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Diversity of CD2 subfamily receptors in cyprinid fishes

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ABSTRACT

CD2 family receptor (CD2f) is evolutionarily conserved and is widely expressed by various types of leukocytes. To elucidate the phylogenetic diversity of the CD2f, we characterized CD2f in teleosts using ginbuna crucian carp and zebrafish. The identified CD2f isoforms of the ginbuna carp (caauCD2f) exhibited high sequence similarity to the mammalian CD2 subsets CD48, CD244, and CD319, but it was difficult to classify them into their respective mammalian CD2f based on sequence similarity, the presence of an immunoreceptor tyrosine-based switch motif (ITSM), and phylogenetic tree analysis. Although the four caauCD2f isoforms share an extracellular domain with quite high identity (83–94% identity at the nucleic acid level), they differ in the number of ITSM motifs in their cytoplasmic tail. RT-PCR and *in situ* hybridization analyses showed that the caauCD2f isoforms are expressed by different cell populations, suggesting that they, like mammalian CD2f, have diverse roles. Interestingly, immunoglobulin (Ig) domain-like sequences with high identity to caauCD2fs are clustered close together within 0.6 Mbp on zebrafish chromosomes 1 and 2 (at least 8 and 35 sequences, respectively), and many pairs of the Ig domains share more than 90% identity at the amino acid level. Therefore, the teleost CD2fs with considerably high identity have been probably generated from a common ancestral Ig-domain gene by a very recent gene duplication event. These findings suggest that the identified CD2f acquired functional diversification through successive duplications together with the acquisition of ITSM.

1. Introduction

The CD2 family receptor (CD2f), part of the immunoglobulin (Ig) superfamily, includes 11 cell surface molecules in mammals. The CD2f are widely expressed on various types of leukocytes, such as natural killer (NK), T- and B-cells, macrophages, and dendritic (DC) cells, and they have different functions in relation to regulation or activation of immune responses. All CD2f consist of IgV and IgC2 domains, a transmembrane, and a cytoplasmic tail; however, some members only possess a short cytoplasmic tail [3,6,7,14,22]. The CD2 and CD58 genes are on the short arm of human chromosome 1 and the other members are clustered close together on the long arm of chromosome 1 [8]. This structural similarity and genomic localization suggest that the CD2f arose from an ancestral gene through successive gene duplications.

The CD150 (signaling lymphocytic activating molecule: SLAM) subfamily of the CD2f consists of CD150 along with NTB-A, CD319 (CS1), CD84, CD229, and CD244 (2B4). The most characteristic feature of the CD150 family is the presence of 2–4 immunoreceptor tyrosine-based switch motifs (ITSM) in their cytoplasmic tails. The ITSM motif has been shown to interact with small adaptor molecules through Src homology 2 (SH2) domains (examples include SAP, EAT-2A, and EAT-2B (ERT) and their interaction triggers) to induce activating or inhibiting responses of T- and B-cells or NK cells mediated by tyrosine phosphorylation signals [18,22,36,37]. Therefore, the presence of the ITSM motif is important for classification of the CD2f based on their functionality.

Ig, TCR, MHC I, and MHC II as well as other proteins that function in immune recognition have been identified in teleosts in forms resembling their mammalian orthologs [2,4,9,11,23,30]. In addition, novel immune-type receptors (NITRs), which are encoded by clusters of multigene families, have been identified in a number of bony fish species [32,40]. This discovery of NITRs indicates that Ig superfamily (IgSF) receptors diverged extensively before the emergence of teleosts. These studies have shown that fish leukocytes express many types of IgSF receptors for contact with other immune cells. A recent report has also shown that at least seventy CD2-like genes are present in the genome and cDNA databases of *Siurana tropicalis*, suggesting that CD2f genes expanded and diverged in the lower vertebrates [10]. Therefore, it is expected that CD2f genes are also highly diverse in teleosts.

The ginbuna crucian carp, a naturally occurring gynogenetic fish, is a useful model for immunological study [16,26]. Because monoclonal antibodies against CD4 and CD8α have recently been

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produced in this species, the ginbuna carp is the only fish species whose lymphocyte subsets can be purified [34,35]. The zebrafish belongs to the same family as the crucian carp, and its genomic database provides an opportunity for analyzing immune receptor loci in a lower vertebrate [19]. To explore the phylogenetic diversity of CD2f, we cloned and characterized several CD2f genes from the ginbuna crucian carp and identified cell-types expressing the genes. In addition, the genomic organization of the CD2f gene locus was investigated using the zebrafish genome database.

2. Materials and methods

2.1. Cloning and characterization of CD2f genes and SAP from ginbuna crucian carp

Total RNA was extracted with ISOGEN reagent (Nippon Gene) from spleen of clonal ginbuna crucian carp (*Carassius auratus langsdorfii*), a strain (S3n) from Lake Suwa in Nagano prefecture, Japan. The total RNA (1 μg) was then reverse-transcribed with SuperScript II RNaseH-reverse transcriptase (Invitrogen, USA) and used for 5′- and 3′-RACE PCR with a SMART RACE cDNA Amplification kit (Clontech Laboratories, USA) according to the manufacturer's protocol. Briefly, A clone encoding a putative CD2f (FS999292) was found in an expressed sequence tag (EST) library from ginbuna crucian carp infected with crucian carp hematopoietic virus [20]. Gene-specific primers for 5′-RACE were designed based on the partial sequence of the EST clone. First and nested 3′- and 5′-RACE PCR were performed using specific primers for each of the sequences listed in Table 1 and Universal Primer Mix (UPM) or Nested Universal Primer (NUP), respectively.

Amplified fragments were subcloned into a pGEM-T vector (Promega, USA). Plasmid DNA was purified, and both strands were sequenced using a CEQ8800 sequencer (Beckman Coulter, USA). The nucleotide sequences were analyzed by a BlastX homology search of the NCBI database (http://www.ncbi.nlm.nih.gov/Blast.cgi), and deposited in the DDBJ/EMBL/GenBank databases under the following accession numbers: AB666461 (caauCD2f-1), AB666462 (caauCD2f-2), AB666464 (caauCD2f-3), and AB666465 (caauCD2f-4).

The sequences were aligned with ClustalW (www.ddbj.nig.ac.jp/Welcome-j.html) with default setting and phylogenetic tree was developed with Tree View version 0.5.0 (evolution.genetics.washington.edu/phylip/software.html) by the neighbor joining methods.

Table 1
Primers for amplification of CD2f and SAP.

Name	Sequence (5′→3′)
For 5′RACE-PCR	
CD2f-R1	GTCTCATAACAACTTCCCCATGACAC
CD2f-R2	GACATGCGGCATGTAACAAAGGAAGCC
For 3′RACE-PCR	
CD2f-F1	GGAGTGAACTTCAGTATCTACAAG
CD2f-F2	CAAGGAAGTCATTGCAACAGCAGTG
CD2f-F3	GAGTCAACGCAGCTTCTGTCACTTTGAGTT
CD2f-F4	CGCAGCTTCTGTCACTTTGAGTTCTGTTTC
CD2f-F5	GAGTCTAATAGGAAGTCAATGTGGTGGCAG
CD2f-F6	TGTGGTGGCAGTCATTTTGAGTTTTGCTTC
CD2f-F7	GGAGTCTAATAGGAAGTCAATGTGGTG
CD2f-F8	GGAAGTCAATGTGGTGGCAGTCA
For RT-PCR	
CD2f-F9	GACTCTGAAGAACATGCAGAAGACA
CD2f-1-R1	CCGGTCTGGAATGTCATAAG
CD2f-2-R1	CTCTTGTGTCTGGTCTTCAGCCTC
CD2f-F10	CAGATGCTCTCCACAGGAAGTGACA
CD2f-3-R1	CATAAGAGAATGAGGCAACATGGAC
CD2f-4-R1	ATGATCACTGGCACTGCCACCGGAG
SAP-F1	GAAGCGCTGTCTGTGTACCA
SAP-R1	CGTTTAGTGTGACCTGGTGC
For cRNA probe)	
CD2f-1-F1	GAGTGATTCATCTCGCCTGGCTATA
CD2f-1-R2	GTGTCACTGGTGGATATGACAGGAG
CD2fs-F11	GTGCAGACAGGAGCTTCTGTTC
CD2fs-R3	GTCAGGACAGGAGCCTCCACAG

