Schistosoma mansoni TGF-β Receptor II: Role in Host Ligand-Induced Regulation of a Schistosome Target Gene

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Members of transforming growth factor-beta (TGF-β) superfamily play pivotal roles in development in multicellular organisms. We report the functional characterization of the Schistosoma mansoni type II receptor (SmTβRII). Mining of the S. mansoni expressed sequence tag (EST) database identified an EST clone that shows homology to the kinase domain of type II receptors from different species. The amplified EST sequence was used as a probe to isolate a cDNA clone spanning the entire coding region of a type II serine/threonine kinase receptor. The interaction of SmTβRII with SmTβRI was elucidated and shown to be dependent on TGF-β ligand binding. Furthermore, in the presence of human TGF-β1, SmTβRII was able to activate SmTβRI, which in turn activated SmSmad2 and promoted its interaction with SmSmad4, proving the transfer of the signal from the receptor complex to the Smad proteins. Gynaecophoral canal protein (GCP), whose expression in male worms is limited to the gynaecophoric canal, was identified as a potential TGF-β target gene in schistosomes. Knocking down the expression of SmTβRII using short interfering RNA molecules (siRNA) resulted in a concomitant reduction in the expression of GCP. These data provide evidence for the direct involvement of SmTβRII in mediating TGF-β-induced activation of the TGF-β target gene, SmGCP, within schistosome parasites. The results also provide additional evidence for a role for the TGF-β signaling pathway in male-induced female reproductive development.

Introduction

Members of the transforming growth factor-beta (TGF-β) superfamily of secreted polypeptide growth factors play important and diverse roles in cellular growth, differentiation, extracellular matrix formation, and immunosuppression [1–8]. The classical paradigm of TGF-β signaling has been elucidated through numerous studies of different model organisms (for detailed reviews see [9–18]). TGF-β mitogenic effects are mediated through binding to the corresponding plasma membrane receptors of the serine/threonine receptor kinase family. This family of membrane receptors is divided into two subclasses of distinct but structurally related receptors. The type II receptor binds TGF-β and then recruits the corresponding type I receptor to form an active ligand–receptor complex, in which the type I receptor gets phosphorylated and activated by the type II receptor. Phosphorylation occurs on the serine and threonine residues in a domain in the juxtamembrane region called the GS domain [19,20]. The activated type I receptor relays the signal to the next component of the signaling pathway, a member of the Smad family of TGF-β signal transducers, the receptor-regulated Smad or R-Smad. Upon phosphorylation by the type I receptor, the R-Smad forms a hetero-oligomeric complex with a common Smad or a Co-Smad (Smad4) [21,22]. The newly formed Smad complex then translocates into the nucleus where it binds nuclear proteins that direct the Smad complexes to specific promoter sequence(s), where they regulate the transcription of the target gene(s) to exert the specific cellular responses triggered by ligand activation.

Structurally, members of the receptor family consist of relatively short cysteine-rich, three-finger toxin fold extracellular domains [23], single transmembrane (TM) domains, and intracellular regions composed mainly of the serine/threonine kinase domains [24–26]. Not only the specificity of TGF-β signaling is determined by the type I receptor activation by type II receptor, but also the downstream transmission of signals by the type I receptor is dependent on its binding to and subsequent activation by type II receptor. In general, type II receptors are constitutively active kinases [19,27–29] that undergo reorientation of the intracellular domains upon ligand binding, which results in activation of the recruited type I receptor [30].

Flat worms of the genus Schistosoma cause schistosomiasis, a...
In addition to the upstream components such as the type II receptor, the search for TGF-β-responsive gene(s) is important since the modulation of target gene expression dictates the phenotypic outcome of growth factor activation based on the cellular context. To that end, searching protein databases for genes possibly regulated by TGF-β resulted in the identification of a protein homolog to a secreted matrix protein whose expression is greatly induced by TGF-β in human lung adenocarcinoma cell line (Bīg-h3) [40]. The schistosome counterpart is an 86-kDa glycoprotein that is expressed on the surface of adult female worms. In male worms, its surface expression is limited to the gynaecophoric canal (S. mansoni gynaecophoral canal protein [SmGCP]), the site of direct interaction between the mating pair [41]. SmGCP contains multiple short, conserved repeats that exhibit homology to the developmentally regulated neural cell adhesion molecule fasciclin-I from Drosophila melanogaster [42]. In this study, we report the isolation and characterization of the full-length coding sequence of S. mansoni TGF-β type II receptor (SmTβRII) that extends the cDNA sequence an extra 1.5 kilobases (kb) longer than was previously reported (SmRK2a). The extra cDNA sequence encodes the correct C-terminal end of SmRK2a, a 3’-UTR, as well as the polyadenylation signal and poly A tail. We also demonstrate a role for host ligands in regulating schistosome gene expression by showing the direct involvement of SmTβRII in human TGF-β ligand binding. In addition, we report the identification of SmGCP [42] as a TGF-β target gene in the parasitic helminth S. mansoni, and we present evidence for its transcription activation by human TGF-β1 via an SmTβRII-dependent mechanism in the parasite itself.

Results

SmTβRII: Identification, Cloning, and Sequence Analyses of the cDNA and Description of Genomic Organization

Two overlapping expressed sequence tag (EST) clones (CD126244 and CD069595) showed similarity to part of the kinase domain of the TGF-β type II receptor from different organisms and were found to span a region in the intracellular kinase domain of the isolated receptor corresponding to base pairs (bp) 755–1,608 of the submitted cDNA sequence. The use of a PCR product derived from these EST clones as a screening probe for S. mansoni cDNA expression libraries resulted in the isolation of five positive clones. The isolated cDNA clones were sequenced from both strands, and the sequence data were analyzed using the National Center for Biotechnology Information “BLASTX” homology search of protein databases. It was found that these clones belong to TGF-β receptor family of type II subclass. Of these, a 3,932-bp cDNA clone was found to encode a protein of 815 aa, which contains the entire predicted coding sequence of TGF-β type II receptor. Sequence comparisons demonstrated that the schistosome receptor shows highest similarity to mammalian Activin type II receptors (rat, mouse, and human, respectively). These results were based on comparison of the kinase domains of the isolated clone and other members of type II receptor subfamily. However, the N-terminal ECD and the TM domains showed greatest similarity to Activin type II receptors from the spotted green puffer, Tetraodon nigroviridis, and the zebra fish, Danio rerio, and to BMP type II receptor of the giant pacific oyster, Crassostrea gigas. Further analysis using

