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Structural characteristics of zebrafish orthologs of adaptor molecules that associate with transmembrane immune receptors

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Abstract

Transmembrane bound receptors comprised of extracellular immunoglobulin (Ig) or lectin domains play integral roles in a large number of immune functions including inhibitory and activating responses. The function of many of the activating receptors requires a physical interaction with an adaptor protein possessing a cytoplasmic regulatory motif. The partnering of an activating receptor with an adaptor protein relies on complementary charged residues in the two transmembrane domains. The mammalian natural killer (NK) and Fc receptors (FcR) represent two of many receptor families, which possess activating receptors that partner with adaptor proteins for signaling. Zebrafish represent a powerful experimental model for understanding developmental regulation at early stages of embryogenesis and for efficiently generating transgenic animals. In an effort to understand developmental aspects of immune receptor function, we have accessed the partially annotated zebrafish genome to identify six different adaptor molecules: Dap10, Dap12, Cd3ζ, Cd3ζ-like, FcRγ and FcRγ-like that are homologous to those effecting immune function in mammals. Their genomic organizations have been characterized, cDNA transcripts have been recovered, phylogenetic relationships have been defined and their cell lineage-specific expression patterns have been established.
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1. Introduction

The cells of the mammalian immune system rely on an intricate network of signaling pathways in order to differentiate between "self" and "non-self". The activation or inhibition of these signaling pathways relies on specific membrane receptors on immune cells engaging specific ligands. In general, these receptors can be classified as inhibitory or activating based on the functional outcome of ligand recognition. For example, when an activating natural killer (NK) cell receptor binds its ligand, the NK cell is activated to kill the target cell; in contrast, when an inhibitory NK receptor binds its ligand, NK cell-mediated killing is repressed. Similarly, engagement of T-cell antigen receptor (TCR) or Fc receptor (FcεRI) with the appropriate ligand (*e.g.* peptide–MHC complex or IgE, respectively) leads to a direct cell-mediated immune response.

Despite differences in receptor structures, the cytoplasmic signaling utilized by NK receptors, TCR and Fc receptors, is well conserved (Cerwenka and Lanier, 2000; Billadeau and Leibson, 2002; Yokoyama and Kim, 2006). Inhibitory NK and Fc receptors typically possess one or more cytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIMs). In contrast, activating NK,

Abbreviations: FcR; Fc receptor; Ig; immunoglobulin; ITAM; immunoreceptor tyrosine-based activation motif; ITIM; immunoreceptor tyrosine-based inhibition motif; NK; natural killer; TCR; T-cell antigen receptor.

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Fc and other receptors including TCR partner with adaptor proteins, which ultimately transduce signals to the cell nucleus. These receptors physically associate with an adaptor protein *via* oppositely charged residues within their transmembrane domains, *e.g.* a positive charge in the transmembrane domain of the activating receptor and a negative charge in the transmembrane domain of the adaptor protein. The majority of activating NK receptors, including most KIRs and Ly49, utilize the adaptor protein, DAP12; the activating NK receptor NKG2D, another lectin-type receptor, utilizes the adaptor DAP10 (Hyka-Nouspikel and Phillips, 2006; Takaki et al., 2006). TCR and FcR along with other immune related activating receptors, including: CD16, NKp30, NKp46, NKR-P1C and KIR2DL4, utilize FcRγ or CD3ζ (Cerwenka and Lanier, 2000; Tassi et al., 2006). The DAP12, FcRγ and CD3ζ adaptors utilize cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs: $YxxLX_{6-12}YxxL/I$) for signaling (Pitcher and van Oers, 2003), whereas DAP10 uses a YxxM motif similar to CD28 (Wu et al., 1999).

The zebrafish is becoming a more broadly recognized model for infection and immunity and is particularly well suited for examining gene function during embryogenesis (Yoder et al., 2002; Traver et al., 2003a; Trede et al., 2004; Van Der Sar et al., 2004; Phelps and Neely, 2005; Deiters and Yoder, 2006; Lieschke and Currie, 2007). As part of an ongoing effort to characterize immune receptors in zebrafish and to understand the signaling pathways mediated by their immune cells, we have identified and characterized the adaptor molecules: Dap12, Dap10, CD3ζ, CD3ζ-like, FcRγ, and FcRγ-like.

