (Onara et al., 2008). Further, the major histocompatibility complex (MHC) genes have been shown highly polymorphic, not only in salmonid fish (Stet et al, 2000) but also in carp (Rakus, 2008). Recent data indicate a clear potential for the use of MHC genes as quantitative trait loci for disease resistance in Atlantic salmon (Grimholt et al, 2000) and these genes will also be subject of future study in carp. In this thesis we will concentrate on the polymorphism observed for carp transferrin as a gene of prime importance in the innate immune response.

9. Aim and outline of the thesis

The cyprinid common carp (*Cyprinus carpio* L.) is a natural host of the blood parasite *Trypanoplasma borreli* that belongs to the suborder Bodonina, family Cryptobiidae. For comparison; *Trypanosoma brucei* is classified in the suborder Trypanosomatina, family Trypanosomatidae. All kinetoplastid flagellate parasites have a kinetoplastid organelle containing the mitochondrial DNA, a glycosome compartmentalising glycolysis and a mini-exon; a highly conserved short RNA leader sequence trans-spliced onto every messenger RNA. Bodonids such as *T. borreli* have two flagella and can be ecto- or endoparasites of fish. *T. borreli* lives extracellular in the blood and tissue fluids of the host. In nature, transmission of the parasite between fish is achieved via blood-sucking

leeches (*Piscicola geometra* or *Hemiclepsis marginata*) (Lom and Dyková, 1992) that, in the case of *T. borreli* merely act as vector. Infections with *T. borreli* are widespread in farmed populations of common carp. Genetic differences in resistance to *T. borreli* between different carp lines have been described (Wiegertjes et al. 1995). In the laboratory, parasitaemia can easily be established by intraperitoneal injection (Jones et al. 1993) of carp and counted in the blood with the use of a haemocytometer. In this thesis we have applied the *T. borreli* infection model to get more insight in the competition for iron between host and parasite and the importance of Tf polymorphism for modulation of immune function.

In **chapter 1** we introduced iron as an important biological factor in eukaryotic cells and explained the role of transferrin in iron acquisition and transport for both host and pathogen. In **chapter 2** we present a series of challenge experiments on commercially exploited carp lines, using the protozoan *T. borreli*. We genotyped carp used in this experiments showing that DD, DG and DF as the most frequent Tf genotypes in carp breeding lines. We demonstrated significant effects of genetic background of the carp lines on susceptibility to *T. borreli*. This genetic effect was preserved in a next generation. We could detect a significant association of the transferrin genotype with parasitaemia level in the resistant carp breeding lines and the lack of a such an association in the most susceptible line. Upon examination of parasite growth *in vitro*, in culture media supplemented with 3% serum taken from carp with different Tf genotypes, we could show a faster decrease in number of parasites in culture media with serum from DD-typed animals. In **chapter 3** we confirm that Tf is of prime importance for the growth and multiplication of *T. borreli*. We demonstrated that parasite cannot survive in medium supplemented with the Tf-depleted serum while reconstitution with Tf restores normal growth. The addition of purified Tf into incomplete medium (without serum) significantly increased parasite survival. We also confirmed previous finding that Tf polymorphism has a significant impact on *T. borreli* welfare *in vitro*. Cultured parasites die more quickly in an environment containing D-typed transferrin, as compared to medium with G-typed transferrin. We couldn't show an acute phase protein response of Tf during *T. borreli* infection, as was tested by gene expression (RQ-PCR analysis). In **chapter 4** we describe the cloning and cDNA analysis of four allelic forms of transferrin. We concentrated on allelic diversity with respect to differences in sequence, constitutive transcription and three-dimensional structure.

Protein modeling confirmed the overall conservation of the three-dimensional structure of carp Tf. Beside the overall similarity with mammalian Tf, we found that in the N-lobe the majority of iron coordinating residues were not conserved. We speculate that this may have a serious impact on the ability of carp Tf to bind iron. We also show that carp Tf is not a glycoprotein. The distribution of polymorphic sites on the molecule are located outside of the areas which are important for the iron binding ability or ability to bind with the host Tf receptor. We showed that, similarly to other species, the liver is the main organ of Tf production in carp. At the same time we noticed that the basal gene expression of D allele was higher than that of the G allele in other immune related organs, like head kidney, thymus and spleen. Preliminary results with Tf-typed serum suggest a difference in the ability of Tf alleles D and G to modulate the LPS-induced NO production in carp macrophages. **Chapter 5** reports on the isolation of two carp transferrin alleles (alleles D and G) to purity and describes the induction of nitric oxide by cleaved transferring in carp head kidney-derived macrophages. We show that activated macrophages appear to be the source of necessary enzymes required for cleavage of Tf into immunostimulatory fragments. We confirm this by Western blot analyses which shows cleavage products in the activated macrophage cultures, but not in non-activated macrophages. We demonstrate the superiority of the D-type over the G-type Tf in inducing nitric oxide (NO) and confirm that full-length Tf cannot induce NO. We speculate that cleaved Tf fragments are “alarmins”, which function by alerting the immune system to tissue damage or infection. We discuss the possibility that parasites such as *Trypanoplasma borreli* cleave Tf and use Tf fragments to their advantage by modulating the NO induction in carp macrophages. In **chapter 6** we describe the cloning and sequencing of a cathepsin L-like cysteine proteinase from *T. borreli* and the production of a recombinant and biologically active enzyme. We also demonstrate that the *T. borreli* cysteine proteinase is able to digest host proteins, e.g. transferrin. Likely, Tf cleavage fragments are released from the trypanosomes while the iron would remain parasite-associated, possibly contributing to the pathogenicity of the parasite by inducing high amounts of NO in carp macrophages. Finally, in **chapter 7,** we discuss the role of Tf in immunity of common carp and the influence of allelic polymorphism on competition for iron between the host and the pathogen.

2

Genetic resistance of carp (*Cyprinus carpio* L.) to *Trypanoplasma borreli*: Influence of transferrin polymorphisms

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Abstract

In serum most of the iron molecules are bound to transferrin (Tf), which is a highly polymorphic protein in fish. Tf is an essential growth factor for mammalian trypanosomes. We performed a series of experiments with *Trypanoplasma borreli* to detect putative correlations between different Tf genotypes of common carp (*Cyprinus carpio* L.) and susceptibility to this blood parasite. Five genetically different, commercially exploited carp lines (Israelian 'D', Polish 'R2' and 'K', Ukrainian 'Ur', Hungarian 'R0') and a reference laboratory cross ('R3xR8') were challenged with *T. borreli* and parasitaemia measured to determine susceptibility to the parasite. Among the commercial carp lines, Israelian 'D' carp were identified as most and Polish 'R2' carp as least susceptible, and used to produce a next generation and reciprocal crosses. These progenies were challenged with *T. borreli* and parasitaemia measured. We demonstrated significant effects of genetic background of the carp lines on susceptibility to *T. borreli*. This genetic effect was preserved in a next generation. We also observed a significant male effect on susceptibility to *T. borreli* in the reciprocal crosses. Serum samples from a representative number of fish from two infection experiments were used for Tf genotyping by polyacrylamide gel electrophoresis (PAGE), identifying DD, DG and DF as most frequent Tf genotypes. We could detect a significant association of the homozygous DD genotype with low parasitaemia in the least susceptible 'R2' (and 'K') carp lines and the lack of a such an association in the most susceptible 'D' carp line. Upon examination of parasite growth in vitro in culture media supplemented with 3% serum taken from fish with different Tf genotypes, we could show a faster decrease in number of parasites in culture media with serum from DD-typed animals.

Chapter 2

Introduction

There are many factors that affect the growth and multiplication of blood parasites. One of them is iron availability. In serum, under normal conditions, most of the iron molecules are bound to transferrin (Tf). Transferrin is a 80-kDa glycoprotein consisting of two globular domains (N and C), both containing a high-affinity binding site for a single iron molecule (Aisen and Listowsky, 1980). In serum, Tf exists as a mixture of iron-free (apo), one iron (monomeric) and two iron (holo) forms. The relative percentage of each form depends on the concentration of iron (Lieu et al., 2001). The major role of Tfs is transportation of iron that participates in many crucial metabolic processes, among which DNA synthesis and oxygen and electron transport. Most cells acquire iron from Tf by binding to transferrin receptors (TfR). Two holo Tfs are bound by one TfR on the cell surface and thereafter, in the endosome, iron is released from the Tf-TfR complex at acidic pH. The Tf-TfR complex recycles back to the cell surface and the apo-Tf dissociates from the receptor (Dautry-Varsat et al., 1983).

The importance of Tf for blood parasites such as trypanosomes has been clearly shown by studies on *Trypanosoma brucei* (Metakinetoplastida, Trypanosomatida). Transferrin is an essential growth factor for the bloodstream form of *T. brucei* that cannot grow in medium containing Tf-depleted serum (Schell et al., 1991). Transferrin uptake by *T. brucei* involves binding to a heterodimeric Tf-binding protein complex, which is internalised and transported to lysosomes where Tf is proteolytically degraded. The resulting peptide fragments are released from the trypanosomes while iron remains cell-associated (Steverding, 2000). The cyprinid common carp (*Cyprinus carpio* L.) is a natural host of the blood parasite *Trypanoplasma borreli* (Metakinetoplastida, Cryptobiidae) (Wiegertjes et al., 2005). Transferrin is an important component of carp serum (De Smet et al., 1998), which confirms the relevance of the present investigation into the relationship of Tf and carp host susceptibility to *T. borreli*.

Transferrin generally is highly polymorphic in fish. Transferrins of salmonid fish were extensively studied, and associations between polymorphism and resistance to bacterial disease were reported (Suzumoto et al., 1977; Winter et al., 1980). In goldfish (*Carassius auratus*), up to eleven Tf variants were identified (Yang et al., 2004). Both salmonid and goldfish Tfs contain nucleotide regions that seem to have undergone positive selection through evolution (Ford, 2001; Yang and Gui, 2004). This suggests Tf plays an important role in the resistance of fish against pathogens. Although genetic

differences in resistance to *T. borreli* between carp strains have been reported (Jones et al., 1993; Wiegertjes et al., 1995), we studied for the first time the association of Tf genotypes and genetic resistance to *T. borreli* of carp.

We report a series of infection experiments of carp with *T. borreli* to detect putative correlations between different Tf genotypes, as identified by non-reducing poly-acrylamide gel electrophoresis (PAGE), and susceptibility to parasite infection. For the infection experiments we used a number of five commercially exploited carp lines of different genetic background with a record of high or low survival under pond conditions (Pilarczyk, 1998). As a reference, we used a laboratory cross with a record of high parasitaemia after experimental infection with *T. borreli* (Saeij et al., 2003). Serum samples from a representative number of fish from the different carp lines were genotyped for Tf by PAGE and the correlation between Tf genotype and susceptibility to *T. borreli* studied. Genetic differences in susceptibility to *T. borreli* between carp with different Tf genotypes were further examined by studying the parasite growth in culture media supplemented with 3% serum taken from fish with different Tf genotypes. The potential role of Tf as mediating factor in the susceptibility to *T. borreli* is discussed.

Material and methods

Fish

European common carp (*Cyprinus carpio carpio* L.) were the progeny of five commercially exploited carp lines of Polish 'R2' and 'K', Ukranian 'Ur', Hungarian 'R0' and Israelian 'D' origin. In addition, we used a reference carp line 'R3xR8' highly susceptible to experimental infection with *T. borreli* (Saeij et al., 2003). Progenies of all carp lines were raised in recirculating systems at either of two locations: the Institute of Ichthyobiology and Aquaculture of the Polish Academy of Sciences in Poland, or the Wageningen University in The Netherlands and used for three experimental challenges. The initial challenges identified two carp lines, the Polish 'R2' and Israelian 'D' as relatively resistant and susceptible, respectively. A sexually mature dam was taken from each of these two carp lines and crossed with a sire from both carp lines, resulting in 'R2xR2', 'DxD', 'R2xD' and 'DxR2' crosses for subsequent challenge with *T. borreli*. Fish were grown in aquarium systems at 20-23 ^{o}C according to standard procedures and were fed ad libitum. Two weeks prior to each challenge the animals were marked by

tattoo, their weight taken and randomly distributed over new aquaria in the same recirculation system. There were no significant difference in fish weight between carp lines. Carp were 8-9 months old with an average weight of 90 ± 25 g at challenge.

Parasite infection

T. borreli, initially cloned and characterised by Steinhagen et al. (1989) were maintained by syringe passage through susceptible carp ('R3xR8'). Carp were anesthetized with Propiscine 0.2% (Kazuń and Siwicki, 2001) prior to intraperitoneal infection with $1x10^4$ or $2x10^5$ parasites. Blood samples (100 µl) from the caudal vein were taken after anaesthesia at weekly intervals starting the 2nd week post infection (p.i.) to determine parasitaemia. Parasitaemia was determined using a Bürker counting chamber with a minimal detection limit of 10^4 parasites/ml blood. Before counting the blood samples were diluted with PBS 20 times.

In the 1st experiment (Poland), n=15 fish from each carp line (Polish 'R2' and 'K', Israelian 'D', Hungarian 'R0', Ukrainian 'Ur' and reference carp 'R3xR8') were used, equally divided over n=5 aquaria and challenged i.p. with a high dose of $2x10^5$ *T. borreli*/fish. In the 2nd and 3rd experiments, the same carp lines were challenged with a medium dose of 10^4 *T. borreli*/fish. These last two experiments were done simultaneously at two different locations (The Netherlands and Poland) to examine the influence of possible environmental effects on development of parasitaemia. In the experiment located at The Netherlands (2nd experiment), n=15 fish from each line were divided over n=5 aquaria, while in the 3rd experiment (Poland) 2xn=15 fish from each carp line were divided over 2xn=5 aquaria. For the 4th experiment (Poland) a new generation and reciprocal crosses between 'R2' and 'D' carp ('R2xR2', 'DxD', 'R2xD' and 'DxR2') were used (n=30 fish from each cross). Here, animals were equally divided over n=2 aquaria and challenged with a medium dose of 10^4 *T. borreli*/fish.

Identification of transferrin genotypes by polyacrylamide gel electrophoresis (PAGE)

Transferrin genotypes were identified by PAGE electrophoresis (Valenta et al., 1976; Irnazarow and Białowąs, 1994). Serum samples were taken from fish from the 3rd (n=180) and 4th (n=120) experiments. Briefly, serum samples (5 µl) were diluted in 15 µl loading buffer (40% sucrose, 1.5% bromophenol blue; Sigma-Aldrich, St. Louis, MO, USA) and 2 µl of suspension applied on a 6% stacking and 15% polyacrylamide

running gel. Electrophoresis was carried out in running buffer (72 mM Tris, 26 mM boric acid) at 90V for 30 min followed by 250V for 5 h (Smithies, 1955). Protein bands were stained for 1 h with 0.04% Coomassie Brilliant Blue dissolved in 3.5% perchloric acid.

In vitro growth of T. borreli in culture medium containing transferrin-typed carp serum

T. borreli was isolated from the blood by centrifugation (Steinhagen et al., 2000), purified by column chromatography (Overath et al., 1998) and washed in Hank's Balanced Salt Solution. Trypanoplasms were seeded at 10^5 cells/ml in 96-well culture plates and incubated at 26°C. Medium was supplemented with 3% carp serum, pooled from n=20 different individuals taken from crosses between the commercially exploited carp lines mentioned above. The individuals used for the pooled carp serum were typed as DD, DG or GG. Or, as negative control, medium was not supplemented with serum. The number of parasites was determined every 24 hours using a Bürker counting chamber.

Statistical analysis

An analysis of variance of the parasitaemia was completed using the general linear model (GLM) procedure in STATISTICA (version 6.0) with replications and location treated as random effects, and parasite infection dose or genotypes as fixed effects. Means were compared with Fisher's protected least significant difference (LSD) values. Individual parasitaemia records obtained during subsequent 5 sampling points (infection experiments 1-3) or 10 sampling points (infection experiment 4) were expressed as area under the curve (AUC) values. We examined the effects of infection dose by comparing mean parasitaemia of all individuals used in the separate challenge experiments. Putative laboratory-related (location) effect was studied using data of challenge experiments 2 and 3, taking location as a random and carp breeding line as a fixed effect. Second, we analysed Tf genotype (fixed factor) and carp origin (random factor) effects on parasitaemia. The effect of Tf genotype was analyzed in each of the infection experiments separately, except for experiments 2 and 3, where individuals from the same carp lines were pooled. This could be done because there was no significant location-dependent effect on parasitaemia within the carp lines. A separate variance analysis for experiment 4 was run. The parasitaemia differences among the DxD,

R2xR2, DxR2 and R2xD groups was then analysed as a pair combination between parental and reciprocal groups, according to significant effects. These statistical analyses were performed separately for females and males. Significance of differences in Tf genotype distribution within and between carp lines was accessed by Chi squared analysis. Differences were considered significant at $P < 0.05$.

Results

Carp show genetic differences in resistance to T. borreli

Statistical analysis indicated the absence of tank effects and therefore replicates were pooled (data not shown). Parasitaemia was monitored weekly for a period of 5 (experiments 1-3) or 10 (experiment 4) weeks post-infection (w.p.i.). For clarity the data for the carp lines 'R0' (Hungarian) and 'Ur' (Ukranian) are not depicted. The 1st experiment was used to infect carp with a relatively high dose of parasites ($2x10^5$ *T. borreli*/fish) to determine the susceptibility of the commercially exploited carp lines, relative to the reference carp ('R3xR8'). Intraperitoneal injection of *T. borreli* induced a relatively high parasitaemia in two out of five commercial carp lines (Polish 'K' and Ukranian 'Ur'), in contrast to the Polish carp line 'R2' which had a significantly lower parasitaemia ($P = 0.03$) (Fig. 2.1A). Carp of the Hungarian 'R0' and Israeli 'D' lines developed intermediate parasitaemia. The relatively high infection dose of parasites applied in the 1st experiment induced a rapid development of parasitaemia in all carp lines, possibly overruling smaller differences in susceptibility between the carp lines. For that reason, in subsequent infection experiments, the infection dose was lowered to 10^4 *T. borreli*/fish.

The 2nd and 3rd experiments were performed simultaneously at two different locations to study putative laboratory-related effects. In general, peak parasitaemia in response to the lower infection dose was delayed while overall parasitaemia was significantly lower ($P = 0.00$). In the 2nd experiment (location The Netherlands), as also noted in the 1st experiment, the reference carp developed relatively high parasitaemia. Again, the Polish carp line 'R2' developed relatively low parasitaemia (Fig. 2.1B). Carp from line 'D' developed the highest parasitaemia amongst the commercially exploited carp lines, while carp from line 'K' and line 'Ur' developed intermediate-low parasitaemia. Also in the 3rd experiment (location Poland) the reference carp developed high parasitaemia, resulting in some mortality (Fig. 2.1C). Similar to previous experiments, carp from the

Polish line 'R2' showed the lowest and Israeli carp from line 'D' showed the highest parasitaemia amongst the commercial carp lines. Effects of the genetic background on parasitaemia were independent of the laboratory where the challenge was performed.

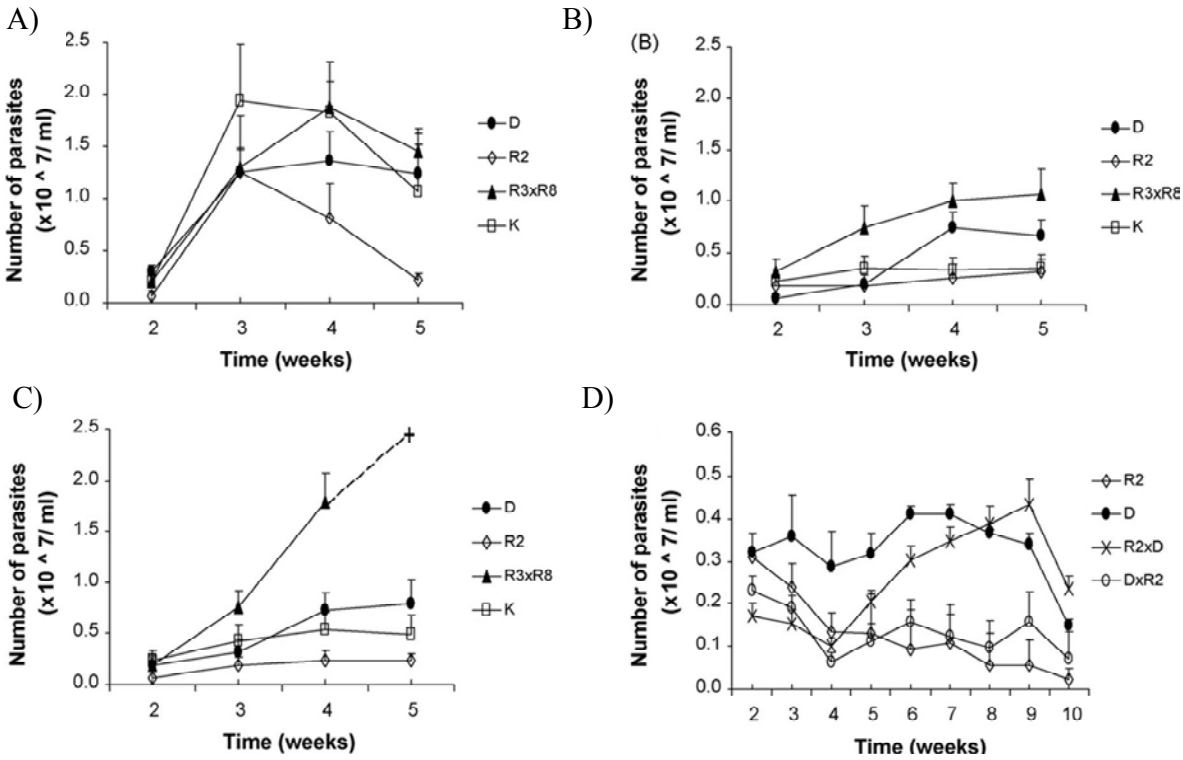

Fig. 2.1. Parasitaemia (*T. borreli*/ml) of a reference laboratory cross (R3xR8), of genetically different European carp lines originating from Israel ('D'), Poland ('K' and 'R2'), or of reciprocal crosses between Polish and Israelian carp ('R2xD' and 'DxR2') during a period of 5 weeks (**A**-**C**) or 10 weeks (**D**) post-infection. Individual parasitaemia was determined as area under the curve (AUC) values. (**A**) 1st experiment: carp (n=15/line) were injected i.p. with a high dose of $2x10^5$ trypanoplasms/fish at the location in Poland. (**B**) 2ndd experiment: carp (n=15/line) were injected i.p. with a medium dose of 10^4 trypanoplasms/fish at the location in The Netherlands. (**C**) 3rd experiment: carp (n=30/line) were injected i.p. with a medium dose of 10^4 trypanoplasms/fish at the location in Poland. '+' during the 5th week p.i. fish of line R3xR8 were dying. (**D**) 4th experiment: carp (n=30/line) were injected i.p. with a medium dose of 10^4 - trypanoplasms/fish at the location in Poland.

We substantiated the genetic effects observed in carp from lines 'R2' and 'D' in the 4th experiment, where not only a next generation of 'R2' and 'D' carp but also carp from the reciprocal crosses 'R2xD' and 'DxR2' were challenged with *T. borreli*. For unknown reasons, overall levels of parasitaemia were lower than in the 2nd and 3rd experiment where fish were injected with the same number of parasites. We did not

observe the characteristic parasitaemia peak at 4th or 5th weeks p.i., therefore the observation was prolonged up to 10th week. Upon close examination, again, carp from line 'R2' developed parasitaemia lower than in carp from line 'D' (Fig. 2.1D). Parasitaemia in the reciprocal crosses showed a clear reciprocal effect manifested as high parasitaemia in 'R2xD' but low parasitaemia in 'DxR2' groups (Fig. 2.1D). In other words, the progeny of the 'R2' male developed a significantly lower parasitaemia than the progeny of the 'D' male (P = 0.00). A sex effect on parasitaemia therefore is suggested for the progeny of the 'DxR2' cross. Lack of significant differences in parasitaemia between 'D' and 'R2xD' and between 'R2' and 'DxR2' indicated a complete dominant, possibly sex-linked effect.

Carp possess polymorphic transferrin

Serum samples from a representative number of fish from the different carp lines were genotyped for Tf by PAGE with the aim to study correlation between Tf genotype and susceptibility to *T. borreli*. As shown in figure 2.2, four Tf genotypes could be identified. Identification of the Tf proteins as D, F or G was according to Irnazarow and Białowąs (1994). The presence of the F allele was unique to Polish carp from line 'K'.

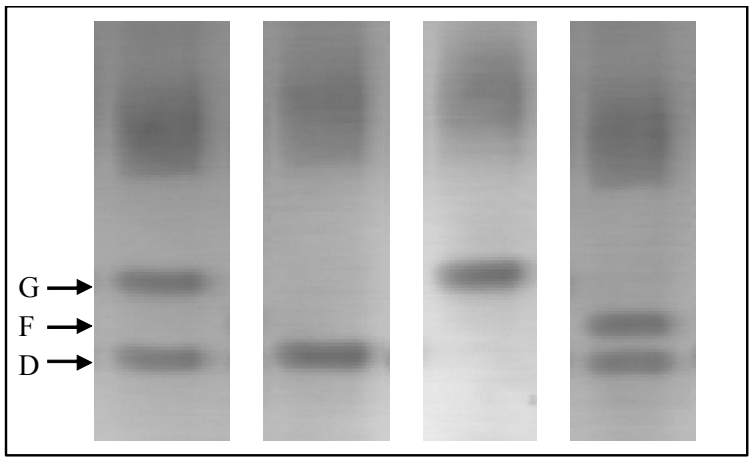

Fig. 2.2. Four different transferrin genotypes as detected by non-reducing polyacrylamide gel electrophoresis (PAGE) in European common carp (from the left: DG, DD, GG and DF). Samples were applied on a 6% stacking and 15% polyacrylamide running gel. Protein bands were stained with 0.04% Coomassie Brilliant Blue dissolved in 3.5% perchloric acid.

Serum samples for Tf genotyping were taken from fish from the 3rd and 4th experiments. All individuals from the reference 'R3xR8' carp line were typed as heterozygous DG and could not be used for association studies, since in these carp the Tf alleles were not segregating. As described above, among the commercial carp lines, Israelian 'D' carp were identified as most and Polish 'R2' carp as least susceptible to *T. borreli*. Three different Tf genotypes (DD, GG and DG) were detected in the Israelian 'D' and Polish 'R2' carp lines. The distribution of the genotypes within lines 'D' and 'R2' was not in accordance with the Hardy-Weinberg equilibrium, manifested by a significant deficiency of DG and GG genotypes in line 'R2' ($P = 0.03$) and a deficiency of the GG genotype in line 'D' ($P = 0.04$) (see next section).

For the 4th experiment a sexually mature dam was taken from each of the Polish 'R2' and Israelian 'D' carp lines and crossed with a sire from both carp lines. In these crosses, three genotypes (DD, DG and GG) were found, indicating the parents were heterozygous DG. In the 'R2xR2' and 'DxR2' crosses the observed frequencies for Tf were in accordance with the expected ratio of 1:2:1, while in the 'DxD' and 'R2xD' crosses we observed a significant deficiency of DG and GG genotypes (P=0.02).

Transferrin genotype can influence parasitaemia in vivo

We observed a clear effect of genetic background on parasitaemia, measured as area under the curve values. Individual marking allowed us to investigate the relation between Tf genotype and parasitaemia. To do so we included carp genetic background as a second factor in the ANOVA and analysed the association between the most frequently occurring genotypes DD, DG and DF and parasitaemia in the commercially exploited carp lines (3rd experiment, carp lines 'R2', 'D' and 'K').

Neither significant differences between DD, DG and DF individuals nor significant Tf genotype-carp line interactions could be detected when Tf genotypes were analysed for all carp lines. However, effects of Tf on parasitaemia were more clear on a uniform genetic background. We analysed separately three carp lines with low ('R2'), intermediate ('K') and high ('D') parasitaemia. In the carp line with low parasitaemia (Polish 'R2': 71% DD, 26% DG, 3% GG) homozygous DD individuals showed a significantly lower parasitaemia than DG heterozygotes ($P = 0.05$) (Fig. 2.3A). In the carp line with high parasitaemia (Israeli 'D': 41% DD, 48%DG, 11% GG), in contrast, homozygous DD individuals showed a significantly higher parasitaemia than DG

heterozygotes (P = 0.02) (Fig. 2.3B). In the carp line with intermediate parasitaemia (Polish 'K': 39% DD, 54% DF, 7% FF), homozygous DD individuals showed a lower parasitaemia than DF heterozygotes, although this observation was not statistically significant (Fig. 2.3C).

The above analysis showed that of all Tf genotypes, DD individuals contributed most to the observed variation in parasitaemia. Analysis of the parasitaemia in the 4th experiment suggested a possible involvement of a sex-related factor because of a strong 'R2' male effect on the variation in parasitaemia. To further get insight in the role of Tf we analysed two factors simultaneously – the parental effect and Tf genotype. Statistical analysis showed no significant differences in parasitaemia between DD and DG genotypes in the two most susceptible crosses ('DxD' and 'R2xD'; progeny of the 'D' male) (data not shown). In contrast, in the two least susceptible crosses ('R2xR2' and 'DxR2'; progeny of the 'R2' male), GG genotypes showed significantly higher parasitaemia than DD and DG genotypes (P = 0.02).

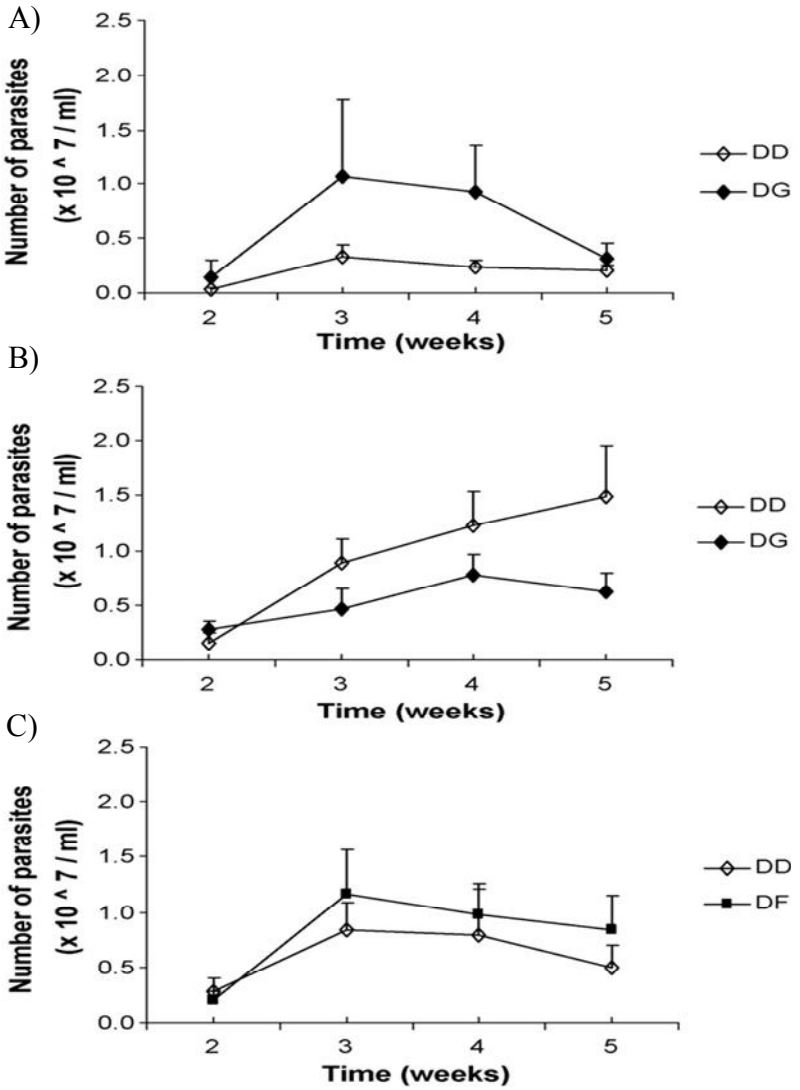

Fig. 2.3. Parasitaemia (*T. borreli*/ml) per carp line (**A-C**), per transferrin genotype (DD, DG or DF) during a period of 5 weeks post-infection. Data shown are from sera from individual fish (n=30) of experiment 3 (Poland), typed for transferrin genotype by PAGE. (**A**) Polish 'R2' carp line. (**B**) Israeli 'D' carp line. (**C**) Polish 'K' carp line.

3.4. In vitro parasite growth is mediated by transferrin genotype

Growth of *T. borreli* was observed only in culture media supplemented with serum, suggesting the presence of serum to be a critical factor for parasite multiplication (Fig. 2.4). Supplementation with 3% carp serum initially resulted in a doubling of the number of parasites during the first 72h, independent of the serum source, but at later time points resulted in a gradual decline in parasite number. There was no difference in the rate of parasite decline between the media supplemented with serum from DG- or GG-

typed fish. In contrast, in media with serum from DD-typed fish the rate of parasite decline was significantly faster ($P = 0.04$).

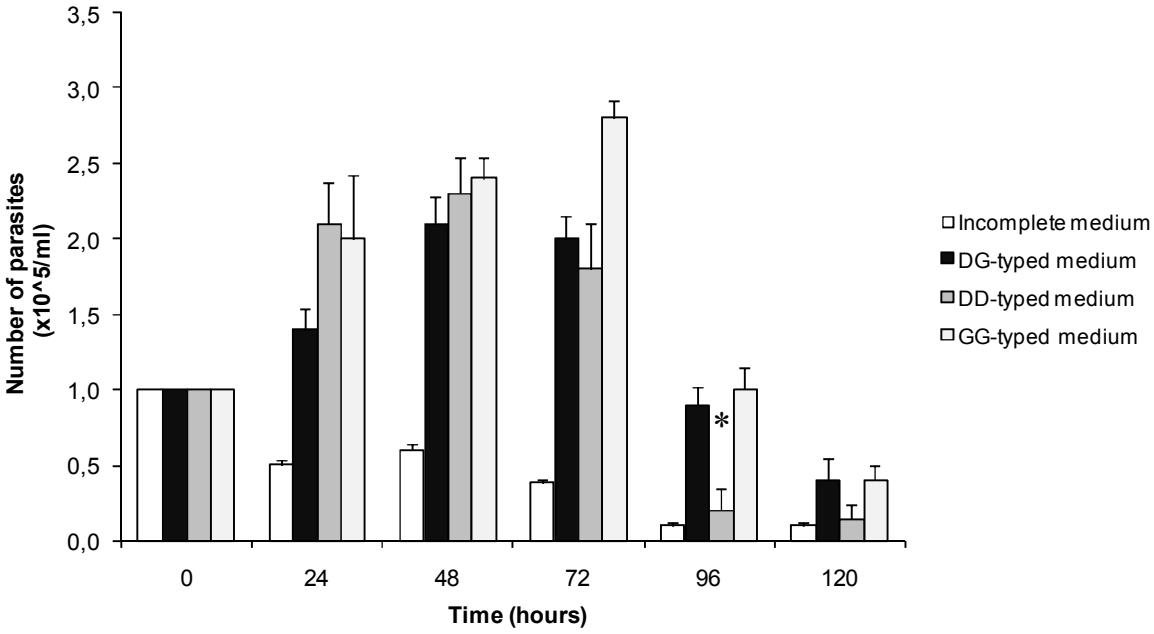

Fig. 2.4. *In vitro* growth of *T. borreli* over a period of 5 days in culture medium (HBSS) supplemented with 3% transferrin-typed carp serum. Media contained serum with transferrin genotypes DG, DD, GG, or no serum as negative control.

4. Discussion

Over the last years evidence has accumulated indicating an influence of genetic factors on resistance of carp to infectious diseases (Price and Clayton, 1999; Shapira et al., 2005). We performed a series of infection experiments with the carp blood parasite *T. borreli* to examine inter-line differences in susceptibility of common carp. The susceptibility of the reference laboratory cross was relatively high compared with the susceptibility of the 'commercial' carp lines, which never died from infection. Increasing homozygosity owing to repeated inbreeding over successive generations could possibly explain the reduced fitness of the laboratory cross. Of more interest were the differences in susceptibility between the commercial carp lines. Although not always pronounced, we could demonstrate a clear pattern of differences in the degree of susceptibility. Carp from the Polish line 'R2' consistently showed the lowest parasitaemia and carp from the Israelian line 'D' were most susceptible. In addition, in

reciprocal crosses, progeny of 'R2' carp showed lower parasitaemia than progeny of 'D' carp. This clearly suggests that even a complex trait such as susceptibility of carp to parasite infection can at least partly be determined by a genetic component. We detected four different transferrin genotypes (DD, DF, DG and GG) in the carp lines tested, confirming the polymorphism for Tf often observed in fish. Among these genotypes, DD and DG were most frequently found. In general, the frequency of homozygous individuals varied among the carp lines and showed an under-representation of FF and GG genotypes. Interestingly, but difficult to explain, agreement with expected frequencies of Tf genotypes was observed only in the progeny of the 'R2' male, and not in the progeny of the 'D' male. Possibly, particular Tf genotypes were eliminated during embryogenesis, but not later during development since we observed no significant mortality after the 1st week post-fertilization. Because the frequencies for the different Tf genotypes varied, we could study correlation with parasitaemia only for the three most frequently occurring genotypes DD, DF and DG. In general, complex genetic traits such as susceptibility to parasite infection cannot easily be ascribed to a single gene or single protein effect. In practice, the relative contribution of a single protein can more easily be detected when a segregating gene of interest is tested on a uniform genetic background. For that reason we examined Tf genotypes in each carp line separately. Parasitaemia in heterozygote (DG and DF) individuals was similar among the different carp lines. Homozygous (DD) individuals, however, showed different reactions depending on the origin of the carp line. We could detect a significant association of the homozygous DD Tf genotype with lower parasitaemia in the 'R2' and 'K' carp lines and the lack of a such an association in the most susceptible 'D' carp line. Also in the crosses where the 'R2' male was used, offspring with the DD genotype had significantly lower parasitaemia, confirming that homozygous DD individuals contributed most to the genetic variation in parasitaemia.

