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Discovery of the DIGIRR Gene from Teleost Fish: A Novel Toll–IL-1 Receptor Family Member Serving as a Negative Regulator of IL-1 Signaling

Yi-feng Gu, Yu Fang, Yang Jin, Wei-ren Dong, Li-xin Xiang, and Jian-zhong Shao

Toll–IL-1R (TIR) family members play crucial roles in a variety of defense, inflammatory, injury, and stress responses. Although they have been widely investigated in mammals, little is known about TIRs in ancient vertebrates. In this study, we report a novel double Ig IL-1R related molecule (DIGIRR) from three model fish (Tetraodon nigroviridis, Gasterosteus aculeatus, and Takifugu rubripes), adding a previously unknown homolog to the TIR family. This DIGIRR molecule contains two Ig-like domains in the extracellular region, one Arg-Tyr–mutated TIR domain in the intracellular region, and a unique subcellular distribution within the Golgi apparatus. These characteristics distinguish DIGIRR from other known family members. In vitro injection of DIGIRR into zebrafish embryos dramatically inhibited LPS-induced and IL-1β–induced NF-κB activation. Moreover, in vivo knockdown of DIGIRR by small interfering RNA significantly promoted the expression of IL-1β and IL-18–stimulated proinflammatory cytokines (IL-6 and IL-1β) in DIGIRR-silenced liver and kidney tissues and in leukocytes. These results strongly suggest that DIGIRR is an important negative regulator of LPS-mediated and IL-1β–mediated signaling pathways and inflammatory responses. The Arg-Tyr–mutated site disrupted the signal transduction ability of DIGIRR TIR. Evolutionally, we propose a hypothesis that DIGIRR and single Ig IL-1R related molecule (SIGIRR) might originate from a common ancient IL-1R–like molecule that lost one (in DIGIRR) or two (in SIGIRR) extracellular Ig-like domains and intracellular Ser and Arg-Tyr amino acids. DIGIRR might be an evolutionary “transition molecule” between IL-1R and SIGIRR, representing a shift from a potent receptor to a negative regulator. These results help define the evolutionary history of TIR family members and their associated signaling pathways and mechanisms. The Journal of Immunology, 2011, 187: 2514–2530.

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regulators in TIR signaling are available, except for a Tollip homolog recently identified from Oncorhynchus mykiss (48). Investigating the extent of the negative feedback mechanisms in ancient vertebrates such as fishes and the evolutionary changes that have occurred in this cytokine regulatory network during vertebrate evolution would be intriguing.

In the current study, the TIRs were identified in three fish species, Tetraodon nigroviridis, Gasterosteus aculeatus, and Takifugu rubripes, and a novel family member, named double Ig IL-1R related molecule (DIGIRR), was successfully characterized. Phylogenetic analysis revealed that DIGIRR was homologous to SIRGIR, but distinct in both protein structure and subcellular localization. In vivo and in vitro functional characterization demonstrated that DIGIRR served as a negative regulator of IL-1–mediated signaling. Importantly, our results also suggest that DIGIRR appears to be an evolutionary “transitional molecule” between IL-1R and SIRGIR and represents a link between the potent receptors and the negative regulators. Our aim is that the current study will contribute to the understanding of the evolutionary history of the TIR superfamily and its associated signaling pathways in both lower and higher vertebrates as a whole.

Materials and Methods

Experimental fish

One-year-old pufferfish, T. nigroviridis, of both sexes and weighing ~4–6 g, were kept in running water at 25°C and fed commercial food pellets twice a day. The fish were held in the laboratory for at least 2 wk prior to experimental use to allow for acclimatization and evaluation of overall health. Only healthy fish, as determined by general appearance and level of activity, were used for these studies.

Sequence retrieval

The sequence of DIGIRR was initially searched on the Tetraodon genome database (http://www.genoscope.cns.fr) using the human IL-1R amino acid sequence (M27492) as a query. When a Tetraodon homolog was found within a DNA contig (Scaffold 14769) in the database, it was further used as a probe to search homologous sequences on Takifugu and Gasterosteus EST/genome databases (http://genome.jgi-psf.org/; fugu6/; fugu6/home.html and http://wwwensembl.org/Gasterosteus_aculeatus). Aided by various software programs, such as Genscan and BLAST (49), a possible fish homolog of human SIRGIR was compiled, and it was used for the design of primers for the cloning of full-length Tetraodon DIGIRR cDNA.

Molecular cloning

Fish were sacrificed after anesthesia, and total RNA was isolated from the whole fish using TRIzol reagent (Invitrogen) and then treated with RNase-free DNase I (Qiagen). The first-strand cDNA was transcribed using an RNA PCR kit (AMV version 3.0; TaKaRa) according to the manufacturer’s instructions. The full-length Tetraodon DIGIRR cDNA was cloned by RT-PCR and RACE-PCR using the specific primers shown in Table 1. The PCR amplification was performed in 10-μl reaction mixtures containing 0.8 μl 10 μM forward and reverse primers, 0.2 μl DNA template, 1 μl PCR buffer (TaKaRa), 0.2 μl deoxyribonucleoside triphosphate mixture (for a final concentration of 2.5 mM each), 7.75 μl distilled water, and 0.05 μl Taq polymerase (5 U/μl; TaKaRa). The cycling protocol was an initial denaturing cycle at 94°C for 4 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final elongation at 72°C for 10 min. The 5′ and 3′ RACE-PCRs were performed according to the protocols provided by the manufacturer (TaKaRa). The open reading frame (ORF) of zebrafish and Takifugu IL-1B cDNA were also amplified by RT-PCR using specific primers listed in Table 1. PCR products were purified by using a gel extraction kit (Qiagen) and then purified into the pGEM-T EASY vector (Promega) and transformed into competent Escherichia coli top10 cells (Invitrogen). Plasmid DNA was purified by using a plasmid Miniprep method and sequenced on a MegaBACE 1000 Sequencer (GE) using the DYEnamic ET Dye Terminator Cycle Sequencing Kit (Pharmacia).

