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Expression analysis of two Eomesodermin homologues in zebrafish lymphoid tissues and cells☆

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Abstract

Eomesodermin (*Eomes*) is a T-box transcription factor that is involved in mesoderm formation in most vertebrates. Eomes is also expressed in $CD8^+$ T cells and NK cells. No information is available on the role of Eomes in the immune system of lower vertebrates to date, although developmental studies on Eomes (*Eomes1*) have been performed in zebrafish. Here we report the identification of a second *Eomes* (*Eomes2*) in zebrafish and compare expression of the two *Eomes* genes in the immune system. Zebrafish Eomes1 and Eomes2, composed of 661 and 534 amino acids, respectively, share 49.3% amino acid identity in their coding regions and 88.7% amino acid identity in their T-box regions. Conserved synteny between regions of the human and zebrafish genomes, gene organization and phylogenetic analysis all indicate that the zebrafish Eomes2 gene is a homologue of mammalian Eomes, as previously found for zebrafish Eomes1. *Eomes1* mRNA was found to be expressed in the gonad, body kidney, spleen and gill, while *Eomes2* mRNA was not detected in any of these tissues. However, strong expression of both *Eomes* mRNAs was detected in the leukocytes from the spleen, followed by those from body kidney and peripheral blood, with expression of *Eomes1* always stronger than that of *Eomes2*. RT-PCR analysis of body kidney cells sorted by FACS revealed that *Eomes1* was expressed strongly in lymphocytes, weakly in blast cells, and was not expressed in granulocytes, while *Eomes2* was expressed weakly in lymphocytes. These results suggest that both *Eomes* genes are involved in the zebrafish immune response, particularly in lymphocyte function as has been found in mammals.

Keywords: Eomes; T-box; Teleost; Immune function; Lymphocytes

1. Introduction

T-box genes, characterized by a region of conserved DNA-binding domain called the T-box, play critical roles as transcription factors in embryogenesis and organogenesis (Wilson and Conlon, 2002; Showell et al., 2004). T-brain1 is a subfamily of T-box genes consisting of three genes in mammals: *T-brain1*, T-box expressed in T-cells (*T-bet*) and Eomesodermin (*Eomes*). Eomes has been shown to be involved in mesoderm formation in most vertebrates (Ryan et al., 1996; Bulfone et al., 1999; Sone et al., 1999) and in trophoblast differentiation in mammals (Russ et al., 2000). Important roles of Eomes during early development have also been reported in zebrafish (Mione et al., 2001; Bruce et al., 2003; Bruce et al., 2005; Bjornson et al., 2005).

A second, critical role for Eomes is in the development of mammalian cell-mediated immunity. Eomes is expressed in $CD8^+$ T cells and NK cells, and is involved in effector differentiation of $CD8^+$ T cells (Pearce et al., 2003; Townsend et al., 2004). Experiments with ectopic expression and loss of function by dominant negative mutation suggest that Eomes induces IFN-γ and cytolytic molecules in cooperation with T-bet. However, no information is available on the role of Eomes in the immune system of lower vertebrates.

Specific cell-mediated immunity has been studied in several teleost species (Nakanishi et al., 2002; Fischer et al., 2006). Recently, mRNA expression of T cell surface marker genes in alloantigen- or virus-specific effector cells has been reported in several fishes, e.g. TCR and CD8 in ginbuna (Somamoto et al., 2005; Somamoto et al., 2006) and rainbow trout (Fischer et al., 2003), and TCR in channel catfish (Stuge et al., 2000, Shen et al., 2004). These findings suggest the presence of cytotoxic T

☆ We identified a second Eomes in zebrafish and analyzed the expression of two Eomes genes in lymphoid tissues and cells. This is the first report in non-mammalian vertebrates to show the presence of multiple isoforms of Eomes and the involvement of Eomes in immune function as well as development.

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lymphocytes (CTL) in fish similar to those of higher vertebrates. Mechanisms of differentiation and maturation of teleost T-cell subsets remain unknown, although T-cell related genes such as TCR, CD3, CD4 and CD8 have been identified in several fish species (see review by Fischer et al., 2006).

It has been well documented that an ancient genome duplication event provided the evolutionary groundwork for generation of variability and diversification of gene function in teleosts (Volff, 2005). Therefore, some genes that exist as a single copy in mammals are present in several copies in teleosts (Venkatesh, 2003). Recently, we discovered an additional *Eomes* gene in teleost genome databases that differs from the known *Eomes* gene of zebrafish. Here we characterize the two zebrafish *Eomes* genes, including expression in various tissues and isolated leukocytes.

