한국어병학회지 제 19 권 제 3 호 (2006)
J. Fish Pathol., 19(3) : 227 ~ 233 (2006)

Cloning and Characterization of DAP10 homologue gene from Olive Flounder, *Paralichthys olivaceus*

Jee Youn Hwang*, Ki Hyuk Kim, Joo Won Kim, Mu Chan Kim and Chan Il Park†

Institute of Marine Industry, The College of Marine Science, Gyeongsang National University, 455, Tongyeong, 650-160, Korea
**Pathology Division, National Fisheries Research and Development Institute, 408-1 Silang, Gijang, Busan 619-902 , Korea*

Olive flounder immunoreceptor DAP10 homologue cDNA was cloned from a peripheral blood lymphocytes (PBLs) cDNA library. The length of the olive flounder DAP10 cDNA is 473bp and it contains an open reading frame of 234bp. The predicted polypeptide sequence is 78 amino acids, consisting of a 22-amino acid leader, an 11-amino acid extracellular domain, a 21-amino acid transmembrane segment, and a 24-amino acid cytoplasmic domain. The amino acid sequence of olive flounder DAP10 has 56%, 50%, 32%, 31%, and 31% sequence identity with zebrafish DAP10, catfish DAP10, cattle DAP10, rat DAP10 and Monkey DAP10, respectively. Olive flounder DAP10 has a conserved aspartic acid in the transmembrane domain and a phophatidylinositol-3 kinase-binding site (YxxM/V) in the cytoplasmic region. Genomic organization reveals that olive flounder DAP10 comprises five exons and four introns. A phylogenetic analysis based on the deduced amino acid sequence grouped the olive flounder DAP10 with other species DAP10. In RT-PCR analysis, DAP10 transcripts were detected predominantly in PBLs, kidney, spleen and intestine.

Key words: Olive flounder, DAP10, Natural killer cells, Phagocytes

Introduction

Cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells play important roles in immune defense by destroying infected tissues, and cytokine production (Bancroft, 1993; Clark *et al*., 1995). These functions can be mediated by many membrane receptors, which are divided into two groups: the C-type lectin family and the immunoglobulin superfamily (Lanier, 1998). NK-cell killing is one such function involving membrane receptors. NK-cell cytotoxicity can be inhibited by many MHC class I receptors containing immunoreceptor tyrosine-based inhibitory motifs (ITIMs); these motifs are defined as I/VxYxxL/I (Blery *et al*., 1997; Daeron *et al*., 1995; Fry *et al*., 1996; Vely & Vivier 1997). Inhibitory NK-cell receptor ligation induces the phosphorylation of the tyrosine residues within the ITIM, resulting in the recruitment of the Src homology 2 (SH2) domain-containing protein tyrosine phosphatase, SHP-1, which mediates the inhibition (Binstadt *et al*., 1996, 1997; Burshtyn *et al*., 1996; McVicar *et al*., 1998). However, certain subgroups of NK receptors (such as Ly49D, CD94/NKG2C, and KIR2DS) lack ITIMs and, furthermore, contain a positively charged amino acid residue in the transmembrane (TM) region (Lanier 1997 Vely & Vivier 1997). These receptors associate with DAP12, which contains an immunoreceptor tyrosine-based activation motif. DAP12 is a type

†Corresponding Author : Chan Il Park, Tel : 055-640-3103, Fax : 055-642-4509, E-mail : vinus96@hanmail.net

I membrane phosphoprotein that can bind to both ZAP-70 and Syk kinases, leading to a cascade of NK-cell and Cytotoxic T lymphocytes (CTLs) activation (Lanier *et al*., 1998a, 1998b; McVicar *et al*., 1998).

Recently, a new membrane adaptor molecule DAP10 was reported to contain a phophatidylinositol-3 (PI-3) kinase-binding site (YxxM) in the cytoplasmic domain (Wu *et al*., 1999). DAP10 forms a complex with NKG2D which is expressed on NK cells, CD8 $\alpha\beta$-T cells, and $\gamma\delta$-T cells (Bauer *et al*., 1999; Wu *et al*., 1999). In this paper, we describe the cloning cDNA, gene structure, and expression pattern of olive flounder DAP10 homologue.

