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The Archaic Roles of the Amphioxus NF-κB/IκB Complex in Innate Immune Responses

Shaochun Yuan,*1 Jie Zhang,*1 Lingling Zhang,* Ling Huang,* Jian Peng,* Shengfeng Huang,* Shangwu Chen,* and Anlong Xu*†

NF-κB transcription factors play important roles in immune responses and the development of the immune system. Many aspects of NF-κB signaling differ significantly among distinct species, although many similarities in signaling exist in flies and humans. Thus, to understand the functional refinement of the NF-κB cascade from invertebrates to vertebrates, the Rel and NF-κB proteins, identified as bbtRel and bbtp105, were characterized in a basal chordate amphioxus. Consistent with the sequence similarities, bbtRel was found to interact with a mammalian κB response element, to move into the nucleus when activated, and to be inhibited by the NF-κB-specific inhibitor helalnin. Similar to the other class I members, bbtp105 could be cleaved into the mature form p58. Such endoproteolysis depends on the GRR sequence and requires both protease degradation and caspase 8 cleavage. Furthermore, we found that bbtIκB and the unprocessed bbtp105 can inhibit the transcriptional activity of bbtRel, whereas bbtp58 forms homodimers or heterodimers with bbtRel to create a mature NF-κB complex. Finally, we found that the survival rate and the expression of bbtIκB and TNF-α-like genes were decreased when adult amphioxus were treated with helalnin before immune challenge, suggesting the archaic roles for NF-κB signaling in innate immune responses in a basal chordate. The presence of the NF-κB–IκB complex in amphioxus indicates that it is a significant feature linking invertebrates to vertebrates and is refined in vertebrates through the expansion and divergence of genes involved in the cascade. The Journal of Immunology, 2013, 191: 000–000.

Nuclear factor κB belongs to a family of inducible dimeric transcription factors that activate the expression of genes involved in normal development, the immune response, inflammation, and apoptosis (1). To date, NF-κB homologs have been found in species ranging from single-cell organisms to mammals, including coral, sea anemone, hydra, horseshoe crab, fruit fly, zebrafish, Xenopus laevis, and human (reviewed in Ref. 2). However, the functional mechanisms of the NF-κB homologs are not consistent between invertebrates and vertebrates. In Drosophila, three NF-κB molecules (Dorsal, Dif, and Relish) and an IκB homolog (Cactus) are responsible for regulating several biological roles (3). Dorsal and Dif are involved in the Drosophila Toll signaling pathway, which recognizes Gram-positive bacteria and fungi, and in the regulation of normal dorsal-ventral patterning during Drosophila embryogenesis (4, 5). The third Drosophila NF-κB–related protein, Relish, is reported to be involved in the immune deficiency (IMD) pathway, which recognizes Gram-negative bacteria (6). However, owing to gene duplication, the mammalian NF-κB family of proteins comprises five members (7). The p65, RelB, and c-Rel proteins constitute the class II proteins, which positively regulate target gene expression through the transcription-activating domain (TAD). The class I proteins, p100 and p105, are precursors of the mature forms, p50 and p52, which lack TADs and therefore form heterodimers with TAD-containing family members to alter their specificities for the κB site (1, 8). Although studies of gene-knockout mice indicate that a lack of the p65 subunit leads to lethal liver degeneration in embryos, roles for NF-κB proteins in dorsal-ventral patterning in mammalian embryos have not been found (9, 10). In contrast, mammalian RelB and c-Rel do play key roles in the development of lymphoid organs, chiefly through response to stimuli in lymphoid cells (9, 11–13).

Regardless of functional differences, the endoproteolytic mechanisms of class I members also differ between flies and humans. The signal-induced endoproteolysis of Drosophila Relish requires the activity of several gene products, including the IκB kinase complex and the caspase 8 homolog Dredd (14). Activated Dredd cleaves off an inhibitory C-terminal ankyrin repeat (ANK) domain from Relish, thereby allowing the translocation of the N-terminal portion to the nucleus, where it induces the expression of AMP (antimicrobial peptide) genes (15–18). In contrast to Relish, the proteasome-mediated processing of p105 and p100 requires a 23-aa glycine-rich region (GRR) (19–21). The GRR-dependent endoproteolytic cleavage does not require specific downstream sequences, whereas Relish requires a downstream sequence in the C-terminal for processing (17, 21).

Moreover, in contrast to Drosophila, which has only one IκB protein, the mammalian IκB family commonly comprises IκBα,
Characterization of Amphioxus NF-κB/IκB complex

IκBβ, IκBe, and Bcl-3, which exhibit structural and biochemical similarities but play unique and nonredundant roles in regulating NF-κB activation (1). For example, IκBo contains a functional nuclear export signal, whereas IκBβ does not, which may result in differences in their subcellular locations and functions (22). IκBo exhibits a rapid response to stimuli and is quickly resynthesized to regulate transient NF-κB activation, whereas IκBβ is less sensitive to stimulus-induced degradation (22). Therefore, the roles of NF-κB signaling in development, the endoproteolytic mechanisms, and transcriptional regulation differ between flies and humans. Because amphioxus occupies the crucial evolutionary position linking invertebrates to vertebrates, characterization of the NF-κB–directed transcription process in this basal chordate will help to define the endoproteolytic mechanism of the class I NF-κB subgroup in the transition from invertebrates to vertebrates as well as demonstrate how NF-κB and IκB have coevolved to participate in embryogenesis and immunity.

