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Evolutional Conservation of Molecular Structure and Antiviral Function of a Viral RNA Receptor, LGP2, in Japanese Flounder, Paralichthys olivaceus

Maki Ohtani,* Jun-ichi Hikima,* Hidehiro Kondo,† Ikuo Hirono,† Tae-Sung Jung,* and Takashi Aoki*†

LGP2 is an important intracellular receptor that recognizes viral RNAs in innate immunity. To understand the mechanism of viral RNA recognition, we cloned an LGP2 cDNA and gene in Japanese flounder (Paralichthys olivaceus). Viral hemorrhagic septicemia virus-induced expressions of LGP2 mRNA were evaluated in vivo and in vitro by quantitative real-time PCR (Q-PCR) using primers based on the clone sequences. The expression of LGP2 mRNA in the kidney dramatically increased at 3 d postinfection. The expression of LGP2 mRNA also increased in the head kidney leukocytes stimulated with artificial dsRNA (polyinosin-polycytidylic acid) in vitro. To evaluate the antiviral activity of the flounder LGP2, three expression constructs containing pcDNA4-LGP2 (full-length), pcDNA4-LGP2ARD (regulatory domain deleted), and pcDNA4-Empty (as a negative control) were transfected into the hirame (flounder) natural embryo (hirame natural embryo) cell line. Forty-eight hours after transfection, the transfected cells were infected with ssRNA viruses, viral hemorrhagic septicemia virus, or hirame rhabdovirus. The cytopathic effects of the viruses were delayed by the overexpression of Japanese flounder LGP2. The Q-PCR demonstrated that mRNA expression levels of type I IFN and IFN-inducible genes (Mx and ISG15) in the hirame natural embryo cells overexpressing LGP2 were increased by polyinosin-polycytidylic acid and viral infections. These results suggest that Japanese flounder LGP2 plays an important role in the recognition of both viral ssRNA and dsRNA to induce the antiviral activity by the production of IFN-stimulated proteins. The Journal of Immunology, 2010, 185: 000–000.

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The nucleotide sequences of Japanese flounder LGP2 cDNA and gene reported in this paper have been submitted to GenBank under accession numbers HM070372 (LGP2 cDNA) and HM100666 (LGP2 gene).

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Abbreviations used in this paper: BAC, bacterial artificial chromosome; CARD, caspase activation and recruitment domain; CPE, cytopathic effect; HINAE, hirame natural embryo; IRF, IFN regulatory factor; ISRE, IFN-stimulated response element; KL, kidney leukocyte; MOL, multiplicity of infection; PAMP, pathogen-associated molecular pattern; PBL, peripheral blood leukocyte; poly I:C, polyinosin-polycytidylic acid; 5′pppssRNA, 5′-triphosphate ssRNA; PRK, pattern recognition receptor; Q-PCR, quantitative real-time PCR; RD, regulatory domain; RLR, RIG-I–like receptor; VHSV, viral hemorrhagic septicemia virus.

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RLRs that bind to viral RNAs contribute to the antiviral activity of the host cells by inducing the expression of type I IFN and other proinflammatory cytokines through the IPS-1 pathway (2).

The mammalian RLR family is composed of three DExD/H box RNA helicases: RIG-I, MDA5, and LGP2 (3–5). The DExD/H box RNA helicase domain includes two ATP-binding motifs (i.e., DExD/H box), which are also known as “Walker motif”. The RNA helicase domain of RIG-I can bind to both dsRNA and ssRNA in an ATP-dependent manner (6). LGP2 differs from RIG-I and MDA5 by the lack of two N-terminal caspase activation and recruitment domains (CARDs) but shares the DExD/H box RNA helicase. The CARD of RIG-I and MDA5 interacts with CARD of IPS-1 (INF-β promoter stimulator 1, also known as MAVS, VISA, and Cardif) (3, 4, 7), and the interaction initiates downstream signaling to produce type I IFNs through promoting phosphorylation of IFN regulatory factor (IRF)-3 and IRF-7 (IRF-3/7) (2, 8) and induction of NF-κB target genes (3). The CARDs usually bind to the regulatory domain (RD; also known as “repressor domain”) to prevent the signal transduction in the absence of viral RNA (6, 9). Recent structural analyses of C-terminal RLRs have shown that the C-terminal RD—which is conserved in RIG-I, MDA5, and LGP2—plays a role in RNA recognition (10–12).

RIG-I is capable of recognizing the uncapped 5′-triphosphate ssRNA (5′pppssRNA) (13, 14) and a short dsRNA (<1 kb) generated by viral replication (3, 15, 16). In contrast, MDA5 has a preference for long dsRNA (>3 kb) containing both artificial (e.g., polyinosin-polycytidylic acid [poly I:C]) and viral RNAs (16). However, LGP2 recognizes dsRNAs (including artificial and viral dsRNAs) and 5′pppssRNA with lower affinity (12). Furthermore, LGP2 binding affinity to the ligands is stronger than those of RIG-I and MDA5 (17).

