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Characterization of a PIAS4 Homologue from Zebrafish: Insights into Its Conserved Negative Regulatory Mechanism in the TRIF, MAVS, and IFN Signaling Pathways during Vertebrate Evolution

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Members of the protein inhibitor of activated STAT (PIAS) family are key regulators of various human and mammalian signaling pathways, but data on their occurrence and functions in ancient vertebrates are limited. This study characterizes for the first time to our knowledge a PIAS4 homologue (PIAS4a) from zebrafish. Structurally, this zebrafish PIAS4a (zfPIAS4a) shares a number of conserved functional domains with mammalian PIAS4 proteins, including the scaffold attachment factor A/B/acinus/PIAS box, PINIT, and RING-finger–like zinc-binding domains and a highly acidic domain in the C-terminal region. Subcellular localization analysis shows that zfPIAS4a is a nuclear-localized protein and that the C terminus of the molecule harbors strict nuclear localization signals. Functionally, zfPIAS4a expression can be dramatically induced by the stimulation of polyinosinic-polycytidylic acid and zebrafish IFNα1. It acts as a critical negative regulator of the TIR domain-containing adapter inducing IFN-β, mitochondrial antiviral signaling (MAVS), and IFN signaling pathways, and it is the first PIAS protein that plays a role in the MAVS-mediated pathway to be identified. The structure and functionality of PIAS4a seem highly conserved from zebrafish to mammals, making zebrafish an attractive model for screens designed to uncover genes involved in IFN-α and inflammatory cytokine-induced signaling pathways. This study provides preliminary evidence that the PIAS regulatory mechanism already existed in fish during vertebrate evolution. It presents valuable clues for improving the understanding of not only the negative regulation of cytokine signaling in fish but also the evolutionary history of the PIAS family from fish to mammals as a whole. The Journal of Immunology, 2012, 188: 000–000.

The protein inhibitor of activated STAT (PIAS) proteins represent one of the most important signal transduction modulator families (1). They regulate transcriptional activities in various signaling pathways, either positively or negatively, but they are mostly associated with repression activity (2). The mammalian PIAS family consists of PIAS1, PIAS2 (PIASx), PIAS3, and PIAS4 (PIASy) (1–3). These member proteins were initially characterized by their ability to interact with and inhibit STAT factors (4, 5). However, the interactions and functions of PIAS proteins were subsequently found to be specific not only to STATs but also to many other transcription factors, viral proteins, oncoproteins, tumor suppressors, and cytokine-induced genes or pathways, such as those dependent on NF-κB, IFNs, SMADs, and androgen receptors (6–9). They are also involved in various biological activities, in particular with immune responses. For example, PIAS3 has also been found to be constitutively activated in diverse human cancer cells and is crucial for the development of the hematopoietic system (10–12).

Five conserved functional domains have been identified in PIAS family members: an N-terminal scaffold attachment factor A/B/acinus/PIAS (SAP) box, a PINIT domain, a RING-finger–like zinc-binding domain (RLD), a highly acidic domain (AD), and a serine- and threonine-rich region at the C terminus (13–16). The PIAS proteins regulate transcription through several mechanisms, including blocking the DNA-binding activity of transcription factors, promoting protein sumoylation, and recruiting transcriptional corepressors or coactivators (5, 7, 9, 17, 18). Among the PIAS family members, PIAS4 has received much attention because it participates in various signaling pathways in different cellular activities, such as hematopoiesis, oncogenesis, and immune regulation. PIAS4 modulates the transcriptional activity of a set of STAT factors, lymphoid enhancer factor 1, and androgen receptors (18, 19). It is particularly important for the negative regulation of TIR domain-containing adapter-inducing IFN-β (TRIF)-induced NF-κB activation and IFN signaling pathways, which limit the strength and duration of proinflammatory cytokines and the responsiveness of type I IFNs (6, 8).

In humans and other mammals, proinflammatory cytokines and type I IFNs are induced through different signaling pathways in...
response to pathogen infection or pathogen-associated molecular pattern stimulation, among which the TLR3- and retinoic acid-inducible gene I (RIG-I)/melanoma differentiation-associated gene 5 (MDA5)-mediated pathways are the two main pathways for the recognition of dsRNA viral infection and dsRNA-induced type I IFN and proinflammatory cytokine production (20, 21). TRIF is thus far the only adapter identified in the TLR3-mediated pathway that directly activates IFN regulatory factor (IRF)-3 and NF-kB, leading to the induction of type I IFN and proinflammatory cytokines or chemokines (22, 23). Mitochondrial antiviral signaling (MAVS), however, is a later-identified adapter in the RIG-I/MDA5-mediated pathway that directly activates IRF-3/7-dependent type I IFN responses and NF-kB–dependent inflammatory gene expression (24–29). Several studies have shown that PIAS4 is a critical negative regulator of the TRIF-induced signaling pathway; in addition, other regulatory molecules, including TNF-α–induced protein 3, NOD-like receptor family member XI, proteasome (prosome or macropain) subunit α type 7, and poly(C) binding protein 2, have been proved to be involved in the MAVS-activated pathway (30–33). However, whether PIAS proteins participate in the MAVS-mediated pathway is still unknown. A recent study showed that TRIF and MAVS are required to produce an immune response to dsRNA and that neither sufficiently functions in the absence of the other (34), indicating possible cross-regulation between the TRIF and MAVS pathways, which suggests that the regulatory mechanisms underlying these two pathways may be more complex than previously known. Therefore, further investigations are still required to clarify the exact mechanisms in the TRIF- and MAVS-mediated pathways.

A number of genes related to the IFN system have been recently identified from different fish species, including the TLRs, the RIG-I–like receptors, the TRIF and MAVS adapters, the IRF members, the JAK/STAT family members, and several IFN-stimulated genes (ISGs), such as protein kinase containing Z-DNA binding domains (PKZ) (35, 36), ISG15 (37, 38), CD40 (39), GTPase Mx protein (40), (ISGs), such as protein kinase containing Z-DNA binding domains like receptors, the TRIF and MAVS adapters, the IRF members, the plexes have been characterized (42, 43). Moreover, TLR3 and TLR22 are predicted in the Ensembl genome database (44, 45). The full 5′-RACE and 3′-RACE core sets (Takara) were used to obtain 5′ and 3′ unknown regions. PCR amplification was performed in 50 µl reaction mixtures containing 4 µl 10 µM forward and reverse primers, 0.5 µl cDNA template, 10 µl PCR buffer (Takara), 1 µl dNTP mixture (for a final concentration of 2.5 mM each), and 0.5 µl PrimeSTAR HS DNA Polymerase (2.5 U/µl; Takara), distilled water was added up to 50 µl. The cycling protocol involved 25 cycles of 98°C for 10 s, 52°C for 15 s, and 72°C for 20 s. The PCR-amplified fragment was then incubated at 72°C for 20 min more with 1 U Taq DNA polymerase (Takara) for the addition of 3′ A overhangs. The products were loaded onto 1.2% (w/v) agarose gel and visualized by staining in 0.1 µg/ml ethidium bromide. The PCR products were purified using a gel extraction kit (Qiagen), ligated into a pUCm-T vector (Takara), and transformed into competent Escherichia coli TOP10 cells (Invitrogen). Plasmid DNA was purified using the Plasmid Miniprep method and sequenced on a MegaBACE 1000 Sequencer (GE Healthcare Life Sciences) using a DYEnamic ET Dye Terminator Cycle Sequencing Kit (Amerssharm Pharmacia Biotech).

