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# Characterization and comparative functional analysis in yeast of a *Schistosoma mansoni* Rho1 GTPase gene<sup>☆</sup>

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## Abstract

Low-molecular weight GTP-binding proteins (LMWGs) of the Ras superfamily are believed to play a role in *Schistosoma mansoni* female development and egg production. Here we describe the characterization of a novel *S. mansoni* gene (*SMRHO1*), highly homologous to Rho-type LMWGs from several other organisms and encoding a polypeptide with 193 amino acids and an estimated molecular mass of 21.8 kDa. *SMRHO1* complemented a *Saccharomyces cerevisiae rho1* null mutant strain even in restrictive temperature and calcium concentration, in contrast with the human RHOA GTPase that was not able to provide complementation in such conditions. Comparison of the amino acid sequence of the  $\alpha$ 3-helix loop7 regions of the two proteins allowed the identification of the proline 96 and threonine 100 amino acid residues of human RHOA as the most probable determinants of the complementation differences. We generated *SMRHO1* mutants (*smrho1*<sup>E97P</sup>, *smrho1*<sup>L101T</sup> and *smrho1*<sup>E97P.L101T</sup>) by site directed mutagenesis and reproduced the conditional lethality phenotype at high temperature, providing strong evidence that the related amino acid positions (Gln<sup>101</sup> and Ile<sup>105</sup>) in the Rho1 GTPase are indeed important for regulation of the cell wall synthesis performed by this protein in yeast. The observation that specific amino acid positions seem to be important for the different functions performed by the Rho GTPases leads to the idea that *SMRHO1* might be a useful target in the development of new anti-schistosomiasis drugs, although it does share high sequence homology with the human RhoA GTPase.

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**Keywords:** *Schistosoma mansoni*; Complementation; Yeast; Rho GTPase; Site directed mutagenesis

## 1. Introduction

*Schistosoma mansoni* is a digenetic trematode that causes schistosomiasis, a disease that afflicts more than 200 million people worldwide [1]. Since 1992, the *S.*

*mansoni* Genome Project has contributed to the discovery of close to 10 000 new genes of this parasite [2]. The study of important genes for *S. mansoni* survival and pathogenicity is the first step for the development of new drugs and vaccines for schistosomiasis control.

Following penetration into the human host skin as cercariae, the parasites mature into adult male and female forms that live in constant copulation in the veins of the portal system and ovulate for the duration of their lives. There is evidence of involvement of low-molecular weight GTP-binding proteins (LMWGs) of the Ras superfamily in the *S. mansoni* female maturation process and egg production [3,4]. This is of great interest for schistosomiasis control because inflammatory reactions triggered by the eggs, and not by the worms themselves, are the cause of liver fibrosis, the most serious pathological lesion of the disease.

**Abbreviations:** GDP, guanine diphosphate; GTP, guanine triphosphate; LMWGs, low-molecular weight GTP-binding proteins; *SMRHO1*, *Schistosoma mansoni* Rho1 GTPase gene; *SMRHO1*, *S. mansoni* Rho1 GTPase protein; *smrho1*, *S. mansoni* Rho1 GTPase mutant gene; UPGMA, unweighted pair group with arithmetic mean.

<sup>☆</sup> **Note:** The nucleotide sequence reported in this paper has been submitted to the GenBank™/EMBL Data Bank with the accession number AF140785.

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The Ras superfamily members (Ras, Rho, Rab, Sar1/Arf, and Ran) are proteins capable, through the binding and hydrolysis of GTP, to create a switch between an active GTP-bound conformation and an inactive GDP-bound conformation [5,6]. The 'on-off' activity of the LMWGs is controlled by several regulatory proteins: the guanine nucleotide exchange factors (GEFs) promote the exchange of the GDP for GTP; the GTPase-activating proteins (GAPs) accelerate the hydrolysis of the GTP to GDP; and the GDP dissociation inhibitors (GDIs) inhibit the dissociation of the GDP from the GTPase [7]. LMWGs receive upstream signals through their regulators and transduce signals to a multitude of effector molecules, while remaining in the GTP-bound form [8–10]. In this fashion, the members of the Ras superfamily perform important regulatory roles, controlling a variety of cellular activities, such as cell cycle, gene expression, cytoskeletal reorganization, vesicle trafficking, nucleocytoplasmic transport and microtubule organization [6,11–14].

