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Short communication

Identification of nuclear factor kappaB (NF-κB) binding motifs in *Biomphalaria glabrata*

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ABSTRACT

Biomphalaria glabrata acts as the intermediate host to the parasite, *Schistosoma mansoni*, and for this reason, the immune system of *B. glabrata* has been researched extensively. Several studies have demonstrated that the transcriptome profile of *B. glabrata* changes following exposure to a variety of pathogens, yet very little is known regarding the regulation of gene expression in this species. Nuclear factor kappaB (NF-κB) homologues have recently been identified in *B. glabrata* but few functional studies have been carried out on this family of transcription factors. The aims of this study therefore were to identify NF-κB binding sites (κB motifs) in *B. glabrata* and examine them via functional assays. Two different κB motifs were predicted. Furthermore, the Rel homology domain (RHD) of a *B. glabrata* NF-κB was able to bind these κB motifs in EMSAs, as well as a vertebrate κB motif.

1. Introduction

The freshwater snail *Biomphalaria glabrata* acts as an intermediate host to the parasite *Schistosoma mansoni*, one of several species of trematode that cause the debilitating disease schistosomiasis in humans (Colley et al., 2014). Studies of the mechanisms by which this snail defends itself against pathogens have provided some understanding of *B. glabrata*'s immune system (Yoshino and Coustau, 2011). At the cellular level, the snail's defense strategies include phagocytosis and cellular encapsulation of pathogens, while humoral factors include lectins, the production of oxygen radicals and a recently identified family of fibrinogen-related proteins (FREPs) (Yoshino and Coustau, 2011). These defense mechanisms likely involve changes in gene expression, and several studies show that the transcriptional profile of *B. glabrata* is modified in response to stressors such as gram-negative and gram-positive bacteria, mechanical wounding, and metazoan parasites (Adema et al., 2010; Hanelt et al., 2008; Hanington et al., 2010; Ittiprasert et al., 2010; Lockyer et al., 2012; Mitta et al., 2005; Zahoor et al., 2014). Despite ample evidence of altered gene expression during immune responses in *B. glabrata*, the regulatory mechanisms responsible are not known. Transcription factors have only recently been identified in *B. glabrata* (Bouton et al., 2005; Kaur et al., 2015; Zhang and Coultas, 2011) and just one promoter region has ever been characterized (Yoshino et al., 1998).

The regulation of gene expression is a complex process involving various protein–protein and protein–DNA interactions, such as the binding of transcription factors to regulatory DNA sequences. One group of transcription factors shown to regulate gene expression in immune and inflammatory responses is the nuclear factor kappa B (NF-κB) family (Liang et al., 2004). NF-κB proteins make up a highly conserved and evolutionarily ancient family of transcription factors, with members having been identified in nearly all animal phyla ranging from prebilateria (sponges) to deuterostomes (mammals) (Gauthier and Degnan, 2008; Gilmore and Wolenski, 2012; Wang et al., 2006). An increasing number of invertebrate and specifically molluscan NF-κB homologues have been reported in recent years, with homologues in *Crassostrea gigas*, *Euprymna scolopes*, *Haliotis diversicolor* supertexta, *Pinctada fucata* and *Chlamys farerri* for example (Goodson et al., 2005; Jiang and Wu, 2007; Montagnani et al., 2004; Wang et al., 2011; Wu et al., 2007). In addition, two NF-κB homologues were recently discovered in *B. glabrata* and they show most similarity to p65 (RelA) and p105 NF-κB proteins (Zhang and Coultas, 2011). Typically p105 is cleaved to a shorter active form, p50, which is often found in a heterodimer with p65 (Gilmore, 2006). Following activation of an

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NF-κB signaling pathway, the NF-κB dimer is released from IκB and translocates to the nucleus to regulate gene transcription. This regulation requires the recognition and binding of kappa-binding (κB) motifs in the regulatory regions upstream of particular genes by the Rel homology domains (RHD) of NF-κB dimers. Kappa-binding motifs are somewhat conserved as evidenced by the identity between the insect consensus κB motif (GGGRNTYYYY) and the mammalian consensus κB motif (GGGRNNYYCC; R is a purine, Y is a pyrimidine and N is any base) (Gilmore, 2006; Kappler et al., 1993). Despite the discovery of several molluscan NF-κB homologues, kappa-binding motifs have so far been identified in only one molluscan species, *C. gigas* (Montagnani et al., 2007). Evidently, little is known regarding how molluscan NF-κB proteins function. Therefore, the aims of this study were to identify putative kappa-binding motifs in representative *B. glabrata* genes and subsequently assess them in functional assays.

2. Materials and methods

2.1. Snail maintenance

B. glabrata snails (BS90 and NMRI strains) were maintained in artificial pond water at 26 °C with a 12:12 h light–dark cycle. They were fed romaine lettuce *ad libitum* and TetraMin® fish flakes, and cuttlefish bone was supplied as a slow release source of calcium.

