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CK-2 of rainbow trout (*Oncorhynchus mykiss*) has two differentially regulated alleles that encode a functional chemokine☆

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ABSTRACT

Rainbow trout chemokine 2 (CK-2) is currently the only known CC chemokine to have a mucin stalk. Further analysis of the mucin stalk region revealed a second, related CC chemokine sequence, denoted here as CK-2.1. This second sequence was determined to be an allele of CK-2 following genomic PCR analysis on several outbred individuals. Furthermore, in both *in vivo* and *in vitro* trials, CK-2 and CK-2.1 were both present, but appeared to have differential tissue expression in both control and PHA stimulated samples. Upon the development of a polyclonal antibody to rCK-2, CK-2 was only observed in the brain, liver and head kidney of PHA stimulated rainbow trout tissues. In comparison, when using the rainbow trout monocyte/macrophage-like cell line, RTS-11, CK-2 protein was observed in both control and PHA stimulated conditions. When studying the function of CK-2, a chemotaxis assay revealed that both peripheral blood leukocytes and RTS-11 cells migrated towards rCK-2 significantly at all concentrations studied when compared to truncated β_2m. Interestingly, this migration was lowest at both the highest concentration and the lowest concentrations of CK-2. Thus, teleostean chemokine receptors may become desensitized when overstimulated as has been observed in mammalian models. The observed chemotactic function was indeed due to rCK-2 as cell migration was inhibited through pre-treatment of both the cells and the polyclonal antibody with rCK-2. As has been observed thus far with all other chemokines, CK-2 does appear to function through binding to a G-coupled protein receptor as chemotaxis could be inhibited through pre-treatment with pertussis toxin. Overall, the results of this study indicate that CK-2 is a functional chemokine that is encoded by two differentially expressed alleles in rainbow trout, CK-2 and CK-2.1.

1. Introduction

The immune system of vertebrates is tremendously complex, involving numerous cell types, barriers and specialized systems which are all used to prevent the entry and colonization of foreign entities throughout the body. The function of this vast network is dependent upon the ability of immune cells to migrate and interact with one another, a role fulfilled by extracellular mediators known as chemokines. Chemotactic cytokines, or chemokines, are a large family of small cytokines responsible for controlling the migratory patterns and positioning of immune cells (reviewed in Griffith et al., 2014). Chemokines were originally believed to have only pro-inflammatory functions but further study has revealed that these small proteins also play critical roles in both tissue homeostasis and development (Tachibana et al., 1998, Gouon-Evans et al., 2002, Virgintino et al., 2013). Regardless of their specific function, all chemokines initiate their actions through binding to appropriate receptors which can be located on a variety of cell types. Of the chemokine receptors discovered thus far, all belong to the family of pertussis toxin (PTX) sensitive, G-coupled protein receptors (GCPR) (reviewed in Rossi and Zlotnik, 2000).

In the highly-studied mammalian model, 46 chemokine genes are currently known (reviewed in Zlotnik et al., 2006, Nomiyama et al., 2010) and are segregated into four families based on differences in their structure and function. The largest family are the CC chemokines, so named because the first two of four cysteine residues are found adjacent to each other in members of this group. In comparison, a second family,

☆ The nucleotide sequence data reported in this paper has been submitted to the GenBank nucleotide sequence databases and have been assigned the accession numbers: AY372431, AY372432 and AY372433.

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Table 1
Primer sequences used in this study.

Primer Name	Sequence (5′-3′)	Annealing Temp.	Application
CK-2 sense	GCAGAAAAGCTGGTGTCGTG	53 °C	RT-PCR, genotyping
CK-2 antisense	GGAAGGTACGGATGGAGAAG		RT-PCR, genotyping
CK-2 stalk sense	GTCTGAGCTCAACATTTCTC	48 °C	Genotyping
CK-2 stalk antisense	AGGAGCTTCAGCCATTAGCA		Genotyping
CK-2 stalk SP2 antisense	GTTCTACAAGCCCCCATAAG		Genotyping
CK-2 stalk SP4 antisense	ATGGGCACATACAATACTGG		Genotyping
EF1-α sense	GAGTGAGCGCACAGTAACAC	54 °C	RT-PCR control
EF1-α antisense	AAAGAGCCCTTGCCCATCTC		RT-PCR control

called the CXC chemokines, has a single amino acid residue located between the first two cysteines. The third family of chemokines, the CX_3C family, consists of a single member, fractalkine. Like the nomenclature of the previous families, the CX_3C group has three amino acid residues separating the first two cysteines of these molecules. The fourth and final family of chemokines has a single cysteine residue and consists of one member, lymphotactin (reviewed in Charo and Ransohoff, 2006). Of these many mammalian chemokines, there are only two known to contain a mucin stalk: fractalkine and CXCL16 (Bazan et al., 1997, Matloubian et al., 2000). The mucin stalk can play an interesting role in chemokine function as it enables both membrane bound and extracellular forms of the attached chemokine depending on cleavage in this region (Haskell et al., 2000, Yoneda et al., 2003).