It has been shown that a reduction of isoprenoid end-products synthesis by mevinolin, an inhibitor of hydroxymethylglutaryl-CoA reductase, was capable of decreasing egg production and blocking the schistosomiasis pathology in infected mice [4] as well as causing loss of parasite viability at higher drug concentrations [15]. Similar effects were observed in schistosome females as the result of prenyltransferase activity inhibition by BZA-5B [16]. This is important because the blocking of prenylation of a 25 kDa group of GTP-binding proteins from schistosomes using BZA-5B led to the speculation that LMWGs could be playing a role in the egg laying mechanism [17]. These observations stimulated the search for LMWGs that might be associated with the regulation of signal transduction pathways in *S. mansoni*. A gene encoding a RAB-related GTP-binding protein was cloned from *S. mansoni* genomic DNA using degenerate oligonucleotide primers in an attempt to identify LMWGs possibly involved in female egg production [16]. The *in vitro* translation product of this *SMRAB* gene is geranylgeranylated and the recombinant protein binds GTP. Using an immunochemical approach, Schussler et al. (1997) showed that the Ras, GAP and MAP kinases displayed sexual and developmental regulation in schistosome extracts [3]. The involvement of these molecules in the female maturation process was not unexpected, since the Ras, GAP and MAP kinases participate in the same signal transduction pathways known to regulate cell proliferation events. More recently, a schistosome cDNA encoding a gene highly similar to K-Ras from various organisms was isolated and characterized [18]. This *S. mansoni* K-Ras homologue gene is overexpressed in females in comparison with male worms and it has been detected in all developmental stages of the parasite. The recombinant SMRAS protein is farnesy-

lated *in vitro* and the native protein was identified by immunocytochemical localization associated with the worm subtegument.

As part of the *S. mansoni* Genome Project, we isolated from a cercariae library a cDNA clone highly homologous to the Rho-type LMWGP genes from several organisms [19]. We report here the complete cDNA and genomic sequences of this gene. Sequence alignments, molecular phylogenetic analysis, and complementation of a *Saccharomyces cerevisiae rho1* null mutant strain allowed us to identify it as a Rho-type LMWGP (*SMRHO1*), the first gene of this class to be identified in *S. mansoni*.

The complementation of yeast containing a *rho1* deletion with *SMRHO1* occurred even in restrictive conditions of temperature and calcium concentration and was much more complete than the only conditional complementation previously observed, with the human RHOA GTPase [20]. Comparison of the  $\alpha$ 3-helix loop7 amino acid sequences of the *S. cerevisiae* RHO1, human RHOA and *SMRHO1* GTPases, allowed identification of Pro<sup>96</sup> and Thr<sup>100</sup> amino acid residues in human RHOA as the most probable determinants of the difference in the complementation ability of *SMRHO1* and RHOA GTPases. Indeed, when we used site directed mutagenesis to replace Glu<sup>97</sup> and Leu<sup>101</sup> residues of *SMRHO1* by Pro and Thr, respectively, *SMRHO1* became only capable of conditional complementation of the *S. cerevisiae rho1* null mutant strain. Consequently, although highly homologous to human RHOA, the *S. mansoni* Rho1 GTPase may prove to be an interesting target for the development of new anti-schistosomiasis drugs.

## 2. Materials and methods

### 2.1. Cloning procedures and DNA sequencing

The proofreading *Pfu* DNA polymerase (Stratagene) was used in PCR amplifications for subcloning. Amplification products used in subcloning or transformation procedures were previously purified using the Wizard PCR-Prep kit (Promega). Cloning into pUC18 vector was done using the SureClone Ligation Kit (Amersham Biosciences). Plasmid purifications were done by alkaline lysis using the Wizard Plus SV Miniprep kit (Promega). DNA sequencing reactions were prepared using Thermo Sequenase Sequencing Kit (Amersham Biosciences) with fluorescent M13 primers and carried out in an A.L.F. DNA sequencer (Amersham Biosciences).

## 2.2. cDNA sequencing

The SMC0220R cDNA clone containing the *S. mansoni* Rho GTPase gene was obtained by random selection from a *S. mansoni* cercariae library as described previously [19]. Initially the 5' and 3' ends of the SMC0220R cDNA clone was sequenced using M13 primers. Subsequently, the PTV3 (5'-TGA TGT TCC CAT TGT CTT AG-3') and PTV4 (5'-AAA TCA AAT CTC AGA CAA TT-3') primers were designed to amplify the internal region of the insert. The 291 bp internal fragment amplified was subcloned into pUC18 vector and sequenced on both strands. In order to obtain a reliable full-length sequence, the ends of the insert were amplified using the M13 reverse/PTV5 (5'-CTA AGA CAA TGG GAA CAT CA-3') and PTV6 (5'-TGT CTA GAT TTG ATT TTC CG-3')/M13 universal primer pairs. The generated fragments of 527 and 234 bp, respectively, were subcloned into pUC18 and sequenced on both strands.

## 2.3. Genomic sequencing

We used the SMRHOF (5'-GGC AAC AAT GGC GAG TG-3') and PTV4 primer pair to amplify the genomic sequence of *S. mansoni* Rho GTPase gene. The PCR amplification generated a fragment of about 700 bp. This fragment was excised from the agarose gel, purified using Gene Clean kit (Bio 101), cloned into pUC18 vector, and sequenced on both strands. In order to obtain a reliable sequence, the genomic clone was PCR amplified into two distinct fragments using the SMRHOF/PTV5 and PTV3/PTV4 internal primer pairs. The generated fragments of 417 and 324 bp were subcloned into pUC18 and sequenced on both strands.