Characterization of zPIAS4a

The comparative gene map positions were determined using MapViewer (http://www.ncbi.nlm.nih.gov/mapview/) and the UCSC Genome Browser (http://genome.ucsc.edu). Gene organizations (intron/exon boundaries) were elucidated by comparing the zPIAS4a cDNA with genome sequences, and the illustrations accompanying this article were drawn using GeneMapper 2.5 (http://genemapper.ogpmole.com). The following species were selected for comparative analyses: human, Homo sapiens; mouse, Mus musculus; Xenopus tropicalis; fugu, T. rubripes; medaka, O. latipes; stickleback, G. aculeatus; tetraodon, T. nigroviridis; and zebrafish, D. rerio. The potential functional motifs in zPIAS4a protein were analyzed using the PROSITE database (http://expasy.org/prosite). The prediction of the tertiary structure of zPIAS4a was carried out in SWISS-MODEL Workspace (http://swissmodel.expasy.org/). The percentages of amino acid sequence identity were calculated using the MEGALIGN program from DNASTAR, and a multiple alignment was generated using the ClustalW program (version 1.83) (49). The phylogenies of the protein sequences were estimated with MEGA5 using parsimony and the neighbor-joining method (50).

Cloning of zPIAS4a TRIF cDNA and MxA promoter

The zebrafish TRIF-encoding cDNA and MxA promoter sequence were cloned to construct an overexpression plasmid and a luciferase reporter vector according to previously reported sequence data (40, 44). Briefly, for TRIF cDNA cloning, RT-PCR amplification was performed using PrimeSTAR HS DNA Polymerase (2.5 U/µl; Takara) followed by the aforementioned protocols. To clone the MxA promoter (1123 bp in length; GenBank: AF532732.1), zebrafish genome DNA was isolated from the whole fish using an AsyPrep Multisource Genomic DNA Miniprep Kit (Axygen Biosciences) according to the manufacturer’s protocol. Two pairs of PCR primers, zMxA pro-outer-F/zfMxA pro-outer-R and zfMxA pro-inner-F/zfMxA pro-inner-R, were designed (Table I). Nested PCR was performed using the genomic DNA

Materials and Methods

Experimental fish

One-year-old male and female wild-type AB zebrafish (Danio rerio) weighing ~0.5–1 g, with body lengths of 1–2 cm, were purchased from National Zebrafish Resources of China, kept in recirculating water at 28°C, and fed with commercial pellets at a daily ration of 0.7% of their body weight. All fish were held in the laboratory for at least 2 wk before use in the experiments to allow for acclimatization and evaluation of their overall health. Only healthy fish, as determined by their general appearance and level of activity, were used in the experiments.

Sequence retrieval

The PIAS4 homologue of zebrafish was searched using WU-BLAST at the Computational Biology and Functional Genomics Laboratory (http://compbio.dfci.harvard.edu/cgi-bin/tgi/Blast/index.cgi) using “human PIAS4 amino acid sequence” as the query (NP_056981.2). A homologue expressed sequence tag (EST; accession number TC302013) was found in the zebrafish EST database. It was then used as a reference for designing primers for the molecular cloning of zfPIAS4a cDNA. The PIAS4 sequences of other fish species, including fugu (Takifugu rubripes), medaka (Oryzias latipes), stickleback (Gasterosteus aculeatus), and tetraodon (Tetraodon nigroviridis), were predicted in the Ensembl genome database (http://www.ensembl.org/index.html).

Cloning of zPIAS4a

The fish were sacrificed after anesthesia and ground in liquid nitrogen. The total RNA was isolated from the whole fish using TRIZol reagent (Invitrogen), with additional DNase I digestion to remove traces of genomic DNA. The RNA concentrations were measured using a spectrophotometer, and their integrity was ensured by analysis on 1.5% (w/v) agarose gel. The zfPIAS4a cDNA was generated by RT-PCR (the primers are shown in Table I). The full 5′-RACE and 3′-RACE core sets (Takara) were used to obtain 5′ and 3′ unknown regions. PCR amplification was performed in 50 µl reaction mixtures containing 4 µl 10 µM forward and reverse primers, 0.5 µl cDNA template, 10 µl PCR buffer (Takara), 1 µl dNTP mixture (for a final concentration of 2.5 mM each), and 0.5 µl PrimeSTAR HS DNA Polymerase (2.5 U/µl; Takara), distilled water was added up to 50 µl. The cycling protocol involved 25 cycles of 98°C for 10 s, 52°C for 15 s, and 72°C for 20 s. The PCR-amplified fragment was then incubated at 72°C for 20 min more with 1 U Taq DNA polymerase (Takara) for the addition of 3′ A overhangs. The products were loaded onto 1.2% (w/v) agarose gel and visualized by staining in 0.1 mg/ml ethidium bromide. The PCR products were purified using a gel extraction kit (Qiagen), ligated into a pUCm-T vector (Takara), and transformed into competent Escherichia coli TOP10 cells (Invitrogen). Plasmid DNA was purified using the Plasmid Miniprep method and sequenced on a MegaBACE 1000 Sequencer (GE Healthcare Life Sciences) using a DYEnamic ET Dye Terminator Cycle Sequencing Kit (Amerssharm Pharmacia Biotech).
template. Then, 3’ A overhangs were added to both PCR-amplified fragments, which were then cloned into a pUCm-T vector (Takara) and confirmed by sequencing analysis on a MegaBACE 1000 Sequencer as described earlier. Only the correct sequences of TRIF cDNA and MxA promoter were used for further purposes.

Plasmid constructions

The open reading frames (ORFs) of zfPIAS4a and TRIF were inserted into pcDNA6/myc-His-B (Invitrogen) between the HindIII and XhoI sites to construct the eukaryotic expression vectors pcDNA6-zfPIAS4a and pcDNA6-zTRIF, respectively. The ORFs of TRIF, MxA, and IFN-1 were inserted into pEGFP-N1 (Clontech) between the XhoI and BamHI sites to construct the eukaryotic expression vectors pEGFP-N1-TRIF, pEGFP-N1-MAX, and pEGFP-N1-IFN1, respectively. The zebrafish MxA promoter (1123 bp) was cloned into pGL3-Basic (Promega) between the KpnI and XhoI sites to construct the IFN signaling reporter vector pGL3-zMxA-1-proloc. The NF-κB luciferase construct was purchased from Clontech (Palo Alto, CA), and the pRL-TK vector was obtained from Promega. The zebrafish MAVS expression plasmid was kindly donated by Stéphane Biachessi (Jouy en Josas, France) (38). The zebrafish IFN1 expression plasmid was a gift from Victoriano Mulero (Department of Cell Biology and Histology, University of Murcia, Murcia, Spain) (45). All primers used for introducing enzyme sites into the plasmid construction are shown in Table I. All constructed sequences were confirmed by sequencing analysis, and the plasmids for transfection and microinjection were prepared free of endotoxin using an EZNA Plasmid Mini Kit (Omega Bio-tek).

Cell culture and transient transfection

The HK293 cells were maintained in DMEM (Biochrom AG, Berlin, Germany) supplemented with 10% (v/v)/FCS (Biowest, Nuaille, France), penicillin (100 U/ml), and streptomycin (100 μg/ml) and were cultured at 37°C in 5% CO2. Cells (1 × 106/ml) were seeded into Multitwell plates (Corning) to allow growth until 70–90% confluence on the day of transfection and then transiently transfected with DNA in Opti-MEM I medium without serum and antibiotics using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer’s instructions. After 4–6 h, the medium was replaced with complete DMEM plus 10% FCS.

Western blot analysis

Zebrafish embryos or HEK293 cells, which were injected or transfected with various expression plasmids or mock control plasmid, were dissolved in 2× SDS sample buffer (~3 μl/embryo) and scattered by pipette agita-
rion, followed by incubation at 100°C for 5 min. The samples underwent centrifugation (10,000 × g at 4°C for 5 min, and the supernatant liquids were collected. The proteins were separated on 10% SDS gels and semidiffusion onto PVDF membranes (Immobilon P; Millipore) according to the manufacturer’s instructions. After staining with Ponceau S for 20 min and fixing with 5% BSA in 0.5% (v/v) Tween 20 in PBS (PBST), the membrane was incubated overnight at 4°C with mouse anti-His mAb (Invitrogen) in blocking buffer. The membrane was washed three times with PBST for 45 min, incubated for 1 h with goat anti-mouse IgA–HRP (Invitrogen) in blocking buffer, and washed three times with PBST for another 45 min. The membrane was then incubated for 5 min in ECL Plus (Amersham Biosciences), and emitted light was detected using a cooled CCD camera (LAS-1000; Fujifilm).

