Published in final edited form as:

Int J Parasitol. 2009 February ; 39(3): 281–287. doi:10.1016/j.ijpara.2008.08.001.

A bone morphogenetic protein homologue in the parasitic flatworm, *Schistosoma mansoni*★

Tori C. Freitas, Euihye Jung, and Edward J. Pearce*

Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, 19143 USA

Abstract

Members of the bone morphogenetic protein (BMP) subfamily of cytokines control many aspects of metazoan development including patterning and organogenesis. Despite the recognition that schistosomes possess key components of a BMP signaling pathway, a BMP-like ligand in the parasitic flatworm *Schistosoma mansoni* remained elusive. Here, we describe the cloning and characterization of an *S. mansoni* BMP (*SmBMP*). *SmBMP* is most closely related to BMP homologues from the free-living flatworms *Schmidtea mediterranea* and *Dugesia japonica*, with 51% and 47% identity at the amino acid level, respectively. Based on reverse transcription-PCR, *SmBMP* is expressed throughout the mammalian life cycle of the parasite in both male and female schistosomes. In support of these results, antibodies to *SmBMP* successfully immunoprecipitated the protein in adult male and female antigen preparations with more protein detected in male parasites. Immunofluorescent studies localized *SmBMP* to the protonephridia of adult parasites, and *SmBMP* was identified in the excretory/secretory products of adult male parasites via immunoprecipitation. With the previous description of a TGF- β subfamily homologue in *S. mansoni*, ligands representing both arms of the TGF- β superfamily have now been described in this trematode.

Keywords

Trematode; Schistosome; Transforming growth factor-beta; Bone morphogenetic protein; Protonephridia

1. Introduction

Over 600 million people are at risk of infection with trematode parasites of the genus *Schistosoma* (Chitsulo et al., 2000). Schistosomiasis is one of the nine neglected tropical diseases that have received considerable attention over the last several years as the causes of extensive morbidity (Hotez et al., 2006). While seven of these neglected diseases are caused by nematode infections, only schistosomiasis is caused by a platyhelminth.

TGF- β signaling in mammals and model metazoan organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster*, are known to mediate a large number of physiological processes including growth and differentiation, cell death, tissue repair and developmental patterning (Massague et al., 2000). Members of the TGF- β superfamily can be split into two main subfamilies based on sequence homology and the different downstream pathways they activate, namely the TGF- β /activin/nodal subfamily and the bone morphogenetic protein/

★Note: Nucleotide sequence data reported in this paper is available in the GenBank database under accession number **EU684544**.

*Corresponding author. Tel.: +1 215 573 3493; fax: +1 215 746 2295. E-mail address: ejpearce@mail.med.upenn.edu.

growth and differentiation factor/Muellerian inhibiting substance (BMP/GDF/MIS) subfamily (Shi and Massague, 2003). The basic TGF- β signaling mechanism involves binding of the extracellular TGF- β homologue to a heterodimeric receptor complex resulting in the activation of specific cytoplasmic proteins (Smads) that eventually translocate to the nucleus to influence transcriptional responses. In mammals, it is known that members of the two subfamilies activate distinct classes of Smad proteins: TGF- β subfamily members activate Smad2 and Smad3 homologues while BMP subfamily members activate Smad1, Smad5 and Smad8 homologues. Several components of TGF- β signaling have been characterized from *S. mansoni* including TGF- β receptors SmRK1 (also known as SmT β RI) (Davies et al., 1998) and SmRKII (also known as SmT β RII) (Forrester et al., 2004; Osman et al., 2006), several Smad proteins (Beall et al., 2000; Osman et al., 2001, 2004; Carlo et al., 2007), and one homologue of the TGF- β subfamily, SmInAct (Freitas et al., 2007).

Previous work elucidating the role of TGF- β signaling in *S. mansoni* has been focused on components of the TGF- β subfamily, where this signaling pathway has been implicated in host-parasite interactions, parasite reproductive development and embryogenesis (Davies et al., 1998; Forrester et al., 2004; Osman et al., 2006; Freitas et al., 2007). The existence of a BMP signaling pathway in *S. mansoni* has been assumed since the cloning and characterization of two *S. mansoni* Smad1 homologues (SmSmad1 and SmSmad1b) (Beall et al., 2000; Carlo et al., 2007), however a ligand for the pathway has not been described. Here, we describe the cloning of an *S. mansoni* BMP homologue, *SmBMP*, and characterize its expression in various stages of parasite development.