When LGP2 was discovered in mammals, in vitro studies suggested that it functions as a negative regulator (9, 17, 18). LGP2-
knockdown and knockout tests showed that the production of type I IFN was enhanced by poly I:C and ssRNA (+) viruses in RIG-I signaling (19). In contrast, LGP2 deficiency resulted in a suppressed IFN response against encephalomyocarditis virus ssRNA (+) viruses, which triggered MDA5-mediated antiviral signaling (20, 21). These results suggest that LGP2 has both negative and positive regulatory effects on RIG-I and MDA5 signaling. Recently, Satoh and colleagues (22) strongly suggested that LGP2 is a positive regulator of both RIG-I- and MDA5-mediated antiviral responses. IFN production in mouse dendritic cells and embryonic fibroblasts obtained from Lgp2−/− mice was severely impaired by various RNA viruses (e.g., picornaviruses), which are recognized by MDA5, but was not impaired by influenza virus RNAs, which are recognized by RIG-I. However, it was shown that the existence of LGP2 was important in the induction of IFN-β production against some viruses such as Sendai virus, Japanese encephalitis virus, and reo virus, which are recognized by RIG-I. Therefore, LGP2 is a positive regulator of both RIG-I- and MDA5-mediated antiviral responses. These data suggest that LGP2 regulates the antiviral responses mediated by RIG-I and MDA5 in viral RNA recognition (8).

In teleosts, several pattern recognition receptors (PRRs) that sense viral RNA have been cloned and characterized. Among the receptors that recognize extracellular signals (i.e., TLRs), the TLR22 gene has been identified in fugu (Takifugu rubripes) and Japanese flounder (Paralichthys olivaceus) (23, 24). The fugu TLR22 recognized dsRNA both in the endoplasmic reticulum and on the cell surface, and it also contributed to IFN production after exposure to poly I:C or dsRNA virus (23). However, RLRs are receptors that recognize viruses in the cytoplasm. The gene for one of these receptors, RIG-I, was cloned from Atlantic salmon (Salmo salar) and the epithelium papulosum cyprinid (EPC) cell line (25). In addition, the CARDs of RIG-I inhibited virus replication in Atlantic salmon (25). Other RLRs, such as MDA5 and LGP2, have not been been characterized functionally, although their cDNAs were cloned from grass carp (Ctenopharyngodon idella) and Atlantic cod (Gadus morhua) (26–28). Furthermore, the LGP2 and Stat35/5 genes were syntenic in zebrfish (Danio rerio), stickleback (Gasterosteus aculeatus), medaka (Oryzias latipes), and fugu (29) as well as in mouse chromosome 11 (5).

The Japanese flounder is one of the most economically important cultured fish species in South Korea and Japan. Understanding the immune response to viral infection may lead to methods for preventing outbreaks of infectious diseases in fish farms. The Japanese flounder genome has two antiviral genes, Mx and ISG15, which possess an IFN-stimulated response element (ISRE) in the promoter regions (30, 31). The ISRE in teleost and mammalian type I IFN promoters is responsive to poly I:C and virus infection (32–35). The transcription of type I IFN genes is regulated by IRF-3 and IRF-7 (36, 37), which suggests that the mammalian antiviral mechanism through type I IFNs conserved in teleosts (38). The Japanese flounder ISG15 gene was found to be induced by poly I:C transfected into the cytoplasm (31), which suggests that Japanese flounder has an intracellular viral recognition system for IFN production. In this study, we report that Japanese flounder has a primordial viral RNA-recognizing receptor, LGP2, that recognizes ssRNA(−) Rhabdoviridae viruses, and whose structure and antiviral function are similar to those in higher vertebrates.

Materials and Methods

Cell lines and viruses

Hirame natural embryo (HINAE) cells derived from Japanese flounder embryo (39) were maintained in Leibovitz’s L-15 (Life Technologies, Carlsbad, CA) medium containing 10% FBS (Life Technologies), 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B at 28°C. A viral hemorrhagic septicemia virus (VHSV) was isolated from Japanese flounder cultured in South Korea. Hirame rhadovirus (HHRV) was supplied by Dr. M. Yoshimizu (40). VHSV and HHRV were propagated in HINAE cells at 15°C until cytopathic effect (CPE) was observed, and then the cultured media was stored at −80°C until use. Virus titers were determined by 50% tissue culture infective dose (TCID₅₀).

Fish

Healthy Japanese flounders obtained from a commercial fish farm in Namhae, South Korea, were kept at 10–28°C, supplied with filtered sea water, and fed commercial food pellets. To collect tissues for RNA extraction, the fish were anesthetized, and the dissected tissues were soaked in RNA Later (Life Technologies) at −80°C. For the challenge test, the fish were immersed in seawater with VHSV (10⁰ TCID₅₀/ml) for 30 min. At 1, 3, and 6 of postinfection, the kidney was individually isolated and stored in RNA Later (Life Technologies) at −80°C for RNA extraction.