Structural characterization of DIGIRR

The comparative gene map positions were determined using Map viewer (http://www.ncbi.nlm.nih.gov/mapview/). Gene organizations (intron/exon boundaries) were elucidated by comparing DIGIRR cDNAs with genome sequences, and illustrations were drawn using GeneMapper 2.5 (http://genemapper.googlepages.com). Selected species included Gallus gallus, T. nigroviridis, G. aculeatus, T. rubripes, Homo sapiens, Mus musculus, and Rattus norvegicus. The potential functional motifs in the DIGIRR protein were analyzed by PROSITE (50), SMART database (51), and the TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) program (52, 53). The signal peptide cleavage sites were predicted with SignalP 3.0 (http://www.cbs.dtu.dk/services/; (54), BLAST (55), and a previously described method (56). Briefly, the genomic DNA (10–15 μg) was digested overnight at 37°C using 100 U of the restriction enzymes HindIII, BamHI, and EcoRI (TaKaRa). Digested DNA was separated by electrophoresis overnight on 0.8% agarose gels, transferred to positively charged nylon membranes (Amersham), and fixed by baking at 120°C for 1.5 h. After prehybridization in 10 ml DIG Easy Hyb solution at 42°C for 2–3 h, hybridization was carried out at 47°C overnight in a hybridization oven. The membrane was washed twice in 2× SSC containing 0.1% SDS at room temperature for 5 min each, and then twice in 0.5× SSC containing 0.1% SDS at 68°C for 15–20 min each. Genomic DNA, hybridized with probe, was immunologically detected by anti-DIG Ab conjugated with alkaline phosphate. The alkaline phosphate substrate CSPD was used in the chemiluminescence assay, and signals were exposed on X-ray film (Kodak) for 5–30 min. Marker Wide Range (500–15,000 bp; TaKaRa) was used to locate the same gel and stained in 1× TBE (0.1 mg/ml; ER) after electrophoresis. Band sizes were calculated by 1D Image Analysis Software (Kodak).

Phylogenetic analysis

The genomic and expressed sequence tag (EST) databases maintained at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), Ensembl (http://www.ensembl.org/), UCSC Genome Bioinformatics (http://www.genome.ucsc.edu/), and TIGR Gene Indices (http://www.tigr.org/tdb/tgi/) were used to search for IL-1R homologs in other species of primate, rodent, bird, and teleost fish. A phylogenetic tree was constructed by the neighbor-joining method with MEGA 3.0 for 1000 replicates (59, 60).

Tissue distribution and expression of DIGIRR

To determine the distribution of Tetraodon DIGIRR in various tissues and organs, fish were i.m. injected with 0.1 ml LPS (LPS from E. coli serotypes 055:B5; 0.01 mg/ml; Sigma). Healthy unstimulated fish served as controls. Brain, heart, gill, intestine, skin, liver, muscle, spleen, and head kidney were carefully removed, flash frozen in liquid nitrogen, and stored at −80°C until use. In another experiment, fish were injected with 0.1 μg/ml LPS for control, 3, 6, 12, 24, and 48 h, and the head kidney tissue was collected and stored in liquid nitrogen. Total RNA was extracted as described earlier, then reversed transcribed into cDNA using an RNA PCR kit (AMV version 3.0; TaKaRa) following the manufacturer’s instructions. Two pairs of specific PCR primers, β-actin-F/β-actin-R and DIGIRR-EF/DIGIRR-ER (Table 1), were designed for the amplification of Tetraodon β-actin (as an internal control) and Tetraodon DIGIRR. The real-time PCR amplification was carried out on the Mastercycler ep realplex real-time PCR system (Eppendorf) using a SYBR Premix Ex Taq kit (TaKaRa) following the manufacturer’s instructions. Briefly, all real-time PCR re- actions were performed in a 10-μl total reaction volume. The experiment protocol consisted of 1) amplification repeated 40 times, using 30 s at 95°C, then 20 s at 60°C, and 2) melting curve analysis using 5 s at 95°C, 15 s at 65°C, then 95°C, and 3) cooling at 40°C for 30 s. Relative gene expression was calculated using the 2^−ΔΔCT method with initial normalization of DIGIRR against β-actin. In all cases, each PCR trial was performed with triplicate samples and repeated at least three times.
Plasmids construction

The ORF of Tetraodon DIGIRR was inserted into pcDNA6/myc-HisB (Invitrogen) between EcoRI and XhoI sites to construct a eukaryotic expression vector named pc6-DIGIRR, in which the C terminus of DIGIRR was fused with a myc-tag. Subcellular localization plasmid was conducted by fusing pcDNA6/myc-HisB with plasmid between EcoRI and XhoI sites of pEGFP-N1 (Clontech); the plasmid was referred to as pEGFPN1-DIGIRR. The cDNA of zebrashish and Tetraodon IL-1β was digested with BamHI and XhoI and subcloned into pCMV-Tag2B (Stratagene) to produce the overexpression vectors pCMV-ZeIL-1 and pCMV-TnIL-1 (Ze, zebrashish; Tn, T. nigroviridis). For preparation of recombinant Tetraodon IL-1β protein, prokaryotic expression vector pET28a-TnIL-1β was constructed by inserting full-length Tetraodon IL-1β cDNA into pET28a (Novagan) between BamHI and XhoI sites. All the primers used for introducing enzyme sites are shown in Table I. Overexpression plasmid pFLAG-CMV-MouseSIGIRR was a gift from Prof. Alberto Mantovani (Istituto Clinico Humanitas, Milan, Italy) (61), and Golgi apparatus subcellular localization plasmid pDsRed2-Golgi was kindly provided by Prof. Thomas Kietzmann (University of Kaiserslautern, Kaiserslautern, Germany) (62). All the plasmids for transfection and microinjection were prepared free of endotoxin by using the Spin Miniprep Kit (Qiagen).

Chimeras and mutant constructions

A chimera molecule was constructed by fusing the extracellular and transmembrane domains of human IL-1R (1–359 aa) with the cytoplasmic DIGIRR region (255–524 aa) to detect whether the intracellular TIR domain in DIGIRR is capable of signal transduction. Two pairs of primers (ChimF1 and ChimR1, ChimF2 and ChimR2) were designed to amplify the extracellular and transmembrane domains of human IL-1R and the cytoplasmic region of DIGIRR, respectively. Both amplified fragments contain 25 overlapping bp. The chimera cDNA was obtained using an overlapping PCR protocol with ChimF1 and ChimR2. Thereafter, it was inserted into pcDNA6/myc-HisB between the EcoRI and XhoI sites, and the resulting plasmid was designated as pc6-Chimera-1. Four chimera mutants (chimera-2, -3, -4, and -5) were constructed by site mutation based on chimera-1 using overlap PCR to investigate the potential role of the amino acids Ser335 and Arg-Tyr420 in the TIR domain of DIGIRR in signal transduction. Chimera-2 was obtained by replacing the Leu420 in chimera-1 with Tyr. For this, two pairs of primers (ChimF1 and ChimR3, ChimF3 and ChimR2) were used to amplify the two fragments (A and B) of chimera-1 before and after the Leu420 site. The mutational fragment C was then generated by introducing the mutation to the 5' terminal of fragment B with the primers ChimF4 and ChimR2. Finally, fragments A and C were fused by overlap PCR using primers Chim1 and Chim2 to generate chimera-2, which was subcloned into pcDNA6/myc-HisB between the EcoRI and XhoI sites. Similar protocols were used for the construction of chimera-3, -4, and -5. In chimera-3, the TIR domain contained two substitutions, with Ala419 replaced with Arg and Leu420 with Tyr. For chimera-4, the TIR domain was generated by replacing Ala419 with Arg. For chimera-5, the Ser335 was mutated into Cys in the TIR domain. In parallel, a human IL-1R–encoding plasmid was constructed by inserting the IL-1RI cDNA into pcDNA6/myc-HisB between the EcoRI and XhoI sites. The human IL-1RI cDNA was kindly provided by Dr. Justin V. McCarthy (University College Cork, Cork, Ireland). A schematic illustration of the chimeras is shown in Fig. 11. All the primers used for chimera and mutant construction are shown in Table I.