2. Materials and methods

2.1. Parasites and animals

The Puerto Rican/Naval Medical Research Institute (NMRI) strain of *S. mansoni* was used in all experiments. Cercariae were collected by exposing infected *Biomphalaria glabrata* to light for 1 h. Adult parasites were recovered by hepatic-portal perfusion of C57BL/6 female mice (Jackson Laboratory, Bar Harbor, ME) infected 8 weeks previously via percutaneous exposure to ~ 60 cercariae. *Schistosoma mansoni* eggs were collected from the livers of infected mice as previously described (MacDonald et al., 2001). The use of mice in this study was approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania.

2.2. SmBMP isolation

The C-terminal deduced amino acid sequence of the *D. melanogaster* Decapentaplegic (DmDPP, **NP 477311**, amino acids 487–588) was used in a *tblastn* search of the Wellcome Trust's Sanger Institute's *S. mansoni* genome scaffolds version 3.1. (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/s_mansoni). A scaffold (Smp_scaff000116) with sequence showing homology to BMP-like ligands was identified and the corresponding putative coding sequence was named *SmBMP*. The 5' and 3' ends of *SmBMP* were isolated using total RNA (1 μ g) from adult parasites and the Superscript III Generacer RACE kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. An *SmBMP*-specific primer was designed for isolating the 3' end (5'-TTGGGTTATTGCACCACAAG-3') and was used with the Generacer 3' primer in reverse transcription (RT)-PCR as per the manufacturer's instructions. The resulting amplicon was cloned into the TA-TOPO cloning vector (Invitrogen, Carlsbad, CA) and sequenced. An in silico analysis of the *S. mansoni* genome aided in the identification of the 5' end of *SmBMP*. The Wellcome Trust's Sanger Institutes *S. mansoni* predicted coding sequence databases (Augustus3, GlimmerHMM, and Twinscan2) were searched for *SmBMP* by performing a *blastn* search of each database with the isolated 3' end of *SmBMP*. Each algorithm suggested different start sites and forward primers were designed corresponding to each (Augustus3 5'-

ATGAAATATGCAAATGTCAGTT-3'; GlimmerHMM 5'-ATGTTTAAATTACATGAACGTCATA-3'; Twinscan2 5'-ATGGGGGAAAAGTCACTTACTCT-3'). Each forward primer was used individually with an *SmBMP*-specific reverse primer (5'-TGGAAATGGACATTGACCTAAACA-3') in an RT-PCR using cercariae cDNA as template. Only the combination of the GlimmerHMM forward primer with the *SmBMP*-specific reverse primer amplified a product of predicted size which was confirmed to be *SmBMP* upon sequencing. Further in silico analysis revealed a long open reading frame (1,027 bp) upstream of the predicted GlimmerHMM 5' end. To isolate the 5' end of *SmBMP*, two *SmBMP*-specific reverse primers were designed within the GlimmerHMM amplified region and were used in 5' Rapid Amplification of cDNA ends (RACE) (5'-CTGGTTCAAAAATGGCTGCGGTTTG-3') and nested 5' RACE (5'-TGCTGCAACTAATTTTCATTTGAAGGT-3') reactions using the Generacer RACE kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The resulting amplicon was cloned and was confirmed by sequencing to be *SmBMP* (GenBank accession number [EU684544](#)).

2.3. Sequence analysis

Sequence comparisons between the deduced amino acid sequence of *SmBMP* and other TGF- β superfamily members were determined using the ClustalW algorithm and the Align 2 sequences (bl2seq) program at the National Center for Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/bl2seq/wblast2.cgi>) using the final ~ 100 amino acid residues of each sequence. An unrooted dendrogram was drawn using the final ~ 100 amino acids within the conserved domain of *SmBMP* and other members of the TGF- β superfamily, and distances were drawn using the Jones-Taylor-Thornton matrix and neighbor joining algorithm in the PHYLIP software package developed by J. Felsenstein (University of Washington, Seattle, Washington). Percentages at branch points were based on 1,000 bootstrap runs.

