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Identification and characterization of five transcription factors that are associated with evolutionarily conserved immune signaling pathways in the schistosome-transmitting snail *Biomphalaria glabrata*

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Abstract

Innate immunity consists of humoral and cellular components that play a vital role in regulation of defense responses to various pathogens in vertebrates and invertebrates. Recent studies have shown that Rel/DIF (dorsal-related immunity factor), Relish, STAT (signal transducer and activator of transcription (STAT), and CREB (cAMP response element-binding protein) transcription factors associated pathways are evolutionarily conserved across the animal kingdom. Although the primary role and general structure of the pathways in immunity have been revealed in many invertebrates, particularly arthropods, almost nothing is known about those pathways in the freshwater snail *Biomphalaria glabrata*, an intermediate host of the human blood fluke *Schistosoma mansoni*, a causative agent of human schistosomiasis. Given the central role of transcription factors (TF) in controlling expression of effector genes, understanding the role of a given TF is essential to obtaining insight into the general function of the corresponding signaling pathway. To better understand the immunity of *B. glabrata*, we investigated five homologues of TFs that have been shown to be associated with multiple prominent immune signaling pathways based on the considerable data reported from a wide phylogenetic range of animals. In this study we identified and characterized cDNAs of five TFs from *B. glabrata*, designated BgRelish, BgRel, BgSTAT1, BgSTAT2 and BgCREB, for the first time. Among the five TFs, Relish is first reported in Lophotrochozoa, one of three superphyla in Metazoa. Our identification of class I (BgRelish) and II (BgRel) NF- κ B in *B. glabrata* suggests the two pathways, Toll-like receptor (TLR) and immune deficiency (IMD)-like pathways, are present in the superphylum Lophotrochozoa. Preliminary expression studies indicate these TF-associated pathways may be involved in the snail's anti-schistosome response. This study not only advances our understanding of the snail defenses, but also provides new perspectives about the evolution of animal immunity.

Keywords

Biomphalaria glabrata; *Schistosoma mansoni*; mollusk; NF- κ B; Relish; STAT; CREB

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1. Introduction

Schistosomiasis, caused by trematodes of the genus of *Schistosoma*, is a chronic and debilitating parasitic disease afflicting more than 200 million people worldwide (Steinmann et al., 2006). The freshwater mollusk, *Biomphalaria glabrata*, is the intermediate host for *Schistosoma mansoni*, one of the human infecting schistosome parasites (<http://www.cdc.gov/parasites/schistosomiasis/>). Schistosomes have a complex life cycle involving a mollusk intermediate host and a vertebrate definitive host. A promising alternative to control of the disease is breaking the parasite's cycle of transmission at the molluscan stage. Thus a better understanding of the mollusk's internal defense mechanisms may lead to the development of novel approaches for the control of schistosomiasis.

The snail *B. glabrata* possesses both cellular and humoral immune elements (Loker, 2010). It has been demonstrated that cell-mediated and humoral immune pathways are regulated by complicated pathways. Thorough investigation on the model organism *Drosophila* has revealed that these pathways that are regulated by the homologues of the transcription factors Rel/DIF (dorsal-related immunity factor), Relish, STAT (signal transducer and activator of transcription) and CREB (cAMP response element-binding protein) are highly evolutionarily conserved. For most non-model organisms it is not feasible to dissect individual components of these very complicated pathways. As TF centers the pathways, an initial effort to investigate the TFs will circumvent the complications. The outcome of such a study should yield valuable insight into the functions of the associated signaling pathways.

The homologue of insect transcription factors Rel/DIF and Relish are collectively called Rel/nuclear factor-kappa B (NF- κ B) transcription factors. Rel/DIF and Relish are the most well studied immune pathways in invertebrates, particularly in *Drosophila* (Lemaitre and Hoffmann, 2007). The primary role of NF- κ B proteins is to control a variety of physiological aspects of immune and inflammatory responses such as the production of antimicrobial peptides. In invertebrates the class I TFs (i.e., Relish) regulate the immune deficiency (IMD) pathway, whereas the class II TFs (i.e., Dorsal/DIF) control the Toll or TLR (Toll-like receptor) pathway (Hoffmann 2003). Both pathways have been shown to be hallmarks of fruit fly immune responses (Hoffmann 2003; Pinheiro and Ellar, 2006; Hetru and Hoffmann, 2009). Recent studies have demonstrated that NF- κ B involved pathways regulate mosquito's defense against malarial parasites and Dengue virus (Frolet et al., 2006; Xi et al., 2009). This suggests these pathways play important roles in insect internal defenses (De Gregorio et al., 2002; Lemaitre and Hoffmann, 2007; Sabin et al., 2010). The main function of NF- κ B pathways in insects are related to humoral responses; for example, a large array of antimicrobial peptides produced by the insects are controlled by these pathways.

The Janus kinase/STAT cascade (Jak/STAT) is another evolutionarily conserved intracellular signaling pathway that plays a vital role in a variety of biological processes such as immunity, hematopoiesis and cell proliferation (Levy et al., 2002; Arbouzov and Zeidler 2006). Disruption of this pathway may lead to immunological diseases (Shuai and Liu, 2003). Although the role of Jak/STAT in humoral responses has been documented in insects, such as regulation of the complement-like thioester-containing proteins (TEPs), the major function of Jak/STAT has been found in cell-mediated responses including phagocytosis, encapsulation processes and hemocyte proliferation (Agaisse and Perrimon, 2004; Shuai and Liu, 2003). Constitutive activation of the Jak/STAT pathway results in the overproliferation of lamellocytes (Sorrentino et al., 2004; Arbouzova and Zeidler, 2006), one of the three lineages of fruitfly hemocytes (plasmatocyte, crystal cell and lamellocyte). Lamellocytes are involved in encapsulating large invaders such as wasp's egg. The immune role of Jak/STAT has also been reported in other invertebrates such as shrimps (Chen et al.,

2008) and mosquitoes (Barillas-Mury et al., 1999; Lin et al., 2004; Gupta et al., 2009); however, it has not been documented in mollusks.

