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Evidence for Evolving Toll-IL-1 Receptor-Containing Adaptor Molecule Function in Vertebrates

Con Sullivan,∗ John H. Postlethwait, † Christopher R. Lage,∗ Paul J. Millard,‡ and Carol H. Kim2∗

In mammals, Toll-IL-1R-containing adaptor molecule 1 (TICAM1)-dependent TLR pathways induce NF-κB and IFN-β responses. TICAM1 activates NF-κB through two different pathways involving its interactions with TNFR-associated factor 6 and receptor-interacting protein 1. It also activates IFN regulatory factor 3/7 through its interaction with TANK-binding kinase-1, leading to the robust up-regulation of IFN-β. In this study, we describe the role of zebrafish (Danio rerio) TICAM1 in activating NF-κB and zebrafish type I IFN. Zebrafish IFN is unique in that it cannot be categorized as being α- or β-like. Through comprehensive sequence, phylogenetic, and syntenic analyses, we fully describe the identification of a zebrafish TICAM1 ortholog. Zebrafish TICAM1 exhibits sequence divergence from its mammalian orthologs and our data demonstrate that these sequence differences have functional consequences. Zebrafish TICAM1 activates zebrafish IFN; however, it does so in an apparently IFN regulatory factor 3/7-independent manner. Furthermore, zebrafish TICAM1 does not interact with zebrafish TNFR-associated factor 6, thus NF-κB activation is dependent upon its interaction with receptor-interacting protein 1. Comparative genome analysis suggests that TICAM1 and TICAM2 evolved from a common vertebrate TICAM ancestor following a gene duplication event and that TICAM2 was lost in teleosts following the divergence of the rayfin and lobefin fishes 450 million years ago. These studies provide evidence, for the first time, of the evolving function of a vertebrate TLR pathway. The Journal of Immunology, 2007, 178: 4517–4527.

The recent discovery of a functional type I IFN in the zebrafish, Danio rerio, provided the first insight into how these ancient antiviral cytokines evolved (1). Fish type I IFNs are unique in that they possess introns and, based upon phylogenetic analyses, cluster away from mammalian type I IFNs (1, 2). The mechanisms underlying activation of fish type I IFNs have yet to be explored. In mammals, transcription of type I IFNs can be initiated by ligand activation of TLR3, TLR4, TLR7, TLR8, and TLR9 (3–9). TLRs transduce signals from extracellular stimuli into the cell through interactions with adaptor molecules. Both TLRs and adaptor proteins possess Toll/IL-1R (TIR)3 domains that facilitate the protein–protein interactions responsible for triggering the signal cascade (reviewed in Refs. 10 and 11). One such adaptor known as TIR domain-containing adaptor molecule 1 (TICAM1, also known as TRIF) plays an essential role in TLR3 and TLR4 signaling, allowing for the up-regulation of IFN-β (12, 13).

Mammalian TICAM1s are comprised of proline-rich N- and C-terminal domains and a central TIR domain essential for interactions with other TIR domains (12, 13). Each domain plays an important role in signaling. The TIR domain is responsible for interacting with TLR3 or TICAM2. The N-terminal domain can interact with TNFR-associated factor (TRAF)6 or form a complex consisting of TANK-binding kinase (TBK)-1, IFN regulatory factors (IRF)3 and 7 (IRF7), TRAF3, IκB kinase-related kinase ε, and NAK-associated protein-1 (14–21). The C-terminal domain interacts with receptor-interacting protein (RIP)1 and RIP3.

In this study, we report the identification of a novel zebrafish TICAM1 ortholog that exhibits unique structural and functional features. Our results provide a glimpse into the evolutionary history of TLR-mediated type I IFN induction in a basally diverging vertebrate TICAM1-mediated pathway. Our findings suggest that zebrafish TICAM1 activates NF-κB and type I IFN through mechanisms not observed in mammalian TICAM1 orthologs and that while zebrafish type I IFN does not group with other avian or mammalian clades (1, 2), its TICAM1-dependent induction indicates the presence of this potent antiviral pathway in early vertebrates.

Materials and Methods

Nomenclature

Nomenclature rules for zebrafish, mouse, and human genes and proteins follow different conventions. To minimize confusion in presenting these data, gene names will be presented in italicized capital letters (e.g., TICAM1) and protein names will be presented in standard capital letters (e.g., TICAM1).
DNA constructs

