Cloning and Expression of *Trypanosoma cruzi* Ribosomal Protein P0 and Epitope Analysis of Anti-P0 Autoantibodies in Chagas' Disease Patients

By Yasir A. W. Skeiky,* Darin R. Benson, Marilyn Parsons, Keith B. Elkon, and Steven G. Reed

From the *Seattle Biomedical Research Institute, Seattle, Washington 98109; the †Department of Pathobiology, University of Washington, Seattle, Washington 98195; ‡The Hospital for Special Surgery, Cornell University Medical College; and the ‡Department of Medicine, Cornell University Medical College, New York 10021

**Summary**

Chagas' disease, caused by the intracellular protozoan parasite *Trypanosoma cruzi*, is a major cause of heart failure in endemic areas. Antigenic mimicry by *T. cruzi* antigens sharing epitopes with host macromolecules has been implicated in the pathogenesis which is thought to have a significant autoimmune component. We report herein on the cloning and characterization of a full-length cDNA from a *T. cruzi* expression library encoding a protein, TcP0, that is homologous to the human 38-kD ribosomal phosphoprotein HuP0. The *T. cruzi* P0 protein shows a clustering of residues that are evolutionarily conserved in higher eukaryotes. This includes an alanine- and glycine-rich region adjacent to a highly charged COOH terminus. This "hallmark" domain is the basis of the crossreactivity of the highly immunogenic eukaryotic P protein family. We found that *T. cruzi*-infected individuals have antibodies reacting with host (self) P proteins, as well as with recombinant TcP0. Deletion of the six carboxy-terminal amino acids abolished the reactivity of the *T. cruzi* infection sera with TcP0. This is similar to the specificity of anti-P autoantibodies described for a subset of patients with systemic lupus erythematosus (SLE) (Elkon, K., E. Bonfa, R. Llovet, W. Danho, H. Weissbach, and N. Brot. 1988. *Proc. Natl. Acad. Sci. USA.* 85:5186). These results suggest that *T. cruzi* P proteins may contribute to the development of autoreactive antibodies in Chagas' disease, and that the underlying mechanisms of anti-P autoantibody may be similar in Chagas' and SLE patients. This study represents the first definitive report of the cloning of a full-length *T. cruzi* antigen that mimics a characterized host homologue in structure, function, and shared antigenicity.

The protozoan hemoflagellate, *Trypanosoma cruzi*, is the causative agent of Chagas' disease, which is endemic in many Latin American countries. During the chronic stage of infection, an abundant inflammatory infiltrate is found in myocardial and nervous tissues (1, 2). This, coupled with the rare detection of parasites in damaged tissues, has led to the hypothesis that autoimmune mechanisms may contribute to tissue injury in Chagas' disease.

Chagas' disease provides an excellent model for exploring the mechanisms of autoimmunity, because the etiology of the disease is known. Regarding humoral response, relatively few antibodies against different self-antigens have been characterized in *T. cruzi*-infected individuals (3–6). To date there have been only two reports on the molecular cloning of *T. cruzi* antigens, one complete (7) and the other a 35-residue peptide (8), containing antigenic epitopes which may induce antibodies that crossreact with self proteins. In general, most of the *T. cruzi* antigens that have been reported are partial sequences comprised mainly of repetitive epitopes (9, 10). Of the identified nonrepeat antigens, complete sequence information is available for only a few (7, 9, 11).

Herein, we report the cloning, expression, and biochemical characterization of a full-length cDNA encoding a 35-kD *T. cruzi* antigen, TcP0, that is constitutively expressed and associated with the ribosomal translation machinery. TcP0 is highly homologous to the human 38-kD type ribosomal P protein HuP0, and is conserved in other *Trypanosoma* species. We show that *T. cruzi*-infected individuals have antibodies against TcP0 which, in most cases, crossreact with the homologous human ribosomal P proteins. Of particular
interest to our study are previous reports which demonstrated that a subset of patients with SLE have autoantibodies against ribosomal P proteins (12, 13).

The uniformity in the target epitope of the autoantibodies against the ribosomal P proteins of humans and mice with SLE (14), of a mouse mAb derived from immunization with heterologous (chick) ribosomes (15), and of TcP0 in T. cruzi-infected individuals, is suggestive of similar mechanisms that lead to the generation of anti-P autoantibodies either spontaneously (human and mouse SLE) or after infection. We propose that exposure to T. cruzi P proteins may result in the generation of autoreactive antibodies which could contribute to the autoimmune pathogenesis characteristic of Chagas' disease.