Materials and Methods

Animals, cells, and reagents

Adult Chinese amphioxus Branchiostoma belcheri tsingtauense were obtained from Qingdao, China. HEK293T (human embryonic kidney) and HeLa cells were grown in DMEM supplemented with 10% FCS and antibiotics. The following inhibitors were used: helenalin (Alexis Biochemicals), Bay 11-7082 (Enzo Life Sciences), and MG132 (Sigma-Aldrich). Antibiotics. The following inhibitors were used: helenalin (Alexis Biochemicals), Bay 11-7082 (Enzo Life Sciences), and MG132 (Sigma-Aldrich).

Cloning of the bbtRel, bbtp105, and bbtIκB cDNAs

Initially, we examined the gene family that contained Rel homology domains (RHDs) and identified two partial sequences in the Branchiostoma floridice genome. Based on these two sequences, gene-specific primers were designed, and partial bbRel and bbtp105 sequences (bbRel and bbtp105 from B. belcheri tsingtauense) were cloned from Chinese amphioxus intestinal cDNA. Next, 5’-RACE and 3’-RACE were performed using the GenRACE Kit (Invitrogen), in accordance with the manufacturer’s protocol, for full-length sequence cloning. The bbtRel cDNA was identified from the amphioxus neurula stage embryo cDNA library. National Center for Biotechnology Information accession numbers KP006939, KP006940, and KP006941 are assigned for bbRel, bbtp105, and bbtIκB, respectively (http://www.ncbi.nlm.nih.gov/).

Plasmid construction

For the expression of bbRel, bbtp105, and bbtIκB, and their truncated mutants in HEK293T cells, PCR fragments encoding amino acids 1-755, 1-350, 351-452, 453-755, and 1-170+453-755 of bbtp105; 1-1024, 1-200, 1-580, 1-2024 of bbRel; and 1-350 of bbtIκB were fused with the Flag or HA tag, and inserted into the expression vector pET-28a (Novagen). The expression vector was constructed by inserting the bbtRel RHD into the expression host BL21 (Novagen). The selected transformant was inoculated in 2 ml of L broth (50 μg/ml) and was stimulated with isopropyl β-D-thiogalactoside at a concentration of 0.5 mM to induce expression at 25˚C. The induced cell suspension was subjected to ultrasonication for 5 periods, each consisting of 90 cycles of 5 s at 5-s intervals. The final product was centrifuged at 12,000 × g for 30 min, and the supernatant was harvested and filtered. The filtered supernatant was applied to an IMAC (Sepharose Fast Flow; GE Healthcare) column that was chelated with Ni2+ and equilibrated with solution A. The column was rinsed with solution A at a flow rate of 2 ml/min to baseline level. Solution B (20 mM Tris-HCl, pH 8.0; 150 mM NaCl, and 100 mM imidazole) was used to wash away the non–specifically bound proteins. The target protein was rinsed with solution C (20 mM Tris-HCl, pH 8.0; 150 mM NaCl; and 200 mM imidazole). The fraction corresponding to the protein peak was collected and applied to a hydrophobic column (G25; GE Healthcare). The column was pretreated with solution D (50 mM NH4HCO3) and was washed and used to establish the baseline value. The corresponding protein fractions were collected and lyophilized under vacuum (Supplemental Fig. 3A). A New Zealand rabbit was immunized with 500 μg purified bbRel-RHD protein in combination with Freund’s adjuvant (Sigma-Aldrich). For the booster immunization, 200 μg recombinant proteins was mixed with an equal volume of Freund’s incomplete adjuvant and injected s.c. into the rabbit’s back. A booster immunization was performed 30 d after the first immunization, with two further injections administered at intervals of 14 d. Whole-blood antisera was obtained by cardiac puncture. Anti-RHD poly-Ab was purified and determined by Western blots (Supplemental Fig. 2F).

Nuclear extraction and EMSA

A biotin-labeled 22-bp duplex bearing the NF-κB binding sequence was synthesized by Invitrogen [NF-κB wt (sense): 5’-AGT TGA GGC GAC TTG TCC CCAG GCC-3’; NF-κB wt (anti-sense): 5’-GCC TGG GAA AGT CCC CTC AAC TAC-3’; NF-κB mutant (sense): 5’-AGT TGA GCC GAC TCC CCC AGG C-3’; and NF-κB mutant (anti-sense): 5’-GCC TGG GAA AGT CCC CTC AAC TAC-3’]. The biotinylated complementary oligonucleotide pairs were annealed to generate double-stranded and biotin-labeled probes (10 pmol/μl) by combination in buffer (10 mM Tris and 1 mM EDTA) at 95˚C for 5 min, followed by gradual cooling to room temperature. The unlabeled complementary oligonucleotide pairs were annealed to generate double-stranded competitor probes (10 pmol/μl) and were used at a 200-fold concentration excess over the labeled probes during the experiments.