Production of Tetraodon IL-1β protein

The pET28a-TnIL-1β was transformed into E. coli BL21 (DE3). One single colony was inoculated into 100 ml Luria–Bertani medium with kanamycin (25 mg/l). The inoculated Luria–Bertani medium was shaken and incubated at 37°C until the OD600 reached value of 0.6, isopropyl β-D-thiogalactoside was added to a final concentration of 1 mM, and the incubation was continued for 6 h. The expression level of the protein was assessed by 10% SDS-PAGE gel followed by Coomassie brilliant blue R250 staining. The recombinant IL-1β was purified using Ni-NTA agarose affinity chromatography according to the QIA Expressionist manual (Qiagen).

Cell culture and transient transfection

The cultured cell lines HEK293T, HepG2, and HaLa cells were cultured in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in 5% CO2. Cells were transiently transfected by Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Cells (1 × 105/ml) were seeded into 24-well plates and transfected with 1 μg DNA in Opti-MEM I medium without serum and antibiotics. At 5 h posttransfection, the medium was changed to DMEM with 10% FBS. At 24 h posttransfection, cells were lysed for reporter gene assays or fixed for laser confocal imaging.

Subcellular distribution

The HEK293T, HepG2, and HaLa cells were subcultured and seeded onto coverslips in 24-well plates before transfection. The next day, the cells were cotransfected with the overexpression plasmids pcDNA6/myc-DIGIRR and/or pEGFPN1-DIGIRR with or without pDsRed2-Golgi, the latter of which contains the human β1,4-galactosyltransferase sequence containing a membrane-anchoring signal peptide that targets the protein to the transmembrane region of the Golgi apparatus. At 24 h posttransfection, cells were washed three times with PBS and fixed for 10 min in 3% (v/v) formaldehyde in PBS. Fluorescence images of pEGFPN1-DIGIRR and pDsRed2-Golgi in cells were obtained using a laser scanning confocal microscope (Zeiss LSM510). In addition, colocalization images of pcDNA6/myc-DIGIRR and pDsRed2-Golgi were obtained using immunofluorescence staining. For this, the fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 15 min. The cells were blocked with 5% (v/v) goat serum at 37°C for 1 h, incubated with rabbit anti-myc Abs (Abcam) at 37°C for 1 h and then with FITC-labeled goat anti-rabbit IgG secondary Abs at 37°C for 1 h. The cells transfected with pEGFPN1-DIGIRR were successively incubated with BODIPY TR C5-ceramide (a Golgi indicator; Molecular Probes) and DiI (a cell membrane probe; Beyotime). For C5-ceramide staining, vital cells were incubated without fixation with 5 mM C5-ceramide at 4°C for 30 min, rinsed several times with an ice-cold medium, and incubated in fresh medium at 37°C for another 30 min. For Dl staining, the cells were fixed with formaldehyde, as described above, and stained with 10 μM DiI at 37°C for 10 min. The fluorescence images of these stained cells were visualized under a laser scanning confocal microscope at ×600 magnification.

NF-kB luciferase assay in mammalian cells

The NF-kB–dependent luciferase assay was performed to examine NF-kB activity and signaling. For this purpose, HELa or HEK293T cells were cotransfected with 400 ng of either pc6-DIGIRR, pFLAG-CMV-MouseSIGIRR, pc6-Chimera1–5, pc6-HumanIL-1R, or empty control vector pcDNA6 along with the reporter plasmid pNF-B-TK-Luc (100 ng) (Clontech) and pRL-TK (10 ng) (Promega). At 24 h posttransfection, the HeLa or HEK293T cells were stimulated for 6 h with human recombinant IL-1β (Invitrogen). Cells were lysed using reporter lysis buffer (Promega) and the luciferase activity was assessed using Dual-Luciferase Assay Reagent (Promega). All results reported represent duplicate experiments from at least three independent transfections.

NF-kB luciferase assay in zebrafish embryos

Zebrafish embryo model was also used to examine NF-kB activity according to the method previously described (63, 64). In brief, expression plasmids (0.1–1.0 ng), including pc6-DIGIRR, pcCMV-ZeIL-1β or pcCMV-TnIL-1β, pcDNA6/pCMV-Tag2B (used to ensure all samples received equal amounts of DNA), firefly and Renilla reporter plasmids (100 and 10 pg/μg), or without 4 μg/μl E. coli LPS (E. coli serotypes O55:B5), were combined with microinjection buffer (0.5% phenol red, 240 mM KCl, 40 mM HEPES, pH 7.4) and injected (2–3 nl) into one-cell-stage embryos using a microinjector (ASI MMP-3). The microinjected embryos were cultured at 28°C in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4). The firefly and Renilla luciferase activities were assayed at 24 h postmicroinjection as described above with 5–10 replicates (each replicate containing the tissue extracts from 30 embryos).

Screening of small interfering RNA against DIGIRR

The small interfering RNA (siRNA) against DIGIRR was initially designed by an siRNA Template Design Tool (Applied Biosystem) following the method previously reported (65). From the identified 46 regions of DIGIRR targeted for siRNA, four regions (Table I) were selected to screen the activity. DNA oligonucleotides for hairpin RNA expression were synthesized by Invitrogen and dissolved in nuclelease-free H2O at a concentration of 3 mg/ml and then mixed with equimolar amounts of complementary sense and antisense strands in annealing buffer (DMEM, firefly and Renilla reporter plasmids (100 and 10 pg/μg), or without 4 μg/μg E. coli LPS (E. coli serotypes O55:B5), were combined with microinjection buffer (0.5% phenol red, 240 mM KCl, 40 mM HEPES, pH 7.4) and injected (2–3 nl) into one-cell-stage embryos using a microinjector (ASI MMP-3). The microinjected embryos were cultured at 28°C in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4). The firefly and Renilla luciferase activities were assayed at 24 h postmicroinjection as described above with 5–10 replicates (each replicate containing the tissue extracts from 30 embryos).

DISCOVERY OF A DIGIRR GENE FROM FISH

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(1 μg) along with overexpression plasmid DIGIRR (1 μg) were cotransfected into HEK293T cells in 12-well plates. The specificity of siRNAs for DIGIRR was determined by real-time PCR.