Production of zebrafish recombinant IFN1

The zebrafish IFN1 expression plasmid was transfected into HK293 cells in 6-well plates (1 × 105 cells per well) as described earlier. The cells were maintained in DMEM supplemented with 10% FCS for the first 8 h posttransfection, after which the medium was changed to serum-free DMEM and the cells were incubated for an additional 24 h. Supernatant liquids were collected, centrifuged at 800 × g for 10 min at 4°C, and filtered through a 0.45-μm filter units (Millipore) and scattered by pipette agita-
rion to remove the cell pellets. Parts of the filter solutions were concentrated using acetone precipitation. Briefly, 1 volume of the cell supernatant liquids and 4 volumes of cold acetone were mixed well, precipitated overnight at –20°C, and centrifuged at 15,000 × g for 15 min at 4°C. The supernatant was carefully discarded, and the precipitate was washed with 1 ml ice-cold acetone and then centrifuged again for 5 min at 15,000 × g and 4°C. The resulting pellets were retained, and the tube was dried by inversion on tissue paper. Finally, the sample was resuspended in a minimal volume of 2× SDS sample buffer, and Western blot analysis was performed using 6× His Tag mAb (Invitrogen) to determine IFN1 expression.

Expression analysis of zfPIAS4

The total RNA from developing embryos and selected tissues, including the heart, spleen, liver, intestine, kidney, gill, brain, skin, and muscle, in normal or experimental fish stimulated with 10 μl polyinosinic-polycytidylic acid [poly(I:C)] (1 mg/ml; Sigma Aldrich), were isolated as described earlier to determine the expression patterns of PIAS4a in embryonic and adult zebrafish. Each fish was i.p. injected with 10 μl of the supernatant liquid from HEK293 cells containing recombinant zfIFN1 to study whether PIAS4a is IFN-stimulated. The supernatant liquid from the HEK293 cells transfected with the empty plasmid (pcDNA6-myc-His-B) was used as the control. Spleenic, liver, intestinal, and kidney tissues from four fish were collected at 4, 18, 24, 30, 48, and 72 h postinjection. Total RNA was isolated using the RNeasy kit and reverse-transcribed using an Eppendorf Thermo 

Subcellular localization

A DsRed2-fused wild-type PIAS4a expression vector (pDsRed2-C1-zfPIAS4a-WT) and a series of RED-fused mutant vectors with various amino acid deletions were constructed to determine the subcellular localization of the zfPIAS4a molecule and identify which domains or regions in the molecule harbor the subcellular localization signals (see Fig. 5A legend, Route 1). These mutants include pDsRed2-C1-zfPIAS4a-ΔM1 (1–46 aa deletion), pDsRed2-C1-zfPIAS4a-ΔPIN1 (104–264 aa deletion), pDsRed2-C1-zfPIAS4a-ΔRLD (296–373 aa deletion), pDsRed2-C1-zfPIAS4a-ΔPP1 (104–264 aa), pDsRed2-C1-zfPIAS4a-ΔCL (265–505 aa deletion), pDsRed2-C1-zfPIAS4a-ΔN1 (1–264 aa deletion), pDsRed2-C1-zfPIAS4a-ΔN2 (1–372 aa deletion). The mutant plasmids were constructed using an overlap PCR method with initial normalization of PIAS4a against β-actin or GAPDH. In all cases, each PCR trial was performed with triplicate samples and repeated at least three times.

Morpholino oligonucleotide and capped mRNA

Translation-blocking morpholino oligonucleotide (MO) was designed and synthesized by Gene Tools and solubilized in water (2 mM). The MO sequence used was as follows: 5′-AATTCGGATGTCTCAGTCTCTTACAA20-3′. To test the binding of the MO, the 5’-untranslated region (UTR) sequence of the zebrafish PIAS4a gene was amplified using the primers zpPIAS4a mo outer F, zpPIAS4a mo inner F, and zpPIAS4a mo R7 and cloned into the pDsRed2-N1 vector, then injected into one-cell-stage embryo together with the EGFP-N1 vector (50 ng/embryo) with or without PIAS4a MO (4 ng/embryo). Red fluorescent protein (RFP) and GFP fluorescence was visualized at 24 h post-microinjection using an Olympus MVX10 MacroView. Capped zebrafish PIAS4a mRNA was synthesized in vitro using a Message Machine kit (Ambion), according to the supplier’s manual, and solubilized in DEPC water for microinjection.
Examination of PIAS4a in TRIF-induced NF-κB activation

NF-κB activation was examined in zebrafish embryos using a luciferase assay as previously described (52). In brief, the NF-κB luciferase reporter gene and the indicated amounts of pcDNA6-zfTRIF and pcDNA6-zfPIAS4a expression plasmids were diluted in microinjection buffer (0.5% phenol red, 240 mM KCl, and 40 mM HEPES, pH 7.4) and injected (0.5–1 nl) into one-cell-stage embryos using a microinjector (ASI MPPI-3). The pRL-TK renilla luciferase reporter plasmid was used as the internal control. An empty control plasmid was added to ensure the same amount of total DNA. After injection, the embryos were rinsed once with E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, and 0.33 mM MgSO₄) in a 28.5˚C incubator. The firefly and renilla luciferase activities were assayed 24 h post-microinjection with 5–10 replicates (each containing the extracts from 50–100 embryos) according to the manufacturer’s instructions. Luciferase activity was normalized to pRL-TK activity and expressed as fold stimulation relative to the control. Western blot analysis was performed to determine zebrafish TRIF and PIAS4a expression.

Examination of PIAS4a in TRIF- and MAVS-activated IFN induction

TRIF- and MAVS-activated IFN induction was examined in zebrafish embryos using quantitative real-time PCR as previously described (42). In brief, expression plasmids, including pcDNA6-TRIF and pcDNA6-MAVS alone or with pcDNA6-PIAS4a, were injected into one-cell-stage embryos as described earlier, and an empty plasmid was injected as the control. RT-PCR and quantitative real-time PCR were performed 24 h post-microinjection with 5–10 replicates (each containing the extracts from 15–30 embryos) for the zebrafish IFN₁ gene. The results are displayed relative to the corresponding GAPDH values to generate the IFN₁ gene copy number. Western blot analysis was performed to determine the expression of zebrafish TRIF, MAVS, and PIAS4a. Furthermore, the effects of PIAS4a on TRIF- and MAVS-activated IFN induction were also determined via mRNA- and MO-mediated overexpression and knockdown experiments. For this, EGFP-fused TRIF- and MAVS-encoding plasmids alone or with capped PIAS4a mRNA or PIAS4a MO were injected into one-cell-stage embryos, with the empty plasmid as the control. Developing embryos (24 hpf) with strong and homogenous EGFP expression were used for analysis. RT-PCR and quantitative real-time PCR were performed for the four IFN reporter genes (ISG15, viperin, PKZ, and CD40) as described earlier.

FIGURE 1. Nucleotide sequence of the zfPIAS4a gene and the deduced amino acid sequence. The asterisk represents the stop codon, and cysteines are encircled. In the 3'-UTR, five RNA instability motifs (ATTTA) are shown in boldface italic, and three poly(A) signals (AATAAA and ATTAAA) are boldface and underlined.
Statistical analysis

Data from three independent experiments were expressed as mean ± SD, and the groups were compared using Student t test for paired samples. The *p values *p < 0.05 and **p < 0.01 were considered statistically significant.