2. Materials and methods

2.1. Cloning zebrafish dap10 (hcst) cDNA

A catfish (*Ictalurus punctatus*) *DAP10* cDNA sequence (GenBank: AAZ16504) was used as the query for a tBLASTn search of the NCBI zebrafish sequence database (Wheeler et al., 2007). Zebrafish bacterial artificial chromosome (BAC) clone DKEY-29H14 (GenBank: BX571853) from chromosome 16 encodes a sequence similar to catfish DAP10 (*E* value = 1e−10). A sequential RACE strategy was used to clone the full-length open reading frame (ORF) of *dap10* (*hcst*) from zebrafish kidney/spleen RNA (GeneRacer, Invitrogen). Initially, 3′ RACE was completed with nested forward primers (designed from the DKEY-29H14 sequence) ZFDAP10-F1 and ZFDAP10-F2 which identified the 3′ untranslated region of the *dap10* mRNA. Subsequently, nested, reverse primers ZFDAP10-3′UTR-R1 and ZFDAP10-3′UTR-R2 were designed within the 3′ UTR of *dap10* and 5′ RACE was completed to generate a cDNA encoding the ORF of *dap10*. Primer sequences are listed in Table 1. PCR products were cloned into pGEM-T easy (Promega) or pCR4-TOPO (Invitrogen) and sequenced.

2.2. Cloning zebrafish dap12 (tyrobp) cDNA

tBLASTn analyses of the zebrafish nucleotide, genome and EST databases, using human DAP12 as a query, failed to identify a similar sequence (no sequence identified with an *E* value < 0.05). As *DAP10* and *DAP12* are adjacent genes in mammals and pufferfish (Guselnikov et al., 2003b), the zebrafish BAC DKEY-29H14, which encodes *dap10*, was examined for the *dap12* sequence. A BLASTx analysis using the nucleotide sequence of DKEY-29H14 as a query identified a short sequence (108 nucleotides) within this BAC with extremely low similarity (*E* value = 1.5) to the transmembrane domain of mouse TCRδ (GenBank: AAH62807) which, like the transmembrane domain of DAP12, includes a negatively charged residue. We predicted that this novel transmembrane domain encoded by BAC DKEY-29H14 would represent an exon of zebrafish *dap12* (*tyrobp*) based on the following observations: 1) the transmembrane domains encoded by this novel zebrafish sequence and mammalian *DAP12* include a negatively charged residue; 2) this candidate zebrafish transmembrane domain is adjacent to *dap10*; and 3) no other sequences within DKEY-29H14 share similarity with *DAP12*. Subsequently, a full-length ORF cDNA of this sequence was cloned (as follows) and confirmed to encode *dap12*. Initially, 3′ RACE was completed from zebrafish kidney/spleen RNA with nested forward primers (designed from the DKEY-29H14 novel transmembrane sequence) ZFDAP12-F1 and ZFDAP12-F2, which identified the 3′ untranslated region of the *dap12* mRNA. Subsequently, nested, reverse primers ZFDAP12-R3 and ZFDAP12-R4 were designed within the ORF of *dap12* and 5′ RACE was completed to generate a cDNA encoding the 5′ UTR of *dap12*. Finally, nested, forward primers ZFDAP12-5′UTR-F3 and ZFDAP12-5′UTR-F4 were designed within the 5′ UTR of *dap12* and a cDNA encoding the ORF of *dap12* was derived by 3′ RACE. Primer sequences are listed in Table 1. PCR products were cloned into pGEM-T easy (Promega) or pCR4-TOPO (Invitrogen) and sequenced.

2.3. Cloning zebrafish FcRγ (fcer1g) cDNA

A zebrafish kidney EST (GenBank BG799671), which encodes FcRγ (*fcer1g*), was identified from a tBLASTn search using a catfish FcRγ sequence (GenBank AF538721) as a query. In order to confirm this sequence, a 5′ RACE strategy was performed as described above using spleen RNA and the nested reverse primers, ZFFcRg-3′UTR-R1 and ZFFcRg-3′UTR-R2, which are complementary to the 3′ UTR of *FcRγ*. Primer sequences are listed in Table 1. PCR products were cloned into pGEM-T easy (Promega) or pCR4-TOPO (Invitrogen) and sequenced.

2.4. Cloning zebrafish FcRγ-like (fcer1gl) cDNA

A zebrafish FcRγ-like (*fcer1gl*) EST sequence was identified (GenBank BC124700) from a tBLASTn search using a catfish FcRγ-like sequence (GenBank AAN38001: Shen et al., 2003) as a query. As this zebrafish EST encoded the entire ORF of *FcRγ-like*, forward and reverse primers, ZFFcRg-B-F, and ZFFcRg-B-R, were designed to amplify the entire ORF from zebrafish spleen cDNA. Primer sequences are listed in Table 1. The *FcRγ-like* ORF was amplified from zebrafish spleen cDNA using 40 cycles and cloned into pCRII-TOPO (Invitrogen) and sequenced.

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