Characterization of the Ras homologue of *Schistosoma mansoni*†,

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**Abstract**

Ras is a member of a super-family of guanine-binding or G-proteins. Ras functions as a molecular switch in the transduction of signals generated by the activation of a variety of cell surface receptors and relays the signals to downstream effectors. Little is known about signal transduction in schistosomes. In order for *Schistosoma mansoni* to survive different immune responses triggered by the host as well as to migrate from the site of penetration at the skin to the final destination in portal circulation, they must receive signals from the host environment and respond to them in a way that allows their survival. We have isolated the schistosome Ras cDNA by using sequence information of the schistosome Ras homologue submitted to the Genbank database. Analysis of the encoded peptide revealed 81% identity and 92% similarity with K-Ras from various species. Ras is a single copy gene as determined by quantitative hybridization experiments. The cDNA was cloned into pGEX-4T and the expressed peptide was used to generate specific antibody reagents. Affinity purified antibodies identified a 23 kDa native protein that localizes to the subtegument. Ras is not associated with the tegument. Ras is expressed in all the developmental stages of the parasite. However, Ras is over-expressed in female worms compared to males. Schistosome Ras was also shown to be post-translationally modified by addition of farnesyl isoprenoid moiety to the cysteine residue in the C-terminal box. Using a schistosome extract in vitro SmRas farnesylation was inhibited by the farnesyl transferase inhibitor, FTI-277, at concentrations comparable to those required to inhibit K-Ras processing. These initial studies on signal

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**Abbreviations:** CNBr, Cyanogen bromide; EGFR, Epidermal growth factor receptor; FPP, Farnesyl pyrophosphate; Ftase, Farnesyl transferase; FTIs, Farnesyl transferase inhibitors; GGPP, Geranylgeranyl pyrophosphate; GGTagase & II, Geranylgeranyl transferases I & II; GAPs, GTPase activating proteins; GDP, Guanosine diphosphate; GTP, Guanosine triphosphate; MAPK, Mitogen-activated protein kinase; MEK, Mitogen-activated Extracellular-regulated kinase; RTK, Receptor tyrosine kinase; sj26 or sjGST, Schistosoma japonicum 26 kDa glutathione-S-transferase; TGF-β, Transforming growth factor-β; TBRI, Transforming growth factor-β receptor kinase I; TNF-α, Tumor necrosis factor-α.

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**Note:** Nucleotide sequence data reported in this paper are available from Genbank™ under the accession number U53177.
transduction in schistosomes should provide a solid basis for improving our understanding of schistosome-host interactions. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Schistosoma mansoni; Ras; Signal transduction; Prenylation

1. Introduction

Schistosoma mansoni has a complex life cycle in which free-living cercariae are able to penetrate intact skin of humans in fresh water that contain them. The head of the cercaria transforms into an endoparasitic larva, the schistosomule. Each schistosomule spends a few days in the skin, enters the venous circulation and eventually migrates to the lungs (5–7 days post penetration). Then, it moves via the circulation to the hepatoportal circulation (> 15 days) where the worms develop into adults (day 28), mate and move to their final niche in the mesenteric circulation where they begin egg production (> 32 days) [1]. For the schistosome parasite to migrate from the skin to the portal circulation and undergo development at the same time, it must receive signals from the host environment and from within that are transduced to regulate cell proliferation and development. Even after maturation, the adult worms receive signals from the host that regulate development. For example, Amiri et al. [2] reported that tumor necrosis factor (TNF) significantly stimulates egg laying by adult female worms. Likewise for S. mansoni to survive as an intravascular parasite in a hostile environment in close contact with host humoral and cellular cytoxic factors, it must respond to signals from the host [3].

In addition to being involved in the interactions between the parasite and its host, signal transduction is implicated in the interactions between the male and female worms, which result in the initiation and maintenance of female worm maturation and egg production [4]. Evidence clearly demonstrates that male worms regulate female gene expression through an unknown signal [5,6].

In order to identify and characterize molecules likely to play important roles during parasite development, we began a study of signal transduction pathways involved in cell proliferation and differentiation. Intracellular signal transduction pathways convey information from the cell surface to the nucleus and this enables the cell to respond to stimuli from its environment by changes in gene expression [7]. The Ras pathway is one of the most extensively studied and best understood signaling pathways. The Ras genes encode a family of closely related guanine nucleotide-binding proteins (G-proteins). They were first identified as oncogenes of RNA tumor viruses [8]. Ras proteins normally play a key role in signal transduction pathways regulating cell proliferation and differentiation. The Ras-mediated signaling pathway is highly conserved among eukaryotes. Ras proteins act as molecular switches which transmit signals from the receptor tyrosine-kinases (RTK), activated at the cell surface by a variety of growth factors and peptide hormones, to downstream effectors, and eventually to the nucleus, where they regulate gene expression and consequently affect cell growth and differentiation.

