Genomic and Functional Uniqueness of the TNF Receptor-Associated Factor Gene Family in Amphioxus, the Basal Chordate

Shaochun Yuan, Tong Liu, Shengfeng Huang, Tao Wu, Ling Huang, Huiling Liu, Xin Tao, Manyi Yang, Kui Wu, Yanhong Yu, Meiling Dong and Anlong Xu

*J Immunol* 2009; 183:4560-4568; Prepublished online 14 September 2009; doi: 10.4049/jimmunol.0901537

http://www.jimmunol.org/content/183/7/4560

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2009/09/14/jimmunol.0901537.DC1

**References**

This article cites 38 articles, 12 of which you can access for free at: http://www.jimmunol.org/content/183/7/4560.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Genomic and Functional Uniqueness of the TNF Receptor-Associated Factor Gene Family in Amphioxus, the Basal Chordate

Shaochun Yuan, Tong Liu, Shengfeng Huang, Tao Wu, Ling Huang, Huiling Liu, Xin Tao, Manyi Yang, Kui Wu, Yanhong Yu, Meiling Dong, and Anlong Xu

The TNF-associated factor (TRAF) family, the crucial adaptor group in innate immune signaling, increased to 24 in amphioxus, the oldest lineage of the Chordata. To address how these expanded molecules evolved to adapt to the changing TRAF mediated signaling pathways, here we conducted genomic and functional comparisons of four distinct amphioxus TRAF groups with their human counterparts. We showed that lineage-specific duplication and rearrangement were responsible for the expansion of amphioxus TRAF1/2 and 3 lineages, whereas TRAF4 and 6 maintained a relatively stable genome and protein structure. Amphioxus TRAF1/2 and 3 molecules displayed various expression patterns in response to microbial infection, and some of them can attenuate the NF-κB activation mediated by human TRAF2 and 6. Amphioxus TRAF4 presented two unique functions: activation of the NF-κB pathway and involvement in somite formation. Although amphioxus TRAF6 was conserved in activating NF-κB pathway for antibacterial defense, the mechanism was not the same as that observed in humans. In summary, our findings reveal the evolutionary uniqueness of the TRAF family in this basal chordate, and suggest that genomic duplication and functional divergence of the TRAF family are important for the current form of the TRAF-mediated signaling pathways in humans. The Journal of Immunology, 2009, 183: 4560–4568.

Tumor necrosis factor receptor-associated factors (TRAFs) are crucial adaptors linking immune receptors and intermediate signal transducers to a variety of immune responses and developmental processes. There are six TRAFs in mammals, three in Drosophila, and one in Caenorhabditis elegans (1). The TRAF family can be classified into three major groups, two ancestral groups, TRAF4 and TRAF6, and the newly evolved group comprising TRAF1, 2, 3, and 5 (2). All TRAF proteins share a similar structure but differ from one another mainly in physiological function.

DTRAF1, the ortholog of human TRAF4, is predominantly expressed during nervous system development and in epithelial progenitor cells (3). It also binds the ste20 kinase msn and specifically activates the JNK apoptosis pathway when triggered by Eiger, a unique TNF homologue in Drosophila (4). However, human TRAF4 is not associated with classical TNFRs but serves as a negative regulator of TRIF (Toll/IL-1R resistance domain containing adaptor-inducing IFN-β)-dependent pathways in the vertebrate TLR system (5). Several studies also implicate its role in human diseases and carcinogenesis (6, 7).

DTRAF2, the null mutant of which shows strong immune deficiencies and affects normal dorsal-ventral (D-V) patterning in Drosophila embryos, physically and functionally interacts with Pelle to mediate the Toll-MyD88-Dorsal pathway (8, 9). Humans TRAF6, the ortholog of DTRAF2, has not only conserved its ancestral role in TLR signal transduction but also adapted to the increasingly complex TNF system, having diverse functions from immunity to development processes, such as the development of lymph nodes, skin, and the CNS (10). In addition, human TRAF6 can be recruited by the IL-1R subfamily that binds specific IL-1-related cytokines through their extracellular Ig-like structures to participate in multiple immunological and inflammatory processes (11).

