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Molecular characterization of purinergic receptor P2X4 involved in Japanese flounder (*Paralichthys olivaceus*) innate immune response and its interaction with ATP release channel Pannexin1

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ABSTRACT

P2X4 receptor (P2X4R) is a member of trimeric ATP-gated receptor channel family. Despite the importance of P2X4R in innate immunity has been addressed in mammals, the immunological significance of P2X4R has not been characterized in fish. In the present study we identified a full-length *P2X4R* cDNA sequence from Japanese flounder *Paralichthys olivaceus* (termed *poP2X4R*) by RT-PCR and RACE approaches and analyzed its gene expression patterns under normal and immune challenge conditions. Qualitative RT-PCR analyses revealed that *poP2X4R* has a widespread distribution in all examined tissues but dominantly expressed in hepatopancreas. In Japanese flounder head kidney macrophages and peripheral blood lymphocytes, *poP2X4R* was rapidly and significantly up-regulated by the immune challenges of LPS, poly(I:C) and zymosan. In addition, *poP2X4R* was up-regulated in spleen, head kidney and gill tissues by *Edwardsiella tarda* infections. Furthermore, we showed that poP2X4R is a membrane glycoprotein which could interact with ATP release channel Pannexin1, an important component in extracellular ATP-activated purinergic signaling pathways involved in Japanese flounder innate immune response. From a comparative immunological point of view, our results have provided new evidence for the involvement of extracellular ATP-gated P2XRs in fish innate immunity.

1. Introduction

Adenosine triphosphate (ATP) is a potent extracellular signaling molecule playing many important roles in host innate immunity through action on purinerigic receptors at the plasma membrane of cells [1]. ATP-gated purinerigic receptors are comprised of ligand-gated receptors (denoted P2XRs) and metabotropic G protein-coupled receptors (denoted P2YRs) [2]. P2X receptors are non-selective cation channels expressed in various immune tissues and cells where they play important roles in control of the innate immune response [3]. Up to date, seven subtypes of P2X receptors, P2X1 through P2X7, have been identified in mammals [4] and are organized as trimeric homomers or heteromers [5].

Our previous studies have revealed that ATP release is significantly induced by PAMP challenges in Japanese flounder *Paralichthys olivaceus* [6] and activation of purinergic receptors by extracellular ATP plays important roles in modulating gene expression of proinflammatory cytokines in *P. olivaceus* [7], suggesting that ATP-gated purinergic signaling may perform important roles in fish innate immunity. However, these studies have not excluded the engagement of other P2XR members besides P2X7R in fish innate immune response. Finding these ATP-activated purinergic receptors thus will improve our understandings of the importance of ATP-dependent signaling in fish innate immunity.

In addition to P2X7R, P2X4R is another important P2X receptor involved in host innate immunity in mammals [8–11]. P2X4R is rapidly activated upon ATP binding, leading to increase in intracellular calcium concentrations, activation of mitogen-activated protein kinases, and production of proinflammatory cytokines

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[12–14]. In contrast, down-regulation of P2X4R by the antisense oligonucleotide decreased the levels of serum IL-1β, TNF-α, IL-6, and IL-17 [15]. P2X4R is also implicated in NLRP3 inflammasome activation [8,10] and P2X7 receptor-dependent macrophage death [16]. In addition, P2X4R plays an important role in an reactive oxygen species generation during microbial infection process [17] and in the controlling of the fate and survival of activated microglia, the resident immune cells of the central nervous system [18]. Furthermore, P2X4R has been indicated as one of the important P2X receptors in responses to hepatitis C virus [19] and *Chlamydia trachomatis* [20] infections.

Compared with the intensive studies in mammals, however, the immunological significance of P2X4 receptor in lower vertebrates has not been revealed yet. In the present study we cloned a *P2X4R* cDNA from *P. olivaceus* (termed *poP2X4R*) and examined its expression patterns under normal and immune challenge conditions. We also investigated the interactions of poP2X4R with Pannexin1 (Panx1) channels, an important component in ATP-dependent signaling complex involved in the Japanese flounder innate immune response [6]. We showed that *poP2X4R* mRNA transcripts were constitutively expressed in all examined normal tissues with dominant expression in hepatopancreas and significantly up-regulated by different PAMP challenges and bacterial infections as well. We also demonstrated that poP2X4R is a glycosylated protein localized on the cell membrane. In addition, co-immunoprecipitation experiments indicated that poP2X4R could interact with the Japanese flounder Panx1 proteins. These observations indicated that, in addition to P2X7R, P2X4R is another important purinergic receptor involved in fish innate immune response. Moreover, the association of P2X4R with ATP release channel Panx1 may form a protein complex to trigger the downstream extracellular ATP-dependent purinergic signaling pathway(s) in fish innate immunity. To the best of our knowledge, the present study investigated the immune significance of P2X4R in fish for the first time.

