Expression and functional characterization of the RIG-I like receptors MDA5 and LGP2 in rainbow trout *Oncorhynchus mykiss*

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ABSTRACT

The retinoic acid inducible gene I (RIG-I) like receptors (RLR) comprise three homologues: RIG-I, melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). They activate the host interferon (IFN) system upon recognition of viral RNA pathogen associated molecular patterns (PAMPs) in the cytoplasm. Bioinformatic analysis of the sequenced vertebrate genomes suggests the cytosolic surveillance system is conserved in lower vertebrates and recent functional studies have confirmed that RIG-I is important to fish antiviral immunity. In this study, we have identified MDA5 and LGP2 homologues from rainbow trout *Oncorhynchus mykiss* and an additional LGP2 variant with an incomplete C terminal domain of RIG-I. Trout MDA5 and LGP2 were constitutively produced in fibroblast and macrophage cell lines and up-regulated by polyI:C, recombinant IFN or infection by RNA viruses (viral hemorrhagic septicemia virus and salmon alpha virus) with a single stranded positive or negative genome. Over-expression of MDA5 and LGP2 but not the LGP2 variant resulted in significant accumulation of Mx transcripts in cultured cells which correlated with a marked enhancement of protection against viral infection. These results demonstrate that both MDA5 and LGP2 are important RLRs in host surveillance against infection of both negative and positive viruses and that the LGP2 variant with a deletion of 54 aa at the C terminus act as a negative regulator for LGP2 elicited antiviral signaling by competing for the viral RNA PAMPs. Interestingly, MDA5 expression was not affected by over-expressed LGP2 in transfected cells, and vice versa, suggesting they likely act in parallel as positive regulators for IFN production.
INTRODUCTION

Toll like receptors (TLR) and retinoic acid inducible gene I (RIG-I) like helicases (RLH) are two major families of pattern recognition receptors (PRRs) that recognize cytosolic viral RNA pathogen associated molecular patterns (PAMPs) in vertebrates (11, 22). For example, TLR-3 and -7 are endosome associated cellular PRRs recognizing viral PAMPs released from the uncoating process. In contrast, RIG-I like helicases sense viral derived RNA molecules in other regions of the cytoplasm. Upon activation, TLRs and RLHs trigger production of type I interferons (IFNs), leading to an enhanced antiviral state of the host cells.

The RLH family contains three members, RIG-I, melanoma differentiation associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2), all of which share homologous core structural domains including a DExD/H box helicase domain, helicase C terminal domain and a C terminal domain (CTD) of RIG-I (11, 22). The central helicase domain contains six conserved DExD/H helicase motifs and is involved in translocation, ATP hydrolysis and RNA binding (36). The CTD, also referred to as the repressor domain or carboxy-terminal regulatory domain (RD), has proven to contain multiple diverse functions such as signaling repression, RNA recognition and protein dimerization (5, 25). RIG-I and MDA5 have additional caspase activating and recruiting domains (CARDs) at the N terminus that are well conserved in proteins involved in immune signaling, cell differentiation and apoptosis (1, 18). Activated RIG-I and MDA5 act on the mitochondria via interaction of CARD domains between RIG-I/MDA5 and mitochondria antiviral signaling protein (MAVS), a mitochondrial receptor which also contains a CARD, facilitating phosphorylation of interferon regulatory factor (IRF) -3 and -7 which are key transcription factors involved in triggering IFN production (12, 26, 35).

Whilst it is certain that RIG-I and MDA5 serve as PRRs for viral PAMPs, the role of LGP2 in mediating an antiviral response is contradictory. Since LGP2 lacks CARD domains at the N terminal region, it is believed that it is unable to interact with MAVS.
and hence may serve as a negative mediator for RIG-I/MDA5 activated antiviral signaling (24, 31, 35). Over-expression of LGP2 was shown to result in decreased IFN production in cells following transfection with polyI:C or viral infection. Furthermore, LGP2 deficient mice exhibited enhanced resistance to viral infection and embryonic fibroblasts isolated from these mice displayed enhanced IFN expression in response to synthetic double stranded RNA. However, positive regulatory roles of LGP2 in RIG-I/MDA5 activated antiviral signaling have also been reported in LGP2 deficient mice which lost the ability to synthesise type I IFNs and were unable to mount efficient antiviral responses against infection with encephalomyocarditis virus (32). A more recent study has provided further evidence that the ATPase domain of LGP2 is required for the LGP2 positive regulatory role in mediating RIG-I/MDA5 dependent antiviral responses (26).

In teleost fish, orthologs of human TLRs which recognize viruses have been reported in zebrafish (4, 16, 21), rainbow trout (19, 23), Atlantic salmon (28), grass carp (29) and gilthead seabream (6). Concerning RLRs, recent studies from our group and others have demonstrated that fish also possess RLR molecules, among which RIG-I and MAVS have been shown to activate the interferon response via a mitochondria associated signaling pathway (2, 14, 36). Over-expression of fish RIG-I and MAVS in cultured cells up-regulates expression of IFN and IFN stimulated genes and enhances resistance against not only RNA viruses but also DNA viruses (2, 14). As in mammals, the CARD domains at the N-terminal region in both RIG-I and MAVS and the CTD in RIG-I are essential for their antiviral activities. In the present study, we identified two other members of the RLR family, MDA5 and LGP2, known in mammals to be involved in mediating antiviral immunity, from rainbow trout (Oncorhynchus mykiss), and demonstrated that they were induced at the transcriptional level by viral infection, exogenous cytosolic double stranded RNA (dsRNA) and stimulation with type I IFN. Induction of MDA5 and LGP2 expression was confirmed at the protein level by Western blot analysis using polyclonal antibodies produced against MDA5 and LGP2 peptides. To further clarify the role of trout MDA5 and LGP2, stable trout cell lines overexpressing these RLR molecules
were generated and shown to exhibit enhanced resistance to infection against a RNA virus (viral hemorrhagic septicemia virus). Strikingly, we found a novel LGP2 variant, containing a shortened CTD with a deletion of 54 amino acid residues at the C-terminus, was unable to trigger antiviral responses and downregulated LGP2 induced Mx gene expression. Furthermore, overexpression of MDA5 had no effect on LGP2 in transfected cells, and vice versa. Taken together, our data indicate that MDA5 and LGP2 act as independent positive regulators of the IFN response in fish cells whilst the LGP2 variant antagonises LGP2 function.