Zebrafish orthologs of TICAM1, RIP1, and TBK1 were identified through basic local alignment search tool sequence analyses of zebrafish genome and expressed sequence tag databases using human and mouse orthologs and available zebrafish sequence data. Each zebrafish ortholog was cloned and deposited in GenBank (zebrafish TICAM1, accession no. DQ848679; zebrafish RIP1, accession no. DQ846680; zebrafish TBK1, accession no. DQ860098). Zebrafish IFN promoter was cloned from a Dvl-destag, zebrafish genomic DNA pool through nested PCR, according to the protocol outlined in the GenomeWalker Universal kit (BD Clontech). First-round PCR used primers API (5’-GTAATACGACTCATATAGGGC-3’) and IFN PRO (5’-GTTATTACGTCCGCGCAAGCAGGGA-3’), and the second-round PCR used primers AP2 (5’-ACTATAGGGGCAGGCGTG-3’) and IFN PRO NESTED (5’-CATTCCGAGATAGCCAGAG-3’). Following overnight incubation, samples were washed eight times in 25 mM Tris-HCl (pH 7.2), 150 mM NaCl, 0.05% Tween 20, and eluted in 40 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 1% Triton X-100 into which a general protease inhibitor cocktail (Roche), peptin hemisulfate (Sigma-Aldrich) was added to 10% of total volume. Following overnight incubation, samples were washed anti-FLAG M2 or anti-HA agarose affinity gel resin (Sigma-Aldrich), eluted for 20 min in ice-cold buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100 into which a general protease inhibitor cocktail containing 4-(2-aminoethyl)benzenesulfonfyl fluoride hydrochloride, benzamidine, bestatin HCl, E-64, EDTA, and leupeptin hemisulfate (Sigma-Aldrich) was added to 10% of total volume. Cell lysates were centrifuged at 16,000 × g for 15 min and soluble fractions were collected. Soluble lysates were transferred to plugged Handee MiniSpin columns (Pierce) containing 10 µl of packed gel volume of washed anti-FLAG M2 or anti-HA agarose affinity gel resin (Sigma-Aldrich). Samples were incubated on a rotating platform overnight at 4 °C. Following overnight incubation, samples were washed eight times in 25 mM Tris-HCl (pH 7.2), 150 mM NaCl, 0.05% Tween 20, and eluted in 40 µl of 2× lane marker nonreducing sample buffer (Pierce) during a 5-min incubation at 100 °C. Samples were reduced in 2-ME, separated by PAGE, and transferred to BioTrace nitrocellulose membranes (Pall Corporation) using the Mini Trans-Blot cell (Bio-Rad). Membranes were blocked in TBS (pH 7.5) with 0.1% Tween 20 containing 5% nonfat dried milk and exposed to rabbit anti-FLAG polyclonal (Affinity Bioreagents), anti-HA polyclonal (Invitrogen Life Technologies), or anti-myc polyclonal Ab (Affinity Bioreagents) overnight at 4 °C. Following overnight incubation, membranes were washed three times in TBST and incubated in goat anti-rabbit secondary Ab conjugated to alkaline phosphatase (Bio-Rad) for 1 h at room temperature. Membranes were washed three times in TBST. CDP-Star AP substrate (Novagen) was applied to membranes according to the manufacturer’s recommendations and the membranes were exposed to film.

Cell culture

293H cells (Invitrogen Life Technologies) were cultured at 37 °C, 6% CO2 in DMEM (high glucose) supplemented with 10% heat-inactivated FBS. ZFL cells were grown in sealed vessels at 28 °C in LDF medium, which consists of 5% Leibovitz’s l-15 medium, 35% DMEM (high glucose), and 15% F-12 nutrient mixture (Ham) supplemented with 5% heat-inactivated FBS, 0.5% heat-inactivated SeaGrow trout serum (East Coast Biologics), 50 ng ml−1 mouse epidermal growth factor, and 1× insulin-transferrin-selenium-X. Unless otherwise noted, all culture products were purchased from Invitrogen Life Technologies.

Luciferase reporter assays

293H and ZFL cells were plated in 24-well plates (Corning) so that they were 90–95% confluent on the day of transfection. Using Lipofectamine 2000 (Invitrogen Life Technologies), 400 ng of TICAM1 construct (mouse TICAM1, zebrafish TICAM1, or indicated deletion construct), 400 ng of reporter construct (NF-kB: pBIIx-luc; ISRE: ISRE-luc; zebrafish minimal Mx (ISRE) promoter: Mx187-luc; or zebrafish IFN promoter: pGL3-IFN Pro), and 10 ng of pRL-CMV, which served as a Renilla luciferase internal control to normalize data, were used to transfect 293H or ZFL cells. At 24 h posttransfection, 293H cells were lysed and luciferase activities were measured using the Dual-Luciferase Reporter Assay system (Promega). At 48 h posttransfection, ZFL cells were lysed and luciferase activities were measured using the Dual-Luciferase Reporter Assay system. All firefly luciferase light outputs were normalized to Renilla luciferase activities. Data are represented as fold induction over empty vector control. Error bars indicate the SEM for ≥3 replicates in a representative experiment.

Coimmunoprecipitation

293H cells were plated in 25-cm2 flasks (Corning) so that they were 90–95% confluent on the day of transfection. Using Lipofectamine 2000, cells were transfected with indicated amounts of plasmids totaling 8 µg (3 µg of zebrafish or mouse TICAM1 construct, 3 µg of interferon construct, 1 µg of pAdV-antagone, and 1 µg of pcDNA3.1-p35). At 48 h posttransfection, cells were washed twice with PBS (Invitrogen Life Technologies) and lysed for 20 min in ice-cold buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100 into which a general protease-inhibitor mixture containing 4-(2-aminoethyl)benzenesulfonfyl fluoride hydrochloride, benzamidine, bestatin HCl, E-64, EDTA, and leupeptin hemisulfate (Sigma-Aldrich) was added to 10% of total volume. Cell lysates were centrifuged at 16,000 × g for 15 min and soluble fractions were collected. Soluble lysates were transferred to plugged Handee MiniSpin columns (Pierce) containing 10 µl of packed gel volume of washed anti-FLAG M2 or anti-HA agarose affinity gel resin (Sigma-Aldrich). Samples were incubated on a rotating platform overnight at 4 °C. Following overnight incubation, samples were washed eight times in 25 mM Tris-HCl (pH 7.2), 150 mM NaCl, 0.05% Tween 20, and eluted in 40 µl of 2× lane marker nonreducing sample buffer (Pierce) during a 5-min incubation at 100 °C. Samples were reduced in 2-ME, separated by PAGE, and transferred to BioTrace nitrocellulose membranes (Pall Corporation) using the Mini Trans-Blot cell (Bio-Rad). Membranes were blocked in TBS (pH 7.5) with 0.1% Tween 20 containing 5% nonfat dried milk and exposed to rabbit anti-FLAG polyclonal (Affinity Bioreagents), anti-HA polyclonal (Invitrogen Life Technologies), or anti-myc polyclonal Ab (Affinity Bioreagents) overnight at 4 °C. Following overnight incubation, membranes were washed three times in TBST and incubated in goat anti-rabbit secondary Ab conjugated to alkaline phosphatase (Bio-Rad) for 1 h at room temperature. Membranes were washed three times in TBST. CDP-Star AP substrate (Novagen) was applied to membranes according to the manufacturer’s recommendations and the membranes were exposed to film.