Results

Characterization of zfPIAS4 gene

The cloned full-length zfPIAS4a cDNA consisted of 3698 bp with a 37-bp 5’-UTR, a 1515-bp ORF encoding a predicted 505-aa polypeptide, and a 2146-bp 3’-UTR that contained five RNA instability motifs (ATTTTA) and three polyadenylation signal (AATAAA and ATTAAA) nucleotides upstream of the poly(A) tail (Fig. 1). Comparative analysis of the zfPIAS4a cDNA sequence and the corresponding genomic sequence characterized the organization of the zfPIAS4a gene (Fig. 2). The zfPIAS4a gene was located within a 12.8-kb genomic fragment on chromosome 22, which seems slightly different from the locations of the human PIAS4 (hPIAS4) gene at chromosome 19 and mouse PIAS4 at chromosome 10. The genes adjacent to the zfPIAS4a locus were retrieved using Genscan and BLAST. With overall conservation among zebrafish and other vertebrate species, most genes adjacent to the zfPIAS4a locus, such as the OAZ1, ATP8b3, ONECUT3, MAP2K2, ZbTB7a, and EEF2, were found to be clustered in zebrafish chromosome 19 and mouse PIAS4 at chromosome 10. The genes adjacent to the zfPIAS4a locus were retrieved using Genscan and BLAST. With overall conservation among zebrafish and other vertebrate species, most genes adjacent to the zfPIAS4a locus, such as the OAZ1, ATP8b3, ONECUT3, MAP2K2, ZbTB7a, and EEF2, were found to be clustered in zebrafish chromosome 22. However, the synteny and organization of these genes were somewhat disordered among different species (even between human and mouse) because transversion of these gene loci usually occurs in different species (Fig. 3). The PIAS4 genes were also predicted in several other fish species, including stickleback (G. aculeatus), medaka (O. latipes), fugu (T. rubripes), and tetraodon (T. nigroviridis), from the EST/genome databases using the zfPIAS4a sequence as a probe. The genomic structure of the zfPIAS4a gene consisted of 11 exons and 10 introns, and it is similar to that of many other species, including human, mouse, rat, and X. tropicalis models, and especially consistent with the predicted PIAS4 of stickleback, medaka, fugu, and tetraodon (Fig. 2). In addition, the chromosomal synteny of the PIAS4 genes among different fish species, such as zebrafish and tetraodon, was well conserved (Fig. 3). Furthermore, another PIAS4-like gene (named PIAS4b, located on chromosome 2), which encodes two alternative splicing isoforms (GenBank: NP_956637.2 and NP_001229871.1), was detected in zebrafish. PIAS4b also shows a conserved synteny with mammalian PIAS4 genes, which suggests that this gene might have arisen from the extra genome duplication in teleosts. However, PIAS4b has lower similarity to PIAS4 sequences from other species compared with PIAS4a. This suggests a functional divergence of PIAS4b, whereas PIAS4a can be expected to retain the ancestral function of their common ancestor, as often observed with pairs of paralogues in teleosts (Table I).

Characterization of zfPIAS4a protein

The mature zfPIAS4a protein consists of 505 aa with a predicted molecular mass of 55.8 kDa. SMART, PROSITE, and multiple sequence alignment analyses show that zfPIAS4a contains various functional domains and motifs typically seen in the PIAS protein family, including a SAP box (35 aa), a PINIT domain (163 aa), an RLD (78 aa), an N-terminal LXXLL signature motif in the poly(A) tail (Fig. 1). Comparative analysis of the zfPIAS4a cDNA sequence and the corresponding genomic sequence characterized the organization of the zfPIAS4a gene (Fig. 2). The zfPIAS4a gene was located within a 12.8-kb genomic fragment on chromosome 22, which seems slightly different from the locations of the human PIAS4 (hPIAS4) gene at chromosome 19 and mouse PIAS4 at chromosome 10. The genes adjacent to the zfPIAS4a locus were retrieved using Genscan and BLAST. With overall conservation among zebrafish and other vertebrate species, most genes adjacent to the zfPIAS4a locus, such as the OAZ1, ATP8b3, ONECUT3, MAP2K2, ZbTB7a, and EEF2, were found to be clustered in zebrafish chromosome 22. However, the synteny and organization of these genes were somewhat disordered among different species (even between human and mouse) because transversion of these gene loci usually occurs in different species (Fig. 3). The PIAS4 genes were also predicted in several other fish species, including stickleback (G. aculeatus), medaka (O. latipes), fugu (T. rubripes), and tetraodon (T. nigroviridis), from the EST/genome databases using the zfPIAS4a sequence as a probe. The genomic structure of the zfPIAS4a gene consisted of 11 exons and 10 introns, and it is similar to that of many other species, including human, mouse, rat, and X. tropicalis models, and especially consistent with the predicted PIAS4 of stickleback, medaka, fugu, and tetraodon (Fig. 2). In addition, the chromosomal synteny of the PIAS4 genes among different fish species, such as zebrafish and tetraodon, was well conserved (Fig. 3). Furthermore, another PIAS4-like gene (named PIAS4b, located on chromosome 2), which encodes two alternative splicing isoforms (GenBank: NP_956637.2 and NP_001229871.1), was detected in zebrafish. PIAS4b also shows a conserved synteny with mammalian PIAS4 genes, which suggests that this gene might have arisen from the extra genome duplication in teleosts. However, PIAS4b has lower similarity to PIAS4 sequences from other species compared with PIAS4a. This suggests a functional divergence of PIAS4b, whereas PIAS4a can be expected to retain the ancestral function of their common ancestor, as often observed with pairs of paralogues in teleosts (Table I).

Characterization of zfPIAS4a protein

The mature zfPIAS4a protein consists of 505 aa with a predicted molecular mass of 55.8 kDa. SMART, PROSITE, and multiple sequence alignment analyses show that zfPIAS4a contains various functional domains and motifs typically seen in the PIAS protein family, including a SAP box (35 aa), a PINIT domain (163 aa), an RLD (78 aa), an N-terminal LXXLL signature motif in the poly(A) tail (Fig. 1). Comparative analysis of the zfPIAS4a cDNA sequence and the corresponding genomic sequence characterized the organization of the zfPIAS4a gene (Fig. 2). The zfPIAS4a gene was located within a 12.8-kb genomic fragment on chromosome 22, which seems slightly different from the locations of the human PIAS4 (hPIAS4) gene at chromosome 19 and mouse PIAS4 at chromosome 10. The genes adjacent to the zfPIAS4a locus were retrieved using Genscan and BLAST. With overall conservation among zebrafish and other vertebrate species, most genes adjacent to the zfPIAS4a locus, such as the OAZ1, ATP8b3, ONECUT3, MAP2K2, ZbTB7a, and EEF2, were found to be clustered in zebrafish chromosome 22. However, the synteny and organization of these genes were somewhat disordered among different species (even between human and mouse) because transversion of these gene loci usually occurs in different species (Fig. 3). The PIAS4 genes were also predicted in several other fish species, including stickleback (G. aculeatus), medaka (O. latipes), fugu (T. rubripes), and tetraodon (T. nigroviridis), from the EST/genome databases using the zfPIAS4a sequence as a probe. The genomic structure of the zfPIAS4a gene consisted of 11 exons and 10 introns, and it is similar to that of many other species, including human, mouse, rat, and X. tropicalis models, and especially consistent with the predicted PIAS4 of stickleback, medaka, fugu, and tetraodon (Fig. 2). In addition, the chromosomal synteny of the PIAS4 genes among different fish species, such as zebrafish and tetraodon, was well conserved (Fig. 3). Furthermore, another PIAS4-like gene (named PIAS4b, located on chromosome 2), which encodes two alternative splicing isoforms (GenBank: NP_956637.2 and NP_001229871.1), was detected in zebrafish. PIAS4b also shows a conserved synteny with mammalian PIAS4 genes, which suggests that this gene might have arisen from the extra genome duplication in teleosts. However, PIAS4b has lower similarity to PIAS4 sequences from other species compared with PIAS4a. This suggests a functional divergence of PIAS4b, whereas PIAS4a can be expected to retain the ancestral function of their common ancestor, as often observed with pairs of paralogues in teleosts (Table I).
are similar to the values for the PIAS4 proteins of human and some other vertebrates (Table II). With the N-terminal segment (1–65 aa containing the complete SAP box) of human PIAS1 (hPIAS1) protein as a template, the three-dimensional structure of the zfPIAS4a N-terminal segment (3–66 aa) was modeled. As shown in the ribbon diagram in Fig. 5B–E, the N terminus of zfPIAS4a exhibits an overall tertiary structure similar to the hPIAS1 N terminus (55). They both adopt a unique four-helix bundle with an up–down–extended loop–down–up topology (α1 to α4 from the N terminus to the C terminus) wherein much of the α1, α2, and α3 helix–extended loop–helix represented the SAP box. Functionally, the hPIAS1 N terminus was found to play a crucial role in recog-

