TcRho1: a Farnesylated Rho family homologue from *Trypanosoma cruzi*

CLONING, TRANS-SPlicing AND PRENYLATION STUDIES

José L. Nepomuceno-Silva¹,²,⁵, Kohei Yokoyama²,⁵, Luiz D.M. Barbosa¹, Sérgio M. Mendonça¹, Júlio C. Paixão¹, Rudi Baron⁴, Jean-Charles Faye⁴, Frederick S. Buckner³, Wesley C. Van Voorhis³, Michael H. Gelb²*, and Ulisses G. Lopes¹*.

¹Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

Departments of Chemistry², Biochemistry², and Medicine³, University of Washington, Seattle, Washington 98195

⁴INSERM U397, ICR 20-24 rue du pont Saint-Pierre, 31052 Toulouse cedex, FRANCE

⁵These two authors contributed equally to this study.

*Address correspondence to Ulisses G. Lopes, Laboratório de Parasitologia Molecular, IBCCF, Universidade Federal do Rio de Janeiro, CCS, Cidade Universitária, RJ, 21949, Brazil; 55 21 012 562 6540, lopesu@biof.ufrj.br

or Michael H. Gelb, Depts. of Chemistry and Biochemistry, Univ. of Washington, Seattle, WA 98195; 206 543 7142, gelb@chem.washington.edu
Abbreviations:

CaaX, carboxy-terminal signal sequence of PFT and PGGT-I substrates (C is cysteine, a is usually, but not always an aliphatic residue, and X is a variety of residues); FPP, farnesyl pyrophosphate; GST, glutathione S-transferase; GGPP, geranylgeranyl pyrophosphate; PFT, protein farnesyltransferase; PGGT-I, protein geranylgeranyltransferase-I; RT-PCR, reverse transcribed-polymerase chain reaction; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; T. cruzi, Trypanosoma cruzi; UTR, untranslated region.
Abstract:

Rho GTPases are members of the Ras superfamily and are involved in signal transduction pathways including maintenance of cell morphology and motility, cell cycle progression, and transcriptional activation. We report the molecular identification in trypanosomatids (*Trypanosoma cruzi*) of the first member of the Rho family. The cloned Rho protein, TcRho1 shares ~40% homology with other members of the Rho family. Southern blot analysis reveals that TcRho1 is a single copy gene per haploid genome, and northern blot assays shows a transcript of 1200 nt in length. Mapping the 5’ untranslated region of TcRho1 transcripts revealed at least five different transcripts derived from differential trans-splicing. Three of the five transcripts contain the trans-splicing site within the coding region of the TcRho1 gene. TcRho1 also contains the C-terminus sequence CQLF (CaaX motif) which is predicted to direct post-translation prenylation of the cysteine residue. A synthetic peptide containing this C-terminus motif, when tested against Q-Sepharose chromatography fractions from *T. cruzi* cytosol, was shown to be efficiently farnesylated but not geranylgeranylated, despite the fact that the CaaX motif with X = F specifies geranylgeranylation by mammalian protein geranylgeranyltransferase-I. Furthermore, immunoblot analyses of epimastigote protein with anti-S-farnesyl-cysteine methyl ester and anti-TcRho1 antisera strongly suggest that TcRho1 is farnesylated in vivo. The farnesylation of proteins such as Rho GTPases could be the basis for the selective cytotoxic action of protein farnesyltransferase inhibitors on trypanosomatids versus mammalian cells.
Introduction

Hydrolysis of GTP to GDP by GTPases functions as a molecular timing mechanism in biological signaling networks (1). The Ras superfamily of small GTPases encompasses several related protein families, whose members are involved in regulation of a diverse set of cellular events (2). Members of this superfamily are ubiquitously found in all branches of eukaryotic lineage. The Rho family of small GTPases is being intensely studied in mammalian cells, due to the critical role of these proteins in maintaining cellular morphology by coordinating the dynamic remodeling of the actin cytoskeleton (3). Other cellular processes under Rho family control are signaling pathways that lead to activation of some transcription factors (4, 5), the control of cell cycle progression (6), and the activation of the NADPH oxidase complex (7). Rho proteins have been found in animals (8), plants (9), fungi (10) and protozoan including *Entamoeba histolytica* (11). However, proteins belonging to this family have not yet been described in deeper lineages of lower eukaryotes.

Amino acid sequences of Ras superfamily GTPases contain five conserved blocks, named G1 to G5, which are essential for GTP binding and hydrolysis (12). These regions are brought together in the globular tertiary structure, forming a cleft where GTP binds (12). Many of these proteins have a hypervariable C-terminus that extends away from the globular core and terminates in a so-called CaaX box (C is cysteine, a is usually but not necessarily an aliphatic amino acid, and X is a variety of different amino acids). The CaaX box serves as a signal for a series of post-translational modifications: 1) farnesylation or geranylgeranylation of the cysteine SH; 2) endoproteolytic removal of aaX; 3) and methylation of the α-carboxyl group of the prenylated cysteine residue. The hydrophobic C-termini of Ras superfamily GTPases are thought to be important for anchoring these proteins to cellular membranes (13, 14). In mammalian cells, farnesylation of CaaX (X = serine, methionine and other residues)
is carried out by protein farnesyltransferase (PFT), whereas protein geranylgeranyltransferase-I (PGGT-I), geranylgeranylates the CaaX when X = leucine or phenylalanine (14).

The family Trypanosomatidae is comprised of obligate protozoan parasites, some of pivotal medical and economic interest. *Trypanosoma cruzi* is the causative agent of Chagas disease, which affects about 17 million people in the American continent (15). Development of new drugs against pathogenic trypanosomatids is needed, thus requiring the characterization of novel potential drug targets. Among them, compounds that impair the function of GTPases, such as PFT inhibitors, are very promising therapeutic alternatives (16).