Growth and maturation. Earlier studies identified members of signal transmission that could be involved in the parasite investigation of this signaling pathway as a plausible means of superfamily on a wide array of cell types stimulated the received from the host. Schistosoma mansoni the parasite. Members of transforming growth factor-beta (TGF-β) superfamily play pivotal roles in development in multicellular organisms. TGF-β signaling requires ligand binding to a specific surface receptor, TGF-β type II receptor. The authors isolated the schistosome TGF-β type II receptor (SmTβRII), which was found to be biologically active and responded to stimulation by host TGF-β. The gynaecophoric canal is a ventral groove in the male worm in which the female must reside for sexual maturity. Gynaecophoral canal protein (GCP) is a protein whose expression in male worms is limited to the gynaecophoric canal and is implicated in female reproductive maturation. GCP expression was found to be regulated by human TGF-β. Knocking down the expression of SmTβRII resulted in a concomitant reduction in the expression of GCP, providing evidence for the direct involvement of SmTβRII-mediated, host TGF-β-induced regulation of schistosome gene expression. This study implicates the TGF-β signaling pathway in worm pairing, a prerequisite for female egg production. Because the eggs produced by the worm pairs are responsible for pathogenesis, the authors’ research identifies potential targets for intervention.

In addition to the upstream components such as the type II receptor, the search for TGF-β-responsive gene(s) is important since the modulation of target gene expression dictates the phenotypic outcome of growth factor activation based on the cellular context. To that end, searching protein databases for genes possibly regulated by TGF-β resulted in the identification of a protein homolog to a secreted matrix protein whose expression is greatly induced by TGF-β in human lung adenocarcinoma cell line (Bīg-h3) [40]. The schistosome counterpart is an 86-kDa glycoprotein that is expressed on the surface of adult female worms. In male worms, its surface expression is limited to the gynaecophoric canal (S. mansoni gynaecophoral canal protein [SmGCP]), the site of direct interaction between the mating pair [41]. SmGCP contains multiple short, conserved repeats that exhibit homology to the developmentally regulated neural cell adhesion molecule fasciclin-I from Drosophila melanogaster [42]. In this study, we report the isolation and characterization of the full-length coding sequence of S. mansoni TGF-β type II receptor (SmTβRII) that extends the cDNA sequence an extra 1.5 kilobases (kb) longer than was previously reported (SmRK2a). The extra cDNA sequence encodes the correct C-terminal end of SmRK2a, a 3’-UTR, as well as the polyadenylation signal and poly A tail. We also demonstrate a role for host ligands in regulating schistosome gene expression by showing the direct involvement of SmTβRII in human TGF-β ligand binding. In addition, we report the identification of SmGCP [42] as a TGF-β target gene in the parasitic helminth S. mansoni, and we present evidence for its transcription activation by human TGF-β1 via an SmTβRII-dependent mechanism in the parasite itself.

Synopsis

Schistosomes are multicellular parasites that infect 200 million people worldwide. Schistosome development in the human host likely involves host molecules that regulate biological processes of the parasite. Members of transforming growth factor-beta (TGF-β) superfamily play pivotal roles in development in multicellular organisms. TGF-β signaling requires ligand binding to a specific surface receptor, TGF-β type II receptor. The authors isolated the schistosome TGF-β type II receptor (SmTβRII), which was found to be biologically active and responded to stimulation by host TGF-β. The gynaecophoric canal is a ventral groove in the male worm in which the female must reside for sexual maturity. Gynaecophoral canal protein (GCP) is a protein whose expression in male worms is limited to the gynaecophoric canal and is implicated in female reproductive maturation. GCP expression was found to be regulated by human TGF-β. Knocking down the expression of SmTβRII resulted in a concomitant reduction in the expression of GCP, providing evidence for the direct involvement of SmTβRII-mediated, host TGF-β-induced regulation of schistosome gene expression. This study implicates the TGF-β signaling pathway in worm pairing, a prerequisite for female egg production. Because the eggs produced by the worm pairs are responsible for pathogenesis, the authors’ research identifies potential targets for intervention.
Dense Alignment Surface method (DAS; http://www.sbc.su.se/~miklos/DAS) [43] predicted a single TM domain spanning aa residues 139 to 164. The protein sequence apparently lacks membrane-anchoring motifs such as GPI (glycosylphosphatidylinositol)-anchoring signals or signal peptides.

The cDNA clone reported in this study, SmTβRII, contains a 64-bp 5' UTR and a 1.4 kb 3' UTR, with a poly A tail and a putative ‘ATTAAA’ polyadenylation signal located 21 bp upstream of the poly A tail. The use of the full-length SmTβRII cDNA sequence to screen *S. mansoni* genomic databases resulted in the assembly of several contigs to construct the genomic gene of TGF-β type II receptor (Figure 1). The 26 kbp gene consists of nine exons and contains about 2.9 and 2.4 kbp of genomic DNA located upstream and downstream of the first and last exon of SmTβRII, respectively. Analysis of the genomic sequence of SmTβRII and comparison to previously identified cDNA clones revealed the presence of two transcripts predicted to produce two different isoforms of type II receptor. One transcript encodes the sequence of the previously reported receptor (550 aa; SmRK2) [39]. A second, longer mRNA translates into the receptor described in this report (SmTβRII). These transcripts are produced from the same gene by alternative splicing of the last two exons (exons 8 and 9) (Figure 1). SmRK2 and SmRK2a were reported to contain 100- and 105-bp 5' UTRs, respectively [39]. Both cDNAs contain an extra 42 bp upstream of what was found in SmTβRII, which is also present in the genomic DNA and likely represents part of the first exon. The rest of the 5’-UTR of SmRK2, SmRK2a, and SmTβRII is almost identical (below).

The 5'-end of SmRK2a shows a 1-bp deletion at position 110, as compared to that of SmRK2 and SmTβRII, which results in a frame shift that deletes amino acids 2 and 3, glutamate and cysteine. The sequence of the corresponding genomic DNA was found to match that of SmRK2 and SmTβRII, but not SmRK2a. Therefore, we believe that the deletion could be attributed to a sequencing error of the SmRK2a cDNA. Regarding the 3'-ends, SmRK2a encodes a 754–aa protein that was proposed to lack the stop codon [39]. The authors explained that this transcript could represent a nonfunctional, rapidly degraded mRNA, or might encode a functional protein albeit lacking the stop codon [39]. DNA sequence data retrieved from genomic DNA databases revealed that the C-termini of SmTβRII and SmRK2a and the 3’-UTR of SmTβRII are encoded by exon 9 of the gene. Thus, these data indicate that the cDNA sequence of SmRK2a is incomplete and that SmTβRII sequence presents the missing 3’-end of SmRK2a.

