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Diversification of IFNγ-inducible CXCb chemokines in cyprinid fish

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ABSTRACT

We earlier identified two CXCL8-like lineages in cyprinid fish, which are functional homologues of the mammalian CXCL8, but with diverged functions. We here investigated whether the carp IFN-γ-inducible CXCb gene, related to the mammalian CXCL9, -10 and -11 chemokines, was subject to a similar diversification. On the zebrafish genome, a cluster of seven CXCb genes was found on chromosome five. Analysis of the promoter of the zebrafish CXCb genes suggests a partially shared, but differential induction. A second CXCb gene, CXCb2, was identified in common carp by homology cloning. CXCb2 is constitutively expressed in immune-related tissues, predominantly in head kidney lymphocytes/monocytes. Interestingly, an induction of CXCb2 gene expression with recombinant carp IFN-γ2 and LPS was observed in macrophages and granulocytes. Finally, difference in sensitivity to LPS, and kinetics of CXCb1 and CXCb2 gene expression during zymosan-induced peritonitis, was observed. These results indicate a functional diversification for cyprinid CXCb chemokines, with functional homology to mammalian CXCL9–11.

1. Introduction

Chemokines are low molecular weight proteins and belong to a large protein family that regulates cell trafficking. Based on a cysteine-motif at the N-terminal side of the protein, chemokines are classified into four groups in mammals, CXC, CX3C, CC and C, whereby in zebrafish an additional fifth group, CX, is found (Bacon et al., 2002; Nomiyama et al., 2008). Chemotaxis is induced upon binding of chemokines to chemokine receptors, CXCR, CX3CR, CCR, CR respectively. These are seven-transmembrane G-protein coupled receptors (GPCRs) that upon activation induce intracellular calcium mobilization and subsequent reorganization of the cytoskeleton (Sanchez-Madrid and del Pozo, 1999). A characteristic of chemokines is their promiscuity and redundancy: one ligand may bind multiple receptors and one receptor may share multiple ligands (Zlotnik et al., 2006). This is reflected by their genomic organization: many chemokine and chemokine receptor genes are localized in (mini)clusters on the genome, a result of repeated (tandem) duplication events. Chemokines located in one cluster often share receptors. Next to multiple duplication events, chemokines are subject to high selection pressures towards diversification and have evolved to a family that can direct migration of multiple cell types (Zlotnik et al., 2006; DeVries et al., 2006).

Chemokines play an important role in the induction and regulation of inflammation, as they orchestrate trafficking of specific subsets of leukocytes towards the site of inflammation. For CXC chemokines, in total sixteen genes are described in humans (Zlotnik et al., 2006). CXCL8, or IL-8, directs migration of neutrophils and monocytes (Baggiolini and Clark-Lewis, 1992; Yoshimura et al., 1987; Walz et al., 1987). Another group of CXC chemokines implicated in inflammation is formed by CXCL9, –10 and –11. Human CXCL9–11 genes are localized in a mini-cluster on chromosome four (Bacon et al., 2002; Zlotnik et al., 2006). These three chemokines are also known by their synonyms: monokine-induced by interferon-gamma (IFN-γ) (MIG/CXCL9), IFN-γ-inducible protein 10 (IP-10/CXCL10) and IFN-inducible T-cell alpha chemoattractant (I-TAC/CXCL11) (Luster et al., 1985; Farber, 1990; Cole et al., 1998). As their names indicate, the expression of all three genes is induced by IFN-γ, which is a type II IFN produced by natural killer (NK) and effector T cells. CXCL9–11 share the same receptor, CXCR3, which is expressed on activated T helper 1 CD4 + cells, effector CD8 + T cells, NK, NKT, plasmacytoid dendritic cells (pDCs) and monocytes/macrophages (Groom and Luster, 2011; Penna et al., 2001; Inngjerdingen et al., 2001; Zhou et al., 2010). Spatial and timely differential expression patterns of CXCL9–11 fine-tune

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T-cell trafficking over the course of an immune response and all three chemokines are implicated in acute and chronic inflammation (Groom and Luster, 2011; Helbig et al., 2009; Loetscher et al., 1996).