Materials and Methods

Cloning and sequencing of cDNA

Olive flounder, *Paralichthys olivaceus*, (weight 600-700 g) that had not been exposed to known antigenic stimulation were used as blood and tissue donors for all *in vitro* experiments. Olive flounder DAP10 homologue cDNA was identified from the analysis of expressed sequence tags (ESTs) of olive flounder leukocytes stimulated with a Con A/PMA cDNA library. The cDNA library used in this study was previously reported (Aoki *et al*., 1999; Nam *et al*., 2000). The nucleotide sequence of plasmid DNA was determined using ThermoSequenase (Amersham Biosciences) with M13 forward and M13 reverse primers and an automated DNA sequencer LC200 (Li-Cor).

A phylogenetic analysis was done as previously reported (Felsenstein, 1996). The determined nucleotide and deduced amino acid sequences, and multiple sequence alignments were analyzed by GENETYX ver. 8.0 (SDC Software Development).

Isolation of the olive flounder DAP10 gene

Two sets of primers were designed to amplify the olive flounder genomic DAP10 sequence. The first set of primers consists of DAP10F (5'-CAGATCAGTCCGAGCTGAGC-3') and DAP10R (5'-TCTTGCATGTGTCATATG-3'). The second set of primers consists of DAP10subF (5'-GGCTGTGGCATTTGCAG-3') and DAP10subR (5'-TCTTGCATGTGTCATATGAAC-3'). Two hundred nanograms of olive flounder genomic DNA was used as a template for amplification. Polymerase chain reaction (PCR) was performed for 35 cycles as follows: 94 ℃ for 1 min, 58 ℃ for 1 min, and 72 ℃ for 2 min. PCR products were isolated from the gel and ligated into the pGEM-T easy plasmid (Promega Corporation, Madison, WI, USA) for sequencing.

Tissue distribution of olive flounder DAP10 gene transcript

Total RNA (50 ng) was extracted from healthy olive flounder brain, heart, intestine, kidney, liver and spleen were reverse-transcribed into cDNA using an AMV Reverse Transcriptase First-Strand cDNA synthesis kit (Life sciences). PCR was performed on the resulting cDNA using the DAP10F and DAP10R specific primer set. β-actin was amplified as a control using the Beta-actin-F (5'-TTTCCCTCCATTGTTGGTCG-3') and Beta-actin-R primers (5'-GCGACTCTCAGCTCGTTG-TA-3') (Katagiri *et al*., 1997). The PCR mixtures were denatured at 94℃ for 2 min and then subjected to 25 cycles of 94℃ for 30 s, 57℃ for 30 s and 72℃ for 1 min. The products were visualized by separation on a 1.5% agarose gel.

Results

Identification of the DAP10 cDNA from olive flounder

The aligned predicted amino acid sequences of the DAP10 of flounder, catfish, zebrafish, rat, cattle and monkey are shown in Fig. 1. The predicted amino acid sequence is 78 amino acid membrane-bound protein that consists of a 22 amino acid signal sequence, a 11 amino acid extracellular region, a single 21 amino acid transmembrane domain, and 24 amino acid cytoplasmic region. The amino acid sequence of olive flounder DAP10 has 56%, 50%, 32%, 31%, and 31% sequence identity with zebrafish DAP10, catfish DAP10, cattle DAP10, rat DAP10 and monkey DAP10, respectively. Like DAP10 sequences in other species examined so far, the olive flounder DAP10 sequence contains three cysteines and a single negatively charged aspartic acid residue at position 41 within the transmembrane domain. Olive flounder DAP10 has a conserved aspartic acid in the transmembrane domain and a phophatidylinositol-3 kinase-binding site (YxxM/V) in the cytoplasmic region.