**MATERIALS AND METHODS**

**Cells and viruses.** The RTG-2 cells were maintained in L-15 medium supplemented with 10% foetal bovine serum (FBS) (Sigma), 100 U/ml penicillin (P) and 100 µg/ml streptomycin (S) at 20°C. The RTS-11 cells were maintained in L-15 medium supplemented with 30% FBS and P/S at 20°C. Salmon alphavirus (SAV) (isolated from a field sample, Inch Kenneth, Scotland; unpublished) was propagated in CHSE-214 cells for 7 days as described previously (15). Viral hemorrhagic septicemia virus (VHSV, isolate DK-F1) was propagated at 15°C on 90% confluent monolayers of BF-2 cells (bluegill fry, ATCC No. CCL-91) and cultured in EMEM supplemented with 5% FBS. Once a full cytopathic effect (CPE) was observed, the virus (TCID<sub>50</sub>) was titrated using BF-2 cells by end point dilution and stored at -80°C in aliquot.

**Cloning and sequencing of trout MDA5 and LGP2.** To search for salmonid MDA5 and LGP2 homologues, expressed sequence tag (EST) sequences in the NCBI database were searched using fish MDA5 and LGP2 protein sequences (37) as bait sequences for BLASTN analysis. Several candidate ESTs were found with high sequence homology to known MDA5s, among which EST sequences GE782837 (Salmo salar) and GE823089 (Oncorhynchus mykiss) aligned well with the N and C terminal sequences of MDA5 respectively. Forward (MDA5-F1) and reverse (MDA5-R1 and MDA5-R2) primers were designed in the 5’ and 3’ end untranslated
region (UTR), to amplify the full length coding sequence of trout MDA5 by semi-nested RT-PCR. For LGP2, the full length sequence of *S. salar* was compiled using the CAP3 sequence assembly program ([http://pbil.univ-lyon1.fr/cap3.php](http://pbil.univ-lyon1.fr/cap3.php)) with the following ESTs: GE781785, DW582483, DW538655, CX355611, DN165806, DW567942 and CK880416, and primers LGP2-F1 and LGP2-R1 located in the 5’ and 3’ end UTR were used for RT-PCR amplification of the full length coding sequence of trout LGP2. Hot start PCR was performed for amplification of both molecules under the following conditions: 1 cycle of 94°C for 5 min; 35 cycles of 94°C for 25 sec, 57°C for 25 sec and 72°C for 2 min, and 1 cycle of 72°C for 10 min. The cDNA template was generated from RTS-11 cells stimulated with 50 µg/ml polyI:C for 4 h. The PCR fragments were cloned into pGEM T Easy vector (Promega) and subsequently sequenced by Eurofins MWG Operon.

**RNA extraction, cDNA synthesis and real time PCR analysis.** Total RNA was extracted using the RNASTAT reagent (AMS Biotechnology (Europe) Ltd) and treated with RNase free DNase I (Fermentas Life Sciences, Germany) and RibolockTM ribonuclease inhibitor (Fermentas Life Sciences, Germany) according to the manufacturer’s instructions. The DNase I treated RNA was reverse transcribed into cDNA using a first strand cDNA synthesis kit (Fermentas Life Sciences, Germany) and kept at -20°C for real time PCR analysis.

Primers used for real time PCR analysis are listed in Table 1. Real time PCR was performed on a Roche LightCycler® 480 using the following conditions: 1 cycle of 95°C/10 min, 45 cycles of 95°C/30 sec, 60°C/30 sec and 72°C/40 sec. Each sample was run in triplicate in a 96 well plate and the mean value recorded. The relative expression of target genes was then normalized to the expression of EF-1α and expressed as arbitrary units. Fold changes were calculated by comparison to the corresponding controls. Three independent experiments were conducted for statistical analysis.

**Construction of MDA5 and LGP2 plasmids.** For expression of recombination proteins, the coding regions of MDA5, LGP2a and LGP2b were amplified by PCR using pQE30MDA5F/pQE30MDA5R, pQE30LGP2F/pQE30LGP2aR and
pQE30LGP2F/pQE30LGP2bR (Table 1), respectively. The PCR products of LGP2a and LGP2b were digested with Bam HI/Kpn I and ligated into digested pQE30 vector, giving rise to plasmids pQE30-LGP2a and pQE30-LGP2b. Similarly, the amplified MDA5 fragment was digested with Sac I/Hind III and cloned into pQE30, generating pQE30-MDA5.

To generate expression vectors of MDA5-GFP and LGP2-GFP fusion proteins for cellular localization studies, the full length coding sequences of MDA5, LGP2a and LGP2b were amplified with primer pairs ptGFPMDAF/GFPMDAR1, ptGFPLGP2F/GFPLGP2aR and ptGFPLGP2F/GFPLGP2bR (Table 1) respectively, and inserted into the Bgl II and Kpn I sites of the pTurboGFP-N vector (Evrogen) to construct pTurbo-MDA5-GFP, pTurbo-LGP2a-GFP and pTurbo-LGP2b-GFP. For over-expression studies of MDA5 and LGP2, an expression vector ptGFP1 was modified from the pTurboGFP-N vector and contained two sets of CMV promoter and SV40 3'UTR to drive expression of the target gene and GFP as separate proteins rather than as a fusion protein. The transfected cells expressing exogenous MDA5 and LGP2 were GFP positive. For anti-viral assays, the full length coding sequences of MDA5, LGP2a and LGP2b were amplified with primer pairs ptGFPMDAF/ptGFPMDAR (for MDA5) and ptGFPLGP2F/ptGFPLGP2R (for LGP2a and LGP2b) respectively, and inserted into the Bgl II and Kpn I sites of the ptGFP1 vector to make constructs ptGFP1-MDA5, ptGFP1-LGP2a and ptGFP1-LGP2b for overexpression in stably transfected RTG-2 cells. Lastly, The coding region of MDA5 was amplified with primers FlagMDA5F/FlagMDA5R, and inserted into the Bgl II/ Kpn I sites of p3xFLAG-CMV™-14 Expression Vector (Sigma) to construct plasmid pMDA5-FLAG.

Expression analysis of MDA5 and LGP2 in RTG-2 and RTS-11 cells by real time PCR. The cells were passaged into fresh cultured flasks 2 days before being stimulated for 4 h and 24 h with either 50 µg/ml polyI:C (Sigma Aldrich), or 20 ng/ml recombinant trout type I IFN2 protein (38). For polyI:C transfection, the RTG-2 cells (5x10⁶ cells in 100 µl nucleofector solution) were electroporated with 5 µg polyI:C using an Amaxa Nucleofector II transfection system (Lonza) under Programme T20,
washed immediately with 5 ml HBSS buffer and cultured at 20°C for 4 h or 24 h. The cells were collected for RNA extraction and real time PCR analysis as described previously (3). The relative expression of target genes was normalized to the expression of elongation factor (EF) -1α and expressed as arbitrary units or fold change relative to the corresponding control group. The mean of three independent experiments was used for statistical analysis, as described below.