2.2. Expression analysis of caauCD2f mRNA in purified leukocytes by RT-PCR

S3n strain of ginbuna crucian carp, weighing 45–57 g, was bled from the caudal vein into heparinized syringes. The blood samples were then layered onto a Percoll (Pharmacia) density gradient of 1.08 g/ml and centrifuged at 350*g* for 30 min at 4 °C to separate out the peripheral blood lymphocytes (PBL). To separate plastic-adherent or non-adherent cells, the PBL were seeded in 48-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark) at 5×10^6 cells/well, and allowed to settle in the wells for 90 min at 25 °C. The non-adherent cells were removed by vigorous pipetting, and the suspended cells were collected by centrifugation at 350*g* for 10 min. The cells remaining in the wells were regarded as adhered cells.

To investigate differential expression of the CD2f isoforms in different lymphocyte subsets, we separated the lymphocyte subsets using anti-CD8α, CD4, and IgM monoclonal antibody (mAb) [34,35]. Kidney cells from the S3n strain of ginbuna crucian carp were dispersed by pressing the tissues through a 150-gauge mesh stainless steel sieve in OPTI-MEM. The cells were washed with OPTI-MEM before layering onto a Percoll density gradient of 1.08 g/ml, and centrifuged at 350*g* for 20 min at 4 °C. Cell layers on the Percoll were collected and washed three times with OPTI-MEM. The cell suspension was incubated with a $1{:}10^4$ dilution of rat anti-ginbuna CD8α mAb (mouse ascites) on ice. The cells were then washed twice with OPTI-MEM-10 and incubated on ice for 20 min with 1 ml of a 1:5 dilution of magnetic bead-conjugated goat anti-rat Ig antibody (Miltenyi Biotec GmbH, Bergisch Glabach, Germany) and then re-washed a further thrice. Surface Ig (sIg)-positive and -negative cells were separated with a magnetic separation system (Mini Macs, Miltenyi Biotec) by applying the cell suspension to a plastic column equipped with an external magnet. The CD8α-positive cells were retained in the column, while the CD8α-negative cells passed through. Both cell fractions were collected and viability was confirmed to be greater than 95% by the trypan blue dye exclusion method. Subsequently, negative cells were incubated with a $1{:}10^4$ dilution of rat anti-ginbuna CD4 mAb (mouse ascites) on ice. The protocol for purification of CD4-positive cells was essentially the same as that described for the CD8α-positive cells. In addition, IgM-positive cells were purified from different fish following a previously described protocol [25].

Total RNA was extracted from these purified leukocyte subpopulations using NucleoSpin RNA II (Machery-Nagel), according to the manufacturer's protocol, and then reverse-transcribed with SuperScript II RNaseH-reverse transcriptase (Invitrogen) and oligo (dT) primer.

RT-PCRs for amplification of caauCD2f were carried out with the following specific primer sets: CD2f-F9 and CD2f-1-R1 for caauCD2f-1; CD2f-F9 and CD2f-2-R2 for caauCD2f-2; CD2f-F10 and CD2f-3-R1 for caauCD2f-3; and CD2f-F9 and CD2f-4-R1 for caauCD2f-4 (see Table 1). AmpliTaq Gold DNA polymerase (Applied Biosystems) was used. The PCR conditions were as follows: 95 °C for 5 min and 36–40 cycles of 95 °C for 15 s, 65 °C for 30 s, and 72 °C for 10 min for amplification of the caauCD2fs; 95 °C for 5 min and 30 cycles of 95 °C for 15 s and 65 °C for 30 s plus 72 °C for 10 min for amplification of SAP; and 95 °C for 5 min

and 36 cycles of 95 °C for 15 s, 65 °C for 30 s, and 72 °C for 10 min for amplification of CD8α.

The specificity of these primers was confirmed by PCR with plasmid DNA carrying each of the four caauCD2fs as an insert (data not shown). Specific primers for CD8α, CD4, Ig, and EF1-α were used as described in previous reports [24].

2.3. Expression analysis of caauCD2f mRNA by in situ hybridization

We prepared two probe sets to investigate caauCD2f-positive cell populations in PBL. A probe was designed to detect the extracellular domain (which is well conserved in all caauCD2fs) to detect all types of caauCD2fs. Another probe corresponded to the cytoplasmic tails of caauCD2f-1 and enabled specific detection of ccauCD2f-1. It was difficult to design specific probes for detecting other caauCD2fs because of high sequence similarity. DNA fragments encoding the two domains were amplified using the primer sets shown in Table 1 and cloned into a pGEM-T vector. Sense and antisense RNA probes of caauCD2f were labeled with digoxigenin (DIG) (Roche Molecular Biochemicals) using the appropriate RNA polymerase (T7 or SP6). *In situ* hybridization was carried out using an ISHR Starting kit (Nippon Gene, Japan) according to the manufacturer's protocol.

PBL were purified using a Percoll (1.09 g/ml) density gradient as described above. The PBL were cytospun onto glass slides, fixed in PBS containing 10% formalin for 10 min, washed in DEPC-treated water for 1 min, and then dehydrated in ethanol for 1 min. Some slides were subjected to Giemsa staining to determine the cell type composition. Proteins were digested by treating the smears with proteinase K (2 μg/ml) for 15 min at 37 °C. The slides were then washed in glycine (2 mg/ml)—PBS for 10 min and immersed in acetylation buffer containing anhydrous acetic acid for 20 min. Prehybridization was performed in 50% formamide containing 4 × standard sodium citrate (SSC) at 45 °C for 30 min. For the hybridization, the cells were overlaid with 70 μl of antisense and sense mRNA probe solution (1 μg/ml) and then incubated in a moist chamber at 45 °C for 16 h. After washing with 4 × SSC, the glass slides were kept in RNase-NTE buffer (20 μg/ml) at 37 °C for 30 min. The slides were blocked with blocking buffer (1% Blocking Reagent [Roche Molecular Biochemicals] in 0.1 M Tris–HCl [pH 7.5] and 0.15 M NaCl) for 0.5 h. The slides were incubated for 1 h with anti-DIG-AP conjugate antibody (Roche Molecular Biochemicals) diluted 1:500 in the blocking solution. After washing with Tris–HCl buffer (0.1 M Tris–HCl and 0.15 M NaCl, pH 7.5), 150 μl of NBT/BCIP solution (Roche Molecular Biochemicals) diluted to 1:200 was reacted for 12–24 h at room temperature in the dark. Finally the reactions were stopped by immersing the slides in 10 mM Tris–HCl 1 mM EDTA for 10 min. The percentage of caauCD2f-positive cells and their cell types were determined by counting a total of 300 cells under a microscope.

2.4. Analysis of zebrafish CD2f genes

BLAST searches of the zebrafish genome database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were performed using the protein sequences of the caauCD2fs as queries. Sequences homologous to caauCD2f were retrieved from the database (reference only) or ESTs using TBLASTN. The zebrafish CD2f genes were then mapped on the chromosomes using NCBI Map Viewer Zv9 (http://www.ncbi.nlm.nih.gov/projects/mapview/) [33,38]. In addition, in order to investigate whether other CD2f genes are present, the TBLASTN analysis was performed using other mammalian CD2f members listed in Fig. 3. A multiple sequence alignment was generated using ClustalW (www.ddbj.nig.ac.jp/Welcome-j.html).