dues in the RLD (indicated by triangles), crucial for binding to Ubc9 and sumoylation, are also present in zfPIAS4a and are completely conserved in different species throughout evolution (53). In addition, the lysine residue in the ΨKXD/E sequence within the SAP box, which is considered a major sumoylation site, is also completely conserved (54). However, the Ser–Thr–rich domain, which is present in other PIAS proteins, was not found in the zfPIAS4a molecule. The isoelectric point (pI) values of the three conserved domains in zfPIAS4a, namely, the SAP box, PINIT domain, and RLD, were predicted to be 11.57, 8.93, and 7.0, respectively, which are similar to the values for the PIAS4 proteins of human and some

<table>
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<th>Primer Name</th>
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<th>Use</th>
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<td>Gene cloning</td>
</tr>
<tr>
<td>zfPIAS4a R1</td>
<td>CTCCTCTCTCTGAGT</td>
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</tr>
<tr>
<td>zfPIAS4a F2</td>
<td>ATTAAGCTTGGCGCGAACGACTG</td>
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</tr>
<tr>
<td>zfPIAS4a R2</td>
<td>ATTCGAGGCATGGGCTAATG</td>
<td>Gene expression</td>
</tr>
<tr>
<td>zfPIAS4a F3</td>
<td>GGAGCGCGAGGAACAGCG</td>
<td>3′-RACE</td>
</tr>
<tr>
<td>zfPIAS4a R4</td>
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<td>3′-RACE</td>
</tr>
<tr>
<td>zTRIF F1</td>
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<tr>
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<tr>
<td>zTRIF F3</td>
<td>ATTAAGCTTGGCGCGAACGACTG</td>
<td>Gene expression</td>
</tr>
</tbody>
</table>

Table I. Primers used for amplifying cDNAs and gene expression analysis
Recognizing both tumor suppressor p53 and adenine–thymine–rich DNA, hPIAS4 was also found to interact with p53 and DNA (19, 56), suggesting that a conserved structural and functional characterization is present in the N terminus (including the SAP box) among the hPIAS1, hPIAS4, and zfPIAS4a proteins. Further functional comparative studies are needed to validate this hypothesis.

Multiple sequence alignment and phylogenetic analysis

The amino acid sequence multiple alignment of the PIAS4 family was prepared encompassing representatives of various species, including humans, mouse, rats, cattle, X. tropicalis, zebrafish, and four other teleost fish. The fish PIAS4 shared an overall sequence identity with other species (Fig. 4). As shown in Table III, zfPIAS4a had an overall 55.4–85.9% amino acid identity with humans and other vertebrates. In general, the homology was much higher between fish species than between fish and mammals. For example, PIAS4 demonstrated 96.5% identity between Tetraodon and fugu, 92.4% identity between fugu and stickleback, 92.4% identity between Tetraodon and stickleback, and 91% identity between stickleback and medaka. These indicate the existence of

![Multiple alignment of zfPIAS4a with other homologues. Residues shaded in black are completely conserved across all the species aligned, whereas residues shaded in gray are similar with respect to side chains. The dashes in the amino acid sequences indicate gaps introduced to maximize alignment. The four conserved domains (SAP box, PINIT domain, RLD, and AD) are indicated above the alignment. The LXXLL signature motif, the ΨKXD/E consensus sequence, and the PINIT motif are boxed. Triangles indicate the two important cysteine residues in the RLD.](attachment://Figure4.png)
a closer genetic relationship between fish PIAS4 proteins than in other classes. In addition, a structure-based sequence alignment analysis of the functional domains (SAP, PINIT domain, and RLD) and the four-helix-containing N-terminal segment between PIAS4 and other PIAS proteins was conducted. The zfPIAS4a SAP box, PINIT domain, and RLD share high sequence identities with those in other PIAS4 proteins (Table IV), which suggest a conserved structural and functional characterization of these domains between different PIAS4 proteins. Meanwhile, the zfPIAS4a N-terminal four-helix domains also show sequence identity with those in hPIAS1 and hPIAS4 proteins (Fig. 5F). Furthermore, the zfPIAS4a SAP box, PINIT domain, and RLD also showed sequence identity with those in other human PIAS proteins. For example, the zfPIAS4a SAP box, PINIT domain, and RLD share 52.8%, 44.7%, and 75.9% sequence identity with those in the hPIAS1 molecule and 58.3%, 44.4%, and 77.2% sequence identity with those in human PIAS3, respectively. These data demonstrate that the PIAS4 proteins share partially conserved structural characterizations between different PIAS proteins, suggesting the presence of a close evolutionary relationship within PIAS family members. A phylogenetic tree was constructed using the neighbor-joining method, which included the PIAS1, PIAS2, PIAS3, and PIAS4 member proteins (Fig. 6). Fish PIAS4s not only clustered together to form an exclusive group but also merged with mammal PIAS4s into a larger group with high bootstrap values. These findings indicate that the evolutionary trend of PIAS4 is in accordance with that of the species.

Table II. The pI values of the three conserved domains in zfPIAS4 and some other PIAS4 proteins

<table>
<thead>
<tr>
<th>Species</th>
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<th>RLD</th>
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<tr>
<td>Rat</td>
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<td>9.36</td>
<td>6.99</td>
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<td>X. tropicalis</td>
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<td>Zebrafish 4a</td>
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</tr>
<tr>
<td>Zebrafish 4b</td>
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</table>

Tissue distribution and expression analysis of zfPIAS4

The mRNA levels in various embryonic developmental stages and in different tissues from poly(I:C)-stimulated, zebrafish IFN-γ-stimulated, and the control fish were assayed using RT-PCR and quantitative real-time PCR to show the expression pattern of PIAS4 in zebrafish. The zfPIAS4a transcripts were detected during the early development of embryos from 6 to 120 hpf, and zfPIAS4a expression decreased from the peak level at 6 hpf to the lowest level at 48 hpf, with persistently low expression from 48 to 120 hpf, and remained stable thereafter (Fig. 7A). In adult fish, the zfPIAS4a transcripts can be detected in all selectively examined tissues (namely, heart, spleen, liver, intestine, kidney, gill, brain, skin, and muscle), and they were expressed at relatively higher levels in the spleen, liver, gill, and brain (Fig. 7B). After receiving i.p. injections of poly(I:C) (10 μg/fish) for 12 h, the zfPIAS4a expression was significantly (p < 0.05) induced in most immune-related tissues examined, including the spleen, liver, intestines, kidneys, and gills (Fig. 7B). The zfPIAS4b transcripts can also be detected in all selectively examined tissues (Supplemental Fig. 1). Zebrafish IFN-γ1 was expressed in HEK293 cells to investigate whether zfPIAS4a expression can be induced by IFN. Western blot analysis shows that the IFN-γ1 recombinant protein is satisfactorily expressed and efficiently secreted into the cultural supernatant liquid (Fig. 7C). After receiving i.p. injections of this supernatant liquid (10 μl/fish), the zfPIAS4a transcripts were significantly (p < 0.05) upregulated in the spleen, liver, intestine, and kidney, albeit at different time points (Fig. 7D–G), which was similar to that of the classical IFN-stimulated gene (viperin) in control groups (Supplemental Fig. 2). These observations revealed that zfPIAS4a is an ISG and that it may play an important role in dsRNA- and IFN-γ1-induced immune responses.