**Production of lentivirus**

Based on data (see Results) showing that pSUPER-DIGIRR-3 was the most effective siRNA construct to suppress the DIGIRR expression, the U6 promoter cassette in lentiviral vector PLB was replaced by the H1-siRNA cassette excised from pSUPER-DIGIRR-3 construct to produce the PLB-DIGIRR lentiviral vector. Lentiviruses were produced by transient transfection of packaging cell line HEK293T cells according to standard protocols (66). In brief, confluent cells were subcultured into 10-cm dishes, and cotransfected with PLB (20 μg), pCMV-VSVG (packaging plasmid for HIV-1–based vectors) (6 μg), and pCMV-dR8.2 (the vesicular stomatitis virus G protein expression plasmid) (15 μg) by Lipofectamine 2000. The lentiviral supernatant was harvested at 48–96 h posttransfection, concentrated via ultracentrifugation at 25,000 rpm for 90 min at 4˚C (SW-41Ti rotor), and dissolved in 100 μl PBS and stored at −80˚C. Lentivirus titers were determined by transduction and flow cytometric analysis of GFP expression in HEK293T cells. The ability of lentiviral supernatant to silence the DIGIRR expression was also evaluated. The HEK293T cells were transfected with DIGIRR, then at 24 h posttransfection, 10 μl concentrated supernatant was added into culture medium, and the silencing efficiency of lentiviral supernatant was detected by real-time PCR.

**In vivo knockdown of DIGIRR**

The lentivirus delivery system developed as described earlier was used for in vivo knockdown of DIGIRR expression in *Tetraodon*. For this purpose, the lentiviruses (5 × 10^9 IVU/ml) concentrated from HEK293T supernatant was repeatedly delivered into fish by muscle injection (20 μl/fish) once a day for 3 d. In parallel, the control group was injected with PBS. Total RNA from liver, kidney, spleen, and leukocytes from head kidney were isolated and reversed transcribed into cDNA. Real-time PCR was conducted to evaluate the efficiency of in vivo suppression of DIGIRR.

**Evaluation of DIGIRR in IL-1–induced inflammation**

Lentiviral supernatant (20 μl/fish) was repeatedly injected into *Tetraodon* as described earlier. At the time of the third injection, some fish were also stimulated with 6 μg of recombinant IL-1β (the control group received PBS). At 24 h after IL-1β challenge, total RNA from liver, kidney, spleen, and head kidney leukocytes was isolated and transcribed into cDNA. The head kidney leukocytes of *Tetraodon* were isolated as described previously (67). Briefly, the head kidney was removed from fish under sterile conditions, washed twice with L-15 medium, and gently passed through a 100-μm nylon mesh. The cell suspension was layered onto a 34/51% discontinuous Percoll (Sigma) gradient and centrifuged at 400 × g for 30 min at 4˚C. Cells at the 34/51% interface were collected for RNA isolation. Real-time PCR was performed to detect the IL-1β–induced inflammatory cytokines (IL-1β and IL-6) using the primers listed in Table I.

**Statistical analysis**

Statistical evaluation of differences between experimental group means was done by ANOVA and multiple Student *t* tests. A *p* value <0.05 was considered to be statistically significant. The sample numbers of each group were always >10 fish of about equal body weight. Data points were from at least three independent experiments.

**Results**

**Identification of DIGIRR cDNA**

Through computational searching of the *Tetraodon* EST/genome databases using the human IL-1R sequence, we discovered one IL-1R–related sequence (named as DIGIRR) homologous to SIGIRR, which had never been characterized. Based on the sequences searched, specific primers (Table I) were designed for the molecular cloning of *Tetraodon* DIGIRR gene. Two ORF fragments of DIGIRR obtained by different primers combined with the fragments obtained from 5′ and 3′ RACE-PCR were compiled together to form a full-length *Tetraodon* DIGIRR cDNA. Similarly, the full-length *Gasterosteus* and *Takifugu* DIGIRR sequences were also predicted by an in silico screen of the EST/genome databases using the *Tetraodon* DIGIRR cDNA sequence as a probe in the same protocols. The nucleotide and deduced amino acid sequences of DIGIRR cDNA from *Tetraodon, Gasterosteus*, and *Takifugu* are shown in Fig. 1 and Supplemental Fig. 1.

The full-length *Tetraodon* DIGIRR cDNA (Fig. 1, GenBank accession number EF095151) is 1916 bp long (excluding the poly-A tail) and consists of a 112-bp 5′ untranslated region (UTR), a 1575-bp ORF encoding a predicted polypeptide of 524 aa, and a 229-bp 3′UTR that contains one polyadenylation signal (AATAAA) 15 nucleotides upstream of the poly-A tail. Similarly, the *Takifugu* DIGIRR cDNA (Supplemental Fig. 1A, GenBank accession number EU305619) consists of a 1569-bp ORF that encodes a 522-aa polypeptide, and the *Gasterosteus* DIGIRR cDNA (Supplemental Fig. 1B, GenBank accession number EU360722) is composed of a 1638-bp ORF that encodes a 545-aa polypeptide. In comparison with the counterparts of DIGIRR in other species, fish DIGIRR genes are 112–135 bp longer than their mammalian homolog SIGIRR. Significantly, SIGIRR was not found in *Tetraodon, Gasterosteus*, or *Takifugu*; rather, we found DIGIRR, which we considered a possible new immune gene in the TIR superfamily.

**Characterization of DIGIRR structures**

Using Genscan and BLAST programs, genes adjacent to the DIGIRR locus were retrieved from *Tetraodon* chromosome 13. Similar to SIGIRR, plakophilin 3, transmembrane protein 16J, phosphatidylserine synthase 2, v-Ha-ras, and Harvey rat sarcoma viral oncogene homolog are all found in the identical order on *Tetraodon* chromosome 13, human chromosome 11, and mouse chromosome 7. Clearly, there is a high degree of conservation of genome synteny between *Tetraodon* DIGIRR and mouse and human SIGIRR (Fig. 2A). By comparing the full-length *Tetraodon, Takifugu*, and *Gasterosteus* DIGIRR cDNAs with the corresponding genomic sequences, the organization of DIGIRR genes was elucidated (Fig. 2D). The *Tetraodon* DIGIRR gene is located within a 3094-bp genomic fragment on chromosome 13, the *Takifugu* DIGIRR gene is located within a 3163-bp genomic fragment of scaffold 198, and the *Gasterosteus* DIGIRR gene is located within a 4313-bp genomic fragment of group XIX. The intron-splicing consensus (GT/AG) is constant at the 5′ and 3′ ends of the introns in all three fish DIGIRR genes.