In the phylogenetic analysis (Fig. 2), olive flounder DAP10 is grouped with vertebrate DAP10 peptides, including zebrafish DAP10. This grouping was well supported by bootstrapping.

Genomic DNA sequence of the olive flounder DAP10 gene

The olive flounder DAP10 gene is approximately 1.3 kb long and consists of five exons and four introns (Fig. 3). Typical intron splice motifs occur at the 5′ (gt) and 3′ (ag) ends of each intron.

Tissue distribution of olive flounder DAP10 gene transcript

Expression of the DAP10 gene in the tissues of olive flounder was detected by RT-PCR. Olive flounder DAP10 transcripts was expressed predominantly in the PBLs, kidney, spleen, and intestine but not in skin or muscle after 25 cycles of PCR (Fig. 4).

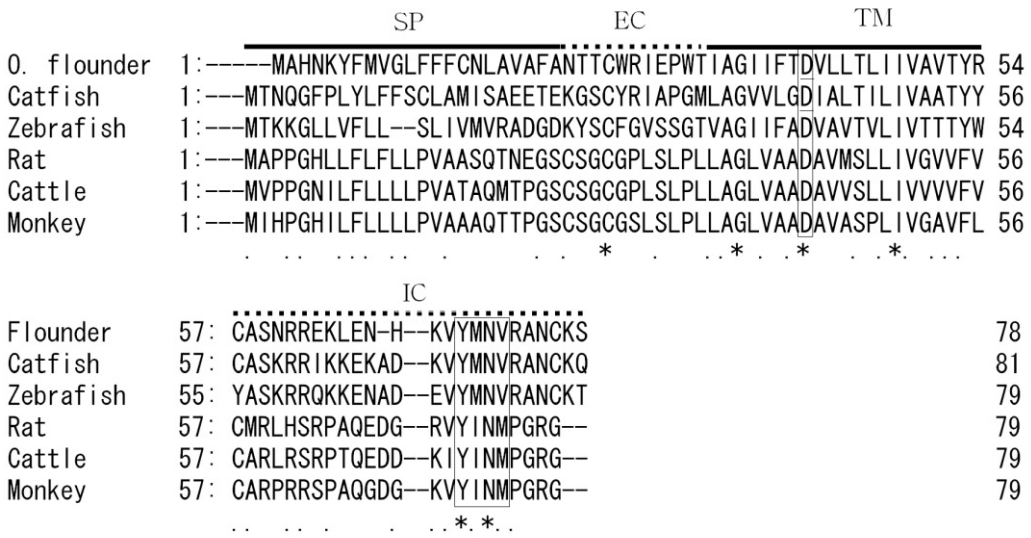

Fig.1. Amino acid sequence alignment of DAP10s. Sequences were obtained from DDBJ/EMBL/GenBank. Amino acids identical to the olive flounder (*Paralichthys olivaceus*) DAP10 are shown by dots. The positions of a single negatively charged aspartic acid residue and the conserved YXXM/V motifs are boxed. Residues that are identical in all sequences are shown as asterisks. Gaps (*dashes*) have been placed to maximize the identity. SP, Signal peptides; EC, Extracellular domain; TM, Transmembrane; IC, Intracellular domain.