The effect of Tf genotype was also tested by culturing *T. borreli in vitro* in medium supplemented with three different Tf genotypes (DD, DG and GG) as provided by pooled carp serum from Tf-typed individuals. Clearly, the *in vitro* culture experiment showed the dependence of *T. borreli* on carp serum for *in vitro* multiplication, as shown previously for related parasites (Schell et al., 1991). The number of parasites initially increased over 72 hrs but then gradually decreased over the next 72 hrs, when apparently the culture media got depleted of essential growth factors. The rate of

decrease in number of parasites was significantly faster in culture media supplemented with DD-typed Tf, which could be supportive for the association of the DD genotype with decreased parasitaemia found *in vivo*. Additional experiments, however, are required to firmly establish the relationship between Tf genotype and growth of *T. borreli*.

Our combined *in vivo* and *vitro* data indicate that fish with the homozygous DD genotype for Tf cannot support the growth of *T. borreli* to the same extent as fish that express the G allele for Tf (DG or GG). Whether this finding would be the direct result of Tf binding to the transferrin receptor of the parasite (Bitter et al., 1998) or an indirect effect of a modulated immune response initiated by the parasite (Stafford et al., 2001; Stafford and Belosevic, 2003), or a combination of both requires further investigations. The Tf D and G alleles identified by PAGE migrate quite differently, suggesting a difference in molecular weight and/or protein charge. Determining sequence differences and identification of three-dimensional properties of both alleles could possibly provide the information required to explain the functional differences observed.

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3

Differences in growth of *Trypanoplasma borreli* in carp serum is dependent on transferrin genotype

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In preparation

Chapter 3

Abstract

Kinetoplastid parasites require transferrin (Tf), being the main source of iron, for growth and multiplication. This group of parasites developed a special receptor-mediated system for acquiring host Tf which bears no structural homology with the host transferrin receptor. *Trypanoplasma borreli*, a blood parasite of common carp, probably uses a similar mechanism to sequester iron from host transferrin.

In this study we demonstrate a critical role of Tf for parasite growth. For *in vitro* studies we isolated and purified Tf from carp homozygous for the D or G allele of Tf. We obtained Tf-depleted serum using specific antibodies to carp Tf and studied gene expression *in vivo* during *T. borreli* infection with Real Time-quantitative PCR.

We demonstrate that *T. borreli* cannot survive in medium supplemented with Tf-depleted serum while reconstitution with Tf restores normal growth. The critical role of Tf for parasite survival was shown in incomplete medium (medium without serum): addition of purified Tf significantly increased parasite survival. We also demonstrate that Tf polymorphism has a significant impact on *T. borreli* multiplication. Cultured parasites die more quickly in an environment containing D-typed Tf, as compared to medium with G-typed Tf. Gene expression during *T. borreli* infection in carp did not show an acute phase response. We could, however, observe an increased transcription of Tf in the head kidney, which may be associated with an immunological function of the Tf protein.

Chapter 3

Introduction

The kinetoplastid parasite, *Trypanoplasma borreli*, which is transmitted by blood-sucking leeches, can infect the cyprinid common carp (*Cyprinus carpio* L.) and multiply extracellulary in the blood and tissue fluids of this host. Similarly to most other living organisms, kinetoplastid parasites require iron for growth (Schell et al., 1991; Steverding, 1998). An important source of iron for kinetoplastids is host transferrin (Tf). Previously, a heterodimeric receptor for Tf uptake was isolated from the bloodstream forms of *Trypanosoma brucei*. This receptor is encoded by the 'homologous expression site associated genes' ESAG6 and ESAG7 (Steverding et al., 1994; Chaudhri et al., 1994; Ligtenberg et al., 1994; Salmon et al., 1994; Steverding et al., 1995; Steverding and Overath, 1996). Most likely, *T. borreli* also requires iron for growth, but the requirement and mechanism for iron uptake by *T. borreli* has not been studied so far.

The mammalian Tf receptor binds two Tf molecules, a process that takes place on the cell surface (Jandl and Katz, 1963). In trypanosomes, the receptor binds a single Tf molecule, a process that occurs in the flagellar pocket (Steverding et al., 1994; Ligtenberg et al., 1994). In both mammals and trypanosomes the ligand-receptor complex is internalised and transported to endosomes where at acidic pH iron is released from the Tf molecule (Maier and Steverding, 1996; Dautry-Varsat et al., 1983). Subsequently, in mammals, the Tf-TfR complex is recycled back to the cell surface (Dautry-Varsat et al., 1983). In contrast, in *T. brucei*, Tf is proteolytically degraded in lysosomes (Steverding et al., 1995), and released as peptide fragments from the trypanosomes into the host bloodstream (Steverding, 2000). Transferrin-dependence has been shown for the bloodstream forms of *T. brucei*, that cannot grow in medium containing Tf-depleted serum (Schell et al., 1991). Similar approaches have not been taken for *T. borreli* yet. However, both parasites belong to the same order of Kinetoplastida, and therefore their metabolism may involve similar processes.

We have previously demonstrated an association between Tf polymorphism and resistance to *T. borreli* infection *in vivo* (Jurecka et al., 2008c). In the same study, we performed a preliminary *in vitro* investigations on the ability of *T. borreli* to survive in media supplemented with serum originated from carp with different Tf genotypes (DD, DG or GG). Although the trypanoplasms initially multiplied to a similar extent in all three Tf-typed media, the decline in parasite number over a period of a few days in culture was strongest in DD-typed medium. The combined *in vivo* and *in vitro*

observations suggest a correlation exists between the presence of particular Tf genotypes and ability of *T. borreli* to multiply. It is not known if this correlation could be related to differences in the ability to sequester iron from different types of Tf molecule or to other factors which could be linked with Tf genotypes. For that reason, experiments that would evaluate the pure effect of Tf were needed.

In the present study, we examined the growth of *T. borreli* in culture media supplemented with complete carp serum or with carp serum depleted of Tf. To do so, we developed a method for Tf depletion using specific antibodies to carp Tf, and compared *T. borreli* multiplication and/or survival in the presence or absence of Tf *in vitro*. We isolated two carp Tf proteins (alleles D and G) to purity using rivanol precipitation and ion-exchange chromatography and confirm that impaired multiplication of *T. borreli* in the presence of Tf-depleted serum could be reconstituted with the addition of purified Tf to Tf-depleted serum. This suggests that *T. borreli* may sequester iron from the carp host serum proteins. In general, a host may react to infection by rapidly modifying the concentration of Tf, lowering the availability of iron for invading pathogens. To study this phenomenon, we examined Tf gene expression during *in vivo T. borreli* infection in different immune-relevant organs by real-time quantitative PCR. We discuss the difference between different Tf genotypes with respect to resistance to *T. borreli*.

Materials and Methods

Animals

European common carp (*Cyprinus carpio carpio* L.) were reared at 23^{o}C in recirculating UV-treated tap water and fed pelleted dry food (Trouvit, Nutreco) daily. R3×R8 carp are the offspring of a cross between fish of Hungarian origin (R8 strain) and of Polish origin (R3 strain) (Irnazarow and Białowąs, 1994, 1995). At the age of 9 months, individual carp were bled and genotyped for Tf. Sera from carp typed homozygous for the D allele of Tf (DD-typed serum) or typed homozygous for the G allele of Tf (GG-typed serum) were used for Tf isolation and purification, and for preparation of parasite culture media. Sera from carp typed heterozygous (alleles D and G) were used for the Tf- depletion experiment.

Identification of transferrin alleles by polyacrylamide gel electrophoresis (PAGE)

Total serum proteins were separated by non-reducing polyacrylamide gel electrophoresis under circumstances particularly suited to visualize Tf proteins (Valenta et al., 1976; Irnazarow and Białowąs, 1994). Samples (5 µl) were diluted in 15 µl loading buffer (40% sucrose, 1.5% Bromophenol Blue; Sigma-Aldrich, St. Louis, MO, USA) and 2 µl of each suspension applied on a 6% stacking and 15% polyacrylamide running gel. Electrophoresis was carried out in running buffer (72 mM Tris, 26 mM boric acid) at 90V for 30 min followed by 250V for 5 h (Smithies, 1955). Protein bands were stained for 1 h with 0.04% Coomassie Brilliant Blue dissolved in 3.5% perchloric acid.

Purification of carp transferrin with ion exchange chromatography

Transferrin for supplementation of parasite media was purified from carp serum (alleles D and G) as described (Sutton and Karp, 1965; Jurecka et al., 2008a). To a quantity of serum of 100 ml about 1 mg $FeCl_3$ was added to saturate Tf with iron. Subsequently, one part of serum was diluted with three parts of 5 mM Tris buffer (pH 8.8) and an equal volume of 0.6% rivanol solution (Ethacridini lactase FPV, FARM-IMEX, Gliwice, Poland) in Tris buffer was slowly added and left overnight at 4^{o}C, while stirring. Rivanol was removed selectively and completely by adding potato starch to the supernatant and by filtration. Ion-exchange chromatography was performed using a Mono-Q column (GE healthcare Life Sciences), pre-equilibrated with 20 mM Tris-HCl (pH 8.0). Filtrates (0.2 µm) were applied to the column in a total volume of 20 ml (4 injections of 5 ml) at a flow rate of 1 ml/min. Unbound proteins were collected in flow-through fractions. Transferrin bound to the column was visible as a brownish-red ring. Elution buffer was applied as a gradient (0-1 M NaCl, 20 mM Tris-HCl) to the column and the transferrin fractions were collected. Finally, samples were dialyzed overnight at 4^{o}C against PBS, adjusted with NaOH to pH 8.0, filter-sterilized and stored at -20^{o}C for further use. Protein concentration was determined by Bradford using BSA as a standard. Purity of isolated Tf samples was analysed by SDS-PAGE. A volume of 6 µl of each Tf sample was heated at 96^{o}C for 10 min with loading buffer containing β-mercaptoethanol and run on a 12.5% polyacrylamide gels. Electrophoresis was carried out in 1 × SDS

running buffer at 80 V for 30 min followed by 100 V for 60 min. Protein bands were stained for 1 h with 0.04% Coomassie Brilliant Blue dissolved in 3.5% perchloric acid.

Production of polyclonal antibodies against carp transferrin

Transferrin was isolated to purity from carp seminal plasma and used for immunization of rabbits to produce specific antibodies against carp Tf (Żmijewski, 2006) as described before (Wojtczak et al., 2007a). Briefly, two rabbits were intradermally immunized with purified Tf (200 µg) emulsified with Freund's complete adjuvant. A second injection with 200 µg of antigen was given 4 weeks later. A control rabbit was injected with PBS instead of Tf. Two weeks after the last injection, the rabbits were bled from the marginal ear vein and the presence of antibodies was determined by double immunodiffusion (Ouchterlony, 1953). The rabbits were then bled to death and the blood allowed to clot at room temperature. To obtain serum, samples were centrifuged for 10 min at 3500 × *g*.

Isolation of IgG from polyclonal rabbit serum

Isolation of the total IgG fraction from the polyclonal rabbit antiserum raised against purified carp Tf was performed as described before (Wojtczak et al., 2007a). Briefly, rabbit serum was warmed to 56°C for 20 min. and solid ammonium sulphate (pH 7.2) was added to 40% saturation under continuous stirring to precipitate the globulin fraction. After centrifugation at 5000 × *g* for 20 min. the supernatant was discarded and the precipitate was re-dissolved in 0.02 M phosphate buffer (pH 7.0). After dialysis over 24 h against the same buffer, the sample solution was filtered through a 0.22 µm pore size syringe filter and applied to HiTrap Protein A HP (1 mL; Amersham Biosciences) column pre-equilibrated with the same buffer. Unbound proteins were eluted with 10 column volumes of the same buffer. Elution of adsorbed IgG was carried out using 0.1 M citric acid (pH 3.0) at a flow rate of 1 mL/min. pH of the eluted fractions containing IgG was brought to 7.0 with 1 M Tris-HCl (pH 9.0). Samples were stored at -80°C for further use. The purity of the IgG fraction was tested using PAGE and SDS-PAGE. PAGE was performed on a separating 10% acrylamide gel according to the method of Laemmli (Laemmli, 1970) using a SE 250 vertical Mighty Small II electrophoresis system (Amersham Biosciences). Gels were stained for proteins with 0.025% Coomassie Brilliant Blue (CBB) R-250 in 40% methanol/7% acetic acid and for

antitrypsin activity using bovine trypsin according to the method of Uriel and Berges (Uriel and Berges, 1968) as described before (Ciereszko et al., 1998). SDS-PAGE was performed on 12.5% gel under reducing conditions (sample was heated at 96^{o}C for 10 min with loading buffer containing β-mercaptoethanol). Electrophoresis was carried out in 1 × SDS running buffer at 80 V for 30 min followed by 100 V for 60 min. Protein bands were stained for 1 h with 0.04% Coomassie Brilliant Blue dissolved in 3.5% perchloric acid.

Western blot

The specificity of the purified IgG fraction from the rabbit Tf antiserum was tested by Western blot on seminal plasma and serum proteins from different fish species. Briefly, 7µl of each sample was separated by SDS-PAGE and blotted onto a nitrocellulose membrane (Sigma-Aldrich, St. Louis, MO, USA). The blot membrane was incubated in anti-carp Tf IgG (1:10,000 v/v in TBS-T (10 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.1 % Tween 20)) and stained with 0.33 mg/ml nitroblue tetrazolinum (Sigma-Aldrich, St. Louis, MO, USA) and 0.17 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (Sigma-Aldrich, St. Louis, MO, USA) in alkaline phosphate buffer (100 mM Tris-HCl, pH 9.5; 100 mM NaCl, 5 mM $MgCl_2$) in dark until color development. The reaction was stopped with 0.2 mM EDTA.

Comparison of concentration of different Tf genotypes in carp serum

A comparison of the concentration of Tf in pooled carp serum (n=10-15 individuals) with different genotypes (DD and GG) was made (Ouchterlony, 1958). To 1% agarose, diluted in deionized water and cooled to 50^{o}C, purified anti-carp Tf IgG was added (1:1000) and the mixture was layered to a Petri dish and allow to cool and polymerize. Four 2 mm holes were punched in the gel and pooled carp sera of DD and GG Tf genotypes were loaded in two dilutions: 10 × diluted and undiluted. The dish was incubated at 4^{o}C in a humid atmosphere for 4 days. The precipitates were visualized by staining for 1 h in 0.04% Coomassie Brilliant Blue dissolved in 3.5% perchloric acid.

Transferrin depletion of carp serum

Total anti-carp Tf IgG (5 mg) was dialyzed overnight against 0.02 M $NaHCO_3$ and lyophilized. The lyophilizate was diluted with coupling buffer 0.2 M $NaHCO_3$ 0.5 M

NaCl (pH 8.3) and coupled to 1 ml HiTrap NHS-activated HP column, according to the manufacturer's protocol (GE Healthcare). This column was used to deplete Tf from carp serum, for in vitro studies on parasite multiplication. After each step, immunoreactivity of the antibody was tested using immunodiffusion. For the Tf-depletion experiment we chose carp serum collected from animals typed as 'DG', because of the presence of both Tf variants. Prior to the application to the immunoaffinity column, carp serum was diluted 20 times with binding buffer (0.05 M Tris-HCl 0.15 M NaCl pH 7.6). Samples (1 ml) were then applied to the column equilibrated with binding buffer and the column was sealed and incubated for 40 min at room temperature to allow ligand binding. Subsequently, unbound proteins were washed from the column with 7 ml of binding buffer and collected as 1 ml fractions. Bound proteins were eluted from the column at low pH with 5 ml of elution buffer (0.5 acetic acid, pH 3), neutralized by the addition of 1 M Tris-HCl, pH 9.0 and also collected as 1 ml fractions. Each fraction was tested using non-reducing PAGE. Unbound fractions (devoid of Tf) were pooled and concentrated using Centrifugal Filter Devices (Millidore, 10 kDa MWCO) at 3780 × *g* for 10 min. Application of samples and buffers was performed using 1 ml and 5 ml syringes, respectively, at a flow rate of about 0.5 ml/min.

In vitro growth of Trypanoplasma borreli

T. borreli was isolated from the blood of infected carp by centrifugation (Steinhagen et al., 2000), purified by ion-exchange column chromatography (Overath et al., 1998) and washed in Hank's Balanced Salt Solution. Isolated parasites ($1x10^7$ parasites/ml) were cultured overnight in complete *T. borreli* medium (45% (v/v) HBSS, 22.5% (v/ v) MEM, 22.5% (v/v) L-15, 10% (v/v) distilled water, 100 IU/ ml penicillin, 100 mg/ml streptomycin, 2mM L-glutamine, 3% (v/v) heat inactivated pooled carp serum (PCS)) at 20^{o}C (Steinhagen et al., 2000). Finally the trypanoplasms were seeded at 10^5 cells/ml in 96-well culture plates and incubated at 20^{o}C.

To *T. borreli* incomplete medium (45% (v/v) HBSS, 22.5% (v/ v) MEM, 22.5% (v/v) L-15, 10% (v/v) distilled water, 100 IU/ ml penicillin, 100 mg/ml streptomycin, 2mM L-glutamine) were added 3% of Tf-typed serum (DG or DD) as positive controls, or 3% Tf-depleted serum with or without purified Tf (100 µg/ml, D or G). Alternatively, trypanoplasms were seeded in incomplete medium and only purified Tf was added (100 µg/ml, D or G), or left untreated as negative control. The number of parasites was

determined every 24 hours using a Bürker counting chamber. To check the influence of the chromatographical procedures on *T. borreli* survival and/or multiplication, we also seeded the parasites in *T. borreli* complete medium containing i) 3% Tf-typed serum (DG) applied to an immunoaffinity column without the presence of rabbit anti-carp Tf IgG or ii) 3% Tf-typed serum (DG) diluted with binding buffer and re-concentrated similar to the treatment applied to obtain Tf-depleted serum.

RNA isolation, cDNA synthesis and Real-Time quantitative Polymerase Chain Reaction (RT-qPCR)

For determination of Tf gene expression during *T. borreli* infection, RNA was isolated from spleen, head kidney and liver of infected (n = 5) and non-infected (n = 5) carp. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Leusden, The Netherlands) including an on-column DNase treatment. Prior to RNA isolation from liver a Proteinase K treatment was also included. RNA concentration and purity was determined at OD260nm/OD280nm. RNA integrity was assessed by electrophoresis on 1% agarose gel. Prior to cDNA synthesis, a second DNase treatment was performed using DNase I, Amplification Grade (Invitrogen, Breda, The Netherlands). Synthesis of cDNA was performed with Invitrogen's SuperscriptTM III First Strand Synthesis Systems for RT-PCR including a non-reverse transcriptase control for each sample. cDNA samples were further diluted 50 times in nuclease-free water before use as template in RT qPCR. cDNA was stored at – 20^{o}C for further use.

Gene expression during *T. borreli* infection was studied by Real Time-quantitative PCR (RT-qPCR) essentially as described previously (Forlenza et al., 2008). RT-qPCR was performed using a Rotor-Gene™ 2000 (Corbett Research, Mortlake, Sydney, Australia) and Brilliant® SYBR® Green QPCR (Stratagene, La Jolla, CA, USA) as detection chemistry. The primers used for RT-qPCR are presented in Table 3.1. Master-mix for each PCR run was prepared as follows: 1 µl of each primer (4.2 µM), 7 µl Master SYBR Green I mix and 5 µl of diluted cDNA. The following amplification program was used: after 15 min of denaturation at 95ºC, 40 cycles of RT-qPCR with three-step amplification were performed: 15 s at 95ºC for denaturation, 30 s at 60ºC for annealing and 30 s at 72ºC for elongation followed by a final holding step of 1 min at 60ºC. A melting step was then performed, and in all cases the amplifications were specific and no amplification was observed in negative controls (non template control and non-

reverse transcriptase control). Fluorescence data from RT-qPCR experiments were analyzed using Rotor-Gene version 6.0.21 software. The cycle threshold Ct for each sample and the reaction efficiencies (E) for the primer set were obtained upon Comparative Quantitation Analysis from the Rotor-Gene version 6.0.21 software (40S: E = 1.75; TF-D = 1.75; TF-G = 1.71). Basal transcription of immune-related genes was calculated as a ratio of reference gene versus target gene in different organs (Pfaffl et al, 2001; Tichopad et al., 2003). The 40S ribosomal protein S11 was used as an internal reference gene.

Table 3.1. Primer sequences applied for real-time quantitative PCR (RT-qPCR). + indicates an LNA modification (underlined).

Primer name	Sequence 5'- 3'
qTfG_fw1	GATCACGCAAAGATGTGGTGAACG
qTfD_fw2	GATGCACGCAAAGATGTGGTAAACA
qTfG_rv1	GGT CCTTT+CAAT+CATGT+CAATGTAC
qTfD_rv2	GGTCCTT+TCAATCA+TG+TCAATGTAG

Statistics

Levels of parasitaemia were tested for significance by a non-paired, two tailed Student's t-test. $P \leq 0.05$ was accepted as significant. Differences in gene expression between infected and non-infected fish in different organs were tested for significance by a t test for independent samples. All analyses were performed using Statistica 6.0 software (StatSoft). Differences were considered significant at $P \leq 0.05$.

Results

Purified rabbit IgG shows a high specificity for carp serum transferrin

From rabbit polyclonal antiserum the total Ig fraction was precipitated by ammonium sulphate precipitation and IgG purified by HiTrap Protein A affinity chromatography. Adsorbed IgG was eluted with citric acid and the purity was tested using PAGE and SDS-PAGE. Under denaturing conditions, two polypeptide chains were observed, a ~80 kDa heavy chain and ~35 kDa light chain, characteristic of rabbit IgG (Fig. 3.1, lane 3). Under non-denaturing conditions we observed a single 150 kDa band (lane 4). The purified IgG fraction was tested against Tfs purified from seminal plasma from different

fish species and was tested against Tf purified from carp serum. Western blot confirmed the specificity of the purified IgG (Żmijewski, 2006).

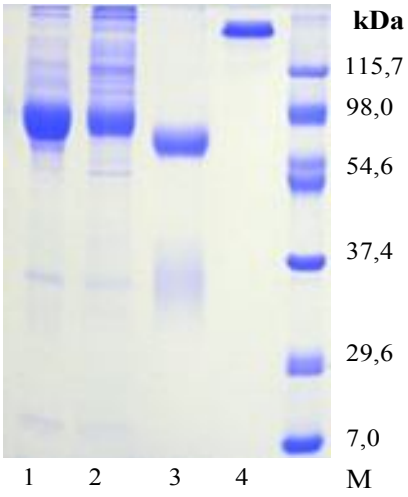


Fig. 3.1. Purification of rabbit anti carp transferrin IgG to deplete transferrin from carp serum. SDS-PAGE of fractions after affinity chromatography (HiTrap Protein A) purification of rabbit IgG against carp transferrin. Lane 1: total rabbit antiserum diluted 5 times. Lane 2: column flow-through fraction. Lane 3: column eluate; purified IgG fraction (reduced). Lane 4: purified IgG fraction under non-reducing (native) conditions. M: molecular weight standards.

Transferrin concentration in serum is equal for different genotypes

We performed Ouchterlony test to examine whether the concentration of Tf in serum differs between particular genotypes. This reaction involves the binding of antigen (Tf in serum) by antibody (anti-carp Tf IgG in gel) with the formation of a visible precipitate. The area of the ring produced by diffusion is proportional to the amount of antigen. As shown in Fig. 3.2, at both dilutions, the precipitation diameter is equal for both Tf genotypes DD and GG.

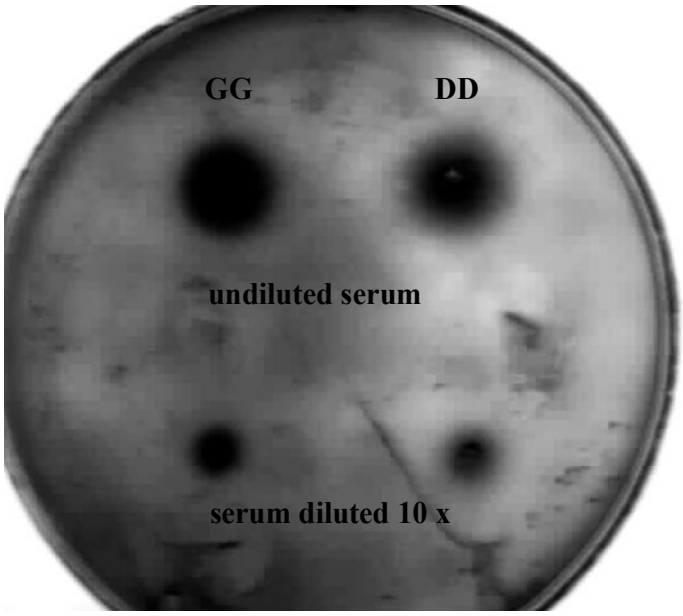


Fig. 3.2 Comparison of different Tf genotypes concentration in carp serum. Mixture of 1% agarose and purified anti-carp Tf IgG (1:1000) was layered to Petri dish. Sera with DD and GG Tf genotypes were loaded into four 2 mm holes in two variants: 10 × diluted and undiluted, and incubated at 4^{o}C in a humid atmosphere for 4 days. The precipitates were visualized by staining in 0.04% Coomassie Brilliant Blue dissolved in 3.5% perchloric acid.

T. borreli require transferrin for multiplication

Tf-depleted serum was obtained by specific removal of Tf from DG-typed carp serum by affinity chromatography using the purified rabbit anti-Tf IgG fraction. Complete depletion of carp serum from Tf was achieved, as shown in Fig. 3.3. The successful depletion required an application of 20 times diluted serum. Application of undiluted carp serum resulted in the loss of other proteins, because of a minimal 4 time on-column treatment. Serum was re-concentrated after treatment.

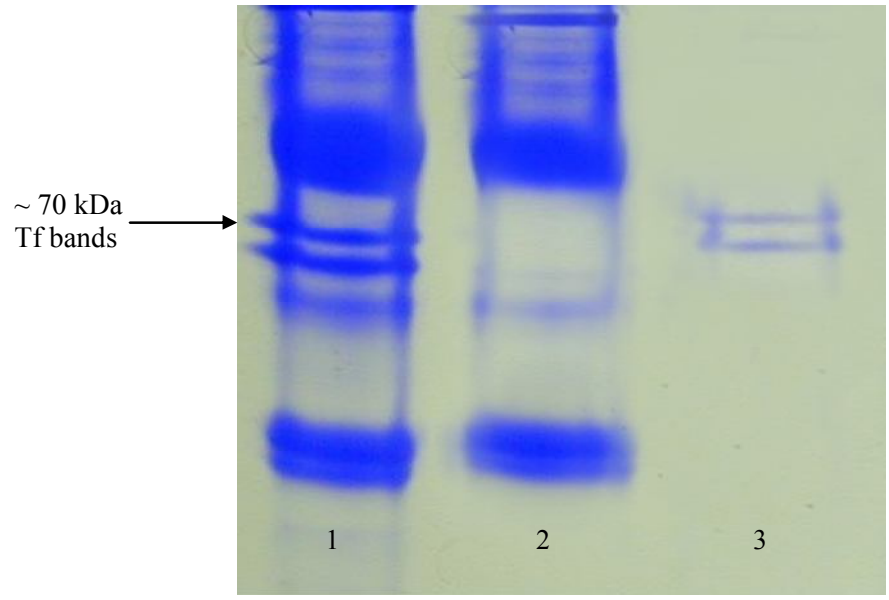


Fig 3.3. Native PAGE showing depletion of transferrin from carp serum using rabbit anti-Tf IgG. Lane 1: untreated carp serum. Lane 2: Tf-depleted carp serum. Lane 3: purified carp transferrin.

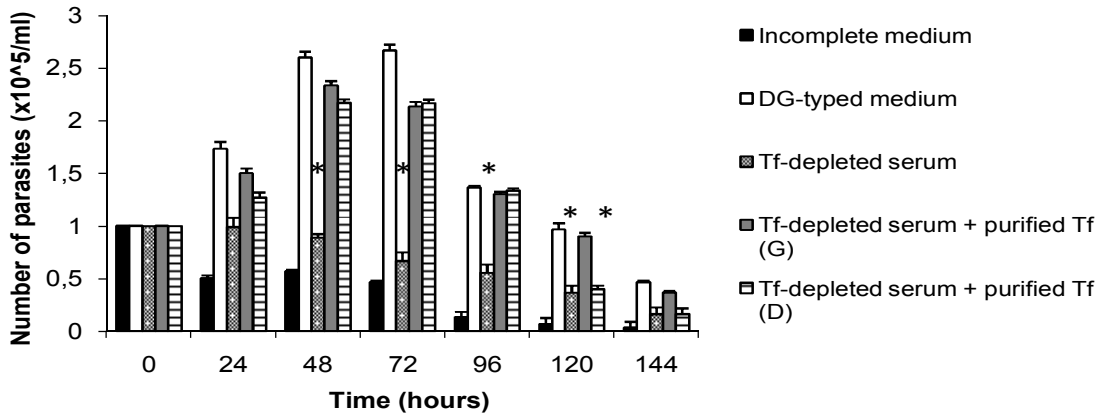
Purified Tf was used to reconstitute Tf-depleted serum for *in vitro* studies on parasite growth. Tf proteins (D and G alleles) were isolated to purity by rivanol precipitation and ion-exchange chromatography as described before (Jurecka et al., 2008a). Carp serum contains 2.5 mg/ml Tf approximately (Wojtczak et al., 2007b). This means that in complete medium containing 3% pooled carp serum, concentration of Tf is approximately 75 µg/ml. Therefore, in subsequent experiments the medium containing 3% of Tf-depleted serum was reconstituted by 100 µg/ml of purified Tf.

Multiplication of *T. borreli* was tested in Tf-depleted serum either reconstituted or not with purified Tf (D or G; 100 µg/ml), or tested in incomplete medium (negative control), or tested in complete medium with 3% DG-typed serum (positive control). As shown in Fig. 3.4A, we observed a significant ($P < 0.004$) decrease in parasite multiplication in Tf-depleted medium compared to the positive control. The difference with the negative control was smaller and not significant. When the Tf-depleted culture medium was reconstituted with purified Tf, parasite multiplication was restored and was comparable with the positive control. We also observed a significant difference between the G and D Tf variants in the rate of parasite decrease at 120h: *T. borreli* were dying faster in medium reconstituted with purified Tf D.

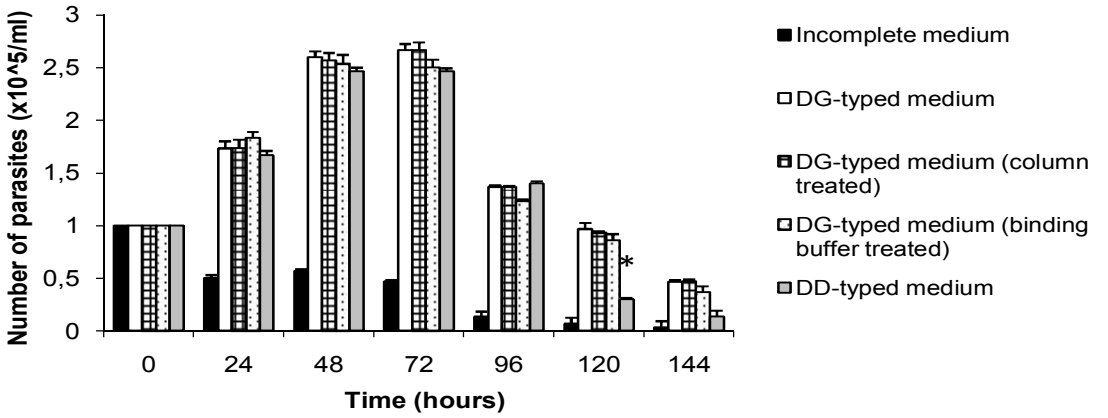
We also examined if the chromatographical procedures influenced the quality of the carp serum and thereby *T. borreli* growth and/or survival. To this end we incubated *T. borreli* in i) media containing carp serum which had undergone column treatment, while in this case the column did not have the anti-carp Tf IgG bound and ii) media containing carp serum which was first 20 times diluted and then reconcentrated. In both cases the serum source was heterozygous DG carp. As a positive control we used two types of complete media, either supplemented with DD or with DG serum. As a negative control, we seeded *T. borreli* in incomplete medium, which resulted in an immediate decrease of the number of parasites. In general, supplementation with 3% carp serum results in a clear increase of the number of parasites during the first 72h, and a gradual decline in parasite numbers after 3 days. There was no effect of column treatment or serum reconcentration on parasite growth and no difference in the rate of parasite decline among the different media was observed (Fig. 3.4B). Interestingly, a difference was noted between media containing serum from DG or DD-typed fish. In latter case the rate of parasite decline was significantly faster ($P < 0.005$) at 120h of incubation (Fig. 3.4B).

To confirm the requirement for Tf for parasite multiplication, in a new set up, trypanoplasms were seeded in incomplete medium and supplemented only with purified Tf (D or G; 100 µg/ml, no serum), not supplemented with Tf (negative control) or grown in DG-typed serum (positive control). The addition of purified Tf to incomplete medium, significantly ($P < 0.01$) enhanced parasite survival (as compared to the negative control), although the levels of *T. borreli* at the peak of multiplication (72h) were significantly lower than in the positive control (serum added, $P < 0.02$) (Fig. 3.4C). Again, we observed a difference in the rate of decrease of parasite numbers between the Tf variants D and G after 120h of incubation ($P < 0.01$) (Fig. 3.4C).

A)



B)



C)

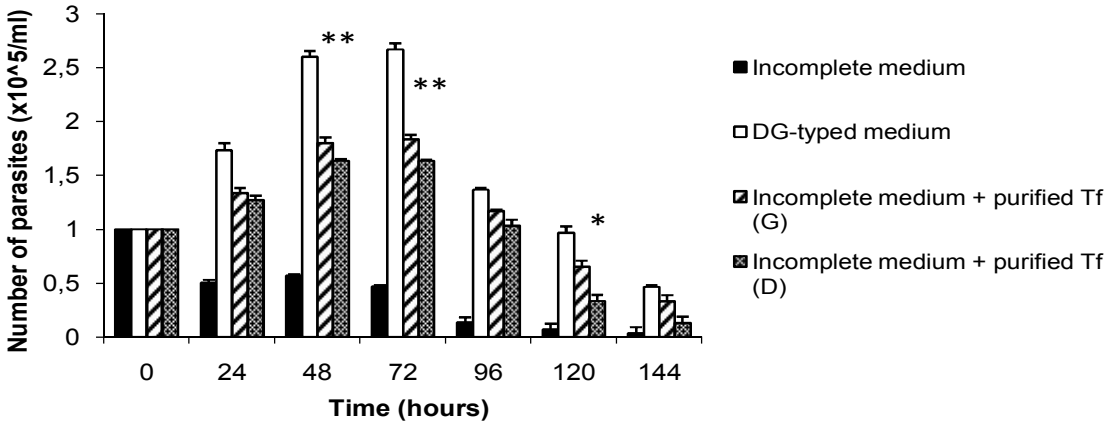


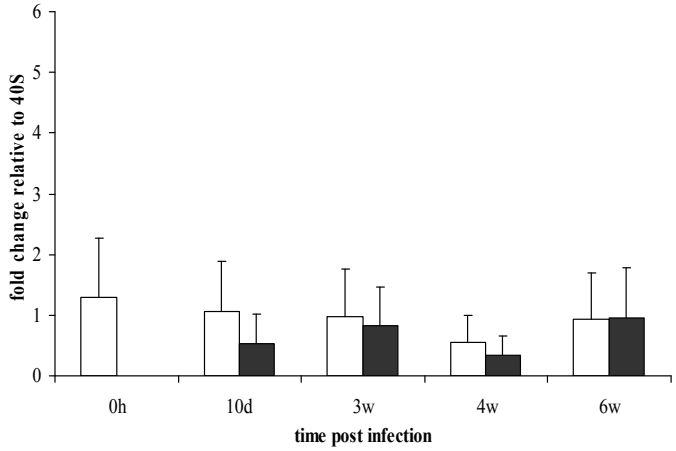
Fig. 3.4 Effect of transferrin on *in vitro* growth of *T. borreli* over a period of 6 days. (**A**) Growth of *T. borreli* in incomplete medium (no serum, negative control), in complete medium (with DG-typed serum;

positive control), in culture medium supplemented with Tf-depleted carp serum either or not reconstituted with purified Tf (D or G; 100 µg/ml). (*) indicate statistically significant difference at $P \leq 0.05$. (**B**) Growth of *T. borreli* in culture medium supplemented with 3% transferrin-typed carp serum. Media contained serum with transferrin genotypes DG, DD, or serum with transferrin genotype DG treated with the chromatographically column or binding buffer, or no serum as negative control. (*) indicate statistically significant difference at $P \leq 0.05$. (**C**) *In vitro* growth of *T. borreli* over a period of 6 days in incomplete *T. borreli* culture medium and supplemented with the purified Tf (D or G; 100 µg/ml), or left untreated as negative control, or grown in DG-typed serum (positive control). (*) indicate statistically significant difference at $P \leq 0.05$.

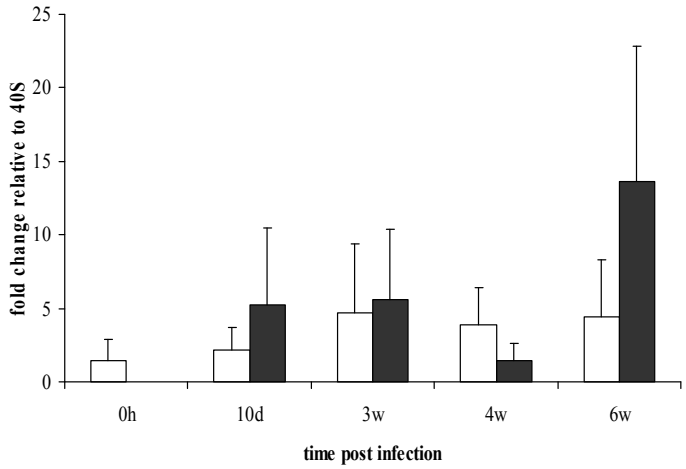
Gene expression of transferrin in immune organs during T. borreli infection

We used allele-specific primers to detect Tf gene expression in carp immune organs during infection with *T. borreli*. We could examine Tf gene expression for the G allele only because we had access only to GG-typed fish for this *in vivo* experiment. A difference in mRNA transcription of carp Tf was not observed in the liver, the main source of Tf (Fig. 3.5A). A small increase in Tf gene expression was noted in the spleen at the 6th week post-infection, but this change was not statistically significant compared to non-infected control fish (Fig. 3.5B). Statistically significant ($P < 0.05$) increase of Tf gene expression, comparing to non-infected control fish, was observed in the head kidney at the 3rd week of infection (Fig. 3.5C).