3. Results

3.1. Cloning and characterization of CD2f receptors and SAP from ginbuna crucian carp

RACE PCR employing specific primers yielded four different CD2fs (caauCD2f-1, caauCD2f-2, caauCD2f-3, and caauCD2f-4) from a single ginbuna carp. Protein domain predictions using SMART server showed that these caauCD2fs consisted of two putative Ig-like domains, a transmembrane, and a cytoplasmic tail (Figs. 1 and 2). Analysis using SMART could not determine whether the Ig domains could be classified as Ig-V or Ig-C2. The extracellular domains of the four caauCD2fs shared 83–94% identity at a nucleic acid level, and encoded single ORFs of 338 (caauCD2f-1), 321 (caauCD2f-2), 256 (caauCD2f-3), and 258 (caauCD2f-4) amino acids (Figs. 1 and 2). A BLAST search with caauCD2f as a query showed high homology to the mammalian CD2f (CD48, Minimum E-value 9E-12; CD84, 9E-09; CD244, 7E-07; and CS1, 3E-06) and zebrafish predicted proteins (LOC100333142). Pairwise alignment using sequences of mouse CD2fs indicated that the extracellular domains of the four caauCD2fs share 20–25% identity and 48–54% similarity with CD48 and CD244 (Table 2). In a phylogenetic tree drawn with the available CD2f sequences, all the caauCD2f isoforms cluster together on their zebrafish homologs, creating a distinct clade from other mammalian CD2f members (Fig. 3).

The cytoplasmic regions of caauCD2f-1 and caauCD2f-2 possess three and two ITSM motifs (TxYxxV/I), respectively, whilst caauCD2f-3 and caauCD2f-4 have no ITSM motifs. caauCD2f-2 possesses a "TVYSTL" sequence that is similar to ITSM in the cytoplasmic tail (Figs. 1 and 2). The cytoplasmic tails of caauCD2f-3 and caauCD2f-4 are short (12 aa) and both contain a conserved tyrosine and several charged residues, but they do not share significant identity.

To obtain the sequence of a putative adaptor protein binding to ITSM in caauCD2f, we searched for SAP in an EST library constructed from the kidney and spleen of a ginbuna crucian carp. Consequently, an EST clone (FS999395) with an open reading frame encoding the putative SAP was found. An amino acid alignment comparing the SAP sequence to mouse and human SAP is shown in Fig. 4. The alignment indicates that *caau*SAP has a SH2 domain that is known to bind to ITSM in mammals. A signal peptide is not present in the sequence of caauSAP, indicating that caauSAP is an intracellular protein like human and murine SAP.

3.2. Expression of caauCD2fs and SAP mRNA in leukocytes

The expression pattern of caauCD2f mRNA in adherent and non-adherent cells is shown in Fig. 5. The cells that adhered to the plastic plate mostly comprised monocytes (more than 89%) and the non-adherent cells comprised lymphocytes (71–75%). caauCD2f-1 and caauCD2f-4 were predominantly expressed by the non-adherent cells, indicating that these caauCD2f isoforms are expressed by lymphocytes. In contrast, caauCD2f-3 mRNA was predominantly expressed by the adherent cells. Also, although caauCD2f-2 mRNA expression in three fish was low or undetectable, it seems that caauCD2f-2 was expressed by both populations. SAP was only expressed by the non-adherent cells, suggesting the co-expression of SAP and caauCD2f-1/caauCD2f-4 in a similar cell population. Collectively, caauCD2f-1, caauCD2f-4, and SAP are dominantly expressed in lymphocytes, and caauCD2f-2 and caauCD2f-3 is expressed by lymphocyte as well as monocytes/macrophages.

RT-PCR analysis using templates from the purified lymphocyte subsets showed that the caauCD2fs were expressed by various lymphocyte subsets (Fig. 6). caauCD2f-1, caauCD2f-2, and caauCD2f-3 were expressed by CD8α- and Ig-positive cells, suggesting that they

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                    Signal peptide                Ig domain
caauCD2f-2      MALNHI-LRLLLCLLTFKTGFSAEISVFVQTGASVQLDIQTQQLPEFELLLWMNNKSESI
caauCD2f-3      MALNHI-LRLLLCLLTFKTGFSAEISVFVQTGTSVQLDIQTQQLPEFDLLFWMNDKSEGI
caauCD2f-1      MTVNRL-LFLLIFIWTSKTGFSAEISVFVQTGASVQLDIQTQQLPEFDILAWTNDKSDSI
caauCD2f-4      MLVNHLELLFLIFLLTFKTGFSAEISVFVQTGDSVQLDIQTQ--EEFEVLYWSNDKSENI
                * :*:: * :*: : * *************** *********   **::* * *:**:.*

                              Ig domain
caauCD2f-2      VRYNSDSKRVTPHDSYKDRVVFNDVTFSLTLKNMQKTDSGLYTARTIGSITRDLVSYRVS
caauCD2f-3      VRYNSDSKRVKPLDSYKDRVVFNDVTFSLTLKNMQKNDSGLYTARISGSINKDLVSYRVS
caauCD2f-1      VRYNSDSKRVTPHDSYKDRVFFNDATLSLTLKNMQKTDSGLYTARTIGLKTTDLVSYRVS
caauCD2f-4      VRYTSETKGVKLHPSYKDRVDFNAQTFSLTLKNMQKTDSGLYTARTSGQLDKNIVTYRLT
                ***.*::* *.   ****** **  *:*********.********  *    ::*:**::

                                  Ig domain
caauCD2f-2      VIDSVEAPVLTVNSNWSSSDSCTVNFTCRAQELMINSSYQNNRCSPQEVTSQINTLILNC
caauCD2f-3      VIDSVEAPVLTVNSNWSSSDSCTVNFTCRAHELMINSSYQNNRCSPQEVTSQINTLTLDC
caauCD2f-1      VIDSVEAPVLTVNSNWSSSDSCTVNFTCRAHELMINSSYQNNRCSPQEVTSQINTLILDC
caauCD2f-4      VIDAVEAPVLTVISNRSSSDSCTVNFTCRTHELKIDSSYQNNSCLREEVTSQINTLILDC
                ***:******** ** *************::** *:****** *  :********* *:*

                                                        Transmembrane
caauCD2f-2      SEEYIICNHSNPVSWNEDRINIKELCIYKE-NELNNVQTVSSLVWPVVCSVALWILIC-L
caauCD2f-3      SKEYIICNHSNPVSWKQDRINIKELCIYKE-DELNNDQSVSSLLWPVVGSVALCILS--L
caauCD2f-1      SDEYIICNHSNPVSWKQDRINITRLCDDK--NGIS--KSDSSRLAIGVGSVAGCIFLLCV
caauCD2f-4      SEKFIICNNSNTVSWKEDIIIITQLCGDNKKNNSKNVQIASSFLGPVVAPVAVPVIILII
                *.::****:**.***::* * *..**  :  :  .  :  ** :   * .**  ::   :

