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BRIEF COMMUNICATION

Kazuhiro Fujiki · Dong-Ho Shin · Miki Nakao · Tomoki Yano

Molecular cloning of carp (*Cyprinus carpio*) CC chemokine, CXC chemokine receptors, allograft inflammatory factor-1, and natural killer cell enhancing factor by use of suppression subtractive hybridization

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Phylogenetic studies on the immune system have revealed that teleost fish, one of the most primitive groups of vertebrates, possess both innate and acquired arms of immunity homologous to those of the mammalian immune system (Magor and Vasta 1998). Genes encoding component molecules of the immune system, including immunoglobulins, T-cell receptor α and β subunits, major histocompatibility complex (MHC), and complement components have been cloned from teleost species. However, the isolation and characterization of cytokines in teleosts has been a slower process. This is probably because similarity cloning using degenerate primers designed from conserved amino acid (aa) sequences of higher vertebrates, in most cases mammals, has not been applicable to cytokines of the phylogenetically distant teleost species. The expressed sequence tag approach allows random cloning, but it needs to be improved in order to isolate cytokines by eliminating housekeeping genes and equalizing the frequencies of the remaining genes.

The suppression subtractive hybridization (SSH) technique, which makes use of suppression polymerase chain reaction (PCR) effects (Diatchenko et al. 1996), has the advantages that smaller amounts of poly(A)$^+$RNA are required, it does not require physical separation of single- and double-stranded cDNAs, and representation of the subtracted sequences are equalized. This allows efficient screening of the subtracted fragments by sequencing. In the work presented here, we performed SSH between carp leukocytes before and after dynamic migration elicited by sodium alginate (Fujiki and Yano 1997) in order to clone cDNA of cytokines and their related proteins.

Nontreated carp head kidney cells (driver) and peritoneal cells (tester) from carp injected with sodium alginate 48 h earlier were prepared as described (Fujiki and Yano 1997). Once extracted, poly(A)$^+$RNA from both cells types was subjected to SSH and selective PCR amplification using the PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, Calif.) according to the manufacturer's protocol. The amplified cDNA fragments were subcloned into pBluescript SK(−) vector (Stratagene, La Jolla, Calif.). Sequencing and BLASTX analysis of 276 randomly selected clones produced 111 clones with significant similarities to known proteins, determined by *E*-values (Gish and States 1993) lower than the threshold (1×10^{-5}). The sequences obtained included 94 distinct sequences that were deposited into GenBank with accession numbers C88358 through C88445. The relative frequency of housekeeping genes, β-actin, and ribosomal protein in the cloned sequences was 0.9%, indicating that the subtraction worked cor-

The nucleotide sequence data reported in this paper have been submitted to the DDBJ, EMBL, and GenBank nucleotide sequence databases and have been assigned the accession numbers C88358 through C88445, AB010468, AB010469, AB010713, AB010959, AB012309 and AB012310. The accession numbers of sequences retrieved in this study are as follows: chicken MIP1β, Q90826; human (hu) MCP1, P13500; huMCP2, P80075; huMCP3, P80098; dog MCP1, P52203; pig MCP1, P42831; huEotaxin, P51671; mouse (mo) eotaxin, P48298; huMIP-1α, P10147; moMIP-1α, P10855; huMIP-1β, P13236; moMIP-1β, P14097; huRANTES, P13501; moRANTES, P30882; rainbow trout CXCR4, AJ001039; huCXCR1, P25024; huCXCR2, P25025; huCXCR3, P49682; huCXCR4, P30991; rabbit (ra) CXCR1, P21109; raCXCR2, P35344; rat CXCR2, P35407; moCXCR2, P35343; moCXCR4, P70658; rat CXCR4, O08565; cattle CXCR4, P25930; huCCR1, P32246; huCCR2, P41597; huCCR3, P51677; huCCR4, P51679; huCCR5, P51681; huAIF-1, P55008; rat AIF-1, P70491; pig AIF-1, P81076; rainbow trout NKEF, Q91191; newt TPx, Q90384; huNKEF-A, P35703; huNKEF-B, P32119

K. Fujiki (✉) · D.-H. Shin · M. Nakao · T. Yano
Laboratory of Marine Biochemistry, Faculty of Agriculture, Kyushu University, Hakozaki 6-10-1, Higashi-ku, Fukuoka 812-8581, Japan
e-mail: fujikik@agr.kyushu-u.ac.jp,
Tel: +81-92-6422896
Fax: +81-92-6422894

rectly. Thirty-four clones related to the immune system were obtained (Table 1), and six clones of particular interest were sequenced in full and designated S-84, L-2, L-56, M-135, L-68 and L-128.