Leukocyte isolation and mitogen stimulation in vitro

Japanese flounder leukocytes were isolated from peripheral blood and whole kidney by Percoll gradients. Blood was diluted 1:10 with Leibovitz’s L-15 medium (Life Technologies) and supplemented with 10 U/ml heparin, 10 nM HEPES, 60 mM NaCl, 5% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B. The whole kidney was dissected and washed once in Leibovitz’s L-15 medium. The tissues were minced in dishes, and large pieces of tissues were passed through a cell strainer (BD Biosciences, San Jose, CA). The filtrate was layered on a 51% iso-osmotic Percoll (GE Healthcare UK, Buckinghamshire, U.K.) solution, and centrifuged at 3000 rpm for 30 min at 4°C. The cells suspended on the 51% Percoll layer were collected and washed by centrifugation in cold Leibovitz’s L-15 medium. The cells were resuspended in the cold Leibovitz’s L-15 medium, and cell numbers were counted.

For stimulation experiments, 1 × 10⁷ cells/ml were cultured in a 24-well plate with 250 µg/ml LPS (Escherichia coli 055:B5; Sigma-Aldrich, St. Louis, MO) or 125 µg/ml polyinosinic-polycytidylic acid (poly I:C; Sigma-Aldrich) at 20°C for 6, 24, and 48 h. The harvested leukocytes were centrifuged at 1600 rpm for 5 min at 4°C, and resolved in RLT plus reagent provided by RNeasy mini kit (Qiagen, Hilden, Germany) for RNA extraction.

RNA extraction and cloning of LGP2 cDNA

For molecular cloning of Japanese flounder LGP2 cDNA, total RNA was isolated from Japanese flounder whole kidney using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. One microgram of total RNA was reverse transcribed with SuperScript III First Strand synthesis system (Life Technologies). To remove the surplus RNA from the original template, reactants were incubated with RNase H (Life Technologies) for 20 min at 37°C. To amplify the Japanese flounder LGP2 cDNA, degenerate primers were designed based on multiple alignments of the published LGP2 cDNA sequences including human (Homo sapiens; GenBank accession number BC014949 under http://www.ncbi.nlm.nih.gov/genbank/), mouse (Mus musculus; NM_031050), African clawed toad (Xenopus laevis; NM_001092446), Atlantic salmon (BT045378), and zebrfish (XM_001920601). PCR amplification for LGP2 fragment was demonstrated with primers WCUP-86 and WCUP-87 (Table I) using Japanese flounder whole kidney cDNA according to the following PCR condition. An initial denaturation step of 3 min at 94°C was followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 30 s, and a final extension step at 72°C, and a final elongation step at 72°C for 5 min. Amplified PCR products were cloned into pCR2.1-TOPO vector (Life Technologies) or pET1.2/blunt vector (Fermentas, Burlington, Ontario, Canada) and then submitted for the nucleotide sequencing. Specific primers were then designed and used for RACE-PCR. For RACE-PCR, the RNA were reverse transcribed using SMARTer RACE cDNA amplification kit (TAKARA Bio, Otsu, Japan) with universal primers provided by the RACE kit. The structure of deduced amino acid sequences of Japanese flounder LGP2 was analyzed using the SMART program (http://smart.embl-heidelberg.de/). The DNA fragment of Japanese flounder LGP2 amplified with primer sets WCUP-174 and WCUP-175 was used as a DNA probe for screening of bacterial artificial chromosome (BAC) clones from Japanese flounder genomic BAC library (41). To determine the nucleotide sequence of the lgp2 gene, the screened BAC clone (#621-B4) was sequenced with 26 primers (Table I), which were designed on each predicted exon sequence. The predicted amino acid sequence was aligned
used ClustalX software, and phylogenetic analysis generated from MEGA 4.0 program. The secondary structure of Japanese flounder LGP2 was predicted using SWISS-MODEL, which has an automated protein structure homology-modeling server (http://swissmodel.expasy.org/) (42–44).

**Construction of plasmid vectors**

The full-length ORF of Japanese flounder LGP2 was amplified from head kidney cDNA by PCR and cloned into pCR2.1-TOPO vector (pCR2.1-LGP2). Initially, the full-length LGP2 (residues 1–682) or the C-terminal RD deleted LGP2 (LGP2RD, residues 1–478) were amplified with primer sets WCUP-226 and WCUP-227 (for full-length) or WCUP-371 (for RD deletion; listed in Table I) from pCR2.1-LGP2 as a template, and then digested with EcoRI (Fermentas) and XhoI (Fermentas). The digested PCR products were ligated with EcoRI and XhoI digested pcDNA4/HisMax expression vector (Life Technologies), which were regarded as pcDNA4-LGP2 and pcDNA4-LGP2RD, respectively.