The chemokine family is estimated to have arisen around 650 million years ago with the emergence of vertebrates and lamprey is the first species in which chemokine genes have been identified (DeVries et al., 2006; Nomiyama et al., 2011; Najakshin et al., 1999). Chemokines are fast evolving genes and few chemokine genes show true orthology between fish and mammals (Huising et al., 2003a). In zebrafish (*Danio rerio*), for which the whole genome is sequenced, a detailed screening enabled identification of the entire chemokine family of ligands and receptors (Nomiyama et al., 2008; DeVries et al., 2006). In common carp (*Cyprinus carpio*), as zebrafish belonging to the cyprinid lineage, but whose whole genome is not yet sequenced, currently six CXC chemokine genes are identified: two CXCL8-like genes (CXCa_L1, CXCL8_L2), one CXCL9–11-like gene (CXCb), two CXCL12 genes (CXCL12, CXCL12b) and one CXCL14 gene (Huising et al., 2004, 2003b; van der Aa et al., 2010, 2012; Abdelkhalek et al., 2009; Savan et al., 2003). The carp CXCL9–11-like gene is the closest homolog of the mammalian CXCL9–11 genes, but in analogy to results earlier shown for CXCa and mammalian CXCL8 (van der Aa et al., 2010, 2012), it could not be validated as a true ortholog of mammalian CXCL9–11 by phylogenetic analysis. We therefore named this gene CXCb (Huising et al., 2003a). We earlier showed that CXCb expression is inducible by recombinant carp IFN-γ2 and that CXCb is expressed during the inflammatory response. Moreover, it was demonstrated that recombinant CXCb induces chemotaxis of carp granulocytes, macrophages and lymphocytes/monocytes (van der Aa et al., 2010, 2012; Arts et al., 2010). In search for a possible diversification of CXCb chemokines in cyprinids, we screened the zebrafish genome for CXCb related genes and found a cluster of CXCb genes on chromosome five. A second CXCb gene was cloned in carp that was named CXCb2. Based on an analysis of the zebrafish CXCb promoter regions and *in vitro* and *in vivo* gene expression studies of carp CXCb1 and CXCb2 genes, we hypothesize that cyprinid CXCb genes have undergone functional diversification and display functional homology with mammalian CXCL9–11.

2. Material and methods

2.1. Bioinformatics, phylogeny and promoter in silico analysis

Carp CXCb1 (Acc nr. AB082985) was used as a query in BLAST search on the zebrafish genome assembly (Zv9, ENSEMBL http://www.ensembl.org). Zebrafish CXCb genes located on chromosome five corresponded with Ensembl gene identifiers ENSDARG00000095747 (CXCL-chr5b), ENSDARG00000092423 (CXCL-chr5g), ENSDARG00000094706 (CXCL-chr5h) and ENSDARG00000093779 (CXCL-chr5i); three genes (CXCL-chr5d/e/f) and two pseudogenes (CXCL-psi1–2) are not yet referenced in Ensembl. The Ensembl gene identifier for the non-CXCb related CXC chemokine CXCL-chr5c is ENSDARG00000075163,while CXCL-chr5a has not yet an Ensembl ID. BLAST searches against EST databases were performed on the website of NCBI (http://www.ncbi.nlm.nih.gov). Multiple sequence alignments were made with ClustalW within the MEGA5 software. Phylogeny trees were constructed with MEGA5 using Neighbor Joining (NJ; Thompson et al., 1994).

The software tool Genomatix MatInspector Matrix library version 8.0 (http://www.genomatix.de) was employed for promoter analysis (Quandt et al., 1995). One kb DNA sequence upstream the predicted start codon was extracted for each studied CXC gene from the zebrafish or human genome assembly (ENSEMBL). DNA sequences were analyzed using the matrixes for general core promoter elements for vertebrates and enhancer elements were used specifically for nuclear factor kappa B/c rel (NF-κB), signal transducer and activators for transcription (STAT), interferon regulatory factors (IRFs) and activating protein-1 (AP-1). Matrix matches with a similarity score >0.80 were accepted and relevant elements were selected.