Materials and Methods

Parasite and Cell Culture. T. cruzi (MHOM/CH/00/Tulahuen C2) and Trypanosoma brucei (EATRO 164, clone IHR1 J) strains were grown and cultured as described (16, 17). K562 human erythroleukemic cells were a gift of Dr. M. Yagi, Seattle Biomedical Research Institute.

Library Construction and Isolation of cDNA Clones. Poly(A+) RNA was purified from total T. cruzi trypomastigote RNA using standard protocols (18). An expression library was constructed with the poly(A+) RNA using the lambda Zap-cDNA unidirectional cloning kit (Stratagene Inc., La Jolla, CA), as suggested by the manufacturer. Approximately 2 x 10^6 plaques were screened in duplicate with ^32P-radiolabeled Leishmania chagasi P0 insert (Sheiky et al., manuscript in preparation) using standard techniques (18). Hybridization was at 55°C using the same cocktail mix as described for the Southern blots (see below). Posthybridization washes were at 55°C for 2 x 15 min with each of 2 x and 0.5 x SSC containing 0.1% SDS. After plaque purification, excision of the pBSK(+) phagemid was carried out according to the manufacturer's protocol (Stratagene Inc.).

Northern and Southern Analysis. Total RNA was extracted by the acid guanidium isothiocyanate method (19), resolved on 1.5% formaldehyde denaturing agarose gels (18), and transferred by capillary blotting onto Zeta Probe membrane (Bio-Rad Laboratories, Richmond, CA) using 50 mM NaOH (20). Genomic DNA was prepared, digested with the restriction enzymes, separated on 0.7% agarose gel, and blotted on to Nytran membrane (18). Hybridization was performed as described (21), except for minor modifications: the salt concentration was 6 x SSC, and dextran sulfate was omitted for Southern blots. Radiolabeled TcP0 cDNA insert was prepared by the random priming method (22), and hybridized overnight at 65°C (Northern blots) or 50°C (Southern blots). Blots were washed twice at 65°C for 20 min with each of 2 x, 0.5 x, and 0.2 x SSC containing 0.1% SDS.

Sequencing. TcP0 cDNA inserts of the pBSK(-) phagemid were excised after restriction with EcoRI and XhoI, and subcloned unidirectionally into the same sites of pBSK(+) vector. Overlapping clones were generated from both the coding (pBSK(+)) and noncoding (pBSK(-)) strands by exonuclease III (23). Single-strand templates were isolated after infection with VCSM13 helper phage, as recommended by the manufacturer (Stratagene Inc.), and sequenced by the dideoxy chain termination method (24) using Sequenase (US Biochemical Corp., Cleveland, OH).

Expression and Purification of Recombinant TcP0 (rpTcP0) Antigens. T. cruzi P0 cDNAs were engineered at the 5' region of the polylinker sequence using appropriate enzymes for in-frame fusion with the amino terminus of β-galactosidase. COOH-terminal deletions were initiated from the 3' end (XhoI) of the TcP0 phagemid insert and treatment with exonuclease III. Recombinant antigens were purified from 500 ml of IPTG-induced cultures (10). The inclusion bodies were sequentially solubilized in two washes each of 10 ml TNE (50 mM Tris, pH 8.0, 100 mM NaCl, and 10 mM EDTA) containing 2, 4, and 8 M urea. Fractions containing the recombinant antigen (usually the 4 and 8 M urea supernatants) were pooled, dialyzed against PBS, and concentrated by precipitation with 30% ammonium sulfate. Purification to homogeneity was accomplished by preparative SDS-PAGE electrophoresis, followed by excision and electroelution of the recombinant antigens as described (25).

Production of Rabbit Antiserum against Recombinant TcP0. An adult rabbit (New Zealand White; R & R Rabbirtty, Stanwood, WA) was immunized with purified rpTcP0 as described (26), except that rIL-1β was excluded.

Antigens. Parasite and cell lysates were prepared by freeze/thaw lysis of pellets in SDS sample buffer, but without glyceral and B-ME. Insoluble material was separated from the supernatant by centrifugation at 10,000 rpm in a microfuge. T. cruzi ribosomes were isolated as previously described for mammalian cells (13), and the final pellet resuspended in SDS sample buffer. Protein concentrations were determined using the BCA protein assay kit (Pierce Chemical Co., Rockford, IL).