Results

Identification of a full-length zebrafish TICAM1 ortholog exhibiting considerable sequence divergence from mammalian orthologs

The TIR domain of the zebrafish TICAM1 was originally described by Meijer et al. (28). The TICAM1 TIR domain cDNA sequence was used to search zebrafish expressed sequence tag and genome resources. A predicted full-length TICAM1 sequence was identified using the online resources of the Zebrafish Genome Project and zebrafish TICAM1 was cloned. The open reading frame of zebrafish TICAM1 is 1701 bp. Comparisons of syntenic regions

Phylogenetic reconstruction

Representatives from a broad range of TIR domain-containing proteins were aligned with DIALIGN (24), a multiple sequence alignment program that compares amino acid sequences via a segment-to-segment approach with no gap penalties imposed. This alignment method is useful in comparing proteins with similar domains that are otherwise unrelated. Molecular phylogenetic analyses were performed using PHYLIP, software version 3.6b (distributed by the author, J. Felsenstein, University of Washington, Seattle, WA at http://evolution.gs.washington.edu/phylip.html (25). Sequences were bootstrapped 1000 times with the program SEQBOOT and these bootstrapped amino acid sequences were used to compute distance matrices with the program PROTDIST, according to the Jones–Taylor–Thornton (26) model of amino acid replacement. Phylogenetic trees based upon these data were generated by the neighbor-joining method (27), using the program NEIGHBOR, and from these trees, an extended majority rule consensus tree was created with the program CONSENSE.

4518 TICAM SIGNALING DIFFERS IN BASILY DIVERGING VERTEBRATES

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among zebrafish, mouse, and human genomes indicate that zebrafish TICAM1 is orthologous to mammalian TICAM1s. Despite this conserved synteny, the zebrafish TICAM1 gene encodes a smaller protein (566 aa) than does the mouse (732 aa) or human ortholog (712 aa). A ClustalW-based alignment (AlignX, Vector NTI; Invitrogen Life Technologies) identified significant gaps in the N and C termini of zebrafish TICAM1 amino acid sequence when compared with human and mouse, raising questions about zebrafish TICAM1’s capacity to transduce downstream signals (Fig. 1A). Furthermore, no identifiable TRAF6-binding motif at consensus positions 265–270 was identified in zebrafish TICAM1. Some sequence divergence was also noted in the BB loop of TIR domain and the RHIM domain (consensus positions 682–720), raising questions about zebrafish TICAM1’s capacity to interact with TLR3 and RHIM domain-containing proteins (RIP1, RIP3), respectively. Zebrafish TICAM1 shares 26% sequence identity and 46% sequence similarity with human and mouse across aligned residues, excluding gaps. Zebrafish TICAM1 is comprised of 318-aa N terminus, a 137-aa TIR domain, and a 110-aa C terminus (Fig. 1B).

FIGURE 1. TICAM1 alignment and domain architecture. A, Alignment of human, mouse, and zebrafish TICAM1 orthologs using AlignX (Vector NTI; Invitrogen Life Technologies), a ClustalW-based algorithm. Dashes indicate gaps in amino acid sequence. The TRAF6-binding motif, TIR domain, and RHIM domain are identified by underlines and annotations. The arrow above the sequence Met-Ile-Gly-Asn at positions 547–550 identifies the amino acid residues targeted by QuikChange mutagenesis to make the Ala-Ala-Ala-Ala RHIM domain mutant. Zebrafish TICAM1 is 566 aa; human TICAM1 is 712 aa; and mouse TICAM1 is 732 aa. B, Both the N- (1–318 aa) and C-terminal (456–566 aa) domains of zebrafish TICAM1 are truncated, calling into question their capacity to interact with homologs of proteins known to interact in mammals.
FIGURE 2. **TICAM1** and **TICAM2** share a close evolutionary relationship. 
A. Zebrafish TICAM1 forms a monophyletic group with mouse and human TICAM1 and mouse and human TICAM2, exhibiting 100% bootstrap support. Representatives from a broad range of TIR domain-containing proteins were aligned with DIALIGN (55), a multiple sequence alignment program that compares amino acid sequences via a segment-to-segment approach with no gap penalties imposed. All bootstrap values <91% are shown while those ≥91% are not shown. 

