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Molecular characterization of three novel chemokine receptors in rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Chemokines signal through a family of seven-transmembrane domain G-coupled receptors in order to regulate both leukocyte mobilization and activate the recruited cells. Although many chemokines have been identified in rainbow trout (*Oncorhynchus mykiss*), only a few chemokine receptors have been reported to date. In this work, we have cloned three novel chemokine receptors in rainbow trout. One of these receptors seems to be a clear orthologue of CCR6, while the second one constitutes a novel CCR9 gene different from the previous CCR9 reported in this species. This gene, which we have designated as CCR9B, represents another lineage of fish CCR9 genes, not previously identified. Finally, a deeper phylogenetic analysis of the third novel chemokine receptor gene, which had been identified on the basis of sequence similarity to CCR3, constitutes a novel lineage of CCR receptors which has no equivalent in humans and that may be teleost-specific. We have designated this novel gene as CCR13, to avoid any possible ascription to mammalian genes. Further transcriptional studies revealed that CCR6 was constitutively transcribed in thymus, gills, hindgut and peripheral blood leukocytes (PBLs), while CCR9B was strongly transcribed in thymus and PBLs but also in spleen, gills, hindgut and brain at lower levels. CCR13, on the other hand, was strongly detected in spleen, head kidney and PBLs and faintly in thymus, gills, brain and gonad. The data provided constitutes a step forward the identification of novel chemokine receptors that may contribute to a future understanding of chemokine signalling in fish.

1. Introduction

Chemokines are a family of small cytokines that regulate immune cell migration under both inflammatory and normal physiological conditions [1,2]. They can be further divided according to the position of conserved cysteines in the amino terminus sequence, with CXC and CC chemokines being the most numerous subfamilies [3], with the other families being the C and CX3C chemokines. Chemokines signal through a family of seven-transmembrane domain G-coupled receptors in order to regulate both leukocyte mobilization and activate the recruited cells. Thus, understanding the specific functions of individual receptors together with their ligands is essential for a complete view of chemokine effects on lymphocyte action during development, homeostasis or disease. These types of studies are still incomplete in mammals due to the great heterogeneity of lymphocyte populations, the high number of chemokines and chemokine receptors acting on each cell type and the promiscuity of chemokine–chemokine receptor interactions [4,5]. Chemokine receptors are classified according to the type of chemokines they bind, thus in mammals CXCR, CCR, CR and CX3CR are present [6]. In fish, only CC and CXC chemokines have been identified in most species, whereas a fish-specific group of CX chemokines in which the third conserved cysteine of the CXC family is retained (in contrast to mammalian C chemokines in which the second cysteine of the CC family is retained) is present in species such as zebrafish (*Danio rerio*). Whether there is a specific receptor group for CX chemokines in zebrafish is still unknown [7].

In fish, despite the great number of chemokine genes identified, the biological role of most of these molecules remains unresolved, since no clear inferences of their functions can be made based on their similarities to potential mammalian counterparts. This is due to the low levels of sequence similarity between trout and mammalian chemokines, especially in the case of CC chemokines. In rainbow trout (*Oncorhynchus mykiss*), although 18 CC chemokine sequences have been reported [8], some aspects of their bioactivity have only been elucidated for CK1 [9], CK6 [10,11] and CK12 [11]. Moreover, the specific cell types on which they are acting and the

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immune functions triggered in these cells upon signalling has not been extensively studied.

In mammals, eleven CC chemokine receptors named CCR1 to CCR11 have been identified in most species [12]. Although not found consistently in the literature, it has been proposed in humans that the *N*-formyl peptide receptor-like 1 (FPRL1) be renamed as CCR12 [13]. This is an orphan receptor cloned originally from a human phagocyte cDNA library, was characterized by nucleotide similarity to FPR [14].

In rainbow trout, to date, only the sequences of CCR7, CCR9, CXCR4 and CXCR8 have been reported [15–17]. CCR9 was originally reported as CCR7 [15], but later phylogenetic analyses have revealed it to be a homologue of CCR9, while a novel CCR7 gene has been identified [17]. Furthermore, no ligands have been designated for these receptors identified, thus in rainbow trout no single chemokine–chemokine receptor pair has been demonstrated to date.

In this study, we describe the identification and characterization of three novel CC chemokine receptors in rainbow trout: a clear homologue of CCR6; a novel CCR9 designated as CCR9B, which represents a distinct lineage of fish CCR9 genes; and a gene designated as CCR13, which constitutes a teleost-specific novel lineage of CCR receptors with no human equivalent. The data provided contributes to increasing the current understanding of chemokine signalling in fish.

2. Materials and methods

2.1. In silico identification of novel rainbow trout CCRs

Human CCR3, CCR6 and CCR9 protein sequences were used as queries against expressed sequence tag (EST) databases from rainbow trout (*O. mykiss*) in the National Center for Biotechnology Information database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using tBLASTn searches.

Using human CCR3, a rainbow trout EST that encoded a CCR3-like sequence was identified (accession number BX862026.3). The sequence had a stop codon, but lacked the start codon within the 5′ end. Therefore 5′RACE was performed to obtain the complete sequence using a cDNA obtained from head kidney, the 5′RACE System for Rapid Amplification of cDNA Ends from Invitrogen and the primers indicated in Table 1. An overlapping fragment containing the initial segment of the CCR3-like coding sequence and the 5′ untranslated region (UTR) was amplified. Primers were then designed to amplify the full coding sequence, which was again sequenced.