**Expression analysis of MDA5 and LGP2 in RTG-2 cells by Western blot.**

Polyclonal antibodies against trout MDA5 and LGP2 were generated in rabbits by Genscript using standard procedures with synthetic peptides for MDA5-1 (CEHLDSRRKEGRPK), LGP2-1 (PRKRFDIVDRRPQDC) and LGP2-2 (CETPEGRKLAKKWK). Other antibodies included: mouse monoclonal anti-Arabidopsis actin (Thermo Scientific), rabbit polyclonal anti-TurboGFP (Evrogen), horseradish peroxidase conjugated goat anti-mouse IgG (Thermo Scientific).

Recombinant proteins of trout MDA5 and LGP2 were produced to verify the polyclonal antibodies generated against the synthetic peptides. For this, the full length cDNA fragments of MDA5, LGP2a and LGP2b were amplified using the primers listed in Table 1, inserted into the pQE30 vector (Qiagen) and expressed in E.coli M15 cells (Qiagen). The recombinant MDA5 and LGP2 proteins were produced as previously described and purified under denaturing conditions (38). The recombinant proteins were separated on a 4-12% pre-cast SDS-PAGE gel (Invitrogen) and transferred onto a polyvinylidene difluoride membrane. Western blotting was performed using a WesternBreeze® Chemiluminescent Western Blot Immunodetection Kit (Invitrogen). The primary antibodies were diluted 400-fold.

To examine the expression of MDA5 and LGP2 at the protein level in RTG-2 cells, the cells were passaged into 25-cm² flasks and cultured at 20°C until they reached 80% confluence (2 days), the culture media were replaced by fresh media and an equal volume of PBS or polyI:C (50 or 100 µg/ml) was added. After 24 h, when the cells were harvested and resuspended in 1×SDS-PAGE sample buffer. Cell lysates were boiled at 95°C for 5 min, centrifuged at 13,000 × g for 1 min and loaded immediately onto the SDS-PAGE gels. After gel electrophoresis, a Western blot was
performed as described previously using polyclonal antibodies against MDA5 and LGP2. Actin protein was detected with mouse monoclonal anti-actin (1:1000, v/v) and the secondary antibody (horseradish peroxidase conjugated goat anti-mouse IgG, 1:2000, v/v) as an internal control to normalize the amount of protein loaded onto the gels.

Kinetics of gene expression in head kidney of fish infected with VHSV. Viral hemorrhagic septicemia virus (VHSV) has a single stranded negative RNA genome and can infect a wide range of marine and fresh water fish species including salmonids. In this study, fish (approx. 15 g) were reared in the Marine Scotland Science Marine Laboratory in Aberdeen, Scotland, and acclimated for 14 days prior to challenge. Fish were stocked in 30 L tanks of freshwater maintained at 10°C, with a flow rate of 50 L/h with aeration. Fish were starved for 24 h prior to the experiment during which they were maintained on a commercial diet. Fish in duplicate tanks were anaesthetised with methane tricaine sulphonate (MS222, Sigma, 0.1 mg/ml) and injected intraperitoneally (i.p.) injected with 100 µl of the isolate DK-F1 (1 x 10^7 TCID₅₀ per fish) or mock-infected with control medium. Head kidney tissues were sampled for preparation of total RNA for real time PCR analysis.

Kinetics of gene expression in TO cells infected with SAV. Salmon alphavirus (SAV), containing a single stranded positive RNA genome, is known to cause sleeping disease in salmonids and has recently been shown to up-regulate IFN expression (3). To determine whether MDA5 and LGP2 are involved in antiviral responses against SAV, we assessed gene expression of MDA5 and LGP2 in Atlantic salmon TO cells infected with SAV. Since the salmon MDA5 and LGP2 (compiled with EST sequences) share significant nucleotide sequence identities in the coding regions (>95.0% for both genes) and the region used for primer design for trout MDA5 and LGP2 expression are almost identical to the corresponding sequences of the Atlantic salmon genes, the trout primers were tested to confirm that they worked for real time PCR analysis of these Atlantic salmon genes. Atlantic salmon TO cells (passage no. P95) (33) were propagated in L-15 medium supplemented with 5% FBS, 2 mM L-glutamine, and 1% non-essential amino acids. Stock cultures were incubated at
20°C for 7–21 days. Cells were passaged at 50–60% confluence and cultured for 24–48 h before viral challenge. Triplicate cultures of TO cells at approximately 80% confluency were infected with the SAV (F93-125 isolate (a subtype 1) (7, 34) at a MOI of 0.1 in 6-well plates at 15°C. Parallel un-infected cells were set up in the same format under the same conditions. At days 1, 2, 3, 4, 6 and 8, the cells were detached with trypsin-EDTA (Invitrogen) and centrifuged at 400 x g for 5 min. The cell pellets were drained and kept at -80°C. RNA was extracted from the infected cell cultures using the Allprep RNA/DNA/Proteins Kit (Qiagen) according to manufacturer’s instructions. The RNA was eluted in 50µl RNase-free dH₂O. RNA was reverse transcribed to cDNA using the TaqMan® Reverse Transcription Reagent kit (ABI) with oligo-d(T)₁₆ as follows: 9.63µl of total RNA (approx. 0.5µg) and 1.25µl 50 µM oligo-d(T)₁₆ were mixed and heated to 70°C for 10 min and chilled on ice. The final volume was adjusted to 25µl by adding Master mix comprised of the following: 1x RT buffer (25 mM Tris-HCl pH 8.3, 37.5 mM KCl, 5.5 mM MgCl₂), 0.5 mM each dNTP, 0.4 U RNase inhibitor and 1.25 U Multiscribe Reverse Transcriptase. The reactions were incubated at 48°C for 90 min, heat inactivated at 95°C for 5 min and stored at -80°C until use. Real time PCR analysis was carried out as outlined above.