Once activated with a specific agonist, RTK promotes the activation of Ras protein by stimulating the exchange of GDP with GTP resulting in the transition of Ras from the inactive state to active conformation. Ras, in turn, passes the signal to the downstream partners by recruiting c-Raf-1, a serine/threonine kinase, to the plasma membrane and phosphorylate it resulting in activating its kinase activity. Raf, then triggers a kinase cascade by phosphorylating Mitogen-Activated Protein Kinase Kinase (MAPK kinase or MEK) which in turn phosphorylates MAPK [9]. The later when phosphorylated, catalyzes the phosphorylation of ribosomal protein S6 kinase (RSK), which phosphorylates ribosomal protein
S6, controlling translational activities. Both MAPK and RSK translocate into the nucleus where they regulate gene expression by phosphorylating transcription factors [10]. The intrinsic GTPase activity of Ras, then, turns the biological event off [11]. This activity is stimulated by Ras-negative regulators, GTPase-Activating Proteins or GAPs.

Many non-tyrosine-kinase cytokine receptor systems are known to mediate part of their signaling via the Ras pathway. Invariably, these receptors associate with tyrosine kinases, which mediate the activation of Ras [12,13]. In addition to the Raf/MEK/MAPK cascade, Ras also activates several other pathways, such as those involving the small G-proteins of the Rho family of Ras-related proteins (e.g. Rac1 and RhoA) [14–16]. These proteins are activators of components of Stress-Activated Protein Kinase (SAPK) pathway [17–19].

Ras is initially synthesized as an inactive, cytoplasmic protein. The first methionine is removed early after translation, then a series of post-translational modifications is signaled by a consensus COOH-terminal tetra-peptide sequence (CAAX box or motif). Cytoplasmic prenyl transferases catalyze the covalent addition of a prenyl isoprenoid group. The mature Ras protein with the attached farnesyl group is predominantly localized to the plasma membrane [20].

In schistosomes, very little is known about signaling pathways [21–25]. Studying the mechanisms by which signals are transported in the parasite tissues should provide insights to parasite biology, site-finding behavior and development. It will also lead to a better understanding of the parasite reaction against different defense mechanisms triggered by the host, and how male signals lead to direct or indirect activation of a number of female-specific genes resulting in female worm maturation and egg production among others. In this report, we present the characterization of S. mansoni Ras. We demonstrate biological activity, localization in adult worms, and expression throughout development.

2. Materials and methods

2.1. Parasites

Schistosoma mansoni NMRI strain is maintained in Biomphalaria glabrata and in Syrian golden hamsters. Recovery of adult worms, as well as 35, 32, 30, 28, 20 and 15 day-old worm pairs, were performed by portal perfusion [26]. Schistosomula, 4 and 7 day-old were recovered from lungs of infected animals and from in vitro cultures [27,28]. Three-hour schistosomula were obtained by mechanical transformation of cercariae [28]. Single sex male and female worms were obtained by portal perfusion of hamsters infected with cercariae from a single snail that had been infected with a single miracidium. All parasite stages were collected and quick frozen in liquid nitrogen and stored at −80°C, for subsequent RNA or protein extractions.

2.2. RNA extraction and cDNA synthesis

RNA from different stages of S. mansoni was prepared using RNA-STAT-60 reagent (Tel-Test Inc.), following the manufacturer’s protocol. RNA was quantified spectrophotometrically and used as templates for cDNA synthesis. cDNA was reverse transcribed from 5 μg total RNA using SuperScript reverse transcriptase (GIBCO-BRL, Gaithersburg, MD) and both oligo (dT)12–18 and random hexamers for priming, following the conditions described by the manufacturer. The cDNA synthesized was used in subsequent PCR amplification for either gene cloning or quantitative PCR analysis (see below).

2.3. Cloning of SmRas coding sequence(cds) and copy number determination

S. mansoni Ras protein homologue cDNA sequence was obtained from Genbank (accession # Y11479 and U53177). The coding sequence of SmRas was amplified using SmRas-cod primer pair (forward primer, 5’-GGAATTCAAAATGACGTAGCTACAAGTATAGT-3’; reverse primer 5’-GCTATTATA ATCATCATTGAGACGC-3’).
They represent bases 15–34 and the complementary sequence of bases 634–658 of the cDNA sequence submitted to the Genbank (U53177), respectively. A restriction endonuclease EcoRI cut site was inserted upstream of the start ATG codon in the forward primer to facilitate subcloning into an expression vector. The reverse primer was designed to be 64 bp downstream of the TGA stop codon. A PCR product with the expected size (~650 bp) was cloned into pCRII 2.1 TOPO-TA vector (Invitrogen, Carlsbad, CA) and fully sequenced using M13 reverse and T7 primers that flank the PCR product.