BLAST searches using human CCR6 identified a rainbow trout EST (accession number CA383964) that encoded a CCR6-like molecule. The sequence lacked a stop codon, therefore 3′RACE was performed to obtain the complete sequence using a cDNA obtained from peripheral blood leukocytes (PBLs), the 3′RACE System for Rapid Amplification of cDNA Ends from Invitrogen and the primers indicated in Table 1. An overlapping fragment was amplified which contained the final segment of the CCR6 coding sequence and the 3′ UTR. Primers were then designed to amplify the full coding sequence, which was again sequenced.

Finally, a CCR9-like sequence, different from that already available in the GeneBank and previously reported as CCR7 by Daniels et al. [15], was identified (accession number NM_001124610). Like CCR3, the sequence had a stop codon, but lacked the start codon within the 5′ end, therefore 5′RACE was performed as described before using the primers detailed in Table 1.

Table 1
Primers used for the cloning and real time RT-PCR expression of the novel rainbow trout CCRs identified.

Gene	Name	Sequence (5′–3′)	Application
CCR3	CCR3-RT-F	GTTCTGTACAACGTCTGGAAGGATT	Real time
CCR3	CCR3-RT-R	ATGGCCAAAGGAAGTAGAAAGAAGA	Real time
CCR3	RACE-R	ACAACTGCCGAGGAGACGCAGGCGTAG	5′RACE
CCR3	RACE-nR	AATGGCATGGACCACAGCCAGGTA	5′RACE
CCR6	CCR6-RT-F	TGCAGAGGAAACAGTTAACAATTCA	Real time
CCR6	CCR6-RT-R	CCAGTAAACCCAGGATACAGATGAC	Real time
CCR6	RACE-F	GTTATGTGCCTGCACACAGCACCATTGG	3′RACE
CCR6	RACE-nF	ATGCTACAACACTGTTTGATCTGGAGAA	3′RACE
CCR9B	CCR9B-RT-F	AATATTTCCAACGTCTGAAACAGGA	Real time
CCR9B	CCR9B-RT-R	CTCACCCAGGACTTATCACACATTC	Real time
CCR9B	RACE-R	CCCAACAACCTCTTCAACCGCACCAAGA	5′RACE
CCR9B	RACE-nR	TTCTGCCTGCCTCTGCTGGTCATGGTT	5′RACE
EF-1α	EF1-RT-F	GATCCAGAAGGAGGTCACCA	Real time
EF-1α	EF1-RT-F	TTACGTTCGACCTTCCATCC	Real time

Table 2
Selected rainbow trout CCR3-like blast search hits using the predicted protein sequence to search the non-redundant protein database (all were within the top 16 matches).

Name	Genbank accession	*e*-Value	% Identity
CC chemokine receptor type 3 [*Salmo salar*]	NP_001167233.1	0.0	86%
CC chemokine receptor type 3 [*Osmerus mordax*]	ACO08921.1	7^{-129}	68%
CC chemokine receptor-3 [*Paralichthys olivaceus*]	BAC87713.1	5^{-157}	68%
CC chemokine receptor family-like [*Danio rerio*]	NP_001093461	3^{-103}	61%
PREDICTED: CC chemokine receptor type 3-like [*Ailuropoda melanoleuca*]	XP_002912807.1	9^{-73}	37%
Chemokine receptor 5 [*Gallus gallus*]	ABS86967.1	1^{-72}	39%
CC chemokine receptor type 1 [*Bos taurus*]	NP_001071307.1	8^{-71}	39%
CC chemokine receptor-3 [*Canis lupus familiaris*]	BAD83841.1	5^{-69}	38%
CC chemokine receptor type 1 [*Canis lupus familiaris*]	NP_001033695.1	3^{-68}	40%

2.2. Sequence analysis

The complete CCR nucleotide sequences were analysed within The ExPASy Molecular Biology server (http://us.expasy.org). Similarity searches were performed using the basic local alignment tool

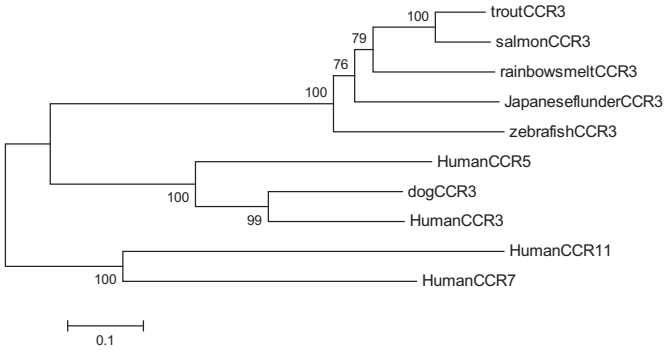

Fig. 1. Evolutionary relationships of CCR3-like sequences. The evolutionary history was inferred using the Neighbour-Joining method [34]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [35]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site [36]. Evolutionary analyses were conducted in MEGA5 [19]. The sequences used are listed in Table 2 or in the legend of Fig. 6.