**Localisation of MDA5 and LGP2 in RTG-2 cells.** The RTG-2 cells were passaged into fresh flasks and cultured for 24 h. Two micrograms of plasmid constructs (pTurboGFP-N, pTurbo-MDA5-GFP, pTurbo-LGP2a-GFP, or pTurbo-LGP2b-GFP) were transfected into 5 x 10⁶ cells using the Amaxa Nucleofector II transfection system (Lonza) under Programme T20. The cells were transferred into 6 well plates and cultured at 20°C with 3 ml L-15 medium supplemented with 10% FBS and P/S. Approximately 24 h post-transfection, the cells were collected for confirmation of LGP2a-GFP and LGP2b-GFP fusion proteins by Western blotting. Subsequently, the cells were examined under a fluorescent microscope and photographed.

**Antiviral activities of MDA5, LGP2a and LGP2b.** The RTG-2 cells were transfected with ptGFP1, ptGFP1-MDA5, ptGFP1-LGP2a or ptGFP1-LGP2b as described above. After 36 h, the cells were cultured in media containing 800 µg/ml
G-418 for 2 weeks to enrich for transfected cells, and then maintained in the media containing 200 µg/ml G-418. The cells were checked under a fluorescent microscope every two weeks and when a minimum ~80% of cells were GFP positive, they were deemed suitable for assessment of their antiviral state. For antiviral assays, the transfected cells were seeded into a 96-well plate (Nunc) at approx. 80% confluency. The cells were left to attach overnight in 100 µl per well L-15 medium (Invitrogen), 10% FBS (Nalgene) and 200 µg/ml G-418 at 23°C and acclimatised at 15°C for 24 h prior to viral infection. Ten µl of supernatant containing VHSV virus (isolate DKF3592) 1 x TCID$_{50}$, previously adapted to RTG-2 cells, was added to column 1 and serially diluted 10-fold in the following columns. The plates were incubated at 15°C for two weeks, drained, fixed in 10% formalin for 10 min at room temperature, and then stained with 0.05% (w/v) crystal violet (Sigma) for 30 min. The plates were photographed under a light box. To quantify stained cells, the crystal violet was dissolved in 100 µl 1% SDS solution for 5 min in an orbital shaker at 150 rpm. The absorbance was read at 562 nm wavelength using a Bio Lab-Tek plate reader. The relative OD was calculated by dividing the average OD of the infected wells (N=6) with that of the uninfected control (N=24). In addition, in parallel un-infected cultures the cells were also collected for examining gene expression of MDA5, LGP2 and Mx by real time PCR.

**Inhibitory activity of LGP2b on LGP2a elicited antiviral responses.** RTG-2 cells (5 x 10$^{6}$ cells) were co-transfected with 5 µg polyI:C and ptGFP1 (2 µg), ptGFP1-LGP2a (2 µg), ptGFP1-LGP2b (2 µg), or ptGFP1-LGP2a (2 µg) plus ptGFP1-LGP2b (2 µg). The cells were washed immediately with culture medium and incubated at 20°C for 6 h. Cells were harvested for extraction of total RNA and real time PCR analysis of gene expression.

**Interaction of MDA5 and LGP2 in RTG-2 cells**

To study whether overexpression of MDA5 affects LGP2 expression, 5 x 10$^{6}$ RTG-2 cells were transfected with 2 µg of p3xFLAG-CMV-14 vector (Sigma), pMDA5-Flag, ptGFP1 or ptGFP1-LGP2a plasmid as described above. The cells were immediately washed with HBSS buffer, cultured in 10% FBS containing L-15
medium at 20°C for 48 h, and collected for protein extraction using cell extraction buffer (Invitrogen). Western blotting was performed to determine trout MDA5 and LGP2 proteins as described above.

**Binding affinity of MDA5 and LGP2 with polyI:C**

Poly(C)-conjugated agarose beads (Sigma) and poly(I) were resuspended in buffer containing 50 mM Tris (pH 7.0) and 150 mM NaCl to a final concentration of 2 mg/ml and mixed at a ratio of 1:2 (v/v). The mixture was incubated at 4°C overnight, centrifuged at 1,000 × g for 1 min and washed once. The beads were resuspended in the same buffer as a 50% slurry and stored at 4°C for use.

Poly(I:C) pull-down assays were performed as described by Sumpter et al. (31). The poly(I:C)-coated beads were equilibrated in binding buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA and 1% NP-40 and resuspended as a 10% slurry. The beads were then incubated with the purified recombinant MDA5, LGP2a or LGP2b at 4°C for 1 h, centrifuged at 1,000 × g, rinsed three times with binding buffer, resuspended in three volumes of 1× SDS-PAGE sample buffer, and boiled for 3 min before being loaded onto a SDS-PAGE gel for Western blotting. For competition experiments, the polyI:C coated beads were incubated with recombinant proteins and 50 µg/ml of polyI:C.

**Statistical analysis.** The relative expression of target genes was normalized to the expression of elongation factor (EF) -1α and expressed as arbitrary units or fold change relative to the corresponding control group. The mean of at least three independent experiments was used for statistical analysis using the paired student t test. P<0.05 was considered to be significantly different.

**RESULTS**

**Sequence analysis of trout MDA5 and LGP2.** The cloned trout MDA5 cDNA contains a 3009 nt open reading frame (GenBank accession No: FN396357), encoding a protein of 1002 aa with a predicted molecular mass of 113.2 kDa and a pI of 5.93. Sequence analysis of the MDA5 protein in the Pfam HMM database (Pfam version
Pfam programme, a distantly related CARD located near the N terminus (aa 12-98) was noticeable when the protein was aligned with the human MDA5. The two CARD domains, the DExD/H box domain, helicase C terminal domain and the CTD domain of trout MDA5 showed a similarity of 58.3%, 77.0%, 80.0% and 69.7% with the corresponding domain of human MDA5, and of 64.2-66.3, 85.9-89.0%, 81.2-89.4%, and 77.0-86.1% with the corresponding domain of fish MDA5. Furthermore, other conserved features include an ATP binding motif (PTGSGKT), an ATPase motif (DECH), and a RNA unwinding motif (TAS) located within the DExD/H domain of trout MDA5. A RNA binding motif (QARGRGRA) was found within the helicase C domain of trout MDA5.