**Antiviral assay**

HINAE cells were cultured in 96-well plates at a concentration of 1 × 10^5 cells per well at 20°C. Before transfection, cells were washed with PBS, and then replaced with Opti-MEM (Life Technologies). Transfection was performed with Lipofectamine 2000 (Life Technologies) according to manufacturer’s instructions. For antiviral assay using 96-well plate, equal molar (1.7 nM) of pcDNA4, pcDNA4-LGP2, or pcDNA4-LGP2RD vectors were mixed with 0.5 μl Lipofectamine 2000 (Life Technologies), and shortage DNA was occupied with pUC19 vector (Life Technologies). The DNA mixtures were transfected to HINAE cells in 50 μl Opti-MEM at 20°C. Forty-eight hours after transfection, cells were infected with VHSV or HIRRV. After 1 h of adsorption, cells were washed with PBS and cultured until the appearance of CPE. After 3 or 4 d, cells were stained with 3% crystal violet for visualizing live cells. For virus titration, 50 μl of culture medium were collected 24 h posttransfection and used for plaque assay. The collected culture media were subjected to 10-fold serial dilution and then added into the monolayer of HINAE cells cultured in 12-well plate. After 1 h of adsorption, cells were rinsed with PBS and overlaid with 0.4% agarose in Leibovitz’s L-15 medium. After 3 d of culture, cells were fixed with 4% paraformaldehyde and stained with 3% crystal violet for counting the plaques.

**HINAE cells cultures**

HINAE cells cultured in 12-well plates at a concentration of 1 × 10^5 cells per well were used for expression analysis with quantitative PCR, and transfection methods are described as follows. For VHSV infection experiments, 8.5 nM pcDNA4, pcDNA4-LGP2, or pcDNA4-LGP2RD vectors were mixed with 0.5 μl Lipofectamine 2000 (Life Technologies) according to manufacturer’s instructions. For antiviral assay using 96-well plate, equal molar (1.7 nM) of pcDNA4, pcDNA4-LGP2, or pcDNA4-LGP2RD vectors were mixed with 0.5 μl Lipofectamine 2000 (Life Technologies), and shortage DNA was occupied with pUC19 vector (Life Technologies). The DNA mixtures were transfected to HINAE cells in 50 μl Opti-MEM (Life Technologies) and then cultured at 20°C. Forty-eight hours after transfection, cells were infected with VHSV or HIRRV. After 1 h of adsorption, cells were washed with PBS and cultured until the appearance of CPE. After 3 or 4 d, cells were stained with 3% crystal violet for visualizing live cells. For virus titration, 50 μl of culture medium were collected 24 h posttransfection and used for plaque assay. The collected culture media were subjected to 10-fold serial dilution and then added into the monolayer of HINAE cells cultured in a 12-well plate. After 1 h of adsorption, cells were rinsed with PBS and overlaid with 0.4% agarose in Leibovitz’s L-15 medium. After 3 d of culture, cells were fixed with 4% paraformaldehyde and stained with 3% crystal violet for counting the plaques.

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** FIGURE 1.** Amino acid sequence alignment of vertebrate LGP2s. Accession numbers: Japanese flounder (flounder), HM070372; fugu, Ensembl number ENSTO000000040292; Atlantic salmon (salmon), BT045378; African clawed toad (frog), NM_001092446; mouse, NM_030150; human, BC014949. The predicted motifs for DExD/H box RNA helicase domains are indicated by numbers (I–VI) and lines. The start position of the regulatory domain is indicated with an arrow head. The two predicted Zn^2+ binding motifs are indicated with black bars. The predicted RNA binding loop is indicated with a box. The conserved and important amino acid residues are shaded, boldface, or both.
Residues forming a disulfide bond are indicated with asterisks under the alignment. In schematics of the secondary structure, predicted α and β structures are shown in black and white boxes, respectively.

FIGURE 2. Secondary-structure prediction of the RDs in Japanese flounder LGP2 with human LGP2, MDA5, and RIG-I. The secondary structure elements of RD corresponded to the alignment in length. Amino acids conserved with the Japanese flounder LGP2 are indicated by black shading, and similar residues are indicated by gray shading. Locations of the Zn²⁺-binding motifs and RNA binding loop are marked above the alignment. Cysteine residues forming a disulfide bond are indicated with asterisks under the alignment. In schematics of the secondary structure, predicted β-sheet and α-helix structures are shown in black and white boxes, respectively.

Vectors were mixed with 2 μl of Lipofectamine 2000, and shortage DNA was occupied with pUC19 vector. Forty-eight hours after transfection, cells were infected with VHSV at 1 × 10⁶ PFU/ml (multiplicity of infection [MOI], 0.1) and cultured for 24 h at 15°C. For intracellular stimulation with poly I:C, vector constructs and poly I:C (0.1 and 1 μg) were mixed with 2 μl of Lipofectamine 2000 per 1.6 μg of total nucleic acids, and the shortage DNA was occupied with pUC19 vector. The mixtures were transfected to HINAE cells in 200 μl of Opti-MEM and then cultured at 20°C. Six hours after transfection, cultured medium were replaced with Leibovitz’s L-15 as culture medium. In the case of extracellular stimulation with poly I:C, the poly I:C (0.1 and 1 μg) was added to the L-15 medium (see the Fig. 10 legend).