## 2.4. Sequence analyses

Sequence alignments and open reading frame (ORF) search were done using the DNASIS program (Hitachi). Homology searches were performed using BLAST programs [21] for sequence similarity search at NCBI. Alignments of amino acid sequences and UPGMA analysis were performed with the software DIALIGN 2.1 [22].

## 2.5. Yeast strains and media

All yeast strains employed are listed in Table 1. The diploid strain BY4727/36 was obtained crossing by replica plating BY4727 with BY4736 strain [23], both of them obtained from ATCC. YPD rich medium was as described [24]. YNB minimal medium was purchased from Difco and 2% glucose or 2% galactose was included as carbon source when required.

Table 1  
Yeast strains used in this study

Strain	Genotype	Reference
BY4727	<i>MAT<math>\alpha</math> his3<math>\Delta</math>200 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 met15<math>\Delta</math>0 trp1<math>\Delta</math>63 ura3<math>\Delta</math>0</i>	[23]
BY4736	<i>MAT<math>\alpha</math> ade2<math>\Delta</math>::hisG his3<math>\Delta</math>200 met15<math>\Delta</math>0 trp1<math>\Delta</math>63 ura3<math>\Delta</math>0</i>	[23]
BY4727/36	<i>MAT<math>\alpha</math>MAT<math>\alpha</math> his3<math>\Delta</math>200 met15<math>\Delta</math>0 trp1<math>\Delta</math>63 ura3<math>\Delta</math>0</i>	This study
SPYD	<i>MAT<math>\alpha</math>MAT<math>\alpha</math> his3<math>\Delta</math>200 met15<math>\Delta</math>0 trp1<math>\Delta</math>63 ura3<math>\Delta</math>0 <i>Arh1::HIS3</i></i>	This study
CMY	<i>MAT<math>\alpha</math>? ade2<math>\Delta</math>::hisG? his3<math>\Delta</math>200 met15<math>\Delta</math>0 trp1<math>\Delta</math>63 ura3<math>\Delta</math>0 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 <i>Arh1::HIS3 pYEDP(SMRH01)</i></i>	This study

## 2.6. Cloning on yeast galactose-inducible overexpression vector

The *S. mansoni* Rho GTPase gene was amplified from the SMC0220R cDNA clone using SMRHOF and SMRHOR (5'-TTA GAG AAC AGT GGA GAT CA-3') ORF flanking primers and cloned into pUC18. The *S. cerevisiae* *RHO1* gene, containing its promoter (0.2 kb) and terminator (0.4 kb) regions, was obtained by direct amplification from yeast colony using primers RHOSACF (5'-ACA TGA GAT CTA CGC AGA TCA-3') and RHOSACR (5'-CAT CGA TCA TTC CTC TGA GTA-3'). The amplified fragment (1226 bp) was previously digested with *Dra*I in order to remove the promoter region, cloned into pUC18 and sequenced on both ends. Thereafter, the fragments containing *SMRH01* and *RHO1* were recovered from pUC18 by digestion with *Bam*HI/*Kpn*I and cloned into the same restriction sites of the pYEDP60-2-ADE2-URA3-*GAL10-CYC1* plasmid (a kind gift of Dr Francisco Nóbrega, UNIVAP, São Paulo) for overexpression in yeast under control of *GAL10-CYC1* promoter induced by galactose [25].

## 2.7. Yeast genetic manipulation methods

Transformations were done with 1  $\mu$ g of purified linear DNA or 0.5  $\mu$ g of plasmid DNA by LiAc/SS-DNA/PEG procedure [26]. The diploid *S. cerevisiae* strain SPYD (Table 1) containing one of the *RHO1* alleles disrupted was obtained by the PCR-mediated gene disruption method [23] using the diploid strain BY4727/36. The 60 mer primers RHO1HIS3F (5'-ATG TCA CAA CAA GTT GGT AAC AGT ATC AGA AGA AAG CTG GCT TCA TTC AAC GTT TCC CAT-3') and RHO1HIS3R (5'-GCT AGA AAT ATG AAC CTT CCA ACA AAA CTG AGG TTG GAG GAG TAT CAT ACT GTT CGT ATA-3') were used to amplify the *HIS3* auxotrophic marker gene cloned into pDIS plasmid (a kind gift of Dr Francisco Nóbrega,