In vitro Translation and Immunoprecipitation. 10 μg of total stage-specific T. cruzi RNA was translated in rabbit reticulocyte lysate in the presence of [35S]methionine, as suggested by the supplier's protocol (Promega Corp., Madison, WI). Typically, 100,000 cpm of the total translated mixture was diluted to 250 μl with solubilization buffer (20 mM Tris, pH 8.0, 50 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 50 μg/ml gentamycin, 5 mM EDTA, 0.1 mM PMSF, and 0.1 mM iodoacetatic acid). 2 μl of anti-TcP0 antiserum was added and incubated on ice for 2 h. Complexes were precipitated by the addition of 40 μl of a 10% vol/vol fixed Staphylococcus aureus (Cowan I) and further incubation for 1 h. After centrifugation through a 1-M sucrose cushion, immunoprecipitates were washed twice each in solubilization buffer and mixed detergent buffers (0.05% NP-40, 0.1% SDS, 0.3 M NaCl, and 10 mM Tris, pH 7.6), and resuspended in 50 μl SDS sample buffer. 10-μl samples were resolved on SDS polyacrylamide gels and prepared for fluorography by treatment with Entensify™ (Du Pont Co., Wilmington, DE), as specified by the manufacturer.

Patient Sera. T. cruzi infection sera were from well-characterized patients (confirmed by both parasitological and serological evaluation) from Brazil (10). Anti-P positive SLE sera were from North American patients and have been described elsewhere (27, 13). Sera from uninfected individuals were from Seattle and nonendemic areas of Brazil.

Immunoblot Analysis. 5-10 μg of parasite or cell extracts or 0.5-1.0 μg of recombinant antigens were separated on 12.5% SDS-PAGE (28), and transferred electrophoretically to nitrocellulose membranes (29). Reactivities of the antisera were assessed as previously described (25) using 125IProtein A, followed by autoradiography.

ELISA. Microtitre plates (Probind™; Falcon Plastics, Cockeysville, MD) were coated overnight with synthetic peptide corresponding to the COOH-terminal 22 amino acids of the human ribosomal P2 protein conjugated with thyroglobulin (27) at a concentration of 250 ng per well in 50 μl of coating buffer (15 mM Na2HCO3, 28 mM NaHCO3, pH 9.6). Control wells were coated with free thyroglobulin. After washing with PBS/0.1% Tween.
20, 50 μl of sera (1:200 dilution) were added and incubated for 30 min at room temperature. Bound antibody was detected using Protein A-horseradish peroxidase (Zymed Laboratories, Inc., Seattle, WA) as described (30).

Results

Cloning and Characterization of the Genomic Organization of the T. cruzi Antigen TePO. As part of a strategy for identifying antigens shared between T. cruzi and Leishmania, we screened a L. chagasi expression library with a pool of sera from individuals with T. cruzi infection. One L. chagasi clone, named LcPO, was isolated (Y. A. W. Skeiky et al., manuscript in preparation) and used to screen a T. cruzi trypomastigote cDNA expression library by crosshybridization. A full-length cDNA clone was isolated and named TcPO (for T. cruzi P0) after comparison with other published sequences (see below).

Fig. 1A displays the entire nucleotide and deduced amino acid sequences of the full-length cDNA insert, a 1073 bp EcoRI/XhoI fragment. The sequence contains the last eight nucleotides of the trnaspliced leader sequence found on the 5' end of all trypanosome nuclearly-encoded transcripts (31), followed by a short (26 nucleotide) 5' untranslated leader seg-

---

**Figure 1.** (A) The nucleotide and predicted amino acid sequences of the full-length TePO cDNA as cloned in the λ uni-Zap expression system (5' EcoRI and 3' XhoI adaptors). The beginning and end of the cDNA are identified by the splice leader (SL) and poly(A) tail. Nucleotide and amino acid numberings are with respect to the first A of the initiation codon (underlined). The termination codon TAA is also underlined. The bacterial expressed fusion protein (which includes the 5' portion of B-gal, the multiple cloning site and the 5' untranslated region of TcPO) is ~5.6-kD larger (40.6 kD) than the coding capacity of the cDNA insert (~35 kD). The location of the restriction sites, PstI (P) and EcoRV (RV), that cut within the insert and were used in the genomic Southern are indicated. (Arrows) Positions of the first residue of the NHz-terminal deletion clone AN222 and the last residues of the COOH-terminal deletion clones AC43, AC33, and AC6, respectively. (B) Nucleotide sequence comparison of two sequenced cDNAs in their 3' untranslated regions. The upper and lower lines show the full-length TePO (top) and the partial-length AN222, respectively. (Vertical lines) Nucleotide identity. Gaps (-) have been introduced to maximize homology. The termination (TAA, underlined) and poly(A) tail (A.) residues are indicated. Numbers indicate the lengths of the 3' untranslated sequences.
ment. An open reading frame of 966 nucleotide encoding a predicted protein of ~35 kD is followed by a 73 nucleotide 3' untranslated portion terminating in a stretch of poly(A) residues.