Recently, there has been a great deal of interest pertaining to the characterization and study of chemokines in teleost species (Laing and Secombes, 2004). As the largest group of vertebrates, consisting of almost 26,000 species (McKenzie et al., 2011), obtaining a deeper understanding of the function and diversification of teleost chemokines could provide insight into the origins and evolution of these small proteins. Since the discovery of the very first teleostean chemokine in rainbow trout (Dixon et al., 1998) several chemokines have been revealed in multiple teleost species including eighteen in rainbow trout, thirty in Atlantic salmon, and eighty-one in zebrafish (Peatman and Liu, 2007, Nomiyama et al., 2008, Bird and Tafella, 2015). As chemokines have been recognized as one of the eight most rapidly evolving proteins (Waterston et al., 2002) clear orthologues between fish and mammalian counterparts are rarely observed. As a result, the function of each newly discovered chemokine in individual teleost species must be experimentally determined.

A unique CC chemokine in rainbow trout is CK-2. The rainbow trout CK-2 gene was discovered in 2002 and is the only CC chemokine known to have a mucin stalk (Liu et al., 2002). In the original description of this gene, northern blots gave two bands, which suggested an additional CK-2 transcript that could have arisen from either alternative splicing or from an allele. In this study, a CK-2 allele has indeed been discovered and is designated CK-2.1. Whether these chemokines are functional is of special interest because of their mucin stalks but to date CK-2 functions have yet to be explored. Therefore, another aspect to our study has been the generation of recombinant CK-2 and a polyclonal antibody to CK-2. Together these reagents have allowed the function and tissue expression of CK-2 to be examined for the first time.

2. Materials and methods

2.1. Fish

Rainbow trout (150–200 g) were obtained from Mimosa Springs Hatchery (Guelph, ON) and kept in 200 l freshwater flow-through tanks at the University of Waterloo. All fish were kept and handled under a permit from the University of Waterloo Animal Care Committee according to CCAC guidelines. Blood was drawn from the caudal vein after anesthesia with 0.01% MS-222 and tissue samples either had leukocytes isolated immediately or were collected in RNA Later and stored at −80 °C for later use. All procedures were performed following guidelines of the Animal Care Committee of the University of Waterloo.

2.2. Maintenance of cell lines

Three cell lines were used for these studies: the gonadal fibroblast RTG2 (Wolf and Quimby, 1962), the spleen monocyte/macrophage RTS11 (Ganassin and Bols, 1998) and the spleen stromal RTS 34 ST (Ganassin and Bols, 1999). The cell lines were routinely maintained in the basal medium L15 with a supplement of fetal bovine serum (FBS) as recently reviewed (Bols et al., 2017).

2.3. Determination of CK-2 genotypes

Genomic DNA was extracted from RTS-11, RTG2, RTS 34 ST and from the whole blood of 55 individual rainbow trout as described by Sambrook et al., 1989. It was previously observed by Liu et al. (2002) that there are at least two copies of CK-2 in rainbow trout. These variants differed in size, thus primers were developed to further explore the origin of this variability. The four primer sets used in this study (Table 1) flanked the mucin stalk which amplified the region containing the size difference between CK-2 and CK-2.1. The PCR parameters were as follows: 95 °C for 5 min; 30 cycles of 95 °C for 30 s (sec), the appropriate annealing temperature for the primer pair for 30 s (see Table 1) and 72 °C for 1 min followed by a final elongation of 72 °C for 5 min. The amplified PCR products were run on a 1.5% agarose gel containing 0.1% ethidium bromide (EtBr) and the DNA bands were visualized with a UV transilluminator containing an EtBr filter. Based on the band sizes, rainbow trout individuals and cell lines were determined to be homozygous for CK-2, homozygous for CK-2.1 or heterozygous.

Upon genotyping of the 55 outbred rainbow trout for CK-2/CK-2.1, the data was assessed to determine whether the alleles were in Hardy-Weinberg Equilibrium using the equation of $p^2 + 2pq + q^2 = 1$ where p represented the frequency of CK-2 and q represented the frequency of CK-2.1.

2.4. Peripheral blood leukocytes (PBL) and head kidney leukocytes (HKL) isolation

Peripheral blood leukocytes (PBLs) were separated by density gradient centrifugation using Histopaque 1077 (Sigma-Aldrich) according to the manufacturer's instructions. Cells were stained with trypan blue to determine viability and counted using a hemocytometer (Hausser Scientific) to give a final concentration of 1×10^7 live cells/ml in L-15 medium supplemented with 20% FBS, 100 U/ml of penicillin and 100 U/ml of streptomycin. Head kidney leukocytes were isolated in the same manner at a density of 1×10^7 cells/ml in RPMI-1640 medium containing 25 mM Hepes, 10000 U/ml penicillin, 10 mg/ml streptomycin and 200 units/ml of heparin sodium salt.