2.4. RNA extraction and RT-PCR

Total RNA was extracted from parasite material using Qiagen's RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Contaminating genomic DNA was removed using Turbo DNA-free endonuclease (Applied Biosystems, Foster City, CA). First strand cDNA was synthesized using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, 200 ng of total RNA from *S. mansoni* eggs, cercariae, adult males and adult females were reverse transcribed using Superscript II Reverse Transcriptase primed with oligo dT. RT-minus controls were performed to confirm the absence of any contaminating genomic DNA (data not shown).

Presence of an *SmBMP* transcript in the various parasite life stages was determined via conventional RT-PCR using Invitrogen's Recombinant *taq* DNA polymerase according to the manufacturer's instructions with 1 μ l of cDNA from each stage tested as template or water as a negative control. *SmBMP* primers were: forward 5'-GGTTGGGCTGGTTGGGTTAT-3' and reverse 5'-TGGAAATGGACATTGACCTAAACA-3'. Paramyosin primers were: forward 5'-CGTGAAGGTCGTCGTATGGT-3' and reverse 5'-GACGTTCAAATTTACGTGCTTG-3'. The cycling program used was 94°C for 2 min, then 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, followed by 72°C for 5 min. Amplified products were resolved on a 2% ethidium bromide stained agarose gel.

2.5. Recombinant *SmBMP* expression and antisera generation

EcoRI (forward) and *XhoI* (reverse) tagged primers were designed to amplify the region corresponding to the last 179 amino acids of *SmBMP* (forward 5'-GGAATTCAGTCAAGATCGTTATTATTA-3' and reverse 5'-CCGCTCGAGTTAACGACAAGCACAACCTT-3'). The cycling conditions were 1 cycle of

94 °C for 2 min, followed by 40 cycles of 94 °C for 30 s, 65 °C for 30 s, and 68 °C for 1 min, ending with one cycle of 68 °C for 5 min using Platinum Pfx DNA polymerase (Invitrogen, Carlsbad, CA). The amplified product was digested with *Eco*RI and *Xho*I and cloned into the bacterial expression vector pET28a (Novagen, Madison, WI), also digested with *Eco*RI and *Xho*I. Sequence analysis confirmed the absence of any mutations. Expression of recombinant SmBMP was induced in *Escherichia coli* BL21(DE3) by addition of 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) when cultures reached an O.D.₆₀₀ of 0.5 at 37 °C followed by further shaking at 37 °C for 3 h. Recombinant SmBMP was expressed in the bacteria as insoluble inclusion bodies and was purified via nickel column chromatography under denaturing conditions (6 M urea) as per the manufacturer's suggestions (Novagen, Madison, WI). Antiserum to SmBMP was generated by Cocalico Biologicals (Cocalico Biologicals, Inc., Reamstown, PA) by s.c. inoculation of 100 µg of purified recombinant SmBMP in FCA, followed by three boosts of 50 µg in Freund's incomplete adjuvant on days 14, 21 and 49, followed by exsanguination on day 64.

2.6. Immunoprecipitation and Western blot analysis

Antigen from adult male and female parasites was extracted by boiling worms in lysis buffer (1% SDS, 200 mM NaCl, 40 mM Tris pH8.8) and sheared via repeated passage through a 23 G needle. Protein concentration was determined by bicinchoninic acid (BCA) Protein Assay (Pierce, Rockford, IL). For immunoprecipitation of SmBMP, 80 µg of male and female antigen were diluted with 7 vol. of 1% NP40 and pre-cleared with 10 µl of anti-Rabbit IgG beads from the Rabbit IgG TrueBlot kit (eBioscience, San Diego, CA) for 1 h on a rocking platform at 4°C. Supernatant was removed and incubated with 3 µg of either pre-immune rabbit IgG or anti-SmBMP IgG at 4°C for 16 h on a rocking platform. Antibodies were precipitated by adding 15 µl of anti-Rabbit IgG beads to the antigen-IgG mixtures and incubated for 1 h at 4°C on a rocking platform. Precipitated antibody-antigen complexes were washed three times with 1% NP40 and prepared for SDS-PAGE analysis by boiling in a reducing SDS lysis buffer (50 mM Tris pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) in a total volume of 25 µl. Samples were separated by 10% SDS-PAGE, electroblotted onto Immobilon nylon membrane (Millipore, Billerica, MA), and probed with anti-SmBMP antiserum (1:10,000). Bound rabbit antibodies were detected using Rabbit IgG TrueBlot antibodies (eBioscience, San Diego, CA) according to the manufacturer's instructions. As a loading control, 20 µg of each adult antigen preparation used in the immunoprecipitation were also separated via 10% SDS-PAGE and electroblotted. This blot was probed with a monoclonal antibody (4B1) against paramyosin (1:10,000) and bound antibodies were detected using affinity purified horseradish peroxidase (HRP)-conjugated horse anti-mouse IgG (Cell Signaling Technology, Danvers, MA). Secondary antibodies were detected using ECL reagents as directed by the manufacturer (GE Healthcare, Piscataway, NJ).