UNIVAP, São Paulo). The 1243 bp amplified product contained at both ends 40 bp of sequence of the *S. cerevisiae* *RHO1* was purified and transformed into BY4727/36 diploid strain for disruption of one of the *RHO1* gene copies through a homologous recombination event. Disrupted mutants were selected on plates of YNB-glucose containing the required nutrients except histidine. PCR amplification of a 788 bp hybrid fragment containing part of *RHO1* and *HIS3* genes using RHOSACF and HIS311 (5'-AAC CCT ATA CCT GTG TGG A-3') primers, confirmed the insertion of the auxotrophic marker in place of one of the *RHO1* genomic copies in the yeast clones (data not shown). The SPYD strain was transformed with the *S. mansoni* Rho and yeast Rho1 genes cloned into pYEDP galactose-inducible plasmid and the clones were selected on YNB-glucose containing the required nutrients. The cells transformed with pYEDP (SMRH01) were induced to sporulate in order to obtain *rho1* null mutant cells complemented with the *S. mansoni* Rho GTPase gene. Sporulation of the diploid cells in liquid medium was induced as described [23]. The sporulated cultures were treated with NovoZym (Sigma) to digest the ascus wall, vortexed vigorously for 2 min to release spores from the asci and subsequently heated at 58 °C for 15 min to kill any remaining vegetative cells [24]. Spores dilutions were spread on YNB-galactose plates supplemented with the required nutrients. The *rho1* null mutant strain CMY (Table 1), complemented by *SMRH01* gene galactose-inducible overexpression, was confirmed by negative PCR amplification of *RHO1* gene using RHOSACF/RHOSACR primers, amplification of *SMRH01* gene using SMRHOF/SMRHOR primers, and amplification of a 788 bp hybrid fragment, containing part of *HIS3* and *RHO1* genes, using RHOSACF/HIS311 primers (data not shown). The PCR products were also sequenced.

### 2.8. Site directed mutagenesis of *SMRH01*

Megaprimer PCR method [27] was used to generate the *smrho1*<sup>E97P</sup>, *smrho1*<sup>L101T</sup> single mutants and the *smrho1*<sup>E97P, L101T</sup> double mutant of *SMRH01* gene. SMRHOE97P (5'-AGT TTT GCA AAC ATT CCA GA-3'), SMRHOL101T (5'-GG ACA CCA GAA ATC CGT C-3') and SMRHO97P101T (5'-T CCA GAA AAA TGG ACA CCA GA-3') primers, containing the mutations E97P, L101T, and E97P-L101T (highlighted in bold), respectively, were used in a first PCR round with SMRHOR primer to produce the megaprimers. SMRHOF primer and the purified megaprimers were used to generate *SMRH01* mutant copies in a second PCR round. *SMRH01* mutant copies were cloned into pUC18 and sequenced on both strands to confirm the presence of the mutations. The 0.2 kb *RHO1* promoter region was PCR amplified from yeast colony using

PROMORHOF (5'-CTG AAG CTT ACC AGC AGG AAT TCG-3') and PROMORHOR (5'-CAG CTG CAG CTT TCT AGT ATA ATT-3') primers containing *Hind*III and *Pst*I sites (showed underlined), respectively. The pYTS plasmid was constructed introducing the amplified *RHO1* promoter region into *Hind*III/*Pst*I gap of the pYEplac112 plasmid [28] (a kind gift by Dr José Miguel Ortega, UFMG, Belo Horizonte). Subsequently, pYTS1, pYTS2, pYTS21, pYTS22, and pYTS23 plasmids were constructed introducing *RHO1*, *SMRH01*, *smrho1*<sup>E97P</sup>, *smrho1*<sup>L101T</sup> and *smrho1*<sup>E97P, L101T</sup>, respectively, into *Bam*HI/*Kpn*I gap, positioned immediately downstream of the *Pst*I site of pYTS. These constructions and pYTS were transformed into CMY strain and the transforming clones were selected at 23 °C in YNB-glucose plates containing only leucine, lysine, and methionine. In these conditions, the galactose-inducible overexpression of *SMRH01* is inhibited and the yeast only express the wild type and mutant genes cloned into the pYTS plasmids under control of the *RHO1* promoter. The presence of wild type and mutant gene copies in the different mutant clones was confirmed by sequencing of the corresponding PCR products.

## 3. Results

### 3.1. cDNA sequence analyses

The partial sequences obtained after sequencing the subclones were aligned and produced a 1016 bp consensus that represents the full-length SMC0220R cDNA clone. We observed, however, that the insert was a chimera of two cDNA molecules joined by the adapters used in the cercarial library construction. The actual cDNA molecule encoding the *S. mansoni* Rho GTPase homologous gene represented 841 bp of this sequence (GenBank accession number AF140785). The most significant ORF found was 579-nt long and encoded a putative polypeptide of 193 amino acids with an estimated molecular mass of 21.8 kDa. The cDNA also contained a long 3'-untranslated portion, which is a known feature of *S. mansoni* transcripts. We named this gene '*SMRH01*' for *S. mansoni* homologue of the Rho1 and RhoA-type LMWGPs. In Fig. 1A, we show the alignment of the amino acid sequence of *SMRH01* with the Rho GTPases from 11 other species. A noteworthy feature was the insertion of a serine residue very close to the N-terminal end, which has not been observed in any of the other Rho GTPase homologs examined (Fig. 1A). Another interesting element was the presence of the polar amino acid aspartate (position 191) substituting for the first (generally aliphatic) amino acid of the C-terminal prenylation motif CAAX (Fig. 1A). Apart of these two