CREB is a transcription factor that regulates diverse cellular responses including proliferation, survival and differentiation. CREB is induced by a variety of growth factors and inflammatory signals and it subsequently mediates the transcription of genes containing a cAMP-responsive element (Shaywitz and Greenberg, 1999; Wen et al., 2010). The role of CREB in innate immunity is the least studied of the previously mentioned pathways. In mollusks, the function of CREB in memory has been studied in the marine snail *Aplysia*, a model organism for neurosciences (Lee et al., 2007). However, recent studies of CREB in oysters suggest that it plays a critical role in the immune response to a rickettsia-like micro-organism (Zhu and Wu, 2008).

With respect to the schistosome-transmitting snail *B. glabrata*, a better understanding of innate immunity is critical in order to further the development of innovative schistosomiasis control strategies and to shed light on the evolution of animal immunity. Considerable efforts and significant progress have been made in the characterization of the snail immunity (Zhang et al., 2004; Guillou et al., 2007; Bayne, 2009; Knight et al., 2009; Adema et al., 2010; Baeza-Garcia et al., 2010; Hanington, et al., 2010, Hanington and Zhang, 2011). In addition, some promising discoveries have been made in snail signaling pathways. Hahn et al. (2001) demonstrated the importance of reactive oxygen and nitrogen species pathways in anti-schistosome responses. Further studies indicated that resistance to the parasite is significantly associated with one allele of the Cu/Zn superoxide dismutase (*SOD*) gene, a gene responsible for detoxification of reactive oxygen species (ROS) (Goodall et al., 2006). Recent studies showed that a mitogen-activated protein kinase (MAPK) pathway is involved in the production of the reactive oxygen and nitrogen species in *B. glabrata* (Humphries and Yoshino, 2008). Moreover, several investigations suggested that MAPK plays a role in the snail hemocyte motility, spreading, phagocytosis, encapsulation and division of hematopoietic cells (Plows et al., 2006; Humphries and Yoshino, 2006; Walker 2006; Salarmat and Sullivan 2009; Zelck et al., 2009).

Despite this wealth of knowledge, almost nothing is known about these prominent evolutionarily conserved pathways in *B. glabrata* except for a report that described the homologues of gram-negative bacteria binding protein (GNBP) and peptidoglycan recognition proteins (PGRP), the extracellular components of the TLR and IMD pathways, respectively (Zhang et al., 2007). This leaves a big gap in our knowledge of immunity of this medically important species.

A fundamental breakthrough in our understanding of the evolution of immunity stems from the first identification of the immune-activating function of the Toll/DIF pathways in *Drosophila* (Lemaitre et al., 1996). Later, with the subsequent discovery of the homologous pathway in mammals (Medzhitov et al., 1997), a new area of human health research began. Further studies of invertebrate immunity may reveal additional basic and surprising features of innate immunity that have so far escaped our attention. This may be especially true for mollusks. Mollusks belong to Lophotrochozoa, one of three superphyla (Deuterostoma, Ecdysozoa and Lophotrochozoa). From the standpoint of evolutionary immunology, much of our current effort in understanding the basic functioning of innate immunity is focused on only two of the three superphyla, the Deuterostoma (vertebrates) and the Ecdysozoa (fruit flies). Because mollusks do not have particularly close affinities to arthropods or mammals, the most intensively studied organisms, some real immunological novelties might be expected from them. Furthermore, mollusks have important value in the economy and medicine since many species serve either as human food resources (e.g., oyster, abalone) or as vectors of human and animal parasitic diseases such as schistosomiasis.

2. Materials and methods

2.1. Snails, cell line, and parasites

There are two *B. glabrata* snail strains, BS-90 and M-line, which have been maintained in our lab. BS-90 snails are resistant to *S. mansoni* whereas M-line snails are susceptible to *S. mansoni*. The parasite, *S. mansoni*, was maintained in lab using mice as the definitive host in maintenance of the life cycle as described by Loker and Hertel (1987). A *B. glabrata* embryonic (Bge) cell line was maintained at our lab as described (Zhang and Loker, 2004).

2.2. Infection and RNA extraction

M-line and BS-90 snails (6-8 mm of shell diameter) were exposed individually to 20 *S. mansoni* miracidia. At 0 (unexposed), 6, 12, 24 and 36 hours post-exposure (HPE) four snails (from each exposure time point) were pooled, frozen and grinded using liquid nitrogen for subsequent RNA extraction (TRIzol Reagent, Invitrogen). A total of 20 individuals (4×5) were sampled at each time point. In addition, RNA was extracted from Bge cells at 6 and 12 HPE in a cocktail of stimuli (lipopolysaccharide, peptidoglycan, and laminarin, from Sigma), which is believed to increase the abundance of TFs, thus making the cloning of the immune genes easier. In addition, an RNAase inhibitor (Ambion) was added to the RNA samples in order to prevent degradation. Finally, the TURBO DNA-free kit (Ambion) was applied to remove DNA contamination from the RNA samples. The concentration and purity of RNA was determined (Nanodrop) and quality RNA samples were used for later cDNA synthesis with random hexamer primers (SuperScript III first strand synthesis system for RT-PCR; Invitrogen).