Copy number of SmRas was determined by performing a quantitative dot blot hybridization using genomic DNA isolated from adult worms and SmRas cDNA as template and radiolabeled SmRas as a probe according to previously published methods [29].

2.4. Expression of SmRas protein and production of specific antiserum

The coding region of SmRas cDNA was ligated into pGEX-4T-1 vector (Pharmacia, Uppsala, Sweden) and overexpressed as a fusion protein with the 26 kDa S. japonicum glutathione-S-transferase (Sj26 or SjGST). A rabbit was immunized by subcutaneous injection with 200 µg of Sj26–SmRas fusion protein emulsified in Freund’s complete adjuvant. The primary injection was followed by 2 booster doses at 4-week intervals using 200 µg fusion protein in Freund’s incomplete adjuvant. The immunized rabbit serum was affinity purified by passing over a Sj26/CNBr Sepharose 4B column. The unbound fractions were then passed over Sj26–SmRas/CNBr Sepharose 4B column. Specific anti-SmRas antibodies were eluted from the column with glycine-HCl, pH 2.8, neutralized with 1 M Tris–HCl, pH 8.0 and used to immunolocalize the native protein in western blots as well as in adult worm cryosections.

2.5. Schistosome antigen preparation

Whole parasite extracts were prepared by homogenizing adult worms (female, male and pairs) on ice in 20 mM Tris–HCl, pH 8.0, containing 150 mM NaCl, 2 mM phenyl methyl sulfonyl fluoride (PMSF), 1 mM Dithiotheritol (DTT) and 50 µg ml⁻¹ leupeptin. Homogenization was completed by ultrasonication for 2 min. Parasite surface-associated antigens (NP-40 extracts) were extracted from male, female, worm pairs and 3-h schistosomula by incubating the parasites in the above buffer containing 0.05% NP-40 for 15 min on ice. The extracts were cleared by centrifugation at 12,000 × g for 15 min [30]. The remaining carcass of the parasites (after NP-40 extraction), was processed as described above for the preparation of whole parasite extracts.

2.6. Western blot analysis

Antigenic preparations of adult female, male, worm pairs and 3-h schistosomula (10 µg each) were separated onto 15% SDS-PAGE [31]. The SDS gels were electrotransferred to PVDF membrane (Millipore, Bedford, MA) [32]. Western blots were probed with the affinity purified anti-SmRas antibodies (2 µg ml⁻¹) and with pre-immune rabbit serum (1:1000 dilution). Alkaline phosphatase-conjugated goat anti-rabbit IgG (whole molecule) (Sigma, St.Louis, MO) was used to probe the bound primary antibodies. A color-substrate reagent (Vector kit II; Vector laboratories, Burlingame, CA) was used to visualize the reactions.

2.7. Immunocytolocalization

The affinity purified anti-SmRas polyclonal antibody (5 µg ml⁻¹) was used as a primary antibody to localize the native SmRas protein in adult worm frozen sections following the protocol described by Mei & LoVerde [33]. Probed sections were evaluated using a Bio-Rad MRC-100 confocal microscope equipped with a krypton laser (Bio-Rad, Hercules, CA).

2.8. Quantitative RT-PCR

In order to determine the expression level of SmRas in different stages of the parasite life cycle, semi-quantitative RT-PCR amplification was per-
formed. A primer pair, SmRasRT-forward (5'-ATGCGCGATCATATGCGTAC-3'), representing bases 213–235 of U53177 cDNA, and SmRas-cod-reverse, shown before, was used to amplify a DNA fragment of 446 bp in size. PCR amplification was conducted in 30 µl reaction volume containing 1X PCR buffer (GIBCO-BRL, Gaithersburg, MD), 1.5 mM MgCl₂, 200 µM of each of dNTP's, 0.75 µM of each of amplification primers, 1U Taq DNA polymerase (GIBCO-BRL, Gaithersburg, MD) and 1µl of RT reaction, described before, as the cDNA template equivalent to 100 ng total RNA. A primer pair spanning a 560 bp DNA fragment of α-tubulin gene [33] was used in the same reaction as an internal control. Amplification was performed for 25 cycles, each consists of three steps, a denaturing step at 94°C for 1 min, an annealing step at 55°C for 1 min 15 s and an extension step at 72°C for 1 min 30 s. That was followed by an extension cycle for 10 min at 72°C. The PCR products were separated onto 1.5% agarose gel, analyzed and quantitated using the Molecular Analyst gel-documentation system (Bio-Rad, Hercules, CA). The use of the α-tubulin gene, a constitutively expressed gene, as an internal control allowed a direct measure of the integrity and quantity of RNA transcripts used in each RT reaction. It also provided a comparable control by which differences in the expression levels, due to under- or over-estimation of the input amount of the RNA template, can be normalized.