The identified trout LGP2 cDNA (named LGP2a) is 2194 bp in length, which encodes a protein of 678 aa (GenBank accession No, FN396358). An alternatively spliced form of 3230 bp (named LGP2b, GenBank accession No, FN396359) was also sequenced, with an intron of 1040 bp retained at the 3' end region of the ORF, resulting in early termination of translation. As a result, LGP2b is 54 aa shorter than LGP2a (Fig. S1B). TMpred analysis showed that the LGP2a and LGP2b proteins contained a transmembrane helix at aa 549-568. Pfam HMM analysis predicted a typical type III restriction enzyme res subunit domain (aa 3-176) at the N terminus, which is highly homologous to the DExD/H helicase domain of MDA5. The helicase C terminal domain (aa 396-477) and the RIG-I CTD domain (aa 552-672) were also detected both in LGP2a and LGP2b (Fig. S1B). The Type III restriction enzyme subunit, conserved C-terminal helicase domain and the CTD domain of trout LGP2a/LGP2b has a similarity of 73.0%, 74.4% and 63.1% with the corresponding domain of human LGP2, and of 62.6-83.9%, 83.1-92.8% and 63.6-74.8% with the corresponding domain of fish LGP2. In addition, a conserved ATP binding motif (PTGGGKKT), an ATPase motif (DECH), an RNA unwinding motif (TAS), and an
RNA binding motif (QASGRARA) were predicted in the trout LGP2a and LGP2b protein.

Expression of trout MDA5 and LGP2 after stimulation with polyI:C or type I IFN. Constitutive expression of trout MDA5, LGP2a and LGP2b was detected in fibroblast like RTG-2 and monocyte/macrophage like RTS-11 cells by real time PCR. The expression level of trout MDA5, LGP2a and LGP2b in RTG-2 cells was higher than that in RTS-11 cells. In RTG-2 cells, MDA5 transcript level was the lowest among the 3 genes, however it was higher relative to LGP2a/LGP2b expression in the RTS-11 cells (Fig. 1A).

To study the effect of the double stranded synthetic RNA, polyI:C, a known activator of MDA5 and LGP2 in mammals, the trout RTG-2 and RTS-11 cells were stimulated by addition of polyI:C (50 µg/ml) into the culture media for 4 h and 24 h. At 4 h post stimulation, polyI:C stimulation resulted in an approx. 7-, 18- and 3-fold increase of transcript level for MDA5, LGP2a and LGP2b in RTG-2 cells and an approx. 4-, 105- and 10-fold increase in RTS-11 cells, respectively (Fig. 1B). The stimulatory effects sustained at 24 h for all three genes in both cell types. It seemed apparent that LGP2 was most inducible in response to polyI:C stimulation. To examine the effect of intracellular polyI:C on MDA5 and LGP2 gene expression, the RTG-2 cells were electroporated with polyI:C, washed with PBS immediately after transfection to remove polyI:C in the culture medium, and incubated for 24 h. MDA5, LGP2a and LGP2b were all induced, with an approx. 9-, 200- and 18-fold increase of expression level respectively (Fig. 1C).

In mammals, activation of RIG-I/MDA5 by viral RNA PAMPs induces IFN expression and these surveillance mechanisms are enhanced by the synthesized IFNs via a positive feedback. In this study, the RTG-2 cells stimulated with 20 ng/ml rIFN2 for 4 h and 24 h exhibited increased expression for MDA5, LGP2a and LGP2b (Fig. 1D). Notably, a 36-fold increase in MDA5 transcript level was detected at 4 h whilst the increase reduced to 5-fold at 24 h.

To confirm induction of MDA5 and LGP2 detected by real time PCR, polyclonal antibodies were raised against synthetic MDA5 and LGP2 peptides for Western blot
analysis. The polyclonal antibodies reacted well with the recombinant proteins produced in bacteria (Fig. 2A). These antibodies were used to analyse MDA5 and LGP2 protein expression in RTG-2 cells after polyI:C stimulation by Western blotting. In control cells, a low level of constitutive expression was detected for MDA5 and the two isoforms of LGP2. Induction of all three proteins after 24 h stimulation with either 50 or 100 µg/ml polyI:C was apparent (Fig. 2B). These data confirmed the transcriptional results obtained by real time PCR.

**Kinetics of trout MDA5 and LGP2 expression in response to viral hemorrhagic septicemia virus (VHSV) and salmon alpha virus (SAV) infection.**

Expression of MDA5 and LGP2 was studied in trout after infection with VHSV, a virus with a single stranded negative RNA genome (Fig. 3A). At day one post-infection, increased transcript level of MDA5, LGP2a and LGP2b was detected. MDA5 expression appeared to stay at a constant level during the 12 day sampling period whilst induction of LGP2a peaked at day 3. Compared with MDA5 and LGP2a, a much bigger increase of gene expression was observed for IFN2.

SAV is a single stranded positive RNA virus which infects salmonid species. Recent studies have shown that SAV infection led to elevation of IFN and Mx expression in TO cells (3, 8). In the present work, the expression of MDA5 and LGP2 was analysed to establish whether these cytosolic PRRs were also involved in antiviral responses against infection of RNA viruses with a positive genome. During the 8 day infection period, expression of MDA5, LGP2a and LGP2b were induced from day 2 and peaked at day 6. Induction patterns were similar for all 3 molecules, but the magnitude of trout LGP2a induction was higher than that of MDA5 or LGP2b (Fig. 3B).

**Localization of trout MDA5 and LGP2 within cells.** To determine their localization within cells, the open reading frames (ORFs) of trout MDA5, LGP2a and LGP2b were inserted into the pTurboGFP-N expression vector, to facilitate over-production of fusion proteins in transfected cells. The RTG-2 cells transfected with plasmids pTurbo-GFP (vector), pTurbo-MDA5-GFP, pTurbo-LGP2a-GFP, or pTurbo-LGP2b-GFP were tested by Western blot analysed for confirmation of
expression of fusion proteins and examined under a fluorescent microscope 24 h after transfection. The presence of LGP2a-GFP and LGP2b-GFP proteins were confirmed using a polyclonal antibody against the GFP protein (Fig. 4A). A global cytosolic distribution was seen for all three GFP fusion proteins (Fig. 4B). However, marked intensified green spots were apparent for the two LGP2 isoforms, most of which were located in the region surrounding the nucleus, possibly the nuclear envelope and endoplasmic reticulum. Control cells transfected with empty vector pTurbo-GFP showed a global cytosolic localization.