With respect to collection of hemocytes, hemolymph was collected from individual snails by cardiac puncture. The hemolymph from 18 snails that were not exposed or exposed to *S. mansoni* infections (20 miracidia per snail; sampled at 6, 12, 24, and 72 HPEs) were pooled and centrifuged at 1,000 g for 5 min. After removal of supernatant (plasma), the hemocytes were transferred to TRIzol for RNA extraction using the same procedures described.

2.3. Generation of full-length cDNA

To obtain full-length cDNA, 5' and 3' rapid amplification of cDNA ends (RACE) (Clontech) were performed on the RNA from the Bge cells. The contiguous cDNA sequences were generated using Sequencher (v. 4.8, Gene Code Corporation). Once the contiguous cDNA was obtained, the primers at the 5' and 3' untranslated regions (UTR) were designed. PCR (Advantage polymerase, Clontech) was then used to amplify the cDNA of target genes, using cDNA sample re-transcribed from RNAs of M-line snails. The cDNA of target genes containing an intact open-reading frame was cloned into the pCR21 vector (Invitrogen) and subsequent sequence analysis was used to determine the final cDNA sequence of individual genes. One clone representing one allele of an M-line snail is presented in this study.

The SMART program (Schultz et al., 1998) was applied to determine the homologues and conserved domains of amino acid sequences.

2.4. Phylogenetic analysis

The Minimum Evolution (ME) method was used for Molecular Evolutionary and Genetic Analysis (MEGA) (4.0.2) (Tamura et al., 2007). All trees were built based on the amino acid sequences.

2.5. Quantitative polymerase chain reaction (qPCR)

Relative gene expression data was obtained using the $\Delta\Delta C_t$ method, with re-transcribed 18S rRNA as the internal standard. The data shown in the corresponding figures are the average value related to those of the control snails and the error bars (standard deviation). A two-tailed homscedastic student *t*-test was used to compare the individual experiment to the control group. Single and double asterisks indicate statistical significance at $P < 0.05$ and $P < 0.01$, respectively. For the data of hemocytes, statistical analysis was not performed because only one biological sample was available. The sequences of primers are listed in Table 1. qPCR was performed on a Sequence Detection System 7000 (ABI) using FastStart Universal SYBR Green (Roche), as described by our previous studies (Jiang et al., 2006; Zhang et al., 2009).

3. Results

3.1. *B. glabrata* class I and class II NF- κ B

Since no reliable homologue of NF- κ B sequence derived from *B. glabrata* was available in public databases at the time of the study, degenerate primers were designed based on the conserved Rel homology domain (RHD) of NF- κ B sequences from the pacific oyster *Crassostrea gigas* and the abalone *Haliotis diversicolor* (Montagnani et al., 2004; Jiang and Wu, 2007). The degenerate PCR primers (see Table 1) targeted the two conserved regions of RHD (amino acid (aa) sequences: CEGRSAGA and DLNVVRLC). Our initial effort to clone the genes was made based on the cDNA derived from the Bge cells after exposure to a cocktail of stimuli (lipopolysaccharide, peptidoglycan, and laminarin) because these microbe-associated molecular patterns have been implicated as critical elements in activation of immune signaling pathways. Two types of RHD were obtained and confirmed. Subsequently, we designed gene-specific primers at the RHD regions and performed 5' and 3' RACE, then cloned and sequenced those PCR products. Computational analyses (NCBI and SMART) further confirmed that both NF- κ Bs possessed an N-terminal conserved RHD, which is the landmark of NF- κ B sequences. Based on the RHD sequences obtained, we indentified further cDNA sequence using RNA derived from M-line snails. Each completed open reading frame (ORF) was derived from one allele of the M-line snails. Interestingly, it was revealed that there are two different types of NF- κ B cDNAs in terms of nucleotide composition and sequence length. Based on characteristics of two different types/classes of NF- κ B (<http://people.bu.edu/gilmore/NF-kB/>), we designated the long (1085 aa) and short forms (610 aa) of NF- κ B as BgRelish (GenBank accession #: **FJ804762**) and BgRel (GenBank accession #: **FJ804761**), respectively. The details for both types are described below.

The long form Relish is called class I NF- κ B. BgRelish contains an N-terminal RHD (residues 58-246), middle IPT (Ig-like, Plexins, Transcription factor, residues 253-355), six C-terminal ankyrin repeats (ANK) (residues 694-907) and a death domain (residues 994-1085) (Fig. 1). There are two conserved motifs in the RHD, the DNA-binding motif (RXXRXXRXXC) and the nuclear localization signal (NLS) (K-K/R-X-K/R) which are the landmarks of all NF- κ B family proteins. BgRelish also possesses six ANKs, which are only found in Relish-like NF- κ B proteins (Fig. 1A, B).

Class II NF- κ B was also identified from *B. glabrata* and named as BgRel. The size of BgRel is 610 aa, consisting of an N-terminal RHD (residues 116-284) and an IPT (residues 289-391). Again, there are two conserved motifs, DNA-binding motif and NLS that are found in the RHD. Unlike BgRelish, neither an ANK repeat nor a death domain has been found in the BgRel (Fig.2A, B).

