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A Unique Feature of Toll/IL-1 Receptor Domain-Containing Adaptor Protein Is Partially Responsible for Lipopolysaccharide Insensitivity in Zebrafish with a Highly Conserved Function of Myd88

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MyD88 and Toll/IL-1R domain-containing adaptor protein (TIRAP) are required for the TLR4 response to LPS stimulation in mammals, but the functions of the two adaptors and their involvement in zebrafish insensitivity to LPS remains unknown. We present a functional analysis of zebrafish Myd88 and TIRap and suggest that Myd88 is more important than TIRap for the activation of Tlr-mediated NF-κB, which may be a novel mechanism of Myd88-dependent TLR signaling in teleosts. Zebrafish TIRap lacks the phosphatidylinositol 4,5-bisphosphate binding motif required for human TIRAP location and has leucine at position 233 rather than the conserved proline of human TIRAP, as well as 105 additional aa at the N terminus. Overexpression of zebrafish TIRap in HEK293T cells did not activate NF-κB and IFN-β, but slightly activated NF-κB in carp leukocyte cells. Zebrafish Myd88 alone strongly induced the activation of NF-κB and IFN-β both in HEK293T and carp leukocyte cells. The function of Myd88 was dependent on its cellular location and the proline in the Toll/IL-1R domain. Although zebrafish TIRap was distributed throughout the cell rather than localized to the cytoplasmic membrane, its impaired ability to activate downstream Tlr molecules was unlikely to be related to its location because chimera TIRAP with a human TIRAP N terminus and membrane-binding domain also did not activate NF-κB. However, the mutation of leucine to proline increased the ability of TIRap to activate NF-κB. We suggest that the zebrafish TIRap needs a longer N terminus to perform its function and could be partially responsible for the resistance to LPS in zebrafish. The Journal of Immunology, 2010, 185: 3391–3400.

Hosts recognize invading microorganisms through germline-encoded pattern recognition receptors (PRRs), components of the innate immune system. The TLR family is among the most important PRRs, recognizing distinct microbial components as pathogen-associated molecular patterns (PAMPs) and activating the immune response. Toll was first identified in Drosophila and was considered to have a role in the development of dorsoventral polarity in the embryo (1). It was later found to also participate in the antifungal response of Drosophila (2). TLR activation not only initiates innate immune defenses, but also regulates Ag-specific acquired immunity in mammals, thus bridging innate and adaptive immunity (3). Thirteen members of the TLR family have been identified in mammals (4). All share a conserved Toll/IL-1R (TIR) domain, which interacts with cytoplasmic adaptors to activate the immune response. Five adaptors have been identified in mammals (5): MyD88, TIR domain-containing adaptor protein (TIRAP), TIR domain-containing adaptor protein inducing IFN-β (TRIF), TRIF-related adaptor molecule, and sterile α-and armadillo motif-containing protein.

The function of TLR4 and the cytoplasm adaptors have been characterized in mammals. TLR4 recognizes diverse microbial components, such as LPS, mannan, glycoconjugates, envelope proteins, and fibronogen (6). It is a central component of LPS-activated immunity. Its recognition of LPS is a critical event in the immune response to Gram-negative bacteria, which is essential to the initiation of endotoxic shock (7). TLR4 is the only receptor in the TLR family requiring two TIR domain-containing adaptors (e.g., Myd88 and TRIF), each of which activates a different pathway in the cytoplasm. The TIRAP- and Myd88-mediated pathways are responsible for the activation of proinflammatory cytokines.

Studies using Myd88-deficient mice have demonstrated that Myd88 is important in LPS-induced TLR4 signaling (8, 9). LPS-induced proinflammatory cytokine production by macrophages and fibroblasts was greatly decreased in Myd88-deficient mice. Furthermore, the cells lacking Myd88 did not respond to peptido-
glycans (PGNs), flagellin, CpG DNA, or ssRNA, indicating that specific TLRs (TLR2, -5, -7/8, -9, and -11) recognize these ligands and then signal directly through MyD88 (10, 11). TIRAP localizes to the plasma membrane through the phosphatidylinositol 4,5-bisphosphate (PIP2) binding domain and recruits MyD88 to the plasma membrane when a ligand binds to the receptor (12–15). TRIF-related adaptor molecule- and TRIF-mediated TLR4 pathways lead to the production of type 1 IFN. TRIF also functions downstream of TRLR3 to generate an immune response to virus. Sterile α- and armadillo motif-containing protein can block the TLR3 signal pathway through inhibition of TRIF (16–19). How TIRAP recruits MyD88 and whether this recruitment is required for the TLR signaling pathway in zebrafish (Danio rerio) remains unknown.

The zebrafish is an important vertebrate model for immunological research (20). Its innate immunity is not well characterized, and only a few studies of TLRs and their signaling in zebrafish have been conducted. In 2004, two independent research groups described 19 putative variants of Tlr in zebrafish (21, 22), including orthologs of mammalian TLR2–5 and 7–9. Zebrafish Tlr4 has two orthologous genes, Tlr4a and Tlr4b. Van der Sar et al. (23) demonstrated that the innate immune response of the developing zebrafish embryo included MyD88-dependent signaling. We and others have revealed that zebrafish Trif, a Golgi-localized protein, participates in IFN induction and NF-κB activation (24, 25).

