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CD4 and CD8 homologues in Japanese flounder, *Paralichthys olivaceus*: Differences in the expressions and localizations of CD4-1, CD4-2, CD8α and CD8β

Goshi Kato, Kana Goto, Ikuyo Akune, Shoichiro Aoka, Hidehiro Kondo, Ikuo Hirono *

Laboratory of Genome Science, Graduate School of Tokyo University of Marine Science and Technology, Konan 4-5-7, Minato-ku, Tokyo 108-8477, Japan

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ABSTRACT

CD4 and CD8 molecules are co-receptors of T cell receptors which interact specifically with MHC class II and I, respectively, during antigen presentation. Here we investigated CD4 and CD8 expression patterns in a fish, Japanese flounder, *Paralichthys olivaceus* in response to infection and tuberculin injection. The CD4-1 mRNA level was gradually and weakly increased in trunk kidney after infection with *Streptococcus iniae*, *Edwardsiella tarda* and viral hemorrhagic septicemia virus (VHSV), while the CD4-2 mRNA level was dramatically increased after *E. tarda* and VHSV infection, but not increased after *S. iniae* infection. CD4-2 mRNA but not CD4-1mRNA increased in the kidney during tuberculin response which is mediated by memory Th1 cells. The patterns for the change of mRNA level in CD8α and CD8β were similar to those of the CD4-2 during the infections and tuberculin response. Fluorescent *in situ* hybridization detected CD4-1 mRNAs on melano-macrophage centers and CD4-2 mRNAs at some cell clusters located near the melano-macrophage centers. CD8α and CD8β mRNAs were detected at the same cell clusters in the spleen and head kidney. These results suggest that CD4-1 and CD4-2 are expressed in different cells and that CD4-2-positive cells, rather than CD4-1-positive cells, have a main role in Th1-related immune responses collaborating with CD8α- and CD8β-positive cells in Japanese flounder.

1. Introduction

In mammals, T lymphocytes, distinguished from other lymphocytes by the expression of T cell receptors (TCRs), are divided into some subsets such as helper T (Th) lymphocytes and cytotoxic T lymphocytes (CTLs). Generally, Th cells and CTLs are defined by the expression of CD4 and CD8 glycoprotein, respectively. CD4 glycoprotein is a 55 kDa monomer expressed on the surface of Th cells and contains four immunoglobulin-like (Ig-like) extracellular domains, a transmembrane domain and a cytoplasmic tail which interacts with p56lck, an src-like tyrosine kinase (Maddon et al., 1985; Clark et al., 1987; Veilette et al., 1988). CD8 glycoprotein exists as either a hetero dimer consisting of an α-chain and a β-chain or a homo dimer consisting of α-chains on the surface of CTLs (Littman et al., 1985; Norment and Littman, 1988). CD8α and CD8β consist of an IgV-like extracellular domain and a transmembrane domain, whereas only the CD8α chain contains a p56lck tyrosine kinase motif in the cytoplasmic tail (Veilette et al., 1988). CD4 and CD8 glycoproteins participate in antigen recognition as co-receptors of TCRs, interacting with MHC class II by their N-terminal domains (domains 1 and 2) and with MHC class I by the IgV-like domain, respectively (Leahy, 1995). The Src kinase, Lck which binds to CD4 and CD8α, is recruited to the TCR-MHC complex in the antigen presentation to enhance the initiation of TCR signaling (Salmond et al., 2009).

In teleost fish, a CD4 homologue containing four Ig-like extracellular domains, designated as CD4-1, was first described in fugu, *Takifugu rubripes* (Suetake et al., 2004), followed by rainbow trout *Oncorhynchus mykiss* (Laing et al., 2006) and carp *Cyprius carpio* (Sun et al., 2007). Teleost fish have a second CD4 homologue, designated CD4-2, that contains two Ig-like domains (Dijkstra et al., 2006; Laing et al., 2006). Both CD4-1 and CD4-2 possess a p56lck tyrosine kinase motif in their cytoplasmic tail and their mRNAs are expressed predominantly in the thymus (Laing et al., 2006; Sun et al., 2007; Dijkstra et al., 2006; Picchietti et al., 2009; Patel et al., 2009). CD4-1 and CD4-2 mRNAs are expressed in IgM^- leukocytes (Laing et al., 2006; Edholm et al., 2007), whereas they are differentially expressed in catfish clonal T cell lines (Edholm et al., 2007). CD4-1^+ leukocytes in ginbuna crucian carp, *Carassius auratus langsdorfi*, share some characteristics such as morphology, tissue distribution and gene expression with mammalian $CD4^+$ leukocytes (Toda et al., 2011). However, the distribution of CD4-1 and CD4-2 in the teleost lymphocytes and the roles of CD4-1 and CD4-2-positive cells are unclear.

