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A novel immune-related gene, microtubule aggregate protein homologue, is up-regulated during IFN-γ-related immune responses in Japanese flounder, *Paralichthys olivaceus*

Goshi Kato, Hidehiro Kondo, Takashi Aoki, 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

Delayed-type hypersensitivity (DTH) response mediated by antigen-specific Th1 cells is used as a test to detect exposure to tuberculosis in humans. Japanese flounder (*Paralichthys olivaceus*) microtubule aggregate protein homologue (PoMTAP) was identified as a gene strongly induced during fish DTH response. In this study, PoMTAP gene was cloned and its expression profile was analyzed. The PoMTAP gene has a transcriptional regulatory region that includes two interferon-stimulated response elements and two IFN-γ activated sites. Expressions of PoMTAP and IFN-γ genes were up-regulated at the same time points during the DTH response, *Edwardsiella tarda* infection and VHSV infection. Furthermore, PoMTAP gene expressing cells also expressed CD3ε, confirming that PoMTAP is expressed by T lymphocytes. These results suggest that PoMTAP is a novel immune-related gene expressed by T lymphocytes that is preferentially induced by IFN-γ and has a role in Th1-mediated immune responses in Japanese flounder.

1. Introduction

The tuberculin response, a delayed-type hypersensitivity (DTH), is defined as a granulomatous inflammation caused by a memory Th1-induced, cell-mediated immune response in mammals. It represents a cutaneous induration and erythema after injection of purified protein derivative (PPD) extracted from culture filtrate of *Mycobacterium tuberculosis*, in individuals previously exposed to *M. tuberculosis* and those vaccinated with *Mycobacterium bovis* Bacillus Calmette and Guèrin (BCG). Upon injection of the antigen to sensitive individuals, Langerhans cells process and present the antigen to local memory Th1 cells or cytotoxic T lymphocytes (CTLs) (Katou et al., 2000). These T cells in concert with activated Langerhans cells and macrophages secrete numerous cytokines including interleukin (IL)-1β, tumor necrosis factor (TNF)α and interferon (IFN)-γ that cause the hallmarks of inflammation (Black, 1999). IL-1β is critical in the development of DTH responses, functioning as T cell co-stimulatory molecules and inducing local inflammation by activating dendritic cells (Nambu et al., 2006). An important role of IL-1β and TNFα in DTH responses is to stimulate the production of chemokines from macrophages to gather DTH-related cells such as neutrophils and mononuclear cells into the region (Kobayashi et al., 2001). IFN-γ is a typical Th1 cytokine that mediates DTH responses, accelerating differentiation and proliferation of Th1 cells and inducing activated macrophages (Harumi and Goldman, 2007). Finally, the cytokine secretions and influx of DTH-related cells into the area result in granulomatous inflammation. Thus, the tuberculin response is used to test whether the individual has an anti-tuberculosis immunity acquired by BCG vaccination or *M. tuberculosis* infection.

We previously used the DTH response in fish to confirm the efficacy of BCG vaccine against pathogenic *Mycobacterium* sp. (Kato et al., 2010). After injection of PPD from *Mycobacterium* sp. culture filtrate into the BCG-vaccinated fish, IL-1β, TNFα and IFN-γ gene expression levels were up-regulated in the kidney. These results suggested that DTH responses to *Mycobacterium* sp. antigen were elicited in BCG-vaccinated fish. In addition, we used a Japanese flounder, *Paralichthys olivaceus*, cDNA microarray to identify fish genes that are associated with activation of Th1-mediated immune responses during the DTH response. One strongly up-regulated gene was a gene of unknown function (Japanese flounder EST clone LA9 (10), GenBank Accession No. C23433).