                                                Cytoplasmic tail
caauCD2f-2      LVFLYFKYKKGAPEEVEGNTIYAQVEAEDQ-------------------TQEKTGNDRPI
caauCD2f-3      LVFLYFKYKKGMSMLPHSL
caauCD2f-1      SAAVCFKYKKGAPEEVEGNTVYAQVEAQEMQKLSSDCNPYDIPDRVQAETQEETGDKQPS
caauCD2f-4      ICTSVFLYRRHKKKDVSQE----------------------------------------
                     * *::

caauCD2f-2      TVYWTLGQHKKPTIPNETDHTVYSEVHKHPNEKLSAISADDT
caauCD2f-1      TTYCTVGQHQNPPVHSETDHTIYSAVFKQQNKKPPVISTSDT
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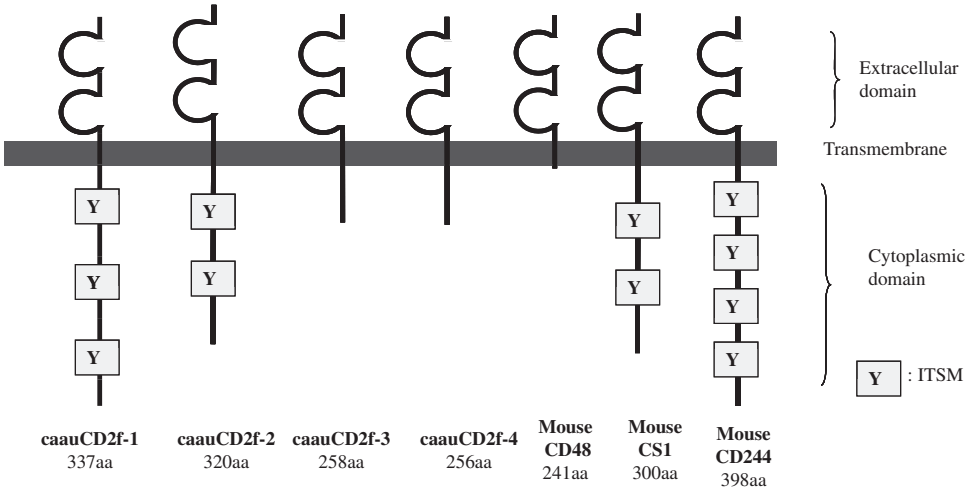
Fig. 1. Multiple amino acid sequences of caauCD2fs. The domains corresponding to the putative signal peptide, IgSF domain, transmembrane, and cytoplasmic regions are shown within the arrowed lines above the sequences. Sequences that are underlined indicate ITSM motifs or sequences similar to an ITSM motif (TVYSTL, underlined with dots). Gaps in the alignment are shown by dashes, conserved or similar amino acids are shown with an asterisk (*) or dot and colon (. and :), respectively.



	caauCD2f-1	caauCD2f-2	caauCD2f-3	caauCD2f-4
caauCD2f-1	-	90.4	89.7	83.5
caauCD2f-2	90.4	-	94.6	83.2
caauCD2f-3	89.7	94.6	-	83.2
caauCD2f-4	83.5	83.2	83.2	-

Fig. 2. Structure of caauCD2f and mouse CD2f. Boxed "Y" indicates ITSM motifs. The numbers shown below each receptor indicate the amino acid number of ORF. The two loops of the extracellular domain indicate IgC2 and IgV of mouse CD2fs or putative Ig-like domains of caauCD2fs, respectively. Table shows identities of each caauCD2f at the nucleic acid level.

are produced in cytotoxic T-cells (CTLs) and B-cells, but not helper T-cells (Th cells). Meanwhile, caauCD2f-4 was expressed by Th cells in addition to CTLs and other lymphocytes. caauCD2f-3 mRNA was detected in CD8- and CD4-negative lymphocytes, suggesting that it is expressed on B cells, monocytes/macrophages and NK cells. *In situ* hybridization analysis showed that a part of the round nuclear cells (probably lymphocytes) and the irregular cells with large cytoplasm (probably monocytes or neutrophils) were caauCD2f-positive (Fig. 7). Hybridizations using a probe capable of detecting all the caauCD2f isoforms showed that caauCD2f-positive cells comprised approximately 17% PBL, and an isoform-specific probe for caauCD2f-1 detected approximately 8.0% of the positive cells. This result indicates that caauCD2f-1-positive cells are a part of the caauCD2f population.

Table 2
Amino acid identity/similarity (%) of caauCD2f among murine CD2 family receptors and cyprinoid NITR.

	caauCD2f-1	caauCD2f-2	caauCD2f-3	caauCD2f-4
CD48	24.5/53.5	22.5/50.1	22.5/50.0	20.5/50.7
CD244 (2B4)	25.3/54.3	22.5/50.0	22.0/48.5	21.5/51.0
CD319 (SLAMF-7)	19.4/42.2	19.4/46.4	20.4/45.5	18.5/40.7
CD229-1[a]	19.0/45.2	19.3/42.1	20.9/45.1	22.1/44.7
CD229-2[a]	18.5/40.5	20.2/40.4	19.2/41.2	18.3/40.4
CD84	18.8/44.6	17.1/41.0	18.1/41.5	16.2/39.1
CD150	16.4/38.5	12.3/37.2	14.4/36.7	12.7/38.6
NTB-A(SLAMF-6)	13.7/46.3	13.2/46.4	14.4/46.0	14.7/45.0
CD2	12.8/36.0	16.0/37.9	14.0/39.8	14.2/36.3
zebrafishNITR	14.2/37.8	17.1/39.4	15.7/36.6	12.8/34.4
carpNITR	14.2/35.1	16.1/37.4	15.6/38.6	11.9/37.6

[a] CD299-1or CD299-2 is a sequence that contains only first and second or only third and fourth Ig-domains, respectively.

3.3. Genomic organization of the zebrafish CD2f genes

To investigate the genomic organization of the teleost CD2f, we surveyed the zebrafish genome database using BLAST searches with the extracellular domain of caauCD2f-1 as a query. As shown in Fig. 8, six sequences from the Ig domain were found on chromosome 1 (Zv9_scafold 51) and 29 of the sequences on chromosome 2 (Zv9_scafold 229 and 231), forming clusters within 0.6 Mbp in each chromosome. Searches performed using the sequences of caauCD2f-2, caauCD2f-3, and caauCD2f-3 yielded similar results (data not shown). The zebrafish sequences (zfCD2f-1.2) had 39–94% sequence similarity and 39–86% identity with the caauCD2f-1 sequence. Two putative Ig-like domains in most gene sets were separated with an intron. Synteny conservation was not found between the mammalian CD2 f and zfCD2f-1.2.