Although knowledge of the functions of DTRAF3 remains elusive, those of TRAF1, 2, 3, and 5 are well documented in humans. Upon differing TNFR engagement, TRAF2 and 5 are involved in the activation of JNK and NF-κB signaling (12, 13), whereas TRAF3 functions as an inhibitor of TRAF2- or 5-mediated activation of the noncanonical NF-κB pathway (14, 15). In addition to the cooperation with classical TNFRs, new studies highlight their crucial roles in the induction of type I IFN, the critical component of innate defense against viral infections. For example, TRAF2 participates in the antiviral action of IFN by rapidly binding to the IFNAR1 subunit (16). TRAF3 directly and specifically interacts with several components of antiviral pathways, such as TRIF, TBK1, and Cardif, demonstrating its...
unique role as a positive regulator of type I IFN production (17–19).

It is clear that functions of TRAFs vary substantially among groups and between Drosophila and humans, thus it is of particular interest to discover how these conserved adaptors increased in number and evolved their protein structures to adapt to the changing functions involved in TRAF-mediated pathways, progressing from Drosophila to humans via intermediate species. One of the key intermediate species, amphioxus, represents the oldest lineage of the chordata and contains 24 TRAFs, 48 TLRs, and a sophisticated TNF system not observed in other invertebrates (20). Thus, investigation into amphioxus TRAFs is not only of importance in revealing the novel functions of this complex defense systems in this primitive species but also will help to shed light on the evolution and development of TRAF-mediated signaling in humans.

Materials and Methods

Adults and embryos, cells, and reagents

Adult Chinese amphioxus (Branchiostoma belcheri tsingtauense (Bbt)) were obtained from Qingdao, China. Samples of the blastula, gastrula, neurula, and larva were collected in the breeding season. Human HEK293T or Hela cells were grown in DMEM (Invitrogen) supplemented with 10% FCS and antibiotics. LPS of E. coli 0111:B4 was purchased from Sigma-Aldrich.

Sequence data

The draft genome sequence of the amphioxus Branchiostoma floridae (Bf) and the corresponding gene predictions can be accessed on the Branchiostoma floridae version 1.0 website of the Joint Genome Institute, U.S. Department of Energy (http://genome.jgi-psf.org/Brafl1/Brafl1.home.html). Sequences of four amphioxus TRAFs were deposited in the National Center for Biotechnology Information database and assigned as BbtTRAF2a (EF210343), BbtTRAF3a (EF210344), BbtTRAF4 (EF210345), and BbtTRAF6 (EF210346).

Cloning of BbtTRAFs cDNAs and genomic sequences

Full-length cDNAs of BbtTRAF2a, 3a, 4, and 6 were obtained by RT-PCR, 5'-RACE and 3'-RACE using gene specific primers according to the GeneRACE Kit (Invitrogen). Genomic sequences of BbtTRAF2a, 3a, 4, and 6 were amplified by specific primers based on their open reading frame (ORFs) (shown in supplemental Table S1).

Southern blot analysis of genomic DNA

Genomic DNA from two individuals was separately digested by the same enzyme and then transferred to Hybond N+ nylon membranes (Amersham Biosciences). DNA probes for BbtTRAF2a and BbtTRAF3a were amplified by primers shown in Table S1 and labeled according to the DIG DNA Labeling System Kit (Roche). After overnight hybridization and stringent washing, the nylon membranes were immunodetected according to the manufacturer’s protocol.

Section and whole-mount in situ hybridization

Digoxigenin-labeled sense and antisense probes for BbtTRAF4 and BbtTRAF6 were synthesized by primers shown in supplemental Table S1 using DIG RNA synthesis kit (Roche). After a series of disposals, the probes of sense or antisense were added to the sections at a concentration of 1 µg/mL, and the sections were hybridized overnight at 42°C. After high-stringency washing, hybridization signals were immunodetected. Whole-mount in situ hybridizations for embryos at different developmental stages were conducted according to the protocol of Holland (21).