2.9. In vitro prenylation assay

An in vitro assay was used to study the post-translational prenyl modification of SmRas protein. The assay used is that described by Hancock [34] with some modifications. Briefly, the coding sequence of SmRas gene was directionally cloned into BamHI and XhoI sites in the pCITE-4a(+) vector (Novagen, Madison, WI). The coding SmRas sequence in the recombinant pCITE vector was transcribed in a coupled in vitro transcription–translation reaction (STP3 system; Novagen, Madison, WI) following the manufacturer’s instructions. The first step of the reaction was the in vitro transcription of the pCITE vector(s), 1 µg, using T7 RNA polymerase. This step was followed by the addition of the translation mix, containing optimized rabbit reticulocyte lysate and one of the following components: 35S-methionine (50 µCi), 3H-Farnesyl pyrophosphate (FPP, 4 µCi), 3H-Geranylgeranyl pyrophosphate (GGPP, 4 µCi) (NEN, Boston, MA). Both FPP and GGPP were used in the presence or absence of GDP (1 mM). The non-recombinant pCITE vector, a non-relevant recombinant pCITE vector, pCITE-RXR, as well as the rabbit reticulocyte lysate with no DNA transcribed were used as negative controls. The translation reactions were incubated at 30°C for 2 h for the 35S-methionine-containing reactions and for 6 h for the reactions containing either 3H-FPP or 3H-GGPP. Aliquots of 2 µl of 35S-methionine-containing reactions and 5 µl of 3H-FPP or 3H-GGPP were removed for analysis on SDS-PAGE. The remaining translation reactions were diluted with an equal volume of a buffer containing 100 mM Tris–HCl, pH 8.0, 300 mM NaCl and 10 mM EDTA. S-protein agarose beads (30 µl) were then added to the reactions, rocked for 1 h at room temp then centrifuged for 2 min and washed five times in 0.5 × of the dilution buffer. The beads were then resuspended in 15 µl of 1 X SDS-loading dye. All samples were size-separated by 15% SDS-PAGE, stained, destained, soaked in ‘Entensify’ reagents (NEN, Boston, MA), dried and exposed to an X-ray film (Kodak, X-OMAT-AR5; Eastman Kodak, Rochester, NY).

2.10. Farnesyl transferase (FTase) inhibition assay

Based on the results of in vitro prenylation, a farnesylation inhibition assay was performed to check the effect of using a farnesyl transferase inhibitor on SmRas prenyl modification. In this assay, SmRas recombinant pCITE vector was transcribed and translated in vitro in the presence of either 35S-methionine or 3H-FPP. A farnesyl transferase inhibitor (FTI-277), kindly provided by Dr J. Bennett, was added to the translation reactions in doses from 50 nm to 2 µm. SmRXR recombinant pCITE vector was used as a negative control. The S-protein immunoprecipitated prod-
ucts were size-separated on 4–15% SDS-gradient gel (BioRad, Hercules, CA). The gel was processed as described above. The in vitro translation was performed in the presence of 1 mM GDP, since SmRas was more efficiently processed in the presence of GDP than in its absence.

2.11. In vitro prenylation assay in schistosome extract

A prenylation assay as above was conducted utilizing schistosome extract to investigate the modification of SmRas using schistosome enzymes. In this assay, adult worm pairs were perfused from an infected hamster under aseptic conditions and washed several times with MEM medium (GIBCO-BRL, Gaithersburg, MD), containing 100 U Penicillin-G and 100 µg Streptomycin ml⁻¹. The worms were then incubated over night at 37°C, 5% CO₂ in MEM medium containing 20% FBS, 100 U Penicillin-G, 100 µg Streptomycin ml⁻¹, and 500 µm mevenolin. The worms (100 pairs) were homogenized in 500 µl buffer containing 50 mm Tris–HCl, pH 8.0, 1 mM MgCl₂, 1 mM DTT, 1 mM EGTA, 1 mM PMSF and 10 µg ml⁻¹ leupeptin. The homogenate was centrifuged at 100,000 g for 1h at 4°C. The cleared homogenate was stored at −80°C until use. The recombinant SmRas-pCITE plasmid was used in a coupled in vitro transcription–translation reactions using cold methionine as described above, then the in vitro synthesized protein was affinity purified with S-protein agarose beads, following manufacturer’s instruction. The purified protein was then incubated for 6 h at 30°C with 50 µl of schistosome homogenate containing 4 µCi of either ³H-FPP or ³H-GGPP, 1 mM GDP and varying amounts of FTI-277. After completion of the reactions, 15 µl aliquots were size separated on SDS-PAGE and the gels were processed as above.