Nuclear extracts from the bbRel overexpressing cells were obtained using the NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Thermo Fisher Scientific). Protein concentrations were measured using the Pierce BCA Protein Assay Kit. The EMSA reaction solutions were prepared in accordance with the manufacturer’s protocol (LightShift Chemiluminescent EMSA Kit, Thermo Fisher Scientific). For binding reactions, amounts equivalent to 10 fmol of the duplex were incubated with 200 ng purified bbRel-RHD protein or 5 μg nuclear extract. The protein–probe mixture was separated using a 10% polyacrylamide 1× native gel and was transferred to a Biodyne B Nylon Membrane (Thermo Fisher Scientific). Migration of the biotin-labeled probes was detected on an x-ray film, using streptavidin–HPR conjugates to bind biotinylated probes and a chemiluminescent substrate. Both were used in accordance with the manufacturer’s protocol.

Embryo collection, section in situ hybridization, Southern blot hybridization, RT-PCR analysis, immunofluorescence imaging, transient transfection, Luciferase reporter assay, and coimmunoprecipitation (co-IP) were performed as described previously (23–25). Primers for hybridization probe preparation and RT-PCR are listed in Supplemental Table I.

Results

Structural and phylogenetic analyses of bbRel, bbtp105, and bbtIκB

To characterize the roles of amphioxus NF-κB cascade in innate immune responses, two genes contained RHDs (named bbtRel and bbtp105) were obtained by RT-PCR and RACE from the Chinese amphioxus (B. belcheri tsingtauense, bbt). BbtRel contains an open reading frame of 2265 bp and encodes a 755-aa protein, which includes an N-terminal RHD and a C-terminal TAD. The two conserved motifs, the DNA binding motif (RXXRXRXXC) and the nuclear localization signal ([NLS]: KKRQK), are in the RHD of bbRel (Fig. 1A). Genomic organization analysis demonstrated that bbRel is encoded by 9 exons, of which exons 2–4 are responsible for the RHD (Supplemental Fig. 1A). Southern blot analysis demonstrated that bbRel is a single-copy gene in the...
amphioxus genome (Supplemental Fig. 1C). A neighbor-joining tree based on the protein sequences of the conserved RHD showed that bbtRel is clustered with the vertebrate class II NF-κB proteins, which include RelA, RelB, and c-Rel, whereas the dorsal-like proteins that belong to invertebrate class II NF-κB are clustered as a separate invertebrate group (Fig. 1B). These results suggest that bbtRel is the ancestor of the vertebrate class II NF-κB group of proteins.

Another NF-κB family member, bbtp105, encodes a 1024-aa protein, which includes a conserved GRR motif (Supplemental Fig. 1B), an N-terminal RHD, and a C-terminal IkB-like domain containing six ANK repeats (Fig. 1A). A death domain (DD) is located at the C terminus, and an NLS is in the IPT domain (Fig. 1A). IPT is a domain that contains an Ig-like fold and is found in cell surface receptors as well as in intracellular transcription factors. A potential PEST domain in amino acids 467–490 (APPDDDLITDSAPTEDTMAMSQA) was predicted by the PEST find Web service (http://emboss.bioinformatics.nl/cgi-bin/emboss/pestfind). Phylogenetic analysis places bbtp105 at the base of the NF-κB clade, indicating that bbtp105 is more homologous to the ancestral NF-κB proteins (p100 and p105) than to the Rel proteins in vertebrates, whereas the Drosophila Relish-like sequence clustered as an invertebrate-specific group (Fig. 1B). Therefore, we propose that all Rel proteins evolved from

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**FIGURE 1.** Sequence analysis of bbtRel, bbtp105, and bbtIkB. (A) Domain topology of bbtRel, bbtp105, and bbtkIkB. The domains were predicted using the SMART database (http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1), whereas the NLS presented in bbtRel (KKRQK) and bbtp105 (KKRK), the caspase 8 recognition site (PxExx[Ac/Ar], x represents any residue) at position 579–582 in bbtp105, and the C-terminal phosphorylation sites of bbtp105 (DSGLEQ) and bbtkIkB (TGLEDS) were manually predicted using sequence alignment in accordance with published sequences. (B) The bbtRel and bbtp105 neighbor-joining trees were constructed using protein sequences from the RHD region. Clustal W 1.83 software was used for multiple alignments. The phylogenetic tree was built using Mega v3.1 software. The neighbor-joining method was used to calculate the trees with 1000 bootstrap tests and handling gaps with pairwise deletion. Aa, Aedes aegypti; Ag, Anopheles gambiae; Cr, Carcinoscorpius rotundicauda; Dm, Drosophila melanogaster; Dr, Danio rerio; Gg, Gallus gallus; Hs, Homo sapiens; Mm, Mus musculus; Sp, Strongylocentrotus purpuratus; Xl, Xenopus laevis. The numbers at the nodes indicate bootstrap values.
a common ancestral RHD-ankyrin structure within a unique superfamily and diverged into the two subgroups that expanded in vertebrates as the result of two whole-genome duplications.