LPS, a constituent of the outer membrane of Gram-negative bacteria, activates macrophages and dendritic cells in the innate immune system. TLR4, together with myeloid differentiation factor-2 (MD-2) and CD14, plays a major role in the recognition of LPS on membranes and initiates a signal that activates NF-κB and IFN regulatory factor. Lower vertebrates, especially fish and amphibians, are tolerant to LPS stimulation (26). Reports have shown that high concentrations of LPS are necessary to elicit an immune response in fish macrophages and mammalian cells (27, 28), which was also observed in an unpublished study from our laboratory.

To identify the differences in TLR signaling of zebrafish and mammals, we studied MyD88 and TIRAP in LPS-induced TLR-mediated signaling. We cloned a full-length cDNA of zebrafish TIRAP, analyzed its expression pattern in tissues, and determined the location of zebrafish TIRAP and MyD88 in HEK293T cells. We also characterized the function of two cytoplasmic adaptors. Our results provide further understanding of the role these adaptors play in the TIR pathway of the innate response of zebrafish.

Materials and Methods

Cloning zebrafish TIRAP

The cDNA for RACE-PCR was prepared by the GeneRacer Kit (Invitrogen, Carlsbad, CA). The TIR gene-specific primers for PCR amplification were designed according to homology to its mammalian counterpart. The gene-specific primer for 5′-RACE and its nested primers were as follows: GeneRacer 5′-Primer: 5′-GGTTGATGTTTGCCTGCGTCT-3′, 5′-gene-specific primer: 5′-TGGTGAACACACACATCATACAAA-3′, GeneRacer 5′-Nested Primer: 5′-TTACCGAGGAAATGGTACGGGT-3′, and 5′-Nested gene-specific primer: 5′-AGTAAGCCGCTGCTCTTCTC-3′. The open reading frame of zebrafish TIRAP was amplified with the upstream primer 5′-CCGGCAT-CTCGTATGATGGTCTCAGT-3′ and the downstream primer 5′-CGGAAATC-CATGATTGTTATGTAAGTCTGACT-3′ using zebrafish cDNA as templates. The PCR products were cloned into the pGEM-T easy vector (Promega, Madison, WI) for sequencing with an ABI 3730 DNA sequence (Applied Biosystems, Foster City, CA).

Aeromonas salmonicida infection and TLR ligand induction

An area of 2 mm² on the dorsal surface of adult zebrafish was scraped with a sterile scalpel prior to experimental groups being placed in water containing 10⁶ CFU/ml Aeromonas salmonicida and a control group placed in sterile water. Skin, gills and internal organs (muscle, eye, brain, kidney, spleen, intestine, heart, liver, ovary, and testis) were dissected from 10 zebrafish 15 h postinfection. The organs mentioned above were treated with TRIzol reagent (Invitrogen) to extract total RNA. The extracted RNA, treated with RNase-free DNase (Takara Bio, Shiga, Japan), was reverse-transcribed into the first-strand cDNA using the ReverTraAce cDNA synthesis kit (Toyobo, Osaka, Japan). The cDNA was prepared for quantitative real-time PCR.

Each of 7 groups of 15 adult zebrafish was i.p. injected with one of the following: zymosan (fungi; 100 μg/g), LPS (Gram-negative bacteria; 100 μg/g), polyinosinic-polycytidylic acid (poly I:C; dsRNA viruses; 100 μg/g), lipopolysaccharide (LTA; Gram-positive bacteria; 100 μg/g), glucan (polysaccharide of D-glucose monomers, Gram-negative bacteria; 50 μg/g), and PGN (Gram-positive bacteria; 100 μg/g) (all purchased from Sigma-Aldrich, St. Louis, MO) or PBS. At 2, 6, 18, 24, and 42 h postinjection, the internal organs of three fish from each treatment were dissected. The organs were treated to extract total RNA, then reverse-transcribed into the first strand cDNA for quantitative real-time PCR.

Quantitative real-time PCR

First-strand cDNA was used as a template. Gene-specific primers for quantitative real-time PCR were designed to generate single-gene-specific amplicons of 140–150 bp fragments. Zebrafish β-actin or GADPH primers were used to normalize the initial quantity of RNA. Each gene was assayed in triplicate for each sampling time with appropriate standards. Quantitative real-time PCR was performed on an ABI Prism 7900 Sequence Detection System (Applied Biosystems) as two-step RT-PCR, using SYBR Premix Ex TaqTM (Takara Bio) according to the manufacturer’s instructions. The threshold cycles and fold inductions were calculated with ABI Prism 7900HD SDS software. Forward primer 5′-CGAAAAGGTTGTAAGGAGTTG-3′ and reverse primer 5′-TGGATTGTGTA-GACGACAGGGAT-3′ were used for zebrafish myd88. Forward primer 5′-GCTGGAACGCGCTCCTCCTT-3′ and reverse primer 5′-TGCTGTTTGA-GTGCTCTCCTCACA-3′ were used for zebrafish Tirap.