The derived amino acid sequence of clone S-84 contained the conserved four-cysteine motif characteristic of the CC chemokine family, in which the first two cysteines are adjacent. This clone showed more sequence similarity to the mammalian monocyte chemotactic protein (MCP) family than to the other CC chemokines, including rainbow trout CK-1 (Dixon et al. 1998) (Fig. 1). MCPs are potent chemoattractants of monocytes in mammals (Baggiolini et al. 1997). The amino acid sequence of S-84 contained an arginine in the position corresponding to Tyr^{28} in human MCP-1, which is conserved in mammalian, chicken, and rainbow trout CC chemokines, and has been shown to play a key role in selectivity of the receptor (Zhang et al. 1994; Wells et al. 1996). Another functionally important feature of CC chemokine is the presence of an aspartate residue in the N-terminal region, important in human MCP-1 for signalling via the receptor (Zhang et al. 1994). The amino acid sequence of S-84 has an N-terminal aspartate one residue behind the equivalent position of mammalian MCPs. Thus while S-84 has some structural similarities to MCPs, functional characterization of the

Table 1 Results of BLASTX searches with *Rsa* I-digested fragments of sodium alginate-elicited carp peritoneal exudate cell cDNA after subtraction by normal fish head kidney cDNA

Clone*	Similar to	Species	Accession number
Cytokines and receptors			
s-63	Pre-B-cell enhancing factor	Human	C88369
s-84	Monocyte chemotactic protein-2	Human	AB010469†
l-68	Allograft inflammatory factor-1	Human	AB012309†
l-128	Natural killer enhancing factor A	Human	AB010959†
m-135	SDF-1 receptor (CXCR4)	Rat	AB012310†
m-141	ErbB-3 receptor protein-tyrosine kinase	Human	C88413
l-2	High-affinity interleukin-8 receptor B (CXCR2)	Rabbit	AB010468†
l-56	High-affinity interleukin-8 receptor A (CXCR1)	Rabbit	AB010713†
Extracellular matrix-associated proteins			
m-95	Metalloproteinase inhibitor 2	Mouse	C88401
m-124	92000 μ_r type IV collagenase	Human	C88404
l-29	Fibronectin	Rat	C88421
l-101	Vitronectin receptor α subunit	Mouse	C88429
EF-hand calcium-binding proteins			
s-69	S100 protein α chain	Rat	C88370
m-3	Calcium-binding protein P22	Rat	C88388
m-34	Calmodulin	Human	C88396
l-4	Grancalcin	Human	C88419
l-38	Calcium-dependent protease, small subunit	Pig	C88423
Lysosome-associated proteins			
s-4	Dipeptidyl-peptidase I	Human	C88359
s-88	*N*-acetylglucosamine 6-sulfatase	Human	C88373
m-125	Cathepsin L	Cattle	C88405
m-126	Proactivator polypeptide	Human	C88406
m-131	Cathepsin B	Cattle	C88410
m-152	Lysosome membrane protein II	Rat	C88415
Cell surface proteins			
s-137	Cell surface antigen MS2	Mouse	C88386
m-112	Platelet-endothelial tetraspan antigen 3	Human	C88402
m-128	Membrane-associated protein HEM2	Mouse	C88408
l-141	G protein-coupled receptor 6H1 from T cells	Chicken	C88435
l-157	CD81 antigen	Human	C88437
Signal transduction-associated proteins			
s-72	Thioredoxin	Chicken	C88371
m-129	Guanine nucleotide-binding protein G(i), α-1 subunit	Chicken	C88409
m-24	Phosphatidylinositol-4-phosphate 5-kinase FAB1	Yeast	C88393
Complements			
m-33	Complement factor B	Human	C88395
Other proteins			
s-46	Pathogenesis-related protein 1C	Tobacco	C88365
m-151	Interferon-inducible protein 1-8U	Human	C88414

* Clones which showed *E*-value below 1×10^{-5} in BLASTX search results and are related to immune system are listed
† Accession number of the corresponding full-length cDNA clone