*T. cruzi* has a digenetic life cycle involving insect and vertebrate hosts. During this cycle, parasites undergo morphological and physiological changes due to different microenvironment stimuli that occur in the insect digestive tract and in the vertebrate host (17). The regulation of such cellular events, which involve cell division, differentiation and host cell invasion, is not well understood. Identifying key molecular regulators in *T. cruzi* is critical for the development of new approaches to control and treat Chagas disease.

Some Ras superfamily proteins have been described in trypanosomatids (18) and functional studies of Rab GTPases in *T. brucei* and *T. cruzi*, reveal similarities between their roles in vesicle trafficking of lower and higher eukaryotes (19-25). Studies in *T. brucei* also reveal an ancestral Ras family protein, which seems to fit in an intermediate position between the Ras and Rap subfamilies (26). Other GTPases from the Ran and Arf families have been found in trypanosomes and *Leishmania* species (18, 27-30). Here we report the characterization of TcRho1, the first Rho family GTPase encoding gene described in *T. cruzi*. As far as we know, this is the most ancestral Rho family sequence found in the eukaryotic lineage.
We have recently shown that inhibitors of trypanosomatid PFTs are much more cytotoxic to *T. brucei*, *T. cruzi*, and *Leishmania mexicana amazonensis* than to mammalian cells (16, 31). The molecular basis for this difference is not known. We have purified *T. brucei* PFT and cloned its α- and β-subunits cloned (32,33). However, no significant PGGT-I activity was detected when *T. brucei* lysate was submitted to ion-exchange chromatography and fractions were assayed using typical mammalian PGGT-I substrates. PFT and PGGT-I share a common α-subunit but have distinct β-subunits. We have not been able to detect the β-subunit of PGGT-I by TBLASTN searching of trypanosomatid genomic databases even though the shotgun coverage of the *T. brucei* genome is currently at 1.5X. These results suggest that proteins which are modified by a single geranylgeranyl chain in mammalian cells may be farnesylated in trypanosomatids. This could explain the selective toxicity of PFT inhibitors to these parasites. Thus, in the present study we also report our results on the prenylation of TcRho1 by protein prenyltransferase present in *T. cruzi* lysates.

**Experimental procedures**

*Parasites.* *T.cruzi* epimastigotes, Dm28c clone and Tulahuen strain, were kindly provided by Dr. S. Goldenberg (DBBM, Fiocruz, Brazil) and Dr. S. Reed (Infectious Diseases Research Institute, Seattle, WA), respectively. Cells were maintained at 28 °C in Liver Infusion Tryptone (LIT) medium (34), supplemented with 10% heat-inactivated fetal calf serum (*Gibco/Brl*) and 0.025 µg/mL hemin (*Sigma*). Metacyclogenesis and purification of Dm28c metacyclic trypomastigotes was performed in TAU-3AAG medium, as described (34).

*Southern and northern blots.* Genomic DNA was prepared from 10⁹ Dm28c epimastigotes. Cells were collected by centrifugation and incubated in 0.5% SDS, 20 µg/mL RNase A and 100 µg/mL proteinase K, at 56 °C for two hours. DNA was extracted using the phenol-chloroform extraction method (36). Five micrograms of genomic DNA was digested with
EcoRI, BamHI, SalI, HindIII and PstI (New England Biolabs). The digested samples were resolved on an 0.8% agarose gel. After electrophoresis, DNA was denatured in 0.5 N NaOH, neutralized, transferred onto nitrocellulose membranes by capillarity through a 20X standard saline citrate solution (3 M NaCl, 0.3M Sodium citrate - SSC) and UV cross-linked (120,000 µJ/cm²) using a UV cross-linker chamber (Ultralum).

Total RNA was prepared from approximately 10^9 cells (99% epimastigotes), according to the methodology described by Perry et al.(37). Poly(A)+ RNA was purified from total RNA by oligo(dT) chromatography, using the QuickPrep mRNA Purification Kit (Amershan Pharmacia Biotech). Twenty micrograms of total RNA and 200 ng of poly(A)+RNA were separated on a 1.5% agarose/formaldehyde gel, blotted onto a nitrocellulose membrane by capillary transfer and UV cross-linked as described above for Southern blots.

Before hybridization, nitrocellulose membranes were blocked during three hours at 42°C in solution containing 50% (v/v) formamide, 5X SSC, 5X Denhardt's solution (1% (w/v) ficoll, 1% (w/v) polyvinylpyrrolidone 1% (w/v) bovine serum albumine), 0.1% (w/v) SDS, 50 mM phosphate buffer (pH 7.0) and 100 µg/mL of denatured salmon sperm DNA. Probes were radiolabeled with the random priming DNA labeling method (38), using either [α-32P]dATP or [α-32P]dCTP (Amershan Pharmacia Biotech). Hybridizations were carried out overnight, at 42°C, in blocking solution containing 10^6 cpm/mL of denatured probe. After hybridization, membranes were washed three times with 0.1X SSC, 0.5% SDS at 55°C and autoradiographed.

Genomic library screening. An λEMBL3 Dm28c genomic library was kindly provided by Dr. W. Degrave (DBBM, Fiocruz, Brazil) and propagated in the KW252 E. coli strain. Approximately 60,000 independent recombinant phages were screened with the [α-32P]dATP labeled pTcrho probe (described in the results section). We used two membrane replicates.
for each hybridization, and plugs containing positive plaques in both of them were selected and submitted to secondary and tertiary screens. After three rounds of selection, three phage clones giving positive hybridization signals were selected. One of them, named $\lambda TcRho1$, was selected for a characterization of TcRho1.