Excretory/secretory products from adult male parasites were collected by culturing 10 worms in each well of a six well plate in a total volume of 4 ml of M199 (Invitrogen, Carlsbad, CA), 10% FCS, 1% Antibiotic/Antimycotic (Gibco), and 1% HEPES in a 37 °C/5% CO₂ atmosphere for 48 h. Harvested culture supernatant was pre-cleared with 30 µl of anti-Rabbit IgG beads for 1 h on a rocking platform at 4°C, split into equal volumes and incubated with 3 µg of either pre-immune rabbit IgG or anti-SmBMP IgG at 4°C for 16 h on a rocking platform. Antibodies were precipitated and antibody-antigen complexes were detected as described above.

2.7. Immunolocalization

To localize SmBMP within adult parasites, 5 µm sections of worms were treated with xylene and rehydrated through an ethanol series ending in PBS. Slides were blocked with 2% normal goat serum (Sigma, St. Louis, MO) for 1 h at room temperature and probed with either rabbit pre-immune serum or anti-SmBMP serum (1:100) overnight at 4°C. Sections were washed

three times in PBS and bound rabbit antibodies were detected by probing with a fluorescein-conjugated F(ab')₂ goat anti-rabbit IgG (1:50) (Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. Slides were washed three times in PBS, dehydrated in ethanol and mounted. Worm sections were photographed using a Leica DMIRB microscope and DC500 camera (Leica, Germany).

3. Results

3.1. Cloning and sequence analysis of SmBMP

SmBMP was identified in the Wellcome Trust's Sanger Institute's *S. mansoni* genome sequence database through a *rblastn* search using the last 100 amino acids of the *D. melanogaster* decapentaplegic (DPP) protein. The 5' and 3' ends of *SmBMP* were isolated via 5' and 3' RACE using *SmBMP*-specific primers designed from within the predicted coding sequence according to the genome sequence and adult parasite cDNA as template. The full-length transcript is 3,013 bp including a 124 bp 5' untranslated region (UTR) and a 102 bp 3'UTR with a poly-A tail. The deduced amino acid sequence of SmBMP is 931 residues and contains many of the molecular characteristics of a BMP homologue including several putative proteolytic cleavage sites at positions 660 (RKPR), 700 (RYKR), 703 (RLQR), 738 (RSRR) and 748 bp (RHNR) where the bioactive, C-terminal domain (268–180 amino acids) could be enzymatically cleaved from the N-terminal pro-domain (Fig. 1). Seven invariant cysteine residues are predicted in SmBMP along with other invariant residues (proline 851 and glycine 861) necessary for proper tertiary structure and dimerization of a functional BMP homologue (Fig. 2). Interestingly, signal sequence prediction software suggests the N-terminus of SmBMP does not contain a secretion signal. Within the C-terminal conserved domain (the last ~ 100 amino acids), SmBMP is 51% and 47% identical to BMP homologues from the free-living flatworms *Schmidtea mediterranea* and *Dugesia japonica*, respectively (Fig. 2). Furthermore, it is 46% identical to *D. melanogaster* DPP and 49% identical to human BMP2 at the amino acid level (Fig. 2). Phylogenetic analysis of SmBMP among other members of the TGF- β superfamily groups this homologue with members of the BMP subfamily (Fig. 3) and most closely with BMP-like ligands from *S. mediterranea* and *D. japonica* (Smed-BMP and DjBMP). This close clustering is expected considering all three worms are members of the phylum Platyhelminthes. Based on this analysis, it cannot be determined whether SmBMP is most closely related to members of the BMP2/4 or BMP5/8 subfamilies.