The 1,029-bp open reading frame in bbtIκB encodes a protein with 343 aa residues and contains several features found in IκB members, such as six ANK repeats at the C terminus and two serine residues (Fig. 1A). The two serine residues in the N-terminal serine-rich region are critical for IκB degradation (Supplemental Fig. 1D). In contrast to human IκBα and IκBβ, a potential PEST domain in amino acids 54–76 (KSCQDDTDCCIESDEDPNESS) is located at the N terminus. Because the PEST region is important for its constitutive phosphorylation and intrinsic stability (26), the different location of the PEST domain suggests that bbtIκB may be regulated by different kinetics of degradation, which need to be further discussed. Southern blot analysis clearly demonstrated that bbtIκB is a single-copy gene in the amphioxus genome (Supplemental Fig. 1C). The genomic sequence of bbtIκB is relatively small, spanning 3.2 kb, and contains six exons ranging in size from 87 to 276 bp and five introns ranging from 208 to 875 bp (Supplemental Fig. 1A). The genomic sequence of human IκBα also contains six exons and is similar in size to bbtIκB, indicating the evolutionarily conserved genomic structure of the IκB family in invertebrates and vertebrates. In addition, the intron/exon boundaries in human IκBα and Bcl3 are similar to those in human IκBα, and individual IκB family members are distributed on different chromosomes (27). Thus, we conclude that IκBα and other family members have not arisen recently through simple gene duplication events but have gradually evolved from an ancestral IκB molecule (Supplemental Fig. 1E).

**Functional characterization of bbtRel**

To define the transcriptional activity of bbtRel, NF-κB reporter assays were performed using the transient expression of bbtRel in 293T cells. In the presence of bbtRel, the transcription of the reporter gene was significantly induced (Fig. 2A). Further reporter assays showed that the truncated bbtRel mutants did not exhibit transcriptional activity (Supplemental Fig. 2A, 2B), indicating that the transcriptional activity of bbtRel depends both on the atypical C-terminal TAD and on the N-terminal RHD. Investigation of the subcellular location demonstrated that the bbtRel-RHD was distributed evenly in the nucleus, whereas the truncated mutants missing the RHD were distributed evenly in the cytoplasm, indicating that the NLS in the RHD region is essential for the nuclear location of bbtRel (Supplemental Fig. 2C).

Next, we assessed whether the NF-κB inhibitors BAY11-7082, evodiamine, and helenalin could block the transcriptional activity of bbtRel. Increased concentrations of helenalin resulted in a dose-dependent reduction in reporter expression; however, this was not true for BAY11-7082 or evodiamine (Figs. 2A, Supplemental Fig. 2D). Then, nuclear extracts of 293T cells in which bbtRel or bbtp105 was overexpressed were used for gel shift assays. The results showed that helenalin can impair the formation of the bbtRel–DNA complex, but not the bbtp105–DNA complex, indicating that helenalin is a specific inhibitor of bbtRel (Fig. 2B).

To eliminate interference from endo-human NF-κB and to investigate whether bbtRel recognizes the κB response element through its RHD in vitro, an EMSA was performed using a recombinant bbtRel RHD (bbtRHD) and the κB element. The results demonstrated that the bbtRHD recognized the mammalian

![FIGURE 2. In vitro characterization of bbtRel. (A) The NF-κB–specific inhibitor helenalin attenuates the transcriptional activity of bbtRel in a dose-dependent manner. At 24 h post transfection, the indicated amounts of helenalin were added to the cell culture medium, the cells were incubated for 12 h, and the reporter expression was measured. Data are shown as the mean ± SD of three samples per treatment. Values were considered significant when p < 0.05. The results were confirmed by at least three separate experiments. (B) Helenalin specifically inhibits the binding of bbtRel to the κB element. At 24 h post transfection, the nuclear extracts were prepared, and binding with κB probes was determined by gel shift assays. (C) Binding activity of the bbtRel protein to the human κB probe in a dose-dependent manner with increasing amounts of bbtRel-RHD protein. (D) EMSA showed that helenalin and MG132 can inhibit the binding activity of bbtRel protein with human κB probe. (E) Increased protein expression and nuclear translocation of bbtRel after bacterial challenge in amphioxus gut tissues. (F) Increased binding activity of bbtRel to the κB probe in cells derived from amphioxus gut tissue after bacterial challenge was measured by EMSA.](http://www.jimmunol.org/issue)
κB element, which was also recognized by human NF-κB and Drosophila Dorsal (Fig. 2C). This interaction was specific because bbRHD did not bind the mutant κB probes and the competitor (unlabeled κB probes) blocked such binding (Fig. 2C). The gel shift complexes were partially reduced by increasing the dose of helenalin or MG132, further suggesting that these two inhibitors impair the interaction of bbtRel with the target sequence (Fig. 2D). To test whether bbtRel can translocate into the nucleus in response to activation, we raised the poly-Ab of bbtRel-RHD, and the subcellular location of bbtRel was tested (Supplemental Fig. 2F). Results showed that following a bacterial challenge, the amount of bbtRel in the nucleus and the binding activity of the bbtRel with the κB probe increased in the cells derived from amphioxus gut tissue, suggesting that nuclear localization of bbtRel after a bacterial challenge is due to its protein activity, but not the cell type (Fig. 2E–F). These data indicate that the specific recognition sequence in NF-κB was acquired early and was maintained during evolution.