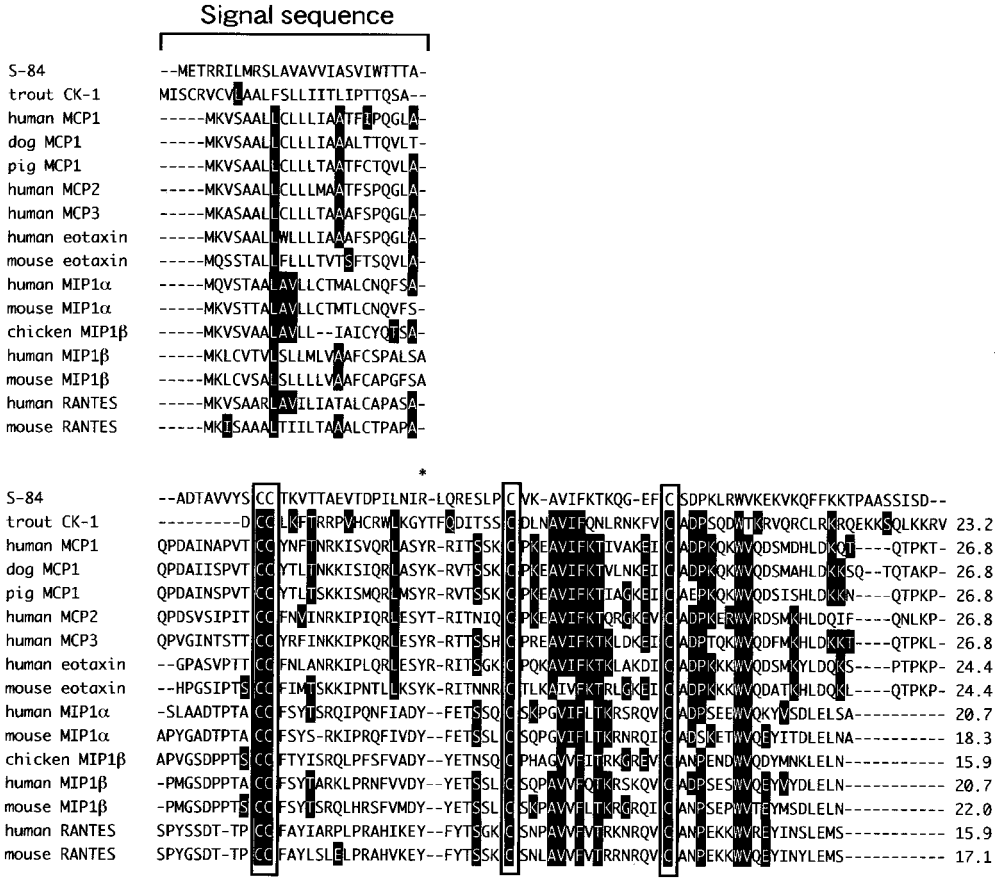


Fig. 1 Alignment of the deduced amino acid sequence of clone S-84 with other vertebrate CC chemokine sequences. Predicted signal sequences and mature proteins were aligned separately by Clustal W. Amino acid residues identical to the S-84 sequence are *shaded. Dashes* indicate gaps introduced for optimal alignment. The conserved four-cysteine motif of the CC chemokine family is *boxed.* The *asterisk* indicates the tyrosine residue conserved among all mammalian CC chemokines. The *number* at the *end* of each sequence represents amino acid similarity (%) to S-84

S-84 protein, including target cell specificity, receptor selectivity, and an analysis of its expression pattern will be needed to prove conclusively that S-84 encodes a chemokine.

Clones L-2 and L-56 are both equally similar to the two known types of mammalian high affinity IL-8 receptors (CXCR1/CXCR2) (Table 2). Both L-2 and L-56 contain the seven transmembrane-spanning motif and the DRY motif which are characteristics of the G protein-coupled receptor family, a group that includes chemokine receptors (Murphy 1994; Leong et al. 1994) (Fig. 2A). The clones also contain aa residues equivalent to Arg^{199}, Arg^{203}, Asp^{265} in the extracellular domains of human CXCR1, which are important for IL-8 binding and signalling, and are shared by all mammalian high-affinity IL-8 receptors (Leong et al. 1994). The aa identity between L-2 and L-56 is 33.4%, suggesting that these two clones represent two distinct subtypes of carp IL-8 receptors.

Clone M-135 is similar to CXCR4, which is a receptor for the CXC chemokine stromal cell-derived factor-1 (SDF-1) (Bleul et al. 1996) (Fig. 2B). Besides being a chemokine receptor and HIV entry co-receptor (Bleul et al. 1997), CXCR4 is critically involved in embryo development, B-lymphopoiesis, myelopoiesis, and cardiogenesis (Ma et al. 1998; Tachibana et al. 1998). This functional importance of CXCR4 probably is indicated by the fact that CXCR4 and SDF-1 are highly conserved among mammalian species (Doranz et al. 1996; Shirozu et al. 1995). Alignment of the carp CXCR4 with recently cloned rainbow trout CXCR4 (Secombes et al. 1998) and mammalian CXCR4 indicates that this molecule is highly conserved not only among mammalian species but also among all vertebrates. This conservation indicates that CXCR4 and its ligand probably also play a critical role in the primary immunological function of teleosts.