In order to understand the relationship of NF- κ Bs, we performed a phylogenetic analysis of NF- κ B molecules was performed. Since the RHD region is highly conserved and other regions are highly variable across animal species, RHD amino acid sequences were targeted for phylogenetic analysis. The phylogenetic tree shows that the sequences are grouped into two clades (Fig.3). Through further analysis of the relationships, it can be seen that the two clades represent the homologues of class I and class II NF- κ B, respectively, but this does not reflect species relationships. This may suggest that the divergence of the two class NF- κ Bs is much older than the divergence of animal species, at least between vertebrates and invertebrates.

3.2. *B. glabrata* *STAT1* and *STAT2*

Since no molluscan STAT sequence has been reported, we used a human STAT aa sequence as a query and identified a homologous EST from *B. glabrata* (CX727805). We then designed gene specific primers based on the EST sequence and performed 5' and 3' RACE on RNA generated from M-line snails. Sequence analyses revealed that two STATs were obtained from *B. glabrata*, designated as BgSTAT1 (GenBank accession #: **FJ804763**) (Fig. 4A, B) and BgSTAT2 (GenBank accession #: **FJ804764**) (Fig. 5A, B). It was confirmed that BgSTAT1 was derived from the EST sequence, but BgSTAT2 did not match any *B. glabrata* ESTs in the public databases.

The two BgSTATs have a similar size (759 and 781 aa, respectively) and possess the same structure; STAT-int, STAT-alpha, STAT bind and SH2 (Src Homology 2 Domain) (see Figs. 4B, 5B).

The overall aa identity of two BgSTATs is 70%. The phylogenetic analysis based on the aa sequences of full-length STATs retrieved from NCBI databases revealed that BgSTAT1 and BgSTAT2 closely cluster together (Fig. 6). There are two main branches of the tree; one includes all invertebrate species mixed with a portion of vertebrate species and the other branch consists only of vertebrate species. Human seven STATs are divided into the two main groups, and the two snail's STATs (BgSTAT1 and 2) are clustered with human STATs 5a, 5b and 6. It seems that the clad that contains the two snail STATs and all other invertebrate's STAT are relatively phylogenetical older than the clads consisting of only vertebrate's STATs.

3.3. *B. glabrata* *CREB*

We used oyster CREB as a query (GenBank accession no: EF405960) to find a homolog of CREB from the *B. glabrata* EST database (GenBank accession no: DT725064). RACE analyses revealed that the homologue of CREB isolated from *B. glabrata* (designated as BgCREB) contains 404 aa (GenBank accession #: **FJ807771**). A C-terminal basic region leucine zipper (BRLZ) region (332-396) was identified in the protein. In addition, an NLS was identified within the BRLZ region (Fig. 7).

Phylogenetic analysis revealed that BgCREB is closely related to the other two gastropod molluscan species, *Lymnaea* and *Aplysia*, but distantly related to bivalve mollusc oyster (Fig.8). This agrees with the phylogeny of molluscan species. No major branches can be seen from the tree.

3.4. Expression analyses

To obtain insight into the functional role of the five TFs identified in this study, we examined the expression of the individual TFs in schistosome-resistant BS-90 and susceptible M-line snails. The expression level of a given TF is a good indication for its activation (Latchman, 1998). Using qPCR analysis, we completed expression studies of five

TFs including BgRelish, BgRel, BgSTAT1, BgSTAT2 and BgCREB from whole body tissues of the two snail strains. In the M-line snails, all five TFs were not found to be significantly up-regulated at 6 hrs post-exposure (HPE). Significant up-regulation of BgSTAT1, BgCREB and BgRel were observed at 12, 24, and 72 HPE. With regard to the two NF- κ Bs, there was only slightly significant up-regulation (~2.5 fold) in BgRel observed at 72 HPE (Fig. 9A). In the BS-90 snails, it is interesting that four TFs, BgRel, BgRelish, BgSTAT1 and 2, were found to be significantly up-regulated at 6 and 12 HPE despite low fold-increase (<4 folds). This up-regulation was followed by significant down-regulation at 24 HPE. In addition, it is intriguing that significant down-regulation in BS-90 snails was observed at 24 HPE in all TFs except for BgRelish (also down-regulated, but not statistically significant) (Fig. 9B). Perhaps the most important observation we found is that after comparing the patterns between the two snail strains, it is apparent that up-regulation in BS-90 snails normally occurs earlier than those of M-line snails.

When focusing on an individual gene, the response of BgRel to infection in BS-90 snails is earlier (6 HEP) than in M-line snails (72 HEP). BgRelish displayed a similar pattern in BS-90 snails but no change in expression was apparent in M-line snails. Regarding the expression pattern of BgSTAT1, there was not a large difference between the two snail strains; up-regulation was noted at 12 HEP for both strains. In the case of BgSTAT2, the up-regulation was observed only in BS-90 snails at 6 HPE, however, no significant changes were found in either snail strain. Intriguingly, the general pattern of CREB is quite different from the other TFs; up-regulation was observed only in M-line snails at 24 and 72 HPE, but not in BS-90 snails. This suggests that the role of CREB may be quite different from the other TFs described in *B. glabrata*.

Moreover, we also examined the expression of individual TFs from the hemocytes of the two snail strains (Figs 10A and B). However, we were not able to perform a statistical analysis because we only had one pooled sample for each HPE. It can be seen that the overall pattern of the TFs tested is generally consistent with those in Figs. 9A and B, with the exception of the fact that the expression level of BgRelish in hemocytes is relatively higher than in whole body tissues. In summary, the expression patterns of the TFs tested show that these TFs and their associated pathways, particularly NF- κ B and Jak/STAT, may be involved in *B. glabrata*'s immune response to schistosome infection.