Expression plasmids

For confocal analyses, the ORFs of four BbtTRAFs were inserted into pEGFP-N1 vector, respectively. For reporter assays and CO-IP tests, the ORFs of four BbtTRAFs (2P, 3P, 4P, and 6P), the truncated mutant without the RING finger domain (6ZT) of BbtTRAF6, and the truncated mutant without the TRAF domain (6ZR) of BbtTRAF6 were all fused with Flag tag and cloned into pcDNA3.0. The TRAF domain only (2CT, 3CT, 4CT, and 6CT) of four BbtTRAFs were fused with Flag tag and HA tag separately and cloned into pcDNA3.0. For functional comparison, the ORFs of human TRAF2, 3, 4, and 6 (h2P, h3P, h4P, and h6P), and segments without the N-terminal RING and zinc fingers of human TRAF2 and 6 (hTRAF2 DN and hTRAF6 DN) were fused with Flag tag and cloned into pcDNA3.0.

Transient transfection and luciferase reporter assay

HEK293T cells (2 × 10⁵ cells per well) were transfected with 1 µg of DNA plasmids, including the indicated amount of detected gene plasmids, 200 ng of indicated luciferase reporter plasmid, 100 ng of β-galactosidase gene, and the supplemented empty vectors. At 24-h posttransfection, cells were lysed, and luciferase activities were measured using the luciferase reporter assay (Promega). The luciferase activity was normalized to β-galactosidase activity and expressed as the fold stimulation relative to empty vector-transfected cells. Values were expressed as mean relative stimulation for a representative experiment from three separate experiments each performed in duplicate.

Comunmunoprecipitation

HEK293T cells in 6-well dishes (5 × 10⁶ cells per well) were transfected with 2 µg of DNA plasmids (1 µg each expression vector). At 48-h posttransfection, cells were lysed and incubated with primary Abs (4 µg of anti-FLAG [M2] (Sigma-Aldrich)) at 4°C overnight, then incubated with protein G-Sepharose for an additional 4 h at 4°C. Analysis was conducted using SDS-PAGE followed by Western blot using the ECL protocol (Amersham Biosciences), with anti-HA (1/1000) and anti-Flag (1/1000) mAb.

Results

TRAF family in amphioxus

A search of the amphioxus genome in our previous study identified 24 TRAF gene models, including one corresponding to
TRAF6, one to TRAF4, four to TRAF3, and 16 to TRAF1/2 (20). The TRAF4 and TRAF6 subgroups represented the most ancient lineages, whereas TRAF1, 2, 3, and 5 lineages diverged from the common ancestor before the amphioxus-vertebrate separation (supplemental Fig. S1). In this study, our analyses suggested that 11 amphioxus TRAF1/2 lineage members were newly generated by lineage-specific duplication, they differed from one another in protein structures (Fig. 1A). Although these TRAF1/2 molecules were newly generated by lineage-specific duplication, they differed from one another in protein structures (Fig. 1A). Protein structures of the N-terminal RING and zinc finger regions were more variable than those of the TRAF domain, indicating that these regions are under more unfixed pressure (supplemental Fig. S2).

Protein structure comparison of four amphioxus TRAFs with their counterparts in Drosophila and human

To gain further insight into this uniquely expanded TRAF family, four representatives from each TRAF group were isolated from the intestinal cDNA library of Chinese amphioxus Bbt and designated as BbtTRAF2a, 3a, 4, and 6, respectively. The similarity among four BbtTRAFs and several classical TRAFs ranged from 23 to 66% (supplemental Fig. S3A). Although all four BbtTRAFs showed similar domain topology with their human counterparts, they were different from those of insects (Fig. 1B). First, both BbtTRAF4 and human TRAF4 contained a RING finger whereas DTRAF1 did not. Second, BbtTRAF6 and human TRAF6 had five zinc fingers, whereas DTRAF2 had only two such domains. In addition, BbtTRAF6 had a large insertion at the linker region between the zinc finger and the N-TRAF region. Similar to the organization in humans, RING finger domains of BbtTRAF2a, 3a, 4, and 6 were organized as CX2CX11,12CX1HX2CX11CX2C, but certain insertions and deletions were observed in the zinc finger regions (supplemental Fig. S3, B and C). Collectively, amphioxus TRAF family members were more similar to their human counterparts than to those in Drosophila, indicating that the TRAF family, in this early chordate, has established a basic structure for the development of TRAF-mediated network signaling in humans.