CXCL accession numbers used for tree construction and promoter analysis were as follows: carp Cyca_CXCa_L1 (CAD13189), Cyca_CXCL8_L2 (AB470924), Cyca_CXCb1 (AB08298), Cyca_CXCb2 (AB08298); CXCL8_L1_Chr1 (XM_001342570), CXCL8_chr17 (EST EH441857); channel catfish CXCL10 (AY335949); rainbow trout Onmy_vig7 (AF483527), Onmy_vig8 (AF483528), Onmy_gIP-10 (AJ417078.); mouse CXCL9 (NM_008599), CXCL10 (NM_021274), CXCL11 (AF179872); human CXCL1 (NM_001511), CXCL2 (NM_002089), CXCL3 (NM_002090), CXCL4 (NM_002619), CXCL5 (NM_002994), CXCL6 (NM_002993), CXCL7 (NM_002704), CXCL8 (P10145), CXCL9 (NM_002416), CXCL10 (NM_001565), CXCL11 (NM_005409), CXCL12 (NM_199168), CXCL13 (NM_006419) and CXCL14 (NM_004887); Zebrafish CXCL8_L1_Chr1 (XM_001342570), CXCL8_chr17 (EST EH441857). Zebrafish CXCb protein sequences were initially obtained from the supplementary data of Nomiyama et al. (2008) derived from the Zv6 assembly, but were checked for congruence with the Zv9 assembly; three of these sequences are also available from GenBank: CXCL-chr5b (NP_001119885.1), CXCL-chr5d (XP_001339307), and CXCL-chr5f (XP_696046).

2.2. Cloning carp CXCb2: 5'RACE-PCR and 3'RACE-PCR

Two oligonucleotide primers, zfccCXC4fw1 and zfccCXC4rv1 (Table 1) were designed based on the zebrafish CXCL-chr5 g sequence. As template for PCR, cDNA from total head kidney leukocytes stimulated with carp rIFN-γ2 Arts et al., 2010 was used. A PCR reaction mixture was prepared with 5 μl of 10x Goldstar buffer (Eurogentec S.A., Seraing, Belgium), 1.5 mM $MgCl_2$, 200 μM dNTPs, 1.25 units of Goldstar DNA Polymerase (Eurogentec, S.A., Seraing, Belgium), 200 nM forward primer, 200 nM reverse primer and filled up to 50 μl with H_2O. PCR, 30 cycles, were carried out under the following conditions: 94 °C for 2 min, 94 °C for 30s and 55 °C for 30s and 72 °C for 1 min, followed by 72 °C for 10 min. A fragment of 152 bp was amplified and cloned into a pGEM-T easy vector (Promega, Leiden, The Netherlands) according the manufacturer's protocol and sequenced using the ABI prismBigDye Terminator Cycle Sequencing Ready Reaction kit and analyzed using 3700 DNA analyzer. Based on this sequence, new primers were designed for 5'RACE-PCR and 3'RACE-PCR, that were carried out with the Gene Racer™ RACE Ready cDNA kit with Superscript III RT (L1502–01, Invitrogen). For the 3'RACE-PCR reaction, RACE-ready cDNA template was prepared from carp kidney RNA according to the manufacturer's protocol. A first round of 3'RACE-PCR was carried out with the gene specific primer fwGSPcarpCXC4. A reaction mixture was prepared containing 5 μl of 10x Goldstar buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 1.25 units Goldstar DNA Polymerase (Eurogentec, S.A., Seraing, Belgium), 200 nM fwGSPcarpCXC4, 600 nM GeneRacer™ 3′Primer, filled to 50 μl with sterile H_20. PCR conditions were: Hot start of 94 °C for 2 min, 94 °C for 30s and 60 °C for 30s and 72 °C for 60s, for 40 cycles, followed by 72 °C for 10 min. The obtained 3'RACE-PCR product was subsequently amplified by a nested 3'RACE-PCR reaction. A similar 3'RACE-PCR reaction mixture was prepared as in the first round, containing fwGSPcarpCXC4nested as forward primer and GeneRacer™ 3' Nested Primer as reverse primer. For nested 3'RACE-PCR, conditions were: hot start of 94 °C for 2 min, 94 °C for 30s and 65 °C for 30s and 68 °C for 30s for 30 cycles, followed by 68 °C for 10 min. 3'RACE-PCR product was isolated from gel and cloned for sequencing.