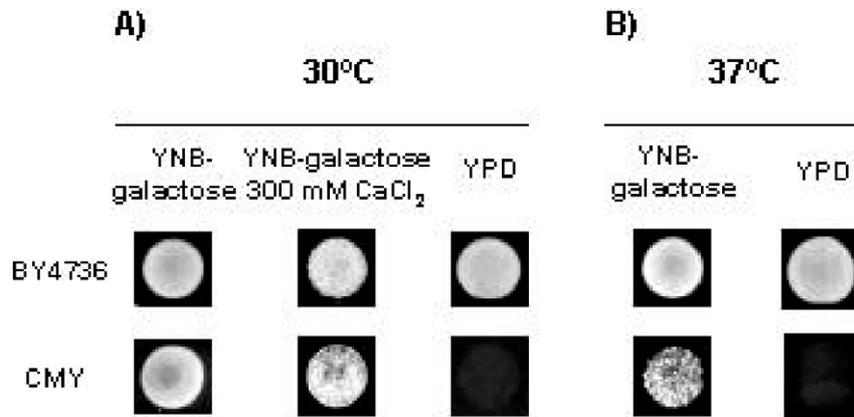


Fig. 2. Complementation of a yeast *rho1* null mutant strain by galactose-inducible overexpression of *SMRH01*. (A) Growth in agar plate at 30 °C of the wild type strain BY4736 containing the empty plasmid pYEDP and of the *rho1* null mutant strain CMY complemented by galactose-inducible overexpression of *SMRH01*. The media used were: YNB-galactose, YNB-galactose with 300 mM CaCl<sub>2</sub> and YPD rich medium containing only glucose as carbon source for galactose-inducible overexpression of *SMRH01* inhibition. (B) Growth at 37 °C of the strains BY4736(pYEDP) and CMY in YNB-galactose and YPD rich medium containing only glucose as carbon source for galactose-inducible overexpression of *SMRH01* inhibition.

and *Candida albicans*) and the second (upper main branch in Fig. 2B) belonging to multicellular organisms. Within the latter, the Schistosome Rho GTPase constituted as an isolated branch (Fig. 1B).

### 3.2. Genomic sequence analyses

Partial sequences obtained from the genomic sub-clones were aligned to produce a 722 bp consensus genomic sequence that contained an ORF identical to the *SMRH01* and three small introns (GenBank accession number AF140785). The first intron has 32 bp and is located between switch I and II domains of the protein. The second intron, of 33 bp, is in the middle of the ORF. The last intron is also 33 bp long and is located in the SAK motif of the protein, which participates in interactions with the guanine of GTP molecule in the active site. All introns interrupt the protein frame. The canonical donor/acceptor splice sites are conserved in all exon/intron junctions.

### 3.3. Complementation of a *S. cerevisiae rho1* null mutant strain by *SMRH01* galactose-inducible overexpression

The *rho1* null mutation in yeast is lethal. For this reason, we obtained first a heterozygous diploid strain with one of the *RHO1* alleles deleted (SPYD strain, Table 1), which was viable at both 30 and 37 °C (data not shown). To investigate the complementation phenotype of *SMRH01* gene in yeast cells, we transformed the SPYD strain with a plasmid (pYEDP) containing the *S. mansoni* Rho1 GTPase gene under control of a galactose-inducible promoter GAL10. After sporulation of the SPYD cells containing the plasmid pYEDP(*SMRH01*), we could then successfully select on plates containing galactose a yeast *rho1* null mutant

haploid strain (CMY, Table 1) complemented by overexpression of the *SMRH01* gene. This complementation could be shown to be due to the presence of *SMRH01* gene, since CMY cells died in YPD rich medium containing glucose as carbon source, in which the expression of the *S. mansoni* GTPase gene in pYEDP was inhibited (Fig. 2A and B). The *SMRH01* gene was able to complement the yeast *rho1* null mutant strain CMY even under temperature and osmotic stress conditions such as at 37 °C and in presence of 300 mM CaCl<sub>2</sub> (Fig. 2A and B). The *S. mansoni* Rho1 gene overexpression in a yeast wild-type strain {BY4736[pYEDP(*SMRH01*)]} did not produce significant alterations in the growth curve of these cells if compared with the yeast *RHO1* gene overexpression in the same wild-type strain {BY4736[pYEDP(*RHO1*)]} (Fig. 3). However, the *rho1* null mutant strain CMY, which is complemented by *SMRH01* overexpression, showed cell enlargement (data not shown) and also a delay in the growth in the same fashion as wild-type strain overexpressing *RHO1* gene {SPYD [pYEDP(*RHO1*)]} (Fig. 3). It occurred probably due to an arrest in the cell cycle, as previously reported for *RHO1* overexpression in yeast [31]. The wild-type strain containing only the plasmid {BY4736(pYEDP)} was used as growth control.