Plasmid construction

The cDNAs encoding hemagglutinin (HA)-tagged full-length zebrafish MyD88 and Tirap with part of 5′ untranslated region (UTR) and single-point mutation at P182 of MyD88 including P188H, P188L, and P188R were cloned into pcDNA3.0. NF-κB luciferase reporter gene vector was kindly provided by Dr. Ping Wang at the University of London (London, U.K.). The IFN-β luciferase reporter gene vector was kindly provided by Dr. Yingqiu Li at the Sun Yat-Sen University (Guangzhou, People’s Republic of China). TIR domain-swap mutants including hN+ZC and ZN+HC were constructed by PCR sewing of cDNA corresponding to aa 1–85 of human TIRAP with aa 184–338 of zebrafish Tirap and aa 1–183 of zebrafish Tirap with aa 86–235 of human TIRAP, respectively. The resulting cDNA was cloned into pcDNA3.1. Wild-type (WT) Tirap, truncated forms of Tirap including ΔN (deletion of aa 1–105), TIR (aa 184–338), and the L233P, L233H, L233PΔN, and L233HAN were subcloned into pcDNA3.1 (Supplemental Fig. 3).

Cell culture and transfection

The HEK293T and HeLa cell lines were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 100 μg/ml penicillin-streptomycin in 5% CO₂ at 37°C. The carp leukocyte (CLC) cell line, which was provided by Dr. Jianfang Gui at the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China), was cultured in M199 supplemented with 10% FBS, 2 mM L-glutamine, and 100 μg/ml penicillin-streptomycin at 25°C. The cells were seeded in a 24-well or 48-well plate and grown for 24 h. HeLa cells were transfected with the indicated expression plasmids with Lipofectamine 2000 reagent (Invitrogen). CLC cells were transfected with jetPEI (poly plus transfection). All of the operations were done by following the user manual.

Immunofluorescence and laser confocal imaging

HeLa and CLC cells were seeded onto cover slips (10 mm x 10 mm) in a 24-well plate and grown for 24 h. Cells were transfected with the indicated expression plasmids. At 24 h posttransfection, the medium was removed, and the cells were fixed for 4% formaldehyde solution. Cells were washed for 3–5 min in PBS three times and permeabilized by treatment for 10 min with PBST (0.1% Triton X-100 in PBS). Cells were blocked 1 h in Ab blocking buffer (1% BSA in PBS), incubated with 5 μg/ml primary Ab in Ab blocking buffer for 45 min, washed 3–10 min in PBST three times, incubated with Alexa Fluor 488/532 Ab (Invitrogen), washed for 3–5 min in PBS three times, labeled with 0.2 μg/ml DAPI (Sigma-Aldrich) in PBS for 1 min, and washed three times in PBS. The specimen was mounted in Mowiol Reagent (Calbiochem, San Diego, CA) and photographed. Imaging of cells was obtained with Leica laser scanning confocal microscopy (Leica Microsystems, Deerfield, IL).
FIGURE 1. A, Multiple alignment of full-length TIRAP. The three main boxes of TIR domains are framed (boxes 1, 2, and 3); the PIP2 binding domain conserved in mammal is underlined. B, Phylogenetic tree based on the alignment of full-length protein sequences of TIRAP by neighbor-joining analyses. Sequences are from the NCBI (www.ncbi.nlm.nih.gov), Takifugu rubripes (accession number BAF91190), Mus musculus (accession number NP_473437), Homo sapiens (accession number AAH32474), Gallus gallus (accession number AAY51675), and Bos taurus (accession number NP_001035051). C, The PI values of zebrafish Tirap and Myd88 compare with those of human TIRAP and MyD88. D, The region does not fit any known structural domain. D, Western blot analysis of WT zebrafish Tirap (cDNA containing partial 5' UTR), human TIRAP, L233P, and ΔN in HEK293T cells. The Western blot analysis of WT zebrafish Tirap and ΔN was also investigated in CLC cells. HA Ab was used for the primary Ab.
Luciferase reporter assay

For reporter assays, cells were plated at a density of 10⁵ cells/well in a 48-well plate. Cells in each well were transiently cotransfected with NF-kB-dependent luciferase reporter plasmid or IFN-β promoter luciferase reporter plasmid and the indicated Myd88 or Tirap expression vectors after the cell was growing for 24 h. All transfections were duplicated. After 24 h, cells were lysed and collected for luciferase reporter assay. Luciferase activity of cell lysates was measured with a luciferase reporter assay (Promega) according to the manufacturer’s instructions. Renilla luciferase reporter plasmid was used as an internal control. Each experiment was performed at least in triplicate, repeated at least three times in all cases.