Nuclear localization of zfPIAS4a

A DsRed2-fused construct (pPIAS4a-WT) with the full-length zfPIAS4a molecule was transfected into HEK293 cells to investigate the subcellular localization of zfPIAS4a. The overexpressed zfPIAS4a fusion protein displayed dot-like signatures in the intracellular region, which completely merged with the DAPI-stained nucleus, suggesting that zfPIAS4a is exclusively localized in the...
nucleus (Fig. 8B), whereas the overexpressed pDsRed2-C1 empty control plasmid showed a smear-like distribution in the cell without any nuclear dot-like localization signals (Fig. 8A). A series of the DsRed2-fused constructs (pPIAS4a-ΔSAP, pPIAS4a-ΔPINIT, and pPIAS4a-ΔRLD) encoding mutants with deletions of conserved domains, including the SAP box, PINIT domain, RLD, as well as N- and C-terminal domains, was transfected into HEK293 cells to analyze which domain in the molecule harbors the nuclear localization signals (NLSs). The overexpressed fusion mutants altered with the SAP box, PINIT domain, or RLD were still localized to the nucleus (Fig. 8C–E). These observations indicate that these three conserved domains might not be essential for the nuclear localization of zfPIAS4a; a DsRed2-fused construct with the PINIT domain alone was selectively constructed to verify this. As anticipated, the location of the PINIT domain-containing protein segment was no longer restricted to the nucleus (Fig. 8F). Similarly, two N-terminal deletion mutants (pPIAS4a-ΔN1 and pPIAS4a-ΔN2) encoding different lengths of C-terminal segments (265–505 and 374–505 aa) still nuclear-localized as the wild-type proteins (Fig. 8H, 8I). In contrast, the C-terminal deletion mutant pPIAS4a-ΔC1 encoding N-terminal segments (1–103 aa) appeared as considerable dot-like signals outside but near the nucleus (Fig. 8G), and the DsRed2 that fused the entire PIAS4a-ΔC2 sequence (1–373 aa) was no longer restricted to the nucleus (Fig. 8J), suggesting that the C terminus of zfPIAS4a (374–505 aa) harbors NLSs, which are responsible for strict nuclear localization.

Effect of morpholino oligonucleotide

To test the efficacy of the MO against PIAS4a, an RFP-encoding construct containing the MO target sequence upstream of RFP was generated. This RFP-MO target sequence fusion construct and the fusion plasmid without the corresponding MO produced strong green or red fluorescence in the injected embryos, respectively, whereas the red fluorescence was effectively inhibited by the corresponding MO. This suggests that the MO against PIAS4a efficiently blocks translation (Fig. 9).

Table IV. Pairwise identity of the three conserved domains in zfPIAS4a with other PIAS4 proteins

<table>
<thead>
<tr>
<th>Species</th>
<th>Human</th>
<th>Mouse</th>
<th>Rat</th>
<th>Cow</th>
<th>X. tropicalis</th>
<th>Fugu</th>
<th>Medaka</th>
<th>Stickelback</th>
<th>Tetraodon</th>
<th>Zebrasfish 4a</th>
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<td>Zebrafish PIAS4a</td>
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<td>98.7</td>
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The accession numbers are provided in the legend to Fig. 5.

zfPIAS4a plays a negative role in TRIF-induced NF-κB activation

In mammals, TRIF is the common adapter in the TLR3 and TLR4 signaling pathways. Overexpression of this adapter leads to the activation of NF-κB signaling, and PIAS4 represses this process (8). The zebrafish pcDNA6-TRIF was constructed and coexpressed with the NF-κB reporter gene into zebrafish embryos to investigate whether zfPIAS4a plays a similar role in TRIF-induced NF-κB activation. Luciferase activity was normalized to pRL-TK activity and expressed as the fold stimulation relative to the negative control (pcDNA6/myc-HisB empty plasmid administered group). Western blot analysis demonstrated that both the overexpressed TRIF and the zfPIAS4a proteins can be detected 24 h postinjection (Fig. 10A, lower panel). Overexpression of the TRIF protein significantly (p < 0.05) activates the NF-κB reporter gene, and the TRIF-induced NF-κB activation is dramatically inhibited (p < 0.05) by zfPIAS4a administration in a dose-dependent manner (Fig. 10A, upper panel). zfPIAS4a thus functions as a negative regulator in TRIF-induced NF-κB activation.

zfPIAS4a plays a negative role in TRIF-mediated IFN-κ1 induction

The observation that zfPIAS4a suppresses TRIF-induced NF-κB activation strongly suggests that this molecule plays an important role in the regulation of proinflammatory cytokine expression. The role zfPIAS4a plays in TRIF-induced IFN production was investigated to clarify this implication. For this purpose, the pcDNA6-TRIF was injected alone or with the pcDNA6-zfPIAS4a into one-cell-stage embryos. An empty control plasmid was added to ensure that the same amount of total DNA was received. Quantitative real-time PCR was conducted to evaluate IFN-κ1 expression at the mRNA level (as determined by IFN-κ1/GAPDH). After pcDNA6-TRIF was administered alone for 24 h, the IFN-κ1 expression level was dramatically upregulated compared with that of the mock plasmid-injected control, suggesting that the zebrafish TRIF induces IFN-κ1 expression. In parallel, after pcDNA6-TRIF and pcDNA6-zfPIAS4a were codispensed for 24 h, the IFN-κ1 expression level was dramatically repressed compared with that of
the TRIF-injected control (Fig. 10B, upper panel). Western blot analysis revealed that the expression of both TRIF and PIAS4a can be detected at this time point (Fig. 10B, lower panel). This result initially indicates that zPIAS4a plays a negative role in TRIF-mediated IFN-γ induction. To verify this conclusion, several further improved protocols were used, including mRNA- and MO-mediated overexpression and knockdown of zPIAS4a, and the EGFP-fused TRIF expression, allowing the screening of embryos with homogenous TRIF protein expression, thereby ensuring the experiments become more stable. The results show that the EGFP-fused TRIF also significantly induced IFN-γ expression, which could be dramatically repressed by PIAS4a mRNA administration. In contrast, PIAS4a knockdown in embryos by MO significantly upregulates IFN-γ expression (Fig. 10C). Taken together, these results clearly demonstrate that zPIAS4a plays a negative role in TRIF-mediated IFN-γ induction. In these experiments, no apparent developmental defects were observed (data not shown).

*zPIAS4a plays a negative role in MAVS-mediated IFN-γ induction*

MAVS is an important adapter protein in the RIG-I/MDA5-mediated pathway; it directly activates the IRF-3/7–dependent IFN-γ pathway. MAVS is an important adapter protein in the RIG-I/MDA5-mediated pathway; it directly activates the IRF-3/7–dependent induction. To verify this conclusion, several further improved protocols were used, including mRNA- and MO-mediated overexpression and knockdown of zPIAS4a, and the EGFP-fused TRIF expression, allowing the screening of embryos with homogenous TRIF protein expression, thereby ensuring the experiments become more stable. The results show that the EGFP-fused TRIF also significantly induced IFN-γ expression, which could be dramatically repressed by PIAS4a mRNA administration. In contrast, PIAS4a knockdown in embryos by MO significantly upregulates IFN-γ expression (Fig. 10C). Taken together, these results clearly demonstrate that zPIAS4a plays a negative role in TRIF-mediated IFN-γ induction. In these experiments, no apparent developmental defects were observed (data not shown).