Genomic rearrangement of amphioxus TRAFs

Southern blot analyses confirmed that BbtTRAF2a and 3a were multiple duplicated genes in the amphioxus genome, whereas the most unexpected finding was that the genomic DNA from two individuals showed different band patterns (Fig. 2A). To investigate the reason for this observation, we conducted genomic comparison of amphioxus TRAF2a, 3a, 4, and 6 in two species, the Bbt and the Bf. The BbtTRAF2a had intron-exon organization similar to that of BfTRAF2a, whereas BbtTRAF3a, 4, and 6 contained fewer exons than those from Bf (Fig. 2B). The significant difference was observed in the pattern of splice junction in the RING and zinc finger domains. We also compared the genomic organization of two newly duplicated

Table 1. Distinct expression patterns of amphioxus TRAF molecules after bacterial challenge

<table>
<thead>
<tr>
<th>Gene Model</th>
<th>Transcript (UC-BC)</th>
<th>TRAF1/2 Lineage</th>
<th>TRAF3 Lineage</th>
<th>TRAF4</th>
<th>TRAF6</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRAF1/2 Lineage</td>
<td>68986</td>
<td>68989</td>
<td>68990</td>
<td>68991</td>
<td>68992</td>
</tr>
<tr>
<td>TRAF3 Lineage</td>
<td>107021</td>
<td>107017</td>
<td>99999</td>
<td>237951</td>
<td>237984</td>
</tr>
<tr>
<td>TRAF4</td>
<td>0</td>
<td>2</td>
<td>19</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>TRAF6</td>
<td>0</td>
<td>2</td>
<td>19</td>
<td>11</td>
<td>0</td>
</tr>
</tbody>
</table>

a Gene model indicated EST sequences matching the corresponding JGI gene models. Transcript difference was obtained by comparing the transcript numbers between the BC library and the UC library.
TRAF3 homologues arrayed on scaffold_251 (models 237951 and 238009). Results showed that, although they had the same protein structure and intron-exon organization, their introns were altered in size (Fig. 2C). In short, our comparisons implied that the genomic region of RING and zinc fingers was less stable than that of the TRAF domain and that the loci of TRAF2 and TRAF3 were unstable and highly polymorphic, facilitating the gene expansion when they were initially derived.

Functional characterizations of amphioxus TRAF1/2 and 3 lineages

Because it was difficult to distinguish among copies of amphioxus TRAF1/2 and TRAF3 homologues through in situ hybridization and real time PCR, we analyzed a total of 400,000 EST sequences from two intestinal cDNA libraries of adult amphioxus Bbt by 454-pyrosequencing, the bacterially challenged (BC) library and the unchallenged (UC) library (S. Huang, unpublished data). First, we identified 11 predicted TRAF1/2 genes and all four TRAF3 homologues. We then compared the transcript differences between these two libraries and found that expression of 3 TRAF1/2 (gene model: 68995, 68989, and 107017) and 2 TRAF3 genes (gene model: 99999 and 237951) were significantly increased in the BC library, whereas transcripts of two TRAF1/2 homologues (gene model: 68986 and 68990) were reduced (Table I and Fig. 1). Thus, these findings confirmed that most of the predicted TRAFs were real-transcribed genes and may play nonredundant roles in antimicrobial infection.

Similar to their human counterparts, the GFP-tagged BbtTRAF2a and 3a were localized mainly to the cytoplasm. Round patches were also observable when cells expressed high amounts of ectopic BbtTRAF2a- or 3a-GFP proteins in Hela cells (see Fig. 5, A and B). We further showed that overexpression of full-length BbtTRAF2a alone did not activate the NF-κB or IFN-β pathway in 293T cells, whereas human TRAF2 induced >50-fold activation of NF-κB and 10-fold induction of IFN-β (see Fig. 5, E and F). Neither BbtTRAF3a nor human TRAF3 alone showed an effect on NF-κB activation or IFN-β induction (data not shown). However, human TRAF3 enhanced IFN-β induction through human TBK1 whereas BbtTRAF3a did not (Fig. 5G).

