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Cloning of Japanese flounder *Paralichthys olivaceus* CD3 cDNA and gene, and analysis of its expression

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**Abstract** Two distinct *CD3* homologue cDNAs, *CD3-1* and *CD3-2*, were isolated from a Japanese flounder leukocyte cDNA library. *CD3-1* consisted of 961 bp encoding 178 amino acid residues, and *CD3-2* consisted of 927 bp encoding 182 amino acid residues. The two deduced amino acid sequences had an identity of 95.1%, and neither had N-linked glycosylation sites. The identities between the Japanese flounder *CD3* and previously reported *CD3* (CD3γ, CD3ζ, or CD3δ) of *Xenopus laevis*, chicken, and various mammals were approximately 25%. The Japanese flounder *CD3* had an extracellular domain, a CXXCXE motif, and an immunoreceptor tyrosine-based activation motif (ITAM), each of which are important characteristics of *CD3* chains. Furthermore, the positions of four cysteine residues in the extracellular domain were preserved in both of the Japanese flounder *CD3* s. A phylogenetic tree based on the amino acid sequences confirmed that the Japanese flounder *CD3* s are closer to *CD3ε* than to *CD3γ* and *CD3δ*. However, the gene structure of Japanese flounder *CD3* is identical to the chicken and *Xenopus CD3γ/δ* genes and the mammalian *CD3δ* gene. Southern blot hybridization and the DNA sequence of the *CD3* gene of homologous Japanese flounder indicated that the *CD3* gene exists as a single copy. Southern blot hybridization also showed the presence of a polymorphic variant of Japanese flounder *CD3*. An RT-PCR analysis detected Japanese flounder *CD3* mRNA in several organs that contained lymphocytes. The proportion of *CD3*-positive cells in the peripheral blood leukocytes was 34.9%.

**Keywords** Japanese flounder · *CD3* · cDNA · Gene · ITAM · Leukocyte

**Introduction**

The antigen recognition signal from the major histocompatibility complex (MHC) is recognized by a T-cell receptor (TCR) in a CD3-TCR complex on T-cell membranes, from where the signal is transmitted to the inside of the cell (Ashwell and Klausner 1990; Klausner et al. 1990). CD3 is indispensable for the expression of the *TCR* genes (Dave et al. 1997; Haks et al. 1998). CD3 has been classified into CD3γ, CD3δ, and CD3ε chains. The sequence and structural homology of the human and mouse CD3γ, CD3δ, and CD3ε genes, and their chromosomal proximity suggested that they arose by duplication of a common ancestral gene. Glycosylation sites are present in the extracellular domain of the CD3γ and δ chains, but not in the extracellular domain of the ε chain. The CD3γ, δ, and ε chains are members of the immunoglobulin superfamily which have the immunoreceptor tyrosine-based activation motif (ITAM), an extracellular CXXCXE motif, and similarly positioned cysteine residues involved in disulfide bonds (Gold et al. 1987; Williams and Barclay 1988).

Nonmammalian *CD3* homologues have been identified in only chicken and *Xenopus* (Berlot and Auffray 1991; Dzialo and Cooper 1997; Göbel and Dang 2000; Göbel and Fluri 1997). CD3γ/δ chains with a structure similar to the structures of both the γ and δ...
chains of mammals have been reported in chicken and *Xenopus* (Bernot and Auffray 1991; Dzialo and Cooper 1997; Göbel and Dangy 2000). The *CD3γ/δ* gene is thought to be an ancestral gene of the mammalian *CD3γ* and *CD3δ* genes (Göbel and Dangy 2000). A chicken CD3δ has also been reported (Göbel and Fluri 1997). However, there have been no reports of CD3 in fish. A comparative analysis of CD3 proteins should include sequences of lower vertebrates such as those of fish for understanding the important amino acid residues and structural features determining the functions of the CD3-TCR complex. Recently, we cloned a partial cDNA fragment of a CD3 homologue by an expressed sequence tag (EST) analysis of Japanese flounder leukocytes (Nam et al. 2000).

In this study, we cloned and sequenced two distinct CD3 cDNAs and a gene from Japanese flounder and characterized their expression patterns.

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**Materials and methods**

**cDNA library screening and sequencing**

The cDNA library used in this study has been previously reported (Aoki et al. 1999; Nam et al. 2000). The cDNA library was screened using a partial cDNA fragment of a CD3 homologue previously identified by an EST analysis (Nam et al. 2000) as a probe for isolation of full-length CD3 cDNA. Hybridization was done as previously reported (Aoki et al. 2000). cDNA clones were sequenced using Thermostable Sequenase (Amersham-Pharmacia) with M13 forward and/or M13 reverse primers and an automated DNA sequencer LC4200 (Li-Cor). Each determined sequence was compared with all sequences available in DDBJ/EMBL/GenBank using BLAST ver. 2.0 (Altschul et al. 1990, 1997).

