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Transcription analysis of two *Eomesodermin* genes in lymphocyte subsets of two teleost species

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ABSTRACT

Eomesodermin (Eomes), a T-box transcription factor, is a key molecule associated with function and differentiation of $CD8^+$ T cells and NK cells. Previously, two teleost *Eomes* genes (*Eomes-a* and *-b*), which are located on different chromosomes, were identified and shown to be expressed in zebrafish lymphocytes. For the present study, we identified these genes in rainbow trout and ginbuna crucian carp. Deduced Eomes-a and -b amino acid sequences in both fish species contain a highly conserved T-box DNA binding domain. In RT-PCR, both *Eomes* transcripts were readily detectable in a variety of tissues in rainbow trout and ginbuna. The high expression of *Eomes-a* and *-b* in brain and ovary suggests involvement in neurogenesis and oogenesis, respectively, while their expression in lymphoid tissues presumably is associated with immune functions. Investigation of separated lymphocyte populations from pronephros indicated that both *Eomes-a* and *-b* transcripts were few or absent in IgM^+ lymphocytes, while relatively abundant in $IgM^-/CD8\alpha^+$ and $IgM^-/CD8\alpha^-$ populations. Moreover, we sorted trout $CD8\alpha^+$ lymphocytes from mucosal and non-mucosal lymphoid tissues and compared the expression profiles of *Eomes-a* and *-b* with those of other T cell-related transcription factor genes (*GATA-3*, *T-bet* and *Runx3*), a Th1 cytokine gene (*IFN-γ*) and a Th2 cytokine gene (*IL-4/13A*). Interestingly, the tissue distribution of *Eomes-a/b*, *T-bet*, and *Runx3* versus *IFN-γ* transcripts did not reveal simple correlations, suggesting tissue-specific properties of $CD8\alpha^+$ lymphocytes and/or multiple modes that drive IFN-γ expressions.

1. Introduction

In mammals, a number of transcription factors involved in T cell maturation and differentiation have been identified. Th-POK and Runx3, for instance, regulate the commitment of common progenitor $CD4^+CD8^+$ double-positive cells to helper and cytotoxic lineages, respectively [1]. Naïve $CD8^+$ and $CD4^+$ T cells can further differentiate into effector cytotoxic T lymphocytes (CTL) and helper T (Th) cells, respectively, expressing lineage-specific transcription factors. The T-box transcription factor, Eomesodermin (Eomes), was originally discovered as a key molecule in *Xenopus* mesodermal development [2], and the involvement of Eomes in T cell functions was only later discovered in mammals [3]. Eomes controls the expression of cytolytic effector molecules and IFN-γ in orchestra with another T-box transcription factor T-bet, and both T-box transcription factors coordinately regulate the differentiation of $CD8^+$ T cells and NK cells [4,5]. In $CD4^+$ T cells, the lineage-specific transcription factors T-bet, GATA-3, RORγt, BCL-6 and Foxp3, control differentiation of Th1, Th2, Th17, T_{FH} and Treg cells, respectively [6].

Specific cell-mediated immunity, which is executed by $CD8^+$ CTL in mammals, has been studied in several teleost species, especially ginbuna crucian carp (*Carassius auratus langsdorfii*), channel catfish (*Ictalurus punctatus*) and rainbow trout

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(*Oncorhynchus mykiss*) [7–10]. The functional conservation of teleost CD8α^+ T cells has been reported in ginbuna by using monoclonal antibodies (mAbs) against CD8α. Ginbuna CD8α^+ T cells can execute alloantigen-specific perforin-mediated cytotoxicity [11–13], and an important role of CD8α^+ T cells in the graft-versus-host reaction (GVHR) was also shown in ginbuna [14]. From expression studies on the transcriptional level, it has been suggested that virus-specific cytotoxic activity can be mediated by CD8α^+ cells in both rainbow trout and ginbuna [15–18]. These findings indicate the presence of cytotoxic T lymphocytes (CTL) in fish similar to those of higher vertebrates. However, the mechanisms of differentiation and maturation of T cell subsets are still largely unknown although T cell-related genes such as TCR, CD3, CD4, CD8, CD28 and CTLA-4 have been identified in several fish species [19].

Around 350 million years ago a fish-specific whole genome duplication (FS-WGD) occurred [20], and some genes, situated as single copy in mammals, are represented by two copies in teleosts. Among T-brain1 subfamily genes, *T-brain1*, *Eomes* and *T-bet*, only *Eomes* was found represented by two loci in teleosts [21]. Eomes-a in zebrafish was shown to play an important role in the embryogenesis, as known for mammalian Eomes [22–26], but the precise function of teleost Eomes-b has not been investigated yet. Interestingly, zebrafish *Eomes-a* shows high expression levels throughout ontogeny, while the *Eomes-b* expression was found to increase gradually during embryonic development [27]. Moreover, the expression of both *Eomes-a* and *-b* was detected in immune cells, especially in lymphocytes [21]. Recently, it was revealed that overexpression of Atlantic salmon (*Salmo salar*) Eomes-a can induce *IFN-γ* and *granzyme A* transcripts [28]. In addition, a positive correlation of salmon *Eomes-a* expression with copy numbers of infectious pancreatic necrosis virus suggested the involvement of Eomes-a in immune responses to viral infection [29]. These observations imply the importance of Eomes-a and -b for the immune system as also known for mammalian Eomes.

