

# TcRho1, a Farnesylated Rho Family Homologue from *Trypanosoma cruzi*

CLONING, TRANS-SPLICING, AND PRENYLATION STUDIES\*

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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 transcription 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 revealed that *TcRHO1* is a single copy gene per haploid genome, and Northern blot assays showed a transcript of 1200 nucleotides 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-terminal sequence CQLF (CAAX motif), which is predicted to direct post-translation prenylation of the cysteine residue. A synthetic peptide containing this C-terminal 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 = Phe specifies geranylgeranylation by mammalian protein geranylgeranyltransferase I. Furthermore, immunoblot analyses of epimastigote protein with anti-S-farnesylcysteine methyl ester and anti-TcRho1 antisera strongly suggested 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.

Hydrolysis of GTP to GDP by GTPases functions as a molec-

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ular 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 protozoa 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) that 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 hyper-variable C terminus that extends away from the globular core and terminates in a so-called CAAX box (where 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 sulfhydryl group, 2) endoproteolytic removal of AAX, and 3) methylation of the  $\alpha$ -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 (where X = serine, methionine, and other residues) is carried out by protein farnesyltransferase (PFT),<sup>1</sup> whereas protein geranylgeranyltransferase I (PGGT-I) geranylgeranylates CAAX when X is leucine or phenylalanine (14).

The family Trypanosomatidae is composed of obligate protozoan parasites, some of pivotal medical and economic interest. *Trypanosoma cruzi* is the causative agent of Chagas' disease, which affects ~17 million people in the American continent (15). Development of new drugs against pathogenic trypanoso-

<sup>1</sup> The abbreviations used are: PFT, protein farnesyltransferase; PGGT-I, protein geranylgeranyltransferase I; bp, base pair(s); UTR, untranslated region; RT-PCR, reverse transcription-polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; GGPP, geranylgeranyl pyrophosphate; FPP, farnesyl pyrophosphate.

matids 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 *Trypanosoma brucei* and *T. cruzi* have revealed similarities between their roles in vesicle trafficking of lower and higher eukaryotes (19–25). Studies in *T. brucei* have also revealed 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, 27–31). 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, 32). The molecular basis for this difference is not known. We have purified *T. brucei* PFT and cloned its  $\alpha$ - and  $\beta$ -subunits (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  $\alpha$ -subunit, but have distinct  $\beta$ -subunits. We have not been able to detect the  $\beta$ -subunit of PGGT-I by TBLASTN searching of trypanosomatid genomic data bases even though the shotgun coverage of the *T. brucei* genome is currently at 1.5 $\times$ . These results suggest that proteins that 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 this 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, clone Dm28c and strain Tulahuen, were kindly provided by Dr. S. Goldenberg (Fiocruz, Brazil) and Dr. S. Reed (Infectious Diseases Research Institute, Seattle, WA), respectively. Cells were maintained at 28 °C in liver infusion tryptone medium (34) supplemented with 10% heat-inactivated fetal calf serum (Life Technologies, Inc.) and 0.025  $\mu$ g/ml hemin (Sigma). Metacyclogenesis and purification of Dm28c metacyclic trypomastigotes were performed in TAU-3AAG medium as described (35).

**Southern and Northern Blots**—Genomic DNA was prepared from 10<sup>9</sup> Dm28c epimastigotes. Cells were collected by centrifugation and incubated in 0.5% SDS, 20  $\mu$ g/ml RNase A, and 100  $\mu$ g/ml proteinase K at 56 °C for 2 h. 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 Inc.). The digested samples were resolved on a 0.8% agarose gel. After electrophoresis, DNA was denatured in 0.5 N NaOH, neutralized, transferred onto nitrocellulose membranes by capillary through a 20 $\times$  SSC solution (3 M NaCl and 0.3 M sodium citrate), and UV-cross-linked (120,000  $\mu$ J/cm<sup>2</sup>) using a UV cross-linker chamber (Ultralium).