*zPIAS4a downregulates IFN signaling*

The zebrafish MxA promoter sequence was cloned, and a zebrafish MxA reporter gene was constructed to investigate the role of zPIAS4a in the IFN signaling pathway. The MxA reporter gene and indicated amounts of IFN-γ and zPIAS4a expression plasmid

---

**FIGURE 7.** Quantitative PCR analysis of zfPIAS4a transcripts in embryos at different developmental stages and various adult zebrafish tissues. (A) Quantification of zfPIAS4a transcripts at the embryonic stage of 6, 12, 24, 36, 48, 72, 96, and 120 hpf against GAPDH. Values are mean ± SD. The relative expression value was averaged from three duplicates (each containing 10–30 embryos). (B) Relative gene expression of PIAS4a in various adult tissues (heart, spleen, liver, intestine, kidney, gill, brain, skin, and muscle) that were stimulated with poly(I:C); healthy fish were used as the control. The relative expression value was averaged from three duplicates, each of which contains four fish. Values are mean ± SD. (C) HEK293 cells were transfected with the pcDNA6-zfIFN-γ expression construct or an empty control plasmid. Approximately 32 h after transfection, the supernatant liquids were collected, part of which was used for Western blot analysis using the His mAb; the rest was used for zebrafish i.p. injection (10 μl/fish), and the zPIAS4a transcript levels were determined using real-time PCR in the (D) spleen, (E) liver, (F) intestines, and (G) kidneys. The gene expression is normalized against β-actin and presented as mean ± SD of four fish. *p < 0.05, **p < 0.01 (t test). The groups without asterisks did not show statistically significant differences with control supernatant-injected fish.
were coinjected into the embryos. After 24 h, the embryos were harvested and lysed for the luciferase assay. Alternatively, the embryos injected with indicated amounts of pcDNA3.1-IFNw1 and pcDNA6-zfPIAS4a were collected for RT-PCR and quantitative real-time PCR analyses. The luciferase assay demonstrated that the MxA reporter gene is significantly stimulated (p, 0.05) in IFNw1-treated embryos compared with the empty plasmid-injected embryos, and after IFNw1 and zfPIAS4a were coadministered, the stimulation of the MxA reporter gene in the embryos was dramatically downregulated compared with that in the IFNw1-stimulated control embryos. Furthermore, the inhibitory effect of zfPIAS4a on the MxA reporter activation was found to be dose dependent (Fig. 12A, upper panel). Western blot analysis demonstrated that both the overexpressed IFNw1 and the zfPIAS4a proteins were detectable 24 h postinjection (Fig. 12A, lower panel). Similarly, the expression of the two IFN-inducible genes viperin and PKZ were significantly stimulated in the IFNw1-treated embryos compared with those in the control embryos. After IFNw1 and zfPIAS4a were coadministered, however, the expression of viperin and PKZ genes in the embryos were dramatically downregulated compared with those in the IFNw1-stimulated control embryos [Fig. 12B (upper panel), 12C]. Western blot analysis was simultaneously performed to determine the expression of IFNw1 and PIAS4a in the embryos (Fig. 12B, lower panel). These suggest that zebrafish IFNw1 shares a signaling pathway similar to that of the human IFN system. Accordingly, the mRNA- and MO-mediated overexpression and zfPIAS4a knockdown were also conducted, and four ISGs (ISG15, viperin, PKZ, and CD40) were analyzed in these experiments. As expected, with IFNw1 and zfPIAS4a mRNA administration to the embryos, the four ISGs became significantly downregulated. In contrast, zfPIAS4a knockdown dramatically upregulated the expression of the ISGs (Fig. 12D–G). Therefore, zfPIAS4a also plays a negative role in the IFNw1 signaling pathway. Similarly, no any apparent developmental defects were observed in the experiments (data not shown).

Discussion

Cytokines use complex signaling cascades to elicit their biological effects. Rapid and efficient attenuation of cytokine signals is crucial in maintaining the homeostasis of immunity and preventing toxic side effects. The PIAS proteins were originally identified as inhibitors of cytokine signaling mediated by STAT family members, and they have since been found to regulate the functions of different proteins, many of which are transcription factors that function in various signaling pathways. Based on this, we hypothesized that a PIAS family member may exist in fish according to its crucial role in immune regulation. In the current study, a PIAS4 homologue (zfPIAS4a) from the zebrafish model that shares many conserved structural hallmarks with the human and mammal PIAS4 proteins was successfully identified. These include similarities in their chromosomal synteny and location, exon/intron organization, sequence identities, and conserved functional domains and motifs, such as the SAP box, the PINIT domain, the RLD, and the AD in the C-terminal region. These observations

FIGURE 8. Localizations of the wild-type and truncated forms of zfPIAS4a in HEK293 cells. In total, 2 x 10^5 HEK293 cells were seeded onto coverslips in 24-well plates 1 d before transfection. Cells were transfected with the red-fused wild type, the nine truncated forms of zfPIAS4a, or the empty control plasmids. After 24 h, fixed cells on the coverslips were stained with DAPI. The images were obtained by fluorescent microscopy. Original magnification ×400. (A)–(J) are the images we obtained after transfecting the cells with the plasmids indicated on the left side of the images, respectively.

FIGURE 9. Control experiments to verify the morpholino-mediated knockdown. Bright field, RFP, and GFP fluorescence micrographs of embryos at 24 hpf. The zebrafish embryos were injected with EGFP-N1 vector, PIAS4a 5′-UTR-RFP plasmid alone (upper panels), or together with a morpholino specific for the zfPIAS4a translation start site (lower panels). Original magnification ×20.
proven that zfPIAS4a is homologous to the PIAS proteins of other vertebrates and suggest that it has been conserved from fish to mammals during vertebrate evolution.

Functionally, the expression of zfPIAS4a can be dramatically induced in most immune-relevant tissues through stimulation with poly(I:C), an analogue of dsRNA polymers commonly used for the induction of type I IFN and proinflammatory cytokines. In addition, it can be induced with recombinant zebrafish IFNγ-1 in selected tissues. These suggest that zfPIAS4a is an ISG and provide initial insights that this molecule might be involved in the feedback regulation of IFN-mediated immunity or inflammatory responses. In humans and mammals, the primary sense dsRNA is mediated by innate pattern recognition receptors, which include TLR3 and RIG-I/MDA5-like receptors at the very least (21). In response to dsRNA stimulation, the TLR3 recruits the adapter protein TRIF, activates NF-κB signaling, and induces IRF-3 activation, followed by IFN-β induction. Meanwhile, RIG-I/MDA5 also triggers IRF-3 activation, followed by type I IFN induction through the adapter protein MAVS. Therefore, the TRIF- and MAVS-mediated pathways are critical for the induction and signaling of IFN and other proinflammatory cytokines (57, 58). The TRIF-mediated pathway has been found to be controlled by various negative regulators, such as sterile alpha and TIR-motif-containing 1 (59), SH2-containing protein tyrosine phosphatase 2 (60), PI3K (61), and PIAS4. However, the regulation of the MAVS-mediated pathway remains poorly understood. In addition, the TLR3 and RIG-I/MDA5 receptor-linked TRIF and MAVS cascades are also identified to play crucial roles in dsRNA sensors, as well as IFN-dependent and NF-κB-dependent signaling pathways in teleost fish, similar to those seen in humans and mammals (47, 62, 63). Nevertheless, the regulatory mechanisms underlying these pathways in fish species is still unclear. The current study provides preliminary evidence that zfPIAS4a is an important negative regulator in both TRIF- and MAVS-mediated pathways in fish and that it plays a negative role in the IFN signaling pathway. These results demonstrate that zfPIAS4a is a multifunctional regulator involved in various pathways and is particularly important for IFN-inducing and IFN signaling networks. Notably, zfPIAS4a participates in the MAVS-mediated pathway. This is the first report to show that a PIAS family member acts as a negative regulator in the MAVS signaling pathway, which is beneficial to understanding the regulatory mechanism underlying the MAVS pathway not only in fish but also in mammals. Moreover, hPIAS4 has been shown to