**Antiviral activity of trout MDA5 and LGP2 in RTG-2 cells.** The RTG-2 cells were transfected with ptGFP1-MDA5, ptGFP1-LGP2a and ptGFP1-LGP-2b and stable cell lines established by selection with G-418. Constitutive over-expression of transfected genes was apparent, with a significant increase of the transcript levels compared with untransfected cells; 12- fold for MDA5, 3- fold for LGP2a and 4- fold for LGP2b, respectively (Fig. 5A). In each case, the expression of Mx was significantly increased in MDA5 and LGP2a transfected cells (P<0.5) but not in LGP2 transfected cells (Fig. 5B). These cells were also examined for their resistance against VHSV infection. The cells transfected with MDA5 and LGP2a were more resistant to VHSV infection than the control cells tranfected with empty vector plasmid (Fig. 6). Over-expression of LGP2b had little impact on the antiviral state of the cells.

**Inhibitory activity of LGP2b on LGP2a activated antiviral response.** To further investigate whether LGP2b has any effect on the LGP2a elicited antiviral responses, RTG-2 cells were co-transfected with 5 µg polyI:C and ptGFP1 (2 µg), ptGFP1-LGP2a (2 µg), ptGFP1-LGP2b (2 µg), or ptGFP1-LGP2a (2 µg) plus ptGFP1-LGP2b (2 µg), and cultured for 6 h. The cells co-transfected with LGP2a and LGP2b exhibited decreased Mx expression compared with cells transfected with LGP2a alone (Fig. 7) (P<0.05), demonstrating that LGP2b suppresses the LGP2a induced antiviral responses.

**Interaction of MDA5 and LGP2 in RTG-2 cells**

It has been recently demonstrated that LGP2 is involved in the primary recognition
of encephalomyocarditis virus upstream of MDA5 during signaling (26). To study whether LGP2 regulates MDA5 expression, MDA5 was over-expressed in transfected RTG-2 cells where LGP2 expression was analysed by Western blotting using polyclonal antibodies. The MDA5 protein was apparently not affected by LGP2 (Fig. 8). Similarly, overexpression of MDA5 did not result in any change of LGP2 at the protein level. These data suggest that MDA5 and LGP2 may serve as parallel positive regulators to activate IFN system.

**Binding affinity of MDA5 and LGP2 with polyI:C**

To determine whether trout MDA5, LGP2a and LGP2b bind to synthetic polyI:C, polyI:C pulldown assay was performed using polyI:C conjugated agarose. All three proteins bound to polyI:C as shown in Fig. 9 and the binding was inhibited by co-incubation with soluble polyI:C. In the case of MDA5 and LGP2b, inhibition by polyI:C was incomplete as a small amount of proteins were still able to bind to the polyI:C agarose. No proteins were detected from poly© conjugated agarose for all three proteins.

**DISCUSSION**

Discovered in 2004, the RLR family of cytoplasmic viral sensors, to date consisting of RIG-I, MDA5 and LGP2, has become a major focus of research into antiviral innate immunity. In this report, we have identified MDA5 and LGP2 homologues from a teleost species and characterised their functions in antiviral defence against RNA viruses. We have demonstrated that LGP2 is an important sensor for viral PAMPs in addition to MDA5, to activate the host IFN system. Moreover, a LGP2 variant with a deletion of 54 aa residues at the C terminus, likely associated with the nuclear envelope/endoplasmic reticulum, possibly acts as a negative regulator of the LGP2 activated antiviral response by competing for viral PAMPs. Furthermore, our results strongly suggest that MDA5 and LGP2 work independently as cytosolic RNA sensors in fish cells. The present work confirms the presence of a
functional RLR system in lower vertebrates and provides an insight into the RLR sensing of viral PAMPs and associated intracellular signaling in teleost fish.

Our previous analysis has shown that the RLR family is well conserved among vertebrates (37). This has been confirmed by recent reports that RIG-I homologues and the associated signaling molecule, MAVS, indeed play an important role in activating fish antiviral responses, in particular the IFN system (2, 14). Like RIG-I homologues in other fish species, the trout MDA5 described here encodes a protein which has a structural domain organization similar to that seen in their mammalian homologues, containing two tandem CARD domains within the first 200 aa region of the N terminus, a DExD/H box helicase domain and a helicase C terminal domain in the middle region, and a C terminal domain of RIG-I near the C terminus. Among the major domains, the two CARDs are least conserved, sharing 34.5% and 33.7% identity with the corresponding human MDA5 CARDs respectively. The second CARD domain of MDA5 (22.4% aa identity with MAVS CARD) is more closely related to the CARD of trout MAVS than the first CARD domain (15.3% aa identity with MAVS CARD). However, both CARD domains have been shown to interact with MAVS in mammals and fish (2, 12, 27). Over-expression of the CARD domains alone in cultured fish cells significantly enhanced type I IFN expression, leading to antiviral protection (2).

The organization of the structural domains in the trout LGP2 is conserved. Like LGP2 molecules in mammals, trout LGP2 has a DExD/H box helicase, a helicase conserved C-terminal domain and a CTD but lacks N terminal CARD domains. All key motifs including the ATP binding motif, the ATPase motif and RNA unwinding motif, are identical, except for the RNA binding loop which is 2 aa shorter in trout LGP2. This RNA binding loop was crucial for the binding specificity and affinity with various types of RNA molecules (31). Interestingly, a splicing variant (namely LGP2b), possibly originated from incomplete splicing of an intron in the RNA transcript, was sequenced. The LGP2 mRNA contained an early translation stop and thus translated into a protein with an incomplete CTD which has a deletion of 54 aa residues at the C terminus. Alignment analysis of the incomplete trout LGP2b CTD
with the human LGP2 CTD showed that the trout LGP2b was devoid of the \(\beta_6-\beta_8\) sheets and the \(\alpha_3\) helix (31).

Modulation of RLRs expression by viral RNA PAMPs has been well documented. RIG-I, MDA5 and LGP2 were induced by foreign double stranded RNA and various types of RNA viruses at an early stage of infection (10, 24). In agreement with the studies in mammals, trout MDA5 and LGP2 were up-regulated in trout RTG-2 cells by transfection with polyI:C, with elevated expression of type I IFN and the antiviral gene Mx. Interestingly, stimulation with extracellular polyI:C also enhanced MDA5 and LGP2 expression in both RTG-2 cells and RTS-11 cells, possibly due to an indirect effect of factors such as IFNs that are induced by polyI:C. In fact, IFNs, as shown in the present study, are known inducers of RIG-I, MDA5 and LGP2 and are capable of amplifying their responses via a positive feedback mechanism (9). However, it cannot be ruled out that Toll like receptors (TLRs) such as TLR22 may also play a role in activation of cytosolic RLRs since TLR22 is known to be one of the cell surface receptors that detect extracellular polyI:C (17). The fact that the MDA5 and LGP2 transcripts were drastically increased at an early stage of VHSV or SAV infection strongly supports the notion that both MDA5 and LGP2 are likely involved in fish innate antiviral immunity in response to both negative and positive RNA viruses. Furthermore over-expression of RIG-I molecules in fish cells conferred strong protection against VHS virus (2). These data demonstrate that RIG-I like RLRs are important sensors in recognizing both single stranded and double stranded RNA viruses in fish.