RT-PCR and quantitative real-time PCR analysis

Total RNA of Japanese flounder tissues were extracted with the RNeasy Mini Kit or Trizol reagent (Life Technologies). One microgram of total RNA was treated with DNase I (Fermentas) to remove the genomic DNA; it was then reverse transcribed with high-capacity cDNA reverse-transcription kits (Life Technologies). RT-PCR was performed using 1 μl of cDNA as a template, 2 μl 10X PCR buffer, 0.75 μl 2'-deoxynucleoside 5'-triphosphate (10 mM), 0.4 μl of primer (10 μM), and 0.1 μl of Taq polymerase (Genet Bio, Seoul, South Korea) to a total of 20 μl. The primer sets WCU5-174 and WCU5-175 were used for LGP2, and WCU5-289 and WCU5-290 were used for β-actin (Table I). The PCR conditions were: an initial denaturation step of 3 min at 94°C, followed by 28 cycles (β-actin) or 35 cycles (LGP2) of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s, and a final elongation step at 72°C for 5 min. PCR products were separated by electrophoresis on a 2.5% agarose gel.

Quantitative real-time PCR (Q-PCR) was performed using Power SYBR Green PCR Master Mix (Life Technologies) or FastStart Universal SYBR Green Master (ROX; Roche, Basel, Switzerland) with the StepOne Plus Real-time PCR System (Life Technologies) according to the manufacturers’ instructions. Primers used for Q-PCR were designed with Primer Express software (Life Technologies). The amplification efficiencies of these primer sets were examined according to the instructions from Life Technologies. These primer sequences and efficiency values are listed in Table I. The specificity of the PCR amplification for all primer sets was verified from the dissociation curves. The relative expression levels for Japanese flounder LGP2, Ms, ISG15, ISG56, and type I IFN mRNA were determined using the Japanese flounder β-actin gene as an internal reference using comparative Ct (2⁻ΔΔCt) methods according to the ABI Prism 7700 User Bulletin #2 (Life Technologies). The statistical p values were calculated by Student t test.

Results

Japanese flounder LGP2 cDNA and gene structure

The full-length flounder LGP2 cDNA was cloned from whole kidney cDNA of Japanese flounder. It consists of 3304 bp including 65 bp of the 5’ untranslated region (UTR), 1190 bp of 3’ UTR, and a 2049 bp of open reading frame encoding 682 aa residues (HM070372). The six important motifs including DEvD/H box for RNA helicase activity were conserved in the RNA helicase region located at the N-terminal region of Japanese flounder LGP2 (Fig. 1). The two Zn²⁺-binding regions and RNA-binding loop in the C-terminus RD were also conserved in Japanese flounder LGP2.

The deduced LGP2 amino acid sequence of Japanese flounder was similar to the LGP2 sequences of fugu (66.6% identity), Atlantic salmon (67.7%), African clawed toad (50.3%), mouse (45.1%), and human (46.9%). The β-sheet and α-helix structures in the predicted secondary structures (Fig. 2) were highly conserved between Japanese flounder and human LGP2 RD, which were also conserved with those of human RIG-I and MDA5. A phylogenetic analysis of LGP2 was conducted with proteins related to the LGP2 sequence, such as RIG-I and MDA5, in several vertebrates (Fig. 3).
3). In the phylogenetic tree, Japanese flounder LGP2 clustered with other fish LGP2s, which were separate from the other RIG-I and MDA5 groups. The genomic organization of the Japanese flounder LGP2 gene (Fig. 4) was determined by direct sequencing using a BAC clone screened from Japanese flounder BAC library. The Japanese flounder LGP2 (HM100666) spanned 5474 bp containing 12 exons and 11 introns. The total length was half that of human LGP2. Comparison of the gene organization between Japanese flounder and human showed that exon 9 in Japanese flounder LGP2 corresponded to two exons (exon 10 and 11) in human LGP2.

**Tissue distribution of Japanese flounder LGP2 expression**

Japanese flounder LGP2 mRNA was expressed in all tissues examined, although it was highly expressed in the head and trunk kidneys, spleen, and heart (Fig. 5, Table I).

**Induction of LGP2 gene expression**

The expression of LGP2 mRNA in whole kidney leukocytes (KLs) increased in a time-dependent manner after LPS and poly I:C stimulation (Fig. 6). However, in peripheral blood leukocytes (PBLs), it was induced with LPS but not with poly I:C. The expression of the Japanese flounder Mx was also induced in KLs and PBLs, but the expression levels in PBLs were lower than those of KLs.