The NLS and DD are important for the localization of bbtp105

For functional characterization of bbtp105, several truncated mutants of bbtp105 were constructed and overexpressed in 293T cells (Fig. 3A). Reporter assay showed that only RHD-IPT and RHD-DD induced reporter gene expression, indicating that the ANK region may play an inhibitory role (Fig. 3A, 3B). To determine the subcellular location of bbtp105, the truncated forms of bbtp105 were transfected into HeLa cells and the locations of the expressed proteins were investigated. The results show that the proteins that contained an NLS and were missing the DD were located around the nucleus, whereas proteins containing the DD were distributed throughout the cytoplasm (Fig. 3C). The Western blots demonstrated that, as with human NF-κB1/2 and Drosophila Relish, bbtp105 was processed spontaneously into the mature form, which was measured at ~ 58 kDa (named bbtp58) (Supplemental Fig. 2E). The Western blot results also suggested that the truncated mutants containing amino acids 260–582 mediated the processing

Figure 3. The NLS and DD are important for the localization of bbtp105. (A) Description of the bbtp105-truncated mutants used in this study. (B) Reporter assays show that, compared with the wild-type bbtp105, proteins without the ANK region enhanced the transcriptional activity of bbtp105. (C) Subcellular localization of the full-length bbtRel, bbtp105, and bbtkB, indicating that the proteins are largely restored to the cytoplasm when inactivated. Overexpression of the bbtp105-truncated mutants indicates that the proteins that contain the NLS and are missing the DD are located around the nucleus, whereas the proteins that contain a DD are distributed throughout the cytoplasm. (D) Amphioxus caspase 8 promotes the endoproteolytic cleavage of bbtp105, whereas the caspase 8 inhibitor zVAD-FMK inhibits the transcriptional activity of bbtp105 and reduces production of the mature bbtp58. (E) Deletion of the GRR sequence at 390–442 aa led to the incorrect processing of bbtp105 and inhibited the translocation of bbtp58 from the cytosol to the nucleus, suggesting that the GRR motif provides a recognition and termination signal for proteolysis. (F) Western blotting shows the inhibition of bbtp105 processing by the protease inhibitor MG132 in 293T cells. (G) Ubiquitination assays show that K63-linked ubiquitination is important for the processing of bbtp105 and that ubiquitination is dependent on the K48 site.
of bbtp105, whereas the truncations containing the DD region were inhibitory (Supplemental Fig. 2E), indicating that the DD is important for stability of the bbtp105 in the cytoplasm. These observations, along with the reporter expression data, suggest that the NLS in the IPT region is essential for nuclear localization of p105; however, when the protein is in the precursor form, the DD is important for its cytoplasmic distribution.

To investigate the processing mechanism of bbtp105, we analyzed the bbtp105 sequence and determined that it contains a GRR at position 391–442 in the N terminus and a conserved caspase 8 recognition motif, DEGD, at position 579–582 (Fig. 1A). Considering that the mature bbtp105 protein is ~58 kDa, it is reasonable to assume that a caspase mediates the cleavage process. Therefore, reporter assays were performed, which demonstrated that, when coexpressed with increasing amounts of bbtcaspase 8 protein [characterized in our previous study (28, 29)], the transcription activity of bbtp105 was enhanced, and the mature bbtp58 was increased. In addition, when the cells were treated with the caspase 8-specific inhibitor zVAD-FMK, the transcriptional activity of bbtp105 and the mature bbtp58 were reduced (Fig. 3D). To determine whether the GRR region is indispensable for the processing of bbtp105, a deletion mutant in which amino acids 391–442 were removed was constructed and expressed in 293T cells. Incorrect processing of bbtp105 occurred, and the translocation of bbtp58 from the cytosol to the nucleus was inhibited, indicating that GRR sequence provides the correct termination signal for the caspase 8-dependent cleavage (Fig. 3E). The proteasome complex and the ubiquitination of bbtp105 are implicated in the degradation of the C-terminal region of bbtp105, as treatment with the proteasome inhibitor MG132 blocked the processing of bbtp105 in vitro (Fig. 3F).