Southern analysis of T. cruzi genomic DNA digested with enzymes that cut both within and outside of TcP0 revealed multiple hybridizing bands when probed with either the full-length TcP0 insert (Fig. 2 A) or a 3' probe (ΔN222; Fig. 1 A). These results indicate that at least two copies of TcP0 are present within the genome. This has also been confirmed from the nucleotide sequences of one other partial TcP0 cDNA. The results revealed that although both cDNA clones have identical TcP0 nucleotide sequences within their coding segments, they differ in the sequences and lengths of their 3' untranslated regions (Fig. 1 B). The weaker hybridizing bands in the EcoRV and PstI lanes of Fig. 2 may reflect on the lengths of the complementary sequence overlap with the uniformly labeled probes or the presence of more divergent members of the TcP0 family. Fig. 2 also illustrates the cross-species conservation between P0 of T. cruzi and other Trypanosoma species including T. brucei and T. lewisi. It is interesting that T. cruzi showed a different PstI hybridization pattern than the other Trypanosoma species.

**TcP0 Is the T. cruzi Homologue of the Evolutionarily Conserved Eukaryotic Ribosomal "Acidic"-type Phosphoproteins.** Comparison of the predicted amino acid sequence of TcP0 with other published protein sequences in the GenBank data base (32), revealed significant homology with members of the family of acidic phosphorylated ribosomal proteins known as the “P” or “A” proteins (12, 13, 33). Fig. 3 A shows alignment of the deduced primary structure of T. cruzi P0 with those of human (HuP0, 33) and yeast (YP0, 34). TcP0 has an overall homology of 58% (36% identity, 22% conservative substitution) with HuP0 and 62% (38% identity, 24% conservative substitution) with YP0. The lengths (322, 317, and 312 amino acids), molecular masses (35, 35.3, and 33.8 kD), and isoelectric points (5.1, 5.8, and 4.6) of TcP0, HuP0, and YP0, respectively, are very similar.

The T. cruzi P0 shows a clustering of residues that are evolutionarily conserved in higher eukaryotes. This includes an alanine- and glycine-rich region adjacent to a highly charged COOH terminus. This domain is the "hallmark" of the eukaryotic P protein family, and is the basis of their immunological crossreactivity (15, 27, 35). Like other P0 proteins, TcP0 has an arginine- and lysine-rich region (located at an equivalent position; residues 42–71). This region is hypothesized to be involved in the binding to rRNA (36). The COOH terminus of the P proteins (P0, P1, and P2) can be divided into two portions: a variable but highly charged region, and the highly conserved hydrophobic COOH terminus (Fig. 3 B).


A

| Species   | P-protein | c-terminus |
|-----------|-----------|------------|
| T. cruzi  | TcP0      | EPEEEDDOOOGMAG-LFP |
| Human     | JL5       | AAAAAEEEDDDGFG-LFP |
| Mouse     | P0        | KEKEEEDDDGFG-LFD  |
|           | P1        | KEKEEEDDDGFG-LFD  |
|           | P2        | KEKEEEDDDGFG-LFD  |
| Shrimp    | P0        | KEKEEEDDDGFG-LFD  |
|           | P1        | KEKEEEDDDGFG-LFD  |
|           | P2        | KEKEEEDDDGFG-LFD  |
| Yeast     | P0        | AEEEDDDGFG-LFP    |
|           | P1        | EEEEDDDGFG-LFP    |
|           | P2        | EEEEDDDGFG-LFP    |
| Drosophila| P2        | KEKEEEDDDGFG-LFP  |
| Dictyostelium | P0       | KEKEEEDDDGFG-LFP  |
|           | P1        | KEKEEEDDDGFG-LFP  |
|           | P2        | KEKEEEDDDGFG-LFP  |

B. Among all the P proteins analyzed thus far, TcP0 is exceptional because of the presence of nonconservative substitutions within the hydrophobic COOH terminus, and the absences of a serine and a terminal acidic residue (Fig. 3 B). The serine residue(s) in this domain have been shown to be phosphorylated in the brine shrimp Artemia salina (37), and in higher eukaryotes by casein kinase II (38). Phosphorylation of these serine residues has been reported to increase their binding affinities for the ribosome (39) or their cellular activities (40).