### 3.4. Comparison among the sequence of the $\alpha$ -3-helix loop7 regions and functional complementation in yeast performed by *SMRH01* and other Rho GTPases

The  $\alpha$ -3-helix loop7 had been previously defined as the region of the human RHOA molecule responsible for its inability to complement yeast *rho1* null mutants at 37 °C or in the presence of 300 mM CaCl<sub>2</sub> [20]. *Drosophila melanogaster* RhoA has its  $\alpha$ -3-helix loop7

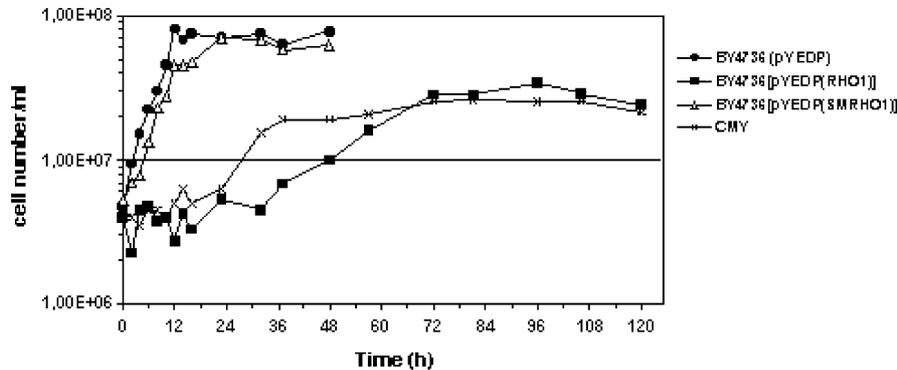


Fig. 3. Growth curves of the *rho1* null mutant strain CMY complemented by galactose-inducible overexpression of *SMRH01* and of the wild type strain BY4736, transformed with the plasmids pYEDP, pYEDP(*RHO1*) or pYEDP(*SMRH01*). The cells were grown at 30 °C in YNB-galactose liquid medium supplemented with the required amino acids, and counted at the indicated times.

region identical to human RHOA and it also cannot complement yeast *rho1* null mutants at 37 °C or in the presence of 300 mM CaCl<sub>2</sub> [32]. In order to try identify the exact amino acid residues responsible for the complementation differences seen between *S. mansoni* and human Rho GTPase genes, we aligned the sequence of *S. cerevisiae* RHO1  $\alpha$ 3-helix loop7 with the corresponding regions of *C. albicans* RHO1, human RHOA, and *SMRH01* GTPases (Fig. 4). The *C. albicans* RHO1 protein was included in the alignment because it fully complements *S. cerevisiae rho1* null mutants under any culture conditions [33]. The *C. albicans* sequence showed very few amino acid alterations in relation to *S. cerevisiae* RHO1, none with apparent functional importance to complementation [33]. However, amino acid differences of the human RHOA and *SMRH01*  $\alpha$ 3-helix loop7 regions, in comparison with *S. cerevisiae rho1*, were in greater number, determining occasionally significant hydrophobic changes (Fig. 4). The discrepancies of the amino acid constitution of the  $\alpha$ 3-helix loop7 regions of the Rho GTPases and the ability of each one to complement *S. cerevisiae rho1* null mutants suggest that proline 96 and threonine 100 at  $\alpha$ 3-helix would be the best candidate amino acids responsible for the conditional complementation by human RHOA.

	$\alpha$ 3-helix	--L7--	I (%)	S (%)
<i>Sc</i> RHO1	95	DSL <sup>*</sup> ENVQEKWIAEVLHFCQGVPI	117	
<i>Ca</i> RHO1	94	DSL <sup>*</sup> DNV <sup>*</sup> TEKWI <sup>*</sup> SEVLHFCQGVPI	116	86 94
<i>Hs</i> RHOA	90	DSL <sup>*</sup> ENI <sup>*</sup> TEKWT <sup>*</sup> EV <sup>*</sup> HFC <sup>*</sup> PNVPI	112	69 73
<i>Sm</i> RHO1	91	DSL <sup>*</sup> FAN <sup>*</sup> TEKWT <sup>*</sup> ET <sup>*</sup> RHFC <sup>*</sup> PDVPI	113	56 73
		** * *** * *** **		

Fig. 4. BLASTP alignment of *S. cerevisiae* (*Sc*) Rho1  $\alpha$ 3-helix loop7 with the corresponding regions of *C. albicans* (*Ca*) Rho1, *Homo sapiens* (*Hs*) RhoA, and *S. mansoni* (*Sm*) Rho1 GTPases. Asterisks indicate identical amino acids, conservative amino acid changes are in light gray and non-conservative amino acid changes are in black. %I, identity; %S, similarity.

### 3.5. The phenotypic effects of the *smrho1*<sup>E97P</sup>, *smrho1*<sup>L101T</sup>, and *smrho1*<sup>E97P, L101T</sup> mutants in yeast