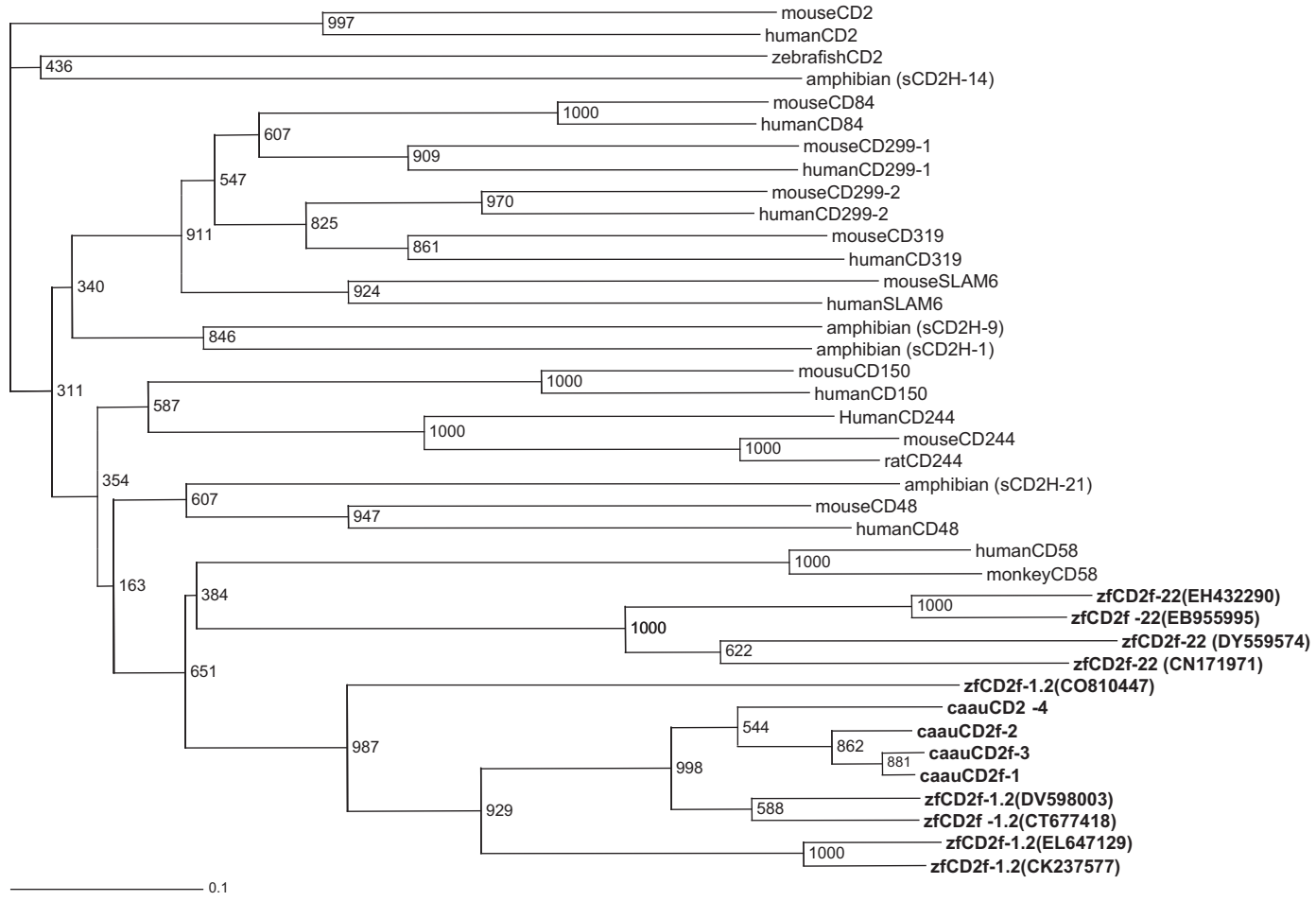


Fig. 3. Phylogenetic tree of CD2f of the zebrafish, ginbuna cursian carp, and mammalian CD2 families. This unrooted tree was constructed by the neighbor-joining method, based on the amino acid alignment (ClustalW) of the extracellular domains. Node values represent bootstrap analysis of 1000 replicates. Teleost CD2f is shown as bold letters. The teleost CD2f accession numbers are noted in the text. Other accession numbers are as follows: mouse CD2 (NM_013486), human CD2 (M16445), mouse CD48 (NM_007649), human CD48 (CR457012), rat CD48 (NM_139103), human CD58 (AL355794), monkey CD58 (XP_002810420), human CD84 (CR541847), mouse CD84 (AB196814), human CD150 (AY040554), mouse CD150 (AF149792), human CD244 (AJ245377), mouse CD244 (DQ167571), rat CD244 (AF156989), CD299 (AF244129), mouse CD299 (AF244130), human CD319 (NP_067004), mouse CD319 (AB196837), human SLAM6 (NM_001184714), and mouse SLAM6 (NP_109635). Sequences of amphibian CD2-like genes (sCD2H) are described in the electronic supplementary material (ESM1) of Ref. [10].

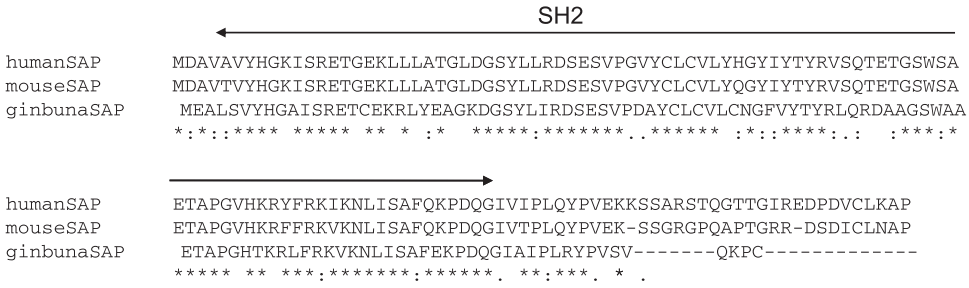


Fig. 4. Multiple amino acid sequences of caauSAP and the mammalian SH2 domain are shown within the arrowed lines above the sequences. Gaps in the alignment are shown by dashes, conserved or similar amino acids are shown with an asterisk (*) or dot and colon (. and :), respectively.

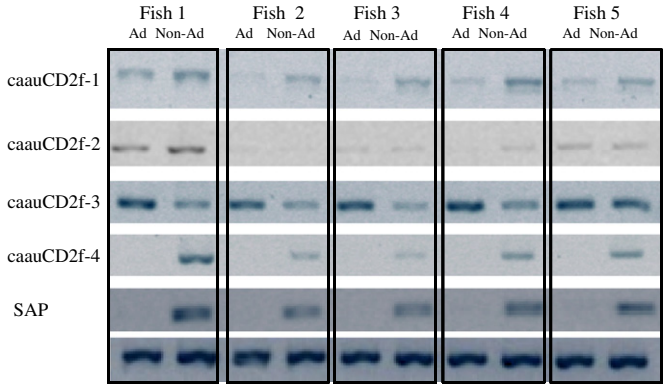


Fig. 5. The expression of caauCD2f and SAP genes in adherent (Ad) and non-adherent (Non-Ad) cell fractions of PBL. The housekeeping gene, EF1-α, was used as an internal control. Results were obtained from five different fish.

According to the sequence similarity of their Ig-domain sequences, the zfCD2f-1.2 genes were classified into eight subgroups (I-VIII). As shown by their alignment (Fig. 9), the sequences showed high identity (78.4–97.8%) within each group, and several pairs (group II and VII) located on both chromosomes 1 and 2 shared substantially high (74.3–78.2%) identity (Fig. 9). Homologous sequences to the genes in each group were found in the EST database (EL647129, CT677417, DV598003, CK237577, CO810447, and CT677418).

In addition, BLAST analysis using mammalian CD2f members as queries showed that additional homologs to CD2f genes (zfCD2f-22) cluster on chromosome 22. The phylogenetic tree showed that these CD2f-22 genes formed a distinct clade from caauCD2fs and zfCD2f-1.2 genes (Fig. 3). The EST clones encoding to the zfCD2f-22 (EB955995, EH432290, CN171971, and DY559574) showed high homology to the mammalian CD2f, such as CD48, CD84, and CD244. As shown in Fig. 3, all the EST sequences formed a clade with teleost CD2f, indicating that the indentified CD2f isoforms diversified in cyprinid fishes. The teleost CD2f genes are not grouped into the same cluster as any of the amphibian CD2f genes described in recent reports [10].

CD2-homologous sequences were found in the zebrafish genome database (NW_003039148) and EST libraries (DT061500, EB987025, CO813765, and EB977172). Three CD2-homologous genes formed a small cluster on a different locus apart from the CD2f clusters in chromosome 1 (Zv9_scaffold12). In a phylogenetic tree analysis, the zebrafish CD2 genes were classified into a different group from the teleost CD2fs and other mammalian CD2f members (Fig. 3).

4. Discussion

It is believed that diversification of IgSF has generated an extremely complex set of proteins with a huge variety of roles including cell–cell interactions and immune functions [8,22,27]. The CD2f, belonging to IgSF, consists of more than 10 cell surface molecules that are predominantly expressed on hematopoeitic cells and involved in various immune responses. In the present study, we identified several teleost CD2fs that possess two, three, or no ITSM motifs in their cytoplasmic tail, and showed that they are differentially expressed by different leukocytes. Multiple CD2f genes are clustered together and at least 35 Ig-like domains corresponding to caauCD2f are present in the zebrafish genome 1 and 2. Although it remains unclear how many CD2f receptors are functional in zebrafish, several zebrafish CD2f genes are indeed functional, as proved by the appearance of a corresponding zebrafish EST. The phylogenetic tree indicates that these CD2f genes are evolutionally distinct from amphibian CD2f genes. Although it is difficult to conclude that all types of CD2f genes in zebrafish and ginbuna crucian carp have been found in the present study, these findings suggest that the identified CD2f receptors have uniquely evolved within cyprinid fishes.