One interesting finding in the present study is that LGP2 appears to act as an activator of the antiviral system in fish, which is in contrast to some reports in mammals where LGP2 was shown to suppress RIG-I and MDA5 activated IFN responses (13, 24). LGP2 lacks CARD domains at the N terminal region but possesses other domains including a DExD/H helicase domain, a helicase C terminal domain and a C terminal domain of RIG-I, and is believed to sequester the viral RNA PAMPs from the RIG-I and MDA5 molecules, hence acting as a negative regulator to control excessive production of IFNs (which is detrimental to the host immune system) and
keeping the IFN level in check when viral infection diminishes. LGP2 knockout mice showed a higher level of IFNs than the wild type animals and were more resistant to vesicular stomatitis virus infection (32). However these LGP deficient mice exhibited impaired type I IFN production in response to infection with a different virus, encephalomyocarditis virus, and thus were more susceptible to this virus (32). Satoh et al. (26) have confirmed that the positive regulatory roles of LGP2 may be attributed to the ATPase domain within the DExD/H. In this study, the trout fibroblast cells stably transfected with the LGP2 construct displayed enhanced resistance against VHSV infection compared with untransfected cells, an outcome of the induced innate antiviral factors such as Mx by LGP2 over-expression in transfected cells.

A trout LGP2 variant (LGP2b) containing an incomplete C terminal domain of RIG-I was also identified. Trout LGP2b was translated from a RNA transcript containing an intron due to incomplete RNA splicing and was up-regulated by polyI:C, IFN and during RNA virus infection (Fig. 2). In contrast to MDA5 and LGP2a, overexpression of LGP2b, lacking the 54 aa C terminal fragment, had little if any effect on Mx expression and cell resistance against VHSV infection in RTG-2 cells (Fig. 6), suggesting it could play an inhibitory role in LGP2a elicited antiviral signaling, perhaps as a dominant negative mediator for the LGP2a elicited IFN response. The similar cellular distribution of LGP2a and LGP2b (Fig. 5) implies that LGP2b may compete for the signaling molecules with LGP2a or directly interact with LGP2a, to block downstream signaling. The CTD of LGP2 is essential for RLR’s function. In mammals LGP2 mutants without the RNA binding domain are unable to activate the MDA5 elicited IFN responses. Viruses such as paramyxoviruses also target the LGP2 and MDA5 pathway by interaction between the CTD and the viral V protein to suppress the host antiviral system (20). It is not clear from this study whether the incomplete CTD was capable of binding viral RNA molecules and/or interacting with downstream signaling adaptor proteins. Trout LGP2b containing an shortened CTD domain with a deletion of 54 aa following the RNA binding motif still had binding affinity with polyI:C, suggesting that the deleted C terminal region is not required for RNA ligand binding. The fact that trout LGP2b antagonised the
stimulatory effect of LGP2a in induction of Mx expression suggests that the C 54 aa terminal region is essential for activating the downstream signaling pathway leading to IFN production and that trout LGP2b functions as a negative regulator possibly by competing with LGP2a for viral RNA PAMPs.

The roles of LGP2 in RIG-I and MDA5 mediated antiviral responses remain patchy and require further investigation. Previous reports indicate that LGP2 may serve as a suppressor to block RIG-I and MDA5 elicited signaling via MAVS in mammals since it lacks the N terminal CARD domains that are required for interaction with signaling proteins. The LGP2 knockout mice produce a higher amount of type I IFNs than wild type mice after stimulation with poly I:C s and vesicular stomatitis virus infection (24). However, other studies have disputed such findings. Venkataraman et al. (2007) found that LGP2 was not the primary negative regulator for IFN production and that LGP2 knockout mice exhibited enhanced resistance to vesicular stomatitis virus infection (32). More recently, LGP2 is suggested to target the signaling pathway upstream of RIG-I and MDA5 (26). It is evident from the present study that both trout MDA5 and LGP2 are able to bind to polyI:C to trigger IFN production (Fig. 9). Furthermore, overexpression of MDA5 in transfected cells had no impact on LGP2 protein level, and vice versa. These findings, together that from previous studies, suggest RIG-I, MDA5 and LGP2 may act in parallel as viral RNA sensors in fish cells (2).

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REFERENCES


FIGURE LEGENDS

FIG. 1. Trout MDA5 and LGP2 are up-regulated at the transcript level in cultured cells in response to intracellular/extracellular polyI:C and type I IFN. (A) The expression of trout MDA5 and LGP2 in normal RTG-2 and RTS-11 cell lines. (B) The effect of extracellular polyI:C stimulation on trout MDA5 and LGP2 expression in RTG-2 and RTS-11 cell lines. (C) The effect of transfected polyI:C on trout MDA5 and LGP2 expression in RTG-2 cells. (D) The effect of recombinant trout IFN2 protein stimulation on trout MDA5 and LGP2 expression in RTG-2 cells. The RTG-2 and RTS-11 cells were stimulated for 4 h with either 50 µg/ml polyI:C or 20 ng/ml trout type I rIFN2 protein. In addition, the RTG-2 cells (5 x 10⁶ cells) were electroporated with 5 µg polyI:C, washed and cultured for 24 h. The cells were collected and used for RNA extraction and real time PCR analysis. The relative expression of target genes was normalized to the expression of EF-1α and expressed as arbitrary units or fold change relative to the appropriate control group. The mean of three independent experiments is shown and bars indicate the SEMs. Asterisks indicate where the increase of gene expression is significant (p<0.05).

FIG. 2. Trout MDA5 and LGP2 are up-regulated at the protein level in RTG-2 cells after stimulation with polyI:C. (A) Verification of rabbit polyclonal antibodies generated against trout MDA5, LGP2a and LGP2b by Western blotting. Four hundred nanogram of purified recombinant trout MDA5 (lane 1), LGP2a (lane 2) and LGP2b (lane 3) was applied and bands of the correct size were visualized after blotting. (B) Protein expression of MDA5, LGP2a, LGP2b and actin in unstimulated cells (lane 1) and cells stimulated with 50 µg/ml (lane 2) or 100 µg/ml of polyI:C for 24 h. Note the actin bands are similar between lanes.