To test the importance of the proline 96 and threonine 100 residues for the conditional complementation of human *RHOA*, we used site directed mutagenesis to obtain *SMRH01* containing E97P, L101T, and E97P-L101T (the discrepancy in residue numbers is due to the serine insertion in *SMRH01* N-terminal). The three mutant *SMRH01* genes, the wild type *SMRH01* and the yeast *RHO1* genes were cloned into the pYTS plasmid under control of yeast Rho1 promoter. After transformation of these plasmids into *rho1* null mutant strain CMY, we examined their phenotypes in YNB-glucose plates, in which the galactose-inducible *SMRH01* overexpression was, thus, inhibited. CMY cells containing only the empty pYTS plasmid were unable to grow under any of the tested conditions, while the cells expressing *SMRH01* and *RHO1* wild-type genes under control of the *RHO1* promoter showed a normal growth even at 37 °C, as expected (Fig. 5). However, the *smrho1*<sup>E97P, L101T</sup> double mutant was unable to grow at 37 °C stress temperature without the presence of 0.5 M sorbitol as osmotic stabilizer (Figure 6 A and B). The single mutant *smrho1*<sup>E97P</sup> grew less well at 37 °C (Fig. 5A) but grew normally at this temperature in the presence of 0.5 M sorbitol (Fig. 5B). The other single mutant, *smrho1*<sup>L101T</sup>, showed less deleterious effects in growth when compared with wild type genes (Fig. 5A and B).

## 4. Discussion

We here describe, the characterization of the first Rho GTPase gene (*SMRH01*) isolated from *S. mansoni*, encoding a protein that is highly homologous to the Rho-type LMWGs of several species (Fig. 1A). The Rho-type LMWGs characteristic motifs [5,29,30] are conserved in *SMRH01*, except for the CAAX prenyla-

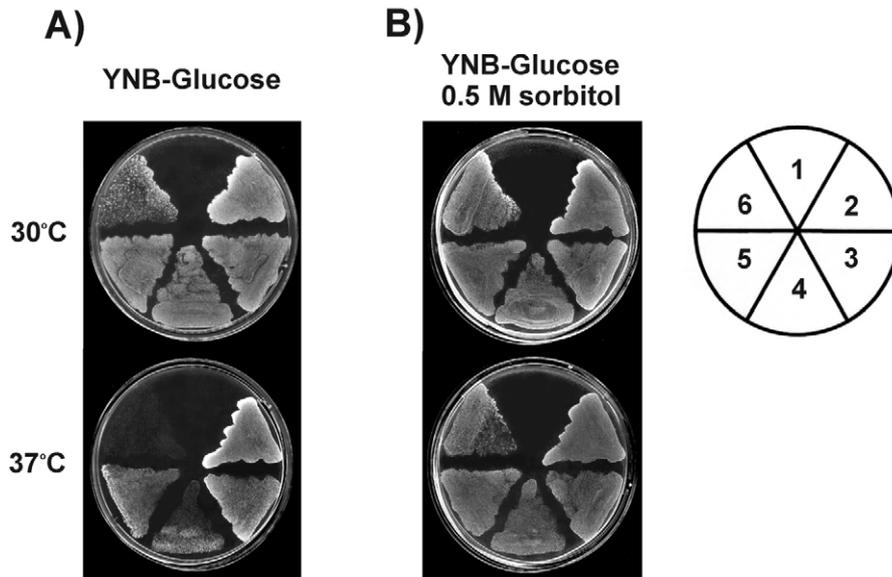


Fig. 5. Conditional complementation of the yeast *rho1* null mutant strain CMY by expression of *SMRH01* mutants under control of *RHO1* promoter. The CMY strain was transformed with the following plasmids (1) pYTS(vector), (2) pYTS1(*RHO1*), (3) pYTS2(*SMRH01*), (4) pYTS21(*smrho*<sup>E97P</sup>), (5) pYTS22(*smrho*<sup>L101T</sup>) and (6) pYTS23(*smrho*<sup>E97P, L101T</sup>). The picture shows the transformant growth after 3 days of incubation at 30 or 37 °C in (A) YNB plates containing glucose but not galactose, to suppress the *SMRH01* overexpression and (B) YNB-glucose plates supplemented with 0.5 M sorbitol as osmotic stabilizer.

tion domain that has a polar amino acid substituting the first aliphatic residue. This amino acid substitution may lead to the lack of prenylation in this protein. Further experiments need to be done to verify if there are other genes of this family coding for a protein with the conserved prenylation motif CAAX. Another peculiarity found in the *SMRH01* deduced amino acid sequence was the insertion of a serine residue close to its N-terminal, which has not been observed in any other Rho-type LMWGP (Fig. 1A). As a consequence of such sequence particularities, *SMRH01* occupies in an UP-GMA phenogram a isolated branch within the cluster of Rho-type GTPases already identified in other multicellular organisms (Fig. 1B).

Additionally, we demonstrated in this work that the *SMRH01* homology (62% identity) to *S. cerevisiae* *RHO1* is sufficient to complement yeast *RHO1* GTPase functions, even when overexpressed in a knock out strain (Fig. 2A). The complementation was adequate to permit survival and growth even under stress conditions such as in the presence of 300 mM CaCl<sub>2</sub> or at 37 °C (Fig. 2). In *S. cerevisiae*, *RHO1* is a regulatory protein required for cell cycle control [34] and also mediates bud growth by controlling polarization of the actin cytoskeleton and cell wall maintenance [35–37]. Consequently, *SMRH01* can apparently perform all these *RHO1* biological functions. However, we observed some phenotypic alterations, such as cell cycle arrest (Fig. 3) and cell enlargement (data not shown) caused by *SMRH01* overexpression in the *rho1* null mutant strain CMY similarly to those previously reported for yeast *RHO1* overexpression [31]. In contrast, the growth was appar-

ently normal when *SMRH01* was overexpressed in the wild-type strain BY4736 (Fig. 3), although enlarged cells were present yet (data not shown). Such results suggest a *RHO1* dominant effect over the *SMRH01* gene indicating that the *S. mansoni* GTPase is not able to perform perfectly all the *RHO1* gene functions. This can be explained by defective molecular interactions of the *SMRH01* with some of the distinct *RHO1* regulatory and effector proteins, due to differences existent among the heterologous and native GTPases.