SLAM family receptors, which are a subfamily of the CD2 receptors, contain multiple copies of the ITSM that recruit SAP [12,13]. The caauCD2fs have different number of ITSM motifs in their cytoplasmic tail, whereas the sequence similarity of the extracellular domains of the four caauCD2fs is very high. The extracellular domains of caauCD2fs show higher similarity to CD48, CD244, and CD319 compared with other CD2 family receptors. CD244 and CD319 are involved in the activation of NK cell-mediated cytotoxicity and possess four and two ITSM motifs, respectively [7]. CD48, a ligand of CD244, possesses no ITSM in its cytoplasmic tail [1,15]. caauCD2f-1 and caauCD2f-2 have two and three ITSMs, respectively, whereas no ITSM motif is found in the cytoplasmic tails of caauCD2f-3 and caauCD2f-4. Therefore, comparison of the primary structures and the presence of ITSMs in caauCD2f and the mammalian CD2 family indicates that caauCD2f-1 and caauCD2f-2 may have functional similarity to CD244 and/or CD319 and caauCD2f-3 and caauCD2f-3–4 may correspond to CD48. The murine CD244 gene encodes two different isoforms (2B4 short and 2B4 long) that arise by alternative splicing, resulting in a different number of ITSMs in their cytoplasmic tails. The murine 2B4 isoforms have been shown to have opposing functions as the long form has an inhibitory function and the short form has an activating function [5,21,29]. However, it seems unlikely that the caauCD2f isoforms have arisen from alternative splicing because the sequences of their extracellular domains are not identical. As the caauCD2f isoforms possess different number of ITSMs, they may exhibit different functions depending on the number of ITSMs similar to murine CD244. Taken together with the results relating to the differential expression of the caauCD2f isoforms, each caauCD2f can be considered as a distinct receptor that has various functions.

In mammals, CD2fs are displayed on various types of leukocytes, such as T-cells, B-cells, NK-cells, macrophages, and DC cells.

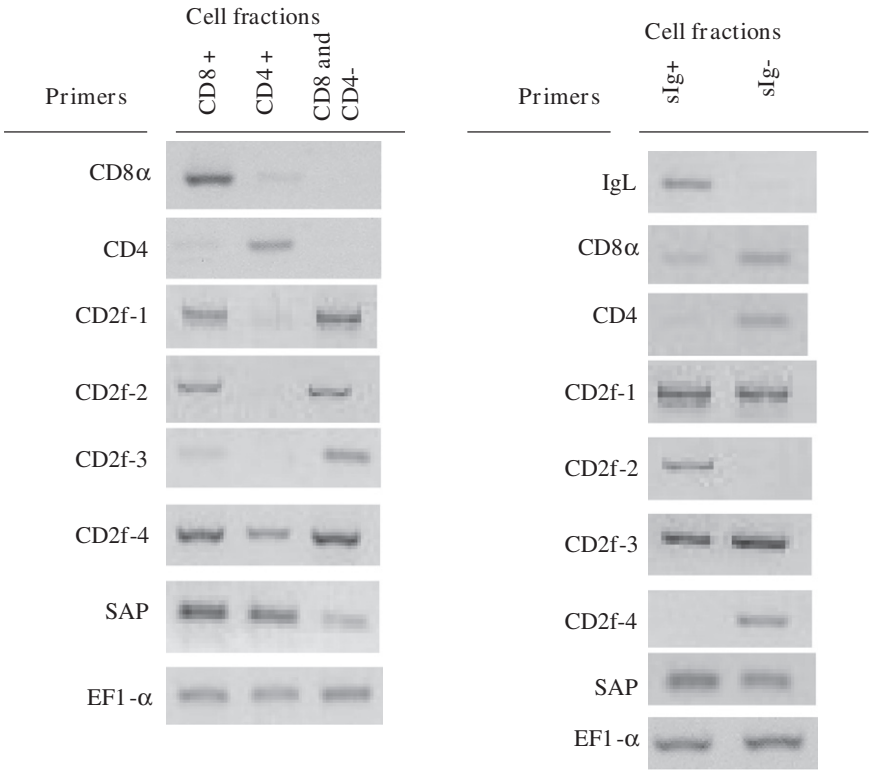


Fig. 6. Expression of caauCD2f and SAP genes in CD8α-, CD4-, and Ig-positive and negative cells. The housekeeping gene, EF1-α, was used as an internal control. Representative data from one of two fish is shown.

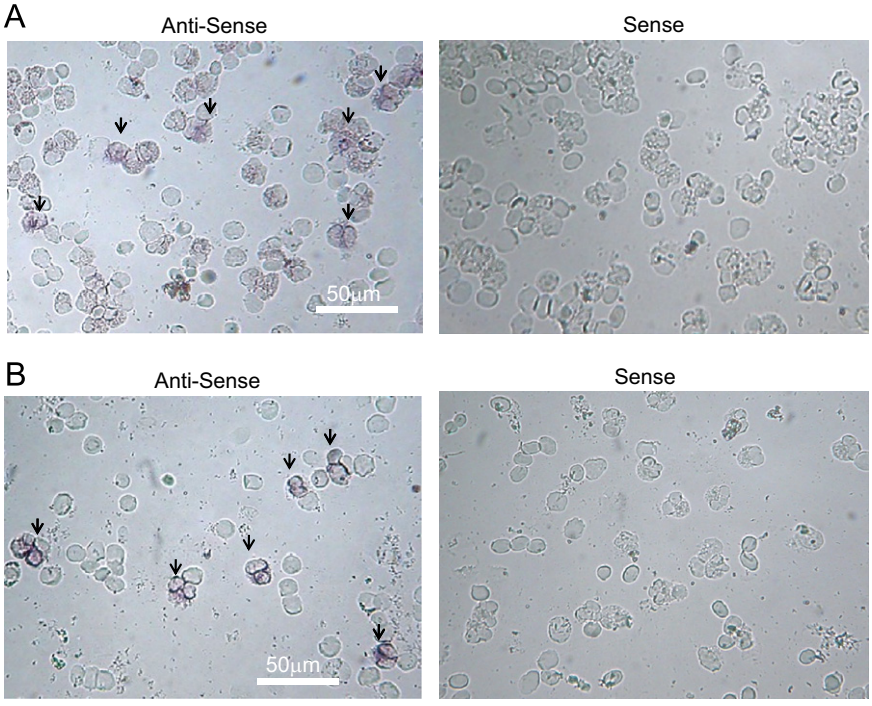


Fig. 7. Expression analysis of caauCDfs by *in situ* hybridization of PBL. caauCD2f-positive cells were detected by a probe that recognized all caauCD2fs (A) or only CD2f-1 (B). Left and right panels show the stained cells hybridized with antisense and sense probes, respectively. Arrows indicate *caau*CD2f-positive cells. All scale bars indicate 50 μm. Representative data from one of two fish analyzed is shown.

caauCD2f-1 and caauCD2f-2, which posses ITSM motifs, are dominantly expressed by CTL and other lymphocytes except for Th cells. In contrast, it is suggested that caauCD2f-4, which has no ITSM, is expressed by Th cells, while it is also expressed on other cells except for Ig-positive cells. Also, the expression pattern of caauCD2fs is different between adherent and non-adherent cells. These findings indicate that each caauCD2f has acquired distinct functions after divergence from their ancestral Ig-like receptors.