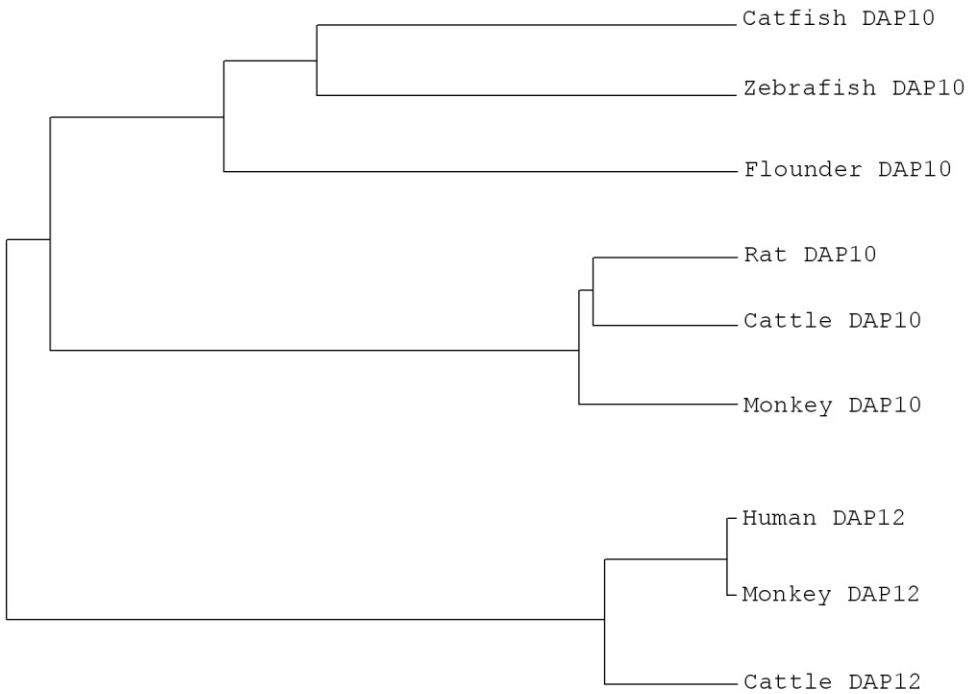

Fig. 2. Phylogenetic tree of amino acid sequences of DAP10, DAP12, and CD3ϵ (out-group). The phylogeny of DAP10s was estimated by the neighbor-joining method of clustering in the PHYLIP program.

```
TCCGGAATTCCCGGGTCGACCCACGCGTCCGTCCAGTCAGCAGATCAGTCCGAGCTGAGCAAGGTATGAGATCTTCATTTACACTGACAG
ACACACTCTTCCTCATCCCAACATTGAACCATTTCATGTCTGTCAAGTCACACCATGATGTGTAGTTTAAGATTGAAGACATGCGCCAAG
AATACTTCACAGCTCTGCCTCATTTTGACTCTGGAGTTGAATTTTCTGTGCTGCACATCATCGACGCTATTGTTTCTAAACGTTCAGGGG
ATTTAACCTTCAAAATGATGCGAAATATAACATGCGAATGATCTTTTGTCTTTCAGAAAGCAGCTAACTGAGCTAAGAAACATGGCACAC
                                                                  M  A  H
AACAAATACTTTATGGTTGGGCTGTTTTTCTTTTGCAGTAAGTCTGATTTTACAGCAGATTTCTCGTCTTTGTGCAAACACACACATGCG
  N  K  Y  F  M  V  G  L  F  F  F  C
CGACACTAGGGTGGTCTCAAACAGGAAAATCAAACCACAAAAACAAAATTGTGCATATGCAGCGGAAACTTAACTGACAGAGGTGTTGCT
GCATAATGTCATTAACAAGCTCTTGTTTCTTTTGTTTTCTTCTCTCAGAATTTGGCTGTGGCATTTGCAGGTGTGTAATTTAAAAGGACAA
                                                N  L  A  V  A  F  A
CTCAGAAATTCAGTTTTTATGAATGTTGCCATTTAGACAAATAGATAAACAGCAACACAAATGCAGAGCCAGACAGACAACCACCTCTTA
TCAGTCAATATTTCCACTTGCACCTACAGAGTTCCTTTCTTTCATCCCACTCTGGTTTTCCCTGTGTGTCTTTGTATACAGACAACACTA
                                                                  N  T  T
CATGCTGGAGGATTGAGCCCTGGACAATAGCGGGCATCATCTTCACAGACGTGTTGTTGACCCTCATCATCGTCGCTGTCACGTACCGTT
  C  W  R  I  E  P  W  T  I  A  G  I  I  F  T  D  V  L  L  T  L  I  I  V  A  V  T  Y  R  C
GTGCCAGCAATCGGCGTGAAAAGTTAGAAAATGGTATGGTAGCAGGGCTGAGAGGATTCTCTCAGAGGGTTTTTACTATTCTATTTTAGT
  A  S  N  R  R  E  K  L  E  N

GCACAGCTCATATTTTGGAAGCATTGATAAGATTTGTTTGTTATTTTTTAAAATTGAATGGCTCCTGTGTCTGTGGTTTATAATGTCTCT
GACAGTACATTGTGTGTCTCTGTCTTCTCCTCTCTCCACAGCTCACAAAGTGTACATGAATGTACGAGCCAACTGCAAGAGCTGATCTAA
                                        H  K  V  Y  M  N  V  R  A  N  C  K  S*
CAATGTGAAGTACATCATGAAATGATGTAGAATTTTTCATATACTGCTTTTTAAAACATATGACACATGCAAGATTAAATTGAACAGATT
TTACATTAAACAATATATACATGTAAAAAAAAAAAAAAAAAAAAA
```

