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Cloning and characterization of a high mobility group box 1 (HMGB1) homologue protein from *Schistosoma mansoni*☆

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Abstract

Mammalian homologue of high mobility group box chromatin protein (HMGB) 1 was identified and cloned from human parasites, *Schistosoma mansoni* and *S. haematobium*. Sequence analyses showed that the parasite HMGB1s has 35–40% identity to human and rodent HMGB1s, and 33% identity to *Caenorhabditis elegans* HMGB1. Parasite HMGB1s also contains an A box and B box domain similar to mammalian HMGB1, however, it lacks the C-terminal tail that is present in mammalian HMGB1s. Analysis of the expression of HMGB1 in various life cycle stages of *S. mansoni* reveal *S. mansoni* HMGB1 (SmHMGB1) as a stage-specific protein, expressed abundantly in egg and adult female stages and at moderate levels in skin-stage schistosomula. Significant levels of SmHMGB1 were also present in excretory secretions of egg stages. Subsequent characterization studies showed that SmHMGB1 is a potent inducer of pro-inflammatory cytokines such as TNFα, IL-1Rα, IL-2Rα, IL-6, IL-13, IL-13Rα1, IL-15 and MIP-1α from mouse peritoneal macrophages. Pro-inflammatory activity, especially production of TNFα-inducing activity, appears to be a function of the B box domain protein. This was confirmed by both real-time reverse transcription PCR and by cytokine ELISA. Thus, results presented in this study suggest that SmHMGB1 may be a key molecule in the development of host inflammatory immune responses associated with schistosomiasis.

Keywords: *S. mansoni*; HMGB1; TNFα; Gene array; Real-time RT-PCR

1. Introduction

Human schistosomiasis is a snail-transmitted, water-borne infection caused mainly by three different species of schistosomes, *Schistosoma mansoni*, *S. japonicum* and *S. haematobium*. Current estimates show more than 200 million people are infected with these parasites worldwide and another 300 million people are at risk [1]. Human infections occur when cercariae, the infective stages of the parasite penetrate intact skin and enter the body [2]. Egg-induced granuloma, mainly in the liver and other internal organs, is the major pathology associated with schistosomiasis [3,4]. Pro-inflammatory cytokines, especially tumor necrosis factor (TNF)α and IL-13 are thought to provide necessary immune priming for the formation of granuloma around eggs [5–8]. Although the actual role of TNFα in schistosomiasis is still debated [9–11], several studies have demonstrated significantly elevated circulating TNFα and soluble TNF receptors (sTNFR I & II) in subjects with hepatosplenomegaly [12–18]. The mechanism for the increased TNFα production in schistosomiasis is not fully understood. During a routine proteomic screening of the secretions of the schistosomula of *S. mansoni*, we identified a protein with significant sequence similarity to high mobility group box chromosomal protein 1 (HMGB1). Studies presented in this manuscript describe cloning and characterization of this protein from *S. mansoni* and a homologue of HMGB1 from a closely related parasite, *S. haematobium*.

HMGB1 is an abundant nuclear protein, highly conserved across several species including mammals, plants, yeast, *C. elegans*, *Drosophila melanogaster* and *Plasmodium falciparum*. Previous studies showed that HMGB1 proteins play an important role in DNA replication, transcription and chromatin assembly [19]. Thus, HMGB1 is primarily an endogenous nuclear protein. However, during a chronic inflammatory reaction, HMGB1

☆ *Note:* Nucleotide sequence data reported in this paper is available in GenBank under the accession number: SmHMGB1—AY485339 and ShHMGB1—AY560894.

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is actively secreted from macrophages through a process that involves acetylation of HMGB1 followed by translocation from the nucleus to the cytoplasm and released extracellularly [20]. HMGB1 can also be passively released from cells undergoing necrosis or death [21,22]. Regardless of either active or passive release, extracellularly released HMGB1 has been recently identified as a delayed mediator of inflammation and is shown to be a potent inducer of TNFα [21]. HMGB1 has been implicated in the pathogenesis of several inflammatory disorders, including bacterial sepsis [23–25], arthritis [26], anorexia [27], metastatic tumor [28] and malaria [29,30], because of their ability to induce TNFα production from macrophages. Significantly higher levels of HMGB1 are present in inflamed synovia during arthritis [31] and in serum during sepsis [32]. Thus, HMGB1 is a cytokine-like molecule with a broad spectrum pro-inflammatory activity [33–37]. In this study, we have evaluated whether the parasite derived HMGB1 has similar pro-inflammatory functions.