A)



B)



C)

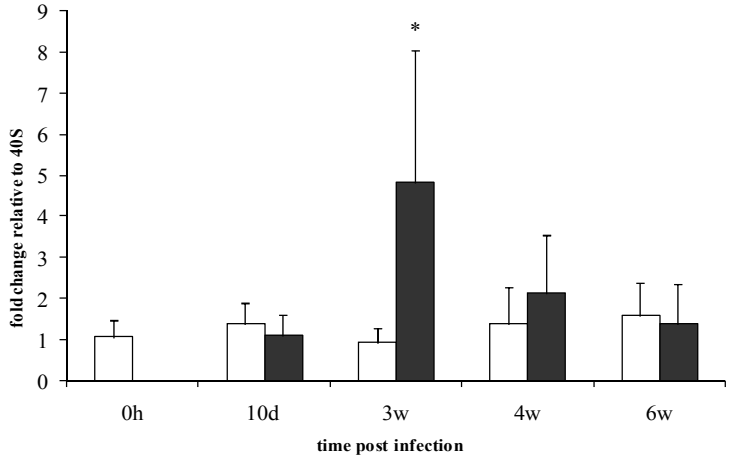


Fig. 3.5 Constitutive gene expression of transferrin allele G during *T. borreli* infection (black bar) and in non-infected fish (white bar) in liver (**A**), spleen (**B**) and head kidney (**C**) of European common carp. Gene expression was analyzed by real-time quantitative PCR and shown relative to the house keeping gene 40S. (*) indicate statistically significant difference at $P \leq 0.05$.

Discussion

In the present paper we describe that Tf-bound iron could be an important factor for the multiplication and survival of *Trypanoplasma borreli*. Our results confirm previous findings that iron transported by Tf is an important growth factor for kinetoplastid parasites (Schell et al., 1991; Steverding, 1998). We observed significant decrease in parasite number when grown in medium with Tf-depleted serum. We could demonstrate that reconstitution of this medium with purified transferrin supported normal growth of

T. borreli. Transferrin alone did not support the growth of the parasite as well as the addition of serum, which suggest that these pathogens utilize more than just iron and that other disturbances in nutrient supply affect homeostasis.

Kinetoplastid parasites such as *Trypanosoma brucei* express receptors for the binding of host Tf (TfR ESAG6 and/or ESAG7). Although probably, it is not known if *T. borreli* parasites also possess similar receptors. So far, the sequence of the *T. borreli* transferrin receptor has not been cloned and sequenced yet. We have tried to obtain cDNA sequences for the *T. borreli* TfR equivalent, and designed primers under the assumption that trypanoplasms and trypanosomes should have similar Tf receptors. PCR primers were designed based on known *T. brucei* sequences of ESAG6 and ESAG7 (GenBank accession nos: AF068702-5 and AF068699) and used to try and amplify *T. borreli* cDNA coding for similar TfR. Although the primers did not amplify specific products, it does not mean that *T. borreli* does not possess TfRs and does not mean that the mechanisms of iron uptake would be completely different. Most probably, Tf receptor identity between *T. brucei* and *T. borreli* is too small to easily design specific primers based on *T. brucei* sequences. Although both parasites are Kinetoplastids, *T. brucei* belongs to the family of Trypanosomatida and *T. borelli* to the family of Parabodonida (Simpson et al., 2006). These families diverged more than 500 million years ago (Fernandes et al., 1993) and sequences can be quite different between these two parasite families. For example, we have recently shown (Ruszczyk et al., 2008) that the nucleotide sequence of the cysteine protease of *T. borreli* and *T. brucei* share 46% identity only. Other approaches than primer design based on conserved sequence stretches need to be applied to find the coding sequence for the receptor(s) used for transferrin binding and iron uptake by *Trypanoplasma borreli*.

Carp Tf is polymorphic and several alleles of carp Tf can be detected by PAGE (Irnazarow and Białowąs, 1994, 1995). We noticed, during the cultivation of *T. borreli*, a consistently faster decline in parasite number in culture media containing D-type transferrin as compared to parasite numbers in culture media with G-type transferrin. This suggests that the G-type Tf might have a superior effect on parasite growth over D-type transferrin. However, bloodstream forms of *T. brucei* require only small amounts of iron for growth (Steverding, 1998). Most likely both D and G alleles of carp Tf would carry enough iron to support growth of *T. borreli*. Indeed, if Tf would serve as a source of iron only, in media with DG-typed serum parasite growth should have been

intermediate between D and G environments. But our previous findings showed that there was no difference in the rate of parasite decline between the media supplemented with serum from DG or GG-typed fish (Jurecka et al., 2008c). Most likely, the differences between the culture media with serum of different Tf genotypes are qualitative and not quantitative, especially since we showed that the concentration of Tf in carp serum does not depend on Tf genotype. But estimation of exact Tf concentration in carp sera with different Tf genotypes is required for the further study. If the G-type of Tf would support parasite multiplication best, it would mean that in DG-typed serum the quantity of G transferrin should be enough for *T. borreli* survival. That could be the reason why we do not observe differences in parasite survival between these DG and GG-typed media. The difference in parasite survival would only be obvious when the parasites have to live in an environment where exclusively the D type of Tf is available.

Transferrin is classified as an acute-phase protein and its concentration can either rise or fall upon the stress or infection, depending on the animal species (Powanda and Moyer, 1981). Upon infection with *T. borreli*, which would need to sequester iron from Tf to survive in serum, the carp host may respond by modulating serum Tf levels. In this case the host would respond by decreasing the levels of Tf to prevent pathogens from sequestering nutrients such as iron. We could not observe an acute phase response when studying gene transcription, in the liver of infected carp. It is possible that the acute phase is regulated at the protein level, which would explain why we did not observe an immediate effect at the gene transcription level. Or, iron depletion is regulated by increasing the concentration of unsaturated Tf in plasma by reducing iron levels via storage in ferritin, which gene expression generally is up-regulated during pathogen invasion (Wooldridge and Williams, 1993; Jurando, 1997). We did not check the gene transcription level of carp ferritin but recent sequence information would allow us to investigate this process in carp in the near future.

During *T. borreli* infection we did observe a significantly higher Tf gene expression in head kidney, which organ contains many macrophages which also are a source of Tf (Haurani et al., 1973). In addition, as shown by Stafford (Stafford et al., 2004), Tf can significantly enhance the killing response of goldfish macrophages exposed to different pathogens or pathogen products. For further studies it would be interesting to see if a similar response occurs also for carp macrophages and can be dependent on Tf genotype. Moreover, determining the cDNA sequences of carp Tf alleles could possibly

provide the necessary information required to explain the functional differences observed.

4

Allelic discrimination, three-dimensional analysis and gene expression of multiple transferrin alleles of common carp (*Cyprinus carpio* L.)

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Chapter 4

Abstract

We cloned and sequenced four different transferrin (Tf) alleles (C, D, F and G) of European common carp (*Cyprinus carpio carpio* L.) and studied allelic diversity with respect to differences in sequence, constitutive transcription and three-dimensional structure. Most of the disulfide bonds were conserved between human and carp Tf, and modeling confirmed the overall conservation of the three-dimensional structure of carp Tf. While the iron-binding sites in the C-lobe of carp Tf were completely conserved, in the N-lobe the majority of iron-coordinating residues were not conserved. This may have a serious impact on the ability of carp Tf to bind iron with both the C- and N-lobe. In contrast to human Tf, we could not detect potential N-glycosylation sites in carp Tf, which does not seem to be a glycoprotein. Comparison of the cDNA of the four Tf alleles of carp indicated 21 polymorphic sites of which 13 resulted in non-synonymous changes. Allelic diversity did not seem to influence the overall conservation of carp Tf. Neither the iron binding sites, nor the receptor binding of carp Tf seemed influenced by allelic diversity. Possibly, interaction with pathogen-associated receptors for Tf could be influenced by allelic diversity. Basal gene expression of Tf alleles D and G was especially high in carp liver. Although we could detect a higher transcription level of allele D than of Tf alelle G in head kidney, thymus and spleen, the differences seem minor with respect to the very high transcription level in liver. Preliminary results with Tf-typed serum suggest a difference in the ability of Tf alleles D and G to modulate LPS-induced NO production in carp macrophages.

Introduction

Serum transferrin (Tf) is a single monomeric glycoprotein of 80-kDa belonging to the transferrin family that also includes lactoferrin in milk, ovotransferrin in avian egg white and melanotransferrin in melanoma cells (Aisen and Listowsky, 1980; Jeltsch and Chambon, 1982; Mezt-Boutigue et al., 1984; Rose et al., 1986). The major role of Tf is transport of iron that participates in a wide variety of metabolic processes, including regulation of the immune system, DNA synthesis and oxygen and electron transport. Serum Tf is synthesized in the liver and secreted into the blood. It exists as a mixture of iron-free (apo), one iron (monomeric) and two iron (holo) forms. The relative percentage of each form depends on the concentration of iron and Tf in plasma (Lieu et al., 2001). Tf also is important for the transport of metals other than iron. Since the metal binding sites of Tf are occupied by iron only for approximately 30%, other metals can be bound without requiring the displacement of the more tightly bound iron. Human Tf consists of two globular domains (N and C lobe) of approximately 330 amino acids each. Both lobes are divided into two subdomains (N-1, N-2, C-1 and C-2), separated by a deep cleft (Hall et al., 2002). Despite the fact that in each of the two lobes an Fe^{2+} cation can be bound, the N-lobe releases iron faster than the C-lobe because of a conformational change initiated by a dilysine trigger, not present in the C-lobe. The N-lobe, therefore, seems to be most important for binding iron, whereas the C-lobe is claimed to be the primary binding site for the transferrin receptor (Zak and Aisen, 2002).

There are a number of reasons to study Tf of common carp (*Cyprinus carpio* L.) in more detail. First, sequence information suggests that Tf of *Cypriniformes* fish might be partially nonfunctional owing to the substitution of important iron- coordinating residues in the N-lobe (Ciuraszkiewicz et al., 2007). Second, in the absence of high concentrations of serum albumin, Tf of common carp has been recognized as the major protein for the transport of metals other than iron, such as cadmium (De Smet et al., 2001). Third, a number of different Tf alleles have been identified by polyacrylamide gel electrophoresis (PAGE) of sera collected from different carp breeding stocks, with some deviations from expected Mendelian frequency distributions noted (Irnazarow and Białowąs, 1995). Last but not least, particular Tf alleles have been associated with resistance of carp to parasite infection (Jurecka et al., 2008c).

We cloned and sequenced four different Tf alleles (C, D, F and G) of carp identified by PAGE. The crystal structures of human lactoferrin (Anderson et al., 1987), bovine lactoferrin (Moore et al., 1997), porcine and rabbit serum Tf (Hall et al., 2002) and, most recently, human serum apotransferrin (Wally et al., 2006) indicate a strong conservation of the three-dimensional structure of the Tf protein family. We have used the crystal structure of human serum apotransferrin as a template to study the carp Tf protein. We describe the implications of non-synonymous amino acid substitutions between the alleles of carp Tf, with respect to overall structure of the protein and to iron- and receptor-binding. In addition, we studied constitutive gene expression of the two most divergent alleles, D and G, in different immune-relevant organs using allele-specific primers by real-time quantitative PCR. We also examined the ability of Tf-typed serum to modulate the induction of nitric oxide by lipopolysaccharide in carp macrophages. We discuss the implications of allelic differences for the role of Tf as immune modulator and as antimicrobial protein

Materials and methods

Animals

European common carp (*Cyprinus carpio carpio* L.) were propagated and grown at the facilities of the Institute of Ichthyobiology and Aquaculture of the Polish Academy of Sciences in Gołysz, Poland. European (*Cyprinus carpio carpio*) and East-Asian (*Cyprinus carpio haematopterus*) common carp are two subspecies that diverged more than 500,000 years ago (Kohlmann et al., 2003; Zhou et al., 2003; Thai et al., 2004). In the present study, we refer to the European common carp subspecies as carp, unless stated otherwise. Fish were healthy and 6-8 months of age at the time of experiments. Serum samples for typing of Tf allelic polymorphism were collected from n = 50 individuals from each of four different carp breeding lines of Hungarian (R0, R7, R8) and of Polish (line K) origin.

Identification of transferrin alleles by polyacrylamide gel electrophoresis (PAGE)

Total serum proteins were separated by non-reducing polyacrylamide gel electrophoresis under circumstances particularly suited to visualize Tf proteins (Jurecka et al., 2008c). Samples (5 µl) were diluted in 15 µl loading buffer (40% sucrose, 1.5% Bromophenol Blue; Sigma-Aldrich, St. Louis, MO, USA) and 2 µl of each suspension

applied on a 6% stacking and 15% polyacrylamide running gel. Electrophoresis was carried out in running buffer (72 mM Tris, 26 mM boric acid) at 90V for 30 min followed by 250V for 5 h (Smithies, 1955). Under these circumstances a number of 6-7 different alleles for carp Tf have been identified and shown to retain the ability to bind iron (Valenta et al., 1976). Protein bands were stained for 1 h with 0.04% Coomassie Brilliant Blue dissolved in 3.5% perchloric acid. Typing of serum for Tf by PAGE was used for determination of allele frequencies. Individual carp with particular Tf alleles of interest were sacrificed using Propiscine 0.2% (Kazuń and Siwicki, 2001) for subsequent RNA isolation from liver.

RNA isolation and cDNA synthesis

Total RNA was isolated from liver of individuals with particular Tf alleles of interest as identified by PAGE analysis of serum collected from different carp breeding lines of Hungarian origin. For determination of basal gene expression, RNA was isolated from different immune organs from carp known to express both D and G alleles (R3xR8 carp, (Irnazarow and Białowąs, 1994)). Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Leusden, The Netherlands) including an on-column DN*ase* treatment. Prior to RNA isolation from liver a Proteinase K treatment was also included. RNA concentration and purity was determined at OD_{260nm}/OD_{280nm}. RNA integrity was assessed by electrophoresis on 1% agarose gel. Prior to cDNA synthesis, a second DN*ase* treatment was performed using DN*ase* I, Amplification Grade (Invitrogen, Breda, The Netherlands). Synthesis of cDNA was performed with Invitrogen's Superscript^{TM} III First Strand Synthesis Systems for RT-PCR including a non-reverse transcriptase control for each sample. cDNA samples were further diluted 50 times in nuclease-free water before use as template in RT-qPCR. cDNA was stored at -20^{o}C for further use.

Cloning, sequencing and genetic analysis

Three overlapping primer sets were designed based on known fish Tf sequences from East-Asian common carp *Cyprinus carpio* Tf variant A (GenBank accession number AF457152), *Carassius auratus gibelio* (AF457151), *Oncorhynchus mykiss* (D89083), *Salmo salar* (L20313), *Oncorhynchus kisutch* (D89084), *Salmo trutta* (D89091), *Salvelinus namaycush* (D89090), *Salvelinus pluvius* (D89088), *Paralichthys olivaceus*

(AF219998, AF219997, D88801) (see Table 4.1 and Fig. 4.2). All PCR reactions were performed in *Taq* buffer using 1.5 units of *Taq* polymerase (Invitrogen, Breda, The Netherlands), 10 ng of liver cDNA, dNTPs (200 µM), $MgCl_2$ (1.5 mM), and primers (200 nM) in a final volume of 50 µl. Thirty-five cycles of amplification were conducted in a Techne Progene PCR system (Cambridge, UK), using the following parameters: 94°C for 1 min, 50-52°C for 1 min, 72°C for 1-2 min, followed by a final extension at 72°C for 7 min.

Ligation of products amplified by PCR was performed using the pGEM-T easy kit (Promega, Leiden, The Netherlands) according to protocol and cloned into JM109 high efficiency competent *Escherichia coli* cells. QIAprep Spin miniprep kit (QIAGEN) was used to isolate plasmid DNA from transformed cells. Both strands of each product were sequenced using the ABI Prism Bigdye Terminator Cycle Sequencing Ready Reaction kit and analyzed on ABI PRISM 377 DNA Sequencer. Search for similar sequences within the GenBank database were performed using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). Comparisons between the sequences were performed using the BioEdit v 7.0.4. software (Hall, 1999), with minor manual optimizations. The ExPaSy Proteomics Tools was used to calculate the theoretical isoelectric points of the different Tf alleles.

Table 4.1. Primer sequences applied to detect the four carp transferrin alleles and for real-time quantitative PCR (RT-qPCR). + indicates an LNA modification (underlined).

Primer name	Sequence 5'- 3'
cDNA	
Tffw1	ATGAACATCCTGCTCA
Tffw2	GGAAAAGCGTTGAGGAGT
Tffw3	GTCATGGTTGAGCAGAGTAAT
Tfrv1	TGCTGTGAATCAGCA
Tfrv2	TCAGGGTATTCCAGGTTAC
Tfrv3	ACTTGATCACATTGTCACCG
RT-qPCR	
qTfG_fw1	GATCACGCAAAGATGTGGTGAACG
qTfD_fw2	GATGCACGCAAAGATGTGGTAAACA
qTfG_rv1	GGT CCTTT+CAAT+CATGT+CAATGTAC
qTfD_rv2	GGTCCTT+TCAATCA+TG+TCAATGTAG

Real-Time quantitative Polymerase Chain Reaction (RT-qPCR)

To study constitutive gene expression of Tf alleles D and G, RNA was isolated from heterozygous carp (n = 5) known to express both D and G alleles but not the C and F alleles (cross R3xR8, (Jurecka et al., 2008c)). Constitutive gene expression was studied by real-time quantitative PCR (RT-qPCR) essentially as described previously (Forlenza et al., 2008). RT-qPCR was performed using a Rotor-Gene™ 2000 (Corbett Research, Mortlake, Sydney, Australia) and Brilliant® SYBR® Green QPCR (Stratagene, La Jolla, CA, USA) as detection chemistry. The primers used for RT-qPCR are presented in Table 4.1. We used LNA modifications to overcome potential problems associated with the inability of Taq polymerase to discriminate between two sequences differing in only one a few nucleotides. Master-mix for each PCR run was prepared as follows: 1 µl of each primer (4.2 µM), 7 µl Master SYBR Green I mix and 5 µl of diluted cDNA. The following amplification program was used: after 15 min of denaturation at 95ºC, 40 cycles of RT-qPCR with three-step amplification were performed: 15 s at 95ºC for denaturation, 30 s at 60ºC for annealing and 30 s at 72ºC for elongation followed by a final holding step of 1 min at 60ºC. A melting step was then performed, and in all cases the amplifications were specific and no amplification was observed in negative controls (non-template control and non-reverse transcriptase control). The melting curve analysis confirmed that the two primer sets consistently amplified two different products, each having two specific and distinct melting temperatures: 77.2 for allele D, and 78.1 for allele G. Fluorescence data from RT-qPCR experiments were analyzed using Rotor-Gene version 6.0.21 software. The cycle threshold C_t for each sample and the reaction efficiencies (E) for each primer set were obtained upon Comparative Quantitation Analysis from the Rotor-Gene version 6.0.21 software (40S: E = 1.75; Tf-D = 1.75; Tf-G = 1.71). Basal transcription of immune-related genes was calculated as a ratio of reference gene versus target gene in different organs (Pfaffl, 2001; Tichopad et al., 2003). The 40S ribosomal protein S11 was used as an internal reference gene.

Three-dimensional modelling of carp transferrins

The structure of human serum apo-Tf with a resolution of 2.7 Å (PDB entry: 2HAU) was used as a template to model the carp Tf C, D, F and G alleles with the program MODELLER (version 9v1 (Sali and Blundell, 1993; Eswar et al., 2003)) using the CVFF force field (Dauber-Osguthorpe et al., 1988). The models were verified after

several rounds of sequence alignment adjustments and energy minimization. Stereochemical quality of the homology models was assessed by PROCHECK (Laskowski et al., 1993). Protein folding quality was verified by PROSAII (Sippl, 1993), which independently evaluates the compatibility of each residue to its environment.

Nitric oxide production of carp macrophages

Carp head kidney-derived macrophages were obtained as described previously (Joerink et al., 2006). Macrophages were stimulated with lipopolysaccharide (0.1 µg/ml; *E. coli*, Sigma L2880) and grown on 5% pooled carp serum obtained from animals homozygous for the Tf allele D or homozygous for the Tf allele G. Nitrite production was measured after 18h incubation at 27°C as described before (Green et al., 1982). Briefly, to 75 µl cell culture supernatant 100 µl 1% (w/v) sulfanilamide in 2.5% (v/v) phosphoric acid and 100 µl 0.1% (w/v) *N*-naphthyl-ethylenediamine in 2.5% (v/v) phosphoric acid were added. The absorbance was measured at 540 nm and nitrite concentrations (µM) were calculated from a standard curve based on known sodium nitrite concentrations.

Statistics

Differences in basal gene expression between Tf alleles D and G in different organs were tested for significance by a *t* test for independent samples. All analyses were performed using Statistica 6.0 software (StatSoft). Differences were considered significant at $P \leq 0.05$. Nitrite production was tested for significance by a non-paired, two tailed Student's t-test. $P \leq 0.05$ was accepted as significant.

Results

Identification of four carp transferrin alleles by PAGE

Polyacrylamide gel electrophoresis of serum proteins showed polymorphism for carp Tf, discriminating four alleles (C, D, F and G, allele designation by Irnazarow and Białowąs, (Irnazarow and Białowąs, 1994)) in different genotypic combinations (Fig. 4.1).

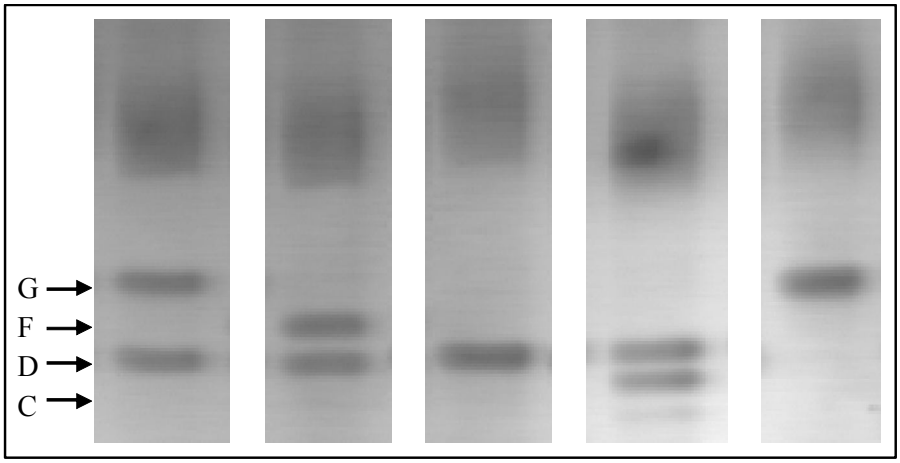


Fig. 4.1. Five different transferrin genotypes (from the left: DG, DF, DD, CD and GG) as detected by non-reducing polyacrylamide gel electrophoresis (PAGE) in European common carp (*Cyprinus carpio carpio* L.). Serum samples were applied on a 6% stacking and 15% polyacrylamide running gel. Protein bands were stained with Coomassie Brilliant Blue.

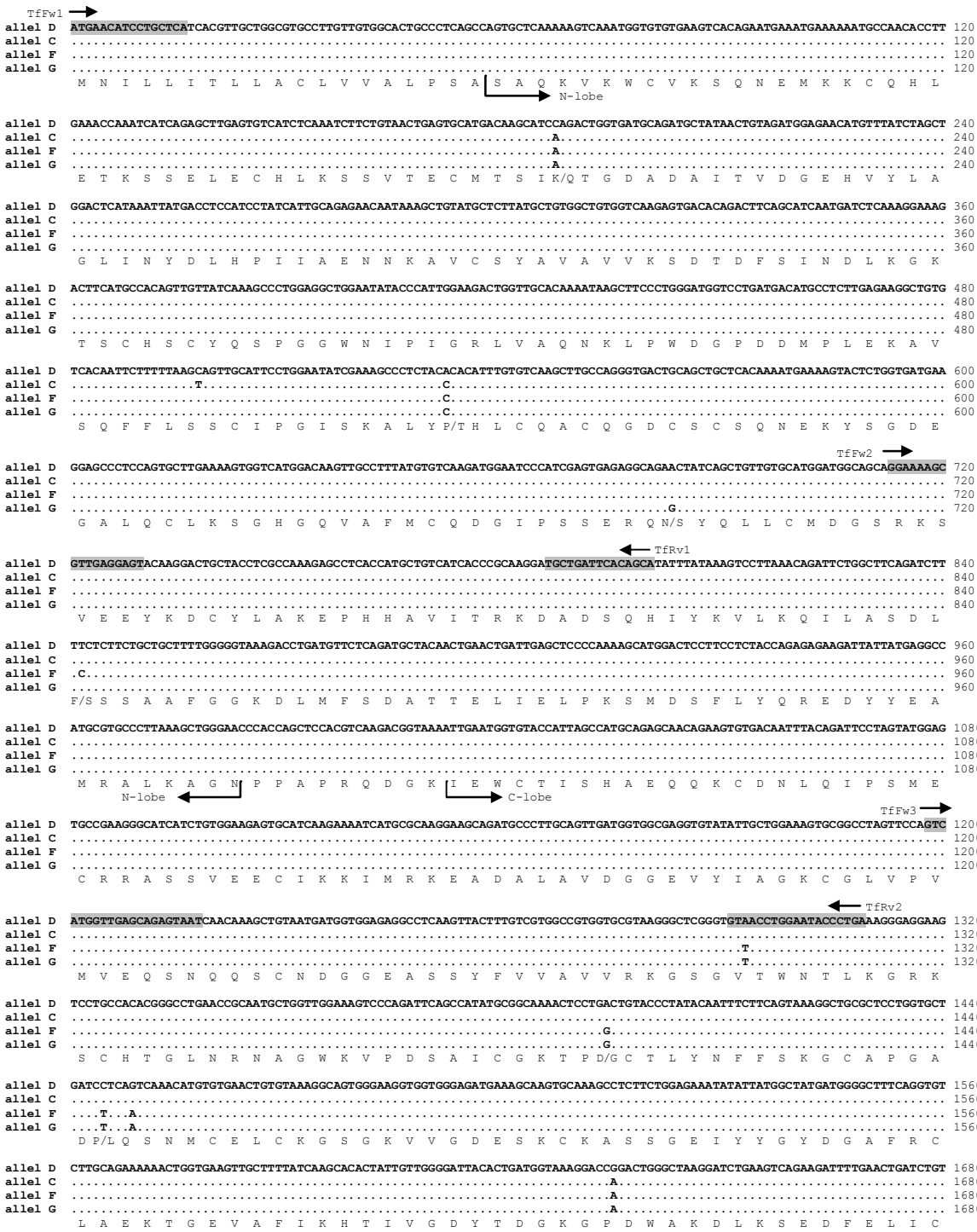
The D and G alleles were most frequent, while the C and F alleles were present only in R0 and K carp lines, respectively (Table 4.2). Four individuals, two homozygotes typed as DD and GG (individuals from lines R7 and R8, respectively) and two heterozygotes typed as CD and DF (individuals from lines R0 and K, respectively), were selected for further sequence analysis. These fish were sacrificed and liver samples were collected for RNA isolation, cDNA synthesis and PCR analysis. First, PCR products of the homozygous individuals (DD and GG) were cloned and sequenced. No sequence variations were detected between three independent PCRs for each of these two genotypes. To obtain the sequence of the less frequent C and F alleles, PCR products of the heterozygous individuals (CD and DF) were cloned and sequenced. This approach allowed us to correlate allelic diversity identified by PAGE to the cDNA sequences. The number of different cDNA sequences obtained from each individual corresponded to its homo- or heterozygous status. The four pI values, predicted as 5.85 for allele C, 5.78 for allele D, 5.92 for allele F and 6.00 for allele G, generally corresponded well to the migration pattern on native PAGE except for the relatively high pI value for allele C. The coding sequences for the four carp Tf alleles were designated C, D, F and G, corresponding to the respective alleles identified by PAGE.

Table 4.2. Allele frequency in breeding lines R0, R7, R8 and K (n=50 individuals/line). Line R7 is characterized mostly by the presence of the D allele, line R8 by G and D. For line R0 and K alleles C and F respectively are present.

Allele Line	C	D	F	G
R0	0.061	0.666	0	0.273
R7	0	0.892	0	0.108
R8	0	0.442	0	0.558
K	0	0.715	0.271	0.014

Carp transferrin has a conserved three-dimensional structure

We obtained full-length 1998 bp mRNA coding sequences and a 246 bp non-coding region (3'end) of four carp Tf alleles C, D, F and G (GenBank accession numbers: EU715322, EU715323, EU715324, EU715325; see also Fig. 4.2). The translated carp Tf sequences are 666 amino acids (aa) in length and share 98.3-99.7% overall identity. Analysis of the predicted aa sequences revealed the presence of a (partial) leader peptide which upon cleavage would release proteins of about 71 kDa. Alignment of the aa sequences with other known Tf sequences showed 84% of overall identity with the East-Asian common carp, 79-76.6% with crucian carp alleles (*Carassius auratus*), 60.0% with zebrafish (*Danio rerio*) and 35.7% with human Tf (Fig. 4.3). Similar to human Tf, carp Tf consists of two globular domains (N and C lobe) of 330 and 327 aa, which again can be sub-divided into two subdomains (N-1, N-2, C-1 and C-2, see also Figs 4.2 and 4.3). Carp Tf does not contain potential N-glycosylation sites.


TfFw1
allel D ATGAACATCCTGCTCATCACGTTGCTGGCGTGCCTTGTTGTGGCACTGCCCTCAGCCAGTGCTCAAAAAGTCAAATGGTGTGTGAAGTCACAGAATGAAATGAAAAAATGCCAACACCTT 120
allel C 120
allel F 120
allel G 120
M N I L L I T L L A C L V V A L P S A S A Q K V K W C V K S Q N E M K K C Q H L
N-lobe
allel D GAAACCAAATCATCAGAGCTTGAGTGTCATCTCAAATCTTCTGTAACTGAGTGCATGACAAGCATCCAGACTGGTGATGCAGATGCTATAACTGTAGATGGAGAACATGTTTATCTAGCT 240
allel C .....A..... 240
allel F .....A..... 240
allel G .....A..... 240
E T K S S E L E C H L K S S V T E C M T S I K/Q T G D A D A I T V D G E H V Y L A
allel D GGACTCATAAATTATGACCTCCATCCTATCATTGCAGAGAACAATAAAGCTGTATGCTCTTATGCTGTGGCTGTGGTCAAGAGTGACACAGACTTCAGCATCAATGATCTCAAAGGAAAG 360
allel C 360
allel F 360
allel G 360
G L I N Y D L H P I I A E N N K A V C S Y A V A V V K S D T D F S I N D L K G K
allel D ACTTCATGCCACAGTTGTTATCAAAGCCCTGGAGGCTGGAATATACCCATTGGAAGACTGGTTGCACAAAATAAGCTTCCCTGGGATGGTCCTGATGACATGCCTCTTGAGAAGGCTGTG 480
allel C 480
allel F 480
allel G 480
T S C H S C Y Q S P G G W N I P I G R L V A Q N K L P W D G P D D M P L E K A V
allel D TCACAATTCTTTTTAAGCAGTTGCATTCCTGGAATATCGAAAGCCCTCTACACACATTTGTGTCAAGCTTGCCAGGGTGACTGCAGCTGCTCACAAAATGAAAAGTACTCTGGTGATGAA 600
allel C .....T.....C..... 600
allel F .....C..... 600
allel G .....C..... 600
S Q F F L S S C I P G I S K A L Y P/T H L C Q A C Q G D C S C S Q N E K Y S G D E
TfFw2
allel D GGAGCCCTCCAGTGCTTGAAAAGTGGTCATGGACAAGTTGCCTTTATGTGTCAAGATGGAATCCCATCGAGTGAGAGGCAGAACTATCAGCTGTTGTGCATGGATGGCAGCAGGAAAAGC 720
allel C 720
allel F 720
allel G .....G..... 720
G A L Q C L K S G H G Q V A F M C Q D G I P S S E R Q N/S Y Q L L C M D G S R K S
TfRv1
allel D GTTGAGGAGTACAAGGACTGCTACCTCGCCAAAGAGCCTCACCATGCTGTCATCACCCGCAAGGATGCTGATTCACAGCATATTTATAAAGTCCTTAAACAGATTCTGGCTTCAGATCTT 840
allel C 840
allel F 840
allel G 840
V E E Y K D C Y L A K E P H H A V I T R K D A D S Q H I Y K V L K Q I L A S D L
allel D TTCTCTTCTGCTGCTTTTGGGGGTAAAGACCTGATGTTCTCAGATGCTACAACTGAACTGATTGAGCTCCCCAAAAGCATGGACTCCTTCCTCTACCAGAGAGAAGATTATTATGAGGCC 960
allel C 960
allel F .C..... 960
allel G 960
F/S S S A A F G G K D L M F S D A T T E L I E L P K S M D S F L Y Q R E D Y Y E A
allel D ATGCGTGCCCTTAAAGCTGGGAACCCACCAGCTCCACGTCAAGACGGTAAAATTGAATGGTGTACCATTAGCCATGCAGAGCAACAGAAGTGTGACAATTTACAGATTCCTAGTATGGAG 1080
allel C 1080
allel F 1080
allel G 1080
M R A L K A G N P P A P R Q D G K I E W C T I S H A E Q Q K C D N L Q I P S M E
N-lobe
C-lobe
TfFw3
allel D TGCCGAAGGGCATCATCTGTGGAAGAGTGCATCAAGAAAATCATGCGCAAGGAAGCAGATGCCCTTGCAGTTGATGGTGGCGAGGTGTATATTGCTGGAAAGTGCGGCCTAGTTCCAGTC 1200
allel C 1200
allel F 1200
allel G 1200
C R R A S S V E E C I K K I M R K E A D A L A V D G G E V Y I A G K C G L V P V
TfRv2
allel D ATGGTTGAGCAGAGTAATCAACAAAGCTGTAATGATGGTGGAGAGGCCTCAAGTTACTTTGTCGTGGCCGTGGTGCGTAAGGGCTCGGGTGTAACCTGGAATACCCTGAAAGGGAGGAAG 1320
allel C 1320
allel F .....T..... 1320
allel G .....T..... 1320
M V E Q S N Q Q S C N D G G E A S S Y F V V A V V R K G S G V T W N T L K G R K
allel D TCCTGCCACACGGGCCTGAACCGCAATGCTGGTTGGAAAGTCCCAGATTCAGCCATATGCGGCAAAACTCCTGACTGTACCCTATACAATTTCTTCAGTAAAGGCTGCGCTCCTGGTGCT 1440
allel C 1440
allel F .....G..... 1440
allel G .....G..... 1440
S C H T G L N R N A G W K V P D S A I C G K T P D/G C T L Y N F F S K G C A P G A
allel D GATCCTCAGTCAAACATGTGTGAACTGTGTAAAGGCAGTGGGAAGGTGGTGGGAGATGAAAGCAAGTGCAAAGCCTCTTCTGGAGAAATATATTATGGCTATGATGGGGCTTTCAGGTGT 1560
allel C 1560
allel F ....T...A..... 1560
allel G ....T...A..... 1560
D P/L Q S N M C E L C K G S G K V V G D E S K C K A S S G E I Y Y G Y D G A F R C
allel D CTTGCAGAAAAAACTGGTGAAGTTGCTTTTATCAAGCACACTATTGTTGGGGATTACACTGATGGTAAAGGACCGGACTGGGCTAAGGATCTGAAGTCAGAAGATTTTGAACTGATCTGT 1680
allel C .....A..... 1680
allel F .....A..... 1680
allel G .....A..... 1680
L A E K T G E V A F I K H T I V G D Y T D G K G P D W A K D L K S E D F E L I C

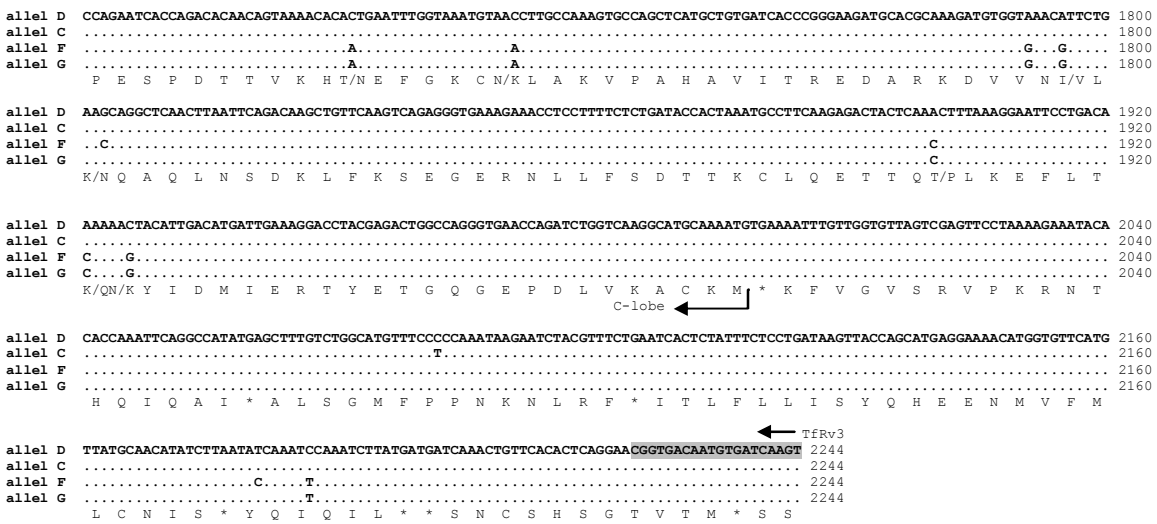
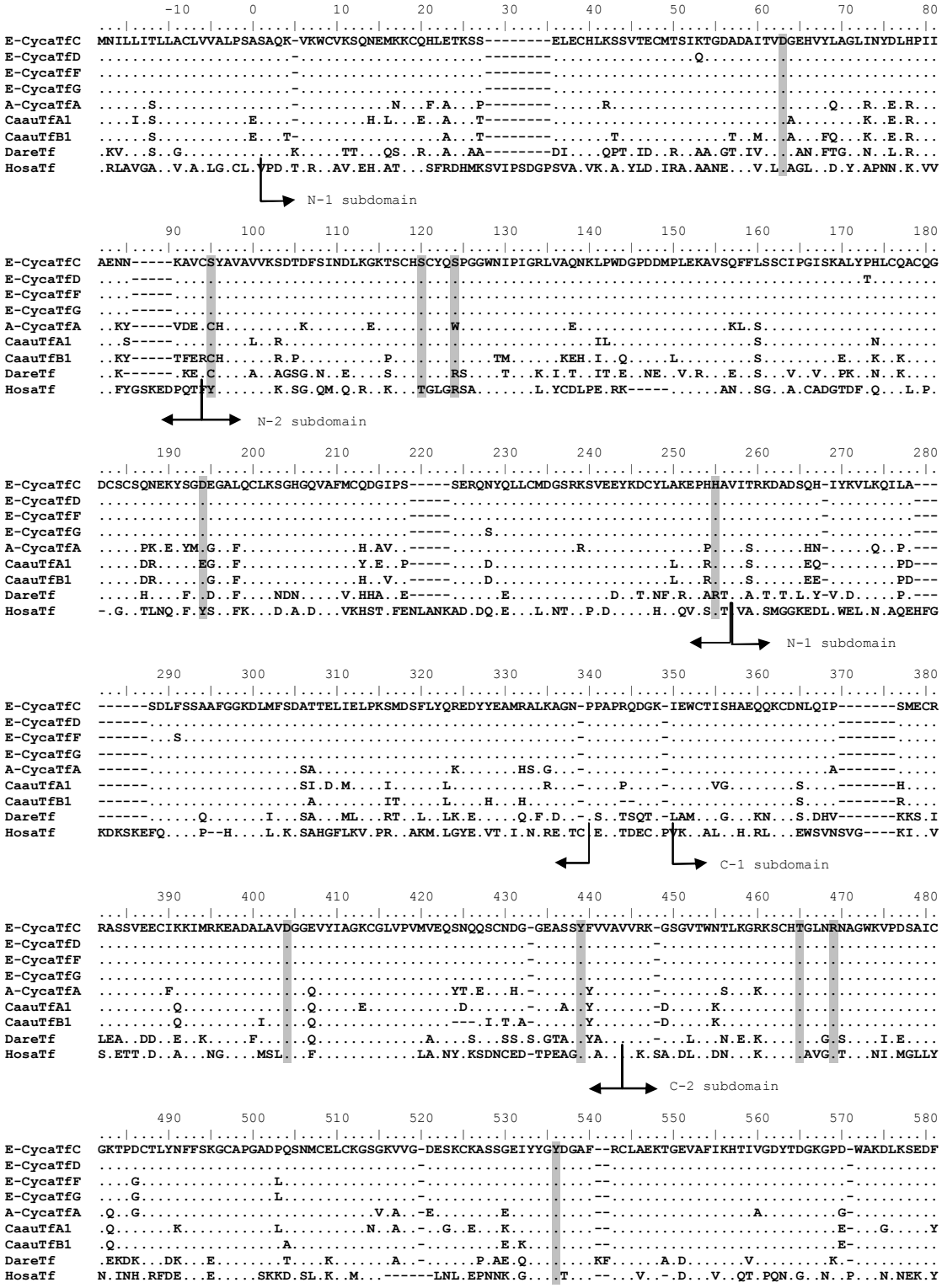


Figure 4.2. Nucleotide sequence alignment of European common carp transferrin alleles C, D, F and G, and deduced protein sequence. Nucleotides are numbered starting with the first nucleotide of the start codon. Dots (.) represent identical nucleotides; arrows border the N- and C-lobes; shadings indicate the position of the primers used for PCR (Fw1, Fw2, Fw3, Rv1, Rv2 and Rv3; see Table 1). Asterisks (*) indicate stop codons.