Next, we showed that neither K48 nor K3 mutants could affect the ubiquitination of bbtp105. However, when K48 and K63 were double mutated, ubiquitination of bbtp105 could not be observed, indicating that both K48- and K63-linked ubiquitination are important for the degradation of bbtp105. Because K63-linked ubiquitination is used to mediate the regulation of gene functions, whereas K48-linked ubiquitination leads to protein degradation, we concluded that the K63-linked ubiquitination may assist the K48-linked ubiquitination of bbtp105 during its processing (Fig. 3G). Taken together, these ideas suggest that the bbtp105 processing mechanism required both caspase 8 cleavage and proteasome degradation. The caspase 8 recognition motif and the GRR sequence may provide the processing signal, whereas the C-terminal DD blocks the processing of this protein.

The relationship among bbtpRel, bbtxkB, and bbtp105

To determine the relationship among bbtpRel, bbtxkB, and bbtp105, reporter assays were performed and results showed that the transcription activity of bbtpRel was severely inhibited by bbtxkB and abolished when bbtxkB was coexpressed (Fig. 4A). ELISA assays further revealed that when bbtpRel was overexpressed in 293T cells, the secretion of human TNF-α was induced in a dose-dependent manner, and such induction could be inhibited when bbtxkB was coexpressed with bbtpRel (Fig. 4B). Co-IP assays demonstrated that bbtpRel interacts directly with bbtxkB and bbtp105, and that bbtp105 formed homodimers (Fig. 4C). We also demonstrated that, in cells overexpressing bbtpRel or in cells treated with MG132, the proteolytic degradation of bbtxkB was inhibited (Fig. 4D). In addition, both K48 and K63 mutants were ubiquitinated in bbtxkB (data not shown). Given the distinct location of the PEST motif of bbtxkB, our results not only indicate that a similar feedback regulation exists between bbtpRel and bbtxkB but also suggests that bbtxkB may depend on another unidentified ubiquitination mechanism.

Because co-IP assays demonstrated that bbtpRel interacts directly with bbtp105 (Fig. 4C), to determine which domain was responsible for the bbtp105–bbtpRel interaction, further co-IP assays with truncated bbtp105 were performed. The data suggest that, in contrast to interaction through the ANK repeats that is observed in mammals, bbtpRel interacts with bbtp105 through the IPT region in amphioxus (Fig. 4E). Next, bbtpRel was coexpressed with different truncated bbtp105 mutants, and reporter assays demonstrated that the IPT region blocked the activity of bbtpRel (Fig. 4F). Therefore, the unprocessed bbtp105 interacts with bbtpRel through its IPT region and to inhibit its transcription activity by its ANK domain. Because the processed mature bbtp58 may form a heterodimer with bbtpRel to create a mature NF-kB complex, and co-IP assay demonstrated that bbtp105 can form homodimers (Fig. 4C), for a more detailed analysis of the p58–p58 homodimer and the p58–bbtpRel heterodimer, we generated constructs that expressed two covalently linked proteins and a flexible peptide linker (Fig. 4G). The 20-aa peptide linker in these constructs was used previously to link multimeric complexes in vivo and in vitro assays (30). The reporter assays demonstrated that the bbtpRel–bbtp58 heterodimer is a more effective transcription factor than is the wild-type bbtpRel or the linked bbtpRel homodimer. In addition, the bbtp58–bbtp58 homodimer induced higher levels of transcription than did the wild-type bbtp105 or bbtp58 (Fig. 4G). Therefore, we can assume that the functional NF-kB complex contains the bbtpRel–bbtp58 heterodimer or the bbtp58–bbtp58 homodimer.

The temporal expression and tissue distribution of bbtpRel, bbtp105, and bbtxkB in the adult amphioxus

Because NF-kB plays important roles in dorsal-ventral patterning in Drosophila, we used whole-mount in situ hybridization to investigate the temporal expression of bbtpRel during amphioxus embryogenesis. The bbtpRel transcripts were abundant from the blastula stage of embryogenesis and were distributed to the dorsal and ventral regions at the neurula stage (Supplemental Fig. 3A). At the larval stage, although still primarily located in the dorsal-ventral region, the bbtpRel transcripts were abundant in the anterior region, suggesting that bbtpRel may be involved in the dorsal-ventral and anterior-posterior patterning of amphioxus embryos (Supplemental Fig. 3A). Moreover, RT-PCR showed that bbtp105 and bbtxkB were also abundant during embryogenesis (Supplemental Fig. 3B).

To determine the tissue distribution of bbtpRel, bbtp105, and bbtxkB, RT-PCR was performed and results showed that transcripts of all the tested genes are abundant in amphioxus digested tissues (Fig. 5B). Further section in situ hybridization was performed, and results showed that bbtpRel transcripts were extensively distributed in the gill slits, hepatic cecum, intestine, and gonads, whereas bbtp105 and bbtxkB transcripts were mainly found in the hepatic cecum and intestine in adult amphioxus that was immune challenged with the Gram-negative bacteria V. vulnificus (Fig. 5A). Because the digestive system is considered the primary line of defense in amphioxus, to confirm the immune significance, real-time RT-PCR analysis was performed to monitor the expression of bbtpRel, bbtp105 transcripts in adults challenged with V. vulnificus. Results showed that transcripts of bbtpRel and bbtp105 were upregulated in 2 h and reached the highest level 6–8 h post infection. The upregulation of bbtpRel is consistent with the result that the amount of bbtpRel is greatly increased after bacterial challenge (Fig. 5C), indicating that bbtpRel may be regulated by other immune-related transcription factors, such as IFN regulatory factors, in amphioxus.
The potential roles of the amphioxus NF-κB cascade in immune defense