The deduced amino acid sequence of an unknown gene LA9 (10) was similar to that of microtubule aggregate protein (MTAP) in mammals. MTAP, a type I interferon (IFN-α and β)-induced protein referred to as interferon-induced protein 44 (IFI44), has a role in aggregating microtubles in hepatocytes during hepatitis C virus (HCV) infection (Honda et al., 1990; Takahashi et al., 1990; Kitamura et al., 1994). MTAP is expressed in cytoplasm and was shown to

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

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inhibit cell proliferation in human cell lines during virus infection (Hallen et al., 2007). MTAP contains a perfect GTP-binding site but has no homology to known GTPases or G proteins (Hallen et al., 2007). In human, the IFI44 gene expression level is sometimes used to an indicator of activation of type I IFN during rheumatoid arthritis, systemic lupus erythematosus (Nzeusseu et al., 2007) and interferon therapy against HCV (Liu et al., 2007). On the other hand, mammals have another paralogous gene, IFI44-like histocompatibility 28 (H28), which is induced by type II IFN (IFN-γ) (Malarkannan et al., 2000). H28 was isolated as an unknown histocompatibility antigen whose gene is located at the polymorphic *H28* locus on chromosome 3 and yields the naturally processed ILENFPRL peptide for presentation to CTL (Malarkannan et al., 2000). However, its function in human immunology is unknown.

In this study, we characterized the structure and expression of Japanese flounder MTAP (PoMTAP) along with type I IFN and IFN-γ, to confirm whether the PoMTAP is associated to the Th1-induced cell-mediated immune responses in Japanese flounder.

2. Materials and methods

2.1. Molecular cloning of PoMTAP cDNA

The full length of PoMTAP cDNA was determined by 3′- and 5′-RACE PCR using a SMART RACE cDNA amplification kit (BD Biosciences, USA) according to the manufacturer's instructions. Primers used in the 3′- and 5′-RACE PCR are shown in 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). The deduced amino acid sequence was compared with all sequences available in DDBJ/EMBL/GenBank in NCBI homepage (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and with all predicted amino acid sequences in Ensembl Genome Browser (http://uswest.ensembl.org/index.html). Putative domains and motifs contained in PoMTAP were predicted using InterProScan (http://www.ebi.ac.uk/interpro/index.html), and sequences of MTAP homologues were aligned using the Clustal W program of DDBJ. A phylogenetic tree was conducted with the Neighbor-joining method (Saitou and Nei, 1987) using the Molecular Evolutionary Genetic Analysis program (MEGA) version 4 (Tamura et al., 2007). A PoMTAP homologue of lancelet was used as an outgroup.

Table 1
Primers used in this study.

Primer name	Sequence of oligonucleotides
RACE-PCR	
MTAP 5′-RACE 1st	5′-CCATCACAACAGCTGAGATGTTGACCAG-3′
MTAP 5′-RACE 2nd	5′-ACCAGATCACGTGATGACGTCACACC-3′
MTAP 3′-RACE 1st	5′-TGCTCTGTCATCACGTCGTTAGCTTGGTGT-3′
MTAP 3′-RACE 2nd	5′-TCAGCTGTTGTGATGGTGCCTTCACGTG-3′
RT-PCR	
MTAP RT S	5′-TTCTCTAATGCCGCCTCCTATC-3′
MTAP RT AS	5′-TGCCTCTTTCAACTTTAACAAACCC-3′
β-Actin S	5′-ACTACCTCATGAAGATCCTG-3′
β-Actin AS	5′-TTGCTGATCCACATCTGCTG-3′
qPCR	
MTAP qPCR S	5′-GTTAGCTTGGTGTGACGTCA-3′
MTAP qPCR AS	5′-CGTGAAGGCACCATCACAAC-3′
Type I IFN qPCR S	5′-GCATCAGCTGAGGATAGACTTGGT-3′
Type I IFN qPCR AS	5′-CAGGGCAGCCACCTCCTT-3′
IFN-γ qPCR S	5′-TGTCAGGTCAGAGGATCACACAT-3′
IFN-γ qPCR AS	5′-GCAGGAGGTTCTGGATGGTTT-3′
IL-1β qPCR S	5′-CAGCACATCAGAGCAAGACAACA-3′
IL-1β qPCR AS	5′-TGGTAGCACCGGGCATTCT-3′
RPL10 qPCR S	5′-GCTCCTCTGGTGCAGTTTGTGA-3′
RPL10 qPCR AS	5′-TGGTGTTTGCTGGCGTCACTCT-3′