A total number of 13 non-synonymous aa substitutions was noted among the four carp Tf alleles, with alleles D and G considered most divergent. The structure of the human apo-Tf (PDB entry: 2HAU) was used as template for homology modelling of the carp Tf alleles. The PROCHECK output showed no residues in disallowed regions, indicating correct dihedral orientation of the backbone residues. The PROSAII check yielded no regions with unusual high energy score, indicative of a good packing of the structure. Four three-dimensional models were generated based on the differences in aa between the four alleles. All models were virtually identical. A ribbon representation of the structure of the two most divergent alleles D and G is shown in Fig. 4.4A. Carp Tf has 17 disulfide bonds while human Tf has 19 disulfide bonds. It is likely that all cysteine residues present in carp Tf form disulfide bonds. The majority (14/19) of the disulfide bonds in human Tf were conserved or had a clear alternative (2/19) in carp (Table 4.3), while in one particular case carp Tf had a unique disulfide bond in the N-lobe without alternative in human Tf (Table 4.3). The overall three-dimensional structure of carp Tf (Fig. 4.4B) was highly conserved, independent of allelic diversity.


N-1 subdomain
N-2 subdomain
N-1 subdomain
C-1 subdomain
C-2 subdomain

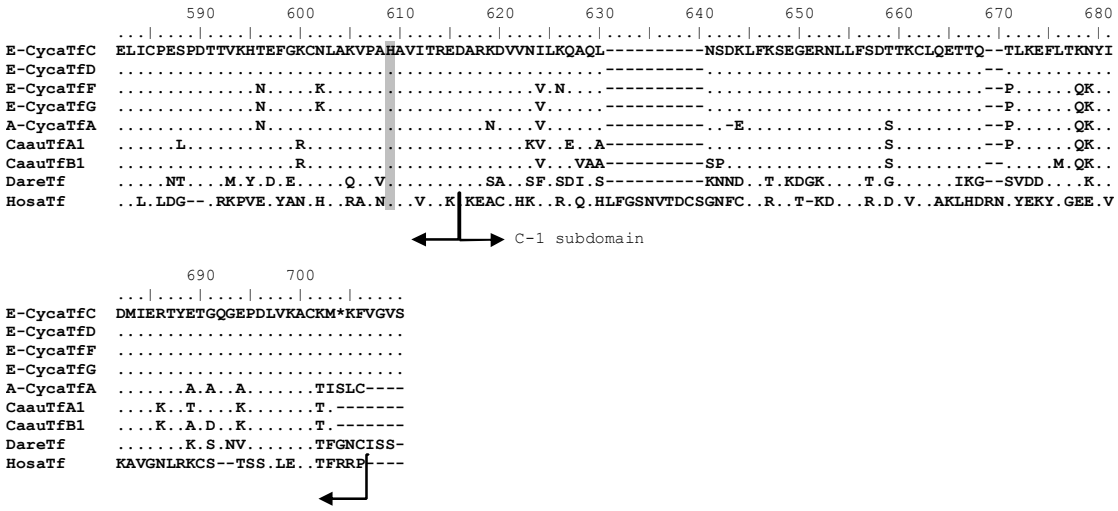


Fig. 4.3. Protein alignment of known transferrin protein sequences. European common carp *Cyprinus carpio* (E-CycaTf) alleles C, D, F and G, East-Asian *Cyprinus carpio* (A-CycaTf) variant A (GenBank accession No. AF457152), *Carassius auratus* (CaauTf) variant A1 (AAM90972), *Carassius auratus* variant B1 (AAM90973), *Danio rerio* (DareTf) (DAA01798) and Homo sapiens (HosaTf) (NP_001054). Dots (.) represent identity to the European common carp Tf C allele. Dashes (-) denote gaps. Arrows indicate the different subdomains of Tf. Iron binding sites are shaded. Numbering is according to mammalian serum transferrin (Lambert et al., 2005).

Allelic diversity of carp transferrin does not affect receptor-binding

In humans, the C-lobe is claimed the primary binding site for the Tf receptor, whereas the N-lobe seems most important for iron binding. Of the human C-lobe only the C-1 subdomain appears to be involved in the interaction with the receptor (Cheng et al., 2004), with a number of n=11 residues in apparent contact with the helical region of the Tf receptor. These residues, situated between His357 and Glu385 (exact positions not shown), are not conserved in the C-1 subdomain of carp Tf.

Of the human N-lobe, both the N-1 and N-2 subdomains seem to contact the Tf receptor, but the N-1 subdomain does not show a convincing pattern of conservation (Wally et al., 2006). For that reason, we did not consider the residues of the N-1 subdomain of carp Tf for receptor-binding. Within the N-2 subdomain of human Tf, there are four residues (Pro142-Arg143-Lys144-Pro145) that appear to make contact with the Tf receptor (Wally et al., 2006). Of these four residues, there are two residues, Pro142 and Pro145 (Pro150 in carp), that are conserved in the N-2 subdomain of carp Tf (Fig. 4.3). These two residues were not different between the different Tf alleles of carp. To get more insight in the receptor contact residues of carp Tf, we used the

structure of the TfR-Tf complex (PDB entry: 1SUV, (Cheng et al., 2004)) as template for homology modelling of carp Tf binding to a Tf receptor. Superpositioning of the carp Tf alleles on the human Tf in the TfR-Tf complex (Fig. 4.4C) showed that of a total of n=13 non-synonymous changes, only one single aa would be in the contact area with the receptor. Although it remains difficult to reliably position carp Tf in a human TfR-Tf complex, it seems reasonable to assume that the allelic diversity of carp Tf will not affect the ability to interact with the receptor.

Table 4.3. S-S disulfide bonds in human and carp Tf. Carp transferrin has 17 disulfide bonds. In one particular case carp Tf had a unique disulfide bond in the N-lobe without alternative in human Tf (number 16 in table). 'No alternative' is indicated as 'na'.

Number S-S bond	Human Tf S-S bond	Carp Tf S-S bond	Alternative S-S bond
1	cys 9 - cys 48	cys 6 - cys 37	
2	cys 19 - cys 39	cys 16 - cys 28	
3	na	cys 78 - cys 196	
4	cys 118 - cys 194	cys 102 - cys 184	
5	cys 137 - cys 331	na	
6	cys 158 - cys 174	cys 147 - cys 163	
7	cys 161 - cys 179		cys 105 - cys 169
8	cys 171 - cys 177	cys 160 - cys 167	
9	cys 227 - cys 241	cys 212 - cys 226	
10	cys 339 - cys 596	na	
11	cys 345 - cys 377	cys 320 - cys 349	
12	cys 355 - cys 368	cys 330 - cys 340	
13	cys 402 - cys 674	cys 374 - cys 643	
14	cys 418 - cys 637	cys 389 - cys 606	
15	cys 450 - cys 523	cys 421 - cys 499	
16	cys 474 - cys 665		cys 445 - cys 439
17	cys 484 - cys 498	cys 455 - cys 479	
18	cys 495 - cys 506	cys 466 - cys 482	
19	cys 563 - cys 577	cys 539 - cys 555	
20	cys 615 - cys 620	na	

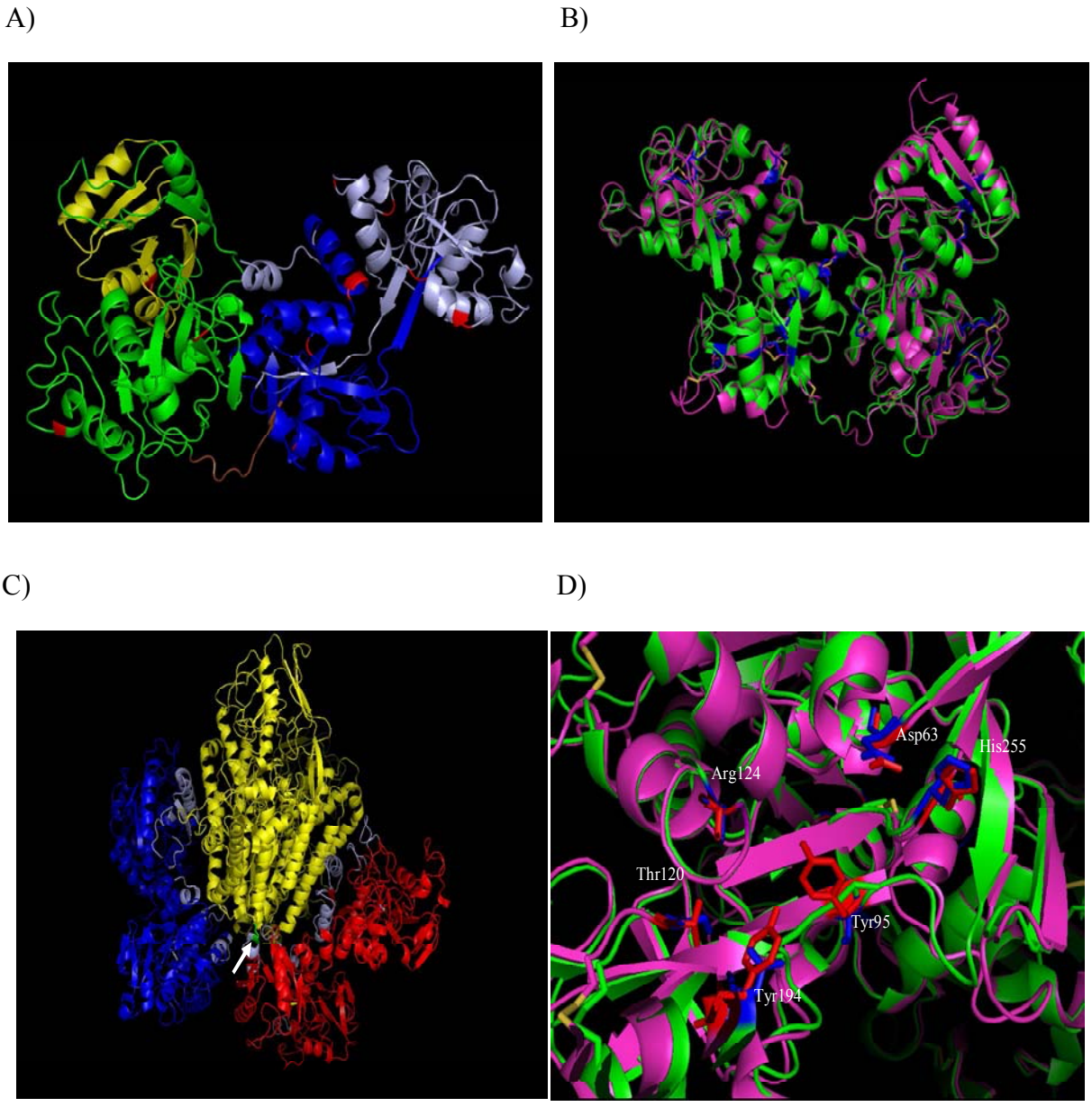


Fig. 4.4. Three-dimensional model of European common carp transferrin.

(**A**) Ribbon representation of carp transferrin allele G. Red colour represents aa substitutions present in allele D. Carp transferrin consists of two globular domains (N and C lobe) of approximately 330 amino acids each. Each lobe is divided into two subdomains (N-1, yellow; N-2, green; C-1, blue and C-2, light blue) and joined by a connecting peptide (brown). (**B**) Carp transferrin allele D (green) modelled on human serotransferrin (violet). Note that the cysteine residues in carp (light brown) and human (dark blue) are in similar positions. (**C**) Ribbon representation of the binding of one molecule of human Tf (dark blue) and one molecule of carp Tf (red) to the human transferrin receptor (yellow) Contact areas are indicated in light blue. Among the four carp Tf alleles only one non-synonymous change is located within the contact area (green colour, indicated with the white arrow). (**D**) N-lobe of carp transferrin allele D (green) modelled on human serotransferrin (violet). Iron-binding sites of carp transferrin are indicated in blue and iron binding sites of human transferrin are indicated in red.

Iron-binding sites are conserved only in the C-lobe and not in the N-lobe and are not affected by allelic diversity

In human Tf both the N- and C- lobe of the Tf molecule have four Fe-binding residues and two anion binding residues. The concomitant binding of a synergistic anion is absolutely necessary for iron binding. All six iron-coordinating residues are fully conserved (with respect to human Tf) in the C-lobe of all carp Tf alleles. In the N-lobe of carp Tf, however, of the six residues (Asp63, Tyr95, Thr120, Arg124, Tyr194 and His255), four (Tyr95, Thr120, Arg124 and Tyr194) are not conserved (Fig. 4.3, Fig. 4.4D). This was true for the N-lobe of all carp Tf alleles. Although the two tyrosine replacements are common to all cyprinid fish, the tyrosines are not always replaced by the same aa, not even when comparing East-Asian and European subspecies of common carp. In European common carp, the two tyrosine residues are replaced with a serine (Ser95) and an aspartic acid (Asp194). Overall, the aa substitutions in the iron-coordinating residues in the N-lobe of carp Tf could have a serious impact on the iron binding capacity. A reduced iron-binding capacity of the N-lobe of carp Tf, however, would not be influenced by the diversity between the carp Tf alleles.

Constitutive gene expression of D and G transferrin alleles in immune organs

Clearly, mRNA transcription of both D and G alleles of carp Tf was especially high in liver (Fig. 4.5). Whereas the internal reference gene (40S) was detectable already after about 18 PCR cycles (not shown), the ratio of Tf versus 40S gene transcription was $\geq$ 30 (!). This indicates that Tf genes are transcribed at very high constitutive levels in liver. In general, the D allele showed slightly higher mRNA transcription than the G allele of carp Tf, with significant ($P \leq 0.05$) differences in all tested immune organs except gut, which could not be ascribed to differences in efficiency between the two primer sets. The difference was minor compared to the level of gene expression.

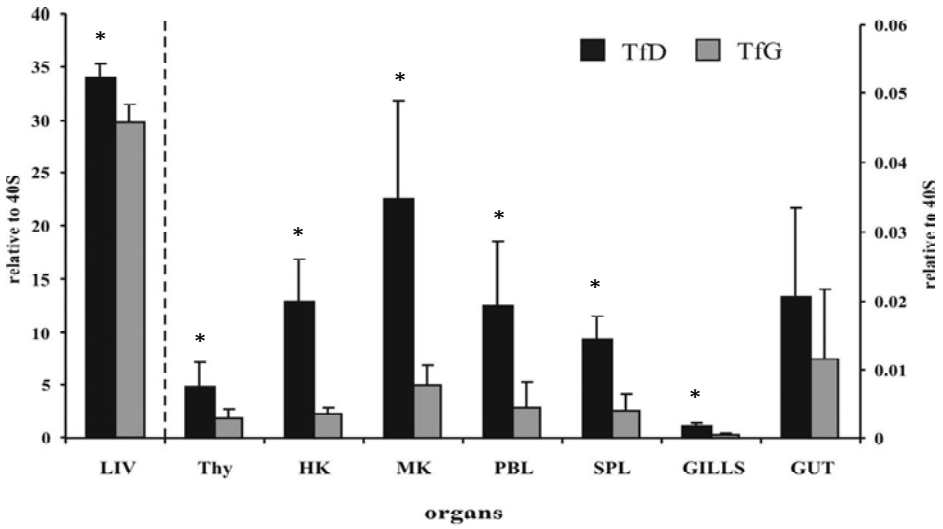


Fig. 4.5. Constitutive gene expression of transferrin alleles D (black bars) and G (shaded bars) in different immune organs of European common carp measured in the same heterozygous fish. Gene expression was analyzed by real-time quantitative PCR and shown relative to the house keeping gene 40S. Y – axes characterize gene transcription in liver (left y-axis) or in other immune organs (right y-axis). Note the difference in scale between the two y-axes. * Statistically significant difference at $P \leq 0.05$. LIV, liver; Thy, thymus; HK, head kidney; MK, mid kidney; PBL, peripheral blood leucocytes; SPL, spleen.

Serum from carp with different Tf genotypes affect the production of nitrite oxide by macrophages

Macrophage cultures (6 days) were grown in medium without serum addition and left untreated (negative control), or grown in medium supplemented with 5% Tf typed carp serum (DD or GG) with co-stimulation with LPS (0.1 µg/ml). Nitrite levels were dependent on the Tf serotype present in the medium, with the highest ($P < 0.03$) induction of NO in the presence of DD Tf-typed serum (Fig.4.6).

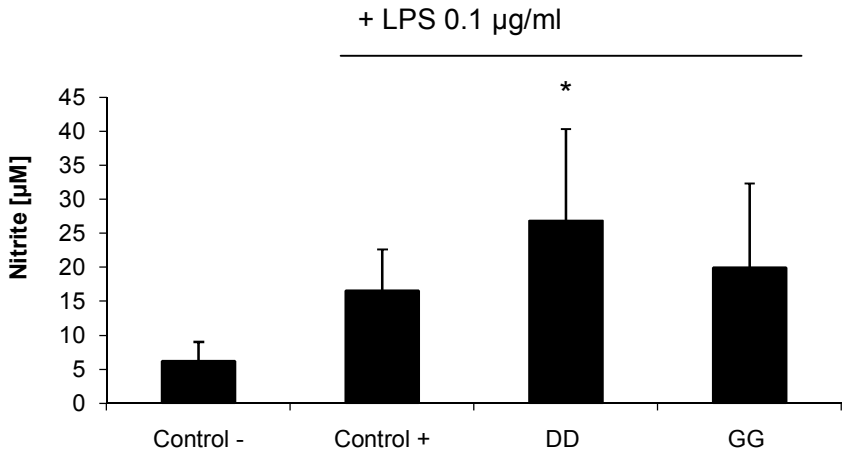


Fig. 4.6. Carp macrophages ($5x10^5$ cells/well) were seeded in triplicate into 96 well culture plates in medium without serum addition or in medium supplemented with 5% transferrin typed carp serum (DD or GG) and left untreated as negative control, or co-stimulated with LPS (0.1 µg/ml), and incubated for 18 h at 27°C prior to determination of NO production. Each bar represents the mean ±SD of five independent experiments.

* Statistically significant difference at $P \leq 0.05$.

Discussion

In this study we identified four different Tf alleles (C, D, F and G) by PAGE which we then cloned and sequenced. We studied the three-dimensional structure of carp Tf and analyzed the sequence differences between the four Tf alleles. In comparison with human Tf, the overall three-dimensional structure of the four alleles of carp Tf is well conserved, except for the iron-coordinating residues in the N-lobe. Allelic diversity did not influence the overall conservation of carp Tf. There were no differences in tissue distribution between the two most divergent alleles (D and G). Although we could detect a higher transcription level of D than of G in head kidney, thymus and spleen, the differences seem minor with respect to the very high transcription level in liver. Preliminary results with Tf-typed serum suggest a difference in the ability of D and G-type Tf to modulate LPS-induced NO production in carp macrophages.

Similar to human Tf, carp Tf consists of two globular lobes (N and C) joined by a short peptide strand. In contrast to human Tf, we could not detect potential N-glycosylation sites in carp Tf, which seems a cyprinid (Yang and Gui, 2004) but not a salmonid (Lee et al., 1998) feature of fish Tf. Although carp Tf did not have equivalents to two disulfide bonds in human Tf and had one unique disulfide bond, the majority of the

disulfide bonds were conserved in carp Tf. The putative existence of an intra-chain disulfide bond in cyprinid fish (Ciuraszkiewicz et al., 2007) was not supported by our modeling study. In general, the models confirmed the conserved three-dimensional structure of carp Tf.

An overall low conservation of iron binding residues in the N-lobe of Tf has been noted for all *Cypriniformes* fish species (Ciuraszkiewicz et al., 2007). Indeed, of the six iron-coordinating residues in the N-lobe, only two residues are conserved in carp. Both globular lobes of human Tf can bind iron, but iron binding is more prominent in the N-lobe than the C-lobe; the latter is thought to play a more important role in binding to the receptor (Zak and Aisen, 2002). Mutagenesis studies on the human Tf iron-binding site, mutating the six aa responsible for iron and anion binding (in the N-lobe), have shown that any destabilization of the site leads to accelerated rates of iron release (reviewed in (Lambert et al., 2005)). This would suggest that carp Tf could have a lower ability to bind iron due to the substitution of the majority of iron-coordinating residues in the N-lobe. Studies on total iron binding capacity (TIBC) in carp serum (Wojtczak et al., 2007b) do not suggest major differences in TIBC from human serum. Possibly, carp Tf serum concentrations could be higher to compensate for a reduced ability to bind and transport iron. Although a reduced iron-binding capacity of the N-lobe of carp Tf would not be influenced by the allelic diversity we observed, surely further study is needed to examine the iron-binding function of carp Tf.

Allelic diversity between the four alleles of European common carp was most obvious for the C-lobe of Tf, with 9 (out of a total of 13) non-synonymous changes. These changes, however, were neither located in the iron binding site, nor (except for a single aa) in the area presumably involved in binding to the Tf receptor. Competition for iron between pathogen and host also could be a contributing factor to Tf polymorphism. Studies in goldfish (Yang and Gui, 2004; Yang et al., 2004) and salmonids (Ford, 2001) identified particular residues subject to positive selection and suggested these residues to be important for interaction with bacterial Tf-binding proteins. Many pathogenic bacteria have evolved systems to acquire iron from Tf. These transport systems consist, among others, of an integral outer membrane receptor/transporter (TbpA) and a lipid anchored co-receptor (TbpB) on the cell surface. Interaction between human Tf and bacterial Tf-binding protein occurs at both the C- and N-lobes of Tf, each interacting with one of the two lobes of TbpB. Interaction between hTf and TbpB is quite

extensive; at least six peptide regions in each lobe of hTf could play a role in the interaction with TbpB (Retzer et al., 1999; Boulton et al., 1999). Although we did not study the potential TbpB-binding sites in carp Tf in detail, it seems that for carp Tf, six (out of a total of 13) non-synonymous changes are located in putative TbpB-interacting regions (data not shown).

Of course, not only competition between bacteria and host could be a contributing factor to Tf polymorphism. The importance of Tf as an essential growth factor for parasites such as the bloodstream form of *Trypanosoma brucei* has been clearly shown (Schell et al., 1991). Transferrin uptake by *T. brucei* involves binding to a heterodimeric Tf-binding protein complex, which is internalised and transported to lysosomes where Tf is proteolytically degraded. We have recently shown that allelic forms of Tf could influence the ability of carp to resist a parasitic infection with the hemoflagellate *Trypanoplasma borreli* (Jurecka et al., 2008c). Homozygous DD individuals showed lower parasitaemia than individuals with other Tf genotypes. Although we now have cDNA and structural information on the differences between particular carp Tf alleles, additional functional studies are required before an association between allelic diversity and resistance to pathogens such as *T. borreli* can be explained.

There might be a further role of Tf that could be a contributing factor to Tf polymorphism. It has been shown that Tf cleavage products can modulate macrophage function by induction of nitrite oxide (NO) in goldfish macrophages (Stafford et al., 2001). Possibly, Tf allelic diversity could be related in function to differences in NO induction, and host-pathogen interaction could be influenced by the amount of NO induced by different Tf alleles. Although the primary cleavage site of Tf (Stafford and Belosevic, 2003) seems to be conserved among the different alleles of goldfish and carp Tf, it may not be the only cleavage site. Therefore, it would be interesting to study whether different Tf alleles could have different influences on NO production by macrophages. In conclusion, we demonstrated that the overall structure of carp Tf is conserved but differs from the human Tf with respect to the iron-coordinating residues in the N-lobe. Allelic polymorphism of carp Tf may be connected with competition for iron with pathogens, or with the ability of Tf to stimulate macrophages. The *T. borreli* infection model could serve to further investigate the functional implications of carp Tf polymorphism.

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5

The induction of nitric oxide response of carp macrophages by transferrin is influenced by the allelic diversity of the molecule

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Chapter 5

Abstract

The central role of transferrin (Tf) as an iron transporting protein has been extended by observations that modified versions of Tf also participate in the regulation of innate immunity. We report on the isolation of two carp Tf proteins (alleles D and G) to purity using rivanol precipitation and ion-exchange chromatography, and describe the activation of head kidney-derived carp macrophages by cleaved Tf. We demonstrate the superiority of the D-type over the G-type Tf in inducing nitric oxide (NO) and confirm previous observations that full-length Tf cannot induce NO in fish macrophages. We believe that cleaved Tf fragments should be considered to be "alarmins". We discuss the possibility that parasites such as *Trypanoplasma borreli* cleave Tf and use Tf fragments to their advantage by modulating the NO induction in carp macrophages.

Introduction

Transferrins (Tfs) are a family of 75-80 kDa iron-binding proteins that are common across many phyla (Aisen and Lystowsky, 1980; Taboy et al., 2001). It is not surprising that Tfs from diverse taxa are highly homologous. For example, the percent of amino acid identity between mouse Tf and that of goldfish, bovine and human molecules is 41%, 63% and 72%, respectively (Baker et al., 2001). The Tf molecule is composed of two relatively homologous lobes (C- and N-lobe; ~ 40% sequence identity), with a single iron-binding site in each lobe. In mammals, Tf is mostly synthesized in the liver and secreted into the blood as a mixture of iron-free (apo-Tf), one iron (monomeric Tf) and two iron (holo-Tf) forms (Dautry-Varsat et al., 1983). The main function of Tf is delivery of iron to cells that express transferrin receptor-I and -II (TfRI or CD71 and TfR-II) via receptor-mediated endocytosis. After endocytosis, iron is released from the Tf N- and C-lobes in the acidic environment of the endosome, and the Tf-receptor complex is recycled back to the surface where at physiological pH (pH 7.4) Tf is released (Wally et al., 2006). Transferrin is also synthesized by macrophages, suggesting a possible role for this molecule in host defense, particularly in inflammatory microenvironments (Djeha et al., 1992).

The central role of Tf as an iron transporting protein has been extended by observations that modified versions of this protein also participate in the regulation of innate immunity. For example, it has been reported that Tf acts as an acute phase protein (Gabay and Kushner, 1999) and that it can create a bacteriostatic environment by sequestering free iron from invading pathogens (Ong et al., 2006). Transferrin fragments are frequently observed in bronchoalveolar lavage (BAL) of cystic fibrosis patients (Britigan et al., 1993) and in the supernatants of mitogen-activated macrophage cultures of lower vertebrates such as bony fish (Stafford et al., 2001; Stafford and Belosevic, 2003), suggesting that Tf fragments may act as danger signals “warning” the immune system of the presence of pathogens or of tissue injury.

Recent evidence suggests that modified Tf is involved in induction of antimicrobial function of macrophages, since immunopurified Tf fragments present in the supernatants of mitogen-stimulated goldfish leukocytes induce significant production of reactive nitrogen intermediates in goldfish *in vitro*-derived macrophages (Stafford and Belosevic, 2003). Similarly, lactoferrin fragments, found in parotid saliva of periodontitis patients, induce the production of interleukin-6 (IL-6), monocytes

chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) (Komine et al., 2007). At inflammatory sites, activated neutrophils release a large number of proteases that can degrade endogenous proteins and membrane bound host proteins that can influence the progression of inflammation. One serine protease produced in copious amounts by activated neutrophils is elastase. Activated neutrophils degranulate and release elastase at the inflammatory site (Bryniarski et al., 2003), suggesting that immunomodulatory Tf and lactoferrin fragments may be generated by a process mediated by this enzyme (Stafford, 2003).

Transferrin is polymorphic in several fish species, among which salmon (Ford, 2001), goldfish (Yang et al., 2004) and common carp, *Cyprinus carpio* L. (Valenta et al., 1976; Irnazarow and Białowąs, 1994; Irnazarow and Białowąs, 1995). Early studies reported associations between Tf polymorphism and resistance to bacterial disease (Suzumoto et al., 1977; Winter et al., 1980), but a direct functional relationship between Tf polymorphism and resistance to fish pathogens has not been established. For both salmonid and goldfish Tfs, nucleotide regions have been identified that have undergone positive selection through evolution (Ford, 2001; Yang and Gui, 2004), supporting a role for Tf in the resistance of fish against pathogens. We employed the infection of common carp with the parasite *Trypanoplasma borreli* as an animal model to study the relationship between allelic diversity of Tf and disease resistance. Previously, we have shown a correlation between allelic diversity of Tf and resistance to *T. borreli* infection (Jurecka et al., 2008c). Trypanosome parasites require iron for growth, and sequester iron from holo-Tf by taking up Tf via receptors in the flagellar pocket. Subsequently, in the endosome, iron is released from Tf at acidic pH, after which the digested Tf is released outside the cell (Steverding, 2000). Infections of carp with *T. borreli* are characterized by high levels of NO (Saeij et al., 2002) and heavily nitrated tissue (Forlenza et al., 2008). Given the immunostimulatory role of fragments of Tf in the induction of an NO response it made sense to examine the role of Tf in infection of common carp with *T. borreli*.

In this paper, we report on the isolation of two carp Tf proteins (alleles D and G) to purity using rivanol precipitation and ion-exchange chromatography, and describe the induction of nitrite oxide response induced by cleaved Tf in carp head kidney-derived macrophages.

Materials and Methods

Identification of allelic diversity for transferrin by native polyacrylamide gel electrophoresis (PAGE)

Total serum proteins were separated by non-reducing PAGE using procedures that discriminate between Tf D and G alleles (Irnazarow and Białowąs, 1994; Irnazarow and Białowąs, 1995; Jurecka et al., 2008c). Samples (5 µl) were diluted in 15 µl loading buffer (40% sucrose, 1.5% Bromophenol Blue; Sigma-Aldrich, St. Louis, MO, USA) and 2 µl of each suspension applied on a 6% stacking and 15% polyacrylamide running gel. Electrophoresis was carried out in running buffer (72 mM Tris, 26 mM boric acid) at 90V for 30 min followed by 250V for 5 h (Smithies, 1955). Protein bands were stained with 0.04% Coomassie Brilliant Blue dissolved in 3.5% perchloric acid for 1 h. Genotyping of individual fish by PAGE was used to select heterozygous DG-typed individuals for obtaining macrophages for nitric oxide (NO) studies, to select individuals for the production of Tf-typed pooled carp sera (from n=10-20 animals per pool) and to select individual Tf-typed fish to isolate Tf to purity from carp serum.

Animals

European common carp (*Cyprinus carpio carpio* L.) were reared at 23^{o}C in recirculating UV-treated tap water and fed pelleted dry food (Trouvit, Nutreco) daily. R3×R8 carp are the offspring of a cross between fish of Hungarian origin (R8 strain) and of Polish origin (R3 strain) (Irnazarow and Białowąs, 1994; Irnazarow and Białowąs, 1995). At the age of 9 months, individual carp were bled and genotyped for Tf. Sera from carp typed homozygous for the D allele of Tf (DD-typed serum) or typed homozygous for the G allele of Tf (GG-typed serum) were used for NO assays, and for Tf isolation and purification.

Purification of carp transferrin with ion exchange chromatography

Transferrins were purified from carp serum (alleles D and G) as described by Sutton (Sutton and Karp, 1965) with the following modifications. Briefly, to a quantity of serum about 1 mg/100ml $FeCl_3$ was added to saturate Tf with iron. Subsequently, one part of serum was diluted with three parts of 5 mM Tris buffer (pH 8.8) and an equal volume of 0.6% rivanol solution (Ethacridini lactase FPV, FARM-IMEX, Gliwice, Poland) in Tris buffer was slowly added and left overnight at 4^{o}C, while stirring.

Rivanol was removed selectively and completely by adding potato starch to the supernatant and by filtration. Ion-exchange chromatography was performed using a Mono-Q column (GE healthcare Life Sciences), pre-equilibrated with 20 mM Tris-HCl (pH 8.0). Filtrates (0.2 μm) were applied to the column in a total volume of 20 ml (4 injections of 5 ml) at a flow rate of 1 ml/min. Unbound proteins were collected in flow-through fractions. Transferrin bound to the column was visible as a brownish-red ring. Elution buffer was applied as a gradient (0-1 M NaCl, 20 mM Tris-HCl) to the column and the transferrin fractions were collected. Finally, samples were dialyzed overnight at 4^{o}C against PBS, adjusted with NaOH to pH 8.0, filter-sterilized and stored at -20^{o}C for further use. Protein concentration was determined by Bradford using BSA as a standard.

Purity of isolated Tf samples was analysed by SDS-PAGE. A volume of 6 μl of each Tf sample was heated at 96^{o}C for 10 min with loading buffer containing β-mercaptoethanol and run on a 12.5% polyacrylamide gels. Electrophoresis was carried out in 1 × SDS running buffer at 80 V for 30 min followed by 100 V for 60 min. Protein bands were stained for 1 h with 0.04% Coomassie Brilliant Blue dissolved in 3.5% perchloric acid.

Isolation of carp head kidney-derived macrophages

Head kidney-derived macrophages were obtained as described previously (Joerink et al., 2006). Briefly, carp were euthanized with 0.25 g/l tricaine methane sulfonate (Crescent Research Chemicals) buffered with 0.38 g/l $NaHCO_3$, and the head kidneys aseptically removed. Head kidney tissue was gently passed through a 100 μm sterile nylon mesh and rinsed with homogenization buffer (incomplete (no serum added) NMGFL-15 medium (Neumann et al., 1998) containing 50 U/ml penicillin, 50 μg/ml streptomycin and 20 U/ml heparin). Cell suspensions were layered on 51% (1.071 g/cm^3) Percoll (Amersham Biosciences) and centrifuged at 450 × *g* for 25 min at 4°C without using the brake. Cells at the interface were removed and washed twice in complete (with 5% pooled carp serum (PCS) and 10% bovine calf serum (BCS) (Invitrogen)) NMGFL-15 medium. Macrophage cultures were initiated by seeding 1.75×10^7 head kidney leukocytes in a 75 cm^2 culture flasks containing 20 ml of complete medium at 27°C. After 6 days, head kidney-derived macrophages were harvested by scraping and washed twice in incomplete medium.

Nitrite oxide assay

Washed 6-day-old cultures of macrophages were used for bio-assays to determine the influence of Tf on nitric oxide production. Nitrite production was measured as described before (Green et al., 1982). Briefly, 75 µl of cell culture supernatants were transferred to separate 96 well plate and 100 µl of 1% (w/v) sulfanilamide in 2.5% (v/v) phosphoric acid with 100 µl of 0.1% (w/v) *N*-naphthyl-ethylenediamine in 2.5% (v/v) phosphoric acid were added. The absorbance was measured at 540 nm and nitrite concentrations (µM) were calculated from a standard curve generated using known sodium nitrite concentrations. To macrophages were added 5% (Tf-typed) carp serum (DD or GG), or LPS (0.1 µg/ml; *E. coli*, Sigma L2880) and/or purified Tf (100 µg/ml, D or G), or macrophages were left untreated as negative control, and incubated for 18 h at 27°C.