Morpholinos and the embryos stimulated with PAMPs

Morpholino (1 mmol/l) was microinjected into embryo yolk at the single-cell phase to knockdown Myd88 and Tirap protein expression. The morpholino-modified antisense oligonucleotides (GeneTools, Philomath, Or) used in this study include 5'-TAGCAAAACCTCTGTAT-CCAGCCG-3' for Myd88 (25) and 5'-CCCTTCTACCTCAGTTACAATTATA-3' for standard control. Additionally, 3 h after morpholino was injected, when the treated zebrafish blastulae were at the sphere stage (4 h postfertilization), PAMPs (10 ng/l) including LPS, LTA, and PGN were microinjected into the intercellular space at the animal pole with PBS as control. One hour later, total RNA was extracted with TRIzol reagent (Invitrogen) from 50–60 embryos, treated with RNase-free DNase (Takara Bio), and reverse-transcripted into the cDNA using the ReverTraAce cDNA synthesis kit (Toyobo). Real-time PCR was performed to detect the mRNA level of IL-1β.

Results

Identification of full-length zebrafish Tirap gene

We cloned the full-length cDNA of zebrafish tirap, which included four exons encoding 338 aa with the TIR domain from aa positions 184–338, by 5’ RACE-PCR. Western blotting (Fig. 1D) showed that Tirap was translated from the first ATG downstream from the TGA in 5’ UTR both in HEK293T and CLC cells (Supplemental Fig. 1). Protein sequence cluster analysis showed that the TIR domain of zebrafish Tirap was well conserved compared with human, mouse, cow, chicken, and Takifugu counterparts, with YD in box 1, RDxxxG in box 2, and FW in box 3. In previous studies, the conserved proline at the tip of the BB loop (RDxxPG) was determined to be functionally important in mammals (29–31). However, in zebrafish Tirap, leucine, not proline, is at the tip of the BB loop (Fig. 1A). Zebrafish Tirap also differs from that of other species with respect to the NH2 terminus with a proline-rich region. From the sequence cluster, we found that mammalian TIRAP had a conserved PIP2 binding motif (KPKLPKMADWFQRTLKKPKP). The four lysines in this motif were important in TIRAP cell localization (15, 32). Although zebrafish Tirap has no PIP2 binding domain, this is also true of chicken and Takifugu. We further constructed the phylogenetic tree based on the full-length TIRAP and found that zebrafish Tirap was clustered with Takifugu TIRAP with the evolutionary trend, in accordance with that of the species (Fig. 1B). We calculated protein isoelectric (PI) point values by different domains on the Expasy Web site; the PI values of zebrafish Tirap and Myd88 were similar to those of human TIRAP and Myd88 (Fig. 1C).

Expression patterns of zebrafish Tirap gene

To identify the expression patterns of zebrafish Tirap, real-time PCR analysis showed that Tirap was expressed in a variety of organs and was especially high in testis (Fig. 2A). We analyzed these organs following infection with A. salmonicida and found Tirap to be highly expressed in kidney, spleen, testis, and ovary (p < 0.05) (Fig. 2A).

We used the TLR ligands zymosan, LPS, poly I:C, LTA, glucan, and PGN to inject zebrafish. At 6 h postinjection, the mRNA level of Tirap was upregulated except for LPS injection. Between 6 and 42 h post PGN and glucan induction, there was a biphasic response, with a dip in expression at 24 h (Fig. 2B).

Zebrafish Tirap does not activate the NF-κB in HEK293T cells

To examine signaling capacities of zebrafish Tirap and its domains, the construct of full-length zebrafish Tirap, the construct lacking the zebrafish Tirap N terminus, and a positive control vector of human TIRAP were overexpressed in HEK293T. Zebrafish Tirap did not activate NF-κB in the HEK293T cell, and the segments of Tirap, including Tirap-ΔN, also showed no such ability, unlike its human counterpart (Fig. 3C). The exchange of zebrafish Tirap with human TIRAP N-terminal portion also did not activate NF-κB (Fig. 3D).

Location is not the only reason for zebrafish Tirap not to activate NF-κB in mammalian cells

We wanted to know where zebrafish Tirap is located, because it lacks the PI(3,4,5)P3 binding motif and, unlike human TIRAP, does not activate NF-κB (p < 0.05). Zebrafish Tirap was expressed in the cytoplasm and nucleus, whereas the expression of human TIRAP is mainly, but not exclusively, in the cytoplasmic membrane (Fig. 3A), which differs slightly from previous reports (15, 32). The truncated segments of zebrafish Tirap, including Tirap-ΔN and Tirap-ΔTIR, also showed the same cell location as Tirap-WT (Fig. 3A). To investigate whether cellular location was the reason that zebrafish Tirap did not activate NF-κB, we exchanged the TIR domain of human TIRAP and zebrafish Tirap. The construct of hN+zC partially localized to the plasma membrane, the cellular location of human TIRAP determined in our study. The chimeric zebrafish Tirap, containing