2.2. Screening of PoMTAP gene

To determine the structure of the PoMTAP gene and its 5′-flanking region, a genomic BAC library of Japanese flounder (Katagiri et al., 2000) was screened using the open reading frame of PoMTAP as a probe labeled with a random primer DNA labeling kit (TAKARA BIO, Japan), according to the manufacturer's instructions. The probes were hybridized as previously reported (Katagiri et al., 2000). Positive clones were picked and then BAC plasmids were extracted using CsCl density-gradient ultra centrifugation. Extracted BAC clones were sequenced by primer walking. Exon–intron construction was estimated using the Spidey program (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/). Transcription factor binding sites were searched for in the 750-bp upstream region of the PoMTAP gene using TFSEARCH ver 1.3 (http://mbs.cbrc.jp/research/db/TFSEARCH.html).

2.3. Tissue distribution of PoMTAP gene expression

Total RNA was extracted from 14 tissues (see Fig. 5 legend) of apparently healthy Japanese flounder (approximately 1 kg) using RNAiso (TAKARA BIO, Japan), following the manufacturer's instructions. First strand cDNA was synthesized with 2 μg of total RNA using MMLV reverse transcriptase (Invitrogen, USA), following the manufacturer's instructions. Peripheral blood leukocytes (PBLs) were isolated using Percoll (GE healthcare, Sweden) as previously reported (Hirono et al., 2000). Total RNA was also extracted from PBLs and cDNA was synthesized as described above. RT-PCR was conducted with the first strand cDNAs as templates using gene-specific primers for PoMTAP and β-actin (NCBI Accession No. HQ386788) as an internal control (Table 1) in a 30 μl reaction volume. PCR products were electrophoresed on a 1% agarose gel and then visualized with ethidium bromide.

2.4. Gene expressions of PoMTAP, type I IFN and IFN-γ in PBLs after immune-stimulation

PBLs were isolated and stimulated with LPS and poly I:C as described previously (Suzuki et al., 2010). Briefly, PBLs were isolated from healthy Japanese flounder weighing approximately 1 kg, and then were re-suspended in primary culture medium (RPMI1640 supplemented with 10% fetal bovine serum, 100 UI/ml of penicillin and 100 μg/ml of streptomycin). Primary cultured PBLs (6.3×10^7 cells) were stimulated with 500 μg/ml LPS (derived from *Escherichia coli* 0127:B8, Sigma–Aldrich, USA) and 50 μg/ml poly I:C (Sigma–Aldrich, USA), phosphate buffered saline (PBS) were also added to primary cultured PBLs as a negative control. Stimulated PBLs were sampled at 1, 3 and 6 h post-stimulation and total RNA were extracted as described above. Primers specific for PoMTAP, type I IFN (AB511962), IFN-γ (AB435094) and ribosomal protein L10 (RPL10, AU050650) as an internal control used in qPCR analyses were designed using primer express software version 3.0 (Applied Biosystems, USA) (Table 1). Reaction mixtures were prepared with fast start universal real-time PCR master mix (Roche Applied Science, USA), according to the manufacturer's instructions. qPCR analyses were performed using a 7300 real-time PCR system (Applied Biosystems, USA), according to the manufacturer's instructions.

2.5. PoMTAP gene expression during fish DTH response

Japanese flounder DTH response was induced by injection of purified protein derivative (PPD) from *Mycobacterium* sp. culture fluid into a BCG-vaccinated fish, as previously described (Kato et al., 2010). Briefly, Japanese flounder weighing an average 8.3 g were vaccinated with 2.4×10^8 CFU/fish of BCG and PBS as a negative control, and then these fish was injected with PPD at