Western blot detection of cleavage products of Tf

Supernatants from activated macrophages co-stimulated with purified Tf were examined by Western blotting for the presence of Tf cleavage fragments. Preparations were run on a 12.5% SDS-PAGE, as described before, electro-transferred to a nitrocellulose membrane (Protrans, Schleicher & Schuell, Bioscience GmbH) and incubated overnight at 4^{o}C in blocking buffer (3% BSA in TBS, 10 mM TrisHCl, pH 7.5, 150 mM NaCl). Membranes were incubated with rabbit anti carp Tf (1:1000) (Stafford and Belosevic, 2003), with mouse anti-carp IgM (WCI-12; 1:50; (Secombes et al., 1983; Koumans-van Diepen et al., 1995)) and with goat anti-mouse HRP (1:1250) in 10% milk powder in TBS. All incubation steps were done for 1 h at RT with shaking (150 rpm). After each incubation step, membranes were washed twice with TBS-Tween/Triton (TBS-Tween, 0.2% (v/v) Triton X-100) and once with TBS, for 10 min at RT. Signal was detected by development with a chemoluminescence kit (Amersham) according to the manufacturer's protocol and visualized by the use of Lumni-fil chemiluminescent Detection Film (Roche). Controls without primary antibody were negative.

Statistics

The Levene test for equality of variances was performed first. Then, nitrite production was compared with a one-way ANOVA (STATISTICA, version 6.0), followed by the unequal N HSD Post Hoc test when differences between means were significant. Differences were considered significant at $P \leq 0.05$.

Results

Allelic diversity of Tf present in carp serum affects the production of nitrite oxide by macrophages

Polyacrylamide gel electrophoresis of serum proteins allowed for typing of allelic diversity of carp Tf, discriminating between three different individual genotypes; DD, DG and GG (see inset in Fig. 5.1). Only individuals with a DG genetic background for Tf were used to culture macrophages for nitric oxide assays. Macrophages were stimulated with Tf-typed carp serum (DD or GG), or grown without serum as negative control, and tested for NO production (Fig. 5.1). Only macrophages stimulated with DD-typed serum (5%) showed a significantly (P = 0.007) higher NO production than macrophages without serum.

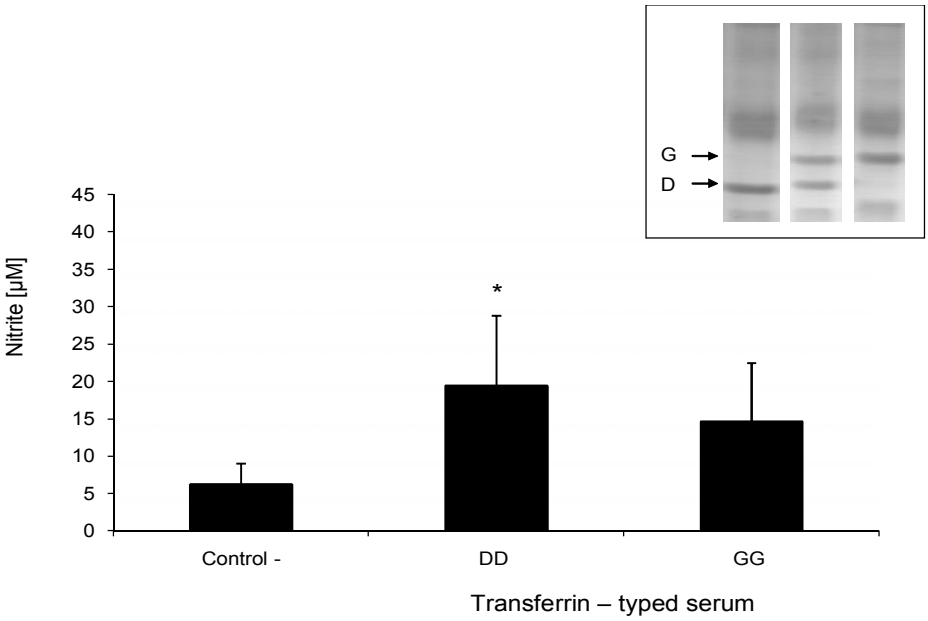


Fig. 5.1 Stimulation of macrophages with Tf-typed serum. Carp macrophages (5×10^5 cells/well) were seeded in triplicate into 96 well culture plates in incomplete medium as negative control or stimulated with 5% transferrin-typed (DD or GG) pooled carp serum, and incubated for 18 h at 27°C prior to determination of NO production. Each bar represents the mean ± SD of five independent experiments. The insert shows three different Tf genotypes (DD, DG, GG) as detected by non-reducing polyacrylamide gel electrophoresis. Symbol (*) indicates a statistically significant ($P \leq 0.05$) difference in the amount of NO between macrophages stimulated with serum and macrophages without serum.

Treatment of macrophages with (Tf-typed) serum as a source of activating factor as well as a source of Tf, confounds the contributing activities of these two treatment regimens to the NO production by macrophages. Therefore, in a new set-up, macrophages were

activated only with LPS, or activated with LPS and co-stimulated with Tf-typed carp serum (DD or GG), or grown without serum and LPS as negative control (Fig. 5.2). Macrophages activated with LPS (0.1 µg/ml) and co-stimulated with 5% DD-typed serum (P = 0.0003) or GG-typed serum (P = 0.01) showed a significantly higher NO production than macrophages stimulated with serum only.

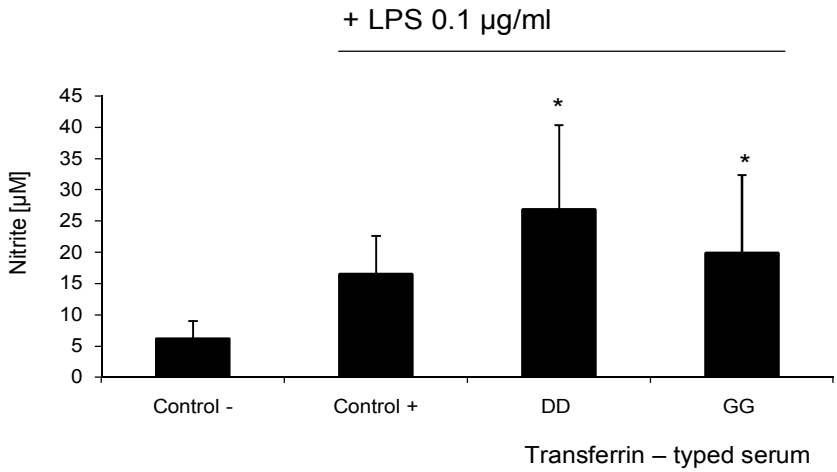


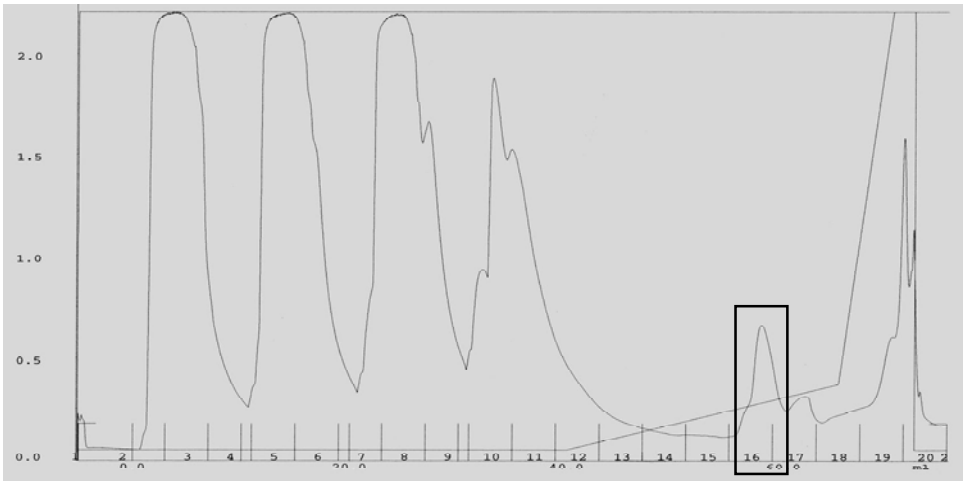
Fig. 5.2 Activation of macrophages with LPS and Tf-typed serum. Carp macrophages (5×10^5 cells/well) were seeded in triplicate into 96 well culture plates in incomplete medium as negative control, or activated with LPS (0.1 µg/ml; *E. coli*, Sigma L2880) as positive control, co-stimulated with 5% Tf-typed (DD or GG) pooled carp serum, and incubated for 18 h at 27°C prior to determination of NO production. Each bar represents the mean ± SD of five independent experiments. Symbol (*) indicates a statistically significant ($P \leq 0.05$) difference in the amount of NO between macrophages activated with LPS and co-stimulated by Tf-typed serum and the negative control (no LPS, no Tf-typed serum).

Cleaved D-typed Tf induces higher NO in carp macrophages than cleaved G-type Tf

We used rivanol precipitation (not shown) and ion-exchange chromatography (Fig. 5.3A) to isolate different Tf proteins (D and G alleles) to purity (Fig. 5.3B). Rivanol (2-ethoxy-6, 9-diamineacridine lactate) is a well-known protein precipitant used for the isolation of Tf from human plasma (Boettcher et al., 1958). The mixing of carp plasma and rivanol resulted in precipitation of most proteins, but not Tf and some other proteins that remained present in the supernatant. Rivanol was removed selectively and completely from the solution by adding potato starch. The intensively-yellow-stained rivanol supernatant was filtrated to clarity, diluted and applied to ion exchange chromatography (Mono-Q column) for purification of Tf. Elution of Tf was achieved at

0.1 M NaCl approximately (Fig. 5.3A). Transferrin purity was examined by SDS-PAGE (Fig. 5.3B). The upper, less intensive, band might be due to an electrophoretic mobility shift induced by a boric acid- or glycine/alanine-Tf complex (Остерман, 1981).

A)



B)

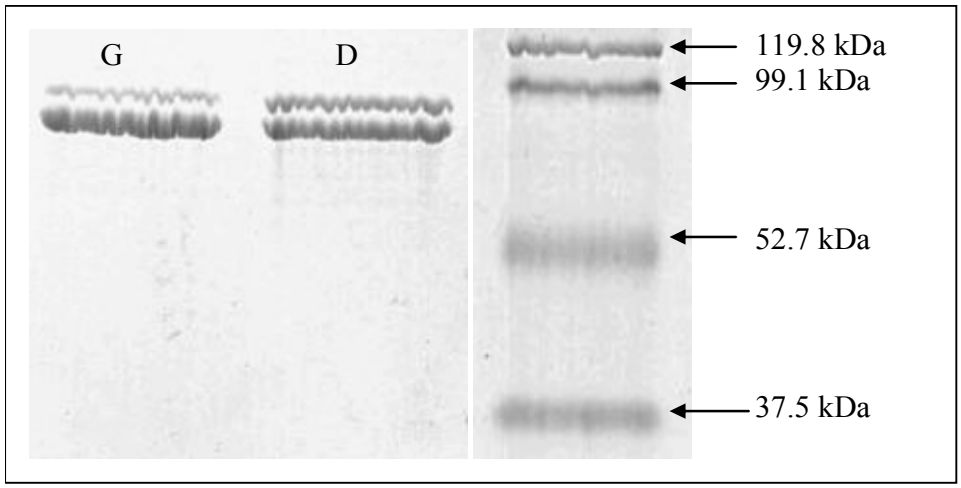


Fig. 5.3 Purification of carp Tf. (**A**) Chromatogram of Tf isolation by MonoQ collumn. Transferrin was eluted (20 mM Tris-HCl buffer, 0-1 M NaCl) with a flow rate of 1 ml/min. The Tf peak is indicated in the boxed area. (**B**) Two carp Tf proteins (alleles G and D) purified by rivanol precipitation and ion-exchange chromatography, separated on 12.5% SDS-PAGE. Protein bands were stained with 0.04% Coomassie Brilliant Blue dissolved in 3.5% perchloric acid for 1 h. Molecular weight is indicated on the right.

Carp serum contains 2.5 mg/ml Tf approximately (Wojtczak et al., 2007b), which means that 5% of pooled carp serum had approximately 125 μg/ml Tf. Consequently, in

subsequent experiments we added 100 µg/ml of purified Tf to the macrophages in the NO assays. First, non-activated (no serum added) macrophages were stimulated with purified native Tf (D or G; 100 µg/ml), or grown without additional Tf (negative control), and tested for NO production. NO production did not increase following the addition of adding native, full-length purified Tf (Fig. 5.4), which confirmed earlier findings that full-length Tf does not activate macrophages (Stafford and Belosevic, 2003).

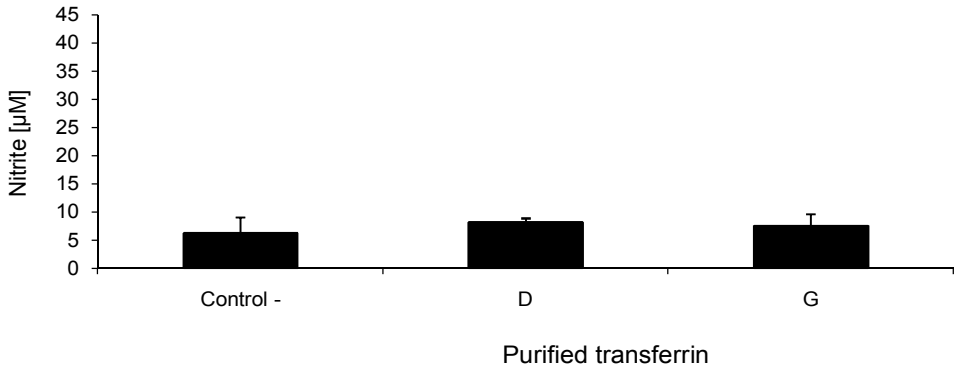
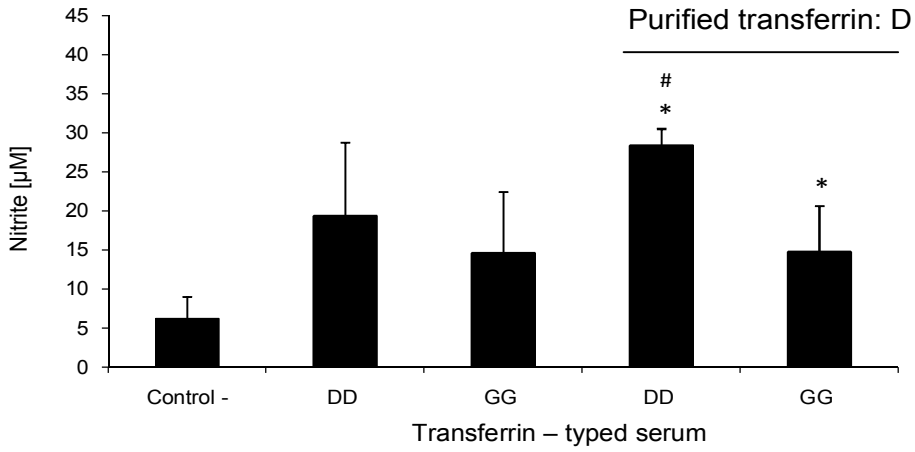


Fig. 5.4 Activation of macrophages with purified Tf. Carp macrophages (5×10^5 cells/well) were seeded in triplicate into 96 well culture plates in incomplete medium and stimulated with purified carp Tf D or G (100 µg/ml), or left untreated as negative control and incubated for 18 h at 27°C prior to determination of NO production. Each bar represents the mean ± SD of five independent experiments.

To examine the requirement for Tf cleavage, macrophages were activated with Tf-typed carp serum (DD or GG) and co-stimulated with purified Tf (D or G), or grown without serum and Tf as negative control. In general, macrophages activated with serum (5%) and co-stimulated with Tf (100 µg/ml) always showed a higher ($P < 0.05$) NO production than unstimulated macrophages. NO production reached highest values in macrophages stimulated with DD-typed serum (Fig. 5.5). Macrophages activated with Tf-typed serum and co-stimulated with purified Tf D showed a significantly ($P = 0.0001$ for DD-typed serum and $P = 0.002$ for GG-typed serum) higher NO production compared to the negative control (Fig. 5.5A). Co-stimulation with Tf also resulted in significantly higher NO production when compared to NO production by macrophages stimulated with serum only but, owing to fish-to-fish differences, this could be shown within individual experiments only (not shown). Macrophages activated with Tf-typed

serum and co-stimulated with purified Tf G showed a significantly (P = 0.003) higher NO production compared to the negative control only when activated with serum typed for Tf as DD (Fig. 5.5B).

A)



B)

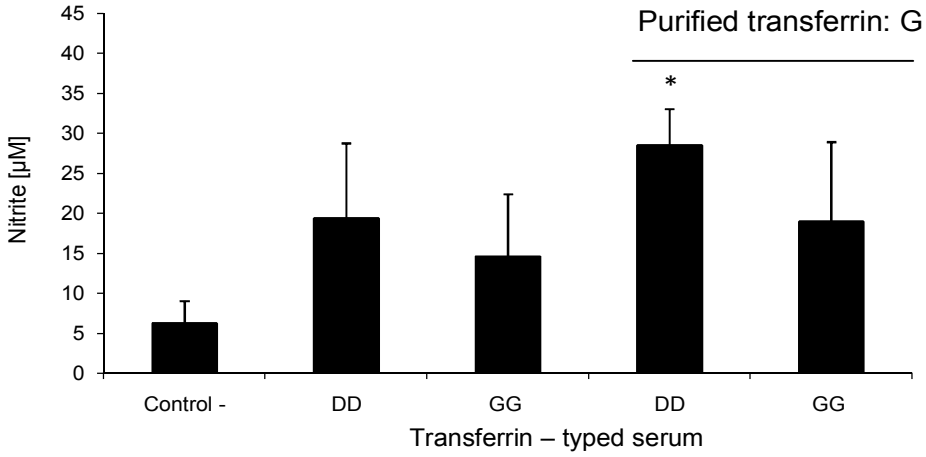


Fig. 5.5 Activation of macrophages with Tf-typed serum and purified Tf. Carp macrophages (5×10^5 cells/well) were seeded in triplicate into 96 well culture plates in incomplete medium and activated only with 5% Tf-typed pooled carp serum (DD or GG), or activated with Tf-typed serum and co-stimulated with purified Tf (100 µg/ml) of allele D (**A**) or allele G (**B**), or left untreated as negative control. Cells were incubated for 18 h at 27°C prior to determination of NO production. Each bar represents the mean ± SD of five independent experiments. Symbol (*) indicates a statistically significant ($P \leq 0.05$) difference in the amount of NO between macrophages activated by Tf-typed serum and co-stimulated with purified Tf and the negative control. Symbol (#) indicates a statistically significant ($P \leq 0.05$) difference in the amount of NO between macrophages co-stimulated with D-type Tf and activated with DD or GG Tf-typed serum.

Additive effects of Tf-typed serum plus purified Tf can be explained by the total presence of 125 µg/ml Tf in 5% carp serum and 100 µg/ml purified Tf.

To examine the role of LPS as macrophage activating factor for the required cleavage of Tf, macrophages were activated only with LPS (0.1 µg/ml), or activated with LPS and co-stimulated with purified Tf (D or G), or grown without LPS and Tf as negative control. In general, macrophages activated with LPS and co-stimulated with purified Tf always showed a significantly higher ($P < 0.05$) NO production than unstimulated macrophages (Fig. 5.6). When compared to the positive control (macrophages stimulated with LPS) the NO induction was significantly higher ($P = 0.002$) only in macrophages co-stimulated with D-type Tf.

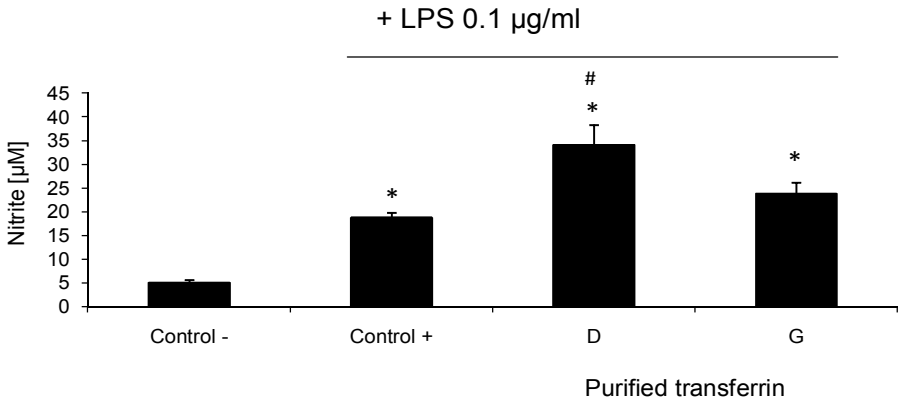


Fig. 5.6 Activation of macrophages with LPS and purified Tf. Carp macrophages (5×10^5 cells/well) were seeded in triplicate into 96 well culture plates in incomplete medium, activated with LPS (0.1 µg/ml; *E. coli*, Sigma L2880) as positive control, activated with LPS and co-stimulated with purified Tf (100 µg/ml) of allele D or allele G, or left untreated as negative control. Cells were incubated for 18 h at 27°C prior to determination of NO production. Each bar represents the mean ± SD of five independent experiments. Symbol (*) indicates a statistically significant ($P \leq 0.05$) difference in the amount of NO between macrophages activated with LPS only or macrophages activated with LPS and co-stimulated with D- or G-type Tf and unstimulated macrophages. Symbol (#) indicates a statistically significant ($P \leq 0.05$) difference in the amount of NO between macrophages co-stimulated with D-type Tf and LPS-activated macrophages.

Detection of Tf cleavage products by Western blot

Purified Tf can induce NO in macrophages only following proper enzymatic cleavage of the full-length protein into immunostimulatory fragments with elastase, or by products released by serum- or LPS-activated macrophages (Stafford et al., 2001; Stafford and

Belosevic, 2003; Stafford, 2003). To confirm the presence of cleaved Tf cleavage products in macrophage culture supernatants, we used an anti-carp Tf monospecific rabbit antibody in Western blot. Full-length Tf protein (70 kDa) was detected in all supernatants (Fig. 5.7), but Tf cleavage products (45 kDa) were detected only in supernatants from (DD-typed serum) activated macrophages co-stimulated with purified Tf (Fig. 5.7, lanes 3-5). Additional cleavage products (~ 28.8 kDa) were detected especially in supernatants from macrophages activated with DD-typed serum and co-stimulated with D-type purified Tf (Fig. 5.7, lane 4), suggesting D type Tf may be cleaved differently than G-type Tf.

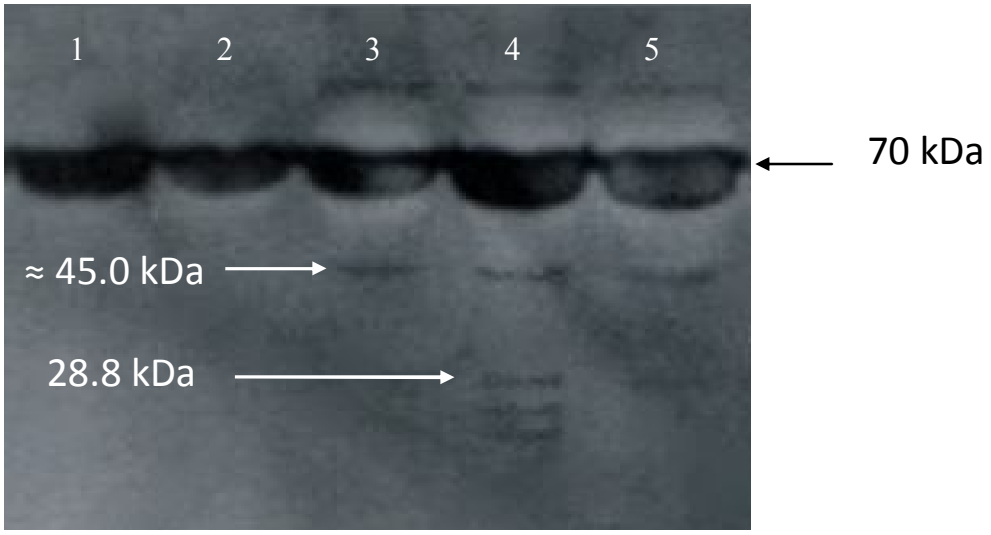


Fig. 5.7 Western blot analysis of stimulated macrophage supernatants using anti-carp Tf antibody. Lanes: supernatants of macrophages stimulated with 1: purified Tf type D (100 μg/ml), 2: purified Tf type G (100 μg/ml), 3: DD-typed serum; 4: DD-typed serum and purified Tf type D (100 μg/ml), 5: DD-typed serum and purified Tf type G (100 μg/ml).

Discussion

In the present paper, we show that immunostimulatory fragments of carp Tf induce a potent nitric oxide (NO) response in carp macrophages, confirming our previous observations for goldfish macrophages (Stafford et al., 2001; Stafford and Belosevic, 2003; Stafford, 2003; Stafford et al., 2004). The ability of carp Tf to activate macrophages was dependent on the cleavage of the full-length protein into immunostimulatory fragments. Cleavage was best achieved by serum- or LPS-activated

macrophages. We isolated two carp Tf proteins (alleles D and G) to purity and demonstrate the superiority of the D-type over the G-type Tf in inducing NO production in carp macrophages. Our results confirm previous observations that full-length Tf cannot induce NO in fish macrophages (Stafford and Belosevic, 2003). In our study, activated macrophages appear to be the source of necessary enzymes required for cleavage of Tf into immunostimulatory fragments. Indeed, only when we activated macrophages with LPS or with carp serum, we observed Tf immunostimulatory activity measured by the nitrite production of treated macrophages. Western blot analyses confirmed the presence of Tf cleavage products in the supernatants of activated macrophage cultures, but not in culture supernatants of non-activated macrophages. The identity of the proteolytic enzyme responsible for cleaving carp Tf into immunostimulatory fragments remains to be determined. One enzyme that is found in copious amounts at inflammatory sites is elastase, which is produced by activated neutrophils and macrophages as well bacteria (Henson, 1971; White et al., 1977). Furthermore, elastase has been shown to cleave goldfish Tf into immunostimulatory fragments capable of inducing the nitric oxide response in goldfish macrophages (Stafford, 2003).

Initial studies predicted the cleavage site for goldfish Tf to lie within the bridge peptide that separates the two lobes of the Tf molecule, a region highly conserved in Tf of different animal species (Stafford and Belosevic, 2003). Not surprisingly the bridge peptide amino acid sequences of carp D and G alleles for Tf were found to be highly homologous (100% identity, (Jurecka et al., 2008b)) confirming the conservation of the predicted putative cleavage site. However, this does not explain the superiority of the D over the G Tf allele in inducing an NO response in carp macrophages. Possibly, the lobes themselves are not entirely responsible for activating the macrophages but represent an initial cleavage event in a series that generates several smaller breakdown products which in turn are responsible for the activation of macrophages. Although the anti-Tf antibody confirmed the initiation of Tf digestion as indicated by Western blot, this antibody may not have the specificity for smaller fragments of Tf. It is probable that different Tf alleles (D versus G) may be differentially cleaved (non-detectable in Western blots) which could explain differences in the induction of nitric oxide response in macrophages. Further research is required to determine the characteristics of these smaller breakdown products, the receptors on macrophages that react to these products,

and to determine the exact reason for the difference between different allelic forms of Tf and the induction of NO.

In early 1990s, Polly Matzinger (Matzinger, 1994) proposed an ingenious model of immunity, the "danger model", that was provocative and has revolutionized the way scientists currently view the innitiation of immune responses. The cornerstone of the "danger model" is that the immune system recognizes danger signals from damaged "self" tissues due to either injury or infection. These endogenous danger signals can be released in response to different tissue damage caused by infection, burns, radiation, nutrient and oxygen deprivation, neoplasia, autoimmunity, and xenobiotics (Zhang and Mosser, 2008). The danger signals are collectively known as "alarmins", which function by alerting the immune system to tissue damage or infection (Oppenheim and Yang, 2005). Recent studies have shown that there is a significant number of endogenous proteins such as heat-shock proteins, hyaluronan fragments, high-mobility group box 1, fibronectin fragments, lactoferrin fragments, modified low-density lipoproteins, extracellular ATP, and myeloid-related protein-8 and -14, that have the capacity to activate the immune system (Zhang and Mosser, 2008).

Current evidence suggests that modified Tf plays an important role in host defense. For example, Tf can significantly enhanced the killing response of goldfish macrophages exposed to different pathogens or pathogen products, including *Trypanosoma danilewskyi* (Stafford et al., 2004). Infections of carp with *Trypanoplasma borreli* are characterized by high levels of NO (Saeij et al., 2002) and heavily nitrated tissue (Forlenza et al., 2008). We have shown in this study that immunostimulatory fragments of Tf can induce an NO response in carp macrophages. Interestingly, activated cysteine proteinase from *T. borreli*, can digest Tf at low pH (Ruszczyk et al., 2008). Probably, similar to other kinetoplastids, *T. borreli* is able to take up Tf in the flagellar pocket and actively digest Tf in the lysosomal compartment. Digestion of Tf allows the parasite to sequester iron which is essential for its growth. In *Trypanosoma brucei* infections, cleaved Tf products are secreted back into the bloodstream of the host (Steverding, 2000). Possibly, Tf products cleaved by *T. borreli* proteinase and secreted back into the bloodstream may contribute to the high NO response typical of *T. borreli* infections. As indicated earlier, Tf fragments have been detected in the bronchoalveolar lavage (BAL) from cystic fibrosis patients also infected with elastase-positive *Pseudomonas aeruginosa* (Britigan et al., 1993). In addition, immunostimulatory Tf fragments have

been identified in the supernatants of mitogen-activated goldfish leukocytes cultures (Stafford and Belosevic, 2003), and more recently a 7.6 kDa Tf fragment was detected in the medium conditioned by super invasive cancer cell line MDA-MB-435S-F/Taxol10p4pSI (Dowling et al., 2007).

Based on the evidence generated in our study, we believe that cleaved Tf fragments should be considered to be "alarmins". Tf fragments may be important alarmins because: (1) Tf is an abundant plasma protein with concentration 2-5 mg/mL in mammals (Regoeczi and Hatton, 1980); (2) of its role in binding iron and sequestering it from invading pathogens (Ong et al., 2006), making Tf a prime target for pathogens to "attack" in order to steal the essential iron ions by means of siderophores (Wandersmann and Delepelaire, 2004) or by enzymatic cleavage (Wolz et al., 1994); (3) in addition to its synthesis in the liver, Tf is produced by the major phagocytic cell of the body, the macrophage (Djeha et al., 1992); and (4) Tf is an acute phase protein (Gabay and Kushner, 1999). Further analysis of the immunomodulatory nature of modified Tf, particularly during inflamatory responses, and identification of the putative receptor(s) for Tf fragments is required to entrench this endogenous host protein in the "alarmin" group of immunostimulatory molecules.

6

Trypanoplasma borreli cysteine proteinase activities support a conservation of function with respect to digestion of host proteins in common carp

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Patrycja Jurecka, Geert F. Wiegertjes

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Abstract

Trypanoplasma borreli is an extracellular parasite that is transmitted by a leech vector and is naturally found in the blood of cyprinid fish. High parasitemia and associated severe anemia together with splenomegaly are typical of infection of common carp, *Cyprinus carpio* L. Papain-like cysteine proteinases expressed by trypanosome parasites contribute to the pathogenicity of trypanosomes, and are considered an important target for the development of new trypanocidal drugs. *T. borreli* is a member of the Parabodonida, sharing a common ancestor with the other Kinetoplastida. We demonstrate the presence of a cysteine proteinase expressed by *T. borreli*. Alignment of the sequence with other kinetoplastid cysteine proteinase sequences supports the phylogenetic hypotheses based on analyses of ribosomal RNA genes. We expressed the *T. borreli* cysteine proteinase in *Escherichia coli*, refolded the purified protein into a biologically active proteinase and showed it has cathepsin L-like activity. Addition of the (non)active proteinase to in vitroderived carp head kidney-derived macrophages did not significantly modulate macrophage activity. Immunization of carp with the recombinant proteinase did induce a very high increase in proteinase-specific antibodies but only slightly lowered parasitemia. Digestion of host hemoglobin and immunoglobulin by the cysteine proteinase likely contribute to the pathogenicity of *T. borreli*. The possibility that digestion by the cysteine proteinase of host discussed. Our findings suggest a conservation of function with respect to cysteine proteinase activity in the Parabodonida in support of the hypotheses on the phylogeny of the Kinetoplastida.

Introduction

The Kinetoplastida is a group of flagellate protozoa that can be subdivided into the two major suborders Trypanosomatida and Parabodonida that diverged some 200–300 million years ago (Stevens et al., 2001). The Trypanosomatida include a number of important mammalian pathogens transmitted by insect vectors, among which are *Trypanosoma brucei*, *T. cruzi*, *Leishmania mexicana* and *L. major*. The Parabodonida are less well studied. In fish, representatives of both suborders can be found. *Trypanosoma danilewskyi*, for example, belongs to the 'aquatic clade' within the Trypanosomatida, while the Parabodonida includes, among others, the fish pathogens *Cryptobia salmositica* and *Trypanoplasma borreli* (Wiegertjes et al., 2005). *T. borreli* is an extracellular parasite that is transmitted by a leech vector and is naturally found in the blood of cyprinid common carp (*Cyprinus carpio* L.). High parasitemia, together with severe anemia and splenomegaly are typical of infection of carp (Steinhagen et al., 1990). The fish respond with an inflammatory reaction that includes a very high nitric oxide production apparently more detrimental to the host than to the parasite (Saeij et al., 2002). Survival of infected carp is related to the production of antibodies (Saeij et al., 2003; Wiegertjes et al., 1995). Important molecules that contribute to the pathogenicity of the Kinetoplastida include various proteinases. In fact, papain-like cysteine proteinases, in particular cathepsin L-like proteinases, expressed by trypanosome parasites are considered as important targets for the development of new trypanocidal drugs and vaccines (Jose Cazzulo et al., 2001; Caffrey et al., 2000; Lalmanach et al., 2002; Schnapp et al., 2002; Zadeh-Vakili et al., 2004). They are thought to be involved not only in pathogenesis (tissue invasion and degradation of proteins) but also in immune response modulation in the host (Aparicio et al., 2004; McKerrow et al., 2006). Cathepsin L-like proteinases B (CPBs) from *L. mexicana* promastigotes facilitate entry into host macrophages while proteinase from amastigotes degrade innate immune signaling components (IkB, NF-kB, CD23, CD25) (Cameron et al., 2004; Pollock et al., 2003). Cathepsin L proteinase from *T. cruzi* (cruzipain) trypomastigotes modulates immune response by releasing kinins from kininogens (Scharfstein, 2006; Monteiro et al., 2006). Cysteine proteinase activities were also detected in fish parasites. In *C. salmositica*, for example, partially purified cysteine proteinases were characterized as important enzymes for intracellular protein catabolism of the parasite and were suggested to be good vaccine candidates (Zuo and Woo, 1998;

Woo, 2003). Although a cathepsin L-like cysteine proteinase from this parasite was recently cloned, no studies on the modulation of the fish immune response were reported (Jesudhasan et al., 2007).

We demonstrate proteinase activity in *T. borreli* lysate and in *T. borreli* excretory–secretory products (ESPs). To study the contribution of *T. borreli* proteinase activity to pathogenicity and immune modulation, we cloned and sequenced the cathepsin L-like cysteine proteinase from *T. borreli* and produced a recombinant biologically active enzyme. Addition of the (non)active proteinase to carp head kidney-derived macrophages did not significantly modulate the production of nitric oxide or gene regulation in vitro. In vivo, immunization of carp with the proteinase induced a very high increase in proteinase-specific antibodies but only slightly lowered parasitemia. Digestion of host hemoglobin, immunoglobulin and transferrin by the cysteine proteinase probably contributes to the pathogenicity of *T. borreli*. Our results characterize cathepsin L-like proteinase as an important enzyme involved in the pathogenesis and immune response modulation during infection of carp with *T. borreli*. Our findings support a conservation of function with respect to cysteine proteinase activity in the Parabodonida.

Materials and Methods

Animals

European common carp (*C. carpio carpio* L.) were reared at 23±2 ^{o}C in recirculating UV-treated tap water and fed pelleted dry food (Trouvit, Nutreco) daily. R3×R8 carp are the hybrid offspring of a cross between fish of Hungarian origin (R8 strain) and fish of Polish origin (R3 strain) (Irnazarow, 1995). Carp were 9 months of age and 150 g average weight at the start of the experiments.

Parasite, parasite lysate and ESPs

T. borreli, initially cloned and characterized by Steinhagen et al. (Steinhagen et al., 1989) was maintained by syringe passage through carp following i.p. injections with 10,000 parasites per fish. Three weeks after infection, parasitemia was monitored using a Bürker counting chamber, setting the minimum detection to 10^4 parasites/ml of blood. For RNA isolation of *T. borreli*, parasites were taken from the buffy coat (Steinhagen et al., 2000) of carp after 3 weeks of infection and purified by ion-exchange column

chromatography (Overath et al., 1998). Parasite lysates were made by washing column-purified parasites (1×10^8 parasites/ml) in carp PBS (cPBS), pH 7.4 (1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, 270 mOsm) and lysing by sonication. Lysates were stored at -80°C until use. For the collection of ESPs, isolated parasites (1×10^8 parasites/ml) were cultured for 1 h as described by Steinhagen et al. (Steinhagen et al., 2000) in *T. borreli* incomplete medium (45% (v/v) HBSS, 22.5% (v/v) MEM, 22.5% (v/v) L-15, 10% (v/v) distilled water, 100 IU/ml penicillin, 100 mg/ml streptomycin, 2mM L-glutamine, without carp serum) at 21°C and centrifuged at 550 × *g* for 10 min. Supernatant with ESPs was stored at -80 °C until use.