**Subcloning of the TcRho1 coding region.** The $\lambda TcRho1$ clone was amplified in the LE392 E. coli strain. DNA from this clone was extracted and purified as described elsewhere (36) and was submitted to Southern blot analysis as described above. A 4.0 Kb EcoRI fragment was selected as an initial target for cloning. Ligation of EcoRI digested genomic cloned DNA with EcoRI digested and 5’ dephosphorylated pKS-II+ Bluescript (Stratagene) followed by transformation of XL1-Blue E. coli, generated several recombinant clones. Plasmid DNA from these clones was extracted as described elsewhere (36), and those containing cloned fragments in the range of 4.0 Kb were selected, and their 5’ and 3’ ends were sequenced. As the TcRho1 coding region was not fully contained in the EcoRI fragment, we further subcloned a 300 bp KpnI fragment from $\lambda TcRho1$ containing part of the 3’ coding region of TcRho1. This clone was radiolabeled and used as a probe to clone a 1.3 Kb EcoRI/PstI fragment from $\lambda TcRho1$ encompassing the remaining TcRho1 sequence.

**Sequence analysis of TcRho1.** Subcloned fragments in pKS-II+ were sequenced using different primers by the dideoxy chain termination method (39) using the T7 Sequencing kit (Amershan Pharmacia Biotech). We used the commercially available T3 and T7 sequencing primers and also three sequencing primers based on the TcRho1 sequence: G2

$$5'CGGAATTCCGGTACCCCGCC3'$$

G4: $$5'CGGTGGGATGACC3'$$

G5:

$$5'CGGTTGGTACATGCAGC3'$$

**Mapping the 5’UTR of TcRho1.** For mapping the TcRho1 5’ UTR and for locating the trans-splicing acceptor sites, we carried out mini-exon, semi-nested RT-PCR against *T. cruzi* RNA. One primer was directed to the mini-exon sequence (ME:...
GGATGGAATTCAGTTTCTGTACTATATTG, kindly provided by Dr. T. Urmeni, IBCCF, UFRJ, Brazil), and the other two primers were directed to sequences within the TcRho1 coding region (G2 \(5^\prime\) CGGAATTCCGGTACCCCGCC\(3^\prime\), and G3: \(5^\prime\) AACTGCAGACCGCAACCCCTTTCA-TTG\(3^\prime\)). Initially 5 µg of total epimastigote RNA was submitted to first strand cDNA synthesis using the SuperScriptII Preamplification System (Gibco/BRL), performed with random hexamers as primers, according to manufacturer's suggested procedures. The first PCR reaction was carried out in the presence of 0.5 mM dNTPs, 1.5 mM MgCl\(_2\), 10 µM of each primer and 5 units of Taq DNA polymerase (Gibco/BRL). To avoid problems derived from differences in primer melting temperatures, a touch-down PCR program was used by decreasing the annealing temperature from 75 °C to 57 °C in 1 °C steps. Then, 20 additional cycles were performed at 93 °C for denaturing, 55 °C for annealing and 72 °C for extension followed by a 10 minutes extension step. On tenth of this first reaction was used as template in the second reaction with the same conditions, but with a conventional thermocycler program (30 cycles consisting of 93 °C for denaturing, 55 °C for annealing and 72 °C for extension, followed by a 10 min extension step). Products of both reactions were resolved on a 2.5% agarose gel. Sites for EcoRI were present in the mini-exon and G2 primers in order to allow ligation of products from the second reaction into pKS-II+. Ligated products were introduced in E. coli XL1-Blue, amplified and sequenced, allowing an accurate mapping of TcRho1 trans-splicing acceptor sites.

Expression of TcRho1. The coding region of TcRho1 was amplified by PCR using 100 ng of total T. cruzi DNA using the primers Terhostart (\(5^\prime\) CGGGATCCTCACAATGGAGGAGACACTG\(3^\prime\)) and Terhoend (\(5^\prime\) CGGGATCCATCAAAAAAGTTGACAGCTCTGTC\(3^\prime\)), both containing BamHI cloning sites. The PCR program consisted of 30 conventional cycles (93 °C for denaturing, 55 °C for
annealing and 72 °C for extension) followed by a 10 min extension step. The 850 bp amplified fragment was cloned in frame with the GST gene in the pGEX-3X vector (Amersham-Pharmacia Biotech), and the resulting construct was used to transform the BL21 E. coli strain. Expression of the recombinant protein was induced with isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.5 mM after cultures reached an A$_{600}$ of ~0.8. Cultures were then maintained for one hr, cells were pelleted, washed twice in phosphate-buffered saline, and the cells lysed by sonication with a Branson sonicator (ten 10 sec pulses interrupted by cooling on ice). The fusion protein was recovered from inclusion bodies using the urea solubilization protocol previous described for the Ras protein (40). Purification of the fusion protein was accomplished by glutathione-sepharose chromatography according to the manufacturer's suggested procedure. The purified protein was cleaved with factor Xa (Amersham Pharmacia Biotech) at 10 U/mg fusion protein to release TcRho1 protein from the GST tag. Protein yield was measured by the Bradford quantification method (41). Preparations were analyzed for purity by 12% SDS-PAGE.

T. cruzi transfection. As described in the Results Section, we desired a strain of T. cruzi that overexpresses a mutant TcRho1 that cannot be prenylated. We had available a mutant of TcRho1 in which the C-terminal CQLF is replaced with FNFFDFA, and this mutant DNA fragment was used to construct the vector for overexpression of mutant TcRho1 in T. cruzi. Overexpression was performed using the T. cruzi expression vector pBS:IL2-CnFc (42). The IL2 encoding insert was excised from the vector with BamHI and replaced with the TcRho1 ORF flanked by BamHI sites. A clone with properly oriented insert was identified, and 5 µg of supercoiled DNA was electroporated into Tulahuen epimastigotes as previously described (42). Transfectants were selected and expanded in 500 µg/ml of G418.