Phylogenies were inferred using the PHYLIP program (ver. 3.5) (Felsenstein 1996), and by distance analysis using the neighbor-joining method. The values supporting each node are derived from 100 resamplings. The radial tree shown in Fig. 2 was created with TreeView software.

**DNA sequencing of a CD3 gene**

A CD3 gene was amplified from genomic DNA of homocloned Japanese flounder by PCR using a set of primers. The PCR primers used in this study were CD3-GF, 5'-etcaagagaacagaagaagtgc-3', and CD3-GR, 5'-tgcatcacagctgacatc-3'. An amplified DNA fragment was cloned and sequenced as described above.

**Southern blot hybridization**

Genomic DNA of a homocloned Japanese flounder and two wild Japanese flounders were isolated as previously reported (Hirono et al., 2000). The isolated genomic DNA was digested with *EcoRI*, *HindIII*, or *PstI*. Southern blot hybridization was conducted as described previously (Hirono et al., 2000).

**RT-PCR analysis**

Total RNA was extracted from healthy Japanese flounder thymus, peripheral blood leukocytes (PBLs), head kidney, trunk kidney, spleen, intestine, heart, liver, stomach, gill, and brain using Trizol (Life Technologies). The purified total RNA (10 µg) was treated with DNase and then reverse transcribed into cDNA using an AMV Reverse Transcriptase First-Strand cDNA Synthesis kit (Life Sciences). The final volume of the cDNA synthesis reaction was 25 µL. The reverse-transcribed sample (1 µL) was used in 50 µL of PCR reaction mixture. The PCR primers used in this study were CD3-F, 5'-catcagttgctgctg-3', and CD3-R, 5'-aggtgtcetgtaactt-3'. The β-actin primer set and TCRα were used for a positive control of RT-PCR (Katagiri et al. 1997; Nam et al. 2000). PCR was performed with an initial denaturation step of 2 min at 95°C, and then 20 cycles were run as follows: 30 s of denaturation at 95°C, 30 s of annealing at 55°C, and 1 min of extension at 72°C. The reacted products were electrophoresed on a 2.0% agarose gel.

**In situ hybridization**

Digoxigenin-labeled sense and antisense RNA probes for CD3-1 were generated with a T7 and SP6 Dig RNA labeling kit (Boehringer Mannheim) with digoxigenin-UTP (Boehringer Mannheim).

In situ hybridization was carried out using a commercial kit (Nippon Gene). PBLs were smeared on a glass slide, fixed in PBS buffer containing 10% formalin for 10 min, washed in DEPC-treated water for 1 min, dehydrated in ethanol for 1 min, pretreated with proteinase K (5 µg/ml) for 15 min at 37°C for protein removal, washed in glycine-PBS buffer (2 mg/ml) for 10 min, soaked in 100 mM triethylamine for 15 min for acetylation, immersed in anhydrous acetic acid for 20 min, and subsequently hybridized in 50% formamide with 4× standard sodium citrate at 42°C for 30 min. For the hybridization, the cells were covered with 100 µl of antisense mRNA probe solution (1 µg/ml) and then reacted in a moist chamber at 42°C for 16 h. After the hybridization, the glass slide was kept in RNase-NTE buffer (20 µg/ml) at 37°C for 30 min. The mRNA was detected with a DIG nucleic acid detection kit (Boehringer Mannheim) as described in the technical manual.

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**Results and discussion**

**Nucleotide sequences of Japanese flounder CD3 cDNA and gene**

Two distinct CD3 homologues, designated CD3-1 and CD3-2, were cloned. CD3-1 consisted of 961 bp encoding 178 amino acid residues, and CD3-2 consisted of 927 bp encoding 182 amino acid residues (AB044572 and AB044572). The identity of the deduced amino acid sequences of CD3-1 and CD3-2 was 95.1%. In the cases of chicken and mammals, CD3 chains (not including the γ chains) are thought to have arisen by duplication of a common ancestral gene (Göbel and Dangy 2000). However, the amino acid sequences of different pairs of CD3s are only 25% identical, although some amino acid sequence features are well conserved in CD3γ, δ, and ε of mammals or γδ and ε of chicken. The high identity of the two Japanese flounder CD3s suggests that they are alleles and not different types of CD3 chains.

The identities of the deduced amino acid sequences of both Japanese flounder CD3s to other known CD3s were low (27%) (Fig. 1). The amino acid sequence alignment (Fig. 1) indicates a conservation of the four