Parasite RNA extraction, cDNA library construction and rapid amplification of cDNA ends

RNA from pelleted parasites was isolated using Trizols (Invitrogen) according to the manufacturer's protocol. RNA concentrations were measured by spectrophotometry (Genequant, Amersham Pharmacia Biotech AB) and 3 ml was analyzed on a 1% agarose gel to check the integrity. RNA was stored at -80°C until use. A λTriplEx2 expression library was constructed from 1 µg of total *T. borreli* RNA using a SMART™ cDNA library construction kit (Clontech). Primers (see Table 6.1) were designed based on a *C. salmositica* cysteine proteinase gene (GenBank accession number AY713477) and used in an anchored PCR in combination with λTriplEx2 primers Tr3 or Tr5. A partial (460 bp) sequence of a *T. borreli* cysteine proteinase gene was identified. The complete sequence was obtained by 50 and 30 rapid amplification of cDNA ends combining Gene Racer™ (Invitrogen) primers and gene-specific primers (Table 6.1). Briefly, *T. borreli* cDNA was reverse transcribed from 5.25 µg of total parasite RNA with Gene Racer™ oligo dT primers after removal of the 5' cap structure and ligated with the Gene Racer™ RNA Oligo. Amplification took place under the following conditions: 94°C for 5 min, 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, for 35 cycles and 72°C for 4 min. New primers were designed (Table 6.1) in order to confirm the obtained sequence and to amplify the full-length mRNA in a single reverse-transcriptase PCR (SuperScript One-Step RT-PCR Invitrogen). Briefly, 0.1 µg of total RNA, forward and reverse primers (200 nM each), 12.5 µl reaction buffer (2×), 20 U RNase inhibitor and 100 U Superscript II RT/Taq mix were mixed and diethyl pyrocarbonate-treated water was added to a final volume of 25 µl. RT-PCR was carried

out under the following conditions: cDNA synthesis at 50°C for 30 min, followed by one cycle of 2 min at 95°C; 30 cycles of 30 s at 94°C, 30 s at 53°C and 1 min at 72°C; and a final extension for 7 min at 72°C.

Table 6.1. Primer sequences applied to detect the *T. borreli* cysteine proteinase sequence. Primer sequences, melting temperatures and efficiencies of the primers for real-time quantitative PCR (RT-qPCR).

Primer	**Sequence 5'-3'**	**Melting temperature**	**Efficiency**
Library			
CPfw	CGCTTGCGGTTCCTGCTGG		
CPrv	GTTGTCCATGAGACCGCC		
Tr5	GTACCCGGGAATTCGGCCATT		
Tr3	ATACGACTCACTATAGGGCGAATTGGCC		
RACE			
TBCPfw1	CATCGAAGGCCAGAACGCGATTGC		
TBCPrv1	CACTCCAATTGACAGCGGTCCATA		
TBCPfw2	CAACCAGTGTGGTCTGAC		
cDNA			
TBCPcfw	CGATTGCAAGTAAGAGA		
TBCPcrv	ATTCTCGTTTCCGCTGCAG		
RT-qPCR			
TNFα fw	GCTGTCTGCTTCACGCTCAA	78	1.72
TNFα rv	CCTTGGAAGTGACATTTGCTTTT		
IL-1β fw	AAGGAGGCCAGTGGCTCTGT	78.8	1.73
IL-1β rv	CCTGAAGAAGAGGAGGCTGTCA		
IL-1R fw	ACGCCACCAAGAGCCTTTTA	76.7	1.72
IL-1R rv	GCAGCCCATATTTGGTCAGA		
IL-12P35 fw	TGCTTCTCTGTCTCTGTGATGGA	77.3	1.78
IL-12P35 rv	CACAGCTGCAGTCGTTCTTGA		
40S fw	CCGTGGGTGACATCGTTACA	78.5	1.75
40S rv	TCAGGACATTGAACCTCACTGTCT		

Cloning, sequencing and phylogenetic analysis

PCR products were purified, ligated and cloned in JM-109 cells using the pGEM-T easy kit (Promega). From each product, both strands of at least six clones were sequenced using the ABI Prism-Bigdye Terminator Cycle Sequencing Ready Reaction kit, and analyzed using an ABI 3730 sequencer. Nucleotide and amino acid sequences were analyzed for identity to other sequences using the GenBank database (Banson et al., 1999). Searches for similar sequences within the database were performed using the Basic Local Alignment Tool (Altschul et al., 1990). The amino acid sequence was assembled using the ExPASy translate tool. The signalP 3.0 Server was used for leader peptide prediction and the PROSITE profile library was used for prediction of protein patterns. Comparison between sequences was performed using ClustalW 1.83 software (Thompson et al., 1994), with minor optimizations made by hand. Percentage identity was calculated using the FASTA program (Pearson, 1990). A phylogenetic tree was constructed on the basis of the proportion of amino acid difference (p-distance) and complete gaps deletion by the neighbor-joining method (Saitou and Nei, 1987) using MEGA 3.1 software (Kumar et al., 2004). Reliability of the tree was assessed by bootstrapping, using 1000 bootstrap replications (Felsenstein, 1985).

Expression, affinity purification and refolding of recombinant cysteine proteinase

A fragment of 939 bp predicted to encode for a *T. borreli* cysteine proteinase gene without leader peptide and C-terminal extension was reverse transcribed from *T. borreli* RNA and cloned into pQE-30UA (Qiagen). Primers were designed to add a *BamHI* restriction site (underlined) to the 5' end
(5' ACCGTAGGATCCGCCCCTACAACCGATG 3') and a *KpnI* restriction site (underlined) to the 3' end
(5' TGCAGCGGTACCTTAAAATCTCCTAGGGAGC 3') to facilitate ligation. The construct was transformed into *Escherichia coli* M15, grown at 30^{o}C in 250 ml of Luria–Bertani media supplemented with 100 µg/ml ampicillin and 25 µg/ml kanamycin, and at OD_{600}=0.6–0.8 protein production was induced with 1 mM IPTG (isopropyl β-D-thiogalactoside) for a further 3–4 h. Bacteria were pelleted (3000g, 30 min, 4^{o}C) and stored at -80^{o}C until use. For recombinant proteinase purification all procedures, unless stated otherwise, were carried out at 4^{o}C. Bacterial pellets (0.2–0.4 g) were resuspended in 4–5 ml of 6 M GuHCl, 0.1 M NaH_2PO_4, 0.01 M Tris–HCl, 0.02 M imidazole, pH 8.0,

sonicated and centrifuged (9300 × *g*, 20 min). The supernatant was collected and mixed with 0.8 ml of Ni-NTA agarose (Qiagen). The suspension was incubated with shaking for 30 min and loaded on a Poly-Preps Chromatography Column (Bio-Rad). The next steps were performed at 300 × *g* for 2.5 min. The column was equilibrated with 2 ml of buffer A (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris–HCl) containing 0.02 M imidazole, 0.01 M 2-mercaptoethanol, 0.1% Triton X-100, pH 8.0, then washed 12× with 2 ml of buffer A containing 0.02 M imidazole, pH 8.0, 12× with 2 ml of buffer A containing 0.03 M imidazole, pH 7.0, and 6× with 2 ml of buffer A with 0.05 M imidazole, pH 6.3. Protein was eluted with buffer A containing 0.25 M imidazole, pH 4.2. For protein refolding the protocol of Sanderson et al. (Sanderson et al., 2000) was modified as follows: sample was 10× diluted with 8 M urea, 0.1 M Tris–HCl, 0.05 M EDTA, pH 8.0, in dialysis bags, transformed in 6 M urea, 100 mM Tris, 5 mM EDTA, 5 mM cysteine, pH 7.6, and dialyzed drop-wise against 3 volumes of 100 mM Tris, 5 mM EDTA and 5 mM cysteine, pH 7.6, until mixing of both buffers was completed. Protein was activated by 4 h incubation with 0.1 M acetate buffer, pH 5.0, at 37°C, concentrated on Vivaspin 15R columns (Vivascience, Sartorius) and dialyzed against cPBS, pH 7.4. Aliquots of activated protein were stored at -80°C until use. Protein levels were determined by the Bradford assay using BSA as a standard.

Proteinase activity of T. borreli lysate, ESPs and recombinant proteinase

Proteinase activities in *T. borreli* lysate and in *T. borreli* ESPs were detected using gelatin-substrate SDS-PAGE electrophoresis. *T. borreli* lysate and *T. borreli* ESPs were loaded on a 12.5% SDS-PAGE containing 1% w/v gelatin, under nonreducing conditions (no heating). Gels were run at 4°C, 80 V for 30 min and 120 V for the following 2 h. Subsequently, gels were washed with 2.5% Triton X-100 (v/v) at room temperature for 1 h and incubated in 0.1 M acetate buffer, pH 5.0, 5 mM DTT for 18 h at 37°C. Gels were stained with Coomassie brilliant blue. Proteinase activities in the *T. borreli* lysate and recombinant proteinase were also quantified by measuring hydrolysis of seven peptide *p*-nitroanilides according to Parama et al. (Parama et al., 2004). For cathepsin L-like cysteine proteinase activity, the *N*-benzyloxycarbonyl-phenylalanyl-arginine pNA (Z–Phe– Arg–pNA, Bachem) substrate was used. For cathepsin B-like cysteine proteinase activity, *N*-benzyloxycarbonyl-arginylarginine pNA (Z–Arg–Arg–pNA, Bachem) substrate was used. Stock solutions of both substrates at 8 mM

concentration were prepared in dimethyl sulfoxide (Merck). Assays were conducted in duplicate in 96-well plates containing 10 µl of parasite lysate or activated recombinant proteinase, 140 µl of buffer and 10 µl of substrate (final concentration 0.5 mM). Plates were incubated at 37°C overnight for lysate and for 2.5 h for the recombinant proteinase. Absorbance was measured at 405 nm using an ELISA reader (Anthos 2020). In order to determine pH optimal for enzyme activity, assays were conducted in 0.1 M acetate buffer, pH 4.0–6.0, 0.1 M phosphate buffer, pH 7.0, and 0.1 M Tris–HCl, pH 8.0. Proteinase activity was additionally measured after incubation for 30 min at 37°C with the cysteine proteinase activator DTT (5 mM; Sigma) or the cysteine proteinase inhibitor E-64 (10 µm; Sigma). Activity of activated recombinant cysteine proteinase against a variety of protein substrates (carp hemoglobin, carp IgM and carp transferrin) was tested. Hemoglobin was prepared according to Knox et al. (Knox et al., 1993) and IgM according to Rombout et al. (Rombout et al., 1993). Transferrin was saturated with iron, incubated with 0.6% rivanol and purified by ion-exchange chromatography. Carp proteins were incubated with activated *T. borreli*- recombinant cysteine proteinase in 0.1 M acetate buffer, pH 5.0, for 15 h at 37°C, run on 12.5% SDS-PAGE gel and stained with Coomassie brilliant blue to detect digestion products.

Macrophage cell culture and nitrite production

Primary cell cultures of macrophages were prepared as previously described (Joerink et al., 2006). Briefly, head kidneys were gently passed through a 100 mm sterile nylon mesh and rinsed with homogenization buffer. Cell suspensions were layered on 51% (1.071 g/cm^3) Percoll (Amersham Biosciences) and centrifuged at 450 × *g* for 25 min at 4°C without braking. Cells at the interface were removed and washed twice in incomplete NMGFL-15 medium (Joerink et al., 2006). Cell cultures were initiated by seeding 1.75×10^7 head kidney leukocytes in a 75 cm^2 culture flask containing 20 ml of complete NMGFL-15 medium (Joerink et al., 2006). Head kidney-derived macrophages were harvested after 6 days of incubation at 27°C by placing the flask on ice for 10 min prior to gentle scraping. For nitric oxide assays 5×10^5 cells were seeded in 100 ml complete NMGFL-15 medium in 96-well culture plates. For measurement of nitrite, cells were stimulated with LPS (50 µg/ml; E. coli, Sigma L2880, positive control), active (pH activated) or non-active *T. borreli* recombinant cysteine proteinase (28 µg/ml), or left untreated and incubated for 18 h at 27°C. Nitrite production was

measured as described before (Green et al., 1982). Briefly, to 75 ml of cell culture supernatant, 100 ml of 1% (w/v) sulfanilamide in 2.5% (v/v) phosphoric acid and 100 ml of 0.1% (w/v) N-naphthyl-ethylenediamine in 2.5% (v/v) phosphoric acid were added in a 96-well plate. The absorbance was measured at 540 nm and nitrite concentrations (µM) were calculated by comparison with a sodium nitrite standard curve.

Real-time quantitative PCR

For gene expression analysis 3×10^6 head kidney-derived macrophages were stimulated with LPS (50 µg/ml, positive control), with active (pH activated) or non-active recombinant cysteine proteinase (28 µg/ml), or left untreated for 6 h at 27°C. RNA from (un)stimulated cells was isolated using the RNeasy Mini kit (Qiagen), including DNase I treatment on the columns, according to the manufacturer's protocol. RNA concentrations were measured by spectrophotometry (Genequant; Amersham Pharmacia Biotech AB), and 1 µl was analyzed on a 1% agarose gel to check the integrity. Routinely, approximately 15-30 µg of RNA was isolated from 3×10^6 cells. RNA was stored at -80°C until further use. For each cDNA synthesis, a negative sample (non-RT), to which no reverse transcriptase was added, and a positive sample containing the reverse transcriptase were included. After a second DNase treatment, 1 µg of total RNA was combined with random primers (300 ng; Invitrogen Life Technologies), 1 µl of dNTP mix (10 mM), 4 µl of first strand buffer (5×), 1 µl of DTT (0.1 M) and 1 µl of RNase inhibitor (40 U/µl; Invitrogen Life Technologies). To each positive sample, 1 µl of Superscript RNase H Reverse Transcriptase II (200 U/µl; Invitrogen Life Technologies) was added and the mix was incubated at room temperature for 10 min and than at 50°C for 60 min. Reactions were stopped by heating at 70°C for 15 min. Water was added up to 100 µl and cDNA stored at -20°C until use.

Real-time quantitative PCR was performed with primers listed in Table 6.1. To 5µl of 5× diluted cDNA, 7 µl of Sybr Green Master Mix (Stratagene), 0.84 µl forward and reverse primer (300 nM each) and 0.32 µl of MilliQ water were added. Quantitative PCR was performed in a 72-well Rotor- Gene centrifugal real-time thermal cycler (Rotor-Gene 2000 Corbett Research). The following cycling conditions were used: one holding step of 10 min at 95°C, followed by 40 cycles of 20 s at 94°C, 30 s at 60°C and 30 s at 72°C; and an incubation for 1 min at 60°C was followed by a melting curve from

60 to 99°C in steps of 1°C with 5 s waiting. At the end of each cycle and during the waiting steps in the melting curve, fluorescence intensities were measured. Raw data were analyzed using the comparative quantitation of the Rotor-Gene Analysis Software version 5.0. Data were further analyzed using the Pfaffl method (Pfaffl, 2001), and average efficiencies per run per gene were used. Gene expression of 40S in each sample was used to normalize the data. Specificity of the amplification was ensured by checking the melting temperature and profile of each melting curve.

Vaccination with recombinant proteinase and detection of specific antibodies

Two weeks before the start of the experiment fish were moved to the infection facilities and acclimatized to 20±2 °C. All fish were individually numbered by tattoo for recognition during the experiment. A number of n = 5 fish were immunized twice i.m. (200 µl) within a 3-week interval with 100 µg of *T. borreli* recombinant cysteine proteinase mixed 1:1 with Freund's incomplete adjuvant. Six weeks after the second immunization, immunized and non-immunized, untreated fish (n = 5 of each group) were challenged i.p. with 10,000 *T. borreli*. Blood samples of 200 µl were collected weekly, starting 2 weeks post challenge, in heparinized syringes.

Plasma samples were checked for antibody levels against *T. borreli* cysteine proteinase by enzyme-linked immunosorbent assay (ELISA). MaxiSorp ELISA plates (Nunc) were coated overnight at 4°C in a humid chamber with shaking (150 rpm) with 100 µl of *T. borreli* recombinant cysteine proteinase (5 µg/ml) per well in cPBS, pH 7.4. All subsequent incubation steps were performed for 1 h at room temperature with shaking (150 rpm). Plates were blocked with 200 µl of 2% sucrose, 0.1% BSA and 0.9% NaCl. Sera were prediluted 50 times in T-TBS (20 mM Tris–HCl, 500 mM NaCl, 0.1% Tween-20, pH 7.4) and measured in three dilutions (100, 200 and 400 times diluted) in 100 µl volume. A standard serum with a high antibody titer was included on every plate to normalize the data. Further incubation steps were with 100 µl mouse anti-carp IgM (1:250) (WCI12, (Koumans-van Diepen, et al., 1995; van-Diepen et al., 1991) and subsequently with 100 µl goat anti-mouse-HRP (1:2000, Dako). In between all incubation steps plates were washed 5× with T-TBS. After the last wash, plates were incubated for 30 min at room temperature in the dark with 100 µl of 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) substrate solution (Roche) and absorbance was measured at 405 nm with 492 nm as reference.

Western blotting

Specificity of immune and pre-immune serum from fish immunized with *T. borreli* recombinant cysteine proteinase was tested by Western blotting on a number of preparations including *T. borreli* recombinant cysteine proteinase prior to and after activation, and *T. borreli* lysate (1.5×10^7, heated to 96°C for 10 min with loading buffer containing β-mercaptoethanol). Preparations were run on a 12.5% SDSPAGE and electro-transferred to a nitrocellulose membrane (Protrans, Schleicher & Schuell, Bioscience GmbH) and incubated overnight at 4°C in blocking buffer (3% BSA in TBS, 10 mM Tris–HCl, pH 7.5, 150 mM NaCl). Immune and pre-immune serum was diluted 1:1000 in 3% BSA in TBS. Membranes were incubated with (pre)immune serum, with mouse anti-carp IgM (WCI12; 1:50) and with goat anti-mouse HRP (1:1250) in 10% milk powder in TBS. All incubation steps were done for 1 h at room temperature with shaking (150 rpm). After each incubation step, membranes were washed twice with TBS-Tween/Triton (TBS-Tween, 0.2% (v/v) Triton X-100) and once with TBS, for 10 min at room temperature. Signal was detected by development with a chemoluminescence kit (Amersham) according to the manufacturer's protocol and visualized by the use of Lumnifil chemiluminescent Detection Film (Roche). Controls without primary antibody were negative.

Statistics

Nitrite production, antibody titer and parasitemia differences were tested for significance by a non-paired, two-tailed Student's *t*-test. Gene expression differences were analyzed after log-transformation by a one-sample Student's *t*-test (SPSS 12.0.1 for Windows). $P \leq 0.05$ was accepted as significant.

Results

Cloning, sequencing and phylogenetic analysis of the T. borreli cysteine proteinase

An anchored PCR on a λTriplEx2 cDNA library of *T. borreli* amplified a 460 bp cDNA fragment coding for a partial cysteine proteinase gene. The full-length coding sequence was obtained by 5' and 3' RACE. The nucleotide sequence of 1383 bp (GenBank accession number EF538804) contained an open reading frame of 1332 bp, flanked by a 5' UTR of 18 bp and a 33 bp 3' UTR. The cDNA sequence translated into a preproprotein of 443 amino acids (Figure 6.1). The predicted molecular weights of the

complete protein and the protein without prodomain were 46.1 and 35.3 kDa, respectively. The preproprotein contained a 17-residue putative signal peptide (preregion), a 96-residue prodomain, a 217-residue catalytic domain and a 113-residue C-terminal extension typical of kinetoplastid cathepsin L-like proteinases. The theoretical cleavage site of the prodomain between the alanine at position 113 and the aspartic acid at position 114 was determined by homology comparison. No polyproline/ threonine hinge linking the catalytic domain with the C-terminal extension, found in other trypanosomatids, could be detected in the *T. borreli* sequence. The prodomain contained a nine amino acid motif (position 28–36) that has been suggested to play a role in a mannose-6-phosphate independent trafficking pathway (Sajid and McKerrow, 2002). The prodomain also contained an ERFNIN-like motif (ERFN, position 41–52) and a GNFD motif (position 69–75), both typical of cathepsin L-like cysteine proteinases but did not contain the occluding loop motif typical of cathepsin B-like proteinases. Conserved features in the catalytic domain included a GCNGG motif (position 175–179), a glutamine at position 132, forming an oxyanion hole and the catalytic triad of cysteine (Cys-138), histidine (His-267) and asparagine (Asn-297) characteristic for thiol proteinases. Amino acids directly flanking the active sites were also highly conserved. The mature protein further contained eight conserved cysteine residues in the C-terminal extension, required for the formation of disulfide bridges. No potential glycosylation sites were identified. The *T. borreli* amino acid sequence of cysteine proteinase was aligned with cathepsin L-like proteinases of other trypanosomatids and was 78% identical to the cysteine proteinase of *C. salmositica*, 41% to *L. mexicana*, 44% to *T. cruzi* and 46% to *T. brucei* (Figure 6.1).

To further study the relationship of the *T. borreli* cysteine proteinase with known cathepsin L-like and B-like proteinases from other kinetoplastid parasites, a phylogenetic tree was constructed using the neighbor-joining method (Figure 6.2). In this tree cathepsin L-like and cathepsin B-like sequences formed two separate clusters. Within the cathepsin L cluster two subclusters could be distinguished, separating the cathepsin L-like proteinases of the Trypanosomatida and the Parabodonida. The *T. borreli* cysteine proteinase clustered together with the cysteine proteinasefrom another fish parasite (*C. salmositica*), both members of the Parabodonida. All branching nodes were supported by high bootstrap values.

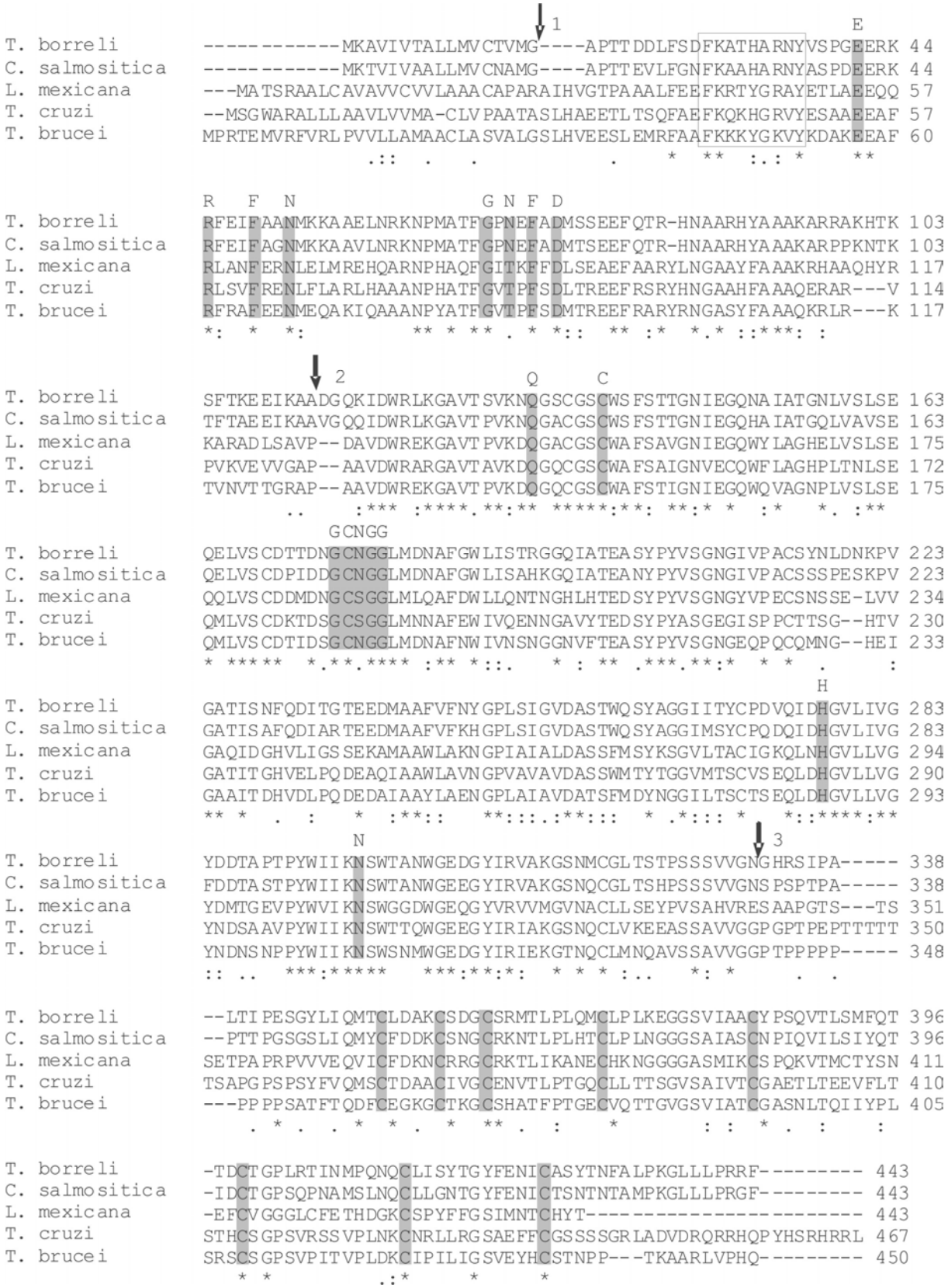


Fig. 6.1. Amino acid sequences of *Trypanoplasma borreli* cathepsin L-like proteinase aligned with cathepsin L-like proteinases from other kinetoplastid parasites. Sequences were aligned by ClustalW 1.83. Asterisks indicate amino acids identical to *T. borreli*, colons and dots denote decreasing degrees of

conservative substitutions, and dashes denote gaps. Arrow 1 denotes the site of signal peptide cleavage (between glycine, 17 and alanine, 18). Arrow 2 denotes the cleavage of the prodomain (between alanine, 113 and aspartic acid, 114), which leaves the mature form of the enzyme. Arrow 3 indicates the border between the catalytic part of the protein and the C-terminal extension (between glycine, 330 and asparagine, 331). The putative motif involved in trafficking of the protein to lysosomes is boxed. The cathepsin L family signatures: ERFNIN (ERFN), GNFD and GCNGG, the glutamine (Q) of the oxyanion hole and the cysteine (C), histidine (H) and asparagine (N) of the catalytic triad are indicated and shaded. Conserved cysteines in the C-terminal extension are also shaded. *T. borreli* cathepsin L proteinase is deposited in the GenBank database under accession number ABQ23398. Proteins were obtained from the GenBank database with the following accession numbers: *C. salmositica*, AY713477; *L. mexicana*, CAA78443; *T. cruzi*, AAF75547; *T. brucei*, XP_845218.

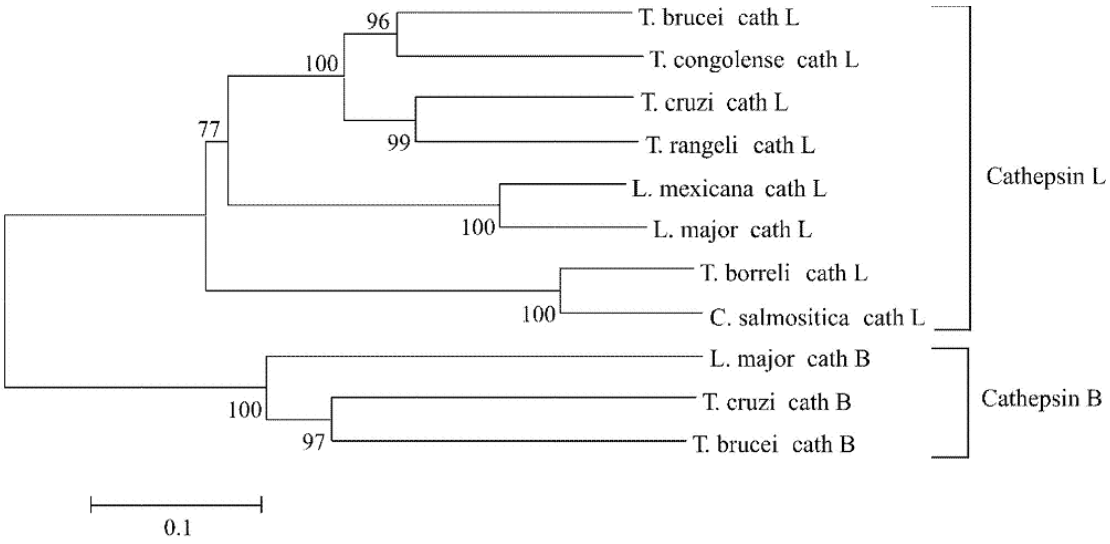


Fig. 6.2. Phylogenetic analysis of *T. borreli* cathepsin L-like proteinase and cathepsin L-like proteinases of other kinetoplastids with cathepsin B-like proteinases as an outgroup. The tree was constructed based on the proportion of amino acid difference (p-distance) and complete gaps deletion by the neighbor-joining method. Numbers at branch nodes represent bootstrap confidence levels of 1000 bootstrap replications. The GenBank accession numbers of the analyzed sequences are as follows: *T. brucei* cath. L, XP_845218; *T. congolense* cath. L, AAA18215; *T. cruzi* cath. L, AAF75547; *T. rangeli* cath. L, 2117247A; *L. mexicana* cath. L, CAA78443; *L. major* cath. L, AAB48120; *T. borreli* cath. L, ABQ23398; *C. salmositica* cath. L, AY713477; *L. major* cath. B, AAB48119; *T. cruzi* cath. B, AAD03404; *T. brucei* cath. B, AAR88085.

Expression, purification and refolding of recombinant T. borreli cysteine proteinase

The promature *T. borreli* cysteine proteinase (protein with prodomain but without C-terminal extension; see Figure 6.1, between arrows 1 and 3) was over-expressed in *E.*

coli M-15 bacteria, which resulted in a high yield (15–20 mg/l) of a 35 kDa protein, corresponding to the predicted 35.4 kDa molecular weight of the His_6-tag fusion protein (Figure 6.3, lanes 2–4). Most of the expressed protein was found in inclusion bodies and purification steps were performed under denaturating conditions using Ni-NTA agarose resin. After purification, the majority of the *T. borreli* recombinant cysteine proteinase had retained the promature form, as judged by relative mobility on SDS-PAGE. Dialysis partly promoted formation of the mature proteinase with the presence of 30 kDa intermediates (Figure 6.3, lane 4). Full activation of the recombinant protein into the mature form (without prodomain) of 23–24 kDa (Figure 6.3, lane 5) was accomplished at low pH (0.1 M acetate buffer, pH 5.0, 4 h, 37^{o}C). Activation was accompanied by further autoproteolysis of the short peptide of the proregion (data not shown). The amount of enzyme recovered from 1 l of *E. coli* culture after refolding and activation was 3–4 mg, approximately. Endotoxin activity was ≤0.01 endotoxin units/µg protein as determined by the Limulus Amebocyte Lysate QCL-1000 from Cambrex (Walkersville, MD).

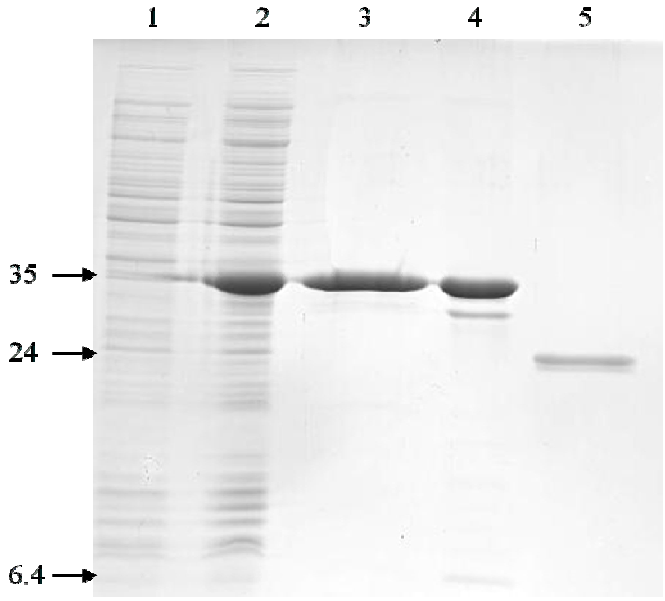


Fig. 6.3. SDS-PAGE analysis of *T. borreli* recombinant cysteine proteinase. Molecular weight is indicated on the left in kDa. Lane 1: non-induced lysate of *E. coli* strain M-15. Lane 2: lysate of *E. coli* strain M-15 induced with IPTG, over-expressed 35 kDa His6-tag fusion protein. Lane 3: 35 kDa His6-tag fusion protein purified on Ni-NTA agarose resin under denaturating conditions. Lane 4: 35 kDa promature protein after dialysis together with 30 kDa intermediates and 10 kDa processed small peptides. Lane 5: 23–24 kDa mature protein after activation in 0.1M acetate buffer, pH 5.0.

Determination of proteolytic activity of T. borreli lysate, ESPs and T. borreli recombinant cysteine proteinase

T. borreli lysate (1×10^8 parasites/ml) and *T. borreli* ESPs (after 1 h of culture of 1×10^8 parasites/ml) were examined for cysteine proteinase activity by gelatin-substrate SDS-PAGE electrophoresis. Gelatin SDS-PAGE performed under conditions optimal for cysteine proteinases (low pH and addition of DTT) showed two bands of activity in both the lysate and the ESPs (Figure 6.4).

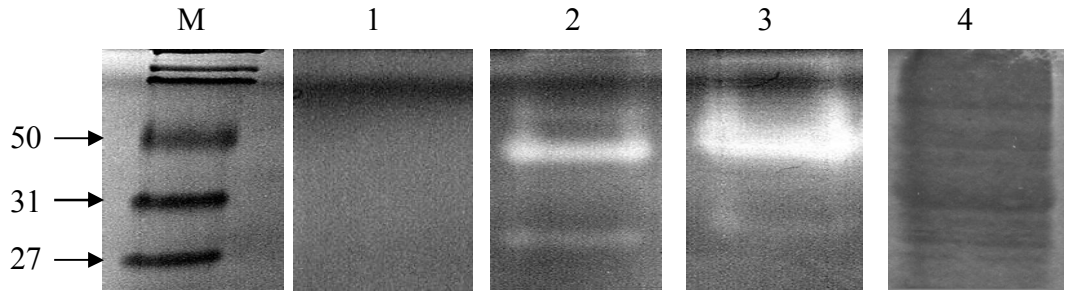


Fig. 6.4. Detection of proteinase activity by gelatin SDS-PAGE analysis. Molecular weight is indicated on the left in kDa. Lane 1: non-digested gelatin SDS-PAGE gel. Lane 2: *T. borreli* ESPs (20 ml). Lanes 3 and 4: *T. borreli* lysate (10 ml). Proteins were resolved on a 12.5% SDS-PAGE containing gelatin (1% w/v) under non-reducing conditions, washed with Triton X-100 (2.5% v/v) and incubated in 0.1M acetate buffer, pH 5.0, 5mM DTT for 18 h (lanes 1–3) and in 0.1M acetate buffer, pH 5.0, 10 mM E-64 for 18 h (lane 4). Gel was stained with Coomassie brilliant blue.

Activities observed in *T. borreli* lysate and in *T. borreli* ESPs suggested that cysteine proteinases are present not only in the parasite itself but can also be secreted. *T. borreli* lysate was also examined for cysteine proteinase activity by hydrolysis of 7 peptide *p*-nitroanilides, which showed high cathepsin L-like activity present in the parasite lysate and very low cathepsin B-like proteinase activity (Table 6.2). Cathepsin L-like activity was present at a broad pH range (pH 4.0–8.0) but highest at pH 4.0 (Table 6.2), suggesting lysosomal localization of the proteinase within the parasite. Proteinase activity was enhanced by the cysteine proteinase activator DTT (≤25% increase at optimal pH) and blocked by the cysteine proteinase inhibitor E-64 (0% activity at optimal pH).

T. borreli recombinant proteinase, after refolding and activation, was examined for cysteine proteinase activity using hydrolysis of 7 peptide *p*-nitroanilides. Hydrolysis of 7 peptide *p*-nitroanilides showed that the activated protein had high cathepsin L-like

activity at a broad pH range (pH 4.0–8.0) with optimal activity at pH 5.0 (Table 6.2). Recombinant cysteine proteinase activity was completely blocked by E-64 (0% activity at optimal pH). Activated recombinant cysteine proteinase was also able to digest purified carp hemoglobin, carp transferrin and carp IgM at pH 5.0 (Figure 6.5).

Table 6.2. Proteinase activity against Z-Phe-Arg pNA and Z-Arg-Arg pNA of *T. borreli* lysate and *T. borreli* recombinant cysteine proteinase.

	pH 4.0	**pH 5.0**	**pH 6.0**	**pH 7.0**	**pH 8.0**
Z-Phe-Arg pNA					
lysate (nmol/ml/min)	44.00±3.04	18.00±5.08	19.37±3.82	19.03±2.00	19.05±0.02
recombinant (nmol/mg/min)	8156±2804	11962±1435	10808±329	10058±1464	10097±4827
Z-Arg-Arg pNA					
lysate (nmol/ml/min)	4.13±0.41	4.06±2.58	5.44±0.37	5.46±0.07	17.69±0.34
recombinant (nmol/mg/min)	0	0	0	nd	nd

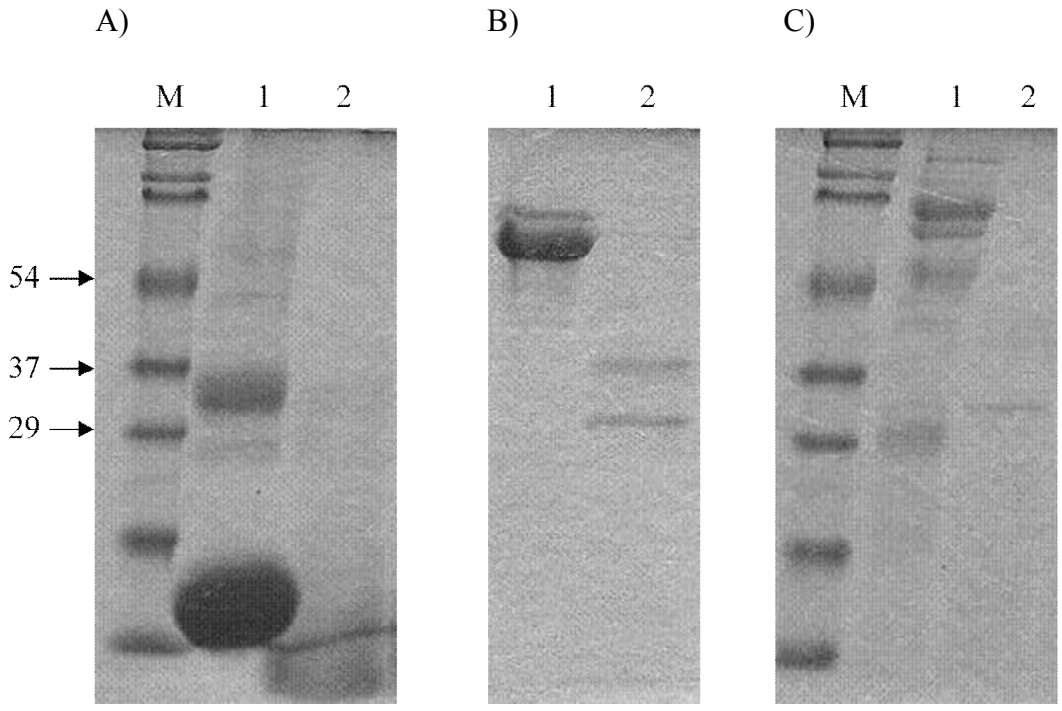


Fig. 6.5. Activity of recombinant cysteine proteinase against a variety of protein substrates. Host proteins: hemoglobin (**A**), transferrin (**B**) and IgM (**C**) were incubated with *T. borreli* recombinant cysteine proteinase in 0.1M acetate buffer, pH 5.0, for 15 h at 37 ^{o}C (lane 2) or incubated only in 0.1M acetate buffer, pH 5.0, for 15 h at 37 ^{o}C as a negative control (lane 1), run on a 12.5% SDS-PAGE gel and stained with Coomassie brilliant blue. Molecular weight is indicated on the left in kDa. Lane 1: undigested protein. Lane 2: digested protein.