3. Results

3.1. Cloning and computer analysis of cDNA coding sequence of SmRas

PCR amplification of SmRas coding sequence using SmRas-cod primer pair resulted in amplification of 664 bp PCR product that encodes 184 amino acid peptide. Sequence analysis of independent clones, showed that the cDNA sequence is identical to that submitted to the Genbank with the exception of one silent base change (434G to T) which encodes the same amino acid (proline 140). The NCBI Blast search, performed without filtering for low compositional complexity regions, showed that SmRas protein has strong homology to K-Ras from different organisms (81% identity and 92% similarity) (1, Panel A). Although the Blast search showed that SmRas is homologous to K-Ras proteins, pileup analysis and multiple sequence alignment (GCG package software, version 9, 1997; Genetics Computer Group, Madison, WI) revealed that the schistosome protein is evolutionary more closely related to N-Ras of the African clawed frog, and chicken, human and rat H-Ras proteins (1, Panel B). It also showed that SmRas has the characteristic features of other Ras proteins (1, Panel A). The 3 GTP-binding sites contained in other Ras peptides are also conserved in SmRas. This includes the ‘A’ consensus sequence [35] or ‘P’ loop [36] which is a glycine-rich region (10-GAGGVGKS-17). The region forms a flexible loop between a beta strand and an alpha helix and interacts with one of the phosphate groups of the nucleotide.

Hybridization experiments to determine the SmRas copy number were performed by quantitative dot blot using a 650 bp cDNA probe. The hybridization data were analyzed with a BioRad phosphoimager. A total of 2.5 pg of the SmRas cDNA gave the same intensity of hybridization as did 2 µg of genomic DNA (data not shown). Given that the S. mansoni genome contains 2.7 × 10⁸ bp [37], the hybridization results indicate that one copy of SmRas is present per haploid genome.

3.2. Western blot analysis

Affinity purified anti-SmRas polyclonal antibody detects a protein of about 23 kDa in extracts of whole parasites and carcass but not in NP-40 soluble proteins (Fig. 2, Panels B and C). Pre-immune rabbit serum did not detect the same protein in total extract of adult worm pairs (Fig. 2, Panel A). In addition, a commercial monoclonal anti-
body directed against human H-Ras protein (anti V-H-Ras; Calbiochem, La Jolla, CA) detected a protein band with same molecular size as that recognized by anti-SmRas rabbit polyclonal antibody (Data not shown). This monoclonal antibody recognizes a peptide sequence in Ras proteins (62-EEYSAMRDQYMRTGE-76), which is conserved in the schistosome homologue (1, Panel A).

3.3. Immunocytolocalization

Anti-SmRas affinity purified antiserum was used to localize the native protein in adult worm cryosections. SmRas was not surface exposed. Fluorescence was observed in both male and female sections. On the ventral side SmRas appears to be associated with bulbous protrusions into the tegument with connections to a network found within the muscle layers connecting to cells below the muscle layers. (Fig. 3, Panels A and B). On the dorsal side SmRas appears to be associated with the tubercles. There seems to be pathways of fluorescence within the body of the parasite (3, Panel C). Sections probed with pre-immune rabbit serum (Panel D) showed only the minimal, commonly observed auto-fluorescence.

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Fig. 1. Pileup analysis and peptide alignment (A) and a dendogram (B) of S. mansoni Ras (sm-ras), Xenopus laevis N-Ras (n-X-laevis), Gallus gallus H-Ras (h-G-gallus), Rattus norvegicus H-Ras (h-R-norvegicus), Homo sapiens H-Ras (h-H-sapiens), and K-Ras proteins of Musculus musculus (k-M-musculus), X. laevis (k-X-laevis), Pleuronectes vetulus (k-P-vetulus), R. norvegicus (k-R-norvegicus), Monodelphis domestica (k-M-domestica), Meleagris gallopavo (k-M-gallopavo), H. sapiens (k-H-sapiens). Black-blocked sequences represent the GTP-binding sites, blocked amino acids are non-conserved, underlined sequences delineate the epitope recognized by the monoclonal antibody (anti-v-H-Ras, Calbiochem) and C-terminal motifs are showed in bold face and upper case. %I and %S are identity and similarity scores, and O is the amino acid overlap length. Panel C is the phylogenetic tree for the 30 C-terminal amino acids of S. mansoni and other Ras peptides. H-Ras and N-Ras peptides are distinguished from K-Ras by letter H or N, respectively, at the end of the species name.
3.4. Quantitative PCR analysis

Analysis of semi-quantitative RT-PCR performed using specific SmRas primers and internal control $\alpha$-tubulin primers revealed that, compared to $\alpha$-tubulin, SmRas exhibits more or less consistent transcription throughout the development of the parasite from the schistosomule stage to the mature adult worm pairs (Fig. 4). However, the transcription levels of SmRas in either mature or immature female worms (Fig. 4, lanes 2 and 4) were higher compared to those exhibited by mature and immature male worms (Fig. 4, lanes 3 and 5) (approximately 3- and 5.5-fold, respectively). In addition, the level of transcription in mature male worms (Fig. 4, lane 3) was about 2-fold higher than that of immature male worms (Fig. 4, lane 5).