Teleost CD8α and CD8β homologues were first reported in rainbow trout (Hansen and Strassburger, 2000; Moore et al., 2005), where they were found to be single-copy genes. Subsequently they

* Corresponding author. Tel./fax: +81 03 5463 0689.
E-mail address: hirono@kaiyodai.ac.jp (I. Hirono).

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were identified in ginbuna (Somamoto et al., 2005) and fugu (Suetake et al., 2006). The nucleotide sequences of many teleost CD8 homologues are now available. The existence of $CD8^+$ CTLs in teleost fish was suggested by up-regulation of TCR and CD8α mRNA during viral infection and allogenic stimulation (Fischer et al., 2003; Somamoto et al., 2006). $CD8\alpha^+$ leukocytes isolated with a monoclonal antibody (mAb) showed high alloantigen-specific cytotoxicity against allogenic cells of the graft donor in ginbuna (Toda et al., 2009). In rainbow trout, the mRNA expressions of two CTL-related genes (perforin and granzyme) in flow-sorted $CD8\alpha^+$ lymphocytes suggest that the expression of CD8α defines CTLs (Takizawa et al., 2011). The authors also suggested that the CD8α and CD8β chains form a hetero dimer.

Japanese flounder, *Paralichthys olivaceus* is an important fish species for aquaculture in Japan. However, various infectious diseases caused severe financial losses in the aquaculture of Japanese flounder. Regarding the problem, a better understanding of its immune system should help to reduce diseases. In addition, Japanese flounder is also a good model for immune-related studies because a number of nucleotide sequences of its immune-related genes are now available. In this study, cDNAs and genes for CD4 and CD8 homologues were cloned from Japanese flounder, in order to investigate the distribution and roles of teleost CD4-1, CD4-2, CD8α and CD8β.

2. Materials and methods

2.1. Fish

Japanese flounder (*P. olivaceus*) obtained from Nisshin Marinetech (Atsumi, Japan) were kept in 500-L recirculating tanks until use. Tissues from adult fish weighing approximately 1.1 kg were used for cDNA cloning, gene cloning, RT-PCR and fluorescent *in situ* hybridization. Juvenile fish weighing approximately 13 g were used for the infection study and vaccination.

2.2. Total RNA, cDNA and genomic DNA preparation

Fish were dissected after anesthetization with 2-phenoxyethanol and tissues were collected and stored in RNA later (Ambion, USA) at −20 °C until use. Total RNA was extracted using RNAiso (TAKARA BIO, Japan) according to the manufacturer's instructions. First strand cDNA was synthesized with 2 μg of total RNA using MMLV reverse transcriptase (Invitrogen, USA), according to the manufacturer's instructions.

A blood sample was collected from an adult fish and genomic DNA was extracted from the blood according to Sambrook and Russell (2001).

2.3. cDNA cloning and sequence analysis of Japanese flounder CD4-1 and CD8β

The full lengths of Japanese flounder (jf) CD4-1 and jfCD8β were determined by 3′- and 5′-RACE PCR using a SMART RACE cDNA amplification kit (BD Biosciences, USA) according to the manufacturer's instructions. Since partial sequences for the jfCD4-1 and jfCD8β were found in the EST analyses, primers used in the 3′- and 5′-RACE PCR were designed based on the partial sequences (Table 1). PCR products were cloned into pGEM T-easy vector (Promega, USA) and sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) with a 3130xl genetic analyzer (Applied Biosystems). Putative signal peptide, transmembrane and cytoplasmic region was predicted using SignalP (http://www.cbs.dtu.dk./services/SignalP/) and TMHMM (http://www.cbs.dtu.dk./services/TMHMM/) software, respectively. N-linked glycosylation sites were predicted using NetNGlyc program (http://www.cbs.dtu.dk./services/NetNGlyc/). Immunoglobulin-like domains of jfCD4-1 and jfCD8β were predicted using InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/). Multiple sequence alignments were generated using ClustalW program in Molecular Evolutionary Genetic Analysis program (MEGA) version 5 (Tamura et al., 2011).

Table 1
Primers used in this study.