For further characterization of the roles of amphioxus NF-κB cascade in immune defense, we treated adult amphioxus with 4 mM helenalin before bacterial challenge. Blocking NF-κB by dietary supplementation of 4 mM helenalin decreased the survival rate of amphioxus following oral infection with V. vulnificus (Fig. 6A). Because IκB and TNF-α are two classical NF-κB target genes, we first cloned the genomic sequences of bbtIκB and bbtTNFα-like. Then the genomic sequences were uploaded to the TESS online prediction program (www.cbil.upenn.edu/cgi-bin/tess/tess) to search whether these regions contain the conserved NF-κB binding motif. The genomic sequence of bbtIκB, including the 2-kb sequence upstream of the ATG and the coding region,
contains three conserved κB binding sites in the first intron of bbtIkB. Furthermore, reporter assays indicated that the 700-bp sequence upstream of the second CDS in bbtIkB responded in a dose-dependent manner, suggesting that this region is indispensable for the interaction with bbtRel (Fig. 6B). Then the expression of bbtIkB following bacterial challenge was determined by RT-PCR, and a significant induction was observed, consistent with studies in Drosophila and humans, in which activation of the NF-κB pathway increased the expression of IκB (Fig. 6C). Similar to the vertebrate TNF-α homolog, the bbtTNFα-like gene contains an NF-κB binding site in its promoter region that responds to the overexpression of bbtRel in a dose-dependent manner (Fig. 6D). Over the same period, bbtTNFα-like, another classical NF-κB target gene required for the innate immune response in vertebrates, was upregulated slightly (Fig. 6E). Moreover, treatment with the helenalin and MG-132 prior to infection consistently suppressed the upregulation of bbtIkB-like and bbtIκB (Fig. 6C, 6E). Because the TLR signaling pathway have been well characterized in amphioxus, the bbtIkB protein was also coexpressed with amphioxus MyD88 or TICAM (31, 32), and the activation of NF-κB mediated by bbtMyD88 or bbtTICAM was inhibited by bbtIkB in a dose-dependent manner (Fig. 6F). Thus, we proposed that the rapid activation of NF-κB in the digestive system of amphioxus provides prompt protection against pathogenic infection, likely by modulating amphioxus TLR signaling to target many immune-related effector genes.

Discussion

The evolution and functional conservation of the NF-κB–IκB cascade

Despite the evolutionary distance between amphioxus and vertebrates, we determined that bbtRel, bbtp105, and bbtIkB contain signature motifs similar to those found in the vertebrate orthologs, notably the DNA binding motif and the NLS in bbtRel, as well as the six ANK repeats and the N-terminal potential phosphorylation sites in bbtIkB. Like human p100 and p105 and insect Relish, bbtp105 is a mosaic protein that contains both the RHD and the inhibitory IκB domain. Despite the sequence similarities among species, the overexpression of bbtIkB and bbtp105 specifically inhibits the translocation of bbtRel to the nucleus and reduces its transcriptional activity, suggesting that bbtIkB and bbtp105 are natural inhibitors of bbtRel. Moreover, the genomic sequence of bbtIkB contains several κB-binding motifs, and the ANK repeats are the site of the bbtIkB–bbtRel interaction, suggesting that the roles of NF-κB and IκB have coevolved and remained conserved throughout the evolution of chordates.

When the Toll pathway is activated, Drosophila Dorsal and/or DIF proteins are released from inhibition and translocate to the nucleus, where they bind to κB response elements and transactivate a specific set of genes, such as the drosomycin gene (33). In this study, we determined that in the presence of helenalin, the survival rate of amphioxus was decreased after an infection challenge. Moreover, we showed that bbtRel recognizes the κB
element and induces the expression of immune-related genes, such as bbtIκB and bbtTNFα-like. Sequence analysis showed that the promoter region of the amphioxus TNFα-like and IκB genes contains conserved κB binding sites that can respond to the activation of bbtRel, further suggesting a role for NF-κB in the amphioxus innate immune response. Because the phylogenetic analysis indicated that bbtp105 and bbtRel are the ancestors of the vertebrate class I and II subgroups, respectively, this study not only demonstrates the archaic roles of the amphioxus NF-κB–IκB cascade in immunity but also provides crucial information regarding the functional refinement of NF-κB molecules during the evolution from invertebrates to vertebrates.