Total RNA was prepared from  $\sim$ 10<sup>9</sup> cells (99% epimastigotes) according to the methodology described by Perry *et al.* (37). Poly(A)<sup>+</sup> RNA was purified from total RNA by oligo(dT) chromatography using the Quick-Prep mRNA purification kit (Amersham Pharmacia Biotech). Twenty

micrograms of total RNA and 200 ng of poly(A)<sup>+</sup> RNA were separated on a formaldehyde-containing 1.5% agarose gel, blotted onto a nitrocellulose membrane by capillary transfer, and UV-cross-linked as described above for Southern blotting.

Before hybridization, nitrocellulose membranes were blocked for 3 h at 42 °C in a solution containing 50% (v/v) formamide, 5 $\times$  SSC, 5 $\times$  Denhardt's solution (1% (w/v) Ficoll, 1% (w/v) polyvinylpyrrolidone, and 1% (w/v) bovine serum albumin), 0.1% (w/v) SDS, 50 mM phosphate buffer (pH 7.0), and 100  $\mu$ g/ml denatured salmon sperm DNA. Probes were radiolabeled by the random priming DNA labeling method (38) using either [ $\alpha$ -<sup>32</sup>P]dATP or [ $\alpha$ -<sup>32</sup>P]dCTP (Amersham Pharmacia Biotech). Hybridizations were carried out overnight at 42 °C in the blocking solution containing 10<sup>6</sup> cpm/ml denatured probe. After hybridization, membranes were washed three times with 0.1 $\times$  SSC and 0.5% SDS at 55 °C and autoradiographed.

**Genomic Library Screening**—An  $\lambda$ EMBL3 Dm28c genomic library was kindly provided by Dr. W. Degrave (Fiocruz, Brazil) and propagated in *Escherichia coli* strain KW252. Approximately 60,000 independent recombinant phages were screened with the [ $\alpha$ -<sup>32</sup>P]dATP-labeled pTcrho probe (described under "Results"). 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 *E. coli* strain LE392. DNA from this clone was extracted and purified as described (36) and was submitted to Southern blot analysis as described above. A 4.0-kilobase pair *EcoRI* fragment was selected as an initial target for cloning. Ligation of *EcoRI*-digested genomic cloned DNA with *EcoRI*-digested and 5'-dephosphorylated pBluescript KS-II<sup>+</sup> (Stratagene) followed by transformation of *E. coli* XL1-Blue generated several recombinant clones. Plasmid DNA from these clones was extracted as described (36); those containing cloned fragments in the range of 4.0 kilobase pairs 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-kilobase pair *EcoRI/PstI* fragment from  $\lambda$ TcRHO1 encompassing the remaining TcRho1 sequence.

**Sequence Analysis of TcRHO1**—Subcloned fragments in pBluescript KS-II<sup>+</sup> were sequenced with different primers by the dideoxy chain termination method (39) using the T7 sequencing kit (Amersham Pharmacia Biotech). We used the commercially available T3 and T7 sequencing primers and also three sequencing primers based on the TcRho1 sequence (G2, 5'-CGGAATTCCTCCGGTACCCCGCC-3'; G4, 5'-GCGTGGGATGACCC-3'; and G5, 5'-GCGTTGGTAACATGCAGC-3').