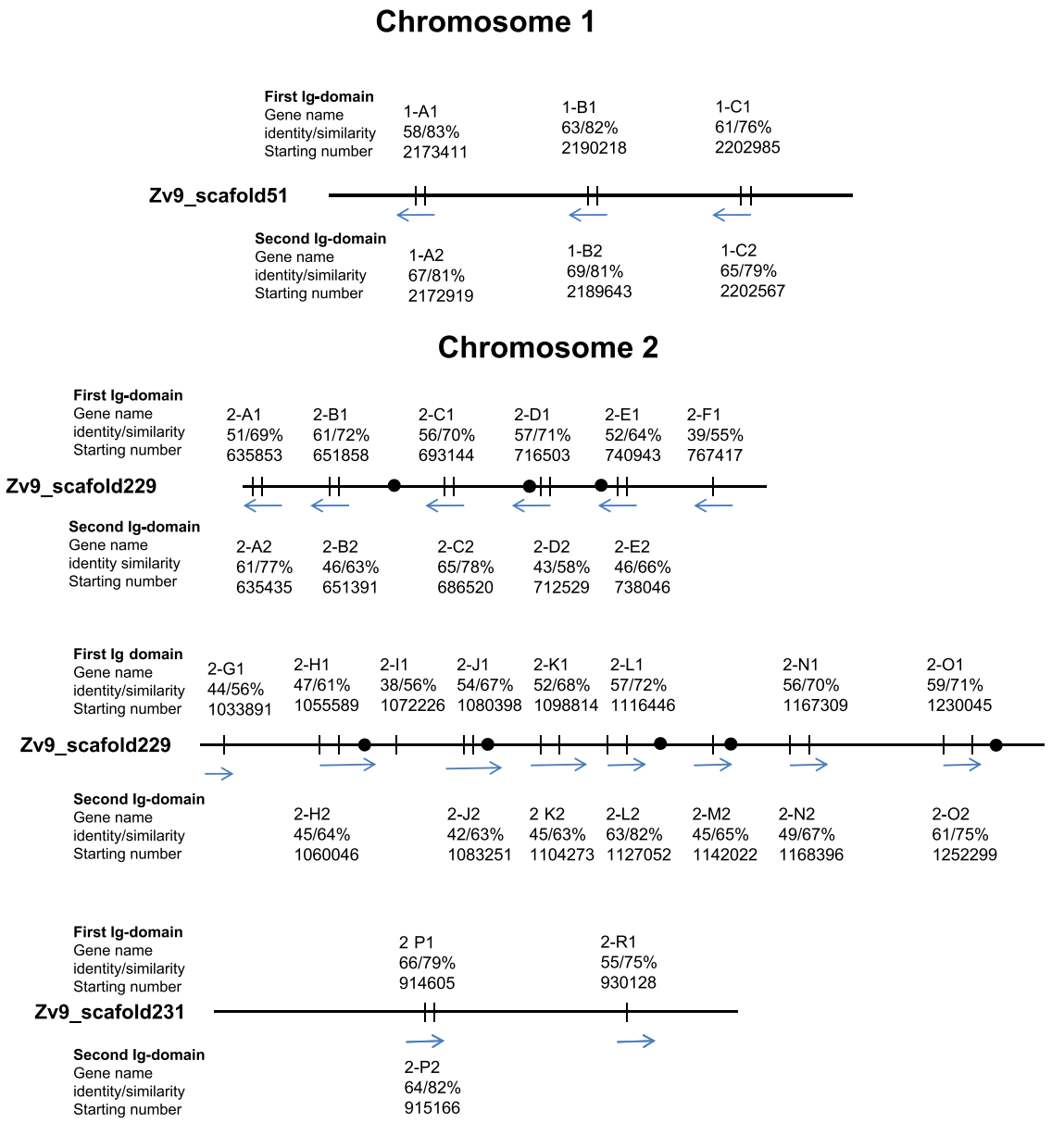


Fig. 8. Localization of CD2f genes on zebrafish chromosomes 1 and 2. Identities and similarities of zebrafish sequences to caauCD2f-1 are shown at amino acid level and subject (zebrafish genomic sequences) and query (caauCD2f-1) base numbers show BLAST hit regions on each zebrafish scaffold. Arrows indicate gene orientations. Black circles show cytoplasmic tails with ITSM motif.

Further functional studies at the cellular level employing this clonal fish and its cell markers would contribute to furthering our understanding of the functional differentiation of the four caauCD2fs.

The genes of the human CD2f were mapped into two clusters [8,22]. CD2 and CD58 are on the short arm of chromosome 1, separated from the other members, which are clustered together on the long arm of chromosome 1. Genomic analysis of zebrafish CD2f genes indicates that the two clusters of the CD2f genes are present on different chromosomes (chromosome 1 or 2). On the other hand, zebrafish CD2-like genes are located on a different locus to the CD2f genes. Three CD2-like genes formed a small cluster on this locus, suggesting that these zebrafish CD2 genes were generated through tandem gene duplications. These findings indicate that CD2 was already separated from the locus of other CD2f members before the emergence of the teleosts.

The genomic analysis of the zebrafish shows that the zfCD2f-1.2 gene clusters are present separately on different chromosomes (chromosomes 1 and 2). As the sequences of the CD2f genes on both chromosomes are quite similar, either locus could have arisen from a retrotransposition event. The retrotransposition of Ig domain-containing receptors has been already implied in teleosts. Yoder et al. [39] reported that part of the NITRs, which possess cytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIMs) and an immunoreceptor tyrosine-based activation motif (ITAM), arose by retrotransposition. These genomic sequences of CD2f and NITR indicate that IgSF genes may be expanded through transposition and that they acquired novel functions by the acquisition of ITSM, ITIM, or ITAM motifs. Above all, the expansion of zebrafish CD2f genes sharing quite high identity strongly supports the hypothesis that the IgSF genes were diversified and generated by successive gene duplication events. In addition, ITAM- or ITIM-containing IgSF receptors, such as leukocyte immune-type receptors (LITR) and novel immunoglobulin-like transcript (NILT), have been identified in several fish species [17,28,31]. It would be interesting to understand the

A

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Gene name                Chr. No.   multiple sequence alignment

                                                              I

zebraEST(EL647129)                  VFVQTEDSVQLDIKTQKLPEFEDLYWINDKSNSIVRYNSETTKVKLYPSYKDRVYFDNETFSLTLVNMQKTDSGRYTARITGESNKDIL
2-A1                          2     LFVQIGDSVQLNTQTQ LPEFEAVFWTSKKSEAIVRYLTESKKVIYQDSYKDRVDFNNKTFSLTLKNMQKADSGLYTARLFGETIKDII
2-B1                          2     VFVQTGDSVQLDIQAQ-LPEFEAVYWTNKKSDSIVRYLTESKKVTCHTSYKDRVDFNNETFSLTLKNMQKADSGLYTAKIFGETNKDIV
                                    :***  *****: ::* ***** ::* ..**::**** :*:.**      ****** *:*:****** ****:*** ***:: **: ***:

                                                             II

zebraEST(CT677417)                  FSAEITVFVQTDS-VQLDLQTQQLPEFDDLFWINDKYEYIVKFSNEYKKIKTYSSYQNRIEFNETSFSLTLKNMQKADSGRYTARINRDLDKDM
1-B1                          1     FSAEITVFVQTGD-SVLDLQTQQLPEFDDLFWINDKYEYIVKYSNEYKKIKTYSSYQNRIEFNETSFSLTLKNMQKADSGRYTARINRDLDKDM
1-A1                          1     FSAEITVFVQTGD-SVLDLQTQQLPEFDDLFWINDKYEYIINYSNEYKKIKTYSSYQNRIEFNETSFSLTLKNMQKADSGRYTARINRDLDKDM
2-P1                          2     FSAEISVFVQTGDSVQLDLQTQPLPEFDYLSWINDKSENIVRYLHESKMIRYHLSYQNRIEFNETSFSLTLKNMQKADSGLYTARISGDLMKDI
                                    *****:*****..   ****** ***** * ***** * *:.: :* * * : :************************* *****. ** **:

                                                            III

zebraEST(DV598003)                  SVFVQTGDSVQLDLQTQPLPEFDDLFWINDKSGNIVKYFHEYKKIRRHPSYQNRIEFNETSFSLTLKNMQKADSGRYTARIIG
2-L1                          2     SVFVLTGDSVELDIQTKL-PEFEELTWIYNKLESIVKYYSKFNIVNSYPAYNNRAIFNETTFSLILENMQKKDSGLYTARING
2-K1                          2     SVFVLTGDSVELDIQTKL-PEIEELTWIYNKLESIVKYYSKFNIVNFYPAYNNRAKMNETTFSLILENMQKKDSGLYTARISG
2-D1                          2     SVFVLTGDSVKLDIQTK--PEFEELTWINTKLESIVKYYSKFKDVNSYPAYNNRAKFNENTFSLILENMQKNDSGLYTARISG
                                    **** *****:**:**:  **::** **  *  .****: ::: :. :*:*:**  :**.:*** *:**** *** ***** *

                                                             IV

zebraEST(CK237577)                  FSAEISKFVQTGASVQLDLQTQPLDLDEITWKNEKTEKVFKYNYETKKIRPYPPYQDRIEFNESSFSLTLKNMQKTDSGLYTVTITGASS
2-O1                          2     FSAEISKFVQTGASVQLDLQTQPPELLYITWKNDKTEKVFNYIHVSKTKTSFPPYQDRIEFNESSFSLTLKNMQKTDSGLYTATISGTSS
2-N1                          2     FSAEISKFVQTGASVQLDLQTQPPELHYIIWRNDKTEKVFNYFPVAQMKTFFPPYQDRIEFNESSFSLTLKNMQKTDSGLYTVTIGGTSS
                                    ********************** :*  * *:*:******:*   ::    :*****************************.** *:**

                                                              V

zebraEST(CO810447)                  VFVQTGQSVKLDPIKTLSGLSRFAWIKDPNANIVRFYNDSNAIKYHSLYKERAKFNNQTFTLMLKNMQKNDGGNYTAKASGQKETVVATY
2-I1                          2     VFVQTGQSVKLDPNKTLSGLSRFAWIKDPNANIVRFYNDSNAIKYHSLYKERAKFNNQTFTLMLKNMQKNDGGNYTAKASGQKETVVATY
2-E1                          2     VFVQTGKSVKLDPNKTLSGLSRFVWVKDPNANIVRFYNDSKEIKYHSSHNDRVEFNTQTFSLTLKNMQKNDSGKYTAKASGQKETVVAAY
                                    ******:****** *********.*:***************: ***** :::*.:**.***:* ********.*:***************
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B

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Gene name                Chr. No.

                                                             VI

ESTclone(EL647129)                  DEVEAPVLTVNSNGSSSELCSFTCSGQNMNISSIYNSSSCPEEEVTSADDHTLRLNCNATFIMCNYSNPLSWKTDIKTVHEL
2-A2                          2     DEVEAPVLTVNLNCSSSELCSFTCSAHNMNISSIYNSSSCPEEEVTSADDHTLRLNCNATFIMCNYSNPLSWKTDIKTVHEL
2-B2                          2     DEVEAPVLTVNSNCSSSELCSFTCSGQNTNISSIYNSSSCPEEEVTSADDHTLRLNCNATFIMCNYSNPLSWKTDIKKVHEL
2-D2                          2     DEVEAPVLTVKSNCSSSELGSFTCSGHNMNISSIYNSSSCPEEEVTSANDHTLRLNCNATFIMCNYSNPVSWKTDIK-VHEL
2-K2                          2     DEVEAPVLTVKSNGSSSKLCSFTCSGQDITMSSIYNSSNCPEEEETSADDHTLRLNCNSTFITCNYSNPVSWKTDIKKVHEL
2-N2                          2     DEVEAPVLTVNSNCSCSELCQFSCSGHNINISSIYNSG-CSKEEVTSADDHILRLNCNTTFIMCNYSNPVSWKTNIKAVHEL
                                    **********: * *.*:* .*:**.:: .:******. *.:** ***:** ******:*** ******:****:** ****

                                                            VII

zebraEST(CT677418)                   DAVEAPVLTVNSI--SSDSCTVNFTCRAQDLIIN SSYQNNNCSPEEVTSQQINTLILF  CSEESIFCNYSNPVSWKKE
1-B2                          1      DAVEAPVLTVNSI--SCDSCTVNFTCRAQDLIINNSSYQNNHCSPEEVTSQQINTLILF--CSEESIFCNYSNPVSWKKE
1-A2                          1      DAVEAPVLTVNSI--SSDSCTVNFTCRAQDLIINNSSHQNNHCSPEEVTSQQINTLILF--CSEESIFCNYSNPVSWKKE
1-C2                          1      DAVEAPVLTVNSI--SSDSCTVNFTCSAQDLIINNSSYQNNQCSPEKETSQQINTLILF--CSEESIFYNYSNPVSWKKE
2-P2                          2      DAVEAPVLTVNSI--SSDSCTVNFTCSAQDLIINNSSYQNNQCSPEKETSQQINTLILF--CSEELIICNYSNPVSWKKE
2-O2                          2      DAVEAPVLTVNSN--SSDSCTVNFTCKTQDLIIN-SSYQINACSPEKVISQKNYTLILSLHCSEESIICNYSNPVSWKED
2-L2                          2      DAVKAPVLTVNSIMFSSNSCTVHFTCRAQDLIIN-SNYQNNTCSQEEVTSQENNTIILS--CSEELIMCNYSNPVSWKKD
2-C2                          2      DAVEAPNLTVNSN  SSDFCMVNFTCRAQDLIIN SSYQNNQCSPEEATLQEMYTLNLS--CSEKSIICNYSNPVSWK--
                                     ***.:* *****   *.: * *:*** :*.***: *.:* * ** *:   *:  *: *   ***: *: *********

                                                           VIII

zebraEST(CO810447)                   DAVDSPVLHEKLIMISVNSCIVDVSCSAHTLQLNNRYYSNNCSQEEVTSSEMQTLTLYCIGKVVVCNYSNPVRRE
2-H2                          2      DAVDSPVLHEKLIMISVNSCIVDVSCSAHTLQLNNRYYSNNCSQEEVTSSEMQTLTLYCIGNVVVCNYSNPVSWK
2-M2                          2      DAVDSPVLHEKSIMISVNSCIVDVSCSAHTLQLNNRYYSNNCSQEEVTSSEMETLTLYCIENVVVCNFSNTVSWK
2-E2                          2      DAVDSPVLHDKSIMISVNSCIVDVSCSAHTLQLNNTSYNNNCSQEEVTSSKMETLTLYCIENVVVCNFSNPVSWK
2-J2                          2      DAVDSPVLNRNFTMISVNSCIVDVSCSAHSFQLNNQYYSDNCSQEEVASSEMQTLTLYCIENVVVCNYSNPVSWK
                                     ********: : ****************::**** *.:**** ***:**:*:******* :*****:**.* :
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Fig. 9. Multiple amino acid sequences of zebrafish CD2f from genomic and EST databases. The groups were classified based on sequence similarity. A partial sequence of putative first Ig domain (A) or second Ig domain (B) are shown separately in A and B, respectively. An EST clone with the highest identity (> 79%) to the genomic sequences has been added to the alignment of each group. The gene names correspond to those in Fig. 8. Gaps in the alignment are shown by dashes, conserved or similar amino acids are shown with an asterisk (*) or dot and colon (. and :), respectively.

phylogenetic relationship between CD2f and these diverse receptors. Since the identified CD2fs share considerably high identity compared to other IgSF that is known so far, they may have been generated from an ancestral Ig-domain gene by more recent duplication event. Further functional analysis of teleost CD2f will advance our understanding of IgSF evolution and diversity.

Acknowledgments

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