2.2. Identification of putative-kappa binding motifs

The upstream regions of immune-related genes were surveyed for the presence of potential kappa-binding (κB) motifs. The genes subjected to this analysis were selected based on their putative roles in the immune system and included: inhibitor of NF-κB (IκB), superoxide dismutase allele b (SOD) and p38 mitogen activated protein kinase (MAPK) (Goodall et al., 2004; Hahn et al., 2001; Humphries and Yoshino, 2006, 2008). In order to acquire the upstream sequences, preliminary data from the *B. glabrata* genome project (http://129.24.144.93/blast_bg/2index.html) was searched via Basic Local Alignment Search Tool (BLAST) using the gene of interest's sequence. The resulting 5′ sequences (~1000–1200 bp in length) were confirmed via PCR using specific primers (Midland Certified Reagent Company Inc., Midland, Texas, USA) and *B. glabrata* genomic DNA as a template. Genomic DNA from BS90, NMRI and BBO2 *B. glabrata* strains were used as templates. The strains were chosen based on either their resistance (BS90) or susceptibility (NMRI) to *S. mansoni* infection, or their use in the *B. glabrata* genome project (BBO2) (Adema et al., 2006). PCR products were sequenced at the DNA Analysis Facility on Science Hill at Yale University (New Haven, CT, USA), and the resulting DNA sequences were then submitted to TFSEARCH (Wingender et al., 1996) in order to identify potential transcription factor binding motifs. Any κB motifs predicted via this approach were used to design oligonucleotide probes for electrophoretic mobility shift assays (EMSAs). In addition, for each gene of interest, the upstream sequences acquired using the three different genomic templates were aligned and compared using Vector NTI® software (Life Technologies, Grand Island, NY, USA).

2.3. Electrophoretic mobility shift assays

BgRHD, a 310 amino acid peptide, representing the Rel homology domain (RHD) of *B. glabrata* p65 NF-κB, (amino acids 83–392; NCBI Accession No. ACZ25559.1) was expressed in *Escherichia coli* using the vector pET-32a (GenScript, Piscataway, NJ, USA). Oligonucleotide probes containing the 10 bp κB motifs predicted upstream of *B. glabrata* genes, as well as the 10 bp 5′ and 3′ of the κB motif, were synthesized (Midland Certified Reagent Company Inc.). In addition, a vertebrate consensus κB motif was synthesized for use in EMSAs. Oligonucleotide probes were labeled with biotin according to the manufacturer's instructions (Biotin 3′ End DNA Labeling Kit, Pierce™, Rockford, IL, USA). Double-stranded biotin-labeled probes (20 fmoles) were each incubated with 0.25 μg BgRHD at room temperature for 20 min in binding buffer (10 mM Tris, 50 mM KCl, 1 mM DTT, pH 9) containing 1 μg polyI:C and glycerol at a final concentration of 5%. This was repeated with the addition of unlabeled probe at 200 fold excess (4 pmoles). Following the incubation, the reactions were run on a 6% non-denaturing polyacrylamide gel for 30 min, and were then transferred to a nylon membrane (type B positive; Fluka Analytical, St. Louis, MO, USA). Biotin-labeled probes were then detected using a Chemiluminescent Nucleic Acid Detection Module according to the manufacturers' instructions (ThermoScientific, Rockford, IL, USA). The results were visualized using a ChemiDoc-It^2 510 Imager (Upland, CA, USA).

The following reagents were provided by the NIAID Schistosomiasis Resource Center for distribution through BEI Resources, NIAID, NIH: *B. glabrata*, Strain NMRI (Unexposed to *Schistosoma mansoni*), NR-21970, Strain BS-90 (Unexposed to *Schistosoma mansoni*), NR-34791, Genomic DNA from *B. glabrata*, Strains BS-90, NMRI and BB02, NR-29375, NR-29377 and NR-29376, respectively.

3. Results

3.1. Identification of putative kappa-binding motifs

The upstream regions of the genes encoding IκB, p38 MAPK and SOD were amplified via PCR, and surveyed for transcription factor binding motifs using TFSEARCH. Consequently, κB motifs were predicted upstream of IκB (GGGCCTTTCC) and p38 MAPK (CGGATTTTCC), beginning at positions −1050 and −263 upstream of the start codon, respectively (see supplementary data). The latter 5 nucleotides of the two κB motifs were identical, but 3 out of 5 nucleotides in the first half of each motif differed. In contrast, a putative κB motif was not identified within the region amplified upstream of SOD. Furthermore, all these upstream regions were amplified using genomic DNA from three different *B. glabrata* strains: BS90, NMRI and BBO2. The resulting sequences were aligned for comparison but no differences were observed among them.

3.2. Electrophoretic mobility shifts assays

EMSAs were carried out to examine the ability of BgRHD to recognize and bind the κB motifs predicted upstream of IκB and p38 MAPK. BgRHD bound to the biotin-labeled oligonucleotide probe containing the κB motif found upstream of IκB. Furthermore, this binding was reduced in the presence of 200-fold of the unlabeled equivalent probe (Fig. 1A). In contrast, binding of the previous labeled wild type (WT) probe by BgRHD was not inhibited by an unlabeled mutant probe (Table 1), supporting the notion that the interaction between BgRHD and the IκB probe is specific. Moreover, no binding was observed between BgRHD and the labeled mutant probe. When using the probe containing the κB motif located upstream of p38 MAPK, the EMSA results were identical to those reported for the IκB probe, likewise indicating a specific interaction between BgRHD and the kB motif upstream of p38 MAPK (Fig. 1B). In addition, EMSAs were carried out using BgRHD and a vertebrate consensus κB motif. Interestingly, the results demonstrated that BgRHD was also capable of binding the vertebrate κB probe, and this interaction could be reduced by the addition of unlabeled vertebrate probe, but not by an unlabeled mutant probe. However,