**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, 5'-GGATGGAATTCAGTTCTGTACTATATTG-3'; kindly provided by Dr. T. Urmeni, Instituto de Biofisica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro), and the other two primers were directed to sequences within the *TcRHO1* coding region (G2 (see above) and G3, 5'-AACTGCAGAACCGCCAACCCCTTCATTGC-3'). Initially, 5  $\mu$ g of total epimastigote RNA was submitted to first strand cDNA synthesis using the SuperScript II preamplification system (Life Technologies, Inc.), performed with random hexamers as primers, according to the manufacturer's suggested procedures. The first PCR was carried out in the presence of 0.5 mM dNTPs, 1.5 mM MgCl<sub>2</sub>, 10  $\mu$ M each primer, and 5 units of *Taq* DNA polymerase (Life Technologies, Inc.). To avoid problems derived from differences in primer melting temperatures, a touch-down PCR program was used by decreasing the annealing temperature from 75 to 57 °C in 1 °C steps. Then, 20 additional cycles were performed at 93 °C for denaturing, at 55 °C for annealing, and at 72 °C for extension, followed by a 10-min extension step. One-tenth of this first reaction was used as template in the second reaction under 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 to allow ligation of products from the second reaction into pBluescript KS-II<sup>+</sup>. 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'-CGGGATCCTCACAAATGGAGGAGACTG-3') and Terhoend (5'-CGGGATCCATCAAAAAAGTTGACAGCTCTGTC-3'), both containing *Bam*HI 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 glutathione *S*-transferase gene in the pGEX-3X vector (Amersham Pharmacia Biotech), and the resulting construct was used to transform *E. coli* strain BL21. Expression of the recombinant protein was induced with isopropyl- $\beta$ -D-thiogalactopyranoside to a final concentration of 0.5 mM after cultures reached  $A_{600} \sim 0.8$ . Cultures were then maintained for 1 h; cells were pelleted and washed twice with phosphate-buffered saline; and the cells were lysed by sonication with a Branson sonicator (ten 10-s pulses interrupted by cooling on ice). The fusion protein was recovered from inclusion bodies using the urea solubilization protocol previously described for the Ras protein (40). Purification of the fusion protein was accomplished by glutathione-Sepharose chromatography (Amersham Pharmacia Biotech) according to the manufacturer's suggested procedure. The purified protein was cleaved with factor Xa (Amersham Pharmacia Biotech) at 10 units/mg of fusion protein to release TcRho1 protein from the glutathione *S*-transferase 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 under "Results," we desired a strain of *T. cruzi* that overexpresses TcRho1 mutant that cannot be prenylated. We had available a mutant of TcRho1 in which the C-terminal CQLF sequence was 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 interleukin 2-encoding insert was excised from the vector with *Bam*HI and replaced with the *TcRHO1* open reading frame flanked by *Bam*HI sites. A clone with a properly oriented insert was identified, and 5  $\mu$ g of supercoiled DNA was electroporated into Tulahuen epimastigotes as previously described (42). Transfectants were selected and expanded in 500  $\mu$ g/ml G418.

Immunoblotting—Antiserum to TcRho1 was raised in a rabbit against the keyhole limpet hemocyanin-conjugated peptide NDNQVVDTSNKQSIEL, 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); and 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 four 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  $A_{280}$  reached a minimum. Antibodies were eluted with 10 volumes of 100 mM glycine (pH 2.5) and neutralizing with 1 M Tris (pH 8.0). The material was dialyzed against 5 mM NaCl and lyophilized.

Tulahuen strain epimastigote cells (log phase) were pelleted in a microcentrifuge tube. The supernatant was removed; the cell pellet was treated with Laemmli sample buffer at 42 °C for 30 min; and a sample from  $10^7$  cells was loaded onto a single lane of a 12.5% Laemmli SDS-polyacrylamide gel. Proteins were electrophoretically transferred to nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech). The membranes were blocked in 5% nonfat powdered milk in Tris-buffered saline containing 1% Tween 20. The blot was incubated for 2 h either with affinity-purified anti-TcRho1 antiserum (1:2000) or with anti-*S*-farnesylcysteine methyl ester antiserum (1:2000) (43) at room temperature. After washing, membranes were incubated for 1 h with a 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 ( $5 \times 10^9$  cells) were collected; washed once with phosphate-buffered saline; and suspended in 1 mM Tris-HCl, 1 mM dithiothreitol, 1 mM EDTA (pH 8.0), and freshly added protease inhibitors (1 mM phenylmethylsulfonyl fluoride; 30  $\mu$ M each tosyllysine chloromethyl ketone and tosylphenylalanine chloromethyl ketone; and 10  $\mu$ g/ml each aprotinin, leupeptin, and pepstatin A). The cells were lysed by sonication with a Branson sonicator (ten 5-s pulses interrupted by cooling on ice). The lysate was diluted to 26 ml and supplemented with the following components at the indicated concentrations: 20 mM Tris-HCl (pH 8.0), 5 mM dithiothreitol, and 5  $\mu$ M ZnCl<sub>2</sub>. This mixture was centrifuged at 120,000  $\times g$  for 80 min at 4 °C, and the resulting super-

natant (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 dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) at 4 °C (three exchanges of 2 liters each).