2. Materials and methods

2.1. Fish and leukocyte preparations

Wild type zebrafish (*Danio rerio*) were obtained from a local pet supplier, maintained in aerated tanks at 28 °C and fed commercial pellet food twice daily. After anaesthetizing fish with benzocaine (Sigma), tails were cut off using a scalpel and peripheral blood was collected from the caudal vessels into heparinized haematocrit tubes. Leukocytes from peripheral blood, body kidney and spleen were obtained as described previously (Moritomo et al., 2003).

2.2. Screening for a second zebrafish Eomes

We used the zebrafish genomic sequence database version 5 at Sanger (http://www.sanger.ac.uk/) to search for zebrafish homologues of mammalian Eomes. In this database, BLASTP analysis with human Eomes protein showed the presence of two types of *Eomes* in the zebrafish genome. One of the two genes (*Eomes1*) had already been reported, but the other gene (*Eomes2*) was novel and we cloned it using specific primers based on the database sequence. We also searched for fugu (*Takifugu rubripes*) and tetraodon (*Tetraodon fluviatilis*) homologues of mammalian Eomes using the Ensembl database (http://www.ensembl.org/index.html).

2.3. RNA extraction and RT-PCR for isolation of zebrafish Eomes2 cDNA

Total RNA was extracted from whole zebrafish with Trizol reagent (Invitrogen, USA) and one microgram was reverse-transcribed to cDNA for 5′- and 3′-RACE PCR with a SMART RACE cDNA Amplification kit (TAKARA BIO INC., Japan). To obtain partial fragments, PCR was performed with the Eomes2-F1/Eomes2-R1primer set (Table 1) using *KOD–Plus*-DNA polymerase (TOYOBO, Japan). The PCR conditions were: one cycle of 94 °C for 2 min, 40 cycles of 94 °C for 15 s, 55 °C for 10 s and 68 °C for 90 s, and finally 68 °C for 3 min. To obtain the complete zebrafish *Eomes2* cDNA sequence, 5′- and 3′-RACE PCR were performed with specific primers that were based on the partial sequence of zebrafish *Eomes2*. For 3′-RACE PCR, 3′-RACE-ready cDNA was amplified in two successive rounds of PCR, first with Eomes2-F2 and 10× universal primer A mix (UPM, supplied in the kit), and then with Eomes2-F3 and nested universal primer A (NUP, supplied in the kit). Likewise, 5′-RACE PCR was performed in two successive rounds of PCR, first with Eomes2-R2 and UPM, and then with Eomes2-R3 and NUP. Both 5′- and 3′-RACE PCR conditions were: one cycle of 98 °C for 1 min, 35 cycles of 98 °C for 10 s, 66 °C for 10 s and 72 °C for 60 s, and finally 72 °C for 5 min. *Phusion* DNA polymerase (FINNZYMES, Finland) was used in all 5′- and 3′-RACE PCR reactions.

2.4. DNA sequencing and gene organization analysis

The amplified fragments were subcloned into the pGEM-T Easy vector (Promega) and selected clones were purified from

Table 1
The primer oligonucleotide sequences and their applications

Primer name	Sequence (5′ → 3′)	Tm[a]	Application
Eomes2-F1	GTAGCGCTGCCCAGTGCGGCTCT	77.71	Partial cDNA cloning
Eomes2-F2	CAGTTCAGCAGTTTGACCCAGCGCTCAGT	77.92	3′-RACE
Eomes2-F3	GCAGTAAACTGGAGCTGACGGCGTACGAG	76.86	3′-RACE
Eomes2-F4	ACAGCCTACCAGAACACTGACATCACTCA	72.1	Gene expression
Eomes2-R1	TCTGGCTTTCTCCGGATCCTCACTGAAGT	75.82	Partial cDNA cloning
Eomes2-R2	AGTCTCGCTCGCTTCGGTCGTCCGA	79.52	5′-RACE
Eomes2-R3	GACAGACTCAAACCCGTCATGTTGAAGCTG	75.6	5′-RACE
Eomes2-R4	AATGCTGGGTCTGCGGCATC	71.67	Gene expression
Eomes1-F	GTAACGGCTTACCAAAACACAGACATCACA	72.04	Gene expression
Eomes1-R	GGCTGCCATAGATGCGAATGCAGAA	74.97	Gene expression
TCRα-F	ACTGAAGTGAAGCCGAAT	57.24	Gene expression
TCRα-R	CGTTAGCTCATCCACGCT	61.61	Gene expression
CD8α-F	AGACGGAAGTCAAGCATAATGCAAATTCGA	73.86	Gene expression
CD8α-R	ATGGGCTTTGCTCCTTTTTTGTCACATAC	71.97	Gene expression
EF1α-F	CGGCAGCTTCAATGCTCAGGTCATC	75.12	Gene expression
EF1α-R	GGGAAATTCATTTGGTCTTGGCAGCCT	74.77	Gene expression

[a] The melting temperature, Tm, is calculated using the nearest neighbor method.