Primer name	Oligo nucleotide sequence
RACE-PCR	
CD4-1 5′-RACE 1st	5′-GTCAGAGATTGTCCAGGCAGCAGAG-3′
CD4-1 5′-RACE 2nd	5′-CAGAGGACCGACTGGGTTCAAACTC-3′
CD4-1 3′-RACE 1st	5′-CAGGTGTTGCAAATCATCTCCTCCC-3′
CD4-1 3′-RACE 2nd	5′-ATTGGTGGTGTACGCTGTACCAGGA-3′
CD8β 5′-RACE 1st	5′-AGGAACTGCAGGATGTTGGGATTGG-3′
CD8β 5′-RACE 2nd	5′-AGAAACAGTCAACAGCGTCCATGCC-3′
CD8β 3′-RACE 1st	5′-AGAAATGCCGCCACCACTTTCAGA-3′
CD8β 3′-RACE 2nd	5′-TGTTCGCTCGGACTGTAACTCCAGC-3′
Gene cloning	
CD4-1 G-S[a]	5′-TGTGATGGAGAAGTTTGTCC-3′
CD4-1 G-AS[b]	5′-TATATGGGAGATATCATGTT-3′
CD4-2 G-S	5′-ATGAACGTCATTGTGTTGTT-3′
CD4-2 G-AS	5′-TCACTCCTTTAGCAGGGGCT-3′
CD8β G-S	5′-GCGTACACACACACACTGGTGAC-3′
CD8β G-AS	5′-CTTCAGGGCATCTGTCTCATCTTC-3′
RT-PCR	
CD4-1 RT-S	5′-CCCATCACCATCCCCTGTTCG-3′
CD4-1 RT-AS	5′-GGGTGCGGGCGAGATTTCAG-3′
CD4-2 RT-S	5′-TGGCTGCGTGGTCGTGGTTC-3′
CD4-2 RT-AS	5′-TGACGATGCCGGATGATGCAG-3′
CD8α RT-S	5′-TGAAGTCGAACCCATCTCCCCTCTC-3′
CD8α RT-AS	5′-CTTCATCTGTTTCCAGGAGCCATCG-3′
CD8β RT-S	5′-CTGGCATGGACGCTGTTGACTGTTT-3′
CD8β RT-AS	5′-ACAGACGGGCTTGACTGGAGGTTTG-3′
β-actin RT-S	5′-ACTACCTCATGAAGATCCTG-3′
β-actin RT-AS	5′-TTGCTGATCCACATCTGCTG-3′
qPCR	
CD4-1 qPCR-S	5′-CCAGTGGTCCCCACCTAAAA-3′
CD4-1 qPCR-AS	5′-CACTTCTGGGACGGTGAGATG-3′
CD4-2 qPCR-S	5′-CACAGCGAGGACGTCAGAAA-3′
CD4-2 qPCR-AS	5′-TCTCTCCCATCACTCCTTTAGCA-3′
CD8α qPCR-S	5′-CCTCTCCCCATACATTGATTCC-3′
CD8α qPCR-AS	5′-CCGAGCTTTGCTGAAGGACTT-3′
CD8β qPCR-S	5′-GATGACACTCAAACCTCCAGTCAA-3′
CD8β qPCR-AS	5′-GCCATCCTGTGCAAAATTCTTC-3′
IFN-γ S	5′-TGTCAGGTCAGAGGATCACACAT-3′
IFN-γ AS	5′-GCAGGAGGTTCTGGATGGTTT-3′
MTAP S	5′-GTTAGCTTGGTGTGACGTCA-3′
MTAP AS	5′-CGTGAAGGCACCATCACAAC-3′
RPL10 qPCR-S	5′-GCTCCTCTGGTGCAGTTTGTGA-3′
RPL10 qPCR-AS	5′-TGGTGTTTGCTGGCGTCACTCT-3′

[a] S indicates sense primer.
[b] AS indicates anti-sense primer.

2.4. Gene cloning and sequence analysis of jfCD4-1, jfCD4-2 and jfCD8β

To determine the gene structures of jfCD4-1, jfCD4-2 and jfCD8β, genomic DNA sequence of the open reading frame of these genes were amplified by PCR using primer sets showed in Table 1. The reaction mixture contained 1 μl of the genomic DNA, 1 μl of the sense and antisense primer (10 μM), 5 μl of 10 × Ex Taq buffer, 4 μl of dNTP mixture (2.5 mM) and 0.5 μl of Ex Taq DNA polymerase (TAKARA BIO, Japan) were adjusted up to 50 μl with distilled water. PCR was carried out for 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 4 min with a final extension of 72 °C for 5 min. PCR products were cloned and sequenced as described above. The exon–intron structure was estimated using Spidey pro-