**TICAM1 and TICAM2 evolved in a gene duplication event**

Mammalian TLRs use four different TIR domain-containing adapter proteins to transduce signals: MyD88, Mal, TICAM1, and TICAM2 (TRAM) (28, 29). All but TICAM2 have been identified in fishes, and accumulating evidence indicates that TICAM2 does not exist in fishes (30). Phylogenetic analysis demonstrates that TICAM1 and TICAM2 form a monophyletic group with 100% bootstrap support (Fig. 2A). These findings are supported by Iliev et al. (30) and suggest that TICAM1 and TICAM2 are sisters from a gene duplication event. The TICAM gene pair is part of a paralogon, or a group of paralogous regions within the same species (31, 32), in the human genome (Fig. 2B). The paralogon includes Hsa19p13 with FEM1A, TICAM1, TMD1, and SEMA6B; Hsa5q21-q23 with FEM1C, TICAM2, TMD7, and SEMA6A; Hsa15q21-q25 with FEM1B, TMD3, and SEMA6D; and Hsa1q21-q22 with SEMA6C and other genes. These four paralogous chromosome segments probably originated in the two rounds of genome duplication that likely preceded the vertebrate radiation (33).

The zebrafish TICAM1 gene on Chr:22 (ENSDART 0000062685) is embedded in one of two zebrafish duplicates of the region of Hsa19p13 that surrounds TICAM1, with orthologous near-neighbors in both genomes (Fig. 3A, bottom panel). The other copy of the human region lies on zebrafish chromosome 2 in the Zv6 version of the zebrafish genome (Fig. 3A, top panel). Both zebrafish duplicate regions include genes slightly further away on Hsa19p13.3 (Fig. 3A, middle panel). The genomic neighborhood of TICAM2 is better conserved in zebrafish than is the TICAM1-containing region, despite the lack of a TICAM2 ortholog (Fig. 3B). Genes that immediately neighbor TICAM2 in the human genome have orthologs that are neighbors in the zebrafish genome (FEM1C and TMED7), but without an ortholog of TICAM2 between them (Fig. 3B). In humans, a sequence appropriate to encode a human membrane-bound isoform of TICAM2 has been identified but not described (GenBank accession no. AY304581). It apparently occurs through an alternative splicing event that combines the open reading frame of TMED7 with TICAM2, its 3' neighbor.
To further test for the presence of a zebrafish ortholog of TICAM2, we performed 3’ RACE to determine whether such a membrane-bound form of TICAM2 could be identified in zebrafish. A single fragment was amplified, but analysis showed it to consist solely of sequences orthologous to TMED7 (data not shown).

Comparative genomic analysis of available sequence data reveals that in the opossum (Monodelphis domestica), a marsupial diverging from the placental lineage ~175 million years ago (34), part of ENSMODT00000019962 on chromosome 6 is orthologous to TICAM2 and part to TMED7; the opossum FEM1C ortholog (ENSMODT00000019962) is unlinked, appearing on chromosome 3. In Xenopus, as in zebrafish, FEM1C (ENSSXETT00000040604) and TMED7 (ENSSXETT00000040594) genes are adjacent to each other, but TICAM2 does not appear between them. In chicken (Gallus gallus), TMED7 (ENSGALT00000034557) is on chromosome Z, but FEM1C (ENSGALT0000013361) is unassigned making it difficult to draw conclusions. The nonvertebrate chordate Ciona intestinalis has an ortholog of TICAM genes (ENSCINT00000022578), of TMED7/3/1 (ENSCINT0000003864), and of FEM1 genes (ENSCINT00000027118). These three genes are not adjacent in the Ciona genome, but the FEM1 ortholog has loci that are close neighbors with loci that are close neighbors of FEM1C and FEM1B in human.

These genomic data suggest the following history for TICAM1 and TICAM2 (Fig. 4). A chromosome in the last pregenome duplication common ancestor of vertebrates possessed FEM1, TICAM, and TMED genes in that order, in addition to SEMA6, KCNN, and other genes (Fig. 4A). After the first round of genome duplication (R1), the TICAM gene on one of the duplicate chromosomes was lost (Fig. 4B), and after the second round of genome duplication (R2), produced what became TICAM1 and TICAM2 (Fig. 4, C and E). In the teleost lineage, the TICAM2 gene was eventually lost, but TICAM1 was retained (Fig. 4D). Some of the other genes in the paralogon are present in duplicate copy in teleosts today, due to a genome duplication event in the teleost ancestry ~300 million years ago (35–39) (data not shown in the figure). According to this proposed history, the current absence of TICAM2 from the genome databases of chicken and Xenopus could be due either to its absence from the animals’ genomes or to a hole in the genome libraries, although the finding that FEM1C and TMED7 are adjacent in the frog would suggest that the gene has been lost from the frog genome in an event independent from the loss from the fish genomes. Given that rayfin fish genomes lack TICAM2 (30), this gene may have been lost early after the divergence of rayfin and lobefin fishes 450 million years ago.

Zebrafish TICAM1 fails to interact with TRAF6 but can associate with TLR3, RIP1, and TBK1

Mammalian TICAM1 interacts with TLR3, TRAF6, TBK1, and RIP1 (15, 17, 40, 41) and these interactions potentiate downstream antiviral signaling, leading to the activation of NF-κB and IFN-β. Zebrafish TICAM1’s unique structural features, particularly the presence of a hydrophilic glutamine residue in the typically hydrophobic θi position, the absence of an obvious TRAF6-binding motif, the truncation of its N- and C-terminal domains, and the sequence differences observed in the RHIM domain (Fig. 1), call into question its capacity to interact with TLR3, TRAF6, TBK1, and RIP1 and transduce vital immune signals. To address zebrafish TICAM1’s capacity to associate with these known interactors, coimmunoprecipitation experiments were performed (Fig. 5).