Immunoblotting. Antiserum to TcRho1 was raised in a rabbit against the KLH-conjugated peptide NDNGVVDTSNKQSIEL, present in the C-terminal hypervariable region.
Antiserum was submitted to affinity purification using the resin prepared by reacting the same peptide used for immunization with CNBr-activated Sepharose 4 Fast Flow (Amersham Pharmacia Biotech). The gel was sequentially washed with 10 volumes of the following buffers: 10 mM Tris, pH 7.5; 100 mM glycine, pH 2.5; 10 mM Tris, pH 8.8; freshly prepared 100 mM triethylamine, pH 11.5. Finally, the gel was washed with 10 mM Tris, pH 7.5 until the pH reached 7.5. Antiserum was diluted 10-fold with 10 mM Tris, pH 7.5 and passed through the column 4 times. The column was washed with 20 volumes of 10 mM Tris, pH 7.5 followed by 500 mM NaCl in 10 mM Tris, pH 7.5 until the OD280 reached a minimum. Antibodies were eluted with 10 volumes of 100 mM glycine, pH 2.5 and collecting eluant in 1 M Tris, pH 8.0 for neutralization. The material was dialyzed against 5 mM NaCl and lyophilized.

Tulahuen strain epimastigote cells (log phase) were pelleted in a microfuge tube. The supernatant was removed, the cell pellet was treated with Laemmlı sample buffer at 42 °C for 30 min, and sample from 10⁷ cells was loaded onto a single lane of a 12.5% Laemmli SDS-PAGE gel. Proteins were electrophoretically transferred to nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech). The membrane were blocked in 5% nonfat powdered milk in Tris-buffered saline containing 1% Tween 20 (TBST). The blot was incubated for two hours either with 1:2000 affinity purified anti-TcRho1 or with 1:2000 anti-S-farnesyl cysteine methyl ester antiserum (43) at room temperature. After washing, membranes were incubated for 1 hour with 1:1000 dilution of horseradish peroxidase-linked anti rabbit IgG and subjected to enhanced chemiluminescence detection (ECL, Amersham Pharmacia Biotech), according to the manufacturer’s instructions.

Partial purification of T. cruzi PFT. T. cruzi Tulahuen epimastigotes from a 1 liter culture (5 x 10⁹ cells) were collected, washed once in PBS and suspended in 1 mM Tris-HCl, 1 mM DTT, 1mM EDTA (pH 8.0), and freshly added protease inhibitors (1 mM...
phenylmethylsulfonyl fluoride, 30 µM each of tosyl-lysine chloromethylketone and tosyl-phenylalanine chloromethylketone and 10 µg/mL each of aprotinin, leupeptin and pepstatin A). The cells were lysed by sonication with a Branson sonicator (ten 5 sec pulses interrupted by cooling on ice). The lysate was diluted to 26 mL and supplemented with the following components to the indicated concentrations: 20 mM Tris-HCl, pH 8.0, 5 mM DTT, 5 µM ZnCl₂. This mixture was centrifuged at 120 000 g for 1 h 20 min at 4 °C, and the resulting supernatant (containing 0.854 mg/mL protein, measured by the Bradford assay) was subjected to protein precipitation with 60% saturated ammonium sulfate at 0 °C. Proteins were collected by centrifugation, and the pellet was dialyzed against ice-cold buffer A (20 mM Tris HCl, pH 8.0, 50 mM NaCl, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride) at 4 °C (3 exchanges, 2 liters each).

The resultant protein solution was loaded onto a column (1 x 8 cm) of Q-Sepharose Fast Flow (Amershan Pharmacia Biotech), previously equilibrated with buffer A. The column was washed with buffer A at a flow rate of 0.5 mL/min for 80 min, and then a gradient of buffer A and buffer B (same as buffer A, but containing 1 M NaCl) was applied as follows: 0 to 4 min, 0 to 15% buffer B, 4 to 124 min, 15 to 55% buffer B, 124 to 128 min, 55 to 100% buffer B, 128 to 152 min, 100% buffer B. Fractions of 1 mL were collected, and the protein elution profile was monitored by measuring the absorbance at 280 nm.

Assay of prenyltransferase activity was carried out by incubating 4 µL of Q-Sepharose fractions at 30 °C for 30 min with 5 µM biotin-QSCQLF (C-terminal peptide of TcRho1, prepared by United Biochemical Research) with 1 µM [³H]FPP (0.3 µCi) or 1 µM [³H] GGPP (0.3 µCi) (both from American Radiolabeled Chemicals) in 20 µL of buffer (30 mM potassium phosphate, 0.5 mM MgCl₂, 20 µM ZnCl₂, 5 mM DTT, pH 7.7). The amount of radiolabeled prenylated peptide was quantified using the avidin-agarose method as
described (44). Fractions were also assayed for PFT activity with 5 µM of recombinant RAS1-CVIM (a generous gift from Dr. C. Omer, Merck) and for PGGT-I activity with 5 µM Ras-CVLL (a generous gift from Prof. G. James, University of Texas), using the glass fiber method (45).

Radiolabeling. For radiolabeling studies, $10^7$ T. cruzi epimastigotes (Tulahuen strain) were cultured for 24 hours in 1 mL of LIT medium containing 100 µCi (1.6 µM) [³H]mevalonolactone (American Radiolabeled Chemicals) and 300 µM simvastatin. Cellular protein was delipidated and submitted to SDS-PAGE as described (16). In some experiments, the PFT inhibitor JJ23 (to be published) was present.

Results

Genomic Organization of TcRho1. We have characterized several cloned RT-PCR fragments amplified from T. cruzi RNA which share homology in their predicted peptide sequences with several Ras superfamily genes (unpublished results). These products were obtained by degenerated RT-PCR, using a primer directed to the mini-exon sequence and a degenerated primer directed to the G3 conserved GTPase domain (DTAGQE). One of the obtained fragments, named pTcrho, shares about 40% similarity with several members of the Rho family proteins. We used pTcrho as a homologous probe in order to characterize TcRho1, a Rho family gene from T. cruzi.