The genomic sequence of the exon representing the region spanning the stop codon and the 3’-UTR reported in SmRK2 [39], shows that what was thought to be a poly A tail in SmRK2 is in fact a stretch of eight adenine (A) nucleotides followed by two guanosines (G) then another three As (Figure S1A, pound [¶] sign) and is not the actual poly A tail of SmRK2.
The faster migrating component is about 62 kDa (Figure 2, Western blots using specific anti-type II receptor antiserum. As expected, two major protein bands were observed in used in these assays can detect both forms of type II receptor. of SmT
affinity-purified IgG directed against the N-terminal 153 aa Detection of Native SmT
location respective to the proposed poly A tail in
transcript. Further evidence for this is that a consensus polyadenylation signal could not be found in an appropriate location respective to the proposed poly A tail in SmRK2 cDNA. It is likely that the approach employed to isolate the full-length cDNA, 3’ RACE (rapid amplification of cDNA ends) using poly dT primer, generated this error. Careful screening of the genomic sequence in search of putative polyadenylation signals for this transcript revealed the presence of a potential signal 309 bp downstream of the reported SmRK2 poly A stretch (Figure S1A, asterisk [*]). In order to determine if this consensus sequence serves as an actual polyadenylation signal, we designed PCR primers upstream and downstream of it (Figure S1A; Table S1). When using cDNA templates, the expected PCR products could be amplified only when a forward primer (F1) was used in combination with either one of two reverse primers located upstream of the putative polyadenylation signal (R1 and R2; Figure S1B, panel I), but not with reverse primers located about 110 bp (R3) and 730 bp (R4) downstream of the proposed signal (Figure S1B, panels I and II). Similarly, no products were obtained when another forward primer (F2; a complementary sequence of R2) was used with reverse primers R3 and R4 (Figure S1B, panel III).

In addition, other PCR reactions were designed to address the relationship of the two transcripts. Primers F1, which is common for both SmRK2 and SmTβRII (exon 7), and F2, which is unique for SmRK2 and located close to the 3’-end of exon 8, were used with a reverse primer (R5), which is unique for SmTβRII (exon 9). Only F1×R5 (Figure S1B, panel II), but not F2×R5, reaction (Figure S1B, panel III), gave the expected PCR product with cDNA templates. These results indicate that the two transcripts are independently represented in the cDNA. Genomic DNA templates, either extracted genomic DNA or isolated bacterial artificial chromosome (BAC) clones, gave the expected products in all the reactions (Figure S1B). All PCR products were sequenced, and sequence information matched that obtained from the database.

The third truncated transcript reported in the Forrester et al. study [39] was described as encoding a 63 aa–long peptide and was suggested to function as a membrane anchor, act as a soluble ligand trap analogous to Follistatin, Noggin, or Decorin, or act as a membrane-bound accessory protein to promote ligand binding. This transcript, indeed, represents the first exon that covers the 5’-UTR and the following sequence that encodes the first 53 aa followed by a 33 bp–long intron that translates into 10 aa followed by a stop codon (Figure 1). The amplification of this DNA fragment could simply be attributed to contamination of cDNA preparations with genomic DNA that was carried over from total RNA extraction steps. This is consistent with the authors’ notion that this sequence may originate from an unspliced intron, since it starts and ends with the classical GT-AG intron boundary sequences [39].

**Detection of Native SmTβRII Protein in Schistosomes**

SmTβRII was detected in schistosome extracts using affinity-purified IgG directed against the N-terminal 153 aa of SmTβRII, SmTβRII-N (41–193). In principle, the antibody used in these assays can detect both forms of type II receptor. As expected, two major protein bands were observed in Western blots using specific anti-type II receptor antiserum. The faster migrating component is about 62 kDa (Figure 2, panel C, arrow 1), whereas the higher molecular weight protein is about 92 kDa (Figure 2, panel C, arrow 2). These data provide further evidence for the presence of two related proteins. The shorter is 550 aa long (SmRK2; 62.2 kDa deduced size) described by Forrester et al., [39], and the larger is 815 aa (SmTβRII; 92.6 kDa deduced size). The detection of the two forms of the receptor in NP-40 extracts indicates that these receptors are associated with the tegument (outer covering) of the parasites. NP-40 extracts probed with pre-immune rabbit IgG, as a negative control, did not show any significant reactivity (Figure 2, panel B).

In immunofluorescence assays, SmTβRII and/or SmRK2 could be detected in adult worm pairs in either cyssectiones (unpublished data) or in live or acetone-fixed whole parasites (Figure 3). Anti-receptor II antibody reactivity with native antigen(s) in live parasites was confined to the parasite surface in female worms (panels F and I) and in male worms, to the tubercles (panel B), as well as in the gynaecophoric canal (panel C). Antibody reactivity patterns in acetone-fixed parasites showed that receptor II is also localized at the interface between the parasite and its host, the oral and ventral suckers (panel K), and the lining tegument of the esophagus (panel L). No specific (far-red) fluorescence could be visualized when pre-immune rabbit IgG was used (Figure S2, panel C).

**Detection of SmTβRII Transcripts**

A RT-PCR semi-quantitative analysis was employed to assess the level of receptor II mRNA throughout schistosome development. Compared to the constitutively expressed control, β-tubulin, SmTβRII exhibited relatively steady expression levels throughout development. On the other hand, SmTβRI showed lower expression levels in developmental stages at earlier than 15-d postinfection, while the overall expression pattern of SmTβRI was significantly lower than that displayed by SmTβRII (Figure 4).

TGF-β type II receptor transcripts were also detected in adult worm pair paraffin sections. By using an anti-sense cRNA probe designed to the N-terminal region of SmTβRII, we detected the transcripts in the vitelline cells and gut epithelial cells (Figure 5, panel IIIC), as well as subtegumental
cells (Figure 5, panel IIIc). As expected, P14, an eggshell precursor transcript employed as a positive control, was localized to the vitelline cells in adult female worm sections with no reactivity in male sections (Figure 5, panel IIC). No specific reactivity could be observed in the negative control sections probed with sense cRNA probe of SmTβRII-N (unpublished data).