Amphioxus TRAF4 not only activates the NF-κB pathway but is also involved in the somite formation

Because TRAF4 is unique among TRAF family in showing a restricted expression pattern during embryogenesis, we performed whole-mount in situ hybridization to detect the temporal expression pattern of this conserved molecule in amphioxus. Transcripts of BbtTRAF4 were first detected in the presomitic mesoderm of the late gastrula and reached the highest level in...
the developing and nascent somites in neurula. Subsequently, the expression of BbtTRAF4 was rapidly down-regulated and maintained in the anterior and posterior somites of the neurula. At 48 h, BbtTRAF4 was detected only in the developing tail bud (Fig. 3). Our data demonstrated that BbtTRAF4 may play a key role in somite formation in amphioxus rather than function in epithelial progenitor cells and nerve system demonstrated for other species.

Section in situ hybridization revealed that transcripts of BbtTRAF4 were concentrated in the digestive system, which is thought to be the first line of defense for adult amphioxus (Fig. 4A). Real-time PCR showed that transcripts of BbtTRAF4 were weakly up-regulated after LPS challenge (supplemental Fig. S4). Moreover, BbtTRAF4 was capable of activating NF-κB responses in 293T cells whereas human TRAF4 could not. J and K, Compared with human TRAF6, BbtTRAF6 activated NF-κB response at a lower level but did not participate in IFN-β production in 293T cells.
The ectopic GFP-tagged human TRAF4 proteins were localized to the cytoplasm and barely detected in nuclear, whereas the endogenous human TRAF4 proteins were found mainly in the nuclear lysates (23). Parallel confocal microscopy showed that the ectopic GFP-tagged BbtTRAF4 proteins also localized to the cytoplasm in a few and round patches in Hela cells (Fig. 5C). However, we could not determine whether endogenous BbtTRAF4 can stay in nuclear because of the lack of amphioxus cell lines at present.

Functional characterization of amphioxus TRAF6

Similar to BbtTRAF4, transcripts of BbtTRAF6 were detected strongly in the epithelial cells of gut villi and mid gut diverticuli and in connective tissue and coelomic cells (Fig. 4, B and C). Real-time PCR also showed that transcripts of BbtTRAF6 were up-regulated after LPS challenge (supplemental Fig. S4). However, whole-mount in situ hybridization of BbtTRAF6 revealed ubiquitous expression, with no clear pattern during embryogenesis (data not shown). Compared with human TRAF6, whose ectopic overexpression resulted in the localization to the general cytoplasm and punctate cytoplasmic structures (24, 25), the GFP-tagged BbtTRAF6 appeared to accumulate to large patches around the nuclear in Hela cells based on confocal microscopy (Fig. 5D). In addition, BbtTRAF6 alone activated NF-κB at a lower level but did not induce IFN-β production (Fig. 5, J and K).

To investigate which domain was essential for the activation of the NF-κB pathway by BbtTRAF6, several truncated mutants were constructed for reporter assays (Fig. 5H). The truncated mutant without the RING finger domain of BbtTRAF6 (6ZT) activated NF-κB response at a lower level than the full-length vector, whereas the truncated mutant without the TRAF domain (6RZ) or only with the TRAF domain (6CT) did not make the same activation (Fig. 6A). In addition, activation through full-length BbtTRAF6 was down-regulated when cotransfected with vector 6CT, but unexpectedly was enhanced by vector 6CT (Fig. 6A). Further coimmunoprecipitation tests confirmed that BbtTRAF6 could form heteromultimer with BbtTRAF2a via their TRAF domain (Fig. 6C). Coimmunoprecipitation test also confirmed that BbtTRAF6 was able to form homomultimer via its TRAF domain (Fig. 6D). In summary, to activate NF-κB responses, BbtTRAF6 has to form homomultimer to exert its function. Moreover, these activities could be regulated by forming heteromultimer with other TRAF protein.