Zebrafish TLR3-HA and zebrafish TICAM1–3×FLAG were overexpressed in 293H cells for 48 h, and following a coimmunoprecipitation experiment, were shown to associate with one another (Fig. 5A, middle panel, lane 1). Empty vector controls were included to show that the interactions were not the result of nonspecific interactions (Fig. 5A, middle panel, lanes 2 and 3). Through coimmunoprecipitation, it was shown that zebrafish TICAM1 associated with zebrafish RIP1 (Fig. 5B, middle panel, lane 1) when overexpressed in 293H cells. Despite its truncated N terminus, zebrafish TICAM1 also is capable of interacting with zebrafish TBK1 (Fig. 5C, lower middle panel, lane 1). Like mammals, this interaction relies on the N terminus of TICAM1, as a TICAM1 protein lacking the N terminus failed to associate with TBK1 (Fig. 5C, lower middle panel, lane 3). Despite the similarity, the functional significance of the TICAM1–TBK1 interaction in zebrafish, based upon the observations described later, is in question. In contrast, following overexpression in 293H cells, zebrafish TICAM1 failed to coimmunoprecipitate zebrafish TRAF6 or mouse TRAF6 (Fig. 5D, middle panel, lanes 1 and 2). Analysis of
zebrafish genome databases fails to uncover a sequence for a second TRAF6 gene, and thus the potential for another TRAF6 co-evolving with TICAM1 in a way that would allow for their protein-protein interaction to be maintained appears extremely unlikely. Zebrafish TRAF6 exhibits 58% identity and 73% conservation with mouse TRAF6. Mouse TICAM1 associated both with zebrafish and with mouse TRAF6 (Fig. 5D, middle panel, lanes 4 and 5). These data, coupled with the alignment data, imply that zebrafish TICAM1 does not interact with zebrafish TRAF6 and that NF-κB activation occurs through an alternative mechanism. Based upon the coimmunoprecipitation data collected, the evidence indicates that NF-κB activation may occur through a TICAM1-RIP1 association.

Zebrafish TICAM1 activates NF-κB in human 293H and zebrafish ZFL cells

Alignment of zebrafish TICAM1 with mammalian orthologs reveals clear sequence differences that may confound its capacity to transduce signals (Fig. 1A). Through overexpression of TICAM1 and various deletion constructs, it has been shown that full-length human TICAM1 can activate NF-κB, IFN-β promoter, and ISRE luciferase reporters and that individual TICAM1 domains (N terminus, C terminus, and/or TIR domains) contribute differentially to this signaling capacity (13, 40, 42). To examine zebrafish TICAM1 and its domains’ signaling capacities, full-length zebrafish TICAM1, along with several zebrafish TICAM1 domain deletion constructs, a zebrafish TICAM1 RHIM mutant construct, and a mouse TICAM1-positive control, were overexpressed in human 293H and zebrafish ZFL cells to assay their effects on NF-κB activation, using the reporter construct pBIIx-luc (43) (Fig. 6, A and B). In 293H cells (Fig. 6A), full-length zebrafish TICAM1 retained a capacity to activate NF-κB (9.3-fold induction), albeit at levels below that of full-length mouse TICAM1 (21.6-fold induction). Similarly in ZFL cells (Fig. 6B), full-length TICAM1 activated NF-κB (3.3-fold induction), but in this instance, mouse TICAM1 exhibited diminished NF-κB activation (1.2-fold induction). Overexpression of a zebrafish TICAM1 N-terminal deletion construct enhanced NF-κB activation over zebrafish full-length TICAM1 in both 293H and ZFL cells, with 24.2-fold (Fig. 6A) and 4.2-fold (Fig. 6B) activations noted, respectively. These data indicate that the TIR and C-terminal domains of zebrafish TICAM1 are responsible for NF-κB activation and are corroborated by the diminished activities noted upon overexpression of deletion constructs missing both the N and C termini (ZFL: 1.4-fold induction; 293H: 1.1-fold induction) or the C-terminal domain (ZFL: 2.0-fold induction; 293H: 2.1-fold induction) (Fig. 6, A and B). Deletion of the TIR domain also diminished NF-κB activation in ZFL cells (1.4-fold induction) (Fig. 6B), but appeared not to disrupt NF-κB signaling in 293H cells (11.2-fold induction), when compared with the full-length TICAM1 result (9.3-fold induction) (Fig. 6A). Furthermore, site-directed mutagenesis of residues 547–550 (Met-Glu-Asp-Asp) in the RHIM domain of zebrafish TICAM1, which correspond to residues 687–690 in human TICAM1 and 688–691 in mouse TICAM1 (Val-Gln-Leu-Gly), to Ala-Ala-Ala-Ala, as previously demonstrated by Meylan et al. (40), led to a diminished capacity for zebrafish TICAM1 to activate NF-κB.