2. Materials and methods

2.1. Animals and maintenance

Japanese flounder *P. olivaceus* were purchased from a local farm in Dagang, Tianjin, China, transported to the laboratory and maintained in an aerated running seawater system at 21 °C for two weeks before experimentation. Fish were clinically examined and only healthy animals without any pathological signs were selected in experiments. For tissue collection, *P. olivaceus* was euthanized with tricaine methane sulfonate (Sigma) as described in previous study [21] and the individual tissue was separated aseptically. All experiments were conducted in accordance with the NIH guidelines for the care and use of experimental animals and the studies were approved by the animal care and use committee of Tianjin Normal University.

2.2. RNA preparation and cDNA synthesis

Tissues were homogenized with a pestle in the presence of liquid nitrogen and total RNA was then isolated by TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Total RNA from cells was extracted using a RNeasy Plus Mini Kit (Qiagen). The integrity of RNA was determined by electrophoresis on a 1.2% formaldehyde-denatured ethidium bromide agarose gel and photographed on a GelDoc™ XR system (Bio-Rad). RNA concentration was measured using a NanoDrop 2000 UV/Vis spectrophotometer (Thermo Fisher Scientific) and subjected to DNase I (Invitrogen, amplification grade) treatment to remove genomic DNA contamination according to the manufacturer's procedure. First-strand cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen) and oligodeoxythymidine adaptor primer (5′-TCGAATTCGGATCCGAGCTCT17V-3′) following the manufacturer's instructions. Mock reverse transcription (without enzyme) for each sample served as negative controls and no PCR products were amplified (data not shown), ruling out the possibility of genomic DNA contamination.

2.3. Cloning of Japanese flounder poP2X4R cDNA

For cloning of Japanese flounder *poP2X4R* gene, a degenerate primer pair (F1/R1, Table 1) was designed and RT-PCR was performed using the cDNA templates from hepatopancreas tissue. PCR products were extracted with a QIAEX II Gel Extraction Kit (Qiagen). The purified PCR products were subcloned into pMD™18-T vector (TaKaRa) and sequenced which showed high amino acid sequence similarity with known P2X4R proteins. The obtained partial nucleotide sequence was used to design gene-specific primers to amplify the 5′- and 3′-untranslated region (UTR) of the Japanese flounder *poP2X4R* cDNA.

The full-length nucleotide sequence of the Japanese flounder *poP2X4R* cDNA was obtained by rapid amplification of cDNA ends (RACE) strategy. The 3′-UTR of *poP2X4R* cDNA end was obtained with a gene-specific forward primer F2 designed based on the sequence obtained above and a universal reverse adaptor primer R2 (Table 1). The 5′-RACE was performed using a SMARTer™ RACE amplification kit (Clontech) by two rounds of nested-PCR amplification. The first-strand cDNA was synthesized and nested PCR was conducted according to the manufacturer's protocol using reverse gene-specific primer GSP (Table 1). The first round of PCR reaction was performed with a forward primer UPM (a mixture of primers UPM-L and UPM-S, Table 1) and a reverse gene specific primer GSP, and then a nested amplification with primer pair NUP/GSP (Table 1) was performed in a MyCycler™ gradient thermocycler (Bio-Rad). Gene-specific primer pair F3 and R3 (Table 1) was designed, and RT-PCR was conducted to obtain the complete open reading frame of the Japanese flounder *poP2X4R* cDNA.

2.4. In silico sequence analyses

Nucleotide sequences were blast against the GenBank database using BlastX algorithm at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast) to determine their sequence identities. The nucleotide and derived protein sequences from *poP2X4R* were compared with other known P2X4R

Table 1
Sequence of primers used in this study.

Primer name	Sequence (5′→3′)
F1	TGTGAAGTGYTCTCRTGGTG
R1	GCTGCASCCARRTTMAYTAT
F2	TCTTGGGCAGGGAAGTGTTA
R2	TCGAATTCGGATCCGAGCTC
GSP	CACACATACCCCACCACATACAGCACCA
UMP-L	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
UMP-S	CTAATACGACTCACTATAGGGC
NUP	AAGCAGTGGTATCAACGCAGAGT
F4	ATGGGCAGGACTGCAAACTG
R3	TTACTGAGTTCCGTAGGCGGT
F5	CGACCTGCGTGGATGATTG
R4	GCTCAGGCAGTTTAGTGTCTATTTC
F6	AGGTTCCGTTGTCCCG
R5	TGGTTCCTCCAGATAGCAC
FLAG-f	CCCAAGCTTGGCAGGACTGCAAACT
FLAG-r	CGGAATTCTTACTGAGTTCCGTAGGCG