Trypanosoma P0 Is Constitutively Expressed. Northern analysis revealed that TcP0 is expressed as a ~1.3-kb transcript during the entire life cycle (epimastigote, trypomastigote, and amastigote) of T. cruzi (Fig. 4 A). An identical hybridization pattern was obtained using poly(A) RNA (not shown). Allowing for the poly(A) tail, the size is in agreement with that of the cloned full-length cDNA. The differences in the lengths of the 3' untranslated portions between the two sequenced cDNA clones (18 nucleotides, Fig. 1 B) are not large enough to expect multiple hybridizing bands. The same Northern also shows that TcP0 probe crosshybridizes with trypanosomes of other sequenced P proteins: JL5 (8); mouse (47); Drosophila (48); shrimp, A. salina (49); yeast, Saccharomyces cerevisiae (34, 50, 51); and Dictyostelium (52). A single gap is introduced to maximize alignment with TcP0. Serine residues within the charged domain are underlined. Differences in the hydrophobic termini are in bold and shaded.
Trypanosoma P0 is constitutively expressed. (A) Northern blot analysis. 5 μg each of the indicated total RNA from the life cycle stages of T. cruzi (E) epimastigote (e), trypomastigote (t), and amastigote (a), as well as T. brucei (Tb) bloodstream (b) and procyclic (pr) form were hybridized with radiolabeled full-length TcP0 insert. Lane c contains 10 μg of RNA derived from the mouse myoblast cell line, L6E9. The mobilities and sizes in kb nucleotides of single-stranded RNA are indicated. (B) Western blot and immunoprecipitation of in vitro translated RNA. Western blot of T. cruzi (Te; e = epimastigote, t = trypomastigote, and a = amastigote) and T. brucei (Tb; b = bloodstream and pr = procyclic form) lysates reacted with rabbit anti-rTcP0 antisera. (Lanerib) Purified polysomal fraction from the epimastigote stage of T. cruzi. (Right) Immunoprecipitation reactions of translation reactions ([35S]methionine labeled) from 5 μg of the indicated T. cruzi RNA using rabbit anti-TcP0 antisera (IVT/IP). Molecular mass markers are shown in kD. Alignment of the composite was based on the mobilities of the markers.

The transcription pattern, we performed Western analysis of T. cruzi lysates using a rabbit antiserum raised against rTcP0. As shown in Fig. 4 B, the antiserum detected a single protein of ~38 kD in both the insect (epimastigote) and mammalian (trypomastigote and amastigote) stages. Thus the expression of TcP0 reflects the transcript abundance. The serum did not crossreact with proteins of sizes expected for the T. cruzi equivalent of P1 and P2. The same blot also shows that purified T. cruzi ribosomes (lane rib) contain TcP0. The postribosomal supernatant, when subjected to similar analysis, also showed an immunoreactive band at 38 kD (not shown). This is in agreement with studies from other species demonstrating the presence of P proteins in ribosome-free cytoplasm (35, 41, 42). Fig. 4 B also shows that rabbit anti-rTcP0 antisera crossreacted with a 38-kD species in both the bloodforms and procyclic forms of T. brucei.

To determine whether endogenous TcP0 is subjected to any posttranslational modification that significantly alters its mobility, immunoprecipitates of TcP0 from in vitro translations using total T. cruzi RNA (Fig. 4 B; lanes IVT/IP) and in vitro transcribed TcP0 cDNA as template (not shown) were performed. The sizes of the precipitated bands were indistinguishable from those revealed in immunoblots of cell lysates. Taken together, these results suggest that, posttranslational events do not significantly affect the size of TcP0, and that the cloned cDNA encodes authentic TcP0 protein. Although the predicted molecular mass of TcP0 is ~35 kD, the endogenous as well as in vitro translated products migrated more slowly (~38 kD). This is attributed to the peculiar secondary structure of the P proteins (33).