FIGURE 5. Zebrafish TICAM1 associates with TLR3, RIP1, and TBK1, but not TRAF6. A, Zebrafish TICAM1 (middle panel, lane 1) coimmunoprecipitates with zebrafish TLR3 (top panel, lane 1). B, Zebrafish RIP1 (middle panel, lane 1) coimmunoprecipitates with zebrafish TICAM1 (top panel, lane 1). C, Zebrafish TBK1 (lower middle panel, lane 1) coimmunoprecipitates with zebrafish TICAM1 (top panel, lane 1). Zebrafish TICAM1 lacking the N-terminal residues (top middle panel, lane 3) fails to associate with zebrafish TBK1 (lower middle panel, lane 3), despite evidence of strong expression in whole cell lysates. D, Zebrafish TRAF6 (middle panel, lanes 1) fails to coimmunoprecipitate with zebrafish TICAM1 (top panel, lane 1), despite strong expression levels in the whole cell lysate (bottom panel, lane 2). Mouse TICAM1 coimmunoprecipitates with both zebrafish TRAF6 (middle panel, lane 4) and mouse TRAF6 (middle panel, lane 5).
Overexpression of zebrafish TICAM1 activates NF-κB and type I IFN promoter, but in an ISRE-independent manner. Luciferase assays demonstrate the effect of mouse TICAM1, zebrafish TICAM1, and zebrafish TICAM1 mutants on NF-κB activation in 293H (A) and ZFL (B) cells. A RHIM domain mutation causes a decrease in NF-κB-luciferase activation relative to the control zebrafish TICAM1 in 293H cells (A). C. Unlike mouse TICAM1, zebrafish TICAM1, and deletion mutants demonstrate limited ISRE-luciferase activation in 293H cells. D and E. In ZFL cells, minimal ISRE-luc and Mx187-luc activation is observed upon overexpression of mouse TICAM1, zebrafish TICAM1, and zebrafish TICAM1 deletion constructs. F. Despite the absence of strong ISRE-luciferase activity, type I IFN promoter-luciferase activation is observed upon overexpression of mouse TICAM1, zebrafish TICAM1, and zebrafish TICAM1 deletion constructs. All firefly luciferase activities are normalized to constitutively active Renilla luciferase levels. Data are presented as fold induction over empty vector. Error bars represent one SEM. Empty, empty vector control; Mouse, full-length mouse TICAM1, Danio, full-length zebrafish TICAM1; ΔN, N-terminal deletion (1–311 aa) of zebrafish TICAM1; ΔC, C-terminal deletion (485–566 aa) of zebrafish TICAM1; ΔTIR, deletion of TIR domain (312–482 aa) of zebrafish TICAM1; ΔNΔC, deletion of N- and C-termini of zebrafish TICAM1 leaving TIR domain (312–482 aa); RHIM Mutant, site-directed mutagenesis of amino acids 547–550 from Met-Ile-Gly-Asn to Ala-Ala-Ala-Ala.

**FIGURE 6.** Overexpression of zebrafish TICAM1 activates NF-κB and type I IFN promoter, but in an ISRE-independent manner. Luciferase assays demonstrate the effect of mouse TICAM1, zebrafish TICAM1, and zebrafish TICAM1 mutants on NF-κB activation in 293H (A) and ZFL (B) cells. A RHIM domain mutation causes a decrease in NF-κB-luciferase activation relative to the control zebrafish TICAM1 in 293H cells (A). C. Unlike mouse TICAM1, zebrafish TICAM1, and deletion mutants demonstrate limited ISRE-luciferase activation in 293H cells. D and E. In ZFL cells, minimal ISRE-luc and Mx187-luc activation is observed upon overexpression of mouse TICAM1, zebrafish TICAM1, and zebrafish TICAM1 deletion constructs. F. Despite the absence of strong ISRE-luciferase activity, type I IFN promoter-luciferase activation is observed upon overexpression of mouse TICAM1, zebrafish TICAM1, and zebrafish TICAM1 deletion constructs. All firefly luciferase activities are normalized to constitutively active Renilla luciferase levels. Data are presented as fold induction over empty vector. Error bars represent one SEM. Empty, empty vector control; Mouse, full-length mouse TICAM1, Danio, full-length zebrafish TICAM1; ΔN, N-terminal deletion (1–311 aa) of zebrafish TICAM1; ΔC, C-terminal deletion (485–566 aa) of zebrafish TICAM1; ΔTIR, deletion of TIR domain (312–482 aa) of zebrafish TICAM1; ΔNΔC, deletion of N- and C-termini of zebrafish TICAM1 leaving TIR domain (312–482 aa); RHIM Mutant, site-directed mutagenesis of amino acids 547–550 from Met-Ile-Gly-Asn to Ala-Ala-Ala-Ala.