FIGURE 2. A. Tissue distribution of zebrafish tirap in normal and A. salmonicida-infected zebrafish. RNA was isolated from skin, gill, muscle, eye, brain, kidney, spleen, intestine, heart, liver, testis, and ovary. Tirap expression pattern was analyzed by quantitated real-time PCR. B. Expression pattern of zebrafish tirap following ligand injection. Adult zebrafish was i.p. injected with either LPS (100 μg/μl), poly I:C (100 μg/μl), LTA (100 μg/μl), PGN (100 μg/μl), zymosan (100 μg/μl), glucan (50 μg/μl), or PBS. At 2, 6, 18, 24, and 42 h, internal organs were dissected, and total RNA was extracted and reverse-transcribed into first-strand cDNA. The fold induction was compared with PBS. *p < 0.05; **p < 0.01.
a TIR domain of human TIRAP, was still expressed throughout the cells (Fig. 3A, Supplemental Fig. 2). A similar result was observed in the CLC cell line. Zebrafish Tirap-WT and Tirap ΔN located in the cytoplasm, whereas human TIRAP and hN+zC localized in the plasma membrane (Fig. 3B). Although hN+zC localized in the plasma membrane as TIRAP both in HeLa and the CLC cell line, it did not activate NF-κB. Thus, we concluded that the location is not the sole reason that zebrafish Tirap does not activate NF-κB.

The zebrafish Tirap mutant with L233P substitution and without the N terminus has the highest activity in HEK293T cells

The site of proline in mammal TIRAP is occupied by leucine in zebrafish Tirap. Using luciferase report assay, we demonstrated that both mutants with L233P and the truncated N terminus showed moderate ability to induce activation of NF-κB, but neither the mutant L233H nor L233H ΔN activated NF-κB (p < 0.05). Interestingly, the mutant with L233P that lacked N-terminal aa 1–105 induced activation of NF-κB 13.1-fold that of the empty vector (Fig. 3E), suggesting that proline on the tip of BB loop and the shortened N terminus act synergistically for the function of TIRAP.

Both zebrafish Tirap and Myd88 activate NF-κB in CLC cells

We transfected zebrafish Tirap-WT, L233P, L233P ΔN, L233H, L233HΔN, hN+zC, zN+hC, and Myd88 to CLC cells. All were observed to activate NF-κB except hN+zC and zN+hC, and, among these activations, Myd88 showed the strongest induction (Fig. 3F).

Conserved zebrafish Myd88 plays an important role in the activation of NF-κB and IFN-β in HEK293T cells

Because of the inability of zebrafish Tirap to activate NF-κB in HEK293T cells, we carried out a luciferase reporter assay to study the function of zebrafish Myd88. Overexpression of zebrafish Myd88 in HEK293T cells led to a strong dose-dependent induction of an NF-κB–dependent luciferase reporter gene (all p values <0.01) (Fig. 4A). In addition, IFN-β was also activated by overexpression of Myd88 in a dose-dependent manner (all p values <0.01) (Fig. 4B). This suggests a crucial role for Myd88 in the activation of NF-κB and IFN-β in zebrafish.

Myd88 is conserved sequentially from teleost to mammal with a death domain in the N-terminal, a TIR domain in the C-terminal, and an intermediary domain between the death domain and the TIR.

FIGURE 3. A, The cellular location of zebrafish Tirap-WT, Tirap ΔN, Tirap-TIR, hN+zC, zN+hC, and human TIRAP in HeLa cells. The cells were fixed and prepared for immunofluorescence staining. HA-tag was used as the primary Ab. The nucleus was stained blue by DAPI; the expression protein was shown in green. B, The cellular location of zebrafish Tirap-WT, Tirap ΔN, Tirap-TIR, hN+zC, and human TIRAP in CLC cells. Original magnification ×1000. C, Unlike human TIRAP, zebrafish Tirap and deletion mutants demonstrate limited NF-κB–dependent luciferase activation in HEK293T cells. D, The chimeras could not activate the NF-κB–dependent luciferase. E, Overexpression of zebrafish Tirap and its mutants including zebrafish TirapΔN, L233P, L233PΔN, L233H, and L233HΔN in HEK293T cells for the NF-κB–dependent luciferase assay. Only L233PΔN showed a little effect to activate NF-κB. F, Overexpression of zebrafish Myd88, Tirap, and its mutants including Tirap ΔN, L233P, L233PΔN, L233H, L233HΔN, hN+zC, and zN+hC in CLC cells for the NF-κB–dependent luciferase assay. Zebrafish Tirap and its mutants could slightly activate NF-κB; zebrafish Myd88 had more powerful function for NF-κB activation. Above data are representative of three independent experiments. *p < 0.05; **p < 0.01.
domain (33). According to the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool, zebrafish Myd88 shows \(~\sim\) 66% identity to human MyD88 (Fig. 5A, 5B). Myd88 was upregulated by PGN and zymosan similar to Tirap. The expression of Myd88 showed a biphasic response, with a dip in the expression at 24 h post PGN or glucan induction, similar to Tirap expression (Fig. 5A-C).