3.2. SmBMP transcript and protein expression and localization

To determine the expression profile of the *SmBMP* transcript, conventional RT-PCR was performed on cDNA from eggs, cercariae, adult male and adult female parasites. As seen in Fig. 4, *SmBMP* is expressed across all four stages tested compared with the reference gene paramyosin. This expression pattern was reproducible between separate RT-PCR experiments with different RNA/cDNA preparations. *SmBMP* was not identified in blast searches of the Sanger Institute's *S. mansoni* expressed sequence tag (EST) databases.

Immunoprecipitation and Western blot analyses using polyclonal antibodies against recombinant SmBMP were performed to investigate the expression of SmBMP protein. Here, we focused on SmBMP expression in adult male and female parasites. An ~ 100 kDa band was precipitated using anti-SmBMP antibodies in male antigen preparations which, based on the predicted molecular weight of the full-length transcript (109 kDa), corresponds to the preprocessed form of SmBMP (Fig. 5). This band was not immunoprecipitated using equivalent amounts of pre-immune antibodies. A smaller band was precipitated from female antigen (~ 85 kDa) as well as a smear of proteins under the 100 kDa band in the male antigen preparation. It is unclear at this time whether these are degraded products or represent alternative forms of SmBMP. A band representing a putative C-terminal, bioactive form of SmBMP with a

molecular weight of ~ 37 kDa was also precipitated from male antigen while overexposing the same blot revealed a second potential bioactive form of SmBMP with a molecular weight of ~ 22 kDa. The ~ 37 kDa band could be the result of a cleavage event at the 660 bp predicted site while the ~ 22 kDa band may be formed by cleavage at either position 738 or 748. Based on the intensities of the pre-processed SmBMP bands, male parasites appear to express more SmBMP than female parasites.

To determine where SmBMP is expressed in the worm, we performed immunofluorescence localization assays on sections of adult parasites. Anti-SmBMP antibodies localized SmBMP to the protonephridia of adult male parasites (Fig. 6). Intense staining was observed in the main tubules of the protonephridia that run laterally on either side of the worm (panel C) as well as within branches of the system (panel D). No positive staining was detected in parasite sections using pre-immune serum.

Using culture supernatant of adult male parasites, we performed immunoprecipitation and Western blot analyses to determine whether or not SmBMP is secreted from the worm. Anti-SmBMP antibodies immunoprecipitated several bands from media exposed to adult males including an ~ 80 kDa band and bands around 40 kDa in size. These bands were not immunoprecipitated using equivalent amounts of pre-immune antibodies.

4. Discussion

It is well established that *S. mansoni* possesses multiple components of active TGF- β signaling, however little focus has been given to the BMP subfamily in this parasitic flatworm. In this study, we report the identification and characterization of *SmBMP*, a BMP homologue in *S. mansoni*. *SmBMP* is expressed in medically important stages of the parasite, namely the egg, cercariae and adult parasites, and localizes to the protonephridia of the adult worm. A recent review by Loverde and colleagues alludes to a BMP homologue in the *S. mansoni* genome (Loverde et al., 2007). Assuming the gene described in this review is that found in Genbank under accession number [EF028064](#), we believe the BMP homologue characterized in this study is the same as that described by Loverde et al. with two exceptions: (i) our clone's deduced amino acid sequence is 402 amino acids longer at the N-terminus and, (ii) the putative cleavage site is not 129 amino acids upstream from the stop codon, but rather 272 amino acids upstream, with a second and/or third potential cleavage site(s) at either 194 or 184 amino acids upstream of the stop codon. Multiple cleavage sites are not atypical of BMP homologues (Cui et al., 2001). This would make the molecular weight of the SmBMP active dimer to be approximately 74 kDa (or ~44 kDa from either of the downstream sites), rather than the previously described 23 kDa (Loverde et al., 2007).

The deduced amino acid sequence of SmBMP is longer than all other full-length BMP homologues analyzed in this study. For example, SmBMP is 530 residues longer than its most related homologue, Smed-BMP, and 535 residues longer than human BMP-2. Most of this increased length is attributed to a longer N-terminus of SmBMP; 659 amino acids lie upstream of the first cleavage site of SmBMP (737 and 747 residues are upstream of the second and third potential cleavage sites, respectively) while 287 make up the N-terminus of Smed-BMP. Functions of the pro-domain in BMP homologues include directing the ligand to the extracellular matrix (Gregory et al., 2005) and in protein stability (Constam and Robertson, 1999). Furthermore, the pro-domain is believed to be essential for proper dimerization and folding of nearly all TGF- β superfamily members (Gray and Mason, 1990). It remains possible that, because different stages of *S. mansoni* live at different temperatures (~24°C in the intermediate snail host and environment and at 37°C in the definitive host), this longer pro-domain may aid in proper folding of the protein regardless of temperature.