Unlike the vertebrate homolog SIGIRR genes with nine encoding-exons, the fish DIGIRR genes have 10 encoding-exons. Particularly, the first exon is much longer (67–70 bp) than that (7 bp) in all higher vertebrate SIGIRR genes. This exon is predicted to encode an unexpected signal peptide sequence, which is not found in SIGIRR, suggesting that the final subcellular DIGIRR localization is different (Figs. 1, 2B). Exon 2, which encodes the first extracellular Ig-like domain (named D1) of the DIGIRR molecule, is unique to the DIGIRR gene. Except for the unique features of the first two encoding exons, however, the size and organization of other exons in DIGIRR genes are well matched with SIGIRR genes. Exons 3–10 in DIGIRR correspond with exons 2–9 in SIGIRR. Exon 3, together with exon 4 of DIGIRR genes, encode the second extracellular Ig-like domain, which confers DIGIRR with a unique two-Ig-like domain structure distinct from SIGIRR (with one) and IL-1R (with three). Exon 5 encodes the transmembrane domain, and the remaining exons (exons 6–10) encode the intracellular regions, including one TIR domain (Fig. 2B). The similar organization between DIGIRR (exons 3–10) and SIGIRR (exons 2–9) genes, together with the conserved genomic synteny, largely provide evolutionary evidence that these two genes probably originated from a common ancestor. The copy number of the *Tetraodon* DIGIRR gene is analyzed by Southern blotting under stringent hybridization and washing.
<table>
<thead>
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<th>Primer Name</th>
<th>Sequence (5′→3′)</th>
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The nucleotide and deduced amino acid sequences of DIGIRR cDNA from *Tetraodon* (GenBank accession number EF095151). The putative signal peptide is underlined, and the transmembrane region is shown in gray. The start and stop codons are in boldface. The three conserved motifs (boxes 1–3) of the TIR domain are underlined and noted. The polyadenylation signal “AATAAA” in the 3'UTR is in bold italic.

![Diagram](image_url)

FIGURE 1. The nucleotide and deduced amino acid sequences of DIGIRR cDNA from *Tetraodon* (GenBank accession number EF095151). The putative signal peptide is underlined, and the transmembrane region is shown in gray. The start and stop codons are in boldface. The three conserved motifs (boxes 1–3) of the TIR domain are underlined and noted. The polyadenylation signal “AATAAA” in the 3'UTR is in bold italic.

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conditions. As shown in Fig. 2C, the digests obtained with three different enzymes displayed one band. As the genomic sequence covered by the probe did not contain any cleavage sites specific to any of the restriction enzymes used here, the results suggest that *Tetraodon* has only one copy of the DIGIRR gene.

Multiple sequence alignment and phylogenetic analysis

An amino acid sequence multiple alignment of the DIGIRR/SIGIRR family encompassing representatives of various species, including primate, rodent, bird, and teleost fish, was prepared using ClustalW. The result shows that the fish DIGIRRs share an overall sequence similarity with other species (Fig. 3). As shown in Table II, the teleost fish DIGIRRs show 31.0–41.2% sequence identity with mammalian SIGIRRs and 35.1–37.9% homology with avian (chicken) SIGIRR. In general, homology is much higher between fish species than between fishes and mammals or between fishes and birds. Indeed, SIGIRRs demonstrate 81.2–89.8% identity among mammals and 61.8–66.2% between mammals and avians, and DIGIRRs show 78.3% identity between Tetraodon and Takifugu, 66.8% between Tetraodon and Gasterosteus, and 68.3% between Gasterosteus and Takifugu. The Tetraodon DIGIRR is clearly more similar to that of Takifugu, indicating the close relationship between Tetraodon and Takifugu. The phylogenetic tree was constructed by using the neighbor-joining method and included DIGIRRs, SIGIRRs, and other IL-1R family members (Fig. 4). The result shows that fish DIGIRRs are not only clustered together to form an exclusive group but also, along with mammal SIGIRRs, merge into a larger group with high bootstrap probability. These findings indicate that DIGIRR and SIGIRR share sequence homology and might originate from a common ancestor molecule.
We then performed a more detailed analysis of the sequence homology between functional domains among DIGIRR, SIGIRR, and other IL-1R family members. When the Ig-like domains are compared in DIGIRR, both newly identified Ig-like domains (named as D1 and D2) are highly conserved among the three fish species (D1, 65.8–73.9%; D2, 54.3–66.4%). However, when D1 and D2 are compared with each other, the identity is lower (13.9–

Table II. Percentages (%) of amino acid sequence identity for SIGIRR and DIGIRR sequences

<table>
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<th>Human</th>
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*DIGIRR sequence.
Tissue distribution and expression analyses

RT-PCR and real-time PCR were used to evaluate the expression patterns of DIGIRR in different tissues in both healthy and LPS-stimulated fish. The result showed that in healthy fish, the DIGIRR mRNA could be amplified in gill, brain, liver, skin, intestine, spleen, head kidney, and leukocytes from blood, spleen, and head kidney, but was totally undetectable in heart and muscle (Fig. 6A, B). After LPS stimulation for 12 h, however, a significant elevation of DIGIRR expression was observed in head kidney, spleen, liver, brain, skin, leukocytes from blood, spleen, and head kidney (all \( p < 0.01 \)), and intestine (\( p < 0.05 \)) compared with that in untreated control fish (Fig. 6C, D). This result indicated that DIGIRR may play a role in inflammatory responses. The expression pattern of DIGIRR appeared different from that previously observed in SIGIRR, which was expressed in most tissues examined in mouse (26), but was significantly downregulated after stimulation with LPS (24). This converse regulation suggests there might be a different mechanism involved in the inflammatory response between DIGIRR and SIGIRR. Furthermore, kinetic expression analysis demonstrated that an upregulation of DIGIRR could be observed after 3-h stimulation with LPS in head kidney. At 6 h and 12 h after LPS challenge, there was a significant (3.3-fold and 4.2-fold) increase in the abundance of DIGIRR mRNA. At 24 h postchallenge, the expression of DIGIRR reached its peak of 6.6-fold higher than that measured in the control group (0 h). After 48 h, however, the expression level decreased (Fig. 6E). This LPS-induced increase in DIGIRR expression mirrors several other IL-1R family members, such as IL-1R, IL-1RAcP, and ST2, which are crucial in the initiation of inflammatory signaling (36, 40, 69, 70).

![FIGURE 4](http://www.jimmunol.org/)
Subcellular distribution of DIGIRR

To investigate the subcellular distribution of DIGIRR, EGFP-labeled or myc-tagged DIGIRR constructs (pEGFPN1-DIGIRR and pcDNA6-myc-DIGIRR) together with the pDsRed-Golgi were cotransfected into HEK293T, HepG2, and HeLa cells. Furthermore, the cells transfected with pEGFPN1-DIGIRR were stained with the Golgi tracker C5-ceramide and the cell membrane indicator DiI. The results show that DIGIRR was clearly distributed in the cytoplasm in all three cell lines examined (Fig. 7). Furthermore, the fluorescence images of both enhanced GFP-(Fig. 7B, 7E, 7F) and the FITC–anti-myc–labeled (Fig. 7C) DIGIRR molecules merged well with the DsRed protein or C5-ceramide signals targeting the Golgi apparatus (Fig. 7D). However, no colocalized signals of the DIGIRR were detected with DiI (Fig. 7A). In contrast, the Tetraodon IL-1RI, a known cell membrane-localized receptor, was clearly demonstrated to be colocalized with the DiI, illustrating that IL-1RI is distributed on the cell surface (data not shown). These observations preclude the presence of DIGIRR on the cell surface and suggest that DIGIRR is a Golgi-distributed protein.