The resultant protein solution was loaded onto a column (1  $\times$  8 cm) of Q-Sepharose Fast Flow (Amersham 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: 0–4 min, 0–15% buffer B; 4–124 min, 15–55% buffer B; 124–128 min, 55–100% buffer B; and 128–152 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  $\mu$ l of Q-Sepharose fractions at 30 °C for 30 min with 5  $\mu$ M biotin-QSCQLF (C-terminal peptide of TcRho1, prepared by United Biochemical Research) and 1  $\mu$ M [<sup>3</sup>H]farnesyl pyrophosphate (0.3  $\mu$ Ci) or 1  $\mu$ M [<sup>3</sup>H]GGPP (0.3  $\mu$ Ci) (both from American Radiolabeled Chemicals) in 20  $\mu$ l of buffer (30 mM potassium phosphate, 0.5 mM MgCl<sub>2</sub>, 20 mM ZnCl<sub>2</sub>, and 5 mM dithiothreitol (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  $\mu$ M recombinant RAS-CVIM (a generous gift from Dr. C. Omer, Merck) and for PGGT-I activity with 5  $\mu$ M 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 h in 1 ml of liver infusion Tryptone medium containing 100  $\mu$ Ci of [<sup>3</sup>H]mevalonolactone (1.6  $\mu$ M; American Radiolabeled Chemicals) and 300  $\mu$ M simvastatin. Cellular protein was delipidated and submitted to SDS-PAGE as described (16). In some experiments, the PFT inhibitor JJ23 was present.

## RESULTS

*Genomic Organization of TcRHO1*—We have characterized several cloned RT-PCR fragments amplified from *T. cruzi* RNA that share homology in their predicted peptide sequences with several Ras superfamily genes. 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 ~40% similarity with several members of the Rho family of proteins. We used pTcrho as a homologous probe 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 labeled pTcrho fragment. Southern blot analysis revealed single bands, suggesting that *TcRHO1* is present as a single copy gene in the Dm28c haploid genome (Fig. 1A). This pattern resembles other characterized trypanosomal 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 overlapping TcRho1 fragments in pBluescript KS-II<sup>+</sup>. 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 a predicted molecular mass of 30,979 Da (Fig. 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<sup>+</sup>TGG) are very close to the optimum initiation sequence (GCC(A/G)CCA<sup>+</sup>TGG) described by Kozak (47), showing conservative substitutions at -5 and -6 and identical bases at +4, -2, -3, and -4.

Homology searching was performed against the Swiss Protein Database using the FASTA program from the GCG Wisconsin Sequence Analysis Software Package (48). The top scoring matches are proteins from the Rho family. The top seven