Fig. 3. Nucleotide and deduced amino acid sequences of the olive flounder (*Paralichthys olivaceus*) DAP10 gene. The 5' (gt) and 3' (ag) ends of each intron are underlined.

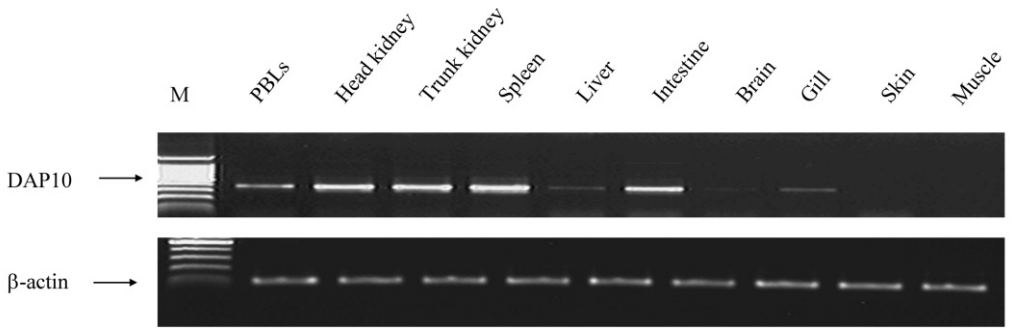

Fig. 4. Expression of DAP10 mRNA levels in various tissues of healthy olive flounder (*Paralichthys olivaceus*) as determined by RT-PCR. β-actin amplification was included as a control. M; 100-bp ladder marker.

Discussion

Cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells are important role in innate immunity, especially tumor elimination, control of infectious diseases, and cytokine production (Bancroft, 1993; Trinchieri, 1989). DAP10 is a component of the activating Ig-like NK cell receptors in humans and of the activating C-type lectin NK cell and cytotoxic T lymphocyte receptors in mouse. It also associates with the Ig like SIRP-1b, TREM and can replace DAP12 in complex with NKG2D, the C-type lectin receptor recognizing the MHC I-like molecules induced by stress (Lanier & Bakker, 2000; Wilson *et al*., 2000; Colonna 2003). DAP10 has so far been described as a component of the NKG2D complex (Diefenbach *et al*., 2002). The genes encoding DAP10 and DAP12 are closely linked in mammals and zebrafish (Wilson *et al*., 2000; Guselnikov *et al*., 2003).

Our search in the EST databases for lower vertebrate homologue of DAP10 resulted in identification of the pig, rat, cattle, catfish and zebrafish cDNAs coding for the DAP10-like proteins. The olive flounder and zebrafish putative DAP10 transmembrane encoding exon is followed by a relatively long exon coding for the cytoplasmic tail containing the YXXV sequence instead of the YXXM motif (Fig. 1, 3). Nevertheless, the accuracy of the DAP10 gene prediction was confirmed by a sequence of the Fugu EST cDNA subsequently deposited in GenBank.