One function of BMP signaling is to establish the dorsalventral (DV) axis in the development of both vertebrates and invertebrates (reviewed in De Robertis and Kuroda, 2004). Recently, RNA interference (RNAi) studies have shown that BMP signaling plays a role in specifying the DV axis in the free-living planaria *D. japonica* and *S. mediterranea* (Molina et al., 2007; Orii and Watanabe, 2007). Considering SmBMP is most closely related to the BMP homologues isolated from these free-living flatworms and that, while *S. mansoni* is a trematode and *D. japonica* and *S. mediterranea* are free-living flatworms, they are nonetheless closely related phylogenetically, it is likely that SmBMP plays a similar role in *S. mansoni*. We treated adult parasites as well as growing schistosomula with double-stranded RNA (dsRNA) corresponding to *SmBMP*, however, while ~40% knockdown was observed using quantitative RT-PCR (data not shown), immediate phenotypes were not observed. This could be due to insufficient knockdown compared with that obtained in *D. japonica* and *S. mediterranea*. It is appealing to speculate that the increase expression of SmBMP in male parasites is a reflection of a potential role for SmBMP in establishing the DV axis. The dorsal and ventral surfaces of male parasites are anatomically and physiologically much more distinct than in females with many tubercles on the dorsal surface and the formation of the gynacophoral canal on the ventral surface, for example.

The localization of SmBMP in *S. mansoni* is different than that observed of the two other platyhelminth BMP homologues where SmBMP is expressed in the protonephridia while DjBMP and Smed-BMP are found along the dorsal midline (Orii et al., 1998; Molina et al., 2007). The protonephridia is believed to serve as a primitive kidney, functioning to regulate the composition of fluids in the organism and in the clearance of metabolic wastes (Wilson and Webster, 1974). Other protein processing and secreting functions have also been proposed for this organ (Finken-Eigen and Kunz, 1997; Skelly and Shoemaker, 2001). One possible explanation for the discrepancy in localization could be the methods employed. We used immunofluorescence on worm sections to localize SmBMP while whole-mount in situ hybridization was used to localize both *DjBMP* and *Smed-BMP* in the free-living worms. Therefore, we could be detecting cells that have simply bound and are responding to SmBMP as opposed to those actually producing it. Additionally, considering the potential of the protonephridia to serve as a means of protein excretion/secretion, localization of SmBMP here may be explained by impending release of SmBMP in excretory/secretory products. To this end, we isolated proteins in the excretory/secretory products of adult male parasites using our antibodies against SmBMP, suggesting SmBMP is secreted by the parasite. This is an interesting finding in that secreted forms of SmBMP will have the opportunity to interact with host receptors. Lastly, BMP signaling is known to play an important role in the development of the vertebrate kidney (reviewed in Cain et al., 2008). Thus, it remains possible that SmBMP functions in the development and/or maintenance of the trematode protonephridia.

To date, two Smad1 homologues have been characterized in *S. mansoni*, SmSmad1 and SmSmad1b (Beall et al., 2000; Carlo et al., 2007). The expression of SmSmad1 has not been localized to specific tissues of the worm while SmSmad1b has been localization to the vitellaria, reproductive ducts and subtegumental tissues of the adult female and in subtegumental and an unidentified tissue of the adult male (Carlo et al., 2007). If SmBMP ligation to its receptor leads to the phosphorylation of both of these Smad1 homologues, SmBMP may have distinct functions in cells that express SmSmad1, SmSmad1b, or a combination of the two, by mediating different transcriptional responses. To our knowledge, only one Smad1 homologue has been identified in free-living flatworms (Molina et al., 2007). Future experiments will be necessary to determine if SmBMP is capable of leading to the phosphorylation of either *S. mansoni* Smad1 homologue.