BbtTRAFs negatively regulate the activities of human TRAF2 and BbtTRAF6

To determine whether BbtTRAFs interact with human TRAF2 and 6 to regulate their activities, human TRAF2 was coexpressed with 2CT, 3CT, and 6CT (Fig. 5H). Results showed that the 2CT and 3CT inhibited the NF-κB activation by human TRAF2 whereas 6CT did not (Fig. 7A). In addition, human TRAF2-DN did not inhibit NF-κB activation through BbtTRAF6 (Fig. 7B). To determine the reason for such inhibition, coimmunoprecipitation tests were performed to demonstrate that BbtTRAF2a and 3a interacted with human TRAF2 via their TRAF domains but BbtTRAF6 did not (Fig. 7C). Likewise, TRAF domain only mutants of BbtTRAF2a, 3a, and 6 suppressed NF-κB activation through human TRAF6, and human TRAF6-DN inhibited NF-κB activation via BbtTRAF6 (Fig. 7, D and E). We also assumed that BbtTRAF2a, 3a, and 6 may form heteromultimer with human TRAF6 via their TRAF domains (Fig. 7F).

Discussion

Expansion and dynamic structure modulation of amphioxus TRAF family

The TRAF family is generally thought to have undergone a routine evolutionary process increasing from three in Drosophila to six in humans. However, this view is challenged by the fact that the TRAF family increased to 24 members in amphioxus, 7 in sea urchin, and 8 in zebra fish. Although these intermediate species all contain a comparable large number of TRAF genes, the huge expansion of the TRAF1/2 and TRAF3 lineages was just observed in amphioxus (20, 26, 27). Usually, a complex genome contains a greater number of gene family members to increase complexity and functional specificity, suggesting that expansion of the TRAF1/2 and TRAF3 lineages at the transition stage from invertebrates to vertebrates may have been due to new functional requirements and/or a new body plan (28). Despite lineage specific expansion, genomic structures of amphioxus TRAFs differed among individuals and between two amphioxus species. The most variable region among various TRAFs appeared in the pattern of splicing junction of the RING and zinc finger domains, which determine the downstream signaling activities for distinct TRAF proteins (29, 30). Thus, the evolutionary development of the TRAF1/2 and TRAF3 lineages is not just restricted to increase in number but also includes dynamic structure modulation, which is important for conveying diverse signaling when recruited by different receptors.

Functional innovation of TRAF1/2 and 3 lineages

Because transcripts of BbtTRAF2a are up-regulated in a Staphylococcus aureus-challenged suppression subtractive hybridization library (31), and transcripts of BbtTRAF3a (gene model: 99999) and the other TRAF3 homologue (model:237951) increase after bacterial challenge by 454 sequencing technology, the TRAF1/2
and 3 lineages should play nonredundant roles in antibacteria defense in amphioxus. Although BbtTRAF2a and BbtTRAF3a alone have no effect on NF-κB activation in human 293T cell lines, they are negative regulators of this conserved pathway when mediated by human TRAF2 or amphioxus TRAF6, much as human TRAF3 serves as a negative regulator for TRAF2 or 5 in the nonclassical NF-κB pathway (14, 15). In addition, members of amphioxus TRAF1/2 and 3 lineages are disparate in domain architectures, whose tiny differences may affect their activities on downstream signaling. Therefore we can assume that some of these duplicated molecules may have positive effort on the NF-κB activation.

Since amphioxus has developed a novel innate defense system by expanding its immune receptors and intermediate signal transducers (20), we can also assume that if the pattern recognition receptors distinguish among pathogen-associated molecular patterns and use distinct TRAF adaptors for downstream signaling regulation, this novel defense system should be comparable to vertebrate adaptive immunity.

Changing physiological and cellular roles of TRAF4

DTRAF1, most closely related to mammalian TRAF4, is predominantly expressed during normal eye development and recruited by Eigen-Wengen pathway (32, 33). Mouse TRAF4 is observed throughout embryogenesis, most notably during ontogeny of the CNS and peripheral nervous system (34). In this study, we found that BbtTRAF4 is involved in somite formation, especially in the posterior somite until the tail bud formed, suggesting that the function of TRAF4 in the nervous system is not conserved in amphioxus.