In the phylogenetic analysis indicated that known zebrafish DAP10 is more closely grouped than rat, cattle and monkey DAP10. This result is possibly due to differences between mammalian and non-mammalian species (Fig. 2).

The five exons and four introns of the olive flounder DAP10 gene are more similar to those in the Fugu DAP10 gene (Guselnikov *et al*., 2003) than to those in the Pig DAP10 gene (Fig. 3) (Yim *et al*., 2001).

Relatively large quantities of olive flounder DAP10 transcript was expressed in pathophysiologically important organs such as PBLs, kidney, spleen and intestine. (Fig. 4). This is in agreement with other study which demonstrated that pig DAP10 mRNAs were found in PBLs, macrophages, spleen, liver, and lymph nodules (Yim *et al*., 2001). Human DAP10 is expressed on NK cells and subpopulation of T cells (Wu *et al*., 1999). Activated mouse macrophages were recently reported to express DAP10 (Diefenbach *et al*., 2000).

Even though the ligands for the olive flounder DAP10 complex is not yet known, the DAP10 complex may play an important role in immune surveillance. Further studies of recombinant protein of olive flounder DAP10 to understand the roles of this gene should provide a better understanding of the fish immune system.

Acknowledgements

I thank Drs. Takashi Aoki and Ikuo Hirono, Tokyo University of Marine Science and Technology, for generously accommodating me for the work on cDNA and gene cloning in their laboratory.

References

Aoki, T., Nam, B.H. and Hirono, I.: Sequence of 596 cDNA clones (565,977) of Japanese flounder Paralichthys olivaceus leukocytes infected with hirame rhabdovirus. Mar. Biotechnol. 1: 477-488, 1999.

Bancroft, G.J.: The role of natural killer cells in innate resistance to infection. Curr. Opin. Immunol., 5: 503-510, 1993.

Bauer, S., Groh, V., Wu, J., Steinle, A., Phillips, J.H. and Lanier, L.L.: Spies T Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science, 285: 727-729, 1999.

Binstadt, B.A., Brumbaugh, K.M., Dick, C.J., Scharenberg, A.M., Williams, B.L., Colonna, M., Lanier, L.L., Kinet, J.P., Abraham, R.T. and Leibson, P.J.: Sequential involvement of Lck and SHP-1 with MHC-recognizing receptors on NK cells inhibits FcR-initiated tyrosine kinase activation. Immunity, 5: 629-638, 1996.

Binstadt, B.A., Brumbaugh, K.M. and Leibson, P.J.: Signal transduction by human NK cell MHC-recognizing receptors. Immunol. Rev., 155: 197-203, 1997.

Blery, M., Delon, J., Trautmann, A., Cambiaggi, A., Olcese, L., Biassoni, R., Moretta, L., Chavrier, P., Moretta, A., Daeron, M. and Vivier, E.: Reconstituted killer cell inhibitory receptors for major histocompatibility complex class I molecules control mast cell activation induced via immunoreceptor tyrosine-based activation motifs. J. Biol. Chem., 272: 8989-8996, 1997.

Burshtyn, D.N., Scharenberg, A.M., Wagtmann, N., Rajagopalan, S., Berrada, K., Yi, T., Kinet, J.P. and Long, E.O.: Recruitment of tyrosine phosphatase HCP by the killer cell inhibitor receptor. Immunity, 4: 77-85, 1996.

Clark, W.R., Walsh, C.M., Glass, A.A., Hayashi, F., Matloubian, M. and Ahmed, R.: Molecular pathways of CTL-mediated cytotoxicity. Immunol. Rev., 146: 33-44, 1995.

Colonna, M.: DAP12 signaling: from immune cells to bone modeling and brain myelination. J. Clin. Invest., 111: 313-314, 2003.

Daeron, M., Latour, S., Malbec, O., Espinosa, E., Pina, P., Pasmans, S. and Fridman, W.H.: The same tyrosine-based inhibition motif, in the intracytoplasmic domain of Fc gamma RIIB, regulates negatively BCR, TCR, and FcR dependent cell activation. Immunity, 3: 635-646, 1995.