DIGIRR downregulates NF-κB signaling in fish

The TIR domain-containing family members were usually found to be positive or negative regulators of two classical (IL-1–mediated and LPS-mediated) NF-κB signaling pathways. To investigate whether DIGIRR participates in these signaling pathways, the involvement of this factor in IL-1–mediated and LPS-mediated NF-κB signaling pathways was examined. For this purpose, zebrafish and Tetraodon IL-1β expression plasmids were constructed, and the typical NF-κB dual-luciferase reporter assay system and the newly developed zebrafish embryo signaling detection model were used. The result showed that injection of zebrafish embryos with both zebrafish and Tetraodon IL-1β expression plasmids induced significant \( p < 0.01 \) activation of
NF-κB signaling (Fig. 8A, 8B). In contrast, coinjection of zebrafish embryos with IL-1–expression and DIGIRR-expression plasmids severely reduced (p < 0.01) the activation of NF-κB signaling (Fig. 8A, 8B). In parallel, little NF-κB activation could be detected after injection of the mock plasmid (pDNA6) in the control group (Fig. 8A, 8B). These results suggested that DIGIRR negatively regulated the IL-1–mediated NF-κB signaling pathway. It has previously been shown that LPS can activate NF-κB signaling in zebrafish embryos (63), so we investigated whether DIGIRR regulated this LPS-mediated NF-κB pathway. As expected, DIGIRR decreased (p < 0.01) the activation of NF-κB signaling induced by LPS (Fig. 8C). Based on these data, we suggest that DIGIRR acts as a crucial negative regulator in both IL-1–mediated and LPS-mediated signaling. In addition, we also examined whether DIGIRR shared a conserved function across species. For this purpose, the inhibitory effect of DIGIRR on IL-1–mediated signaling was examined in HeLa cells. The result showed that ectopic expression of DIGIRR in human cells had no significant effect on IL-1–mediated activation of NF-κB. However, in the positive control cells in which mouse SIGIRR was overexpressed, IL-1–stimulated NF-κB activation was dramatically reduced (Fig. 8D). These results indicated that DIGIRR failed to regulate the IL-1–mediated NF-κB signaling in mammalian cells, suggesting functional diversity exists between DIGIRR and other family members, including SIGIRR, in different species.

**In vivo knockdown of DIGIRR by siRNA**

In an attempt to silence the expression of DIGIRR for in vivo functional studies, we developed a lentivirus-based siRNA delivery system for fish. Forty-six potential siRNAs, targeted to different regions of DIGIRR, were initially predicted by a template design program, from which four siRNAs (Table 1) were selected for further evaluation. Of the four generated constructs (pSUPER-DIGIRR-1 to -4), the pSUPER-DIGIRR-3 was identified to be the most effective (75%) in inducing DIGIRR mRNA degradation (Fig. 9A). This siRNA encoding sequence was inserted into a lentiviral vector (PLB), and the construct was transfected into HEK293T cells to produce lentiviruses together with pCMV-VSVG and pCMV-dR8.2. The titer of the concentrated viruses reached ~5 × 10^5 TU/ml. The siRNA expression efficiency of the lentiviruses detected in HEK293T cells reached above 90% using the expression of GFP as an indicator (Fig. 9C), and the interference efficiency of this system reached above 74% (Fig. 9B). These results demonstrated that the recombinant lentiviruses could silence the DIGIRR expression in vitro. To investigate further whether this lentiviral system could be used for in vivo knockdown purpose, we administered the recombinant viruses into *Tetraodon* once a day for 3 d. Real-time PCR was performed to test the silencing of DIGIRR in different tissues. The result showed that DIGIRR expression could be dramatically downregulated (46–78%) in liver, kidney, and in leukocytes from head kidney, although it seems not obviously reduced in spleen (Fig. 10A). The development of this in vivo knockdown technology might represent a new method to deliver siRNAs into fish and greatly benefit functional studies of DIGIRR in vivo.

**DIGIRR downregulates IL-1–induced inflammation**

The observation that DIGIRR suppressed IL-1β–induced and LPS-induced NF-κB signaling strongly suggested that DIGIRR may modulate inflammatory responses. To clarify this effect further, we explored the function of DIGIRR in IL-1–mediated inflammatory responses. We delivered the lentiviruses into *Tetraodon* once a day for 3 d, followed by coadministration with recombinant IL-1β protein (see Supplemental Fig. 2) on the last day. Quantitative PCR data showed that, after induction by IL-1β stimulation, the expression of the proinflammatory cytokines IL-6 and IL-1β were dramatically upregulated in the liver, kidney, and head kidney leukocytes from DIGIRR-silenced fish relative to IL-1β–stimulated control fish (Fig. 10B, 10C). We also found that the DIGIRR-silenced fish with no external IL-1β stimulation had higher basal expression of IL-6 and IL-1β compared with control fish (Fig. 10B, 10C). These observations strongly suggested that DIGIRR-silenced fish were hyperresponsive to either exogenous or endogenous IL-1β–stimulated inflammatory responses and further confirmed that DIGIRR served as a negative regulator in IL-1–mediated signaling.

**FIGURE 7.** Subcellular distribution analysis of DIGIRR in cultured cells. A, HeLa cells were transfected with plasmid pEFPN1-DIGIRR, fixed, and stained with the cell membrane probe DiI, which demonstrates that DIGIRR was distributed in the cytoplasm, and no colocalized signals of DIGIRR were detected with DiI. B, HeLa cells were cotransfected with pEFPN1-DIGIRR and pDsRed-Golgi, which shows that the enhanced GFP-labeled DIGIRR merged well with the DsRed protein. C, HeLa cells were cotransfected with pDNA6-myc-DIGIRR and pDsRed-Golgi, fixed, and stained with FITC-anti-myc Abs, showing that DIGIRR merged well with the DsRed protein. D, HeLa cells were transfected with pEFPN1-DIGIRR and stained with the Golgi tracer C3-ceramide, which demonstrates that DIGIRR merged well with the C3-ceramide signal. E and F, HEK293T and HepG2 cells were cotransfected with pEFPN1-DIGIRR and pDsRed2-Golgi, showing DIGIRR and DsRed proteins were colocalized in the Golgi apparatus. Scale bars, 10 μm (original magnification ×600).
**NF-κB signaling of chimeras with amino acid replacements**