2. Materials and methods

2.1. Identification of SmHMGB1

N-terminal amino acid sequences of a purified protein from secretions of *S. mansoni* schistosomula showed significant sequence similarity to mammalian HMGB1 and to *S. japonicum* HMGB1. Subsequently, we searched the EST databases of *S. mansoni* with *S. japonicum* HMGB1 homologue (GenBank™ accession number AY223388) at the parasite genome BLAST server (www.ebi.ac.uk/blast2/parasites.html). This search identified 49 ESTs: 37 from germball stage, 5 from schistosomula stage, 4 from miracidium, 2 from adult stages and 1 from egg stages. After aligning the sequences, a forward primer specific to *S. mansoni* HMGB1 was designed (5′-ATGGCTGAAGACAAGGGTAAG-3′) from *S. mansoni* germball EST (EMBL:CD1332 23). As the ESTs were partial, 3′-end of HMGB1 could not be identified. Using 5′-end of SmHMGB1 sequence as forward primer and T7 promoter primer located downstream to the multiple cloning sites as reverse primer, a PCR reaction was performed on *S. mansoni* schistosomula cDNA library. PCR parameters were as follows: 30 cycles of denaturation for 30 s at 95 °C, primer annealing for 30 s at 58 °C, primer extension for 3 min at 72 °C and a final extension of 5 min at 72 °C. PCR products were cloned in pST-Blue vector (Novagen, Madison, WI), and DNA insert was sequenced on both strands to confirm authenticity of the gene. Sequences obtained were confirmed by BLAST analyses (www.ncbi.nlm.nih.gov) and deposited in the GenBank as SmHMGB1 under the accession number AY485339. SmHMGB1 homologue was then PCR amplified from cDNA preparations of adult *S. haematobium* using *S. mansoni* forward primer (5′-ATGGCTGAAGACAAGGGTAAG-3′) and oligo(dT) reverse primers. DNA sequencing was performed on both strands of the PCR products as above and sequences obtained were deposited in GenBank as ShHMGB1 under the accession number AY560894.

2.2. Construction of full length, A box and B box domains of SmHMGB1 expression vectors

Open reading frame (ORF) of SmHMGB1, A box and B box domains were cloned in T7 expression vector, pRSET A (Invitrogen, Lajolla, CA). Forward PCR primer corresponded to the beginning of SmHMGB1 ORF with addition of an upstream in-frame *Bam*HI restriction site (5′-CGCGGATC-CATGCTGGGGATCGAATTC-3′). Reverse primer corresponded to the 3′-end of SmHMGB1 ORF flanked by *Hin*dIII restriction site (5′-CCCAAGCTTCTAATCGTCAGACTCT-GA-3′). Forward primers for A box and B box domain corresponded to beginning of ORFs with upstream *Bam*HI restriction sites (5′-CGCGGATCCCCAAAAGGTGCTATGAATGCT-3′; 5′-CGCGGATCCCCTAAAAAGGCACTGTCAGCA-3′). Reverse primers corresponded to beginning of ORFs flanked by a *Hin*dIII restriction site (5′-CCCAAGCTTTTCATAA-TGTTCCATCTCAC-3′; 5′-CCCAAGCTTTTTGTATTTTTG-CATCGCTTT-3′). PCR parameters were 30 cycles of denaturation for 30 s at 95 °C, primer annealing for 30 s at 55 °C, primer extension for 30 s at 72 °C and a final extension of 5 min at 72 °C before storing samples at 4 °C. PCR products obtained were digested with *Bam*HI and *Hin*dIII and ligated to similarly digested T7 expression vector pRSET A. Insert DNA was sequenced to ensure authenticity of cloned nucleotide sequence on both strands.

2.3. Expression and purification of recombinant full length, A box and B box domains of SmHMGB1

Recombinant constructs of SmHMGB1, A box or B box domains in T7 expression vector were maintained in XL-1 Blue strains (Stratagene, Lajolla, CA). For expression, recombinant plasmids were transformed into BL21(DE3) containing pLysS (Invitrogen) to minimize toxicity. When OD of cultures reached 0.6, 1 mM IPTG (isopropyl thio-β-D-galacto pyranoside) was added to induce gene expression and incubated for an additional 3 h. Total proteins were separated in 12% SDS-PAGE gels and presence of histidine tagged protein was confirmed using penta-His antibody (Qiagen, Valencia, CA). Subsequently, histidine tagged recombinant proteins were purified under denaturing conditions in 8 M urea by immobilized cobalt metal affinity column chromatography (Clontech, Mountain View, CA) using an imidazole elution gradient as per the manufacturer's recommendations. Purified proteins were dialyzed against PBS at 4 °C to remove urea. Absence of urea in the final purified preparations was confirmed by a colorimetric kit (Sigma, St. Louis, MO). Purity of recombinant proteins was subsequently determined by separating each protein in a 12% SDS-PAGE and staining with SilverSNAP stain kit II (Pierce Biotechnology, Rockford, IL). Contaminating LPS in the samples were removed by passing purified recombinant proteins through a polymyxin B affinity column (Pierce Biotechnology) and levels of endotoxin/LPS in the final preparations were determined using an E-TOXATE kit (Sigma) as per manufacturer's instructions.