Genomic DNA from Dm28c epimastigotes was digested with several restriction enzymes and probed with the pTcrho labeled fragment. Southern blot analysis revealed single bands, suggesting that TcRho1 is present as a single copy gene in the Dm28c haploid genome (Figure 1A). This pattern resembles other characterized trypanosome GTPase genes, reinforcing the hypothesis of preferential organization of small GTPases in trypanosomes as single copy genes (18).
Cloning and sequence analysis of the TcRho1 gene. The genomic clone \( \lambda \)TcRho1 was obtained from a Dm28c genomic library and was used for subcloning of three TcRho1 overlapping fragments in pKS-II+. Sequencing of a 1021 bp region through three subclones revealed an open reading frame of 831 bp for the TcRho1 gene, coding for a 277 amino acid protein with predicted molecular weight of 30,979 Da (Figure 2). The coding region is 51.9% GC, which is consistent with the average GC content of 56% found in \( T. cruzi \) genes (46). The nucleotides around the ATG initiation codon at +1 (ATCACAA\(^{+1}\)TGG) are very close to the optimum initiation sequence (GCCA/GCCA\(^{+1}\)TGG) described by Kozak (47), showing conservative substitutions at -5 and -6 and identical bases at +4, -2, -3, -4.

Homology searching was performed against the SWISSPROT database, using the FASTA program, from the Genetics Computer Group Wisconsin Sequence Analysis Software Package (GCG) (48). The top scoring matches are proteins from the Rho family. The top 7 matches were selected and aligned with TcRho1 by the GAP program (GCG). The highest identities and similarities levels are shown in Table 1.

Sequence database searching using the tBLASTn algorithm (49) against ESTs from diverse organisms detected a \( T. cruzi \) EST (28j18 clone, accession number AI075525) sharing 100% identity with TcRho1. This clone spans part of the 3’ region of TcRho1. A multiple alignment of TcRho1 and its closest homologues was performed using the CLUSTAL X program (50) (Figure 3). As noted for many cloned sequences from trypanosomatids (27, 33), TcRho1 contains insertions, thus accounting for its increased molecular weight when compared with other proteins of the Rho family. The conserved G1-5 domains required for GTP binding and hydrolysis were found in the TcRho1 sequence. The consensus sequence in the G5 domain of TcRho1 (TCSSK) differs from that found in most Rho family sequences, in which the residue in the second serine position is an alanine. However, we have found that Rho family members identified in plants have a serine instead of an alanine residue. The
asparagine residue in the G2 domain (Asn 46), a hallmark of proteins from this family and the target for ADP ribosylation catalyzed by C3 botulinic toxin, is also conserved. An unusual insertion in the carboxyl terminal variable region is present in TcRho1, and it is followed by a CaaX motif, indicating that TcRho1 is a potential target for post-translational modification by trypanosomal protein prenyltransferases.

Phylogenetic analyses were conducted using the MEGA program package (version 2.0) (51). When compared with other Ras superfamily proteins, using the Neighbor-Joining algorithm (52), TcRho1 diverges within the Rho family branch, with a 98.9% bootstrapping value, providing a reasonable degree of confidence for identifying TcRho1 as a member of the Rho family (Figure 4). Phylogenetic analysis using parsimony and distance algorithms produced similar tree branching patterns. Interestingly, TcRho1 apparently diverges before Rho family branching into the Rho and Rac/Cdc42 subfamilies. Comparing TcRho1 sequence with several Rho sequences and Rac/Cdc42 sequences, we find at least four different positions that are conserved only in Rho group proteins (F-26, E-45, D-130 e R-219) and another four positions that are conserved only in the Rac/Cdc42 group (V-17, E/D-38, F-123 e T-172). These findings, together with the phylogenetic branching, suggests that TcRho1 is probably an ancestral Rho family member, arisen before Rho family division in the two subgroups.

**RNA analysis.** Northern blot analysis using up to 20 μg of total RNA shows that the TcRho1 transcript is not abundant in epimastigotes, but a diffuse band around 1200 bp is evident when 200 ng of poly(A)+ RNA was loaded on the gel (Figure 1B).

We mapped the 5’untranslated region of TcRho1 mRNA using a mini-exon semi-nested RT-PCR approach with a sense oligonucleotide directed to the mini-exon and antisense oligonucleotides directed next to the G3 region (in the first reaction) and to the G2 region (in the second reaction). Electrophoresis of PCR products revealed three major bands,
corresponding in size to the two splice-leader acceptor sites in the 5’UTR of TcRho1 mRNA (Figure 5A). These bands were not seen in a negative control using epimastigote RNA not submitted to reverse transcription (not shown). White arrows indicate the specific products generated in the reaction. PCR products were further cloned and sequenced, showing that the two largest fragments indeed correspond to the acceptor sites located at -85 and -39 in the TcRho1 5’UTR. Three other transcripts were sequenced and these corresponded to splice acceptor sites within TcRho1 coding sequence, at positions +6, +9, and +25 (Figure 5B and 5C). The significance of these products is obscure, as they are not translatable into TcRho1 protein.

Prenylation studies. TcRho1 contains the C-terminal CaaX motif, CQLF, suggesting that this protein is post-translationally modified with either a farnesyl or geranylgeranyl group as observed with mammalian and yeast homologues of Rho family GTPases. Mammalian proteins containing the C-terminal CaaF motif have been shown to be preferentially geranylgeranylated in in vitro assays (44). To examine whether TcRho1 is farnesylated or geranylgeranylated, we carried out a prenyltransferase assay using the N-terminally biotinylated peptide corresponding to the C-terminus of TcRho1, biotin-QSCQLF as a prenyl group acceptor substrate and fractionated cytosolic T. cruzi epimastigote proteins as a source of T. cruzi protein prenyltransferases.

The 0-60% ammonium sulfate precipitate of cytosolic proteins from Tulahuen strain epimastigotes was fractionated by Q-Sepharose chromatography. As observed with T. brucei cytosolic fraction (53), a single peak of PFT activity was detected with RAS-CVIM/[3H]FPP as substrates, indicating the existence of PFT in T. cruzi (Figure 6). No significant PGGT-I activity was detected in these fractions when tested with substrates of mammalian PGGT-I (Ras-CVLL/[3H]GGPP). The biotinylated C-terminal peptide of TcRho1, biotin-QSCQLF, was efficiently farnesylated in the same fractions that contained the enzyme activity
farnesylating RAS-CVIM. In contrast, geranylgeranylation of biotin-QSCQLF in the presence of \[^{3}H\]GGPP could not be detected in any of the Q-Sepharose fractions. The level of *T. cruzi* PFT activity measured with biotin-QSCQLF was about two fold higher than that measured with RAS-CVIM; the latter is one of the best substrates for trypansomatid PFT found to date. These results suggest that TcRho1, which possess the C-terminal CQLF motif, is farnesylated by PFT in *T. cruzi*.