To observe in vivo induction of LGP2 gene expression, Q-PCR analyses were done using whole kidney cDNAs from three fish infected with VHSV (Fig. 7). Three days postinfection, the expression of LGP2 mRNA in whole kidney increased >200-fold compared with the control (0 d). Although the expression level of type I IFN was increased 15-fold 3 d postinfection, the induction was lower than in others.

**Antiviral activity of flounder LGP2**

The infection of HINAE cells transfected with the empty vector, by VHSV at $1 \times 10^4$ PFU/ml (MOI: 0.1) or HIRRV at $1 \times 10^5$ PFU/ml (MOI: 1.0), led to a complete CPE in 3 d (Fig. 8B, 8D). These CPE appearances were decreased with virus concentration in a dose-dependent manner. In contrast, the transfection of the HINAE cells with pcDNA4-LGP2 fully protected them against both VHSV and HIRRV in 3 d. Interestingly, the transfection of HINAE cells with pcDNA4-LGP2ΔRD failed to protect the HINAE cells infected with the viruses, although the pcDNA4-LGP2ΔRD-transfected cells slightly protected them against HIRRV (Fig. 8D). Based on the VHSV titers, replication of the virus in HINAE cells transfected with pcDNA4-LGP2 was significantly repressed compared with the control (Fig. 8C), whereas replication in HINAE cells transfected with pcDNA4-LGP2ΔRD was not repressed at all.

**Induction of IFN-inducible cytokine gene expressions by LGP2 overexpression**

To understand the gene expression profiles for LGP2 response postinfection by a virus, type I IFN expression level is an important factor because it triggers the subsequent antiviral responses, including the induction of IFN-inducible genes containing Mx, ISG15, and ISG56. Thus, type I IFN, Mx, ISG15, and ISG56 were tested for the experiments in Figs. 9 and 10. The expression of all examined genes was significantly induced by LGP2 overexpression in the presence of VHSV, although ISG56 gene expression was induced more weakly than others (Fig. 9). The overexpression of empty or mutated constructs failed to induce those genes.

To observe the response of cytokines after recognition of dsRNA by Japanese flounder LGP2, HINAE cells were cotransfected with pcDNA4-LGP2 and various concentrations of poly I:C. Mx gene expression in HINAE cells overexpressing LGP2 increased ~5-fold in the presence of 1.0 μg poly I:C (Fig. 10A), although it was well below the expression level in the absence of poly I:C (250-fold increase). Expression of the ISG15 gene was induced by transfected poly I:C, and this was increased upon LGP2 overexpression. The weak expression of Mx and ISG15 genes were induced by addition of poly I:C (Fig. 10B). Furthermore, HINAE cells overexpressing LGP2 were not influenced by extracellular poly I:C (Fig. 10C). In these experiments, however, type I IFN and ISG56 mRNA were not increased by cotransfection with poly I:C and LGP2.
Discussion

So far, the LGP2 gene has been functionally characterized only in mammalian species, although functionally uncharacterized sequences have been described in Atlantic cod (EU371924, a partial cDNA) (28), grass carp (27), and Atlantic salmon (BT045378). To elucidate the evolutionary origins and primordial functions of LGP2 as one of the receptors that recognize PAMPs, our studies focused on the discovery of RLR family orthologs in teleosts. Therefore, in this study, the Japanese flounder LGP2 gene was completely sequenced. In addition, we report the antiviral function of a nonmammalian LGP2. The exon-intron boundaries of Japanese flounder LGP2 gene were highly conserved with those of the human gene (Fig. 4), although introns 2 and 10 in the human gene were deleted in Japanese flounder LGP2 gene. Furthermore, the DExD/H box RNA helicase motifs and RD were also conserved in Japanese flounder LGP2 gene (Figs. 1–3).

Table I. Oligonucleotides sequences

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<th>Target (GenBank Accession Number)</th>
<th>Purpose</th>
<th>Name</th>
<th>Sequence of Oligonucleotide (5’ → 3’)</th>
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<td>WCUP-86</td>
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<td>WCUP-227</td>
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<td>Real-time PCR (98%)</td>
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<td>Type I IFN (AB511962)</td>
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<td>WCUP-379</td>
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a Amplification efficiency for real-time PCR analysis.
flounder LGP2 has functional abilities and also explains the antiviral function of Japanese flounder LGP2 (Fig. 8). Moreover, mutation studies revealed that a lysine (Lys) residue in motif I of the RNA helicase in human LGP2 and RIG-I (Lys30 in LGP2 or Lys270 in RIG-I) are involved in signal transduction (6, 17). In the motif I of Japanese flounder LGP2, Lys30 of human LGP2 appears to be conserved in Japanese flounder LGP2, where it is Lys33 (Fig. 1), which suggests that Lys33 is also involved in signal transduction.