Two sites of conserved amino acids (Ser and Arg-Tyr) in the TIR domains of the IL-1R family are suggested to be extremely important for receptor signal transduction. Substitutions at these two sites by any other amino acid in molecules such as SIGIRR and IL-1RAcP results in the interruption of IL-1R–mediated NF-κB activation, and they become negative regulators in the signaling pathways. A series of chimeras with functional domains or amino acid replacements were constructed to investigate whether one substitution at the Arg-Tyr site may functionally transform DIGIRR. An IL-1–mediated NF-κB reporter assay was used for this purpose. As shown in Fig. 11, the chimera with the extracellular and transmembrane portions of human IL-1RI and the cytoplasmic region of DIGIRR (chimera-1) exhibited no response to IL-1 stimulation in the HEK293T cells, in which the NF-κB activity was kept at the basal level, similar to that of the control group (empty vector pcDNA6). In contrast, a significant, ∼4-fold increase ($p < 0.01$) in NF-κB activation was observed in the positive group, in which the human IL-1RI dramatically responded to IL-1. This result indicates that the DIGIRR TIR domain with an Arg-Tyr site mutation at the Arg-Tyr site may functionally transform DIGIRR. An IL-1–mediated NF-κB reporter assay was used for this purpose. As shown in Fig. 11, the chimera with the extracellular and transmembrane portions of human IL-1RI and the cytoplasmic region of DIGIRR (chimera-1) exhibited no response to IL-1 stimulation in the HEK293T cells, in which the NF-κB activity was kept at the basal level, similar to that of the control group (empty vector pcDNA6). In contrast, a significant, ∼4-fold increase ($p < 0.01$) in NF-κB activation was observed in the positive group, in which the human IL-1RI dramatically responded to IL-1. This result indicates that the DIGIRR TIR domain with an Arg-Tyr site mutation at the Arg-Tyr site may functionally transform DIGIRR.

**Discussion**

The IL-1R family is a subgroup of the TIR superfamily. The IL-1Rs are characterized by extracellular Ig-like domains and a conserved TIR domain in the intracellular region. In most cases, the extracellular portion of IL-1R family members is highly conserved, with three Ig-like domains, except for SIGIRR, which consists of only one Ig-like domain. In the current study, we identified a novel member of the IL-1R family that displayed a high degree of sequence homology in the cytoplasmic TIR domain but expressed two Ig-like domains in the extracellular region. This novel molecule was referred to as DIGIRR based on this unique extracellular structure. The novel DIGIRR protein also displayed another unique characteristic not observed in other known IL-1R family members: subcellular distribution within the Golgi apparatus. Thus, our study has added a new member to this family (DIGIRR, which could also be named TIR9 after SIGIRR/TIR8).

Most characterized members of the TIR superfamily serve as positive regulators in various TLRs-mediated and IL-1–mediated signaling pathways (1–3, 8), whereas negative regulators in these signaling pathways are rare. In recent years, several negative regulators, such as IL-1RII, ST2, and SIGIRR, have been identified in humans and other mammals, which provide preliminary...
insight into the origin, occurrence, and evolution of negative regulatory mechanisms in TIR signaling pathways. Structurally, these negative regulators share many similarities with their positive counterparts. However, distinct alterations or mutations in key

FIGURE 9. Screening of siRNAs targeting Tetraodon DIGIRR expression and detection of siRNAs produced by lentivirus. A, Screening of effective siRNAs. Four different siRNAs against DIGIRR were designed to target distinct parts of the DIGIRR mRNA and inserted into siRNA-expressing vectors to construct four recombinant plasmids (pSUPER-DIGIRR-1 to -4). The HEK293T cells were cotransfected with pSUPER-DIGIRR or control plasmid (pSUPER) together with overexpression plasmid pC6-DIGIRR. The inhibitory efficiency of siRNAs was measured by real-time PCR. The pSUPER-DIGIRR-3 was found to be the most effective in silencing DIGIRR, so this siRNAs encoding sequence was then subcloned into lentiviral vector pLenti4.1. Lentiviruses were produced by cotransfection with pLenti4.1, pCMV-ROSA, and pCMV-dR8.2 into 293T cells. B, The efficacy of DIGIRR knockdown by lentiviruses was also evaluated by real-time PCR. C, The siRNA expression efficiency of the lentiviruses detected in HEK293T cells exceeded 90%, as revealed by GFP fluorescence under laser scanning confocal microscopy (Zeiss LSM 510).

FIGURE 10. DIGIRR-silenced fish showed enhanced inflammatory responses to IL-1β. A, The DIGIRR expression could be dramatically downregulated in liver, kidney, and leukocytes from head kidney. B and C, DIGIRR-silenced fish showed greater induction of the proinflammatory cytokines IL-6 and IL-1β after administration of recombinant IL-1β. The lentivirus with siRNAs was repeatedly delivered into fish by muscle injection once a day for 3 d and followed by coadministration with recombinant IL-1β on the last day. Twenty-four hours after IL-1β challenge, relative gene expression of DIGIRR, IL-1β, and IL-6 was calculated and normalized to β-actin expression. Each sample was quantified by PCR, and results are expressed as the mean of three independent experiments. Expression level is normalized to the control group (“No siRNA + PBS”). Bars represent SEMs. Statistical significance is denoted with an asterisk as compared with controls: *p < 0.05, **p < 0.01.
functional domains are observed in these molecules. Therefore, it seems reasonable to suggest that these negative and positive regulators might be derived from a common ancestor but that negative regulators have lost distinct functional elements. The SIGIRR molecule may be an example. It appears to be derived from its potent counterpart, the IL-1R. IL-1R possesses three Ig-like domains in its extracellular region and one intact TIR domain with two conserved amino acids sites (Ser and Arg-Tyr) in its intracellular region, which are required for signal transduction in all TIRs (25, 27, 68). The SIGIRR, however, contains only one Ig-like domain in its extracellular portion, whereas the TIR domain has two substitutions at the conserved sites of amino acids, Cys222 and Gln-Leu305. As a result, the SIGIRR lost the ability directly to interact with IL-1α, IL-1β, or IL-1R antagonist and to transduce signals from IL-agonist binding (24, 25, 71). It is therefore possible that SIGIRR became a negative regulator through loss of two extracellular Ig-like domains and by two mutations in the TIR domain. This is supported by the isolation of DIGIRR from fishes, ancient lower vertebrates that are an indispensable model organism for evolutionary studies.

In this study, we suggest that DIGIRR bears the hallmarks of a "transitional molecule" between IL-1R and SIGIRR; in other words, DIGIRR may be an early example of the transition from potent receptor to negative regulator during vertebrate evolution. A model for the evolutionary relationships among all TIRs, DIGIRR, and SIGIRR is proposed (Fig. 12). This conclusion is supported by several observations as discussed below.