In Vitro Interaction of SmTβRII with SmTβRI

Schistosome TGF-β receptor interactions in vitro were evaluated by co-immunoprecipitation. The degree of receptor interactions were expressed as the percentage values of the precipitated radiolabeled product compared to the input material. Incubation of the N-terminal domains of the two receptors resulted in a 2-fold enhancement (Figure 6, panel A, lane 3) over background precipitation of 35S-SmTβRI-N with S-protein agarose beads (Figure 6, panel A, lane 2). Non-specific background precipitation was removed in later experiments (Figure 6, panel B) by pre-absorbing 35S-labeled SmTβRI-N with S-protein agarose beads. Compared to the interactions in the absence of ligand, adding 1 nM of recombinant human TGF-β1 (Figure 6, panel A, lane 4 and panel B, lane 5) or, to a lesser extent, 5 nM recombinant human Bone Morphogenetic Protein-2 (BMP2; Figure 6,
identification of SmGCP as a S. mansoni TGF-β target gene

SmGCP was found to exhibit homology to three related proteins: D. melanogaster fasciclin I [44]; mouse and OSF2 (human osteoblast specific factor 2) [45], a homophilic adhesion molecule that plays a role in bone formation; and lastly the βig-h3 [40]. Each contains repeated, short, highly conserved regions within more divergent homologous domains (fas1 domain) of approximately 140–150 aa [40,44]. SmGCP contains two of the fas1 domains between residues 43–306 and is localized to the adult parasite surface (restricted to the gynaecophoric canal in male worms), although it lacks a consensus TM domain or a GPI-anchoring signal [42]. The first step in evaluating the SmGCP gene as a possible target for TGF-β regulation was to determine its expression pattern throughout development. Semi-quantitative RT-PCR was performed in which SmGCP-specific primers were used to amplify a PCR product of approximately 300 bp. Figure 8A shows that SmGCP exhibits an expression peak at 28 d postinfection (lane 7), which coincides with worm mating. A similar expression level was observed in adult male worms (lane 11), although relatively low levels are observed in 15-d-old or earlier schistosomules and parasite eggs (lanes 3–5).
The responsiveness of SmGCP to induction by members of the TGF-β family was also examined. Total RNA was extracted from adult worms that were incubated in culture media supplemented with either 1 nM of TGF-β1 or 5 nM of BMP2, and subjected to semi-quantitative RT-PCR. Transcript levels for SmGCP and identified members of TGF-β signaling pathways were examined using this approach. Compared to untreated worm controls (Figure 8B, lane 1), an approximately 2-fold increase of SmGCP expression was observed in adult worms treated with human TGF-β1 (Figure 8B, lane 2), although BMP2 treatment resulted in a slight decrease in SmGCP levels (Figure 8B, lane 3). No significant differences were detected in levels of SmTβRI, SmSmad1, SmSmad2, or SmSmad4 compared to untreated controls (unpublished data).

The above data indicate that SmGCP expression is modulated by human TGF-β. To determine whether this is dependent on stimulation of the TGF-β signaling pathway or a secondary event incited through stimulation of a different signaling network as part of a more generalized cellular response, we investigated the effect of blocking TGF-β signaling on the expression of SmGCP, by employing RNA interference (RNAi) to knock down type II receptor as the initial event of TGF-β signaling.

Freshly perfused 26-d-old and 33-d-old worm pairs transformed with TGF-β type II receptor–specific short interfering RNA (siRNA) were left untreated or treated for 24 h with 1 nM human TGF-β1. RT-PCR data demonstrated that SmTβRII-specific siRNA treatment did diminish the levels of SmTβRII mRNA about 3- to 4-fold (25%–30% of the levels of untreated samples; Figure 9, panel B, lanes 3, 4, 7, and 8). Moreover, not only did SmTβRII-siRNA treatment result in a concomitant reduction in SmGCP levels (there was 2- to 3-fold reduction in levels of SmGCP compared to untreated samples; Figure 9, panel D, lanes 3 and 7), but SmGCP also failed to respond to human TGF-β1 induction (Figure 9, panel D, lanes 4 and 8) as opposed to what was observed in non-transformed parasites (Figure 8B, lane 2, and Figure 9, panel D, lanes 2 and 6). None of the other cDNA species that were used as controls for PCR amplification (SmTβRI,

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**Figure 5.** Localization of SmTβRII mRNA Transcripts in Tissue Sections of *S. mansoni* Adult Worms by FISH

Column A represent phase-contrast fields, Column B represent non-specific autofluorescence fields observed in vitelline lobules (V) using green fluorescence filter (522 nm) and Column C shows specific probe reactivity as represented by far-red fluorescence using 680 nm filter. Row (I) shows sections of a male (♂) and a female (♀) worm probed with the positive control cRNA probe (the antisense strand of eggshell protein P14). Specific fluorescence could be observed in the vitellaria (V) of the female worm. As expected, no specific fluorescence could be observed in male worm sections. Specific reactivity of SmTβRII antisense probe could be seen in vitelline cells (V) and gut epithelial cells (G) in a female worm section (panel IIC) and in subtegumental cells (STC) in a male worm section (panel IIC). No significant fluorescence could be seen in the negative control reaction using SmTβRII sense cRNA strand (unpublished data).

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SmSmad1, SmSmad2, and SmSmad4) showed any significant variation that could be attributed to different treatment regimens (Figure 9, panels C, E, F, and G), indicating that the responses were specific and triggered by SmTβRII-siRNA treatment. These gene products, although part of the same pathway as SmTβRII, are not directly regulated by TGF-β, as mentioned above.

Discussion

In this study, we report the identification of type II TGF-β receptor in S. mansoni, which represents a crucial step in elucidation of the source and subtype of the ligand(s) that regulate the biological events stimulated by these pathways and subsequently determine the ultimate outcome in terms of phenotype. Genomic DNA sequence information gathered from S. mansoni databases and validated by sequencing SmTβRII-positive genomic clones isolated from the BAC library revealed the genomic organization of the SmTβRII cDNA cloning and gene. RT-PCR data demonstrated the presence of two independent transcripts that give rise to two different TGF-β type II receptor isoforms. These transcripts are products of the same gene generated by alternative splicing of the last two exons. The amino acid sequence and BLAST search data show that SmRK2 [39] encodes a receptor that lacks the last 40 aa of the putative kinase domain. Consequently, the S. mansoni TGF-β type II receptor undergoes alternative splicing to produce transcripts that give rise to different length receptors that might exhibit different kinase activities. These different type II receptors may signal in different cells or developmental stages cooperating with SmTβRI or another unidentified type I receptor. Previous studies on Xenopus laevis activin receptor II, ActRII [46], and mouse ActRII gene [47], as well as human TGF-β type I receptor SKR2 [48], reported multiple mRNA variants that give rise to C-terminally truncated isoforms for each receptor. This led Xu and his coworkers [48] to propose that the addition of the poly A tail to alternative exons at the carboxyl-terminal coding exon–intron junction could represent a common feature of both TGF-β type I and type II receptor genes. Therefore, alternative splicing generates transcripts encoding products that vary in length and subsequently in their kinase activities and target phosphorylation sites, a feature that may add to the heterogeneity of biological effects of individual ligands.