4. Discussion

4.1. NF- κ B transcription factors and potentially associated pathways

Rel/NF- κ B proteins can be divided into two classes based on C-terminal sequences to the RHD domain. Members of one class (the mammalian NF- κ B proteins p50, p100, p105 and *Drosophila* Relish) have long C-terminal domains that contain multiple copies of ankyrin repeats, which act to inhibit these molecules. The second class (the Rel proteins) includes c-Rel (and its retroviral homologue v-Rel), RelB, RelA (p65) and the *Drosophila* Dorsal/Dif proteins. This second class of Rel proteins contains C-terminal transcription activation domains. Despite its ability to activate transcription in a variety of species, these domains are not normally conserved at the sequence level.

In the context of innate immunity, the primary role of NF- κ B proteins is to control a variety of physiological aspects of immune and inflammatory responses. After activation, NF- κ B translocates from the cytoplasm to the nucleus and binds to the promoter region of a target gene, which results in the target gene being transcribed. The NF- κ B associated signaling pathways (i.e., Toll/TLR and IMD pathways) are evolutionarily conserved. NF- κ B homology has been identified in a variety of organisms from non-Bilateria animal phyla (i.e., the Cnidarian *Nematostella vectensis*) (Sullivan et al., 2007, 2009) and the

demosponge *Amphimedon queenslandica* (Gauthier and Degnan, 2008) to Bilaterian animals (i.e., the horseshoe crab) (Wang et al., 2006; Fan et al., 2008), the model organism *Drosophila* and humans (Lemaitre and Hoffmann, 2007). Interestingly, in *Caenorhabditis elegans*, an Ecdysozoa, the NF- κ B protein is absent. It was hypothesized that some of the functional homologues (e.g., TLR and Cactus) present in the genome may not be involved in innate immunity (Pujol et al., 2001). A recent study however suggested TOL-1, a single TLR in *C. elegans*'s genome, plays a role in anti-pathogenic responses, implying that part of the TLR-mediated immunity might have been evolutionarily conserved (Tenor and Aballay, 2008). Nevertheless, it is unclear how the NF- κ B and associated pathways have evolved across different animal phyla (Hoffmann, 2003).

In this study we identified two NF- κ Bs (BgRel and BgRelish) from *B. glabrata*. The implication of our study is that TLR and IMD-like signaling pathways may exist in this species that is the intermediate host in the transmission of schistosomiasis. This notion is further supported by our previous studies showing homologues of the extracellular components of the pathways, gram-negative bacterial binding proteins (GNBPs) and peptidoglycan recognition proteins (PGRPs) for this species (Zhang et al., 2007).

Remarkably, no Relish-like or human NF- κ B p100/p105 homologs that contain ankyrin repeats have been reported in the superphylum Lophotrochozoa, including in recent report of *Capitella capitata* and *Helobdella robusta*, two species of Annelida belonging to Lophotrochozoa (Davidson et al., 2008). Also, no Relish-like NF- κ B has been reported in mollusks despite several class II NF- κ Bs that have been documented in mollusks, including oysters, abalone and squid (Montagnani et al., 2004; Goodson et al., 2005; Jiang and Wu, 2007). Annelida and mollusca are the two main phyla in the superphylum Lophotrochozoa. Our identification of BgRelish in *B. glabrata* demonstrates that Relish associated pathway may also be present in the Lophotrochozoa superphylum. Identification of a single NF- κ B gene in the genome of the demosponge *Amphimedon queenslandica*, a representative of an even earlier branching Metazoan lineage, has demonstrated that the Rel/NF- κ B family originated at the dawn of the Metazoa (Gauthier and Degnan, 2008). No evidence of RHDs in fungal and choanoflagellate genomes has been found, suggesting that the RHD is a Metazoan-specific innovation. In the demosponge, it has been shown that a single NF- κ B gene contains ankyrin repeats exists (Gauthier and Degnan, 2008), however, ANK-containing NF- κ B has not been described in the Cnidarian *Nematostella vectensis*. Thus, it has been hypothesized that ankyrin repeats was lost over evolutionary time (Gauthier and Degnan, 2008). Given that the Demospongiae is a lineage branching earlier than the Cnidaria, the domain arrangement observed in NF- κ B of *N. vectensis*, with only the RHD present, could have resulted from a secondary loss of the ankyrin repeat domain. Domain shuffling during early Metazoan evolution has been suggested to contribute to the generation of metazoan protein diversity (Adamska et al. 2007). Together, it can be suggested that Relish-like TFs might be lost in some phyla, which may lead to disappearance or disruption of associated pathways. Whether a similar scenario has occurred in the Lophotrochozoa is a very interesting question. Further studies on more species of Lophotrochozoa may reveal the mystery. Nevertheless, our identification of class I and II NF- κ B in the snail *B. glabrata* suggests the presence of TLR and IMD-like immune signaling pathways in the Lophotrochozoa.

In addition to an accumulation of recent data on *Drosophila* (Lemaitre and Hoffman, 2007; Hetru and Hoffman, 2009), there is mounting evidence suggesting a functional role of NF- κ B in the immune responses of other invertebrates. In the horseshoe crab, both classes of NF- κ B were found to play a role in infection (Wang et al., 2006; Fan et al., 2008). Recent studies have shown that NF- κ B involved pathways play important roles in mosquito immune defense against malarial parasites and Dengue virus, mosquito-borne human pathogens

(Frolet et al., 2006; Xi et al., 2008). All these findings imply an important role of the two pathways in insect internal defenses (De Gregorio et al., 2002; Lemaitre and Hoffmann, 2007; Sabin et al., 2010).