DTRAF1 is restricted to utilization by the Eiger-Wengen-JNK pathway, whereas vertebrate TRAF4 is not involved in classical TNFR signaling. Only one study has reported that human TRAF4 may participate in the TRIF-dependent pathway (5). Our current study did not indicate whether amphioxus TRAF4 is directly involved in TNFR or TLR signaling. What we have found was that amphioxus TRAF4 alone already has obtained its NF-κB activity.

which has not been observed in Drosophila. In addition, transcripts of BbtTRAF4 were abundant in the amphioxus digestive system and up-regulated after LPS challenge, indicating that BbtTRAF4 may be a component of amphioxus defense system by activating NF-κB pathway. In summary, our observations indicated that physiological roles of TRAF4 have changed and differed widely among phyla, although it is thought to be the most conserved adaptor in the TRAF family.

Functional similarities and differences of TRAF6 among Drosophila, amphioxus, and humans

Upon stimulation by distinct TLRs, cascades through human TRAF6 can result in anti-bacterial (TLR4) and anti-viral defense (TLR7/8/9) responses but have no apparent roles in normal development (10, 35). In Drosophila, the MyD88-Pelle-DTRAF2 cascade specifically relays the signal for anti-bacterial defense and normal D-V axis patterning but not for antiviral response (8). At the transition stage, amphioxus TRAF6 was found to be abundant in the digestive system and up-regulated after bacterial challenge, suggesting an antibacterial function. However, its function in D-V axis patterning could not be established, because we did not detect an expression pattern of BbtTRAF6 related to the D-V patterning during amphioxus embryogenesis.

Similar to its human counterpart, amphioxus TRAF6 alone could activate the NF-κB pathway and form heteromultimer with other TRAF proteins to attenuate their effect on NF-κB activation. However, the TRAF domain of BbtTRAF6 dose not show a dominant-negative effect but instead activates the NF-κB at a level comparable to that observed with full-length BbtTRAF6. The similar situations were also observed for DTRAF1 in the JNK pathway and for human TRAF6 in selective activation of p65 homodimer reporter (4, 36). In addition, the TRAF domain of BbtTRAF6 alone dose not inhibit signaling mediated by amphioxus MyD88 in 293T cells (data not shown), whereas the similar construct of human TRAF6 acts as dominant negative (37). It is possible that the interaction between the exogenously expressed TRAF domain and endogenous human proteins may be responsible for this seemingly irregular activation of signaling. Thus, although the protein structure of TRAF6 and its role in NF-κB is conserved during evolution, its mechanism and physiological role are not always the same for different species at different evolutionary stages.

The evolution of TRAF-mediated signaling pathways from basal chordate

In Drosophila, DTRAF1 and 2 mediate two separate pathways, the primitive TNF pathway (Eiger-Wengen-JNK) and Toll-MyD88-Dorsal signaling (8). In vertebrates, the TNF and TNFR have expanded into two large gene families (38, 39). Concurrently, additional TRIF-dependent signaling and other antiviral pathways have developed in the vertebrate innate system (35). Corresponding to these evolutionary events, members of the TRAF family increased during amphioxus TLR7/8/9, TRIF, or IRF3/7, and related molecules in amphioxus were not found to mediate the production of IFN-β.

In summary, our findings not only suggest strong evolutionary conservation of vertebrate and invertebrate TRAFs but also highlight the functional divergence and innovation of TRAF families in the protochordate, providing fundamental background for further investigation into the innate immune system of amphioxus. Furthermore, our genomic and functional comparisons suggest that the increase in members of the TRAF family, with functional differentiation, is critical to the current form of vertebrate TRAF-mediated signaling pathways, corresponding to the extension of TNF and TLR systems (Fig. 8).

Acknowledgments

We thank Dr. Hong Tang for his gift of human TBK1, human TRAF2, 3, and 6 plasmds. Vectors related to these genes were reconstructed based on these original constructions. We also thank Dr. Hong Tang for his constructive comments on the functional comparison.

Disclosures

The authors have no financial conflict of interest.

References