FIG. 3. Trout MDA5 and LGP2 expression is induced by infection with negative and positive single stranded RNA viruses. (A) Rainbow trout were intraperitoneally challenged with VHSV or medium (mock infected) and head kidney tissues were sampled at days 1, 3, 7 and 12 for gene expression analysis; (B) Atlantic salmon TO
cells were infected with salmon alpha virus (SAV) or medium (mock-infected), and collected at days 1, 2, 3, 4, 6 and 8 for gene expression analysis. Gene expression of trout MDA5, LGP2a, LGP2b and IFN2 (A only) was analyzed by real time PCR and their expression level normalized to the expression of EF-1α. Fold change relative to the appropriate control group is presented. The mean of three independent experiments is shown and bars indicate the SEMs.

FIG. 4. Trout MDA5 and LGP2 are localized in the cytoplasmic region of RTG-2 cells. The RTG-2 cells were transiently transfected with pTurboGFP, pTurbo-MDA5-GFP, pTurbo-LGP2a-GFP or pTurbo-LGP2b-GFP. After 24 h, cells were collected for analysis. (A) Western blot confirmation of expression of fusion proteins. Lane 1: cell lysate transfected with pTurbo-LGP2a-GFP; lane 2: cell lysate transfected with pTurbo-LGP2b-GFP. (B) Cellular localisation of MDA5, LGP2a and LGP2b in transfected cells under a fluorescent microscope. Note the cytoplasmic expression of MDA5 and LGP2.

FIG. 5. Overexpression of trout MDA5 and LGP2 enhances Mx gene expression in RTG-2 cells. The RTG-2 cells were transfected with ptGFP1, ptGFP1-MDA5, ptGFP1-LGP2a or ptGFP1-LGP2b. Gene expression of MDA5, LGP2a, LGP2b (A) and Mx (B) was determined in stably transfected cells. The mean of three independent experiments is shown and bars indicate the SEMs. Asterisks indicate where the increase of gene expression is significant relative to ptGFP1 transfected cells (p<0.05).

FIG. 6. Overexpression of MDA5 and LGP2a but not LGP2b in RTG-2 cells enhances protection against VHSV infection. The RTG-2 cells stably transfected with ptGFP1, ptGFP1-MDA5, ptGFP1-LGP2a or ptGFP1-LGP2b were infected with 10 fold diluted VHSV for two weeks and stained with crystal violet. The plates were photographed and one row of cells from the 96 well plates is shown (N=6) (A). Stained cells were subsequently dissolved in 1% SDS solution and density (OD\textsubscript{562}) was measured. The relative OD was calculated by comparing the average OD of the infected wells (N=6) with that of the uninfected control (N=24) (B).

FIG. 7. The LGP2 variant lacking a 54 aa C terminal region (LGP2b) suppresses
LGP2-induced gene expression of antiviral factors. The RTG-2 cells were transiently co-transfected with polyI:C and ptGFP1, ptGFP1-LGP2a, ptGFP1-LGP2b, or ptGFP1-LGP2a plus ptGFP1-LGP2b, washed and cultured for 6 h before cell collection for RNA extraction. The mean of three independent experiments is shown and bars indicate the SEMs. Asterisks indicate where the increase of gene expression is significant (p<0.05).

FIG. 8. Overexpression of MDA5 and LGP2 in RTG-2 cells does not affect gene expression of either molecule at the protein level. (A) The RTG-2 cells were transfected with plasmid p3xFLAG-CMV-14 vector (lane 1) or pMDA5-Flag (lane 2) and analysed for LGP2 expression using rabbit polyclonal anti-LGP2 antibody. (B) The RTG-2 cells were transfected with ptGFP1 (lane 1) or ptGFP1-LGP2a (lane 2) and analysed for MDA5 expression using rabbit polyclonal anti-MDA5 antibody.

FIG. 9. Binding affinity of MDA5 and LGP2 with polyI:C. Purified recombinant proteins were incubated with polyI:C coupled agarose beads. After washing, the beads were subject to Western blot analysis using polyclonal antibodies against MDA5 and LGP2. Competition of polyI:C binding to MDA5, LGP2a and LGP2b was conducted by inclusion of soluble 50 µg/ml polyI:C in the reaction. (A) Binding affinity of MDA5 with polyI:C. Lane 1: MDA5 pulled down from polyI:C coupled agarose; lane 2: MDA5 pulled down from co-incubation with soluble polyI:C; lane 3, MDA5 pulled down from polyC coupled agarose. (B) Binding affinity of LGP2 and LGP2b with polyI:C. Lanes 1 and 3: LGP2a and LGP2b pulled down from polyI:C coupled agarose; lanes 2 and 4: LGP2a and LGP2b pulled down from co-incubation with soluble polyI:C; lanes 5 and 6, LGP2a and LGP2b pulled down from polyC coupled agarose.
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Fig. 1

A

B
Fig. 1

C

D
Fig. 2
Fig. 2

1          2          3
MDA5
LGP2a
LGP2b
Actin

B
Fig. 3

A

Fold change relative to control

Days post infection

MDA5
LGP2a
LGP2b
IFN2
Fig. 3

Fold change relative to control

Days post infection

MDA5
LGP2a
LGP2b
Fig. 4

A

kDa
120
100
80
60
50
40
30

M          1          2

LGP2a-GFP
LGP2b-GFP
Fig. 4

- pTurbo-MDA5-GFP
- PTurbo-LGP2a-GFP
- PTurbo-LGP2b-GFP
- pTurboGFP
Fig. 5
A
1
10
100
1000
ptGFP1-MDA5
ptGFP1
ptGFP1-LGP2a
ptGFP1
ptGFP1-LGP2b
ptGFP1
Gene expression relative to EF-1a
*
*
*
MDA5                            LGP2a                           LGP2b
Fig. 5

B

Gene expression relative to EF-1a
Fig. 6

A

VHSV infected  |  uninfected

ptGFP1-MDA5

ptGFP1-LGP2a

ptGFP1-LGP2b

ptGFP1

0  10  10^2  10^3  10^4  10^5  virus dilution fold
Fig. 6

B

![](image)

OD (562nm) relative to uninfected control

ptGFP1
ptGFP1-MDA5
ptGFP1-LGP2a
ptGFP1-LGP2b
Fig. 7

Gene expression relative to control

A
Fig. 8

A

1  2

LGP2a
actin

B

1  2

MDA5
actin
Fig. 9