The semi-quantitative RT-PCR results show that the type II receptor exhibits a relatively constant transcription level throughout the parasite lifecycle that is 3- to 5-fold higher than that displayed by SmTβRI. This suggests the presence of a second type I receptor that may cooperate with SmTβRII in the stages in which SmTβRI shows a significantly lower expression level, such as the early stages of development [35], and stages involving the intermediate host. Furthermore, SmTβRI was found not to mediate the signals transmitted

Figure 6. In Vitro Interaction of Amino- (N-) Terminal Domains of SmTβRI and SmTβRII

Interaction of the in vitro translated non-labeled N-terminal domain of SmTβRII with 35S-labeled N-terminal domain of SmTβRI, in the presence or absence of different TGF-β ligands (panels A and B) or varied amounts of different TGF-β1 (panel B). Ligand concentrations (in nM) are shown at the top of each lane. Reactions were precipitated using S-protein agarose beads (EMD Biosciences, Novagen). Precipitated products were separated by SDS-PAGE and subjected to autoradiography. In vitro translated products (5% and 20%) of input radiolabeled products are shown in the left lane of panels A and B, respectively. Background precipitation of 35S-labeled-SmTβRI-N by S-protein agarose beads is shown in lane 2, panel A. The background reactivity was removed in later experiments by pre-clearing 35S-SmTβRII-N by treatment with the S-protein agarose beads (panel B) prior to use in interaction assay. Percentage values of precipitated reactive radiolabeled product of each reaction are shown at the bottom of each lane.

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through SmSmad1 [36,38], a fact that supports the presence of a second SmTβRI receptor capable of interacting with and activating SmSmad1. An understanding of the performance of the different type II receptor isoforms (SmRK2 and SmTβRII) in different signaling contexts and their cellular localization and expression patterns are issues that await further investigation. The phenomenon of alternative splicing, of different exons at the C-terminal end, was reported before in S. mansoni SER, an epidermal growth factor receptor ortholog [49].

The detection of both TGF-β type II receptor isoforms in the NP-40 extracts of adult worm pairs, which represents a pool of outer tegument proteins, provides evidence that both isoforms are in fact expressed in the adult stage. They are present in the tegument where the type II receptor, as the initial signaling component in the TGF-β pathway, is anchored into the surface and may respond to both host and self signals. Such expression profiles and the localization of TGF-β type II receptor at the interface between the parasite and its host was further confirmed by immunofluorescence assays on whole-mount adult worms as well as on adult worm sections. The detection of either or both of the two isoforms on the surface of live parasites (tubercles and gyneacochoric canal in male worms and general surface localization in females) and in the lining of the esophageal canal of fixed parasites (Figure 3), highlights the fact that the TGF-β type II receptor is actively engaged in dynamic signaling processes at the host–parasite interface. Our results are in agreement with localization data reported for SmRK2 that showed intense surface staining in both female and male worm sections, which indicated that SmRK2 exhibits vegetal expression [39]. It can be concluded from the report of Forrester et al. [39] that the observed expression pattern could be representative for either or both isoforms of type II receptor since the utilized antibody reagents used in that study were generated against an N-terminal synthetic peptide that is shared in both isoforms.

The fluorescent in situ hybridization (FISH) assay (Figure 5) provided an additional method for detection and localization of type II receptor transcript. The detection of the transcripts in the subtegumental cells is consistent with the production of SmTβRII in the tegument and its insertion into the parasite’s surface. In addition, the type II receptor transcripts were also detected in gut epithelia and vitelline cells. This may indicate that the receptor could be involved in other signaling processes leading to sexual and digestive tissue development and differentiation. The type II transcript and protein distribution profiles are consistent with previous studies that reported the localization of components of TGF-β signaling pathway, SmTβRII [50], SmSmad2 [37], and SmSmad4 [38], to digestive tract and male and female reproductive tissues. Furthermore, the RT-PCR results demonstrate that type II receptor shows little variation throughout the parasite lifecycle, an observation that may indicate its involvement in parasite growth and development in different stages.

Interaction assays were designed to identify the domain(s) involved in receptor complex formation. Our results demonstrate that in the absence of ligand, full-length SmTβRI and SmTβRII showed no significant interaction. However, the ECD of the two receptors exhibited ligand-dependent interaction. Furthermore, the kinase domains of the receptors displayed autonomous interaction, whereas the C-termini showed no affinity toward each other. These data are in agreement with previous studies, which reported that the interaction between the TGF-β receptors depends on the ligand binding to either type II receptor followed by the recruitment of receptor I, as in the case with TGF-β and activin [19,51,52] or to both receptors as in the case of BMPs [53–55]. Several reports have also shown the interaction of the kinase domains of receptor I and receptor II for members of the TGF-β superfamly [56–58].

Furthermore, our results demonstrate that the presence of the appropriate ligand (TGF-β1 and to a lesser extent BMP2) stimulated the association of the ECD of receptor I and receptor II, whereas Activin A or BMP4 had no affect. These results were surprising since the BLAST search data categorize the S. mansoni type II receptor as belonging to the Activin type II receptor subfamily. A similar situation was found in Drosophila type II receptor, punct, which was initially classified as belonging to the Activin type II receptor subfamily, based on sequence similarities and ligand-binding properties [59]. However, this receptor was later found to bind to and mediate signals of the decapentaplegic (Dpp), a Drosophila ortholog of mammalian BMP, and functionally respond to members of the BMP family [53]. Thus, determination of the functional ligand can not be predicted based only on sequence similarities of the kinase domain. Moreover,
Beall and Pearce [36] reported that human TβRII appears to facilitate TGF-β binding to SmTβRI and that a chimeric receptor composed of the extracellular domain of SmTβRI and the intracellular domain of human TβRI could bind human TGF-β ligand and activate a luciferase reporter system in response to TGF-β1 and TGF-β3 but not BMP7.