To date aforementioned transcription factors associated to T cell development have been identified in several fish species, but the expression analysis in teleost T cell subsets remained to be investigated. In the present study, we have cloned two *Eomes* genes, *Eomes-a* and *-b*, from rainbow trout and ginbuna crucian carp, which serve as experimental models for teleost cell-mediated immunity. Expression analysis comparing several organs of rainbow trout and ginbuna showed that both *Eomes* genes are mainly transcribed in lymphoid organs. Moreover, the expression profiles of both *Eomes* genes as well as T cell-related transcription factors (*T-bet*, *Runx3* and *GATA-3*) and cytokines (*IFN-γ* and *IL-4/13A*) were examined in rainbow trout CD8α^+ lymphocytes from non-mucosal and mucosal lymphoid tissues, revealing inconsistencies between Th1 transcription factors (*T-bet*, *Eomes-a* and *-b*) and *IFN-γ* expression, suggesting that the CD8α^+ lymphocyte populations are distinct between individual tissues.

2. Materials and methods

2.1. Fish

Homozygous isogenic rainbow trout strain C25, established at Nagano Prefectural Experimental Station of Fisheries, Japan, and clonal triploid ginbuna crucian carp from Lake Suwa (S3N clone) were used for *Eomes-a* and *-b* sequence analysis. Rainbow trout strain "Born" obtained from the Landesforschungsanstalt für Landwirtschaft und Fischerei, Born, Germany, and clonal triploid ginbuna from the island of Okushiri (OB1 clone) were used for expression analysis. Rainbow trout were maintained in 400 l tanks at 15 °C in a partially recirculating water system and fed with commercial dry pellets. Ginbuna were maintained in a recirculation system with filtered water disinfected by ultraviolet light at 25 °C and fed pelleted dry food once daily.

2.2. cDNA preparations and sequencing of Eomes-a and -b in rainbow trout and ginbuna

Total RNA was extracted from splenocytes of rainbow trout and ginbuna by using NucleoSpin RNA II system (Macherey–Nagel) and RNeasy mini kit (Qiagen), respectively. One microgram of total RNA was reverse-transcribed into cDNA for 5′- and 3′-RACE PCR with a SMARTer RACE cDNA Amplification Kit (TAKARA BIO INC). PCR reactions for RACE and ORF cloning were performed with Phusion Hot Start High-Fidelity DNA Polymerase (New England BioLabs) according to the manufacturer's guidebook. Cloning and sequence analysis were performed as described in previous reports [30,31]. The sequence of primers for cDNA cloning and expression analysis is noted in Supplementary Tables 1 and 2. The Genbank accession numbers are as follows: rainbow trout *Eomes-a-1*, JF719911; rainbow trout *Eomes-a-2*, JF719912; rainbow trout *Eomes-b*, JF719913: ginbuna *Eomes-a-1*, JF719914; ginbuna *Eomes-a-2*, JF719915; and ginbuna *Eomes-b*, JF719916.

2.3. Expression analysis of two Eomes mRNAs in rainbow trout and ginbuna tissues

Rainbow trout tissues indicated in Fig. 2(A), Supplementary Figs. 3 and 4 were homogenized by using Tissue lyser (Qiagen), after which total RNA was extracted by NucleoSpin RNA II system (Macherey–Nagel). RT-PCR was performed with OneStep RT-PCR Kit (Qiagen), gene-specific primers (Supplementary Tables 1 and 2), and 10 ng total RNA per 12.5 μl reaction mixture according to the manufacturer's guidebook. Total RNA of ginbuna tissues indicated in Fig. 2(B) was extracted with RNAiso (Takara Bio Inc.). One μg of total RNA from the various tissues was reverse-transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Expression analysis by PCR with the specific primer sets was performed with BIOTAQ (Bioline).

2.4. Expression analysis of flow-sorted cells

Leukocyte preparation from rainbow trout and ginbuna is described in previous publications [30,32]. Single cell suspensions of rainbow trout pronephrocytes were incubated with a rat anti-CD8α mAb [32] and a combination of mAb 4C10 and N2 (mouse origin), that bind to IgM heavy chain [33] and light chain [34], respectively, for 30 min, followed by staining with FITC-conjugated goat anti-rat IgG (H + L) and R-PE-conjugated anti-mouse IgG subclass specific antibodies (Jackson ImmunoResearch) for 20 min, respectively. The stained leukocytes were separated into CD8α^+, surface IgM$^+$ (sIgM) and CD8α^-/sIgM$^-$ cells with a MoFlo high-speed cell sorter (Dako). Rainbow trout leukocytes from thymus, blood, pronephros, spleen and mucosal tissues were likewise stained with a rat anti-CD8α mAb and FITC-conjugated goat anti-rat IgG (H + L) and were subsequently sorted into CD8α^+ and CD8α^- lymphocytes using a MoFlo (DakaCytomation) high-speed sorter. Ginbuna pronephrocytes were separately stained with anti-IgM, anti-CD8α or anti-CD4-1 mAbs. After washing, cells were incubated with biotinylated anti-mouse IgG + M goat IgG (Jackson Immunoresearch) followed by staining with streptavidin-PE (Serotec). In ginbuna, separate single staining was necessary because fluorescence intensity of mAb$^+$ cells was not intense enough to properly discriminate more than one positively stained subpopulation in double staining. Six populations, sIgM, CD8α^+ and CD8α^- lymphocytes or CD4-1$^+$ and CD4-1$^-$ lymphocytes were