Reactivity of Chagas' Patients with T. cruzi and Human P Proteins. To address the question of whether individuals with T. cruzi infection produce antibodies against TcP0, sera from 10 patients were tested on immunoblots (Fig. 5 A) containing purified rTcP0 (lanes A), as well as total trypomastigote lysate (lanes B). All ten patients (1–10) showed binding to rTcP0. The smear of bands towards the upper portion of the gel (lanes A) is the result of aggregation of rTcP0 after purification. The specificity of the reactivity of patient sera on rTcP0 is demonstrated below (Fig. 6). Thus, T. cruzi-infected individuals produce anti-TcP0 antibodies. Pooled sera from uninfected individuals showed no reactivity. To determine whether Chagas' patients produce antibodies reactive to human P proteins, we tested the same 10-patient sera described above by ELISA using an available synthetic peptide corresponding to the COOH-terminal 22 amino acid residues (C-22) of the human P2 protein (35). In human P proteins, the C-22 terminal residues are identical for P0, P1, and P2, with the exception of a single conservative substitution in P0 (an aspartic to glutamic acid) at position nine with respect to the COOH terminus (Fig. 3 B). This substitution was shown to have no effect on the binding competence of patient sera (13). Fig. 5 B shows that 9 of 10 sera reacted with the human peptide with absorbance values ranging from 3.1 to 24-fold higher than the mean of sera from uninfected controls. These findings indicate that most Chagas' patient sera with anti-T. cruzi P protein antibodies crossreact with self P proteins.

Epitope Mapping of Anti-TcP0 Antibody. To determine the TcP0 epitope(s) recognized by T. cruzi-infected individuals, we tested the reactivities of patient sera on truncated versions of rTcP0. Fig. 1 indicates the end points of the clones used, all of which were expressed in Escherichia coli as fusion proteins with β-galactosidase. TcP0ΔN222 lacks the amino 222 residues and contains only the 100 COOH-terminal portion amino acids of TcP0. When expressed in E. coli, this COOH-terminal fusion protein maintained reactivity with a pool of Chagas' sera (Fig. 6 A). The weaker and lower bands are probably the result of partial degradation of the fusion protein. Therefore, major antigenic determinant(s) of TcP0 recognized by Chagas' sera reside within the COOH terminal of the molecule. To map the epitope(s), we performed 3' deletions resulting in clones with COOH-terminal truncations of 6 (TcP0ΔC6), 33 (TcP0ΔC33), and 43 (TcP0ΔC43). All three clones maintained the same fusion amino acid residues as TcP0 until their respective deletion junctions. Coincidentally, their reading frames continued past the P0 sequence in to the plasmid for an additional 59 amino acid (~9 kD). Immunoblotting of TcP0ΔC33 and TcP0ΔC43 with a pool
Figure 5. T. cruzi-infected individuals contain antibodies that react with TcP0 and human P proteins. (A) Immunoblots showing reactivities of Chagas' patient sera with recombinant TcP0. The blots were probed with sera from individual patients (1-10) infected with T. cruzi using the following sera dilutions: 1 = 1:400; 2 = 1:200; 3 = 1:200; 4 = 1:400; 5 = 1:400; 6 = 1:400; 7 = 1:400; 8 = 1:800; 9 = 1:800 and 10 = 1:400. Strips from patients 7-12 were exposed twice as long (12 h) as those of 1-6. (B) Normal sera pool from four uninfected individuals (1:100 dilution each). Sera from rabbits immunized with rTcP0 (rP0) was used at a dilution of 1:1000. Lanes A contain 0.5 μg of recombinant TcP0, and lanes B, 5 μg of trypomastigote lysate. (Left) Size markers in kD. (B) ELISA showing the reactivities of the same ten individual sera (see above) on human C22 peptide. The sera were used at a 1:200 dilution. Background absorbance (no serum controls) was subtracted from all samples. Relative absorbance = absorbance value of test sera + mean of the absorbance of sera from uninfected individuals.