In our previous work, we reported that SmSmad2 associates with SmSmad4 upon phosphorylation and activation of the former by type I receptor, SmTβRI. Only the constitutively active mutant form of SmTβRI (SmTβRI/Q-D), but not the wild-type receptor, could achieve this activation in the absence of ligand or receptor II [38]. In this study we included the type II receptor and the ligand to evaluate Smad complex formation as a secondary event following receptor complex formation and receptor I activation of R-Smad. Our data show that TGF-β1 induced SmSmad complex formation comparable to what was observed with SmTβRI/Q-D, whereas in the presence of BMP2, SmSmad2/SmSmad4 association was at background level (in absence of receptors). These data demonstrate that host TGF-β1 not only induced receptor complex formation, but also propagated the signal to R-Smad causing its activation and association with Smad4. This is consistent with the results of Lagna et al. [21] who reported that agonist-induced activation of human Smad1 (by BMP) and Smad2 (by TGF-β and activin) led to their association with Smad4. On the other hand, the lack of interaction between the non-phosphorylatable mutant of SmSmad2 (SmSmad2-MH2/AAA) with SmSmad4 is in agreement with a previous report that showed that in the presence of BMP, mutation of the C-terminal phosphorylation motif of Smad1 abolished transduction by preventing its association with Smad4 [60].

The SmGCP exhibits surface expression in adult parasites especially in male worms in which expression is limited to the gynaecophoric canal and is almost absent in unisexual male worms [42]. Interestingly, the transcription profile of SmGCP shows its peak expression in 28-d-old worms, a time that coincides with worm pairing. This distribution pattern emphasizes the potential role of SmGCP in male–female interactions and the male-stimulated reproductive maturation of the female schistosome [34]. TGF-β1-treated worms displayed an enhanced expression level of SmGCP. In order to investigate the mechanism of SmGCP expression induction in response to TGF-β, we knocked down SmTβRII expression to prevent initiation of the TGF-β signaling pathway. We employed RNAi to achieve silencing of the SmTβRII gene, as the successful use of this technique to knock down gene transcription in *S. mansoni* sporocysts had been previously...
demonstrated [61,62]. In this study, we report the effective use of siRNA as a tool to knock down a target gene expression in late parasitic stages (28- and 35-d-old worms). The chosen delivery method, the particle bombardment, proved effective, as was shown in different studies reporting the use of biolistic technique to transfect *S. mansoni* with different constructs [63–68] and in our standardization experiments using an HcRed-encoding plasmid. The observed concomitant reduction of SmGCP and SmTβRII expression in worms treated with SmTβRII siRNA suggests that SmGCP closely follows the expression pattern of TGF-β receptor II. In fact, the failure of TGF-β1 to induce the expression of SmGCP in worms in which the type II receptor was knocked down indicates a direct relationship between the classical TGF-β pathway as represented by type II receptor and SmGCP as a potential target gene. However, SmGCP could represent a primary TGF-β target gene or it could be a target gene for another gene product that primarily induced by TGF-β pathway.

**Materials and Methods**

Identification, cloning, and sequence analyses of SmTβRII. Mining of *S. mansoni* DNA sequence databases resulted in the identification of two overlapping EST clones (CD126244 and CD069595) that show similarity to different members of TGF-β type II receptors [72]. Based on the EST sequences, a primer pair was designed that amplified an detailed investigation involving the assessment of schistosome Smad complex binding to the promoter region of SmGCP will be necessary to address this point at the molecular level. In either case, it is obvious that induction of SmGCP expression constitutes part of the mitogenic effect exerted by the host TGF-β ligand in schistosome.

In schistosomes, an interesting biological interplay has evolved such that male schistosomes, via an unknown stimulus, regulate female-specific gene expression and thus female reproductive development and egg production [34]. In order for the male to stimulate and to maintain female reproductive development, there must be direct contact between the male and the female. This is accomplished by the female residing in the gynecophoric canal of the male. One scenario is that the female first stimulates the male, and the male in turn produces signals that regulate female development [34]. The coinciding of peak expression of SmGCP with worm pairing, localization of SmGCP in male worms only in the gynecophoric canal [41] and the requirement for the male to maintain contact with the female, and induction of SmGCP by TGFβ implicates the TGFβ pathway as an important signaling component for worm pairing.

![Figure 9. Silencing of TGF-β-Induced Expression of SmGCP by Knocking Down SmTβRII Expression](image)

Semi-quantitative RT-PCR analyses for transcripts of SmGCP as well as various components of schistosomal TGF-β signaling pathways in 35-d-old and 28-d-old worm pairs, untransformed and transformed with SmTβRII-siRNA, and either left untreated or treated with TGF-β1 (1 nM). The top panel shows the agarose gel separation of the PCR products of SmTβRII (panel B), SmGCP (panel D), SmSmad4 (panel E), SmSmad2 (panel F), SmSmad1 (panel G), and the constitutively transcribed control, α-tubulin (panel A). The lanes are labeled to show detailed treatment of each sample. The bar graph representation shows the percentage values of the optical densities in pixels of the PCR bands for each gene compared to the corresponding band of α-tubulin control from the same stage. Values were calculated from three independent PCR amplifications (Error bars represent the standard deviation). DOI: 10.1371/journal.ppat.0020054.g009

**Figure 9. Silencing of TGF-β-Induced Expression of SmGCP by Knocking Down SmTβRII Expression**

Host tumor necrosis factor-α (TNF-α), secreted as a part of the host response to eggs trapped in liver tissues, was found to significantly stimulate egg laying by adult female worms [69]. In addition, human EGF induced phosphorylation of native SER protein, the *S. mansoni* homolog of EGFR. In response to human EGF stimulation, SER was also shown to be able to activate a Ras-responsive reporter system in epithelial canine cells and mediate EGF responses in *Xenopus* oocytes [70]. The effects of thyroid hormone, interleukin-7, and insulin on schistosome development in the mouse host have also been demonstrated [71].

The demonstration that schistosome type II receptor is able to bind human TGF-β ligand provides strong evidence for the utilization of host ligand in parasite growth and development. However, this, in fact, does not rule out the involvement of a self ligand or a set of ligands, as localization studies for components of schistosome TGF-β signaling pathways indicate that TGF-β signaling is active in sexual tissues, a fact that implies active engagement of TGF-β signaling in internal parasite organs as well as at the interface with its host. This observation has been reported previously in a study that showed type II receptors have diverse ligand-binding abilities, and this phenomenon offers an explanation for the wide variety of biological responses that can be elicited by members of TGF-β family [53].

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The induction of SmGCP expression by TGF-β1 provides support for the hypothesis that schistosome gene expression can be regulated by host ligands. It further implicates the host ligand as a stimulator for parasite–parasite interactions via regulating the expression of a gene that is likely involved in such biological events. Several studies have demonstrated involvement of host molecules in regulating biological processes in schistosomes. Host tumor necrosis factor-α (TNF-α), secreted as a part of the host response to eggs trapped in liver tissues, was found to significantly stimulate egg laying by adult female worms [69]. In addition, human EGF induced phosphorylation of native SER protein, the *S. mansoni* homolog of EGFR. In response to human EGF stimulation, SER was also shown to be able to activate a Ras-responsive reporter system in epithelial canine cells and mediate EGF responses in *Xenopus* oocytes [70]. The effects of thyroid hormone, interleukin-7, and insulin on schistosome development in the mouse host have also been demonstrated [71].