Mammalian LGP2 RD (containing two Zn$^{2+}$-binding motifs and one RNA-binding loop) plays an important role in the ability of LGP2 to bind specifically to viral RNAs, including both dsRNA and 5’-ppp ssRNA (11, 12). LGP2 RD has a higher affinity for RNA than RIG-I RD and MDA5 RD (11). These affinities are dependent on the structure of a large basic surface formed by the RNA binding loop (11). Lys and phenylalanine residues in the RNA binding loop region are especially important for RNA-binding specificity (12). Comparison between Japanese flounder and human LGP2 RDs indicated that these Lys and phenylalanine residues in the Japanese flounder RNA binding loop is conserved (Figs. 1, 2). Furthermore, in mammalian RIG-I RD, mutation of the cysteines causes a reduction of RIG-I binding activity, while the two cysteine residues (CxxC motif) of the two Zn$^{2+}$-binding motifs are important for LGP2 to bind RNA as zinc-coordinating cysteines (10). In the first Zn$^{2+}$-binding motif, the CxxC motif was totally conserved in the Japanese flounder LGP2. In contrast, the structure of two cysteines in the second Zn$^{2+}$-binding motif differed from that of mammals by having an additional two residues between the two conserved cysteines (i.e., CxxxxC) (Fig. 1). Thus, these cysteine residues in the Zn$^{2+}$-binding motifs in Japanese flounder LGP2 RD are probably important for RNA binding. These findings raise the possibility that Japanese flounder LGP2 RD, like human LGP2 RD, binds both ssRNA and dsRNA.

Our finding that Japanese flounder LGP2, but not RD-deleted LGP2, had antiviral activity (Fig. 8) indicates that the RD is required for the antiviral response. In mammals, dsRNA and 5’-ppp ssRNA were found to bind to the basic surface and RNA binding loop of LGP2 RD (11). An analysis of the secondary structure of Japanese flounder LGP2 RD showed that the structure of Japanese flounder LGP2 RD is highly similar to that of human LGP2 RD (Fig. 2). Therefore, these results, and the fact that Japanese flounder and human LGP2 RD have similar structures (Fig. 2), suggest that the C-terminal RD of Japanese flounder LGP2 is required for the antiviral response. Recently, Satoh and colleagues (22) indicated that mammalian LGP2 was essential for type I IFN production in response to various RNA viruses, including ssRNA (+), ssRNA (−), and dsRNA viruses, and was necessary for RIG-I– and MDA5-mediated antiviral responses. The high degree of conservation of Japanese flounder LGP2 RD suggests that it has a function similar to that of mammalian LGP2 RD. The finding that Japanese flounder LGP2 induces IFN-inducible genes (Figs. 9, 10) is consistent with this suggestion.

The finding that Japanese flounder LGP2 was expressed strongly in head kidney, trunk kidney, spleen, and heart (Fig. 5) is interesting because these organs have important roles in the immune response of teleosts. The head kidney has many leukocytes (46), and the heart has many phagocytes for removing foreign particles (47).

Expression of the Japanese flounder LGP2 gene in the head kidney was induced by poly I:C stimulation (Fig. 6) and VHSV-infection (Fig. 7) as well as type I IFN and Mx gene expression. In mammals, LGP2 expression is also induced by poly I:C (19), which is known as a strong inducer of the expression of type I IFN genes. Interestingly, LPS increased the expression of LGP2 mRNA in both KLs and PBLs (Fig. 6). The mechanism by which LGP2 mRNA was upregulated by LPS stimulation is unknown, although human RIG-I was induced by LPS stimulation in vitro (48, 49). LPS also induces the expression of mammalian IFN-β through TLR4 signaling (50, 51), which suggests that LPS upregulated Japanese flounder LGP2 mRNA probably by upreg-
ulating the expression of type I IFN. Based on these results, we hypothesize that the strong responses of Japanese flounder LGP2 expression could be regulated by the ISRE motif, which is responsive to type I IFN.

Expression of the Mx gene is induced not only by type I IFN but also by IRF3 and IRF7 binding to ISRE in the Mx gene promoter. ISRE motifs are widely conserved from fish to mammals, including Japanese flounder (30, 34, 35, 37, 52, 53). Mx protein binds to the viral nucleocapsid protein and inhibits the trafficking in cells (54). ISG15 is known as a ubiquitin-like protein and inhibits the IPS-1 signal transduction by interaction of the ISG15 polymeric form to RIG-I (55). ISG56, a known IFN-inducible negative regulator of IFN production in mammals (56), forms a complex with eIF3 and inhibits viral protein translation (54). All these gene expressions are regulated by the ISRE motifs, which are capable of binding to IRF-3 and IRF-7. In mammals, the promoters of the ISG15 and ISG56 genes have an ISRE sequence (57, 58). The promoter of the Japanese flounder ISG15 gene, like that of the Mx gene, has ISRE motifs (31). Therefore, the expressions of genes in Japanese flounder are regulated by IRF-3/7 binding to the ISRE motif.