The functional effect caused by amino acid sequence differences can be addressed by comparing the results of *SMRH01* and human *RHOA* complementation studies in *S. cerevisiae* *rho1* null mutants. In contrast to the complete complementation provided by *SMRH01*, human *RHOA* overexpression is lethal and its expression under control of the yeast *RHO1* promoter produces phenotypes sensitive to high temperatures such as 37 °C [20]. However, in the presence of 0.5 M sorbitol this temperature-sensitive phenotype was suppressed, providing the first evidence that one of the functions performed by the *S. cerevisiae* *RHO1* should be the maintenance of the yeast cell osmotic integrity [20]. Indeed, *RHO1* has been identified as an essential regulatory subunit of 1,3-β-glucan synthase, the multi-enzymatic complex that synthesizes 1,3-β-linked glucan, a major structural component of the yeast cell wall [35–37].

Analyses of human *RHOA*/yeast *RHO1* chimeras demonstrated that the α3-helix loop7 region of the *RHOA* molecule was determinant of the conditional complementation phenotype observed [20]. A simple

comparison of this region in *C. albicans* [33], human [20] and schistosome Rho GTPases led us to identify proline 96 and threonine 100 as candidate residues for the functional difference between human RHOA and SMRHO1 during heterologous expression in yeast (Fig. 4). The presence of a proline in position 96 of the human protein may determine a conformational alteration in the  $\alpha$ 3-helix, reducing its interaction capacity with effector molecules of *S. cerevisiae*. It is well-known that proline can disrupt  $\alpha$ -helices in water-soluble proteins [38,39] and, thus, influence the native structural conformation and protein function and interactions. For instance, the mutation of a glutamic acid to proline disrupts the interaction between cancer-related papillomavirus E6 protein and E6AP cellular protein, providing the first evidence that the E6-binding motif of the E6AP must be helical when bound to E6 [40]. Similarly to proline, threonine is also common in bend regions of kinked alpha helices [39]. The presence of a threonine residue in the position 100 of RHOA could, therefore, produce a conformational difference that would have functional repercussions.

Our results with the *smrho1*<sup>E97P</sup>, *smrho1*<sup>L101T</sup>, and *smrho1*<sup>E97P, L101T</sup> mutants confirmed the importance of proline 96 and threonine 100 for the temperature-sensitive phenotype seen in yeast *rho1* null mutant complemented by the human RHOA GTPase. This was most evident when we observed the phenotypic effect of complementation with the *smrho1*<sup>E97P</sup> and *smrho1*<sup>E97P, L101T</sup> mutants. The double mutation had a particularly drastic effect onto the capacity of cell wall synthesis regulation under high temperature (Fig. 5A), probably due to an important conformational alteration induced in the  $\alpha$ 3-helix. However, the single replacement of a leucine by a threonine at position 101 did not have such a severe phenotypic effect at 37 °C (Fig. 5A).

Recently, a set of high temperature-sensitive *RHO1* mutants were generated by random PCR and arbitrarily divided in two groups (*rho1A* and *rho1B*) according to their functional defects [41]. It is interesting to observe that the *smrho1*<sup>E97P</sup> and *smrho1*<sup>L101T</sup> mutants produced in our work are located near to the W104R and E102K mutations grouped as *rho1B*. This group is made up specifically of mutants that showed defects in activation of the 1,3- $\beta$ -glucan synthase, the RHO1 effector enzyme responsible for the yeast cell wall synthesis.

In conclusion, even though the Rho GTPases from different species are small molecules that share a high degree of homology, subtle amino acid differences in certain regions can determine their interaction specificity with the multiple regulatory and effector proteins of each organism. Hence, there seems to be a direct relationship between specific regions of Rho-type LMWGs and the different functions performed by these proteins. This is in good agreement with previous observations made on *S. cerevisiae* RHO1 [34,41]. The

SMRHO1 gene may, thus, provided an attractive target for the development of new antischistosomiasis drugs, in spite of its high sequence identity with human *RHOA* gene.

The Schistosome Genome Project has already generated sequences of many genes and requires intensive efforts on functional genomics [2]. Nevertheless, such endeavor can be restricted by the technical difficulties of parasite genetic manipulation. The use of *S. cerevisiae* and other model organisms constitutes a powerful tool for investigation of Schistosome gene function, as demonstrated in this work.

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