The aggregation location is important for zebrafish Myd88 function

Histidine substitution for proline in the TIR domain of TLR2/4 leads to a dominant dysfunction (34, 35). We wanted to know if this substitution in the TIR domain of Myd88 would result in the same dysfunction. Zebrafish Myd88-P188H and P188R still activated NF-\(\kappa\)B in HEK293T and CLC cells, but the mutant with Myd88-P188L did not (Fig. 6C). The activity of the mutants was coincident with the cellular location of Myd88. Myd88-P188H and P188R showed a similar cellular location as the Myd88-WT in HeLa (Fig. 6A) and CLC cells (Fig. 6B). The cellular location of Myd88-P188L expressed throughout the cytosol both in HeLa and CLC cells (Fig. 6A, 6B), suggesting the importance of the cellular location of Myd88 for the activation of NF-\(\kappa\)B. The same result was observed for the activation of IFN-\(\beta\) (Fig. 6C). The ability to activate NF-\(\kappa\)B or IFN-\(\beta\) was not determined by the character of amino acid composition on the tip of the BB loop, but by the condition of Myd88 intracellular aggregation. The more Myd88 aggregated inside cells, the stronger its effect.

Myd88 is related to localization of Tirap in cells

As we showed before, zebrafish Tirap and its mutated Tirap\(\Delta\)N were expressed in cytoplasm and nucleus evenly (Fig. 3A), and Myd88 aggregated in a condensed area within cytoplasm (Fig. 6A). When Tirap and Myd88 were overexpressed simultaneously, Tirap became aggregated and colocalized with Myd88 (Fig. 7A). It was the same case for Tirap\(\Delta\)N (Fig. 7B).

**FIGURE 4.** Overexpression of zebrafish Myd88 activated NF-\(\kappa\)B and IFN-\(\beta\) promoter in HEK293T cells. A, HEK293T cells were cotransfected with the pcDNA3.0 empty vector or with increasing amounts (5, 10, 25, and 50 ng) of expression vector for zebrafish Myd88 together with the NF-\(\kappa\)B luciferase reporter vector (50 ng) versus control Renilla (5 ng) expression vector. B, Zebrafish Myd88 expression vector (5, 10, 25, and 50 ng) or empty vector was cotransfected with the IFN-\(\beta\) luciferase reporter vector (50 ng) versus control Renilla expression vector (5 ng) into HEK293T cells. After 24 h, luciferase activities in cell lysates were measured and expressed as the fold stimulation. Above data are representative of three independent experiments. All \(p\) values <0.01.

**FIGURE 5.** A, Multiple alignment of full-length MyD88. B, The phylogenetic tree was constructed based on the alignment of full-length protein sequences of MyD88 by neighbor joining analyses. Sequences are from the NCBI (www.ncbi.nlm.nih.gov/), T. rubripes (accession number NP_001106666), M. musculus (accession number AAC53013), H. sapiens (accession number AAC50954), G. gallus (accession number NP_001026133) and B. taurus (accession number AAY22119), Xenopus tropicalis (accession number NP_001016837), and Drosophila melanogaster (accession number AAF58953). C, Expression pattern of zebrafish Myd88 following ligand injection. \(p < 0.05\); \(**p < 0.01\).
Myd88 plays an essential role in PGN- and LTA-induced IL-1β expression

When embryos were treated with PGN and LTA, IL-1β mRNA expression level was enhanced ~3–5-fold. As the embryos were treated with Myd88 morpholino to knockdown the protein expression of Myd88, the induced expression of IL-1β was decreased significantly (p < 0.05) (Fig. 8A, 8B). The IL-1β expression level did not change after LPS stimulus. Consistent with the results published recently (36, 37), we found that the zebrafish embryo was also insensitive to LPS. Knockdown of Myd88 had no effect on IL-1β expression in the embryos stimulated with LPS (Fig. 8C).

Discussion

The innate and adaptive immune systems of zebrafish are highly conserved compared with those of mammals. Pathogen recognition is a basic and important feature of the immune system. TLRs are the most crucial PRRs, recognizing PAMPs to induce specific signaling cascades and proinflammatory responses. TLR4 is critical in LPS-induced septic shock in mammals. From our previous research and that of others, we noted that zebrafish are insensitive to LPS, as are other teleosts. To understand the differences in TLR signaling of zebrafish and mammals, we investigated MyD88 and TIRAP in LPS-induced TLR-mediated signaling.

Protein sequence alignment showed that MyD88 is conserved from teleost to mammal. The identity between zebrafish Myd88 and human MyD88 is 66%, which is in agreement with the reports by Jault et al. and Meijer et al. (21, 22). The PI value of zebrafish Myd88 was also similar to human MyD88 (Fig. 1C), but the N terminus and the length of zebrafish Tirap differed from mammalian TIRAP. The TIR domain and the PI value of zebrafish Tirap were similar to human TIRAP (Fig. 1C). The N terminus of human TIRAP is a proline-glutamate-serine-threonine segment containing a PIP2 binding domain (4), but zebrafish Tirap N terminus is much longer, with a proline-rich region. Leucine, but not proline, was present at the tip of the BB loop in the TIR domain of zebrafish Tirap. Both Myd88 and Tirap showed a biphasic response with a dip in expression at 24 h post PGN or glucan injection. We consider the biphasic response to be a normal phenomenon. The mRNA expression pattern of TLR3 (a dip in expression 18 h post zymosan induction) and retinoic acid-inducible gene I/mitochondrial antiviral signaling (a dip in expression 24 h post poly I:C induction) in our previous study showed a similar pattern (24). PGN is a component of G+ bacterium, and glucan is a component of G− bacterium, whereas zymosan and...
Tirap IS PARTIALLY RESPONSIBLE FOR LPS INSENSITIVITY

Overexpression of zebrafish Myd88 in HEK 293T and CLC cells resulted in strong activation of both NF-κB and IFN-β, indicating that downstream of Myd88-dependent TLR signal pathway was conserved in zebrafish and mammals, because TLRs need to use MyD88 directly to convert signaling to NF-κB and IFN-β in zebrafish.