Diefenbach, A., Jamieson, A.M., Liu, S.D., Shastri, N. and Raulet, D.H.: Ligands for the murine NKG2D receptor: expression by tumor cells and activation on NK cells and macrophages. Nat. Immunol., 1: 119-126, 2000.

Felsenstein, J.: PHYLIP (phylogeny inference package), version 4.0. Department of Genet-

ics, University of Washington, Seattle. 1996.

Fry, A.M., Lanier, L.L. and Weiss, A.: Phosphotyrosines in the killer cell inhibitory receptor motif of NKB1 are required for negative signaling and for association with protein tyrosine phosphatase 1C. J. Exp. Med., 184: 295-300, 1996.

Guselnikov, S.V., Najakshin, S.M. and Taranin, A.V.: Fugu rubripes possesses genes for the entire set of the ITAM-bearing transmembrane signal subunits. Immunogenetics, 55; 472-479, 2003.

Katagiri, T., Hirono, I. and Aoki, T.: Identification of a cDNA for medaka cytoskeletal beta-actin and construction for the reverse transcriptase-polymerase chain reaction(RT-PCR) primer. Fish. Sci., 63: 73-76, 1997.

Lanier, L.L.: Natural killer cell receptors and MHC class I interactions. Curr. Opin. Immunol., 9: 126-131, 1997.

Lanier, L.L.: NK cell receptors. Annu. Rev. Immuno.,l 16: 359-393, 1998.

Lanier, L.L., Corliss, B.C., Wu, J., Leong, C. and Phillips, J.H.: Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. Nature, 391: 703-707, 1998a.

Lanier, L.L., Corliss, B., Wu, J. and Phillips, J.H.: Association of DAP12 with activating CD94/NKG2C NK cell receptors. Immunity, 8: 693-701, 1998b.

Lanier, L.L. and Bakker, A.B.: The ITAM-bearing transmembrane adaptor DAP12 in lymphoid and myeloid cell function. Immunol. Today, 21: 611-614, 2000.

McVicar, D.W., Taylor, L.S., Gosselin, P., Willette-Brown, J., Mikhael, A.I., Geahlen, R.L., Nakamura, M.C., Linnemeyer, P., Seaman, W.E., Anderson, S.K., Ortaldo, J.R. and Mason, L.H.: DAP12-mediated signal transduction in natural killer cells: a dominant role for the Syk protein-tyrosine kinase. J. Biol. Chem., 273: 32934-32942, 1998.

Nam, B.H., Yamamoto, E., Hirono, I. and Aoki, T.: A survey of expressed genes in the leukocytes of Japanese flounder, Paralichthys olivaceus, infected with hirame rhabdovirus. Dev. Comp. Immunol., 24: 13-24, 2000.

Trinchieri, G.: Biology of natural killer cells. Adv. Immunol. 47: 187-376, 1989.

Vely, F. and Vivier, E.: Conservation of structural features reveals the existence of a large family of inhibitory cell surface receptors and noninhibitory/activatory counterparts. J. Immunol. 159: 2075-2077, 1997.

Wilson, M.J., Lindquist, J.A. and Trowsdale, J.: DAP12 and KAP10 (DAP10)-novel transmembrane adapter proteins of the CD3 family. Immunol. Res. 22: 21-42, 2000.

Wu, J., Song, Y., Bakker, A.B., Bauer, S., Spies, T., Lanier, L.L. and Phillips, J.H.: An activating immunoreceptor complex formed by NKG2D and DAP10. Science, 285: 730-732, 1999.

Yim, D.S., Jie, H.B., Lanier, L.L., Kim, Y.B.: Molecular cloning, gene structure, and expression pattern of pig immunoreceptor DAP12. Immunogenetics, 51: 436-442, 2000.

Manuscript Received : August 23, 2006
Revision Accepted : October 2, 2006
Responsible Editorial Member : Jung-Woo Park (Ulsan Univ.)