Our preliminary studies indicated that there is no significant up-regulation of TFs in M-line snails after exposure to *S. mansoni* within 24 HPE. However, we do see a slight up-regulation at 72 HPE. Interestingly, in the schistosome-resistant BS-90 snails, significant up-regulation was observed at 6 HPE followed by a significant decrease in expression at 24 HPE (except for Relish). Later there was a progression back to normal levels of expression. Since *S. mansoni* are killed in BS-90 snails at 1 to 3 DPE (Lie et al., 1980; Sullivan and Richards, 1981), this suggests that these resistant snails might be capable of mounting an earlier immune response. Interestingly, this finding is consistent with the expression patterns we observed with NF- κ B in the resistant snails infected with schistosomes. This may suggest that NF- κ B pathways are involved in the defense against schistosome parasites. In *Drosophila*, production of antimicrobial peptides, the downstream products of the NF- κ B signaling pathways, are significantly up-regulated in 3 hrs post-infection (De Gregorio, et al., 2002). Thus it seems that early response of defense pathways is critical for successful anti-pathogen defenses.

A similar pattern was observed in hemocytes. However, the response of BgRelish is stronger than that of BgRel. This may indicate that the function of BgRelish is related to hemocytes. With respect to activation of TF activation, the expression level of a given TF is only one indication for its activation. There are different ways indicating TF activation expression level, such as phosphorylation and translocation between the cytoplasm and nucleus (Latchman, 1998). Further functional studies are necessary to verify and further define the function of the BgNF- κ B and the other TFs described in this paper.

4.2. STAT1 and 2 transcription factors

The Jak/STAT cascade was originally discovered as a cytokine-induced signaling pathway required by myeloid and lymphoid cell lineages (reviewed by Ihle, 1995). Its role is indispensable in a variety of biological processes such as immunity, hematopoiesis and cell proliferation (Levy et al., 2002; Arbouzov and Zeidler 2006). STATs were identified as a family of cytoplasmically located transcription factors that are activated by phosphorylation, where upon dimerization they translocate to the nucleus and act upon target genes. The activation of STATs occurs as a result of the action of receptor-associated Janus tyrosine kinases or through the action of receptors that possess intrinsic tyrosine kinase activity. The mammalian STAT family consists of seven members that are highly related to one another (Levy et al., 2002). The most evident functions identified to date are cell-mediated defenses that include phagocytosis and encapsulation processes as well as hemocyte proliferation (Agaisse and Perrimon, 2004; Shuai and Liu, 2003). The immune role of Jak/STAT has also been documented in other invertebrates. In *Drosophila* STAT92E was found to control the regulation of apoptosis (Betz et al., 2008), hemocyte maturation and differentiation (Minakhina et al., 2011), and defense (Agaisse and Perrimon, 2004). In shrimp the Jak/STAT pathway has been shown to play a role in defense against white spot syndrome virus (WSSV) (Chen et al., 2008). Finally, in mosquitoes it has been shown that STATs are involved in response to microbes and malaria (Barillas-Mury et al., 1999; Lin et al., 2004, Gupta et al., 2009).

Although STAT molecules and their associated pathway have yet to be explored in mollusks, the second largest superphylum next to insects, the discovery of two STATs from *B. glabrata* will enable us to investigate the role of the Jak/STAT pathway in snail defense responses. We show here that *B. glabrata* possess at least two STATs. In BS-90 resistant snails up-regulation was observed in BgSTAT2 and 1 at 6 and 12 HPE, respectively. We

found both BgSTATs were down-regulated at 24 HPE. This peculiar pattern of down-regulation at 24 HPE has been shown in shrimp (Chen et al., 2008) and is an unusual pattern that remains to be investigated.

4.3. CREB transcription factor

CREBs have been extensively studied in the mollusk *Aplysia*, a model organism for the studies of brain and memory. With regard to immunity, it has been found that oyster CREB is involved in the defense response. In this study we identified one BgCREB that has similar structure to CREB in other species. However, we did not observe up-regulated expression in BS-90 resistant snails after exposure to the schistosome. An up-regulation at 24 and 72 HPE in M-line snails was observed. This is contrary to down-regulation patterns of immune suppression perhaps due to parasitic establishment in schistosome susceptible snails (Hanington et al., 2010). In addition, no up-regulation has been observed in any hemocyte samples of the two snail strains. This may suggest that CREB does not function in an immune defense context in *B. glabrata*.

5. Concluding remarks

This study provides us with a repertoire of information on the five transcription factors, from *B. glabrata* and paves a way to elucidate role of the evolutionarily conserved immune pathways in the snail immunity. Our first report of the Relish-like TF in the snail suggests that both NF- κ B pathways (TLR and IMD) are present in the superphylum Lophotrochozoa. Our preliminary data indicates that the snail's NF- κ Bs and STATs may play a role in the anti-schistosome responses. Due to the fact that the activation of TFs can be exhibited through different fashions (i.e., expression level, translocation and phosphorylation), the expression pattern described in this paper is just one of the ways of indicating TF activation. Moreover, it is also understood that non-up-regulation responses may not necessarily suggest inactivation. Despite preliminary data provided, further functional assays are needed to verify the data and elucidate the role of the TF-associated pathways in the snail's immune defenses. Functional assays underway include the production of recombinant proteins and antibodies in our lab. Furthermore, it would be interesting to know the relationship between the TF associated immune pathways and important defense effectors, such as fibrinogen-related proteins (FREPs) (Hanington and Zhang, 2011). Nevertheless, this study paves the way towards uncovering the depths of the immune pathways utilized by this medically important species, which in-turn may yield novel insights into the development of innovative control strategies for schistosomiasis. In addition, this work enhances our understanding of the evolution of animal immunity.