Effects of T. borreli recombinant cysteine proteinase on nitric oxide induction and gene expression in macrophages

T. borreli infections of carp in vivo are typically characterized by high levels of nitric oxide (Saeij et al., 2002). We examined the *in vitro* potential of *T. borreli* cysteine proteinase to induce nitric oxide in head kidney-derived macrophages of carp. Activated *T. borreli* recombinant cysteine proteinase (28 µg/ml) did not significantly induce production of nitric oxide higher than seen in non-activated control samples (Figure 6.6A). To further characterize the modulatory potential of the *T. borreli* cysteine proteinase *in vitro*, we examined gene expression in head kidney-derived macrophages with real-time quantitative PCR, 6 h after addition of (non)activated proteinase (Figure 6.6B). Activated recombinant cysteine proteinase did not significantly induce expression of IL-1b, IL-1R and IL-12P35 different from the non-activated control.

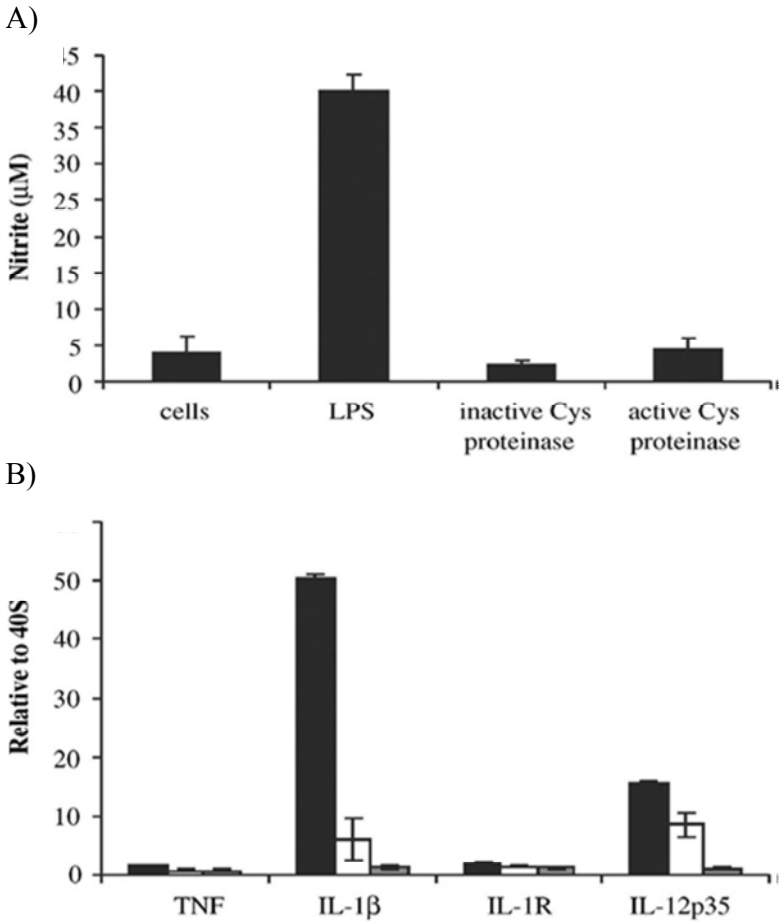


Fig. 6.6. Nitrite production (**A**) and gene expression (**B**) of head kidney-derived macrophages after stimulation with *T. borreli* recombinant cysteine proteinase. (**A**) Macrophages, 5×10^5 per well were stimulated with LPS (50 µg/ml), (non)-active *T. borreli* recombinant cysteine proteinase (28 µg/ml) or

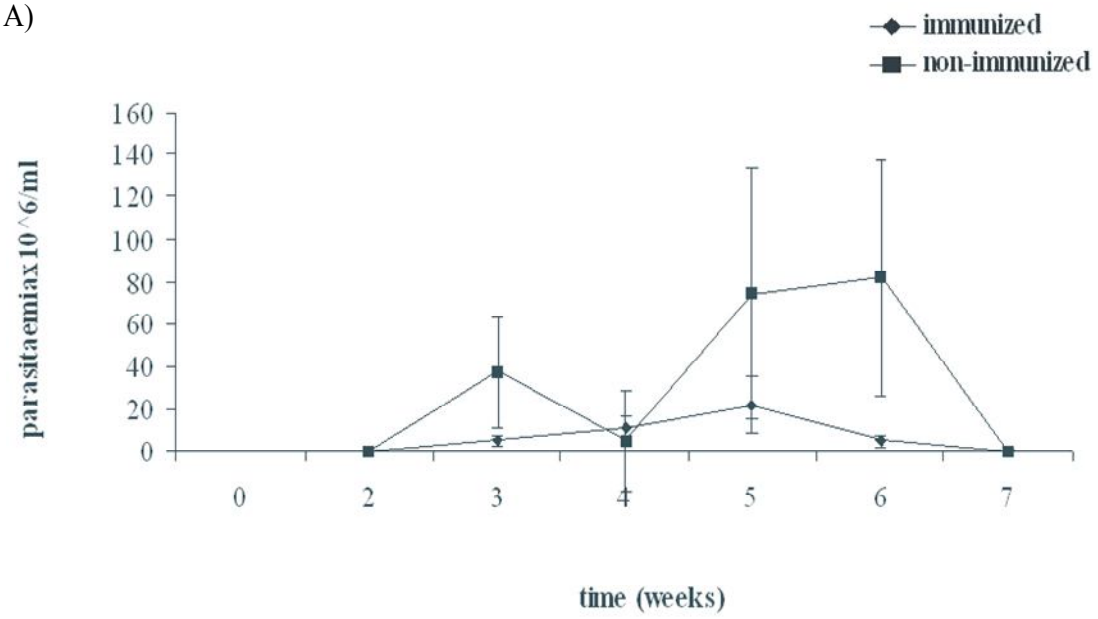
left untreated as control for 18 h at 27 °C and nitrite level was measured. Data are from a representative experiment out of three independent experiments with similar results, average and SD of duplicate measurements are given. (**B**) Macrophages, 3×10^6, were stimulated with LPS (50 µg/ml, closed bars), nonactive *T. borreli* recombinant cysteine proteinase (28 µg/ml, open bars) or active *T. borreli* recombinant cysteine proteinase (28 µg/ml, grey bars) for 6 h at 27 °C and gene expression was measured by real-time quantitative PCR. Average and SD of n = 2 fish are given. Values are expressed relative to unstimulated cells.

Immunization with T. borreli recombinant cysteine proteinase

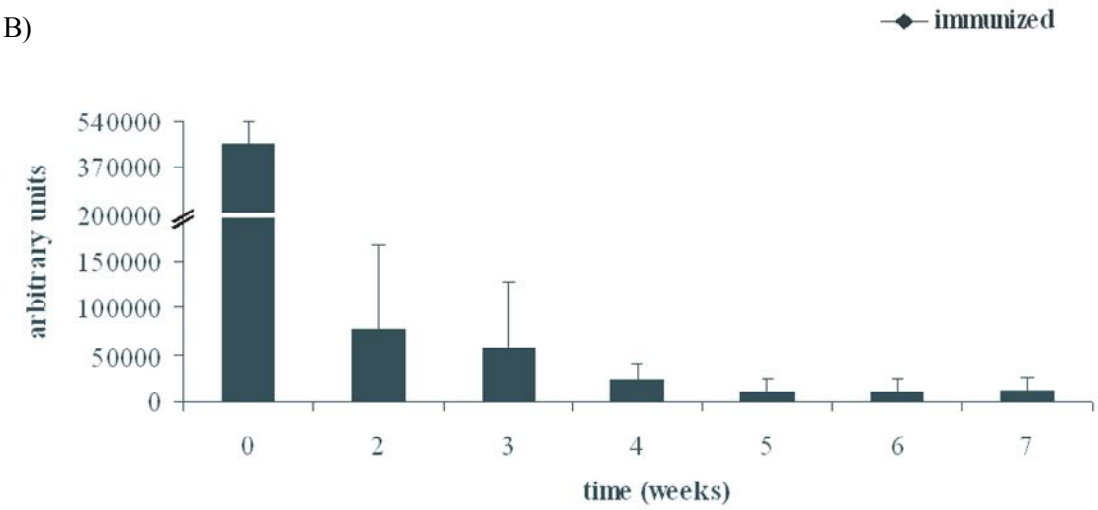
Fish were immunized twice with *T. borreli* recombinant cysteine proteinase and challenged with live *T. borreli*. Serum levels of antibodies specific against *T. borreli* recombinant cysteine proteinase in (non)immunized fish were measured by ELISA. Parasitemia in immunized fish was slightly but not significantly lower than in non-immunized fish (Figure 6.7A). Immunization with *T. borreli* recombinant cysteine proteinase induced a very high antibody response already after first immunization (data not shown) with specific antibody titers remaining elevated until the day of challenge with *T. borreli* (Figure 6.7B). During challenge, however, antibody levels decreased. In contrast, in non-immunized but challenged fish the antibody titer was low at the moment of challenge and it gradually increased to higher levels (<400 units), which were, however, still lower than those observed in immunized fish (>400,000 units) (Figure 6.7C).

Immune serum specifically recognized not only the recombinant cysteine proteinase, both before and after activation (Figure 6.8, lanes 1–2, immune serum), but also the native cysteine proteinase present in *T. borreli* lysates (Figure 6.8, lane 3, immune serum). Pre-immune control serum did not recognize *T. borreli* cysteine proteinase (Figure 6.8, lanes 1–3; non-immune serum).

A)


immunized
non-immunized
parasitaemiax10^6/ml
160
140
120
100
80
60
40
20
0
0
2
3
4
5
6
7
time (weeks)

B)


immunized
arbitrary units
540000
370000
200000
150000
100000
50000
0
0
2
3
4
5
6
7
time (weeks)

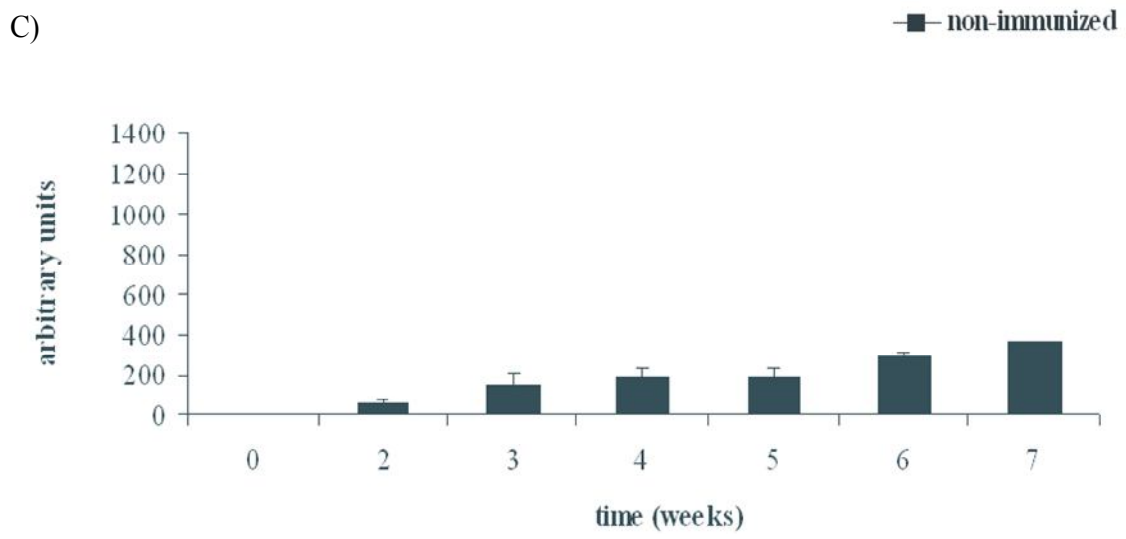


Fig. 6.7. Parasitemia and antibody titers in (non)immunized fish infected with *T. bor*reli (10,000 parasites/fish, i.p.). (**A**) Parasitemia. Blood samples were taken weekly from week 2 until week 7 post infection and parasitemia was monitored with a Bürker counting chamber. (**B**) Specific antibody titer (ELISA) against recombinant *T. borreli* cysteine proteinase in sera of fish immunized with recombinant antigen and challenged with parasites. Fish (n = 5) were immunized i.m. twice with a 3-week interval with 100 µg of recombinant cysteine proteinase with Freund's incomplete adjuvant. Fish were challenged 6 weeks after the second immunization. Blood samples were taken at week 0 and weekly from week 2 until week 7 post infection. (**C**) Specific antibody titer (ELISA) against recombinant *T. borreli* cysteine proteinase in sera of fish not receiving a prior immunization (control fish) and challenged with parasites. Blood samples were taken at week 0 and weekly from week 2 until week 7 post infection (n = 5 fish).

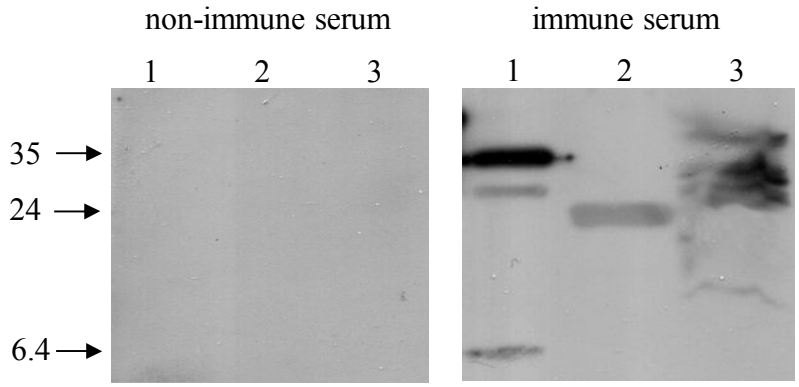


Fig. 6.8. Western blot analysis of (non)immune serum against *T. borreli* recombinant cysteine proteinase. Molecular weight is indicated on the left in kDa. Lane 1: *T. borreli* recombinant cysteine proteinase prior to activation. Lane 2: *T. borreli* recombinant cysteine proteinase after activation. Lane 3: *T. borreli* lysate. Proteins were resolved on a 12.5% SDS-PAGE, electro-transferred to nitrocellulose and probed (1:1000) with (non)immune serum from non-immunized fish and fish immunized twice with *T. borreli* recombinant cysteine proteinase.

Discussion

Our data showed the presence of proteinase activity in lysates of *T. borreli*. Proteinase activity in whole *T. borreli* lysate could be inhibited by E-64 and stimulated by DTT, confirming cysteine proteinase activity. The specificity of this activity was further investigated using hydrolysis of seven peptide *p*-nitroanilides. Specificity of enzymes belonging to the papain-like family is primarily determined by P1/S2 interactions, which exhibit marked preference for bulky hydrophobic or aromatic residues. Proteinases of cathepsin B-like and cathepsin L-like subfamilies can be distinguished by their ability to degrade small peptides that vary at the P2 position. We used Z–Phe–Arg–pNa (Z-FR-pNa; cathepsin L) and Z–Arg–Arg–pNa (Z–RR–pNa; cathepsin B) peptides to investigate the substrate preference of the proteinase(s) present in whole *T. borreli* lysates. Proteinases in whole parasite lysate degraded both substrates but with substantially higher activity towards Z–FR–pNa (cathepsin L). The presence of cathepsin L-like cysteine proteinase activity in lysates of kinetoplastids is more common than the presence of cathepsin B-like activity (Mackey et al., 2004).

We cloned and sequenced the cysteine proteinase of *T. borreli* from an expression library. Sequence alignment with other kinetoplastid cathepsin L- and cathepsin B-like proteinases strongly supports the phylogeny of the Kinetoplastida (Sakanari et al., 1997). The protein shows all the characteristics of cathepsin L-like proteins of other protozoan parasites, including ERFNIN-like, GNFD and GCNGG motifs, the oxyanion hole (Gln) and the active triad formed by Cys, His and Asn (Sajid and McKerrow, 2002). Cathepsin L-like proteinases often exist as multiple isoenzymes encoded by a tandem array of several genes (Souza et al., 1992). Different isoenzymes, although conserved, can show activity differences (Juliano et al., 2004; Boulange et al., 2001; Lima et al., 2001). We did not examine the genomic organization of cysteine proteinases in *T. borreli* but, recently, we did find two additional cDNAs, suggesting that also for *T. borreli* different isoenzymes exist.

The protein also showed the typical nine amino acid motif in the prodomain, which plays a crucial role in a mannose-6- phosphate independent trafficking pathway (Sajid and McKerrow, 2002) where the prodomain is cleaved in late Golgi vesicles (*T. cruzi*) or in the flagellar pocket (*L. mexicana*), and active enzyme subsequently is delivered to the lysosome (Brooks et al., 2000; Engel et al., 1998). The presence of this motif

suggests the same mechanism may be used for targeting of *T. borreli* cysteine proteinases to lysosomes.

We successfully expressed the *T. borreli* cysteine proteinase in *E. coli*, but only when we excluded the C-terminal domain in our construct. Initial attempts to express the fulllength protein did not result in an active enzyme probably due to misfolding (unpublished data). Cysteine proteinases are rich in disulfide bridges of which 50% approximately are present in the C-terminal domain. Although the C-terminal may be highly immunogenic and possibly involved in immune evasion, it is not necessary for enzyme activity (Sanderson et al., 2000; Boulange et al., 2001; Eakin et al., 1992). We did include the proregion of the enzyme in our construct to allow for its role in the correct folding and secretion of the protein (Vernet et al., 1990). Our approach supports similar successful attempts to produce active recombinant proteinases from other kinetoplastid parasites (Sanderson et al., 2000; Boulange et al., 2001). In contrast, however, we were able to produce active recombinant enzyme after purification with Ni-NTA agarose resin, which was not true for *L. mexicana* (Sanderson et al., 2000). The electrophoretic mobility of our complete fusion protein corresponded well with the predicted molecular weight, which was not completely true for the mature enzyme. Activation of the *T. borreli* recombinant proteinase was initiated by purification, supported by the refolding procedure and completed by acidification. Production of the mature form of recombinant cysteine proteinases involves several hydrolytic events that result in removal of the prodomain; however, the (number of) cleavage sites differ between parasites (Sanderson et al., 2000; Eakin et al., 1992). Indeed, during the activation process several intermediate forms of active protein were present and possibly the deviation from the expected molecular weight noticed for the mature protein could be due to incomplete processing of the mature enzyme. Nevertheless, bioactivity of the mature recombinant enzyme was very high compared with the activity present in parasite lysate, suggesting that a potential partial presence of the proregion does not influence bioactivity through an inactivation of the active site.

Cathepsin L-like proteinases of trypanosomes contribute to parasite pathogenesis but also to immune response modulation in the host (McKerrow et al., 2006). CPBs from *L. mexicana* suppress Th1 and/or stimulate Th2 immune responses, depending on the mouse strain (Pollock et al., 2003; Buxbaum et al., 2003). Cruzipain, the cathepsin L-like cysteine proteinase from *T. cruzi* stimulates macrophages from mice into an

alternative activation state in vitro and induces a Th2 immune response in vivo (Giordanengo et al., 2002). We used the *T. borreli* active recombinant cysteine proteinase to investigate its immunomodulatory effect on carp head kidney-derived macrophage cultures (Joerink et al., 2006). Infection of carp with *T. borreli* typically is associated with a high nitric oxide production and associated inflammatory reaction (Saeij et al., 2002). *In vitro*, we could not detect a clear modulation of macrophage activity by the active *T. borreli* recombinant protease, as compared to the non-active negative control. Neither did we detect a significant induction of nitric oxide production (innate activation of macrophages), nor did we detect an alternative activation (unpublished data) as evaluated by arginase activity (Joerink et al., 2006). Of course, this does not rule out that the *T. borreli* cysteine proteinase could contribute to the high NO levels oberved *in vivo*. To further assess, *in vitro*, the modulation of carp macrophages by *T. borreli* proteinase we measured gene transcription induced by the (non)active recombinant protein. Although gene transcription of IL-1b and IL-12P35 (Huising et al., 2006), but not IL-1R (Mezt et al., 2006) and TNFa (Garcia-Castillo et al., 2004; Bridle et al., 2006; Chang et al., 2005), seemed modulated by the addition of recombinant protein, there were no significant differences between the active and non-active proteinase. Therefore, it remains difficult to mimic the macrophage activation seen during *T. borreli* in an *in vitro* situation.

Activated *T. borreli* cysteine proteinase could digest carp hemoglobin, immunoglobulin and transferrin. Probably, similar to other kinetoplastids, *T. borreli* is able to take up host proteins in the flagellar pocket and actively digest the ingested proteins with the proteinase in the parasite lysosomal compartment (Berasain et al., 2003; Mbawa et al., 1992; Troeberg et al., 1996). However, further studies are needed to confirm if digestion of hemoglobin might contribute to the severe anemia associated with *T. borreli* infections (Steinhagen et al., 1990) and if digestion of carp IgM might contribute to an initial immunosuppression period postponing the moment where high levels of circulating IgM contribute to the clearance of *T. borreli* (Pal et al., 2003). Digestion of transferrin might allow the parasite to sequester iron as an essential growth factor. In *T. brucei* infections, cleaved transferrin products are secreted back into the bloodstream of the host (Steverding, 2000). Interestingly, enzymatically cleaved transferrin products can induce a potent nitric oxide (NO) response in fish macrophages (Stafford et al., 2004). Possibly, transferrin products cleaved by *T. borreli* proteinase and secreted back

into the bloodstream could contribute to the high NO response typical of *T. borreli* infections.

To investigate if *T. borreli* cysteine proteinase induces protective immunity we immunized fish with the recombinant enzyme and challenged with the live parasite. During the challenge, antibody levels rapidly decreased from 450,000 units at the day of challenge to 13,000 units 7

weeks post challenge (Figure 6.7B). This decrease cannot completely be explained by the half-life of carp IgM of 22.5 days (Overath et al., 1999); the possibility that digestion of carp IgM by *T. borreli* might contribute to this rapid decline in antibody level (Pal et al., 2003) deserves further attention. Total parasitemia was slightly but not significantly lower in immunized versus nonimmunized fish. Cathepsin L-like proteinases from mammalian kinetoplastid parasites are considered attractive vaccine candidates depending on the adjuvant (Schnapp et al., 2002; Zadeh-Vakili et al., 2004; Pollock et al., 2003). However, we cannot exclude that vaccination along with an effective adjuvant could lead to protection.

In this study we produced a recombinant cathepsin L-like cysteine proteinase from a parabodonidid parasite, with highest activity at low pH, suggesting lysosomal origin. Digestion by the cysteine proteinase of host hemoglobin, immunoglobulin and transferrin probably contribute to the pathogenicity of *T. borreli*. Immunization with the recombinant proteinase induced a high antibody response but was not protective. Sequence alignment and our bioactivity studies suggest a conservation of function with respect to cysteine proteinase activity in the Parabodonida in support of the hypotheses on the phylogeny of the Kinetoplastida.

Acknowledgments

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7

General discussion

Chapter 7

This study focused on the role of transferrin (Tf) in innate immunity of common carp (*Cyprinus carpio* L.). Specifically, we studied Tf polymorphism in relation to resistance to disease, through *in vitro* and *in vivo* approaches. As an animal disease model, we used experimental infection of carp with the blood parasite *Trypanoplasma borreli* (Order Kinetoplastida). This Order also includes mammalian trypanosomes, such as *Trypanosoma brucei* and *Trypanosoma cruzi*, which cause sleeping sickness in human and cattle or 'Chagas' disease, respectively. In farmed populations of cyprinids, infection with *T. borreli* can occasionally contribute to relatively high fish mortality, ranging from 75 to 100% (Steinhagen et al., 1990).

Although antibiotics and other chemotherapeutants can be cost-effective in controlling some diseases and have become an integral part of modern (intensive) aquaculture, important alternatives and certainly more sustainable approaches are vaccination and immunostimulation by feed additives. These alternatives, however, are not always cost-effective in semi-intensive culture systems for carp. Genetic selection for a higher innate immune response seems to be the most promising approach to disease control in semi-intensive carp pond farming. Relevant polymorphic genes, such as Tf that is of immunological importance, could be used as markers in future marker-assisted selection programs for increased resistance to diseases.

Transferrin plays a major role as iron-binding molecule in most living organisms. It provides the host with iron, sequestered via transferrin receptors on host cells. However, Tf may also be used by pathogens sequestering iron via their own Tf receptors. Indeed, for trypanosomes, iron is a growth factor of critical importance and mechanisms for iron acquisition via Tf receptors have been investigated in great detail. *T. borreli*, as a parasite related to the trypanosomes, most likely is characterized by a similar requirement for iron during its journey through the fish host. Our study on the effect of Tf and Tf polymorphisms on the multiplication of *T. borreli* and the carp immune defense mechanisms will lead to a better understanding of host-parasite interactions in fish.

In this chapter we discuss: 1) The capacity of carp Tf to act as iron-binding protein; 2) The evolutionary drive for maintaining Tf polymorphism in common carp; 3) The role of Tf in the immune response and influence on host-pathogens interactions; 4) The use of the polymorphic protein Tf as marker to find correlation for disease resistance.

1. Iron binding sites in carp transferrin are not conserved

During the last 10-20 years Tf-encoding genes from a variety of animals were cloned, sequenced and their three-dimensional structures modeled (Anderson et al., 1987; Moore et al., 1997; Hall et al., 2002; Wally et al., 2006). These studies showed strong conservation between Tf family members (transferrin, ovotransferrin, lactotransferrin and melanotransferrin) and between the two lobes (N and C) of the Tf protein (Lambert et al., 2005, review). We confirmed the conserved general structure of carp Tf with respect to domains/subdomains homology and confirmed the overall similarity of the carp three-dimensional structure to Tfs of other species (**chapter 4**).

In general, the coordination of iron in each lobe of Tf involves four amino acid ligands (two tyrosines, a histidine and an aspartic acid) with two additional ligands provided by a synergistic anion. In some cyprinid species, including zebrafish, grass carp, crucian carp and goldfish, residues involved in iron binding in the N-lobe are not conserved. Therefore, it was suggested that the substitutions in the N-lobe of cyprinid Tf make this lobe non-functional (Ciuraszkiewicz et al., 2007). For European common carp, the iron binding part of the C-lobe is completely conserved as compared to human. However, the iron binding part of the N-lobe of carp Tf showed that four out of the six crucial residues were not conserved (**chapter 4**).

The substitution of the majority of iron-coordinating residues of the N-lobe could result in a lower ability to bind iron. In fact, we recently studied the capacity of carp Tf to bind iron, not only by comparison of the degree of conservation of the relevant amino acids, but by examining the total iron binding capacity of carp serum. These experiments confirmed that, in comparison to human serum, Tf in carp serum can bind only half the amount of iron (Wojtczak and Jurecka, unpublished data).

Tf also is an important protein for the transportation of metals other than iron. Under normal circumstances Tfs are occupied by iron for approximately 30% only, and other metals such as cadmium can be bound without requiring the displacement of the more stable iron. Interestingly, carp Tf is able to bind two cadmium ions (De Smet et al., 2001). This would suggest that the substitutions in the iron binding sites of the N-lobe of carp Tf did not result in a complete loss of metal ion binding ability.

2. Transferrin polymorphism may be related with differences in disease resistance

We identified four different Tf alleles (C, D, F and G) by PAGE in European common carp, which we then cloned and sequenced (**chapter 4**). Subsequent analysis indicated that the evolutionary pressure was different on the N- and C-lobes of carp Tf, as noted from the distribution of the number of substitutions between the two lobes. Comparison of the four carp Tf alleles revealed the presence of 21 polymorphic sites, of which 13 polymorphic sites resulted in non-synonymous changes (4 in the N-lobe and 9 in the C-lobe).

At the protein level, conserved parts usually show lower substitution rates than parts which are not under strong purifying selection. If the two lobes of the carp Tf molecule would be functionally equal, i.e. they both would bind iron to the same extent, one would expect the degree and pattern of polymorphism between the N- and C-lobes to be similar. Comparison of the amino acid similarity of the two lobes by the Neighbor-joining method using p-distance (MEGA vs 4; Tamura et al., 2007) showed that the branching order of the C-lobe reflects the history of speciation more correctly than the branching order of the N-lobe (Fig.7.1).

When the number of nonsynonymous amino acid substitutions is higher than the number of synonymous substitutions, it reflects positive selection, which means that selection is driven towards an increased polymorphism of a gene. In contrast, when synonymous substitutions outnumber nonsynonymous substitutions, this usually reflects purifying selection, typical for conserved and functionally important parts of a molecule. Positive selection can be quantified using Tajima's D statistics (Tajima, 1989), which compares the number of segregating sites per site with the nucleotide diversity. Average heterozygosity π and level of polymorphism θ was higher for the C-lobe than for the N-lobe (Table 7.1). This indicates that especially the C-lobe of carp Tf is under positive selection.

Table 7.1. The Tajima test statistic estimated for the full length transferrin (N- and C-lobes), N-lobe and C-lobe. The abbreviations used are as follows: **S** – number of segregating sites, **θ** – level of nucleotide polymorphism, **π** – nucleotide diversity and **D** – Tajima test statistic.

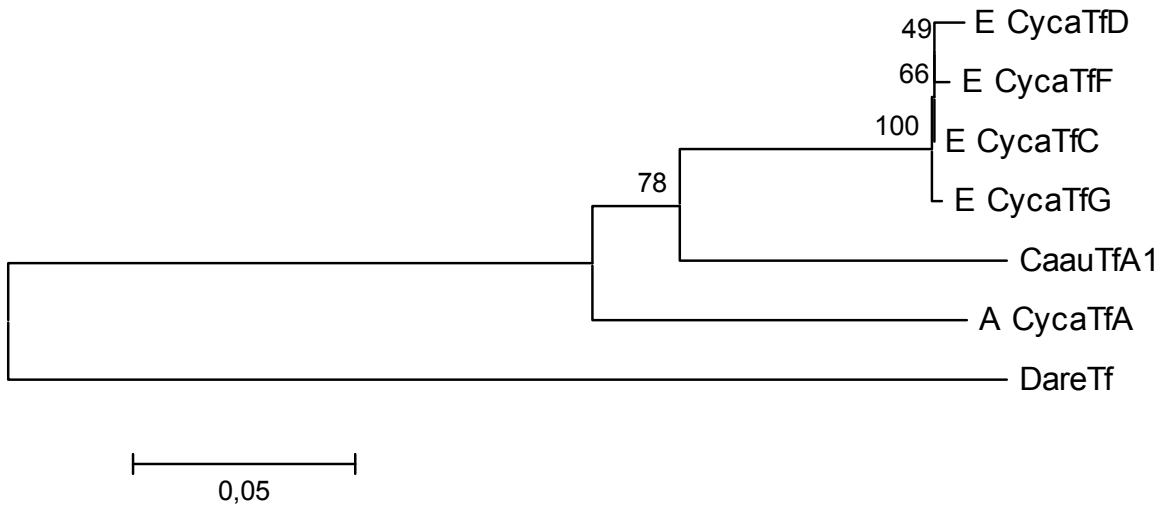
	S	**θ**	**π**	**D**
N-and C-lobes	21	0.005105	0.005570	0.936769
N-lobe	5	0.002755	0.002525	-0.796844
C-lobe	13	0.007206	0.008469	1.772604

Previous studies on goldfish Tf suggested eleven residues to be under positive selection, with five residues located at the N-lobe that could possibly indirectly influence the capability of iron binding or release (Yang and Gui, 2004). The authors suggested that the five selected sites would converge in the interdomain of the N-lobe and could potentiality form hydrogen bonds with those directly bonded to iron and anion. Consequently, variation of these five selected sites might indirectly influence the capability of goldfish transferrin to bind or to release iron. However, the sites in the N-lobe of goldfish that would be directly involved in iron binding are comparable to the sites we found in common carp, i.e. four residues are substituted as compared to the human Tf N-lobe. Thus, it seems likely that variation at the five selected sites in the goldfish N-lobe is not connected with iron binding but maybe with other functional properties of the N-lobe.

In our studies, none of the aa substitutions in carp Tf were located close to iron binding sites, neither in the N-lobe nor in the C-lobe. This suggests that in common carp the observed aa substitutions and related polymorphism would not be connected with differences in iron binding capacity. Similarly, in salmonids, analysis of the sites identified under positive selection, also did not show a correlation with iron binding function. Instead, the majority of the residues were located within the area interacting with bacterial Tf-binding proteins (Ford, 2001).

The main role of bacterial Tf-binding proteins is acquiring iron from host Tf. This includes an integral outer membrane receptor/transporter (TbpA) and a lipid anchored co-receptor (TbpB) on the cell surface. Both C- and N- lobes of human Tf are involved in the interaction with bacterial Tf-binding protein. Interaction between hTf and TbpB is quite extensive; at least six peptide regions in each lobe of hTf could play a role in the interaction with TbpB (Retzer et al., 1999; Boulton et al., 1999). In carp, Tf analysis of non-synonymous changes showed that six, out of a total of 13 non-synonymous changes were located in putative TbpB-interacting regions (**chapter 4**). Further conclusions with respect to pathogen-driven diversity of carp Tf, however, should preferably be based on functional studies. Transferrin uptake by parasitic protozoa includes binding to a heterodimeric Tf-binding protein (**chapter 1**). It could be interesting to use the animal infection model of *T. borreli*, which was shown to require iron for growth (**chapter 3**) and presumably acquires iron via a Tf-binding receptor, for further studies on the ability of this parasite to bind different alleles of carp Tf to a different extent.

A)



B)

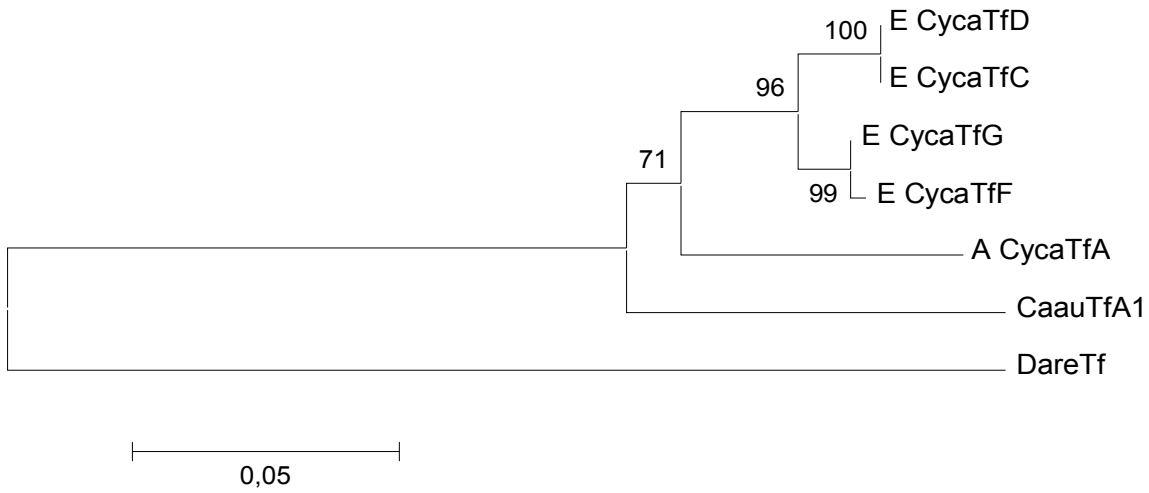


Fig. 7.1. Neighbor-joining tree of N-lobes (**A**) and C-lobes (**B**) of European common carp *Cyprinus carpio* (E CycaTf) alleles C, D, F and G, East-Asian *Cyprinus carpio* (A CycaTf) variant A, *Carassius auratus* (CaauTf) variant A1 and *Danio rerio* (DareTf) Tf protein sequences. Numbers at branch nodes represent bootstrap confidence levels of 1000 bootstrap replications.

3. The role of Tf in the immune response and influence on host-pathogens interactions

Investigations over the last few decades discovered novel roles for Tf, mainly connected to the immune response to infectious agents. Tf now is classified as an acute-phase protein and upon stress or infection its concentration can either rise or fall, depending on the animal species (Powanda and Moyer, 1981). For example, the concentration of

lactoferrin can increase in response to infection and deprive pathogens of iron (Kontoghiorges and Weinberg,
1995). Bovine lactoferrin has been shown to enhance the respiratory burst of rainbow trout macrophages *in vitro* (Sakai et al., 1995) and to increase resistance to bacterial infection after oral administration (Sakai et al., 1993). In our studies an important reason for using infection with *Trypanoplasma borreli* as an animal infection model was the well-known dependence for growth of kinetoplastid parasites on Tf availability (Schell et al., 1991; Wilson and Britigan, 1998; Steverding, 2000). This served an excellent tool to investigate the role of Tf in the host-parasite interactions.

