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Short communication

Identification and characterization of ATP-gated P2X2 receptor gene dominantly expressed in the Japanese flounder (*Paralichthys olivaceus*) head kidney macrophages

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ABSTRACT

P2X2 receptor (P2X2R) belongs to the family of purinergic receptors that have been shown to play important roles in regulating host innate immune response. Although the immunologic significance of P2X2R has been studied in mammals, the presence and immune relevance of P2X2R in fish remains unclear. In this study we extended our previous observations by identifying and characterizing a *P2X2R* ortholog (termed *PoP2X2R*) from Japanese flounder (*Paralichthys olivaceus*). Quantitative real-time PCR analysis revealed that *PoP2X2R* mRNA transcripts are widely distributed in all examined normal tissues and are dominantly expressed in hepatopancreas tissue. In addition, we for the first time showed that multiple P2XR subtypes, including *P2X2R*, *P2X4R* and *P2X7R* are co-expressed in the Japanese flounder head kidney macrophages (HKMs) and peripheral blood lymphocytes (PBLs), indicating that they may assemble into hetero-receptor complex or interact in the form of homotrimers to trigger diverse purinergic signaling in the Japanese flounder immune cells. Compared with the known Japanese flounder *P2X4* and *P2X7* receptors, however, *PoP2X2R* is much more abundantly expressed in the Japanese flounder HKM cells, suggesting that *PoP2X2R* may play an important role in this type of immune cells. Glycosylation and immunohistochemistry analyses revealed that PoP2X2R is a glycoprotein expressed on the plasma membrane. Immune challenges experiments showed that *PoP2X2R* was significantly induced by LPS, poly(I:C) and zymosan stimulations in the HKM and PBL cells, and by *Edwardsiella tarda* infections in spleen and gill tissues as well. Taken together, we have identified and characterized a new P2X2R member that is involved in fish innate immune response.

1. Introduction

Accumulating lines of evidence have demonstrated that purinergic signaling has a profound effect on multiple immune cell responses such as intracellular pathogen removal and apoptosis, T lymphocyte proliferation, cytokine release, chemotaxis, phagocytosis, Ag presentation and cytotoxicity, and nitric oxide production [1,2]. Purinergic receptors that recognize extracellular nucleotides and adenosine are important components for the purinerigc signaling system and are divided into three types: P1, P2 and adenosine receptors [3]. P2 receptors mediate the actions of extracellular nucleotides in cell-to-cell signaling and can be further functionally classified into two subtypes: ligand gated cation channel P2X receptors (P2XRs) and G-protein coupled P2Y receptors [4].

P2X receptors are activated by extracellular ATP and are formed by the assembly of three of the seven subunits (P2X1–7) upon ATP binding, in a homomeric or heteromeric configuration [5]. Among the seven known P2XR subtypes, P2X2R has the second longer intracellular C-terminal domain [6] and shows slow desensitization properties [7,8]. The presence of P2X2R in mammalian immune cells has been studied by real-time PCR, western blot and immunocytochemical assays [9,10] and knockout of P2X2R was

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associated with an increase in the number of immune cells, perhaps as a consequence of a compromised immune system and chronic infection [11]. Previous study also indicated that P2X2R is one of the important P2X receptors that are involved in hepatitis C virus (HCV) induced liver pathogenesis and are up-regulated by HCV structural proteins [12]. Although the engagement of P2X2R in innate immunity has been investigated in mammals, it remains unclear whether P2X2R is also implicated in fish innate immune response. In this study, to examine the immunological importance of P2X2R in fish, *P2X2R* ortholog (termed *PoP2X2R*) was identified and characterized from the flat marine fish Japanese flounder (*Paralichthys olivaceus*) in which several important molecular components for the extracellular ATP-activated purinergic immune signaling systems have been characterized [13–16]. Our findings provide the first evidence that multiple *P2XR* subtypes are co-expressed in Japanese flounder immune cells and tissues, indicating that they may assemble into heteroreceptor complex to trigger more complicated purinergic signaling in fish immune cells. Our results also emphasized that diverse and complicated purinergic signaling pathway(s) may exist in fish innate immunity.