Acknowledgments

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Highlights

>Almost nothing is known about evolutionarily conserved immune signaling pathways in the freshwater snail *Biomphalaria glabrata*, a medically important species, despite a large number of studies reported in a wide phylogenetic range of animals.

>Given the central role of transcription factors in regulation of the pathways, we identified and characterized cDNAs of five transcription factors from *B. glabrata*, designated BgRelish, BgRel, BgSTAT1, BgSTAT2 and BgCREB, for the first time.

>Among the five transcription factors, Relish is first reported in Lophotrochozoa, one of three superphyla in Metazoa, suggesting presence of the two conserved NF- κ B signaling pathways (Toll-like receptor (TLR) and immune deficiency (IMD)-like pathways) in the superphylum Lophotrochozoa.

>Gene expression studies indicate these transcription factor-associated pathways may be involved in the snail's anti-schistosome responses.

A

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ATGATGGCTATGTCATTTCTGACGCCGATCTTGATTGGGAGATTGCAAAATATGAACAGCAGTGAATTGATGAGATTGTTGAGAACATGAATGATGAT 102 nt
M M A M S F S D A D L D L G D L Q N M N S S E L M R F V Q N M N D D 34 aa
ACTGCTGGGCAACTGATCAATCAAAATGCAGCAGATTCAATCCCTCCACAGAAATAACTAACTCCACCCCTGTTCACTCTGTGCAAGATTCCCACATCAT 204
T A G Q L I N Q M Q Q I H S P P Q N I T N S H P V H P V Q D S H H H 68
GCTATGCAGCAGTACTCAATAACCTGCTGTTTATGCAGCACCAGTCTATTACCCATGCAGCCACCCAGCAACCAACAACAGCCACAACCCAG 306
A M Q Q Y S N N P A V Y A A P V Y Y P M Q P P Q Q P Q Q Q P Q P Q 102
CTTCCACCCCATGTTCAAATGCTGCTAGCACCCCTTACGTAGAATATTAGAGCAACCCAAAGTCTAGGGGATTGCGCTTCCGCTATGAATGTGAAGGGCGT 408
L P P H V Q T A A S T P Y V E I L E Q P K S R G L R F R Y E C E G R 136
TCTGCTGGGAGTGTGCCAGGAGAAAACAGTACTAATGATCACAGGACATACCCACAATCAAAATCCACAACATAATGGGCCAGCAATTATTGTTGTGCT 510
S A G S V P G E N S T N D H R T Y P T I K I H N Y N G P A I I V V S 170
TGTGTAACCAAGGAGAACCCACCTCACTGTAAGCCGATCCACATGCTATTGTTGGCAGGAGTGAAGAAAGGAGTGTGTACATTGCGTGTAAAGATACA 612
C V T K E N P P H C K P H P H A I V G R D C K K G V C T L R V K D T 204
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S D K I V F P Q I G I Q C A K K K D V E S S L R L R K E I N V D P Y 238
CAAACCTGGGTTTGTATCAGCTCAGTCCAATATTGACCTGAATGTTGTTAGGCTGTGTTTCAAGTTTCTTACCTAATGAACAGGGCAAAGTGACAGGGTT 816
Q T G F D H A Q S N I D L N V V R L C F Q V F L P N E Q G K V T R V 272
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V P P V C S H P I H D K K S S K D L V I C R V D K S S G K A R G G D 306
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E V F L L C D K V N K E D I K V R F Y E E N E Q G M V V W E D L G D 340
TTTGGCCAGGGTGATGATAGGCAGTATGCTATTGCTTCCAGAACCATCTTATCACAATACAGAAATCACAGCTCCAGCAGAGGTTCTGTATGACGTTA 1122
F G Q G D V H R Q Y A I V F R T P S Y H N T E I T R P A E V L M Q L 374
CAGAGGCGCTGTATGGGAACTAGTGAACCTATACCATTTACTTACATGCCTGAAGACCCAGATCTGACAGAATCGCAGAAAAGAGAAAAAGAAAGGCT 1224
Q R P S D G E T S E P I P F T Y M P E D P D P D R I A E K R K R K A 408
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Q R F L E Y W N S T G V A D Y K R G A S G A S V R G R L H T M L K A 442
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T R R I K K D P D V I P G L P G F M D T S G V D G V N G V G A V G G 476
GCTGATGCCAATGAGTCATGTGACTGTGACAACTCTCCATGGGAGTGTCACTGTCTCTCTCTCAGCTGGCAGCTGGATTCCAATGTCAATGCT 1530
A D A T M S H V T A D N S S M G S V T V S S F S A G S L D S N V I A 510
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E L S G L S S S D L S I V S T E N G Q L V I S V S G G E G S G L S V 544
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D S I N L P P N I L S G E L M N Q I D I Q Q L N A Y L Q D Q G S G S 578
GAGATTTTATGGCAATGACCCATCAACCAATTCATATCTTAGATCATGATCCTGAAACAGAGGCAGCACTTAGAGGATTAAATCTCAATTTGA 1833
E I L L G N D P N Q P I H I L D H D P E T E A A L R G L N S Q I . 610

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B **Fig.2.**

A) Nucleotide and deduced amino acid (aa) sequences of BgRel (#FJ804761). **B)** Schematic diagram of BgRel. Denotation of double and single lines is same as Fig.1.