The expression of type I IFN mRNA was induced by VHSV infection in live fish (Fig. 7) and HINAE cells (Fig. 9), but not by poly I:C stimulation in HINAE cells (Fig. 10A, 10B). In salmonid, Mx and ISG15 expressions are induced by infectious salmon anemia virus infection through two pathways: an IFN-dependent manner and an IFN-independent manner (59, 60). These findings suggest that Japanese flounder Mx and ISG15 induction by poly I:C transfected into HINAE cells (Fig. 10A) could be mediated through the IFN-independent pathway. In contrast, Japanese floun-

**FIGURE 8.** Schematic representation of transfected Japanese flounder LGP2 constructs and antiviral activity of Japanese flounder LGP2 in HINAE cells against VHSV and HIRRV. The full-length Japanese flounder LGP2 and RD-deleted LGP2ΔRD were constructed (A). Both constructions and control pcDNA4 were transfected into HINAE cells. The cells were cultured for 48 h at 20˚C and then infected with VHSV (B) or HIRRV (D) in duplicate at the indicated densities. Three or 4 d postinfection, cells were fixed with 4% paraformaldehyde and stained with 3% crystal violet. C, The virus titer was calculated by plaque assay. Twenty-four hours postinfection with VHSV, the cultured medium was collected and infected to new HINAE cells in a six-well plate. Error bars indicate the SD for the results of three individual experiments. Asterisks indicate the significant differences of virus titers between control, LGP2 and LGP2ΔRD. *p < 0.0001, Student t test.

**FIGURE 9.** Expression of type I IFN and IFN-inducible genes in LGP2-transfected HINAE cells infected with VHSV. One microgram of expression plasmid encoding full-length LGP2, RD-deleted LGP2ΔRD, or pcDNA4 (as control) was transfected into HINAE cells. Forty-eight hours after transfection, cells were infected with VHSV at 1 × 10⁵ PFU/ml and incubated at 15˚C for 24 h. Total RNA was extracted and processed for Q-PCR analysis using primers targeting Mx, ISG15, ISG56, and type I IFN (primers shown in Table I). The β-actin gene was used as an internal control to normalize the cDNA template and for Q-PCR calculations. Expression levels are shown as fold increase values relative to the mock or VHSV-infected pcDNA4-empty vector transfected cells. Error bars indicate the SD for the results of three experiments. Student t test was used for statistical analysis, and asterisks indicate significant differences between control plasmid-transfected HINAE cells and LGP2 or LGP2ΔRD plasmid-transfected HINAE cells. *p < 0.01; **p < 0.001.
der ISG56 expression was not induced by poly I:C transfection (Fig. 10A), because it could be regulated by the IFN-dependent pathway alone.

Furthermore, another possibility could be discussed. Fish type I IFNs have been classified into groups I and II, and their expression patterns were varied (61, 62). In group I, zebrafish and rainbow trout possess an alternative-splice transcript lacking the leader peptide, the expression level of which was much weaker than that of another transcript that includes the leader peptide after poly I:C stimulation (61, 63). Therefore, the Japanese flounder type I IFN tested in this study might be an alternative-splice variant with a weak response to poly I:C stimulation.

The Mx and ISG15 induction levels shown in Fig. 10B (poly I:C stimulation from the extracellular) were much lower than those shown in Fig. 10A (poly I:C stimulation in the cytoplasm), which were increased upon LGP2 overexpression. These results suggest that Japanese flounder LGP2 is involved in the induction of IFN-inducible genes (e.g., Mx and ISG15). Furthermore, the induction of Mx and ISG15 genes in HINAE cells overexpressing LGP2 were not influenced by extracellular poly I:C stimulation (Fig. 10C). These results suggest that the strong expressions of type I IFN and IFN-inducible genes after VHSV-infection and poly I:C cotransfection with excessive LGP2 expression are mostly induced by the RLR pathway within the cytoplasm.

In conclusion, this study represents the molecular cloning and a functional characterization of an ancestral PAMP recognition receptor: an LGP2 ortholog in a teleost fish. Our results show that the expression of Japanese flounder LGP2 is induced by virus and poly I:C, and that LGP2 inhibits two kinds of Rhabdoviridae virus infection, and simultaneously induces type I IFN and IFN-inducible gene expression. This finding indicates that the type I IFN induction by LGP2 is a primordial function that has been conserved in fish and mammalian LGP2s throughout several hundred million years of evolution. Furthermore, LGP2 is a positive regulator of RLR signaling in teleosts, and the RD of Japanese flounder LGP2 plays a key role in viral RNA recognition. From the evidence between fish and mammals, further study using various types of fish pathogenic viruses will be necessary to understand the virus specificity of LGP2.

Disclosures
The authors have no financial conflicts of interest.
ANTIVIRAL ACTIVITY OF LGP2 IN TELEOSTS


17. Antiviral activity of LGP2 in teleosts

References