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859-bp PCR product from adult worm cDNA, which was cloned in pCR2.1-TOPO vector (Invitrogen, Carlsbad, California, United States) and sequenced. The DNA fragment was labeled using the MegaPrime DNA labeling system (Amersham Biosciences, Piscataway, New Jersey, United States) and employed to screen an S. mansoni adult worm pair × ZapII cDNA expression library [73]. Isolated clones were excised from agarose gel using a ZAP II plasmid isolation kit (Stratagene, Mountain View, California, United States). One of those clones was found to contain the entire coding sequence of the type II TGF-β receptor. DNA and protein sequence analyses were performed using programs in the Wisconsin Package Version 10.3 (Accelrys, San Diego, California, United States).

DNA sequence analysis revealed the presence of two SpHl cut sites, one preceding the start ATG codon, while the second is about 325 bp downstream of the start ATG codon (at bp 59 and 592 of the submitted cDNA sequence). Two sets of primers were designed to amplify the cDNA fragment corresponding to the N-terminal domain of the receptor (193 aa); a forward primer that represented DNA sequence from bp 65–817 and had a BamHI site cut site inserted upstream of the start ATG codon and a reverse primer, which represented the sequence complementary to bp 625–645 of the submitted cDNA sequence. The PCR product was cloned into the pCR-TOPO vector (Invitrogen) giving rise to pSmTgRII-N(1-193)/TOP20-II that was sequenced to confirm the absence of PCR-generated introns and to separate each cDNA sequence from the parental BlueScript-SK+ vector that was digested with the multiple cloning sites of the parent vector pBlueScript-SK+ to excise the SmTgRII cDNA fragment suitable for cloning in several vectors designed with BamHI and either XhoI or SalI at the 5′- and 3′-ends of the multiple cloning sites, respectively.

**Genomic organization of SmTgRII gene.** S. mansoni genomic DNA databases from The Wellcome Trust Sanger Institute and The Institute for Genomic Research (TIGR), were searched using the type II receptor cDNA sequence to identify contigs that constituted the entire gene. In addition, full-length cDNA was excised from the parent vector, vector primed-labeling, and used to screen an S. mansoni baculovirus genomic library [74] to isolate BAC clones that harbor the genomic sequence of receptor II. The isolated BAC clones were used as templates in PCR reactions to validate the information obtained from searching genomic DNA databases. The order of the assembled contigs was confirmed by PCR on genomic DNA extracted from S. mansoni cercariae as well as SmTgRII-positive BAC clones isolated from BAC library screening, using primers located on the 5′- and 3′-ends of each contig and the following one. In addition, the intron positions were confirmed by PCR amplification of DNA fragments that included exon–intron–exon junctions of the multiple exons, when such exons were interrupted by small introns.

**Production of polyclonal antibodies against SmTgRII.** The pSmTgRII-N(1-193)/TOP20-II was used to amplify a cDNA fragment encoding 153 aa from the N-terminal domain of the receptor and cloned into the prokaryotic expression vector, pMAL-c2X (New England Biolabs, Ipswich, Massachusetts, United States), downstream and in frame with the MBP (maltose-binding protein) coding sequence, pSmTgRII-N(1-193)/pMAL. A 67-kDa MBP fusion protein was expressed, purified following the manufacturer’s instructions, and used to immunize New Zealand rabbits. Affinity-purified IgG was used for immunoprecipitation, immunofluorescence, and Western blot analyses.

**Immunological assays.** Purified IgG fractions were used to detect the native protein in parasite extracts and in cryosections following protocols described in previous studies [37,38]. Immunofluorescence assays on whole-mount adult worms, either live or acetone fixed, were also conducted, following a protocol described in previous studies. Western Blot analyses were performed on parasite extracts and on whole-mount adult worms, either live or fixed for 5 min in ice-cold acetone, were incubated for 2 h at room temperature in MEM medium supplemented with 10% fetal bovine serum (Invitrogen), used as a blocking reagent. Incubation medium was replaced with test and control primary antibodies (IgG fractions), 5 μg/ml diluted in the above medium, and incubated overnight at 4 °C. Samples were washed with 1× PBS for four times, 5 min each, and then anti-primary, biotin-conjugated secondary antibodies (5 μg/ml; Molecular Probes, Invitrogen) was used to visualize the reactive antigen antibodies. Affidouve fluorescence attributed to secondary compounds could be visualized at yellow-green fluorescence wavelength (530 nm), but not at far-red fluorescence (680 nm). Cryosections and whole-mount parasites were examined using a Bio-Rad MRC-1024 confocal microscope equipped with Krypton-Argon laser and 522-nm and 690-nm filters (Bio-Rad, Hercules, California, United States). Micrographs representing different laser sections of the samples were chosen to delineate the localization of specific and nonspecific fluorescence in the examined samples.

**Detection of SmTgRII transcript using RT-PCR and FISH analyses.** Two approaches were employed to detect SmTgRII mRNA. A semi-quantitative analysis to estimate relative SmTgRII mRNA levels in different developmental stages was carried out by RT-PCR as previously described [38]. In addition, gene transcripts were localized by FISH. In the semi-quantitative RT-PCR assay, PCR products, amplified with each reverse primer pair, were separated by electrophoresis in 2% agarose gels, stained with ethidium bromide, analyzed using a gel-documented system (GelDoc1000; Bio-Rad), and quantified using Quantity One software (version 4.2.3; Bio-Rad). Negative control PCR reactions using reverse transcription reaction mix lacking reverse transcriptase were also included. Reaction mixtures included 1 μg of total RNA and 0.3 μmol primers for SmTgRII, which are described previously (544–1444 and the complementary sequence of bp 777–801 as forward and reverse primers, respectively, yielding 378-bp PCR product) were used to amplify a PCR product that served as a constitutively transcribed control [75]. A specific primer pair for SmTgRII (forward primer, starts at bp 584; reverse primer, ends at bp 724) was used that yields a 236-bp PCR product. Primer pairs specific for SmTgRII (forward primer starts at bp 1,608, reverse primer ends at bp 1,894), and SmGCP (forward primer starts at bp 818, reverse primer ends at bp 1,114), were also used to amplify the corresponding fragments of these genes transcripts yielding products of 287 and 297 bp long, respectively. In addition to the above genes, SmSmad1, SmSmad2, and SmSmad4 were similarly processed as control genes. The PCR primers and reaction conditions that were used to amplify those products were as previously described [38]. Due to differences in the quality of RNA preparations included in this assay, the volumes of input cDNA templates of each stage were varied according to normalization data using β-tubulin control. Also, due to differences in the abundance of the assayed cDNA species and in order to ensure that the amplification products were analyzed in the exponential phase and that there was no primer dimers formation, at least three independent PCR reactions were used to amplify each cDNA was also varied. For β-tubulin, 24 cycles were used, whereas 27 cycles were used to amplify the PCR products for SmTgRII, SmSmad4, and SmGCP, and 28 and 29 PCR cycles were used in the case of SmSmad1 and of SmTgRII, respectively. All variables were considered and compensated for in data analysis.