With the isolation of a BMP homologue from *S. mansoni*, we now have representatives from each major subfamily of the TGF- β superfamily in this parasitic flatworm. The current version

of the *S. mansoni* genome sequence suggests *SmBMP* and *SmInAct* are the only two TGF- β -like ligands present. TGF- β signaling is believed to be present in all metazoans providing a pathway that helps to regulate aspects of development key to being a multicellular organism, such as cell proliferation, differentiation, organization and death (Massague and Gomis, 2006). Considering *D. melanogaster* has seven TGF- β -like ligands and the nematode *C. elegans* has four, it will be interesting to determine how many functions the two TGF- β homologues in the platyhelminth *S. mansoni* may fulfill.

Acknowledgments

Schistosome life stages used in this research were supplied by the National Institute of Allergy and Infectious Diseases (NIAID) Schistosomiasis Resource Center at the Biomedical Research Institute (Rockville, Maryland, United States) through NIAID Contract NO1-AI-30026. This work was supported by NIH grant R01AI075226 to E.J.P. T.C.F. was supported by grant number F32AI071417 from the National Institute of Allergy and Infectious Disease.

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Fig. 1.

Genomic organization of *SmBMP*. Boxes represent exons with Roman numerals above each box indicating the exon number. Dashed lines represent introns. Exon length is indicated within each box while intron length is indicated above each dashed line. The solid lines flanking the coding region of *SmBMP* represent the 5' and 3' untranslated region (UTR) of the transcript. Arrows indicate potential cleavage sites (RXXR) where asterisks specify those sites with experimental evidence.

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SmBMP   CQRRELIINFDVAGWAGWVIAPQAYNARYCLGQCPFPLSTHYNTTNHAVLL 621
Smed-BMP CQRYSLIVTFKEVGWSKWIIPQNYNAYYCKGNCPYPLSDNFNATNHAIQ 350
DjBMP   CQRYPLIVTFKEVGWSKWIIPQNYNAYYCKGNCPYPLSDNFNATNHAIQ N/A
DmDPP   CRRHSLYVDFSDVGWDDWIVAPLGDAYYCHGKCPFLADHFNSTNHAVVQ 537
HsBMP2  CKRHPLYVDFSDVGWNDWIVAPPGYHAFYCHGECPPFLADHLNSTNHAIQ 346
      *.: * : * . *** *.:** *.* ** *.**.*: : :*.***.:

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SmBMP   QLVHLLDVARISGPCCVPHQLSSQSLLYHSQNGDVVLRVYEDMVVESACR 672
Smed-BMP LLIHGLKD-SIPKPCCVPYNLRPETLLYLNREGDALLREFKMSVSSCSCH 400
DjBMP   LLVHGLKDL SIPKPCCVPYHLHPETLLYLNNEG DALLREFKMSVSSCSCH N/A
DmDPP   TLVNNMNP GKVPKACCVPTQLDSVAMLYLNDQSTVVLKNYQEMTVVGCGR 588
HsBMP2  TLVNSVN-SKIPKACCVPTELSAISMLYLDENEKVVLKNYQDMVVEGCGCR 396
      *.: :. :. .*** * . :.* . .*: :.* * .*:

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Fig. 2.

Multiple sequence alignment of the deduced amino sequence of the C-terminal region of SmBMP and other members of the BMP/GDF/MIS subfamily of TGF- β proteins. Shaded residues represent the invariant cysteine, proline and glycine moieties of all BMP homologues. Numbers to the right indicate the position of the last amino acid in the row within each sequence. In the case of DjBMP, a full-length sequence is not available, therefore the positions are not applicable. Asterisks under the alignment indicate exact matches across all five sequences, “.” represents strong similarity, “:” represents weak similarity, and a lack of a symbol indicates no similarity among residues.

Fig. 3. Phylogenetic representation of SmBMP among other members of the TGF-β superfamily. SmBMP (boxed) is shown clustering among members of the BMP/GDF/MIS subfamily (solid line) and not with members of the TGF-β subfamily (dashed line). Conserved amino acids in the C-terminal region (the last ~100 amino acids) of each protein was used in the analysis. Percentages at branch points are based on 1,000 bootstrapping events. Accession numbers for sequences used in the analysis are: *Acropora millepora* decapentaplegic (AmDPP), **AAM54049**; *Actinia equina* BMP2/4 (AeBMP2/4), **AAK20912**; *Amphiura filiformis* afuni (Afuni), **AAX54512**; *Ancylostoma caninum* TGH-1 (AcTGH1), **AAX36081**; *Branchiostoma floridae* BMP2/4 (BfBMP2/4), **AAC97488**; *Brugia malayi* TGH-1 (BmTGH1), **AF012878**;