**Zebrasfish TICAM1 activation of type I IFN occurs through N-terminal-independent and ISRE-independent mechanisms**

Mammalian TICAM1 has been shown to play an essential role in TLR3- and TLR4-mediated IFN-β activation through an IRF3/IRF7-dependent mechanism (12, 16, 44, 45). TBK1 is brought into a complex with TICAM1 at its N terminus via TRAF3 and phosphorylates IRF3 and IRF7, allowing for its eventual nuclear translocation and up-regulation of IFN-β (15, 17, 19–21). Overexpression of zebrafish TICAM1 led to minimal activation of an ISRE-luciferase reporter in 293H cells (2.6-fold above empty vector) (Fig. 6C) and no activation in ZFL cells (Fig. 6D). Similarly, using the zebrafish-derived Mx187-luc construct, which is a minimal IFN-inducible Mx promoter construct possessing two ISREs, a 3.4-fold induction over empty vector control was observed in ZFL cells (Fig. 6E). In contrast, mouse TICAM1 overexpression robustly activated ISRE-luciferase reporter in 293H cells (136-fold above empty vector) (Fig. 6C); however, when compared with the 293H data, it exhibits minimal activation of ISRE in ZFL cells (ISRE-luc: 1.2-fold above empty vector; Mx187-luc: 4.9-fold above empty vector) (Fig. 6, D and E). Despite the unexpected absence of robust ISRE activation in ZFL cells, as seen in 293H cells, zebrafish type I IFN was activated by zebrafish TICAM1 (9.5-fold induction) (Fig. 6F). Mouse TICAM1 strongly activated the zebrafish type I IFN promoter (22.9-fold induction) (Fig. 6F). Overexpression of the zebrafish TICAM1 N-terminal deletion construct led to an even more enhanced type I IFN activation (16.9-fold induction) than with full-length zebrafish TICAM1 (Fig. 6F). These results correlated with the observed NF-κB activation, in which the N-terminal deletion construct exhibited stronger activation (Fig. 6, A and B). The C-terminal deletion retained a capacity to activate IFN promoter (Fig. 6F) (10.8-fold induction), but it appeared not to be strongly ISRE-induced in ZFL cells (ISRE-luc: 1.7-fold induction; Mx187-luc: 3.4-fold induction) (Fig. 6, D and E). Interestingly, site-directed mutagenesis of the C-terminal RHIM domain at residues 547–550 from Met-Ile-Gly-Asn to Ala-Ala-Ala-Ala disrupted IFN promoter activation (4.0-fold induction over empty vector control), leading to a 2.4-fold reduction from the
wild-type TICAM1 and 2.7-fold reduction from the C-terminal deletion TICAM1 construct (Fig. 6F). The apparent difference in IFN promoter activation capacity between the C-terminal deletion TICAM1 construct and the RHIM mutant indicates not only an activating role for the RHIM domain in IFN activity, but also a possible inhibitory role for other portions of the C terminus. Constructs lacking the TIR domain retained a capacity to activate the IFN promoter construct (6.7-fold induction) (Fig. 6F), but again exhibited minimal ISRE activation in 293H (1.9-fold induction) (Fig. 6C) or ZFL (ISRE-luc: 1.5-fold induction; Mx197-luc: 2.0-fold induction) cells (Fig. 6, D and E). Overexpression of the combined N-terminal, C-terminal deletion, which consists largely of the TIR domain alone, resembles the data presented by Yamamoto et al. (13). The IFN promoter construct was activated 2.4-fold in ZFL cells (Fig. 6F); the ISRE-luc reporter was induced 1.5-fold in 293H cells (Fig. 6C) and 1.3-fold in ZFL cells (Fig. 6D); the Mx187-luc reporter was activated 1.1-fold above empty vector control (Fig. 6E). These data show that the TIR domain of zebrafish TICAM1, when expressed alone, has a dominant-negative effect.

Discussion
Phylogenetic analyses imply that fish type I IFNs form clades distinct from those of other mammalian and avian type I IFNs and thus cannot be classified as being α- or β-like, except by function (1, 2). These findings, coupled with the unique gene structure of fish type I IFNs, raise interesting questions about how they are triggered to signal and whether TLR-mediated pathways are responsible for their induction. In describing the first fish type I IFN, which we cloned from the zebrafish, our laboratory noted its antiviral potential, as it was inducible, in ZFL cells, by the TLR3 agonist polyinosinic-polycytidylic acid (poly(I:C)) and was protective of cells infected with snakehead rhabdovirus (1). Subsequently, we noted that zebrafish type I IFN exerts its antiviral effects through the induction of antiviral proteins like Mx (22) and can itself be induced in vivo by snakehead rhabdovirus (46).

The findings described herein illustrate a TICAM1-dependent mechanism by which fish type I IFN expression can be induced. In mammals, TLR3 and TLR4 signaling through TICAM1 leads to the specific up-regulation of IFN-β. Direct IFN-α up-regulation through TICAM1-dependent pathways has yet to be described and indeed may not occur. Furthermore, based upon the current literature, direct IFN-α induction appears restricted to MyD88-dependent signaling pathways (6). These findings are interesting in light of the fact that zebrafish type I IFN, as already discussed, cannot itself be induced in vivo by snakehead rhabdovirus (46).

TLR3 recognizes dsRNA (3) and endogenous cellular RNA (47) and unleashes a potent MyD88-independent antiviral pathway (12, 13). Human TLR3 is a 904-aa long protein expressed on the cell surface of human fibroblast epithelial cells (48) and within dendritic cells (49, 50). It possesses a horseshoe-shaped ectodomain consisting of 23 leucine-rich repeats (51, 52), a transmembrane domain, and a TIR domain required for downstream signaling (3). TLR3 orthologs have been cloned from a broad range of vertebrates, from zebrafish to humans (53). The actual role of TLR3 in mediated antiviral responses is under scrutiny (54); however, specific evidence does indicate a real immunological function. Specifically, TLR3 is believed to interact with dsRNA intermediates of single-stranded RNA viruses like respiratory syncytial virus (55) or West Nile virus (56). Interactions were also noted between TLR3 and mouse CMV, a DNA virus (57), and TLR3 and reovirus, a dsRNA virus (3, 54).