In order to examine the type of prenyl group attached to TcRho1 in vivo, we used a recently described antiserum that recognizes S-farnesyl-cysteine methyl ester but fails to recognize the S-geranylgeranylated compound (43). This immunological method was used because of the impracticality of obtaining sufficient amounts of native TcRho1 from *T. cruzi* for direct prenyl group structure determination by radiometric or mass spectrometric methods (54) (RNA analysis described above suggests that TcRho1 is present at low levels in epimastigotes). For these experiments, we prepared a stable *T. cruzi* transfectant that overexpresses a mutant TcRho1 that cannot be prenylated (CQLF replaced with FNFFDFA, already available in our lab as described in Methods). This mutant protein serves as a gel position marker of TcRho1 from whole parasites and also serves to confirm the specificity of the anti-S-farnesyl-cysteine methyl ester for the farnesyl portion of TcRho1.

As shown in Figure 7, the immunoblot using anti-TcRho1 antiserum detects a protein band from whole parasites that co-migrates with recombinant TcRho1 produced in *E. coli*. The observed apparent MW for these bands is ~39-kDa (predicted MW 31-kDa). The immunoblot from parasites that overexpress the TcRho1 mutant (Figure 7) shows a ~10-fold increase compared to non-transfected parasites in amount of protein detected at the ~39-kDa position, thus supporting the assignment of this band as TcRho1. As shown in Figure 7, the immunoblot analysis of non-transfected parasites with the anti-S-farnesyl-cysteine methyl ester antiserum clearly shows a band at ~39-kDa, which co-migrates with the band detected
with anti-TcRho1 antiserum. The intensity of this band did not increase when transfected parasites were analyzed with the anti-S-farnesyl-cysteine methyl ester antiserum (Figure 7). This latter result shows that the farnesyl group but not the protein component of wild type, endogenous TcRho1 is being detected. These immunological studies strongly support the farnesylation of TcRho1 in vivo, which is consistent with the in vitro data with T. cruzi PFT.

As shown in Figure 8, immunoblot analysis of T. cruzi proteins with the anti-S-farnesyl-cysteine methyl ester antiserum reveals several bands in the 39-80 kDa range. Similar sizes of radiolabeled proteins are seen in the fluorograph of proteins from T. cruzi that was grown in the presence of [3H]mevalonolactone to label their prenyl groups (Figure 8). The fluorograph shows that the most intense radiolabeled proteins are in the range 25-33 kDa. These are not detected with the anti-farnesyl-cysteine methyl ester antibody, suggesting that they are not farnesylated. Trypanosomatids are known to contain several Rab GTPases (18), which are likely to be geranylgeranylated like their mammalian homologues. Treatment of T. cruzi with the PFT inhibitor JJ23 caused a decrease in the radiolabeling of specific proteins with MW > 34 kDa, with less effect on the amount of tritium incorporated into the 25-33 kDa proteins [similar to the pattern seen with T. brucei (16)]. This result further supports the proposed geranylgeranylation of most of the 25-33 kDa proteins. All together, the results suggest that the anti-farnesyl-cysteine methyl ester antiserum detects farnesylated but not geranylgeranylated proteins in T. cruzi, as shown previously with mammalian cells (43).

**Discussion**

TcRho1 is the first Rho family member from trypanosomatids to be identified, albeit some of Ras superfamily genes have been cloned in these organisms (18). TcRho1 has conserved GTPase motifs and a C-terminus CaaX motif that is a target for post-translational prenylation. Phylogenetic analysis shows that Tchro1 clearly belongs to the Rho family clade...
of GTP binding proteins, however, it does not seem to branch within Rho or Rac/Cdc42 subgroups, apparently having diverged from the clade before the division between Rho and Rac/Cdc42 proteins. As trypanosomatids are believed to have branched early on eukaryotic evolution, this GTPase may be an ancestral Rho family member of higher organisms. Another monomeric GTPase protein, the Ras/Rap protein found in *T. brucei*, also branched in a similar way (27).

Interestingly, five trans-splicing sites were mapped in TcRho1 mRNA, three of them lying inside the coding region (Figure 5). As far as we know, these are the first naturally occurring trans-splicing sites found inside a coding sequence, although mutation induced trans-splicing in a coding region has been described (55). There is another open reading frame downstream of the internal trans-splicing sites. Initiation at the first ATG downstream of the spliced leader sites interior to the ORF predicts a small protein of 54 amino acids, and no significant homology was detected to this putative protein in sequence databases. Poly-pyrimidine tracks in *T. cruzi* RNA are thought to regulate trans-splicing of RNA (56). The TcRho1 5’ UTR has two poly-pyrimidine tracks. The upstream track may direct trans-splicing to the two “functional” splice sites, and the other small tract may direct trans-splicing to the downstream sites. As gene expression in trypanosomatids relies mainly on post-transcriptional events (57), and the transcription rates of most genes do not seem to undergo drastic changes (57), as observed for higher eukaryotes, the production of truncated and untranslated RNA molecules may be a way to reduce protein production. It would be interesting to investigate whether the parasite is able to alter the ratio of trans-splicing to the upstream sites versus the downstream sites, in order to modulate TcRho1 expression. There is no apparent difference in the trans-splicing profile of TcRho1 RNA in epimastigotes, metacyclic trypomastigotes, and in amastigotes (data not shown), although other conditions were not tested such as heat shock stress or reduced pH.
Rho proteins have been shown to be pivotal regulators of actin cytoskeletal remodeling in mammals and in yeast (3). Trypanosomatids, however, do not contain any obvious microfilamentous structures. Although these protozoa have conserved actin genes and also proteins related to actin, such as profilin and spectrin (58-63), all attempts to highlight F-actin in these organisms have been unsuccessful (64, 65). The actin-myosin system is believed to play an important (but still unknown) role in parasite physiology. Mammalian Rho proteins have also been shown to be involved in the control of signaling pathways leading to activation of transcription factors. It is difficult to assume a similar role for TcRho1, since the unusual transcription machinery of trypanosomatids seems to be under modest control, with genes lacking promoter sequences and defined transcription initiation sites. Wiese (1998) hypothesized that the traditional signaling pathways leading to transcription activation in trypanosomatids could be shifted to the regulation of post-transcriptional events, such as trans-splicing, mRNA stability, and translation (66). It would be interesting to verify whether TcRho1 is an upstream regulator of such signaling pathways.

