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

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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 allele 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.

1. 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 [1–4]. 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 [5]. 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 [6]. 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 [7].

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 [8]. 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 [9]. 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

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[10]. Last but not least, particular Tf alleles have been associated with resistance of carp to parasite infection [11].

We cloned and sequenced four different Tf alleles (C, D, F and G) of carp identified by PAGE. The crystal structures of human lactoferrin [12], bovine lactoferrin [13], porcine and rabbit serum Tf [6] and, most recently, human serum apotransferrin [14] 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.

2. Materials and methods

2.1. 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 [15–17]. 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.

2.2. 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 [11]. 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 90 V for 30 min followed by 250 V for 5 h [18]. 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 [19]. 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% [20] for subsequent RNA isolation from liver.

2.3. 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 (R3 × R8 carp [21]). 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™ 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 °C for further use.

2.4. 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 1 and Fig. 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) [22]. Comparisons between the sequences were performed using the BioEdit v. 7.0.4. software [23], with minor manual optimizations. The ExPaSy Proteomics Tools was used to calculate the theoretical isoelectric points of the different Tf alleles.

2.5. 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 R3 × R8, [11]). Constitutive gene expression was studied by real-time quantitative PCR (RT-qPCR) essentially as described previously [24]. RT-qPCR

Table 1
Primer sequences applied to detect the four carp transferrin alleles and for real-time quantitative PCR (RT-qPCR).

	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

+ indicates an LNA modification (underlined).