According to the previous research (29), the BB loop (RDxvF2, F2v) extends away from the rest of the TIR domain. Amino acid substitution at the tip of BB (F2v) loop will not disrupt the TIR domain structure of TLRs but the direct point of contact with other molecules containing the TIR domain. Studies of proline mutation in the TIR domain of TLRs and TIRAP have revealed that this site is important for signaling (30, 35). The mutation analysis in our study further substantiated that proline at the tip of the BB loop of the TIR domain is important for signaling (30, 35). The mutation analysis in our study indicated that the amino acid character at aa 188 was not the decisive factor in zebrafish Myd88 cellular location. However, the cellular location of Myd88 was the decisive factor in its function in the activation of NF-κB and IFN-β in zebrafish.

Human TIRAP only carries its function upstream of Myd88 in TLR2/4 signaling, but, in our study, the overexpression of zebrafish Tirap did not activate NF-κB in HEK293T cells and only slightly activated NF-κB in CLCs. Based on the sequence differences of zebrafish Tirap and human TIRAP, we tried to determine why zebrafish Tirap impaired the function of this pathway. When we deleted the N-terminal amino acids not present in human TIRAP to generate the chimera with the TIR domain/no-TIR domain of human TIRAP, unlike other proximal TLR signaling members, as well as leucine in zebrafish Tirap were factors contributing to Tirap dysfunction in HEK293T cells. Therefore, the additional amino acids in the N-terminal region as well as leucine in zebrafish Tirap were factors contributing to Tirap dysfunction in HEK293T cells. The experiments performed in CLC showed that zebrafish Tirap and the chimeras did activate IFN-β (Supplemental Fig. 4) but slightly activate NF-κB, suggesting that Tirap is functional in teleosts but not as strong as mammalian TIRAP. The selective pressure on TIRAP, unlike other proximal TLR signaling members, differed from that in mammals throughout evolution, which may have resulted in a partial function attenuation of Tirap in zebrafish. The sequence difference also affected the cellular location of Tirap. Human TIRAP is localized constitutively at the cytoplasmic membrane through the PIP2 binding domain in the proline-glutamate-

**FIGURE 7.** Myd88 is related to localization of Tirap in cells. A. Immunofluorescence microscopy of HeLa cells transfected for 24h with Tirap-GFP and Myd88-HA, then stained with anti-HA and Alexa Fluor 532 donkey anti-mouse IgG Ab. B. Immunofluorescence microscopy of HeLa cells transfected for 24h with Tirap ΔN-GFP and Myd88-HA, then stained with anti-HA and Alexa Fluor 532 donkey anti-mouse IgG Ab. A and B. Original magnification ×1000. Tirap-GFP, GFP-labeled Tirap; Tirap ΔN-GFP, GFP-labeled Tirap ΔN.

**FIGURE 8.** IL-1β mRNA expression level in Myd88 knockdown embryos induced with PAMPs. IL-1β mRNA expression in Myd88 knockdown embryos stimulated with PGN (A), IL-1β mRNA expression in Myd88 knockdown embryos stimulated with LTA (B), and IL-1β mRNA expression in Myd88 knockdown embryos stimulated with LPS (C). *p < 0.05.

activated NF-κB (30). We obtained the same result for the substitution of P188H in zebrafish Myd88 with respect to the activation of NF-κB. P188R reduced the Myd88 function, and P188L impaired the function of Myd88 severely. Thus, the TIR domain in zebrafish Myd88 may perform a different function from the TIR domain in TLRs and TIRAP. The mutation in zebrafish Myd88 also altered its cellular location. Human Myd88 has been shown to be localized in the cytoplasm of 293T cells as large condensed forms with morphologies, such as spot, star, string, ring, and cocoon (31). Previous studies have indicated that some TLRs are localized in the endoplasmic reticulum, Golgi apparatus, endosomes, and other cytoplasmic compartments (38–41). Nishiya and coworkers (31) proposed that human MyD88 is localized to these cytoplasmic compartments, but the specific organelle in which MyD88 resides is yet to be determined. In our study, we found that zebrafish Myd88 was localized in cytoplasm in condensed spots in both HeLa and CLC cells. P188H and P188R substitution slightly alter the cell location of zebrafish Myd88, but P188L showed different cell locations throughout the cytoplasm. Small hydrophobic residues (proline, alanine, valine, and isoleucine) are usually present at Φ2 in several TIR domains (29). Proline and leucine are hydrophobic amino acids, whereas histidine and arginine are hydrophilic. The results of our study indicated that the amino acid character at aa 188 was not the decisive factor in zebrafish Myd88 cellular location. However, the cellular location of Myd88 was the decisive factor in its function in the activation of NF-κB and IFN-β in zebrafish.

poly I:C represent fungus and virus, respectively. Because the biphasic response did not occur solely in PGN/glucan injection, it may be a general phenomenon, but the alteration in response may occur at different times depending on the ligand. It is possible that the initial period of injection is characterized by a stress reaction. The microorganisms or ligands are eliminated by the host gradually and in different levels, resulting in a variation in time of the reduction in response. Following the initial response, the remaining ligands or microorganisms may induce a more intense reaction.