SLE Patients Contain Antibodies that Crossreact with TcP0. Approximately 10-20% of patients with SLE possess anti-ribosomal antibodies. These antibodies react predominantly with three of the ~80 ribosomal proteins: P0, P1, and P2 (12, 13). Sera from these patients also react with the homologous antigens present in rats, shrimp, and yeast (12). We therefore tested the binding of eight SLE sera previously characterized as anti-human P positive (27, 14) on TcP0. We found that although all eight SLE sera reacted with rTcP0 with varying intensities, they either showed no reactivity or bound weakly to truncated rTcP0ΔC6. Fig. 7 (A and B) is representative of two individual SLE sera, showing their variable reactivities with rTcP0 (lanes 1) and rTcP0ΔC6 (lanes 2). The sera also reacted with a 38-kD band in T. cruzi lysate (lanes 4) with intensities proportional to their respective reactivities on rTcP0. Fig. 7 A, lane 5 is a control showing that the band detected by the rabbit anti-rTcP0 sera on T. cruzi lysate comigrates with the 38-kD band detected by SLE sera. As expected, the SLE sera reacted with proteins with migrations characteristic of the P protein family in human K562 cell ly-
The position of human and TcP0, respectively. Lanes 3 and 4 contain 2.5 and 10 μg each of total K562 lysates, in containing parasite lysate (as in lane 4) but reacted with rabbit anti-TcP0 sera. The position of human and T. cruzi P0 is indicated, as are those of human P1 and P2.

Figure 7. Patients with SLE contain antibodies that crossreact with TcP0. Immunoblots showing reactivities of two SLE sera (1:250 dilution) on TcP0 (A and B). Lanes 1 and 2 contain 2.5 μg of purified rTcP0 and ΔC6, respectively. Lanes 3 and 4 contain 2.5 and 10 μg each of total K562 and T. cruzi trypomastigote lysates, respectively. Lane 5 is a control containing parasite lysate (as in lane 4) but reacted with rabbit anti-TcP0 sera. The position of human and T. cruzi P0 is indicated, as are those of human P1 and P2.

sates (lanes 3). The SLE sera containing anti-human P activity also reacted with other species in T. cruzi lysates, including a very strong ~15-kD band and two weaker bands in the ~17-kD range. Any of these might correspond to the T. cruzi equivalent of P1 and P2.

Discussion

We report on the cloning and characterization of a T. cruzi full-length cDNA, TcP0, identified as the parasite equivalent of the ribosomal phosphoprotein P0. The evidence supporting the identity of the cDNA as TcP0 is the homology (~60%) to other eukaryotic P0 proteins in sequence, size (~38 kD), acidic isoelectric point, and ribosomal localization. TcP0 is present as multiple copies within the genome, and for at least two sequenced cDNAs, they have identical coding segments, but diverge markedly in their 3' untranslated regions. TcP0 is transcribed as a ~1.3-kb mRNA that is constitutively expressed in all stages of the parasite life cycle. Cross-species studies demonstrated that P0 is highly conserved in other Trypanosoma species at both the nucleotide and protein levels, as demonstrated by Southern and immunoblot assays.

The 38-kD TcP0 protein, like the mRNA, is constitutively expressed and is associated with the ribosomal translation machinery. This constitutive expression coupled with the cross-species conservation strongly suggests that TcP0 serves a housekeeping function, as expected for a P0 ribosomal protein. Immunoblot analysis with ten randomly selected sera from T. cruzi-infected individuals, revealed that all contained IgG antibodies against TcP0, albeit of varying levels.

In higher eukaryotes, the P0 protein family comprises three antigenically crossreactive proteins P0, P1, and P2 (12, 13, 15, 33). P0, the largest protein of the family, has an apparent molecular mass of ~38 kD, while P1 and P2 migrate as a doublet in the 14–19 kD on SDS-polyacrylamide gels. These proteins all possess an alanine- and glycine-rich region of 20–30 residues adjacent to a highly charged COOH terminus, but show much lower homology in the remainder of the protein. P1 and P2 are believed to be functional homologues of the bacterial proteins L7/L12, and P0 is thought to be the homologue of the L10 protein. These proteins are known to play an essential role in the elongation step of protein synthesis (39, 43). The proteins form a pentameric complex composed of two molecules each of P1 and P2, and one of P0 (33, 35, 44). The carboxy 17 amino acid residues of the P proteins are shared both within and across species (33, 35). In addition, a mouse mAb raised against chicken ribosomes was shown to bind to all three of the human P proteins (27, 15).