Interestingly, TLR3 was shown to be activated in response to nonviral dsRNAs as well. dsRNAs from eggs of the helminth parasite Schistosoma mansoni were shown to trigger an immune response through the TLR3 signal transduction cascade (58). TLR3 activation by ligands appears to occur in the acidic compartments of early phagolysosomes or endosomes, as an acid pH is required (59). Disruption of acidic pH blocked TLR3 activation by poly(I:C). Additionally, TLR3 must form multimers to be active (59). Upon activation, the antiviral signal cascades through the adaptor molecule TICAM1 (12, 13). The TLR3-TICAM1 interaction requires their respective BB loops. The consensus sequence for the BB loop, a conserved sequence contained within the TIR domain and shown to be important for TLR and TICAM1 signaling, is RDXϕxϕ2G, where x represents any residue and ϕ represents any hydrophobic residue. The mutation of the ϕ2 residue from proline to histidine results in the inability of the human TICAM1 to interact with human TLR3 and thereby up-regulate human IFN-β (12). Similarly, when the ϕ2 position of human TLR3 was mutated from alanine to histidine, TLR3 failed to interact with human TICAM1 and therefore failed to up-regulate IFN-β (12). The BB loop of the putative zebrafish TICAM1 differs from the consensus sequence and from the mammalian TICAM1s (Fig. 1A). The proline at ϕ2 is conserved in known mammalian TICAM1s. In the putative zebrafish TICAM1, this position contains an alanine instead. In addition, in zebrafish, ϕ2 is occupied by a glutamine, and the x position contains an alanine. We believe that the differences noted in these positions may be important to TLR3-TICAM1 signaling in zebrafish; however, our coimmunoprecipitation data indicate that TLR3 and TICAM1 can associate and thus this interaction appears conserved despite sequence differences (Fig. 5A). It is noteworthy that human and mouse TLR3 contain alamines in the ϕ2 positions, while human and mouse TICAM1 contain prolines in their ϕ2 positions. These occurrences
are reversed in zebrafish, where TLR3 possesses a proline in its $\phi_2$ position and putative TICAM1 possesses an alanine in its $\phi_2$ position. This “complementary switch” may be noteworthy from an evolutionary perspective, but may also have practical impacts on TLR3-TICAM1 interactions and signaling.

In mammals, TBK1 interacts with the N terminus of TICAM1 and then phosphorylates IRF-3 and IRF-7, leading to up-regulation of IFN-$\beta$ (15–18). Although zebrafish TICAM1 retains the capacity to communoprecipitate TBK1 through its N terminus (Fig. 5c), indicating this interaction can occur, it is unclear what role the TBK1 interaction plays in the up-regulation of zebrafish type I IFN. In fact, upon deletion of the N terminus responsible for the TICAM1-TBK1 interaction in mammals, enhanced IFN activation was noted (Fig. 6f), indicating that the N terminus, and thus potentially the TBK1 interaction, has one or both of the following effects: it may facilitate an inhibitory portion of the zebrafish TICAM1 pathway, or it may sterically hinder TICAM1’s interaction with other protein partners that are yet to be described.

It was noteworthy that zebrafish TICAM1, lacking an apparent TRAF6-binding motif with the consensus PxxExxA/CxAr (P represents proline, x represents any amino acid, and [Ac/Ar] represents an acidic or aromatic amino acid), failed to communoprecipitate zebrafish TRAF6 (Fig. 5d). This finding confounds TRAF6’s role in mediating a NF-$\kappa$B response, at least in the zebrafish, and supports the assertion by Gohda et al. (60) that the TICAM1-TRAF6 interaction may be nonessential in the mammalian TLR3 pathway. Our finding is in contrast to reports by Sato et al. (15) and Jiang et al. (41), who showed that TICAM1-TRAF6 interaction is important for NF-$\kappa$B activation in human 293 cells. Furthermore, overexpression of an N-terminal deletion zebrafish TICAM1 in 293H and ZFL cells, causing enhanced NF-$\kappa$B activation relative to full-length zebrafish, was noted (Fig. 6, A and B). These data lend additional credence to an aforementioned potential inhibitory role the N terminus plays in zebrafish TICAM1. Indeed, our data indicate that TICAM1 signaling is driven by the TIR domain and C terminus, with the RHIM domain playing an important role. Our finding that zebrafish TICAM1 and RIP1 can interact with one another (Fig. 5b), and that mutation of the TICAM1 RHIM domain at residues 547–550 from Met-Ile-Gly-Asn to Ala-Ala-Ala resulted in a diminished capacity for IFN activation (Fig. 5a), suggests that this activation occurs because of this association, as is the case in mammals (40, 42).

The evolutionary history of TLR-mediated induction of type I IFN responses provides essential perspective into the origins and complexities of antiviral immunity. The data presented herein represent the first insights into the underlying mechanisms basally diverging vertebrates use to counter pathogens through TICAM1-dependent signaling. Our findings also demonstrate that TICAM1-dependent induction of type I IFNs is an ancient mechanism, although the means by which TICAM1 induces this particular zebrafish type I IFN is through an apparently alternative signaling pathway. Although the complete details of how TICAM1 induces this zebrafish type I IFN are yet to be determined, our findings may be predictive in defining undiscovered TICAM1-dependent, IRF3/7-independent type I IFN induction schemes in mammals, perhaps through a RHIM-dependent mechanism. In mammals, the TICAM1-TBK1 interaction is essential to type I IFN activation (15). Our data show that while this interaction also occurs in zebrafish (Fig. 5c), its importance in mediating the TLR-mediated antiviral response is unclear. In particular, our surprising finding that a TICAM1 construct lacking the N-terminal domain necessary for interaction with TBK1 exhibited an enhanced activation of the zebrafish type I IFN promoter (Fig. 6f) indicated that other parts of the TICAM1 protein play a role in this activation. Although deletion of the C terminus of TICAM1 did not alter IFN promoter activation in ZFL cells, mutation of the C-terminal RHIM domain at residues 547–550 from Met-Ile-Gly-Asn to Ala-Ala-Ala-Ala resulted in a diminished capacity for IFN activation (Fig. 6f). These findings show a clear role for the RHIM domain in IFN activation but also indicate that other portions of the C terminus may negatively regulate IFN activation. Further investigation of the zebrafish pathway may provide the context necessary to resolve some of the discrepancies noted in characterizing the role and importance of TICAM1’s domains in tetrapods.

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Disclosures
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References