Clone L-68 shows close identity to mammalian allograft inflammatory factor-1 (AIF-1) (Fig. 3). Carp AIF-1 is the first reported nonmammalian homologue of this gene. AIF-1 was originally identified as a cytokine abundant in rat heart allografts (Utans et al. 1994) and was later found to be constitutively expressed mainly in macrophage lineages from various tissues in mammals (Utans et al. 1995, 1996; Chen et al. 1997). As pointed out for rat and human AIF-1, mammalian AIF-1 proteins contain an EF-hand-like motif (Strynadka and James 1989), although unlike in calcium-binding proteins, mammalian AIF-1 proteins have only a single repeat of this motif, in which the conserved calcium-binding loop segment has a substitution at position 12 (Utans et al. 1995; Autieri 1996). Carp AIF-1 also contains an EF-hand-like motif but the region corresponding to the conserved loop segment has another substitution at position 5 (Gln) in addition to the one at position-12 (Gly). This sequence diversity of the EF-hand-

Table 2 Identities of the deduced amino acid sequences of clones L-2, L-56, and M-135 with rainbow trout and human chemokine receptors

Clone	Identity (%)										
	L-56	Trout CXCR4	Human CXCR1	Human CXCR2	Human CXCR3	Human CXCR4	Human CCR1	Human CCR2	Human CCR3	Human CCR4	Human CCR5
L-2*	33.4	–	34.9	35.2	30.4	25.3	25.8	25.5	24.5	28.1	25.0
L-56*	–	–	40.3	38.8	28.8	28.3	26.0	27.3	24.5	27.0	28.6
M-135†	–	64.8	30.2	32.0	28.9	59.1	27.0	26.0	25.5	28.9	25.2

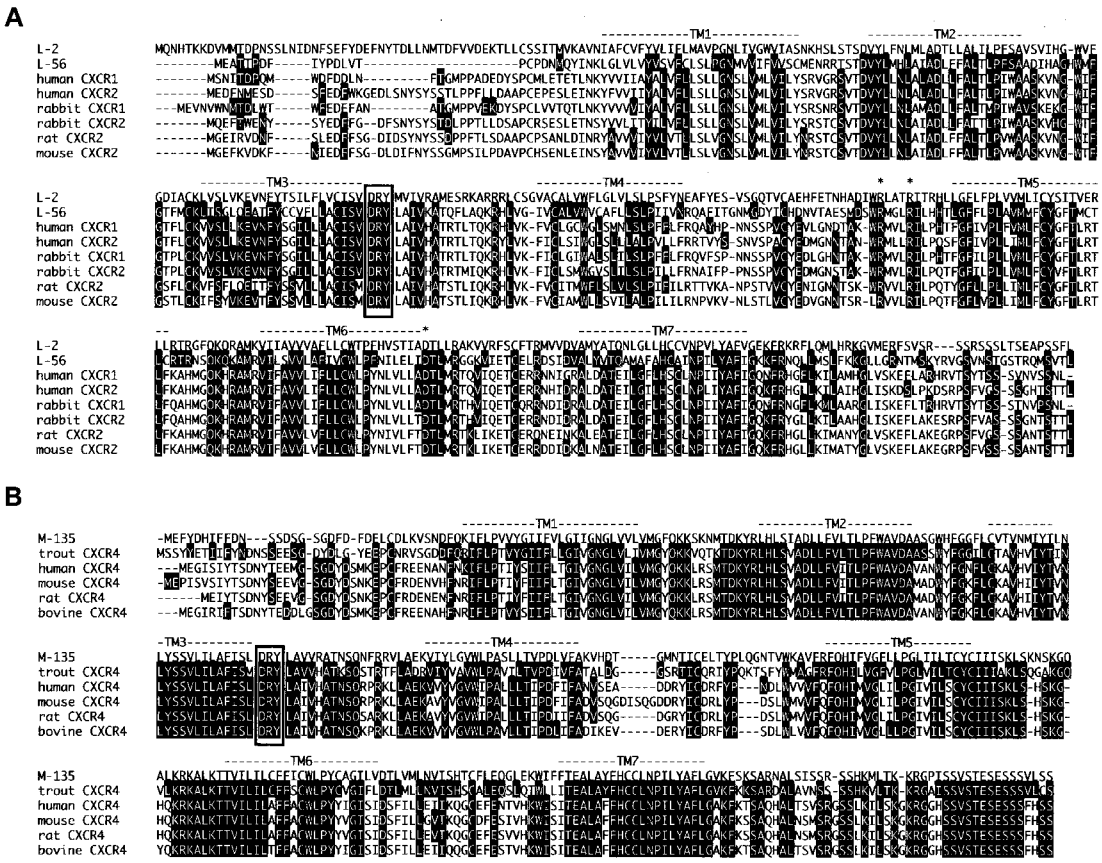
* Identities of L-2 and L-56 were calculated on the basis of multiple alignment in which all sequences except for M-135 were aligned by Clustal W