Fig.3.

A phylogeny of RHD aa sequences from various NF- κ B sequences. BgRelish and BgRel are bolded and boxed. The GenBank accession number for a given sequence is provided in the parenthesis. The opened and filled circles showed on the branches indicate bootstrap values of 51-80% and 81-100%, respectively. All these apply to Figs. 6 and 8 below as well.

A

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ATGTCACAGTGGGCCAAGGTTGAGCGTAGCTCAAACATCTTCCGGCAATGCAACAGTATTATCCACCTCATTCCCTTTGGAAGTGGGTACTTTTATGT 102 nt
MSQWAKVERSSNIFRQMQQYYPPHFPLEVRYFLC 34 aa
GAATGGATTGAGGAACAAGTGGGATGAAATAGATGAAATTAATCCTGAGCATGCAAAACAAGTTTGGACATAATGTTGGATTGCTACGCCAGAAAGCA 204
EWWIEEQQWDEIDEINPEHAKQVLDIMLDDLRLQKA 68
GAAAGTCAAGAGCATTTCATTGTCGATTAAAGTTGGAGGAAATTTATCAAAATCTACAGAACTATATTGAAACCAATGCATTAGGAATGGTTCAAGTGATT 306
ESQEHFIVRIKLEEIYQNLQNYIETNALGMVQVI 102
AAAACTGTTTGGCAGCAGAAATAAAATTTATAGAACAAAGCTGAGCAAGCCATGCAAGCAGCAGCAGATTCAAAAAGCTTTGCTGAGCATCACAGGATCAAT 408
KNCLEAAEIKFIEQAQAQQQQQIQKALSEHHRIN 136
GATCTAATTATGAATGAAATTCAGAACTTAACAGGAGATTGAGAGCTCAGAAAATGATGTTAAAACTTACAACATAAGCAAGAAATGTGTATCATAGAA 510
DLIMNEIQNLTRRLQSSSENDVKNLQHKQEMCIIE 170
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YNKMQKQKAGLLQLLSNMPNSQEKINKKEAQQLKQK 204
GAACAGATTGATGACAAGTTAAACAAATGGCTCAAGAGATTCTTCGTATAGAAATAGATTGTCTAAAAAGTTTGGCAGACTATGTTAACTTCAAGAG 714
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GTTATCTATGAGTCACTGAAACTCATTGCAGAAATGGAAGAACTTCCATTAAGCCCCAATATGAATCAGGAAGTACACAGACTTTCCCAACATGATG 2244
VIYEESTENS LQNMEELEPLSPNMMNQELDTDFPNMM 748
GCAGACCTCCTACTGAGTAGCATTATGTCATCTTGA 2380
ADLLLS SIMSS . 759

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B

Fig.4.

A) Nucleotide and deduced amino acid (aa) sequences of BgSTAT1 (#FJ804763). B) Schematic diagram of BgSTAT1. SH2:Src Homology 2 Domain.

Fig.5.

A) Nucleotide and deduced amino acid (aa) sequences of BgSTAT2 (#FJ804764). B) Schematic diagram of BgSTAT2.

Fig.6. Phylogenetic analysis of STAT aa sequences collected from public databases. The tree was constructed based on the full-length aa sequences.

Fig.7.
A) Nucleotide and deduced amino acid (aa) sequences of BgCREB (#FJ807771). The shaded area represents the BRLE region. NLS is underlined. **B)** Schematic diagram of BgCREB.

Fig.8.
Phylogenetic analysis of CREB aa sequences. Full-length amino acid sequences were used to build the tree.

Fig.9.

Expression of five TFs in whole body tissues of M-line (**A**) and BS-90 (**B**) snails after exposure to *S. mansoni*. The error bar denotes the standard derivation. Single and double asterisk indicate $P < 0.05$ and $P < 0.01$, respectively. Hrs: hours.

Fig. 10. Expression of five TFs in pooled hemocytes of M-line (A) and BS-90 (B) snails after exposure to *S. mansoni*.

Table 1

Primers used in this study

Name	Sequence (5'-3')
BgRel-F1	TGTGAGGG(A/G)(C/A)G(C/A)TC(A/T)GC(T/G)GG(T/C)AG
BgRel-R1	ACA(G/C)AG(A/T)GC(G/T)(A/C)(A/C)(T/C)A(T/C)(G/A)TT(C/A)AG(A/G)TC
qRelish-F1	GAACACCAACATAGACAAGGCAGTA
qRelish-R1	CTTTGGGCTCGCTGACTTCT
qDIF-F1	TGCTGGGCAACTGATCAATC
qDIF-R1	AGGATGAACAGGGTGGGAGTTA
qSTAT1-F	TCCCAACAAACCCAAAGATGAT
qSTAT1-R	GAGTGATGGGATGTAGCCATTTT
qSTAT2-F	TGAGCATCAACCACCAAGTAT
qSTAT2-R	ATTAAGCTTTCCCCGACAAG
qCREB-F	AAGTACCTCATCTGTATGTCGCAAGAG
qCREB-R	TCAAGCTGCTCCACAATAACATG
q18S-F	CGCCCGTCGCTACTATCG
q18S-R	ACGCCAGACCGACACCAA