3.5. In vitro prenylation assay

Prenylation is the first post-translational modification of Ras. It helps anchor Ras proteins to the inner side of the cell membrane. Ras is modified by attaching an isoprenoid group, usually C$_{15}$ farnesyl isoprenoid and to a lesser extent C$_{20}$ geranylgeranyl group, via a thioether bond to the cysteine residue that is three amino acids away from the C-terminal extremity. The isoprenoid group used to modify Ras protein depends primarily on the sequence of its C-terminal motif. SmRas has the C-terminal motif, CCIQ (Fig. 1).

This sequence fits the requirements for peptide modification either by farnesylation, using FTase, or by geranylgeranylation, catalyzed by GGTaseII [38].

In order to find out what kind of prenyl group was being used to modify the SmRas protein, SmRas mRNA was transcribed and translated in vitro, $^3$H-FPP or $^3$H-GGPP was added in separate translation reactions in the presence or absence of GDP (see Section 2). The results of this assay showed that only $^3$H-FPP is incorporated in Sm-Ras (Fig. 5, Panel A, lanes 5 and 7). Furthermore, the incorporation of the labeled farnesyl was greater in the presence of GDP than in its absence (Fig. 5, Panel B, lanes 5 and 7). No incorporation of the $^3$H-label was obtained when $^3$H-GGPP was used in either the presence or absence of GDP (Fig. 5, Panel A, lanes 6 and 8). The negative controls (vector alone or vector with an irrelevant protein) showed no incorporation of $^3$H-FPP nor $^3$H-GGPP (Fig. 5, Panels A and B, lanes 10, 11, 13 and 14).

3.6. FTase inhibition assay

Addition of FTI-277 to the translation reactions revealed that SmRas farnesylation is inhibited by FTI-277 in a dose-dependent manner. Fig. 6 shows a decrease in the $^3$H-labeled SmRas as a result of increase in the concentration of FTI-277 in the translation mix (lanes 5–9). SmRas farnesylation was almost abolished at concentrations of FTI-277 $\geq$ 2 $\mu$M.

3.7. SmRas prenylation in schistosome extracts

When schistosome homogenate was used as a source of prenyltransferases, SmRas was found to be farnesylated. Fig. 7 shows a composite fluorograph of in vitro synthesized and prenylated SmRas in which a schistosome extract was used as the source of modifying enzymes. The incorporation of $^3$H-label was found in FPP-containing reactions but not with GGPP (Fig. 7, lanes 1 and 7). The use of FTI-277 produced an inhibition pattern (Fig. 7, lanes 2–5) comparable to that
obtained with rabbit reticulocyte lysate. Quantitatively, the use of 50 nM of FTI-277 (Fig. 7, lane 2) produced approximately 60% inhibition of the 3H-FPP incorporation as determined by scintillation counting of the labeled bands excised from the SDS-gels. The farnesylation reaction was completely inhibited at concentrations of FTI-277 ≥ 500 nM (lanes 4 and 5). A weakly labeled band of about 23 kDa was detected when using 3H-FPP (Fig. 7, lane 1). Based on molecular size, this band likely represents native SmRas that was farnesylated. No incorporation of 3H-GGPP was detected when recombinant SmRas was treated with equal doses of FTI-277 (data not shown).

4. Discussion

To optimize reproductive success under the limitations imposed by conditions within an individual host, parasitic helminths, such as S. mansoni, have evolved mechanisms that allow them to detect and respond to host factors [39]. For example, the host cytokine TNFα, which is secreted in response to schistosome ova and promotes granuloma formation, has been shown to work as a positive regulator of fecundity for female parasites [2]. Silva et al. [40] have demonstrated the presence of a 130 kDa protein that binds host complement ‘C3’. This protein was found to be

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Fig. 4. Expression of SmRas throughout schistosome development. Photograph of an agarose gel that shows the amplification products of α-Tubulin and Sm-Ras PCR reactions. Panel I shows the adult worm stages: worm pairs (lane 1), females (lane 2), and males (lane 3). Panel II, lanes 4–10 shows the immature worm stages: single sex females, single sex males, 35, 32, 28, 20 and 15-day worm pairs. Panel III, lanes 11–13 shows schistosomula stages: 7-d, 4-d and 3-h schistosomules. M = molecular size markers (1 kb ladder, LifeTechnologies).