For the FISH assay, the SmTgRII RNA probes, a test probe and a negative control probe were prepared by in vitro transcribing and labeling the anti-sense strand of the N-terminal domain of SmTgRII-N(1-193)/TOP20-II cDNA. Cloned 177 RNA polymerases (Ambion, Austin, Texas, United States), respectively, and digoxigenin-11-uridine-5′-triphosphate (DIG-11-UTP; Roche Applied Science, Indianapolis, Indiana, United States), following the manufacturer’s suggested instructions. An RNA probe representing the anti-sense strand of the coding sequence of the pSmTgRII-N(1-193)/TOP20-II cDNA. DIG-labeled probe detection was achieved by using an anti-DIG monoclonal antibody (0.25 μg/ml; Roche Applied Science), followed by a biotin-conjugated goat anti-mouse antibody (Molecular Probes, Invitrogen), and the antibody complexes were visualized using streptavidin-conjugated Fluoro-488 (Molecular Probes, Invitrogen) in concentrations similar to that used in the immunofluorescence assays. Sections were examined by Bio-Rad MRC-1024 confocal microscope using 522-nm and 680-nm filters.
Silencing of SmTβRI gene expression by siRNA treatment of S. mansoni adult worms with human TGF-β1 and BMP2.

Adult culture media and supplements were purchased from Invitrogen, unless otherwise stated. Freshly perfused adult worms were collected from infected golden hamsters (77), washed, and then incubated overnight at 37 °C, 5% CO2 in MEM medium supplemented with 1 mM sodium pyruvate, 1X non-essential amino acid solution, 1X amino acid solution, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 μg/ml amphotericin B (antibiotic antimycotic solution), and 2 mM Glutamax-I. Recombinant human TGF-β1 and BMP2 (R&D Systems) were added individually to about 25 pairs of adult worms to final concentrations of 1.0 nM; Activin A, 2.0 nM; and BMP2 and BMP4, 5.0 nM (R&D Systems, Minneapolis, Minnesota, United States). Reaction volumes were brought up to 50 μl with 1X IPP buffer (150 mM NaCl, 29 mM Tris-HCl, [pH 7.5], 2% glycerol). The reactions were incubated for 1 hour at room temperature after which protein complexes were precipitated using 1X IPP buffer containing 0.1% NP-40. Protein-bound beads were resuspended in 1X SDS sample buffer, boiled, and separated by SDS-PAGE. The 12% gels were stained, destained, treated with the Fluorographic reagent (Amplify; Amersham Biosciences), dried, and then exposed to X-ray film.

Supporting Information

Figure S1. TGF-β Receptor II Gene Showing the Relative Locations of the Primers and the Obtained PCR Products Used to Elucidate the 3'-End of SmR2k cDNA

(A) A sketch represents part of TGF-β receptor II gene (exons 7 and 8, and partial sequence of 9) showing the relative locations of the primers used to elucidate the 3'-end of SmR2k cDNA. The pound sign (#) and asterisk (*) show the relative locations of the proposed poly A stretch in the Forrester et al. study (39) and the putative polyadenylation signal at the 3'-end of exon 9. (B) Agarose gels showing PCR products amplified from S. mansoni adult worm pair cDNA templates (lanes 2), which were prepared from a total RNA that had been treated with DNaseI, genomic DNA extracted from S. mansoni adult worm pairs (lanes 3), and a SmTβR-II-positive BAC clone (lanes 4). A minus RT control (lanes 1) was also included to verify absence of any genomic DNA contaminations in cDNA preparations. Forward primers used in these experiments are indicated to the left of each set of amplification reactions, and reverse primers are listed above lane numbers. Molecular size marker (M) was run, and sizes are indicated in kb to the right of each panel. Product sizes are listed in Table S2.

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Figure S2. Whole-Mount Live S. mansoni Adult Worm Immunofluorescence (Negative Control)

Live S. mansoni adult worms were probed with pre-immune rabbit IgG, followed by biotin-conjugated anti-rabbit IgG. Samples were incubated with streptavidin Alexa Fluor 647 conjugate and analyzed with Bio-Rad MRC1024 confocal laser microscope. (A) represents the phase-contrast field of a male worm (♂) and a female worm (♀). (B) represents an immunofluorescence field viewed at 322 nm. (C) represents the far-red fluorescence field (680 nm). Please note, due to lack of specific fluorescence in panel (C) that results in the lack of visible details at this wavelength, the micrograph was set at high brightness to reveal the adult worms in the field. O, esophagus; Os, oral sucker; V, vitellaria.

Found at DOI: 10.1371/journal.ppat.0020054.sg002 (2.2 MB TIF).

Table S1. Sequences of Primers Used in Mapping the 3'-End of SmRk2 cDNA and Their Locations Respective to Either cDNA or Genomic Gene

Found at DOI: 10.1371/journal.ppat.0020054.s001 (31 KB DOC).

Table S2. Expected Sizes of PCR Products of cDNA or Genomic DNA Resulting From Using Different Primer Combinations to Elucidate the Structure of the 3'-End of SmRk2 cDNA

Found at DOI: 10.1371/journal.ppat.0020054.s002 (28 KB DOC).
Accession Numbers

Sequence data reported in this manuscript are available from GenBank (http://www.ncbi.nlm.nih.gov/GenBank) under accession number AY651287. Other GenBank accession numbers for the genes or genetic material discussed in this paper are as follows: ß-tubulin (M80214); Sm(GP) (U74862); Sm(RK)2 (AY550912); Sm(RK)2a (AY285784); and [deleted repeat of Sm(RK)2] SmTF1K (AF031557).

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Author contributions. AO, EGN, and PTL conceived and designed the experiments. AO, EGN, SV, and PTL analyzed the data. SV and PTL contributed reagents/materials/analysis tools. AO, EGN, SV, and PTL wrote the paper.

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Competing interests. The authors have declared that no competing interests exist.
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