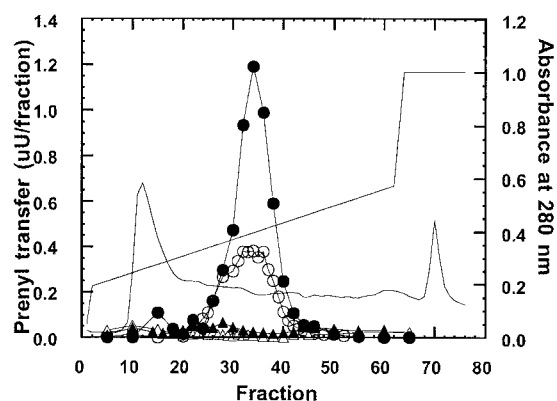


FIG. 6. Q-Sepharose chromatography of *T. cruzi* PFT. The 0–60% ammonium sulfate fraction of epimastigote cytosolic proteins (21 mg of protein) from 1 liter culture was fractionated on a Q-Sepharose column (1 × 8 cm). Elution with an NaCl gradient was performed as described under “Experimental Procedures.” Protein prenyltransferase assays were carried out with biotin-QSCQLF and [<sup>3</sup>H]farnesyl pyrophosphate (●) or RAS-CVIM and [<sup>3</sup>H]farnesyl pyrophosphate (○) for farnesylating activity and with biotin-QSCQLF and [<sup>3</sup>H]GGPP (▲) or Ras-CVLL and [<sup>3</sup>H]GGPP (△) for geranylgeranylating activity. 1 micro-unit (*uU*) is the amount of enzyme that produces 1 pmol of product/min using the assay conditions given under “Experimental Procedures.”

within TcRho1 coding sequence at positions +6, +9, and +25 (Fig. 5, B and C). 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 fractions (53), a single peak of PFT activity was detected with RAS-CVIM and [<sup>3</sup>H]farnesyl pyrophosphate as substrates, indicating the existence of PFT in *T. cruzi* (Fig. 6). No significant PGGT-I activity was detected in these fractions when tested with substrates of mammalian PGGT-I (Ras-CVLL and [<sup>3</sup>H]GGPP). The N-terminally biotinylated 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 [<sup>3</sup>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 ~2-fold higher than that measured with RAS-CVIM; the latter is one of the best substrates for trypanosomatid PFT found to date. These results suggest that TcRho1, which possesses the C-terminal CQLF motif, is farnesylated by PFT in *T. cruzi*.

To examine the type of the prenyl group attached to TcRho1 *in vivo*, we used a recently described antiserum that recognizes *S*-farnesylcysteine 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

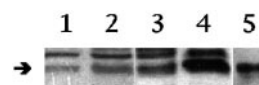
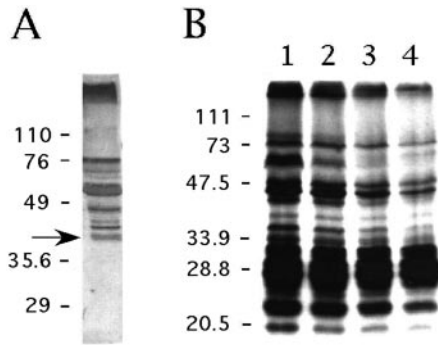


FIG. 7. Western blot analysis of *in vivo* prenylation of TcRho1 in *T. cruzi* epimastigote cells. Total cell proteins of *T. cruzi* Tulahuen epimastigotes ( $1 \times 10^7$  cells) (lanes 1–4) and purified recombinant TcRho1 expressed in *E. coli* (lane 5) were resolved by SDS-PAGE on a 12.5% gel. Lanes 2 and 3, wild-type *T. cruzi* cells; lanes 1 and 4, transformed cells overexpressing mutant TcRho1 (C-terminal CQLF sequenced replaced with FNFFDA). Lanes 1 and 2 were probed with anti-*S*-farnesylcysteine 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.

prenyl group structure determination by radiometric or mass spectrometric methods (54). (The 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 TcRho1 mutant that cannot be prenylated (CQLF replaced with FNFFDFA, already available in our laboratory as described under “Experimental Procedures”). 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*-farnesylcysteine methyl ester antiserum for the farnesyl portion of TcRho1.