Fig. 5. In vitro prenylation of SmRas. Fluorograph SDS-PAGE of in vitro translated (left panel) and S-protein agarose-immuno-precipitated (right panel) products of rabbit reticulocyte lysates with no DNA (lanes 1, 2, and 3), pCITE-Ras (lanes 4, 5, 6, 7, 8), pCITE-RXR (lanes 9, 10, 11), non-recombinant pCITE (lanes 12, 13, 14). Reaction components were added according to the illustrated key at the top of the photographs. Arrow points to in vitro synthesized SmRas protein. 35S-Met, 35S-methionine; 3H-FPP, 3H-Farnesyl pyrophosphate; 3H-GGPP, 3H-Geranylgeranyl pyrophosphate; GDP, Guanine diphosphate.

responsible for renewal of the parasite envelope, which overlays the apical plasma membrane, upon binding to C3. The identification of various growth factor receptors in S. mansoni, represents further evidence that schistosomes are responsive to stimuli emanating from the host environment. A transforming growth factor-β serine-threonine receptor kinase I (TβRI), was recently identified in S. mansoni. It was shown to be expressed at the parasite surface and this expression was up-regulated following infection of the mammalian host [39]. Epidermal growth factor receptor (EGFR), a tyrosine kinase receptor, is another example of growth factor receptors that are expressed on the surface of parasites [41]. Together, these observations suggest the presence of signaling pathways by which the parasites can detect host signals and respond to them in a way that presumably increases their survival.

Fig. 6. Farnesyl transferase inhibition assay of SmRas prenylation. In vitro translation reactions were performed in the presence of 35S-methionine (lanes 1 and 3), or 3H-FPP (lanes 2, 4–9). SmRXR was used as negative control (lanes 1 and 2). In vitro synthesized SmRas mRNA was used as a template (lanes 3–9). FTI-277 was added in increasing amounts, 0.05, 0.1, 0.5, 1.0 and 2 μM (lanes 5–9). The samples were size-separated by SDS-PAGE of S-protein agarose-immuno-precipitated products and fluorographed.
Ras is an example of an intracellular signal transducer. Ras plays a key role in modulating a wide diversity of signaling pathways, as evidenced by its transient activation by growth factors, hormones, neurotransmitters and cytokines [42,43]. Upon activation, Ras relays the stimulus from activated RTKs by activating a cascade of protein kinases (e.g. Raf/MEK/MAPK) which in turn initiates further cytoplasmic and nuclear events resulting in alterations of cell morphology [44], cellular communications [45], and mitosis [46].

Using an EST representing a schistosome homologue of the Ras superfamily (GenBank accession U53177) as a probe, we obtained a cDNA containing the entire coding region. NCBI blast search with the SmRas peptide showed a high degree of homology with K-Ras from different organisms. However, pileup analysis of the deduced peptide revealed that the phylogenetic organization of SmRas is also closely related to N-Ras of African clawed frog and H-Ras of chicken, rat and human (Fig. 1). However, in a comparison of the 30 C-terminal amino acid residues, SmRas peptide is more similar to K-Ras proteins. This observation suggests that schistosome Ras is more primitive, which might perform the biological activities displayed by H-Ras and N-Ras proteins in different species, while its C-terminal posttranslational modifications are similar to those of K-Ras proteins.

Western blots and immunolocalization data showed that the native schistosome Ras is not exposed on the parasite surface. The native protein could be detected in whole worm but not NP-40 extracts. The immuno-fluorescence data is also in agreement with the western analysis where the native protein was found not to be tegument associated. This was not unexpected as Ras proteins are known to be intracellular transducers, which are not exposed on the surface but mediate signals received from surface-exposed receptors.

Quantitative RT-PCR analysis showed that SmRas is expressed throughout parasite development. Obviously Ras could be involved in normal growth, differentiation and proliferative processes in various stages that result in the growth of the parasite from less than 400 microns to about 15 mm in size. Interestingly, compared to male worms, Ras is over-expressed in female worms. This may be attributed to the involvement of Ras protein in female worm maturation, particularly vitelline cell maturation and/or egg laying. This suggestion is supported by previous reports that prenylated proteins are implicated in egg production in S. mansoni [47–49]. However, SmRas was also found to be transcribed at same level in immature females as in mature female worms. This plus the fact that both mature and immature male worms showed the lowest levels of Ras transcription likely point to a number of functions for Ras during development. Schussler et al. [22] recently reported the detection of Ras protein in the parasite extracts using a commercial antibody. The signals detected in mature female worms were stronger than immature females. This may reflect...
differences in post-translational processing rather than transcriptional regulation.