2. Materials and methods

2.1. Experimental animals and maintenance

Japanese flounder *P. olivaceus* were obtained from a local farm in Dagang, Tianjin, China. Animals were transported to the laboratory and cultured in an aerated running seawater system at 21 °C for two weeks before experimentation to acclimate the laboratory conditions. Only healthy animals without any pathological signs were selected in experiments. The procedure for collection of the individual tissues of *P. olivaceus* was stated in the previous study [17]. Japanese flounder *P. olivaceus* was not an endangered species and not required for any special approval from local or central government.

2.2. RNA preparation and cDNA synthesis

Total RNA from Japanese flounder tissues and cells was extracted using TRIzol reagent (Invitrogen) and the PureLink® RNA Mini Kit (Invitrogen), respectively, according to the recommendations by the manufacturer. The integrity of purified RNA was examined by electrophoresis on a 1.2% formaldehyde-denatured agarose gel and visualized with ethidium bromide staining. The purified RNA was quantified with a NanoDrop 2000 UV/Vis spectrophotometer (Thermo Fisher Scientific) and subjected to DNase I treatment (Invitrogen, amplification grade) to digest residual genomic DNA followed by first-strand cDNAs transcription using SuperScript III reverse transcriptase kit (Invitrogen) or Transcriptor First Strand cDNA Synthesis kit (Roche) according to the manufacturers' instructions.

2.3. RT-PCR and RACE PCR

The internal fragment of *PoP2X2R* cDNA was amplified using the degenerate primer pair F1/R1 (Table 1) and the cDNA templates synthesized from Japanese flounder spleen tissue. PCR products were separated by agarose gel and inserted into pMD18-T vector (TaKaRa). The identities of PCR products were determined by DNA sequencing. The obtained internal sequence was utilized to design the gene-specific primers to amplify the 5′- and 3′-sequences of *PoP2X2R* cDNA using rapid amplification cDNA ends (RACE) strategy. The 3′-RACE PCR was performed with a gene-specific forward primer F2 designed based on the internal sequence obtained above and a universal reverse adaptor primer R2 (Table 1). A two-step touch-down PCR was applied to amplify the 5′-end of *PoP2X2R* cDNA by a SMARTer™ RACE amplification kit (Clontech): 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, followed by a nested amplification with primer pair NUP/GSP (Table 1) performed in a MyCycler™ gradient thermocycler (Bio-Rad). Finally, the complete open reading frame (ORF) sequence of *PoP2X2R* cDNA was amplified by a confirmation PCR using Taq2000 DNA polymerase (Stratagene) with gene-specific primer pair F3/R3 (Table 1) targeted on the 5′-UTR and 3′-UTR of *PoP2X2R*, respectively, which were designed based on the sequences obtained by RACE PCR. The PCR products with expected molecular size were inserted into pDM18-T vector. Three independent colonies were randomly selected and sequenced with both directions which showed 100% sequence identity.

2.4. Sequence analyses

The identities of PCR products were determined by blast against

Table 1
Sequence of primers used in this study.

Primer name	Sequence (5′→3′)	Purpose
F1	TGGGACTAYGARACYCCMAA	gene cloning
R1	GTAATATTTRGCAAACCTG	gene cloning
F2	ATATGCCAAACAAGCGCAA	3′-RACE
R2	CGAATTCGGATCCGAGCTC	3′-RACE
GSP	TCATCTTTGGAGTCTCATAGTCCCA	5′-RACE
UMP-L	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	5′-RACE
UMP-S	CTAATACGACTCACTATAGGGC	5′-RACE
NUP	AAGCAGTGGTATCAACGCAGAGT	5′-RACE
F3	GACCTTGACAACAATTCCGA	gene cloning
R3	GTCACTGAGCTGTCGCAAG	gene cloning
F4	CCCAAGCTTGGTGAAGTATTTCATACGTTC	DNA construct
R4	CGGGGTACCTCACAGCGGTACACCGTCTG	DNA construct
P2X2-f	TCAGAGGGAGGTGATGTGATTAGTA	real-time PCR
P2X2-r	CCCCTTGGACACAGTCAGAATC	real-time PCR
P2X4-f	CGACCTGCGTGGATGATTG	real-time PCR
P2X4-r	GCTCAGGCAGTTTAGTGTCTATTTC	real-time PCR
P2X7-f	CCGAAATACTCCTTCAGACGC	real-time PCR
P2X7-r	CGCCTGTCCGAACACCAT	real-time PCR
β-actin-f	AGGTTCCGTTGTCCCG	real-time PCR
β-actin-r	TGGTTCCTCCAGATAGCAC	real-time PCR