The mature NF-κB complex in amphioxus exhibits both similarities and differences

Our study demonstrated that bbtp105 inhibits bbtRel, suggesting that, in activated cells, bbtp105 is processed into p58, which results in the coactivation of bbtp105 and bbtRel. We also determined amphioxus caspase 8 as the bbtp105 endoprotease, suggesting that this caspase-mediated endoproteolytic process is similar to the Dredd-mediated process in *Drosophila*. However, unlike *Drosophila* Relish, a direct interaction between bbtp105 and bbtcaspase 8 was not observed. It is reasonable to presume that this interaction is instantaneous and difficult to detect by overexpression. Alternatively, the interaction may require other unidentified proteins for endoproteolysis to proceed. When the GRR was deleted, endoproteolysis did not occur, regardless of the presence of bbtcaspase 8, suggesting that the GRR motif is a separate recognition and termination signal for proteolysis. We also demonstrated that, as in vertebrate class I NF-κBs, the endoproteolytic processing required the K63-linked ubiquitination of bbtp105 and the degradation by a protease. Therefore, we can assume that the protease-dependent mechanism was established before the evolution of chordates, and, because of improvements to the process, the caspase 8–dependent processing was ultimately lost in vertebrates, although it is still conserved in protochordates.

However, a number of obvious differences were also observed. First, unlike Relish, in which the C-terminal 107 aa are required for endoproteolysis and signal-dependent phosphorylation by *Drosophila* IκB kinase β (15), the C-terminal sequence of bbtp105 is the inhibition signal for processing of the protein to mature p58. Second, the N-terminal serine-rich region of Relish and the PEST domain negatively regulated Relish activation, whereas the ANK repeats were responsible for the negative regulation of bbtp105 in amphioxus. Third, the IPT region is the foundation for the heterodimerization between bbtRel and the mature p58 form of the

FIGURE 6. Potential roles of the amphioxus NF-κB in immune defense. (A) The survival rate of amphioxus after a challenge with Gram-negative bacteria *V. vulnificus* in the absence or presence of 4 μM helenalin. The bacterial challenge was performed at 1 h post treatment with DMSO (gray curve) and helenalin (black curve). The results are presented as the survival rates from two parallel experiments. (B) The reporter assays demonstrated that the 2-kb sequence upstream of the ATG did not respond to the overexpression of bbtRel, whereas the 700-bp sequence upstream of the second CDS in bbtIκB responded in a dose-dependent manner. (C) RT-PCR analyses of the expression patterns of bbtIκB after a challenge with Gram-negative *V. vulnificus* in the presence or absence of helenalin. (D) Sequence analysis indicated that the 1-kb sequence upstream of ATG contains one κB binding site in the bbtTNFα-like gene, and reporter assay confirmed that bbtRel can bind to this region. (E) RT-PCR analyses of the expression patterns of bbtTNFα-like after a challenge with Gram-negative *V. vulnificus* in the presence or absence of helenalin. The bacterial challenge was performed at 1 h post treatment with DMSO (blue curve), helenalin (green curve), or MG-132 (red curve). The results are presented as fold induction in the abundance of mRNA relative to that in samples injected with DMSO and were determined using the 2^ΔΔCt method from two parallel experiments performed in triplicate. The endogenous control for normalization is cytoplasmic bbtα-actin. (F) Reporter assays showed that bbtIκB inhibited the activation of NF-κB, as mediated by bbtMyD88 and bbtTICAM in a dose-dependent manner. All reporter assay data are shown as the means ± SD of three samples per treatment, and values were considered significant when *p* < 0.05. The results were confirmed by at least three separate experiments.
protein. Moreover, the IPT region significantly inhibits the transactivation activity of bbtRel and controls the nuclear location of bbtRel105, suggesting that when bbtRel forms a heterodimer with the mature p58, p58 not only assists the nuclear translocation of bbtRel but is critical for its binding to the κB element. Therefore, although not as many members of the NF-κB family are found in amphibians as are found in vertebrates and insects, the functional bbtRel–bbtp105 heterodimer and the bbtp58–bbtp58 homodimer in amphibian eukaryotic cells that function as powerful as those found in other species.

**Amphioxus NF-κB facilitates an understanding of the evolution of the immune system**

In our previous studies of amphibious TLRs and related signaling molecules, we provided compelling evidence for a functional intracellular TLR–NF-κB signaling cascade (25, 31, 32, 34). Amphioxous expresses >48 TLRs and >40 TIR-containing adaptors, which suggests an expanded repertoire of adaptors and intermediate signal transducers in its TLR signaling pathways. In the current study, we determined that the amphioxus genome contains only two NF-κB family members, bbtRel and bbtRel105. Moreover, the NF-κB complexes in amphibious are simpler than those in Drosophila, which further supports our previous observation that ancestral chordates contain a highly elaborate cognate recognition and cytoplasmic regulation system, but not on transcriptional effectors.

In vertebrate evolution, along with the innovation of adaptive immunity did not originate in vertebrates, but rather in a common ancestor of basal chordates. Further evidence that vertebrate-specific adaptive immunity did not exist in basal chordates. Although not as many members of the NF-κB family are found in amphibious as in vertebrates and insects, the functional bbtRel–bbtp105 heterodimer and the bbtp58–bbtp58 homodimer in amphibious exert functions that are as powerful as those found in other species.

**Disclosures**

The authors have no financial conflicts of interests.

**References**