As shown in Fig. 7, the immunoblot analysis using anti-TcRho1 antiserum detected a protein band from whole parasites that comigrated with recombinant TcRho1 produced in *E. coli*. The observed apparent molecular mass for the band is ~39 kDa (predicted molecular mass of 31 kDa). The immunoblot from parasites that overexpress the TcRho1 mutant (Fig. 7) shows an ~10-fold increase compared with non-transfected parasites in the amount of protein detected at the ~39-kDa position, thus supporting the assignment of this band as TcRho1. As shown in Fig. 7, the immunoblot of non-transfected parasites with the anti-*S*-farnesylcysteine methyl ester antiserum clearly shows a band at ~39 kDa that comigrated 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*-farnesylcysteine methyl ester antiserum (Fig. 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 Fig. 8, immunoblot analysis of *T. cruzi* proteins with the anti-*S*-farnesylcysteine methyl ester antiserum revealed 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 [<sup>3</sup>H]mevalonolactone to label their prenyl groups (Fig. 8). The fluorograph shows that the most intense radiolabeled proteins are in the 25–33-kDa range. These were not detected with the anti-*S*-farnesylcysteine 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 molecular masses >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)). These results further support the proposed geranylgeranylation of most of the 25–33-kDa proteins. All together, the results suggest that the anti-*S*-farnesylcysteine methyl ester antiserum detects farnesylated (but not geranylgeranylated) proteins in *T. cruzi*, as shown previously with mammalian cells (43).



**FIG. 8. Analysis of prenylated proteins in *T. cruzi* epimastigotes.** A, total cell proteins from *T. cruzi* epimastigotes ( $1 \times 10^7$  cells) were resolved by SDS-PAGE on a 12.5% gel, and the gel was subjected to Western blotting with anti-S-farnesylcysteine methyl ester antiserum. The arrow shows the migration position of TcRho1. B, shown are the results from radiolabeling of *T. cruzi* proteins with [ $^3$ H]mevalonolactone and inhibition of protein prenylation by the CAAX mimetic JJ23. *T. cruzi* epimastigotes ( $1 \times 10^7$  cells) were labeled for 24 h with 6.7  $\mu$ M [ $^3$ H]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 (lane 4)  $\mu$ M. Radiolabeled proteins were analyzed by SDS-PAGE on a 12.5% gel and visualized by fluorography. The gel was exposed to x-ray film at  $-80^\circ\text{C}$  for 16 days.

#### DISCUSSION

TcRho1 is the first Rho family member from trypanosomatids to be identified, albeit some of the Ras superfamily genes have been cloned in these organisms (18). TcRho1 has conserved GTPase motifs and a C-terminal CAAX motif that is a target for post-translational prenylation. Phylogenetic analysis shows that TcRho1 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 in 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 (Fig. 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 codon downstream of the spliced leader sites interior to the open reading frame predicts a small protein of 54 amino acids, and no significant homology to this putative protein was detected in sequence data bases. Polypyrimidine tracks in *T. cruzi* RNA are thought to regulate trans-splicing of RNA (56). The *TcRHO1* 5'-UTR has two polypyrimidine 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 to modulate TcRho1 expression. There is no apparent difference in the trans-splicing profile of TcRho1 RNA in epimastigotes, metacyclic trypomastigotes, and 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 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 (66) 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. Hydroxymethylglutaryl-CoA reductase has been characterized in *T. brucei* and *T. cruzi* (67, 68). Incubation of trypanosomatids with radiolabeled mevalonate in the presence of hydroxymethylglutaryl-CoA reductase inhibitors leads to metabolic labeling of a collection of proteins in the 20–30-kDa range (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 hydroxymethylglutaryl-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 anticancer and anti-parasite chemotherapy (32, 51, 70). 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 (32, 33), and we have shown in this study that PFT enzyme 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* (32). 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, Leu and Phe, seem to specify farnesylation in *T. cruzi* and *T. brucei*.

It is impractical to obtain TcRho1 from *T. cruzi* in an amount sufficient for direct determination of the structure of its prenyl group. However, the immunoblot analysis using anti-S-farnesylcysteine methyl ester antiserum (Fig. 7) strongly supports the farnesylation of TcRho1 in epimastigotes. The use of over-expressed 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 of 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 (71) and that geranylgeranylated Rho family proteins are implicated in cell cycle progression in some cell types (72). This could be one of the reasons why PGGT-I inhibitors are much more toxic to mammalian cells than are PFT inhibitors (73). 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* is not apparent, parasite transfection studies with dominant-positive and dominant-negative TcRho1 variants are being carried out to explore the functions of this GTPase.

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