The aim of our first experiments performed *in vivo* was to study the relation between Tf polymorphism and carp resistance to infection with *T. borreli* (**chapter 2**). Although experimental infections with *T. borreli* had been shown to result in very high infection rates that can range between 0 – 100 %, depending on the carp strain used (Jones et al., 1993; Wiegertjes et al., 1995), a thorough investigation of genetic variability that could influence carp susceptibility to this parasite, had never been performed. Our results indicated that Tf genotype may influence the susceptibility to (parasitic) pathogens, which is consistent with previous findings on associations between Tf polymorphism and resistance to bacterial disease in salmonids (Suzumoto et al., 1977; Winter et al., 1980). We observed a significant association of the DD genotype of Tf with low parasitaemia in two resistant carp lines (Polish 'R2' and 'K'), but a reverse association in the most susceptible line 'D' (**chapter 2**).

We also showed that higher/lower resistance to *T. borreli* could be controlled by sex related genetic factors (**chapter 2**). Specifically, an effect of the parental male was shown to be significant in reciprocal crosses. Similar studies were performed in mice where sex-dependent resistance to *Trypanosoma rhodesiense* infection was examined. Female mice occurred to be more resistant as compared to male mice. But this effect was not inherited by males in F1 reciprocal crosses (Greenblatt and Rosenstreich, 1984).

Our data confirmed that particular Tf alleles can play an important role in carp susceptibility to infection with *T. borreli*. We then proceeded to investigate the influence of different Tf genotypes on parasite growth and multiplication. We continued with *in vitro* tests using parasite cultures and culture media supplemented with serum of different Tf genotype origin. Parasites were dying more quickly in culture medium

supplemented with DD (Tf-typed) serum, but we did not see a difference in parasite decline between culture media supplemented with serum from DG or GG-typed fish (**chapter 2**). Because all carp Tf alleles are expected to bind the same amount of iron (**chapter 4**), the differences in parasite multiplication could not easily be explained by an excess or a lack of Fe ions delivered to *T. borreli*.

We then performed a depletion of Tf from carp serum which allowed us to further examine to what extent Tf-bound iron, and the difference between the D and G allele of carp Tf, would be an important factor for parasite growth (**chapter 3**). It had not been proven that *T. borreli* indeed utilizes host Tf to obtain iron, although these studies had been performed for the bloodstream form of *T. brucei* (Schell et al., 1991). The *T. borreli* parasites were dying most quickly in culture media that were supplemented with either DD-typed serum or with purified D-typed Tf (**chapter 3**). The faster decrease of parasite number in the presence of D-type Tf *in vitro* seems in accordance with our *in vivo* findings (**chapter 2**), where we showed a lower susceptibility of DD-typed fish to parasite infection. Our data support a biological significance for the observed polymorphism of carp Tfs.

We could not amplify the sequence of any Tf-binding receptor in *T. borreli* similar to ESAG (**chapter 3**), possibly because the sequence identity between TfR of trypanosomes and putative TfRs of trypanoplasms would be too low to design primers for PCR. However, it is not unlikely that *T. borreli* does sequester iron via specific receptors for Tf. If true, Tf might end up in the lysosomal compartment of the parasite where it would encounter a cysteine proteinase able to degrade the Tf protein into smaller cleavage products (**chapter 6**). Interestingly, digestion of Tf by activated recombinant cysteine proteinase from *T. borreli* results in different digestions profiles for the D and G alleles (Fig. 7.2). Digestion of Tf by the cysteine protease resulted in a number of peptide fragments among which a 45 kDa band, characteristic for D allele. The latter 45 kDa protein band could also be seen in macrophage cultures activated with Tf-typed serum (DD) and co-stimulated with purified Tf (**chapter 5**).

Probably, similar to other kinetoplastids, *T. borreli* is able to take up host proteins in the flagellar pocket and actively digest the ingested proteins such as Tf with help of the cysteine proteinase in the parasite lysosomal compartment. The iron would remain cell-associated and the Tf fragments would be released back to the bloodstream and may act as danger signals “warning” the immune system for the presence of pathogens. Most

likely, the parasite would use these signals to modulate the immune response to its own advantage.

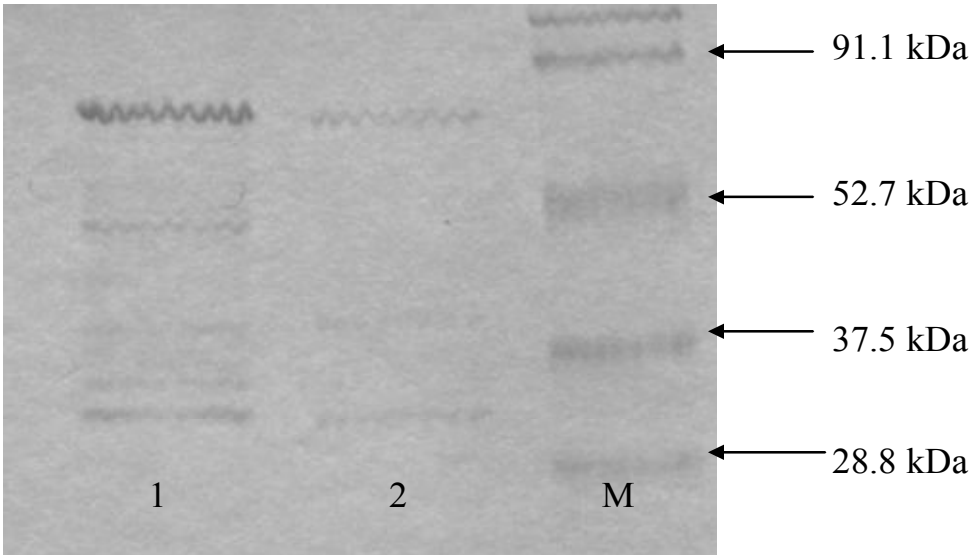


Fig. 7.2. Transferrin was incubated with activated *T. borreli* recombinant cysteine proteinase in 0.1M acetate buffer, pH 5.0, for 15 h at 37 1°C, run on 12.5% SDS-PAGE gel and stained with Coomassie brilliant blue to detect digestion products. Lane 1 represents digestion of purified transferrin D allele, line 2 represents digestion of purified Tf G allele. Molecular weight is indicated on the right in kDa.

Transferrin cleavage products can act as alarmins and induce nitric oxide (NO) production in fish macrophages (Stafford and Belosevic, 2003). Transferrin cleavage products can be generated by activated macrophages themselves (**chapter 5**) but maybe also by *T. borrreli* upon cleavage of Tf by the parasite's cysteine proteinase (Fig. 7.3). Nitric oxide is an important messenger molecule involved in many physiological and pathological processes, which are both beneficial and detrimental (Hou et al., 1999). Nitric oxide is a key element in host defense against invasive pathogens being toxic to bacteria and parasites, however many pathogens have evolved mechanisms for NO resistance (Janeway, 2005). Infections of carp with *T. borreli* are characterized by high levels of NO (Saeij et al., 2002). Interestingly, the parasite seems to use the high NO levels to their own advantage. NO was shown to have cytostatic (reduced motility) but not cytotoxic (unchanged viability) effects on the parasites (Forlenza et al., 2008). The fact that *T. borreli* is not nitrated suggests that the NO-inducing ability of this parasite may be an adaptation strategy by *T. borreli* adopted to survive and evade the immune response of the host (Forlenza et al., 2008). Possibly *T. borreli* can bind G-type Tf more

easily, as suggested by the *in vitro* multiplication studies, and maybe *T. borreli* induces more or different alarmins from D-type Tf, as discussed in **chapter 5,** inducing more NO. Future research should concentrate on experiments to find out if, indeed, *T. borreli* can use Tf cleavage products to modulate the host immune response to its advantage. It would be interesting to study the production of NO induced during parasite infection of DD-typed fish as compared to GG-typed individuals.

Immunostimulatory fragments from carp Tf seemed different for D- and G-type Tf alleles, as identified by Western blotting of supernatants of stimulated carp macrophages (**chapter 5**). Transferrin cleavage products were able to induce a potent NO response. The level of nitrite was significantly higher for Tf cleavage products from the D-type Tf allele (**chapter 5**). Full-length Tf could not induce NO in fish macrophages (Stafford and Belosevic, 2003; **chapter 5**). The enzyme responsible for the cleavage of (goldfish) Tf into immunostimulatory fragments capable of inducing NO in macrophages is elastase (Stafford et al., 2003). Elastase digests Tf in many fragments among which is a 31 amino acid peptide located in the N2 subdomain of Tf, named TMAP (transferrin macrophage-activating peptide) (Haddad and Belosevic, 2008). Upon comparison of the sequences of the carp Tf alleles we could detect a single amino acid difference between the Tf alleles D and G within the TMAP area. Western blot analysis showed the presence of a peptide band of ~ 15 kDa similar to TMAP only in supernatants of carp macrophages that were stimulated with the D allele of the Tf (**chapter 5**). Probably, this peptide could be responsible for high NO induction in carp macrophages. Synthesis of the TMAP areas of carp Tf alelles D and G could maybe demonstrate differences in the ability to induce NO in macrophages.

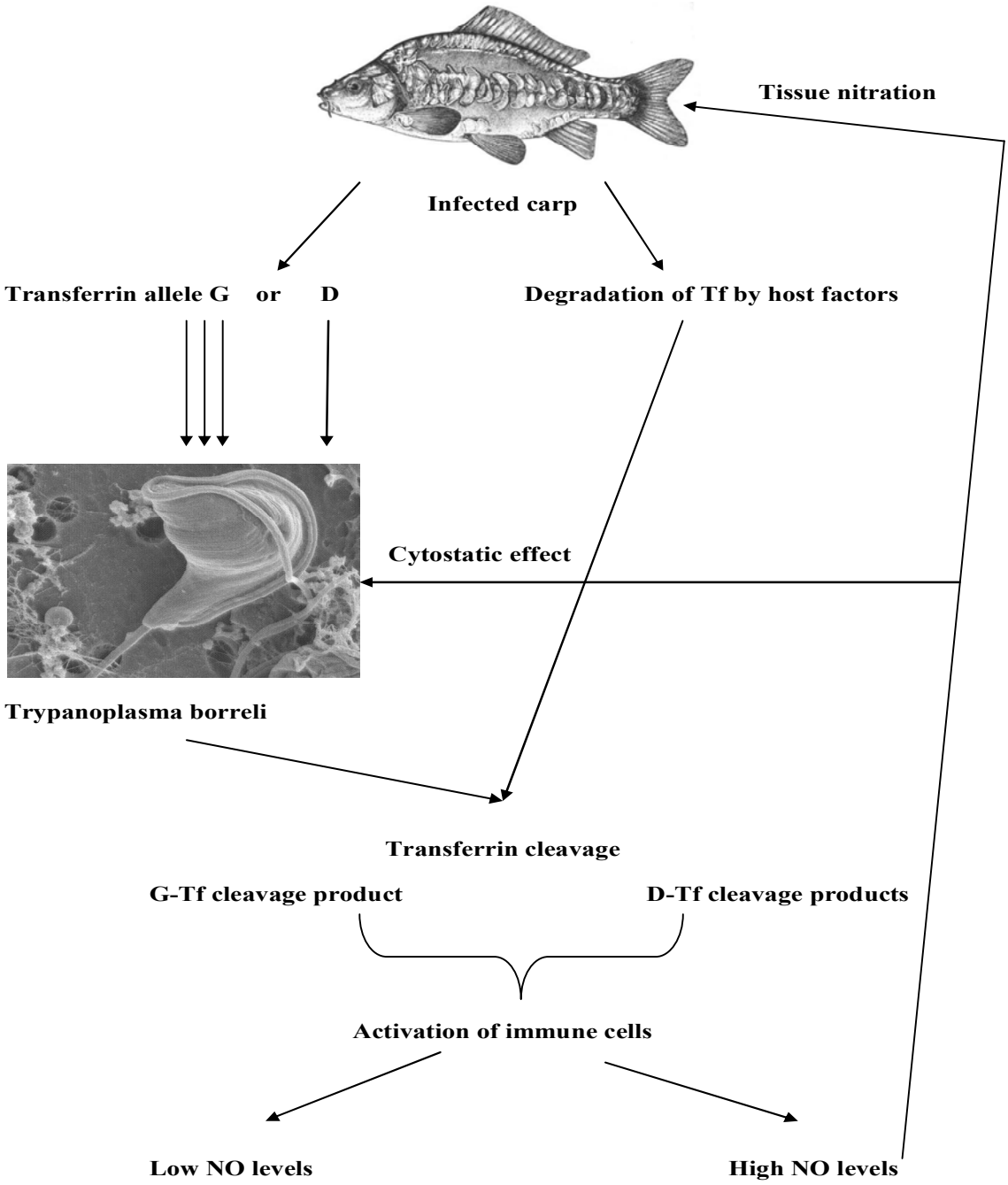


Fig. 7.3. Theoretical pathway of processes that can occur during infection of carp with *T. borreli*. Parasites bind host transferrin to obtain iron but the process is more prominent for the Tf G allele (three arrows) than for the Tf D allele (single arrow). After degrading Tf, the cleavage products are released back into the bloodstream. At the same time, possibly, as a reaction of the host to infection, transferrin is degraded by host enzymes (e.g. elastases released by macrophages and neutrophils). Tf cleavage forms, different for Tf D and G alleles and derived from both processes, activate immune cells being 'alarmins'. Only fragments from Tf D alleles are able to induce high NO levels in the bloodstream which cause host tissue nitration. There may be more Tf G allele-derived fragments, which cannot induce high levels of NO.

4. Transferrin polymorphism and correlation with disease resistance

Transferrins are polymorphic in most vertebrate species examined. The reasons for maintaining Tf genetic polymorphism are still unknown and many studies have been dedicated to solve this question. There are a number of publications that present an association between Tf polymorphism and differences in disease resistance. For example, in humans, Alzheimer's disease is associated with particular Tf alleles (Namekata et al., 1997; Zhang et al., 2003) but there is lack of a similar association with schizophrenia (Maeno et al., 2007). In Parkinson's disease, genetic variations in the control of iron metabolism may be involved in the pathogenesis by contributing to disturbances of iron metabolism in the brain (Borie et al., 2002). Interestingly, one particular Tf genotype in humans showed a significant correlation with resistance to malarial infection (Thakur and Verma, 1993). Finally, already mentioned in this thesis, associations between Tf polymorphism and resistance to bacterial disease in salmonids (Suzumoto et al., 1977; Winter et al., 1980) and resistance to parasite infection of carp (Jurecka et al., 2008c) have been reported.

Another group of publications report on the influence of Tf polymorphism on biological functions of the organisms, among which growth. It has been shown that iron metabolism in humans, who express over 30 different genetic variants of Tf, is not affected by the Tf protein polymorphism (Lee et al., 1999). This is a very important observation, confirming our observations that evolutionary pressure maintaining Tf variability does not seem to be connected with the ability to bind iron. In domestic fowl, a strong correlation between certain Tf alleles and body weight was shown (Rashid, 1982). Also in carp, fry with certain heterozygous genotypes exhibited a greater growth rate than homozygotes (Walawski, 1987).

Some publications report on the influence of Tf polymorphism on reproduction. In pigeons, having two Tf alleles, heterozygous females hatch a larger percentage of their eggs than homozygous females. Probably ovotransferrins from heterozygous females inhibit microbial growth better than ovotransferrins from either of the homozygous types, or those from a mixture of homozygous types (Frelinger, 1972). In mammalian reproduction, Tf plays an important role transporting iron to germ cells inside the blood-testis barrier (Sylvester and Griswold, 1994). The level of Tf in plasma of mammals is an important marker for correct function of Sertoli cells (Barthelemy et al., 1988). Transferrin was found as a main component of carp seminal plasma, suggesting that it

may play an antibacterial role, limiting the access of bacteria to iron (Wojtczak et al., 2005). When we analyzed sperm motility we could show significant differences between DG and DD Tf genotypes. Spermatozoa of DD males were characterized by higher VCL (curvilinear velocity) and ALH (lateral head displacement) but lower STR (straightness of movement) and LIN (linearity) values as compared to DG males (Wojtczak et al., 2007). In other words, spermatozoa from DD males swam faster than those of DG males, but the trajectory of their movement was more curved.

Differences in sperm quality could be a reason for maintaining Tf genetic polymorphism, and studies to examine if differences in sperm motility influence fertilizing ability are needed. We frequently observe an under-representation of GG genotypes in carp populations reared in an open environment (see also **chapter 2, 4**). This could be indirect evidence for an effect of Tf genotype on the breeding success of common carp.

5. Conclusions

Competition for iron between carp and pathogens could be a very important factor influencing Tf polymorphism. We proved that *T. borreli* cannot maintain a high level of multiplication in an environment depleted of Tf, suggesting a requirement for iron for growth. We were not yet able to detect the DNA sequence for the parasite Tf receptor, and further studies are required to detect this receptor, if present, and compare its organization to TfRs of other Kinetoplastid parasites such as *T. brucei*. Sequence analysis of four carp Tf alleles did not show differences between the alleles with respect to iron- or receptor- binding sites. This suggests that evolutionary pressure maintaining Tf variability may not be connected with the ability of Tf to bind iron. Surprisingly, it is highly probable that Tf of the *Cypriniformes*, including all carp Tf alleles in this study, could be partly non-functional with respect to Fe binding capacity of the N-lobe.

Both our *in vitro* and *in vivo* experiments showed that the same Tf allele was responsible for a faster decline in parasite numbers in culture and enhanced resistance of carp to *T. borreli*. It remains to be solved whether these differences are due to different effectiveness of Tf alleles or whether there are other factors involved, acting at the level of degraded Tf proteins. Probably the host – parasite interaction is influenced by several factors, which all together modulate the immune response of fish during infection. Kinetoplastid parasites take up host iron and can digest Tf with, for example, cysteine

proteinases. Transferrin fragments released by *T. borreli* back into the host bloodstream of carp could act as danger signals, modulating the immune system of the host. This may be similar to the process where “alarmins” are derived from cleavage of Tf by host factors, e. g. elastases secreted by macrophages and neutrophils. Both, pathogen- and host-derived cleavage forms of Tf would induce nitric oxide in macrophages. The level of NO induced in macrophages was shown to be dependent on Tf genotype. The same Tf allele D, responsible for carp resistance to *T. borreli* and a higher decline of parasite number *in vitro*, induces a high level of NO in carp macrophages.

In summary, we have provided evidence for Tf polymorphism in common carp to be related to disease resistance, implying that Tf could be a useful genetic marker for marker-assisted selection programmes for increased resistance to diseases. Before Tf can be applied as a genetic marker in breeding programmes, further investigations are required to compare our laboratory-based results to survival of carp with different Tf genotypes under semi-intensive pond conditions in aquaculture.

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Summary

Summary

Iron is fundamental to the biology of eukaryotic cells since it plays a key role in many metabolic functions. Iron concentrations are tightly regulated, for example by ferritin, because excessive iron leads to tissue damage. Iron cannot cross cellular membranes directly and most cells acquire iron from the iron transporting protein transferrin (Tf), via transferrin receptors. During nutritional immunity the body reacts with a metabolic adjustment in order to render important nutrients unavailable to invading microorganisms. However, pathogens also have evolved a range of mechanisms to acquire iron from the host (**chapter 1**).

In the study described in this thesis, we used a natural host-parasite model of common carp (*Cyprinus carpio* L.) infected with *Trypanoplasma borreli*, a protozoan kinetoplastid, extracellular blood parasite of carp to get more insight in the competition for iron between host and parasite. Transferrin of common carp is highly polymorphic with several alleles identified according to differences in electrophoretic mobility. We studied the implications of Tf polymorphisms for iron binding and modulation of immune function.

We performed a series of challenge experiments infecting five genetically different, commercially exploited carp lines with *T. borreli*. Our results indicated that Tf genotype may influence the susceptibility to pathogens. We observed a significant association of the DD genotype of Tf with low parasitaemia in two resistant carp lines (Polish 'R2' and 'K'), but a reverse association in the most susceptible carp line 'D' (**chapter 2**). We also showed that variation in resistance to *T. borreli* could be controlled by sex-related genetic factors. Examination of parasite growth *in vitro*, in culture media supplemented with 3% serum taken from fish with different Tf genotypes, showed a faster decrease in number of parasites in media supplemented with serum from DD-typed animals (**chapter 2**).

In general, pathogens also have mechanisms to acquire iron from the host. We developed a method for Tf depletion of carp serum using specific antibodies to carp Tf, and compared *T. borreli* multiplication and survival in the presence or absence of Tf *in vitro*. Parasites were dying in medium containing Tf-depleted serum, which clearly showed that Tf is essential for parasite growth and multiplication (**chapter 3**). We isolated two allelic forms of carp Tf (alleles D and G) to purity using rivanol precipitation and ion-exchange chromatography (**chapter 5**). We showed that parasite growth *in vitro* could be reconstituted by the addition of purified Tf to Tf-depleted

serum (**chapter 3**). We observed differences in *T. borreli* multiplication and survival in culture media containing different sera typed differently for Tf genotypes (**chapter 3**).

We identified four complete coding sequences for common carp Tf alleles C, D, F and G, and confirmed the overall similarity of the carp Tf three-dimensional structure to Tfs of other species. We could show that carp Tf differs significantly in critical iron-binding sites in the N-lobe of the molecule, as compared to other non-cyprinid fish species (**chapter 4**). The substitution of a majority of the iron-coordinating residues in the N-lobe indeed seems to affect the ability to bind iron, which may be compensated for by higher serum concentrations of Tf (**chapter 7**). Comparison of constitutive gene expression of two Tf alleles D and G showed a comparably high gene expression level in liver and small but consistent differences in gene expression for allele D over allele G in other immunologically important organs (**chapter 4**). Our data suggest that the allelic polymorphism is not related to differences in iron binding and/or binding to the host Tf receptor but could be linked with other factors, such as competition for iron with pathogens (**chapter 4**).

Transferrin itself may also exert effects that are not directly linked with maintaining iron levels and Tf cleavage products have been shown to stimulate macrophages to produce large amounts of nitric oxide (NO). To study the induction of NO in carp head kidney-derived macrophages, we isolated two allelic forms of carp Tf (alleles D and G) to purity and showed that the level of activation of macrophages by Tf was different for the D and G allele (**chapter 5**). Differences in NO levels induced could be related to different cleavage forms of the two alleles D and G, as shown by Western blot, confirming that full-length Tf cannot induce NO. The D-type Tf cleavage products induced significantly higher nitric oxide (NO) production than cleavage products of G-type Tf. (**chapter 5**).

Transferrin uptake by trypanosome parasites involves Tf binding to a receptor. The TfR-Tf complex then is internalised and transported to lysosomes, where Tf is proteolytically degraded. We described the cloning and sequencing of a cathepsin L-like cysteine proteinase from *T. borreli* and production of a recombinant and biologically active enzyme (**chapter 6**). We demonstrated that the *T. borreli* cysteine proteinase is able to digest host transferrin. Likely, Tf cleavage fragments are released from the trypanosomes while iron would remain parasite-associated, possibly contributing to the

pathogenicity of the parasite by inducing high amounts of NO in carp macrophages (**chapter 7**).

Our study dealt with different aspects of Tf polymorphism, discussing the role of Tf in immunity of common carp and the influence of allelic polymorphism on competition for iron between host and pathogen (**chapter 7**). Further investigations should shed more light on the selective advantage of particular alleles to provide a basis for incorporating Tf as a genetic marker in marker-assisted selection programmes for increased resistance to diseases. This could contribute to improved survival of carp kept under semi-intensive farming systems in ponds.

Streszczenie

Streszczenie

Żelazo pełni podstawową rolę w wielu funkcjach metabolicznych komórek eukariotycznych. Ponieważ nadmiar żelaza jest toksyczny dla komórek, jego stężenie w organizmie musi być ściśle regulowane. Żelazo transportowane jest we krwi poprzez wiązanie do transferyny (Tf), a kompleks taki pobierany jest przez komórki za pośrednictwem receptorów transferynowych (TfR).

Jednym z ważnych procesów angażujących metabolizm żelaza jest odpowiedź na inwazję patogenów. Z jednej strony w organizmie dochodzi do zmian metabolicznych mających na celu ograniczenie dostępności dla patogenów ważnych składników odżywczych, np. żelaza. Z drugiej strony patogeny wykształciły odpowiednie mechanizmy ułatwiające im pobór żelaza w organizmie żywiciela (**rozdział 1**).

W celu zbadania mechanizmów konkurencji o żelazo pomiędzy patogenem a żywicielem, wykorzystano model chorobowy z zastosowaniem naturalnego, żyjącego we krwi i płynach ustrojowych karpia (*Cyprinus carpio* L.), pasożyta, *Trypanoplasma borreli.*

U karpia transferyna jest wysoce polimorficznym białkiem, którego poszczególne allele można zidentyfikować podczas natywnej elektroforezy w żelu poliakrylamidowym. Obecne badania miały na celu skorelowanie polimorfizmu Tf z funkcją wiązania żelaza oraz jej wpływu na przebieg odpowiedzi immunologicznej.

W badaniach *in vivo*, pięć genetycznie odległych linii hodowlanych karpia zakażono *T. borreli*. Otrzymane rezultaty wskazują, że genotypy Tf mogą wpływać na odporność. Zaobserwowano statystycznie istotny związek genotypu DD transferryny z niskim zapasożyceniem w dwóch odpornych liniach karpia (Polskich ‘R2’ i ‘K’) oraz odwrotną zależność w najwrażliwszej linii ‘D’ (**rozdział 2**). Ponadto stwierdzono, że różnice w odporności karpia na *T. borreli* mogą być powiązane z płcią.

Hodowla pasożytów *in vitro* w pożywkach hodowlanych z dodatkiem surowicy pochodzącej z ryb o różnych genotypach Tf wykazała szybszy spadek liczby *T. borreli* w pożywkach z dodatkiem surowicy pochodzącego od ryb z genotypem transferryny DD (**rozdział 2**).

Patogeny posiadają odpowiednie mechanizmy pozyskiwania żelaza od żywiciela. Przy użyciu specyficznych przeciwciał przeciwko Tf karpia, otrzymano surowicę pozbawioną transferyny, którą następnie wykorzystano w badaniach *in vitro* namnażania się i przeżywalności *T. borreli*. Pasożyty żyły krócej w pożywce hodowlanej pozbawionej Tf, co potwierdziło, że transferyna jest kluczowym elementem

procesu namnażania się pasożytów (**rozdział 3**). Ponadto, przy zastosowaniu chromatografii jonowymiennej, wyizolowano i oczyszczono dwie alleliczne formy transferyny karpia (D i G) (**rozdział 5**) i wykazano, że *in vitro* tempo namnażania pasożytów może być przywrócone poprzez dodatek czystej Tf do surowicy wcześniej jej pozbawionej (**rozdział 3**). Obserwowano także istotne różnice w namnażaniu się i przeżywalności *T. borreli* w pożywkach z dodatkiem surowic pochodzących od ryb o różnych genotypach Tf (**rozdział 3**).

Zidentyfikowane zostały cztery kompletne sekwencje cDNA, kodujące transferyny karpia (allele C, D, F i G). Przy zastosowaniu techniki modelowania białek potwierdzono, że trójwymiarowa (trzeciorzędowa) struktura Tf karpia jest identyczna ze strukturami Tf innych gatunków. Pokazano także, że transferyna karpia znacząco różni się w krytycznych miejscach wiązania żelaza w podjednostce N w porównaniu do Tf innych ryb nie należących do rodziny karpiowatych (**rozdział 4**). Zamiana większości aminokwasów odpowiedzialnych za wiązanie żelaza w podjednostce N prawdopodobnie wpływa na mniejszą zdolność wiązania Fe, co prawdopodobnie jest równoważone przez wyższe stężenie Tf we krwi karpi w porównaniu do innych gatunków zwierząt (**rozdział 7**). Porównanie ekspresji genów Tf allela D i G pokazało wysoki poziom ekspresji tych genów w wątrobie i niski poziom ekspresji w innych organach powiązanych z układem odpornościowym (**rozdział 4**). Nasze rezultaty sugerują, że polimorfizm transferyny karpia nie jest determinowany zdolnością wiązania Fe do receptorów gospodarza, ale może zależeć od innych czynników, np. współzawodnictwem z patogenami o żelazo (**rozdział 4**).

Rola transferyny nie ogranicza się do funkcji wiązania i transportu żelaza we krwi. Pokazano, że produkty rozpadu Tf stymulują makrofagi do produkcji tlenku azotu (NO). Wykazano między innymi, że wyizolowana i oczyszczona transferryna karpia stymuluje makrofagi pochodzące z nerki głowowej karpia do produkcji NO, a poziom tlenku azotu jest zależny od typu transferyny jakiej użyto (**rozdział 5**). Różnice w poziomie NO najprawdopodobniej wynikają z faktu powstawania różnych produktów rozpadu dwóch alleli Tf D i G, bowiem natywna transferyna nie wpływa na wzrost poziomu NO. Z kolei produkty rozpadu transferyny typu D indukowały istotnie wyższy poziom tlenku azotu w porównaniu do produktów rozpadu transferyny typu G (**rozdział 5**).

Streszczenie

Pobór transferyny przez pasożyty z rodzaju Trypanosoma następuje poprzez wiązanie Tf żywiciela do receptorów pasożytów. Kompleks TfR-Tf transportowany jest do lizosomów, gdzie transferyna jest proteolitycznie degradowana, a żelazo zostaje wchłonięte. Ważnym dla przebiegu tego procesu enzymem jest proteinaza cysteinowa podobna do L-katepsyny. W obecnej pracy sklonowano i zsekwencjonowano L-katepsynę z genomu *T. borreli* oraz otrzymano rekombinowany biologicznie aktywny enzym (**rozdział 6**). Pokazano, że proteinaza cysteinowa *Trypanoplasmy borreli* może trawić transferynę karpia. Prawdopodobnie produkty rozpadu Tf są usuwane z lizosomów do krwioobiegu żywiciela i przyczyniają się do indukcji NO w makrofagach (**rozdział 7**).

W prezentowanej pracy rozważaliśmy różne aspekty polimorfizmu transferyny oraz przedyskutowaliśmy jej rolę w odporności karpia i wpływ na współzawodnictwo o żelazo pomiędzy gospodarzem i patogenem (**rozdział 7**). Dalsze badania, związane z zastosowaniem transferyny jako markera genetycznego w selekcji ryb, dostarczyć mogą cennych informacji związanych ze zwiększoną odpornością na choroby, co wpłynie na zmniejszenie strat hodowców karpi.

Samenvatting

Samenvatting

IJzer is fundamenteel voor eukaryotische cellen en speelt daarom een belangrijke rol in vele metabolische functies. Omdat een teveel aan ijzer kan lijden tot weefselschade worden ijzerconcentraties strak gereguleerd, bijvoorbeeld door ferritine. IJzer kan niet direct de celmembraan passeren en daarom verkrijgen de meeste cellen ijzer van het transporteiwit transferrine (Tf), via de transferrine receptoren. Het lichaam kan, via een metabolische aanpassing, er voor zorgen dat belangrijke nutriënten zoals ijzer niet beschikbaar zijn voor invasieve micro-organismen (nutrionele immuniteit). Pathogenen, op hun beurt, hebben een aantal mechanismen ontwikkeld om ijzer van de gastheer te verkrijgen **(hoofdstuk 1)**.

In het, in dit proefschrift beschreven onderzoek, hebben we gebruik gemaakt van een in de natuur voorkomend gastheer-parasiet model. Karpers (*Cyprinus carpio* L.) werden geïnfecteerd met *Trypanoplama borreli*, een eencellige extracellulaire kinetoplastide bloedparasiet, om meer inzicht te krijgen in de competitie om ijzer tussen gastheer en parasiet. Karper transferrine is sterk polymorf, dat wil zeggen, er zijn verschillende allelen geïdentificeerd op basis van verschillen in electroforetische mobiliteit. We bestudeerden de implicaties van Tf polymorfisme voor ijzer binding en modulatie van het immuunsysteem.

We voerden een serie infectie experimenten met *T. borreli* uit op vijf genetisch verschillende commercieel gebruikte karperstammen. Onze resultaten lieten zien dat het Tf genotype mede de vatbaarheid voor dit pathogeen beïnvloedt. We zagen een significante associatie van het Tf DD genotype met lagere aantallen parasieten in twee resistente Poolse karperstammen (‘R2’en ‘K’), maar een tegengestelde associatie in de meest vatbare karperstam ‘D’ **(hoofdstuk 2)**. We toonden ook aan dat de variatie in resistentie tegen *T. borreli* onder invloed staat van sexe-gerelateerde genetische factoren. Onderzoek naar de groei van deze parasiet *in vitro,* in kweekmedium verrijkt met 3% serum van vissen met een verschillend Tf genotype, toonde een relatief snelle afname aan van de groei van parasieten in medium verrijkt met serum van DD-getypeerde karpers **(hoofdstuk 2)**.

Veel pathogenen hebben mechanismen om ijzer te verkrijgen van hun gastheer. We ontwikkelden een methode voor het verwijderen van Tf uit karperserum, gebruik makende van specifieke antilichamen tegen karper Tf, en vergeleken de groei van *T. borreli* in de aan- of afwezigheid van Tf *in vitro*. De parasiet kon niet groeien in medium zonder Tf, hetgeen duidelijk liet zien dat Tf essentieel is voor de groei en

vermenigvuldiging van deze parasiet **(hoofdstuk 3)**. Vervolgens isoleerden we twee allelische vormen van karper Tf (allelen D en G) via precipitatie met rivanol en opzuivering via ion-uitwisselings chromatografie **(hoofdstuk 5)**. Hiermee toonden we aan dat de parasietgroei *in vitro* hersteld kon worden door toevoeging van gezuiverd Tf aan kweekmedium zonder Tf **(hoofdstuk 3)**. We zagen verschillen in de vermeerdering en overleving van *T. borreli* in kweekmedia met verschillende Tf genotypes **(hoofdstuk 3)**.

We hebben vier coderende gensequenties gevonden voor karper Tf allelen, namelijk C, D, F en G en konden een duidelijke overeenkomst aantonen tussen de drie-dimensionale structuur van karper Tf en Tf's van andere diersoorten. Karper Tf verschilde echter significant in een aantal kritische bindplaatsen voor ijzer in de N-lob van het molecuul, vergeleken met Tf van andere, niet cyprinide, vissoorten **(hoofdstuk 4)**. Dit verschil in ijzer-coördinerende residuen in de N-lob liet inderdaad een verschil zien in de mogelijkheid om ijzer te binden, hetgeen echter gecompenseerd lijkt te kunnen worden door hogere serum concentraties van Tf **(hoofdstuk 7)**. Vergelijking van de basale waarden aan genexpressie tussen de twee allelen D en G liet een vergelijkbaar sterke genexpressie zien in de lever, maar ook een minder sterk maar wel consistent verhoogd verschil in genexpressie voor allel D ten opzichte van allel G in enkele andere immunologisch belangrijke organen **(hoofdstuk 4)**. Het lijkt erop dat het allelisch polymorfisme niet gerelateerd is aan het verschil in ijzerbinding en/of binding met de gastheer Tf receptor, maar heeft dit te maken met andere factoren zoals de competitie om ijzer met pathogenen (**hoofdstuk 4)**.

Transferrine zelf kan ook effecten hebben die niet direct gekoppeld kunnen worden aan het reguleren van ijzer niveaus. Tf afbraakproducten kunnen makrofagen stimuleren tot een hoge productie van stikstofoxide (NO). Om de inductie van NO in karper makrofagen van de kopnieren te bestuderen hebben we twee allelische vormen van karper Tf (allelen D en G) opgezuiverd. Hiermee konden we aantonen dat het activatieniveau van makrofagen verschillend was voor de allelen D en G **(hoofdstuk 5)**. Verschillen in NO productie konden gerelateerd worden aan verschillende afbraakproducten van de twee allelen D en G. Western blots lieten vervolgens zien dat compleet Tf geen NO kan induceren. Afbraakproducten van het D-type Tf induceren de stikstofoxide productie tot significant hogere waarden dan de afbraakproducten van G-type Tf **(hoofdstuk 5)**.

Voor de opname van Tf door typanosoom-achtige parasieten is binding met een receptor nodig. Het TfR-Tf complex wordt dan binnen de cel gebracht en getransporteerd naar de lysosomen waar Tf proteolitisch wordt afgebroken. We hebben een cathepsine L-achtig cysteine proteinase van *T. borreli* gekloneerd en de gensequentie beschreven, alsmede de productie van een biologisch actieve recombinante vorm van dit enzym **(hoofdstuk 6)**.

We konden aantonen dat *T. borreli* cysteine proteinase gastheer Tf kan afbreken. Het is goed mogelijk dat Tf afbraakproducten worden vrijgegeven door de parasieten terwijl het ijzer parasiet- geassocieerd blijft. De afbraakproducten kunnen mogelijk bijdragen aan de pathogeniciteit van de parasiet door het induceren van verhoogde NO niveaus in karper makrofagen **(hoofdstuk 7)**.

Samenvattend, hebben we onderzoek gedaan naar verschillende aspecten van Tf polymorfisme. We hebben gezocht naar een verklarende rol van Tf in de immuniteit van karper en de invloed van allelisch polymorfisme op de competitie om ijzer tussen gastheer en pathogeen **(hoofdstuk 7)**. Vervolgonderzoek moet meer inzicht geven in het selectieve voordeel van bepaalde allelen, met als doel het gebruiken van Tf polymorfisme als genetische merker in merker-gebaseerde selectie programma's voor verhoogde ziekteresistentie. Dit zou kunnen bijdragen tot een verhoogde overlevingskans van karpers gehouden in semi-intensieve visteelt systemen.

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Curriculum vitae

Patrycja Magdalena Jurecka (née Matysek) was born on 17 September 1974 in Warszawa, Poland. She grew up in Cieszyn, a small town bordering with the Czech Republic, where she graduated high school. In 1995 Patrycja admitted to Jagiellonian University in Krakow, where she was studying the chemistry, specializing in biological chemistry. In 2000 she obtained a Master degree in chemistry. At the same year she was employed by the Institute of Ichthyobiology and Aquaculture of the Polish Academy of Sciences in Gołysz. She became a member of the research group which worked on genetic aspects of disease resistance of common carp. In 2001 Patrycja joined the Cell Biology and Immunology Group at the Wageningen University as PhD sandwich student. During her time in the Netherlands she attended several PhD courses at Wageningen University. She also took part in several international conferences, workshops and discipline-specific meetings, presenting posters and giving oral presentations as well.

List of publications

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PARITY Mid – term Review Meeting (2004)

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