Modification of protein by farnesyl or geranylgeranyl groups has been shown to be indispensable for membrane targeting and cellular functioning of many GTPases in mammals and yeast. The mevalonate pathway, which provides precursors for prenyl groups, has been identified in trypanosomatids. HMG-CoA reductase has been characterized in T. brucei and T. cruzi (67, 68). Incubation of trypanosomatids with radiolabeled mevalonate in the presence of HMG-CoA reductase inhibitors leads to metabolic labeling of a collection of proteins in the range of 20–30 kDa (major group of prenylated proteins), suggesting that Ras superfamily GTPases are prenylated in these parasites (16, 53, 69). T. brucei bloodstream and procyclic forms undergo drastic morphological changes when treated with HMG-CoA reductase and PFT inhibitors (16, 69). As Rho proteins are known to be involved in maintaining cellular architecture, it is conceivable that the impairment of TcRho1 prenylation
may be one of the causes for these morphological alterations. Impairing post-translational processing of Ras and Ras-related proteins, by blocking PFT, has been proposed as a promising target for anti-cancer and anti-parasite chemotherapy (32, 70, 71). CaaX mimetic inhibitors have been shown to prevent growth of *T. brucei*, *T. cruzi* and *L. mexicana* (16, 32).

*T. brucei* PFT has been purified and cloned (33), and it is shown in the present study that PFT enzymatic activity also occurs in *T. cruzi* epimastigotes. *T. brucei* PFT shows a strong preference for CaaX substrates ending in glutamine or methionine when tested against a library of SSCALX (X = all 20 amino acids) (33). However, the C-terminal peptide of TcRho1 QSCQLF, is an excellent substrate for *T. cruzi* PFT. This plus the observation that SSCALF is a poor substrate (33) indicates that the identity of the aa dipeptide unit can also affect substrate specificity. In contrast no significant geranylgeranylation activity could be detected when Q-Sepharose fractions of *T. cruzi* cytosol were assayed with the TcRho1 peptide and radiolabeled GGPP. These results strongly suggest that TcRho1 is prenylated by *T. cruzi* PFT rather than by a minor reaction of PGGT-I. In addition, the C-terminal peptide of the Ras/Rap-like protein found in *T. brucei* (27) has the C-terminal sequence CTML and only farnesylation of this peptide was detected using Q-Sepharose fractions derived from *T. brucei* (33). Again, no PGGT-I activity could be detected in *T. brucei* cytosol. Thus, the two X residues of CaaX that specify geranylgeranylation in mammalian cells, L and F, seem to specify farnesylation in *T. cruzi* and in *T. brucei*.

It is impractical to obtain sufficient TcRho1 from *T. cruzi* in an amount sufficient for direct determination of the structure of its prenyl group. However, the immunoblot analysis using anti-farnesyl-cysteine methyl ester antiserum (Figure 7) strongly supports the farnesylation of TcRho1 in epimastigotes. The use of overexpressed mutant TcRho1 that cannot be prenylated because it lacks a CaaX motif shows that the antiserum detects only the farnesyl portion of TcRho1.
The results in this study could explain why PFT inhibitors are highly cytotoxic to trypanosomatids (16, 32). In fact, Rho family proteins are important regulators of mammalian cell growth and morphology, and it has been shown that mammalian cell growth is much more sensitive to PGGT-I inhibitors than to PFT inhibitors (67) and that geranylgeranylated Rho family proteins are implicated in cell cycle progression in some cell types (68). This could be one of the reasons that PGGT-I inhibitors are much more toxic to mammalian cells than are PFT inhibitors (69). This difference in sensitivity of trypanosomatids and mammalian cells to PFT inhibitors provides a basis for the development of PFT inhibitors as anti-trypanosomatid therapeutics.

Since the role of TcRho1 in the physiological functions of T. cruzi are not apparent, parasite transfection studies with dominant positive and negative TcRho1 variants are being carried out to explore the functions of this GTPase.
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Table I:
Identity and similarity levels of different Rho members with TcRho1.

The analysis was carried out with the GAP program from the GCG computer package software.  S.c., Saccharomyces cerevisiae; S.p., Schizosaccharomyces pombe; D.d., Dictyostelium discoideum; E.h., Entamoeba histolytica; C.e., Caenorhabditis elegans; H.s., Homo sapiens; and D.m., Drosophila melanogaster

| Protein | Accession | Identity (%) | Similarity (%) |
|---------|-----------|--------------|----------------|
| [Organism] | Number |              |                |
| RHO1   | [S.c.] p06780 | 50.5         | 63.7           |
| Rho1   | [S.p.] q09914 | 53.3         | 61.4           |
| RacC   | [D.d.] p39149 | 47.4         | 60.8           |
| RacA   | [E.h.] q24814 | 49.2         | 60.2           |
| RhoA   | [C.e.] q22038 | 46.5         | 60.0           |
| Tc10   | [H.s.] p17081 | 46.9         | 57.5           |
| Cdc42  | [D.m.] p40793 | 46.3         | 57.6           |
Figure legends

Figure 1. (A) Southern blot analysis of T. cruzi DNA, probed with the pTcrho fragment under high stringency conditions. Dm28c DNA was digested with EcoRI (E), BamHI (B), SalI (S), HindIII (H) and PstI (P). Arrowheads indicate DNA markers in kb. (B) Northern blot analysis of TcRho1 transcript. Poly(A)+ RNA (200 ng) from Dm28c epimastigotes was hybridized against the TcRho1 KpnI clone as probe under high stringency conditions. Migration of rRNA was used as molecular markers.