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serine-threonine segment, which acts as a sorting adaptor to recruit MyD88 to the cytoplasmic membrane and is associated with TLR4 to activate the downstream signaling pathway and induce activation of NF-κB (15). The chimera with the TIR domain of human TIRAP replaced with the corresponding region of zebrafish Tirap showed the same cellular location as human TIRAP both in HeLa and CLC cells, indicating that the N terminus is the region mainly responsible for the cellular location. This chimera did not activate the NF-κB pathway, indicating that cellular location is not sufficient for zebrafish Tirap to activate NF-κB. When Tirap and Myd88 were overexpressed simultaneously, Tirap changed its cellular location to colocalize with Myd88. Thus, it was likely that zebrafish Tirap was still potential to interact with Myd88, although it lacked conserved proline at position 233.

It has been reported that teleosts are not sensitive to LPS induction (27, 42). It is accepted that in mammals, LPS–TLR signaling requires binding of TIRAP to the plasma membrane and recruitment of MyD88. In this study, we demonstrated that in zebrafish, molecules downstream of the MyD88-dependent signal pathway were available for activation. Thus, lack of sensitivity to LPS in zebrafish may be determined by the molecules upstream of MyD88. In mammals, MD-2 and CD14 may facilitate the recognition of LPS, and TIR domain-containing adaptor molecule 2 functions as a bridge between TLR4 and TRIF. However, these proteins have not been found in teleosts, and studies have suggested that their absence could be responsible for insensitivity to LPS in the teleost (7). The absence of those molecules not only attenuates the recognition of LPS, but also inhibits TLR4 induced TRIF-dependent signaling and consequently reduces the production of IFN. Another two studies revealed that zebrafish TLR4 orthologs negatively regulated the MyD88-dependent signaling pathway (36), and the lack of responsiveness to LPS was most likely due to the inability of the extracellular portions of zebrafish Tlr4a and Tlr4b to recognize the molecule (37). These two studies provided the reasons for LPS insensitivity of the level of cellular membrane receptor recognition, which may partially explain the reasons. Zebrafish has specific mechanisms of innate immunity and a different immune response from mammalian (43). The cytosolic signaling molecules, especially TIRAP and MyD88, have been demonstrated to be indispensable in LPS-induced signaling transduction in mammals in previous studies. They are also very important for LPS-induced immune response in zebrafish.

Zebrafish Tirap did not function in HEK293T cells (human cell line) but was active in CLC cells (teleost fish cell line). The zebrafish Tirap mutant with an L223P mutation and without aa 1–105 in the N terminus, which is more similar to human TIRAP, does induce activation of NF-κB both in HEK293T and CLC cells, indicating that evolutionary pressure resulted in the sequence change of human TIRAP and enhanced function. Therefore, WT zebrafish Tirap may be another component contributing to LPS insensitivity. The conformation of the zebrafish Tirap molecule may weaken its function of recruiting MyD88 to transfer the signal from TLRs to downstream molecules, resulting in low sensitivity to LPS.

Overexpression of zebrafish Myd88 activated NF-κB and IFN response promoter, suggesting that the elements downstream of Myd88 in the TLR pathway are conserved in both zebrafish and mammals. Additional evidence was also attained in knockdown experiments.

It is well known that LTA and PGN are recognized by TLR2 in mammals. The morpholino oligonucleotide knockdown experiment applied in zebrafish embryos indicated that Myd88 also plays an essential role in PGN- and LTA-induced IL-1β upregulation.

The sequence difference in the N terminus of zebrafish Tirap and human TIRAP resulted in failure of Tirap to localize to the cytoplasmic membrane for contact of TLRs with downstream molecules for the activation of NF-κB. This resulted in resistance to LPS-induced endotoxic shock, suggesting that the TLR4-mediated TIRAP-dependent pathway is important in teleosts and may be the underlying mechanism of LPS insensitivity.

It appears that zebrafish Tirap, in addition to the absence of MD-2, CD14, and TIR domain-containing adaptor molecule 2, plays a significant role in the insensitivity of zebrafish to LPS, which may be the result of its longer N terminus along with the 223 leucine. Zebrafish Tirap seems to reflect the evolutionary development of TLR pathways among teleosts, which became structurally modified during evolution to optimally fit into the TLR-mediated NF-κB pathway in mammals. The imperfect function of Tirap is important for the activation of TLR-mediated NF-κB in zebrafish.

Disclosures

The authors have no financial conflicts of interest.

References