B. malayi TGH-2 (BmTGH2), **AF104016**; *Caenorhabditis elegans* DAF-7 (CeDAF7), **NP 497265**; *C. elegans* DBL-1 (CeDBL1), **NP 504709**; *Ciona intestinalis* TGF- β superfamily signaling ligand (CiBMP), **NP 001072008**; *Crassostrea gigas* GDF (CgGDF), **CAA10268**; *Drosophila melanogaster* decapentaplegic (DmDPP), **NP 477311**; *D. melanogaster* glass bottom boat (DmGbb), **NP 477340**; *D. melanogaster* Screw (DmScrew), **NP 524863**; *Dugesia japonica* BMP (DjBMP), **BAA32087**; *Homo sapiens* BMP-2 (HsBMP2), **NP 001191**; *H. sapiens* BMP-4 (HsBMP4), **NP 001193**; *H. sapiens* BMP-5 (HsBMP5), **NP 066551**; *H. sapiens* BMP-6 (HsBMP6), **AAH99625**; *H. sapiens* BMP-7 (HsBMP7), **NP 001710**; *H. sapiens* BMP-8 (HsBMP8), **XP 001716617**; *H. sapiens* TGF- β 1 (HsTGF β 1), **CAA29283**; *H. sapiens* TGF- β 2 (HsTGF β 2), **NP 003229**; *Nematostella vectensis* BMP2/4 (NvBMP2/4), **AAR13362**; *N. vectensis* BMP5-8 (NvBMP5/8), **ABC88372**; *Paracentrotus lividus* univin (PIUnivin), **ABG00200**; *Platynereis dumerilii* decapentaplegic (PduDPP), **CAJ38807**; *Ptychodera flava* BMP2/4 (PfBMP2/4), **BAA89012**; *Saccoglossus kowalevskii* BMP2/4 (SkBMP2/4), **ABD97262**; *S. kowalevskii* BMP5/8 (SkBMP5/8), **ABD97263**; *S. mansoni* TGF- β (SmInAct), **ABI64156**; *Schmidtea mediterranea* BMP (Smed-BMP), **ABV04322**; *Strongylocentrotus purpuratus* (SpUnivin), **NP 999793**; *Strongyloides stercoralis* (SsTGH1), **AY662390**.

Fig. 4.

Expression of *SmBMP* transcript. First strand cDNA from various *Schistosoma mansoni* stages was used in reverse transcription-PCR. Ethidium bromide-stained gel of products after 35 cycles of amplification. Numbers to the right of each gel represent the amplicon length for each transcript. The life-cycle stage is indicated at the top of each lane.

Fig. 5.

Expression of native SmBMP protein. Antigen extracts of adult male and female parasites were immunoprecipitated with antibodies from either rabbit pre-immune serum or antibodies from rabbit serum after immunization with recombinant SmBMP. SmBMP was only precipitated when anti-SmBMP antibodies were used. The bracket indicates the pre-processed form of SmBMP (~ 100 kDa) while the arrow points to a processed form of SmBMP (~ 37 kDa). Heavy and light chains of the rabbit IgG molecules used in immunoprecipitation are seen at ~ 50 and 25 kDa, respectively. This blot was over-exposed and the region surrounding ~ 25 kDa is shown to the right with an arrow pointing to a second potential processed form of SmBMP (~ 22 kDa). Equivalent parasite antigen was loaded on a separate gel and probed with anti-paramyosin antibodies.

Fig. 6.

Immunofluorescence localization analyses. Panels A and B are differential interference contrast (DIC) images and panels C–F are fluorescence images of adult male sections. Arrows point to the localization of SmBMP in the main tubules (panel C) and branches of the protonephridia (panel D). G, gut; D, dorsal; V, ventral. Scale bar length indicated in panels A and B are the same as panels C and E, and D and F, respectively. Pictures shown are representative of two separate experiments.

Fig. 7.

Secretion of SmBMP from adult male parasites. Culture supernatants from male parasites were immunoprecipitated using antibodies from either rabbit pre-immune serum or antibodies from rabbit serum after immunization with recombinant SmBMP. The arrow points to an 80 kDa band specifically immunoprecipitated with anti-SmBMP antibodies while the bracket corresponds to bands ~ 35–42 kDa in size also immunoprecipitated with anti-SmBMP antibodies.