First, DIGIRR shares sequence identity and functional domains with SIGIRR and IL-1Rs, indicating the existence of evolutionary relationships among these TIR superfamily members. Second, the cysteines involved in the first Ig-like domain in DIGIRR, which were absent in SIGIRR, were well conserved in fish. This confirmed that the two Ig-like domains were common structures in fish DIGIRR proteins. Remarkably, the numbers of the Ig-like domains in the extracellular region of IL-1R, DIGIRR, and SIGIRR were in turn three, two, and one. The IL-1Rs with three Ig-like domains were shown to be potent receptors that have the ability to bind IL-1α, IL-1β, or IL-1R antagonist, whereas SIGIRR, with only one Ig-like domain, was unable to bind these cytokines. Thus, the decreased number of Ig-like domains among IL-1R, DIGIRR, and SIGIRR suggested that the DIGIRR may represent a link between the potent receptor (IL-1R) and the negative regulator (SIGIRR) that lacks two extracellular Ig-like domains necessary to recognize ILs. It is generally believed that all three Ig-like domains in mammalian IL-1Rs are necessary for IL-1α or IL-1β binding (72), but an exception was found in fish. For example, the trout IL-1R1, with only two Ig-like domains, had the ability to bind IL-1β (73), indicating that the fish DIGIRR with two Ig-like domains may still bind ILs. Functional studies are needed to evaluate this possibility. Such studies could also define which one of these two Ig-like domains is the more crucial for the recognition of ligand.

Third, two conserved amino acids sites, Ser and Arg-Tyr, within the TIR domains of the IL-1R family important for the signaling of TIRs (25, 27, 68), were changed by two in SIGIRR and by one in DIGIRR, underscoring the possibility that DIGIRR is a transitional molecule between IL-1R and SIGIRR. In SIGIRR, the Ser and Arg-Tyr were changed to Cys and Gln-Leu, leading to a receptor that cannot mediate signal transduction (24, 25, 72). However, only one amino acid site substitution occurred in the DIGIRR intracellular domain (Ala-Leu420 replaces the Arg-Tyr536), whereas Ser was conserved in IL-1R and other family members. Notably, the Tyr in the Arg-Tyr site was replaced with an aliphatic residue, such as Leu (in SIGIRR and DIGIRR) and Val (in IL-1R).
have a putative 16–19 aa signal secretion peptide at the N-terminal region, whereas there is no predictable signal peptide in all SIGIRRs. However, similar signal peptides were also found in other IL-1R subfamily members, such as IL-1RI, IL-1RII, IL-1RAPL, TIGIRR, ST2, and IL-18R, providing further evidence for an evolutionary link between IL-1R and DIGIRR and suggesting that fish DIGIRR is translocated into the membrane through a conserved pathway that is similar to IL-1R subfamily members. Finally, IL-1R possesses three Ig-like domains and an entire TIR domain, with no amino acids substitutions, implying that IL-1 binding and signal transduction requires these elements. It is the loss of the Ig-like domains and crucial amino acid changes that transformed SIGIRR into a negative regulator (24, 27, 71). The results demonstrated that SIGIRR exerted its inhibitory role by disturbing the heterodimerization of IL-1R and IL-1RAcP by binding to these molecules through the extracellular Ig domain. Also, the altered intracellular TIR domain inhibited signaling by attenuating the recruitment of receptor-proximal signaling components, such as MyD88, TNFR-associated factor 6, and IRAK, to the receptor (71). Most members of the IL-1R family, including SIGIRR and Tetraodon IL-1RI (data not shown), are distributed on the cellular membrane (74). In contrast, DIGIRR is localized in the cytoplasm and may be a Golgi-distributed protein. Therefore, we presumed that there might be different posttranslational processing and subcellular targeting for DIGIRR and SIGIRR. Most likely, DIGIRR suppresses IL-1R–mediated signaling pathways by interacting with adapter proteins like MyD88 and TNFR-associated factor 6 or directly with the IL-1R complex through its Arg-Tyr–mutated TIR domain. Further investigation is needed to clarify the detailed molecular mechanisms of negative regulation of IL-1R signaling by DIGIRR.

There are still very few protocols available for functional studies of genes in fish. As suggested in preceding reports, morpholino demonstrated effective loss of function of embryonic genes in several species, such as zebrafish (75). However, technologies for the suppression of genes in adult fish tissues are undeveloped. The RNA interference (RNAi) method has emerged as a powerful technique to inhibit the expression of specific genes in living cells. It is mediated by siRNAs produced from long dsRNAs of exogenous or endogenous origin by the endonuclease Dicer (76–79). Generally, siRNAs can be obtained by chemical synthesis, interference plasmids, or viral plasmids (80, 81). It has also been reported that short hairpin RNAs, precursors to siRNA, can be expressed using lentivirus, allowing for RNAi in a variety of cell types (82). The lentiviruses, such as the HIV, are capable of infecting both dividing and nondividing cells and integrating into the host cell chromosome. Indeed, siRNAs produced by lentivirus are widely applied. In vitro delivery of siRNAs into cultured cells can be achieved by several protocols, including transfection with liposome (78). However, in vivo delivery appears far more difficult and therefore receives much attention. Recently, protocols for in vivo siRNA delivery have been developed or improved,
including i.v. or orally delivery synthetic siRNA or siRNA-expressing plasmids with various modifications (83). These protocols have been used in mouse models and may prove beneficial for drug delivery in clinical application (80, 84–87). However, the delivery of siRNAs into specific adult fish tissues is still elusive. In the current study, we have successfully used a lentivirus vector to deliver the DIGIRR-silencing siRNA into fish by i.m. administration. The results demonstrated that expression of DIGIRR could be effectively reduced by 46–78% in immune-relevant tissues (liver and kidney) and cells (leukocytes). This was effective in silencing DIGIRR, and suppression resulted in a hyperresponse to IL-1β, strongly supporting the idea that DIGIRR functioned as a negative regulator in IL-1 signaling and inflammation. The successful application of this lentivirus-based siRNAs delivery in fish immune system could greatly benefit further functional studies of both DIGIRR and other genes in fish immunity.

In conclusion, we report in this study a novel DIGIRR gene from a model teleost fish, T. nigroviridis. Structural and functional characterizations showed it represents a previously unknown TIR family member that serves as a negative regulator in IL-1 signaling. We propose that DIGIRR and SIGIRR might originate from an ancient IL-1R–like molecule through the loss of one and two extracellular Ig-like domains and mutation of intracellular Ser and Arg-Tyr amino acids. DIGIRR might be an evolutionary "transitional molecule" between IL-1R and SIGIRR, representing the connector between the potent receptor and the negative regulator. This study also demonstrates the importance of all Ig-like domains and Ser and Arg-Tyr amino acids for IL-1R-mediated signaling modulation. Our results not only add a new member to the TIR superfamily but also contribute to a better understanding of the evolutionary history of this family and its associated signaling network.

Disclosures

The authors have no financial conflicts of interest.

References


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