Prenylation is the first step in the post-translational modification series that allows the Ras protein to be anchored to the inner side of the plasma membrane. The C-terminal CAAX sequence of Ras determines if the substrate will be modified by farnesyl transferase or geranylgeranyl transferase. For example, the Drosophila Ras1 is geranylgeranylated [50] whereas the yeast Saccharomyces cerevisiae Ras1 and Ras2 proteins are farnesylated [51]. Most Ras family members have a CAAX consensus sequence that allows one to predict whether it will be farnesylated or geranylgeranylated [52,53]. The schistosome Ras has the sequence CCIQ which could be prenylated by either farnesyl or geranylgeranyl. SmRas was found to be farnesylated. However, its C-terminal processing may differ from that of other Ras proteins such as H- and N-Ras, since these proteins are palmitoylated at the cysteine residue(s) upstream of the C-terminal box [54]. Schistosome Ras which lacks cysteine residues in the 50 amino acids preceding its C-terminus, is not likely to be palmitoylated. In addition, the polylysine stretch present in K-Ras proteins (six consecutive lysine residues) is conserved in the schistosome protein with the substitution of two lysine residues with the closely related basic amino acid, ‘arginine’. This suggests that SmRas is processed and anchored to the plasma membrane by its farnesyl isoprenoid moiety along with a polybasic region similar to other K-Ras proteins. This supports the notion that SmRas is likely a more primitive family member. In addition to the results obtained from the in vitro prenylation assay, which utilized rabbit lysates, schistosome extract was used in order to test the prenyl modification in the parasite in vitro. Schistosome worms were pretreated with mevenolin, an inhibitor for hydroxymethylglutaryl (HMG)-CoA reductase, the rate-limiting enzyme of the prenoid biosynthetic pathway [55] and tested as a source of FTase and GGTases. This pretreatment of worms was performed to block the synthesis of endogenous (unlabeled) prenyl groups and consequently increase the incorporation of the labeled groups in the modified proteins. Schistosome extract prepared from mevenolin-treated worms was shown to be able to farnesylate exogenous SmRas as in Fig. 7. The presence of another 3H-labeled protein band of about 23 kDa is thought to be the native SmRas, which is 23 kDa.

Farnesyl transferase inhibitors (FTIs), have evolved as potential agents capable of interfering in Ras functions by inhibiting its farnesylation and consequently its correct cellular localization and biological activities. FTIs have demonstrated a potent and specific ability to block Ras processing, signaling and transformation in transformed and tumor cell lines both in vitro and in animal models [56]. SmRas farnesylation was inhibited in vitro, when using rabbit reticulocyte lysate as a source of modifying enzymes, by the FTase inhibitor, FTI-277, although at a relatively higher concentration than that reported to inhibit H-Ras farnesylation. However, a 60% inhibition level of farnesylation reaction in schistosome extract was achieved using 50 nM of FTI-277 (IC50 ≤ 50 nM). This indicates a greater sensitivity of the schistosome enzyme to the effect of the inhibitor than that of the mammalian enzyme. However, this inhibition level is still relatively higher than that required to inhibit H-Ras farnesylation. FTI-276, a closely related FTase inhibitor (FTI-277 is the methyl ester derivative of FTI-276), inhibited H-Ras processing in vitro with an IC50 of 500 pM [57]. Lerner et al. [57] reported that treatment of H-Ras transformed NIH-3T3 cells with FTI-277 potently inhibited H-Ras processing and the constitutive activation of MAPK (IC50 = 100 pM). However the dose required to achieve a comparable effect in the K-Ras4B-transformed cells was 100-fold higher (~ 10 μM). Taking into consideration the close similarity between the C-terminal ends of SmRas and other K-Ras proteins, such relatively high concentration of FTI-277 required to inhibit SmRas farnesylation might be expected. The relative insensitivity of K-Ras proteins to the effect of FTIs, in general, may have arisen from the higher affinity (10–20-fold) of their C-termini for FTase enzyme [58]. Another explanation that may account for this observation is that, under normal conditions, K-Ras proteins are farnesylated, but can undergo modification by GGTaseI in vitro, in the presence of FTIs [59], and in vivo,
when cellular FTase is inhibited [60]. However, our data indicates that, under these experimental conditions, SmRas is not processed by schistosome GGTases in vitro.

FTI-277 exerts its effect in blocking H-Ras activation of MAPK, in NIH-3T3 transformed cells, by blocking recruitment of Raf to the plasma membrane where it would be activated [57]. The net effect involves the induction of apoptotic pathways resulting in cell death [61]. Whether similar paradigms apply to schistosomes and thus result in the use of FTIs as antischistosomal drugs remains to be tested. In this regard it has recently been demonstrated that the FTI, FTI-745,631, inhibited the growth of the cultured Plasmodium falciparum by preventing maturation beyond the early trophozoite stage [62].

It has become clear that the Ras-mediated signaling pathway is not a single linear cascade. Instead, different signaling pathways or complexes are connected by a signaling network. Cross-talk between different pathways may vary from tissue to tissue and from one developmental event to another, and thus, may provide additional regulation for specific functions [63]. Therefore, interfering with the biological activities of schistosome Ras may give rise to new control methods that work in synergy with the currently ongoing strategies to hamper this globally threatening parasitic infection. In this regard, we demonstrate an effective in vitro assay for screening SmRas inhibitors.

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