Figure 2. Nucleotide sequence of the TcRho1 gene with flanking regions shown in lower case. The peptide sequence is shown in upper case. Poly-pyrimidines stretches are in bold and start and stop codons are underscored. This sequence was deposited in GenBank™ under the access number AF177587.

Figure 3. Multiple alignment of Rho family sequences, performed by Clustal X. G1 to 5 domains and the CaaX motif are in black boxes. Asterisks represent identical residues, while single points indicate low conserved substitutions and double points highly conserved substitutions. Dashes represent gaps in sequences. Dm, Drosophila melanogaster; Sp, Schizosaccharomyces pombe; Hs, Homo sapiens; At, Arabidopsis thaliana; Eh, Entamoeba histolytica; and Sc, Saccharomyces cerevisiae. GenBank™ accession numbers as follows: 1, I45716; 2, A55924; 3 B34386; 4, P15154; 5, AAF40241; 6, NP001656; 7, JC4932; 8, NP015491; and 9, P08134.

Figure 4. Phylogenetic tree showing the families that comprise the Ras superfamily of small GTPases. The bar represents 0.1 amino acid changes per site. Hs, Homo Sapiens; Mm, Mus musculus; Sc, Saccharomyces cerevisiae; Gg, Gallus gallus; Eh, Entamoeba histolytica; Gl,
*Giardia lamblia; Rn, Rattus norvegicus; and Ce, Caenorhabditis elegans.* Accession numbers as follows: 1, P16587; 2, AAB52968; 3, P09527; 4, B34323; 5, P01112; 6, S03180; 7, AAC33178; 8, CAA56682; 9, NP013330; 10, Jc4931; 11, P42558; and 12, P38543.

**Figure 5.** Mapping the 5' region of the TcRho1 transcript. (A) Ethidiun bromide stained agarose gel of the semi-nested RT-PCR reaction described in the text: lane 1, 123 bp molecular weight marker; lane 2, first reaction using epimastigote RNA; lane 3, second reaction (semi nested) using epimastigote RNA; lane 4, first reaction using metacyclic trypomastigote RNA, lane 5, second reaction using metacyclic trypomastigote RNA. (B) Nucleotide sequence corresponding to cloned products of reaction in lane 3 in (A). Mini-exon sequences are represented in bold italics. AG dinucleotides are represented in underscored boldface. The ATG initiator codon is circled. (C) TcRho1 trans-splicing scheme (not drawn to scale). The black box indicates the TcRho1 open reading frame, the thin lines indicate the flanking regions, and the hatched boxes indicate the poly-pyrimidine stretches. The arrows represent mapped trans-splicing sites in the TcRho1 5' region.

**Figure 6.** Q-Sepharose chromatography of *T. cruzi* PFT. The 0-60% ammonium sulfate fraction of epimastigote cytosolic proteins (21 mg protein) from 1 L culture was fractionated on a Q-Sepharose column (1 x 8 cm). Elution with a gradient of NaCl concentration was performed as described in "Materials and Methods". Protein prenyltransferase assays were carried out with Biotin-QSCQLF/[3H]FPP (●) or RAS-CVIM/[3H]FPP (○) for farnesylating activity and with Biotin-QSCQLF/[3H]GGPP (▲) or Ras-CVLL/[3H]GGPP (△) for geranylgeranylating activity. One µU is the amount of enzyme that produces 1 pmol of product per min using the assay conditions given in Experimental Procedures.
Figure 7.  Western blot analysis of \textit{in vivo} prenylation of TcRho1 in \textit{T. cruzi} epimastigote cells.  Total cell proteins of \textit{T. cruzi} Tulahuen epimastigotes (1 x 10^7 cells) (lanes 1-4) and purified recombinant TcRho1 expressed in \textit{E. coli} (lane 5) were resolved by SDS-PAGE on a 12.5% gel: wild type \textit{T. cruzi} cells (lanes 2 and 3) and transformed cells overexpressing mutant TcRho1 (C-terminal CQLF replaced with FNFFDA) (lanes 1 and 4). Lanes 1 and 2 were probed with anti-S-farnesyl-cysteine methyl ester antiserum, and lanes 3-5 were probed with anti-TcRho1 antiserum.  ECL detection was carried out after incubation with horseradish peroxidase-linked anti-rabbit IgG.  The arrow shows the migration position of TcRho1.

Figure 8.  Analysis of prenylated proteins in \textit{T. cruzi} epimastigotes.  (A) Total cell proteins from \textit{T. cruzi} epimastigotes (1 x 10^7 cells) were resolved by SDS-PAGE (12.5% gel), and the gel was subjected to western blotting with anti-S-farnesyl-cysteine methyl ester antiserum.  The migration position of TcRho1 is shown.  (B) Radiolabeling of \textit{T. cruzi} proteins with \[^3\text{H}\text{]mevalonolactone and inhibition of protein prenylation by the CaaX mimetic JJ23. \textit{T. cruzi} epimastigotes (1 x 10^7 cells) were labeled for 24 h with 6.7 \mu M \[^3\text{H}\text{]mevalonolactone (100 \mu Ci) in the presence of 300 \mu M simvastatin. JJ23 was tested at 0 (lane 1), 5 (lane 2), 25 (lane 3), and 100 \mu M (lane 4). Radiolabeled proteins were analyzed by SDS-PAGE (12.5% gel) and visualized by fluorography.  The gel was exposed to X-ray film at -80\textdegree C for 16 days.
5'P

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