† Identities of M-135 were calculated on the basis of multiple alignment in which all sequences except for L-2 and L-56 were aligned by Clustal W

like motif makes unlikely that AIF-1 is a member of the EF-hand family.

Clone L-128 (975 bp) encodes a sequence of 199 aa. The predicted aa sequence shows 72.9–78.7% identity to rainbow trout, newt, and human natural killer cell enhancing factors (NKEF), respectively. NKEF was originally purified as a human red blood cell-derived cytokine with NK cell enhancing activity (Shau et al. 1993). It was subsequently found to be identical to thioredoxin peroxidase (TPx) which reduces H_2O_2, utilizing electrons from thioredoxin (Chae et al. 1994). Expression of carp NKEF by peritoneal cells probably indicates that a redox reaction was required in the sodium alginate-elicited inflammatory response because a

Fig. 2 **A** Alignment of the deduced amino acid sequences of clones L-2 and L-56 with mammalian interleukin-8 (IL-8) receptor sequences. Amino acid residues identical to the L-2 sequence are *shaded. Dashes* indicate gaps introduced for optimal alignment. *Asterisks* indicate the residues important for IL-8 binding. **B** Alignment of deduced amino acid sequence of clone M-135 with rainbow trout and mammalian CXC chemokine receptor-4 (CXCR4). Amino acid residues identical to the M-135 sequence are *shaded. Dashes* indicate gaps introduced for optimal alignment. The DRY motif characteristic of the G protein-coupled receptor family is *boxed.* The lines *above* the sequences marked with TM indicate each transmembrane domain



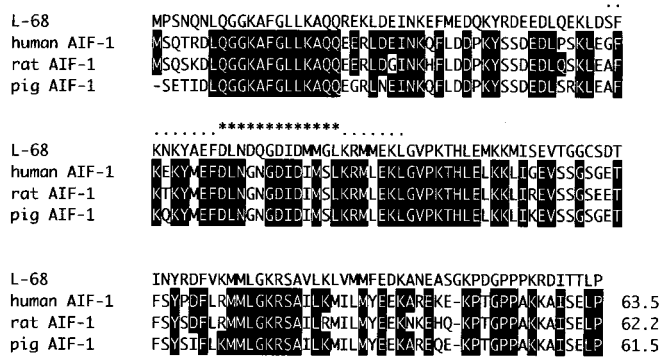


Fig. 3 Alignment of deduced amino acid sequence of clone L-68 with human, rat, and pig allograft inflammatory factor-1 (AIF-1). Amino acid residues identical to the L-68 sequence are *shaded. Dashes* indicate gaps introduced for optimal alignment. The EF-hand-like motif of L-68 is shown by *dots* and *asterisks* (the region corresponding to the conserved loop segment) *above* the sequence. The *number* at the *end* of each sequence represents amino acid identity (%) to L-68

thioredoxin gene fragment (s-72) was also obtained. A recent report that TPx seems to be involved in reducing H_2O_2 generated by mammalian cells in response to some cytokines (Kang et al. 1998) also supports this assumption. Human NKEF contains two known subtypes, A and B, and only NKEF-A has NK-enhancing activity (Sauri et al. 1996). Carp NKEF is more similar to NKEF-A (74.9% aa identity) than to NKEF-B (72.9% aa identity), suggesting that carp NKEF has NK enhancing activity as well as thioredoxin peroxidase activity; however, a test of this hypothesis must wait until carp NK cells can be clearly identified and isolated, which is not currently possible.

The results presented here prove the usefulness of SSH for cloning genes encoding cytokines and related proteins from teleosts. SSH using other sets of appropriately selected combinations of tester and driver cells could accelerate the identification of teleost cytokines and other genes expressing small amounts of mRNA.

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