**FIGURE 10.** PIAS4a negatively regulates the TRIF-dependent pathway. (A) One-cell-stage zebrafish embryos were injected with NF-κB luciferase reporter plasmids, TRIF vectors, and indicated amounts of PIAS4a expression plasmids. The pRL-TK renilla luciferase reporter plasmids were used as the internal control. Empty control plasmids were added to ensure the same amounts of total DNA. The firefly and renilla luciferase activities were assayed 24 h post-microinjection according to the manufacturer’s instructions with 5–10 replicates (each containing the extracts from 50–80 embryos). Luciferase activity was normalized to pRL-TK activity and expressed as the fold stimulation relative to control. Values represent mean ± SD. Western blot analysis was performed to determine the expression of zebrafish TRIF and PIAS4a (lower panel). (B) One-cell-stage embryos were injected with pcDNA6-TRIF (40 pg/embryo) alone or with the pcDNA6-zfPIAS4a (40 pg/embryo) into their cytoplasm, and empty plasmids (pcDNA6-mycHiB B) were injected as the control. The figure shows the levels of the zfIFNγ1 mRNA relative to GAPDH expression, measured using quantitative RT-PCR from whole embryos 24 h after injection with 5–10 replicates (each containing 15–30 embryos) (upper panel). Values represent mean ± SD. Western blot analysis was performed to determine the expression of zebrafish TRIF and PIAS4a (lower panel). (C) One-cell-stage embryos were injected with EGFP-TRIF expression plasmid alone (50 pg/embryo) or with capped PIAS4a RNA (200 pg/embryo) or MO against PIAS4a (4 ng/embryo), and empty plasmids (pEGFP-N1) were injected as the control. The figure shows the zfIFNγ1 mRNA levels relative to GAPDH expression, measured using quantitative RT-PCR from whole embryos 24 h after injection with 5–10 replicates (each containing 15–30 embryos). Values represent mean ± SD. *p < 0.05. **p < 0.01.

**FIGURE 11.** Effects of zfPIAS4a on MAVS upregulated zfIFNγ1. (A) pcDNA6-MAVS (40 pg/embryo) alone or with pcDNA6-zfPIAS4a (40 pg/embryo) was injected into one-cell-stage embryos, and empty plasmids (pcDNA6-mycHiB B) were injected as the control. Quantitative RT-PCR was performed 24 h later with 5–10 replicates (each containing 15–30 embryos) for zfIFNγ1 mRNA. Values represent mean ± SD. Western blot analysis was performed to determine the expression of zebrafish TRIF and PIAS4a (lower panel). (B) EGFP-MAVS vector (80 pg/embryo) alone or with capped PIAS4a RNA (200 pg/embryo) or MO against PIAS4a (4 ng/embryo) was injected into one-cell-stage embryos, and empty plasmids (pEGFP-N1) were injected as the control. Quantitative RT-PCR was performed 24 h later with 5–10 replicates (each containing 15–30 embryos) for zfIFNγ1 mRNA. Values represent mean ± SD. *p < 0.05. **p < 0.01.
function as a SUMO ligase, and the SAP box, PINIT domain, and RLD are found to be essential to its molecular functionality in sequence- and structure-specific DNA binding, nuclear retention, and SUMO E3 ligase activity, respectively (14, 19, 64). Further studies are needed to clarify whether these domains in the zfPIAS4a molecule have similar functions and to advance the current understanding of the molecular and functional evolution of the PIAS family.

Specific intracellular localization is essential for proper protein functions in signaling pathways. Although there are examples of cytoplasmic regulation in which PIAS proteins are involved, most of the PIAS protein interactions reported to date occur with transcription factors or other proteins linked to nuclear regulation in mammals (65, 66). Further studies are needed to clarify whether these domains in the zfPIAS4a molecule have similar functions and to advance the current understanding of the molecular and functional evolution of the PIAS family.

Figure 12. PIAS4a negatively regulates the IFN-γ-dependent pathway. (A) One-cell-stage zebrafish embryos were injected with zfMxA-pro-luc vectors and indicated amounts of pcDNA3.1-zfIFNγ1 and pcDNA6-PIAS4a expression plasmids; the pRL-TK renilla luciferase reporter plasmids were used as the internal control. Empty control plasmids were added to ensure the same amounts of total DNA. The firefly and renilla luciferase activities were assayed 24 h post-microinjection according to the manufacturer’s instructions with 5–10 replicates (each containing the extracts from 50–80 embryos). Luciferase activity was normalized to pRL-TK activity and expressed as the fold stimulation relative to control. Values represent mean ± SD (upper panel). Western blot analysis was performed to determine the expression of zebrafish IFNγ1 and zfPIAS4a (lower panel). (B and C) Effects of zfPIAS4a on zfIFNγ1 upregulated ISGs: (B) viperin and (C) PKZ. pcDNA3.1-zfIFNγ1 (40 pg/embryo) was injected alone or with pcDNA6-PIAS4a (40 pg/embryo) into the cytoplasm of one-cell-stage embryos, empty plasmids (pcDNA6-mycHis B) were injected as the controls, and quantitative RT-PCR was performed 24 h later with 5–10 replicates (each containing 15–30 embryos). The levels of viperin and PKZ mRNA relative to GAPDH expression are shown. Values represent mean ± SD (upper panel). Western blot analysis was performed to determine the expression of zebrafish IFNγ1 and zfPIAS4a using the His mAb (lower panel). (D–G) Further verification of the effects of zfPIAS4a on zfIFNγ1-upregulated ISGs: (D) ISG15, (E) viperin, (F) PKZ, and (G) CD40. EGFP-IFNγ1 vector (50 pg/embryo) was injected alone or with capped PIAS4a RNA (200 pg/embryo) or MO against PIAS4a (4 ng/embryo) into one-cell-stage embryos, empty plasmids (pEGFP-N1) were injected as the controls, and quantitative RT-PCR was performed 24 h later with 5–10 replicates (each containing 15–30 embryos). The levels of ISG15, viperin, PKZ, and CD40 mRNA relative to GAPDH expression are shown. Values represent mean ± SD. *p < 0.05, **p < 0.01.
nuclear localization. This result is generally consistent with that obtained for the hPIAS4 protein, suggesting that PIAS4 family members share a conserved subcellular localization mechanism from fish to mammals. Further studies are nevertheless needed to determine the exact amino acids that compose the NLSs of this 374–505 aa C-terminal segment.

IFNs are important members of the innate immune system that play critical roles in both innate and acquired immunity. Teleost IFNs were recently discovered in zebrafish, and they have since been found in many fish species (70–72). The predicted protein sequences of fish IFNs show low (<20%) similarity to mammalian and avian type I IFNs. Significant differences between fish and higher vertebrate IFNs, such as structural features, gene organizations, and type of receptors, were also observed. However, the fish IFNs share functional characterizations similar to those of mammals. For example, they upregulate the activity of MxA, PKZ, virepin, stat1, and IL-1b (45). Fish and mammals have gene orthologs among JAK/STAT family members and a number of IFN-induced proteins. Previous data and the current results suggest that the IFN-activated signaling pathways and the key regulators in the IFN signaling network originated from fish, and they were conserved from teleosts to mammals during vertebrate evolution. In addition, the fact that PIAS4 is present in fish suggests that this molecule is a primitive member of the PIAS family. However, further investigations are still needed to clarify the exact evolutionary relationship among the family members.

In summary, this study has pioneered the identification of a PIAS family member from teleost fish. Functional characterization demonstrated that zPIAS4 acts as a critical negative regulator in the TRIF- and MAVS-mediated signaling cascades and the IFN signaling pathway. The data significantly contribute to the current knowledge on the negative regulation of cytokine signaling in fish, as well as to the evolutionary history of the PIAS family, and they help to understand better the regulation of TLR-, RLR-, and IFN-triggered innate inflammatory responses.

Disclosures

The authors have no financial conflicts of interest.

References