We mapped the antigenic epitope(s) of TcP0, and demonstrated that the reactivities of sera from T. cruzi-infected individuals require a single linear determinant. Deletion of the carboxy six hydrophobic residues abrogates the immunological reactivity of TcP0 on Western blots. This is analogous with the binding properties of SLE anti-P autoantibodies where the only required epitope has been mapped to the carboxy 11 residues (27). We demonstrated that anti P-positive SLE sera do in fact recognize TcP0. The variable degree of reactivities of the SLE sera on TcP0 is in agreement with previous studies on human P proteins which revealed that different SLE patients have variable specificities and reactivities with shorter, as well as modified peptides of the C11 residues (27). Given that the hydrophobic COOH-terminus of TcP0 is different from those of the human P proteins, it is surprising that anti-P SLE sera reacted as well as they did on TcP0. More interesting, is the finding that, like sera from T. cruzi-infected individuals, the reactivity of SLE sera on TcP0ΔC6 is either abrogated or greatly reduced. In addition, the SLE sera also showed reactivities on parasite lysates with proteins of sizes characteristic of P0, P1, and P2. Given that the hydrophobic terminal residues of T. cruzi P1 is identical to those of the human P proteins (see below and Fig. 3 B), it is very likely that the strong reactive ~15-kD 'band' detected by the SLE sera on T. cruzi lysate is TcP1 and/or TcP2. The SLE sera also reacted with two additional bands of ~50 kD (above P0) on parasite lysates. Whether these represent modified forms or complexes of T. cruzi P proteins, or different T. cruzi antigens that are shared with human and T. cruzi, remains to be determined. The reciprocal assay using the antigenic C22 peptide of human P2 demonstrated that most T. cruzi-infected individuals possess antibodies that crossreact with self-ribosomal P proteins. However, their reactivities did not correlate with the results of the immunoblots on rTcP0, indicating that different T. cruzi-infected individuals show variable crossreactivities for a substituted COOH-terminal domain.

The results of the Western blot and immunoprecipitations indicate that either TcP0 is not antigenically related to T. cruzi P1 and P2, or that the specificity of the rabbit anti-TcP0 serum does not include the conserved COOH terminus. The COOH terminus of TcP0 (which shows differences in the linear arrangement of the residues with other P proteins), may also be different from that of the T. cruzi P1 and P2. This is not unreasonable given that a cloned T. cruzi sequence encoding a 35-residue peptide named JL5 (8) has a COOH
terminus that is different from TcP0, but almost identical to that of the human P proteins (12/13 identical). JL5 does not represent a cloned fragment of TcP0 since it differs in sequence, and detects an 0.7-kb transcript too small to encode P0, but adequate to encode P1 or P2. It is different from P2 (Skeiky et al., unpublished results) and appears to represent the COOH-terminal portion of P1 (45). This could explain the lack of immunological crossreactivity between TcP0, TcP1, and TcP2 of T. cruzi using the rabbit anti-TcP0 serum. The differences in the specificity of the human and rabbit sera may reflect outcomes of antigenic stimulation initiated by T. cruzi infection versus immunization with purified monovalent rTcP0.

Levitus et al., (46) recently demonstrated that sera from Chagas' heart disease patients crossreact with recombinant human ribosomal P1 and P2 proteins. In addition, it was previously shown (8) that immunoselected antibodies against JL5 recognize a predominant 38-kD antigen on T. cruzi lysate which, presumably, is TcP0. However, it remains unresolved whether the JL5-immunoselected reactivity was initiated through recognition of antigenic determinant(s) of TcP0 or JL5. Nevertheless, this lends further support to our argument that, despite the differences in the fine organization of the COOH-terminal residues of TcP0, antibodies directed against it can crossreact with the COOH-terminal region of the prototype P proteins both within and across species. We demonstrated that the reciprocal also holds true.

It was also shown that 57% (of 44 patients) of selected T. cruzi-infected individuals (chronic Chagas' heart disease) reacted with the C13 terminal residues of JL5 (46). In our studies, we found that all ten randomly selected T. cruzi infection sera recognized TcP0. The combined results suggest that the antibodies against T. cruzi ribosomes can have unique, as well as shared specificities for the P protein family both within, as well as across species.

The possibility that TcP0 antibodies found in sera of T. cruzi-infected individuals are the result of leakage of self P proteins and subsequent immunization is unlikely since not all sera reacted with the human C22 peptide, although they all reacted with TcP0. Hines et al. (14) demonstrated that in the autoimmune MRL mice, anti-P autoantibodies could be induced by immunization with xenogenic A. salina ribosomal P proteins, but not by syngenic mouse ribosomes. In addition, it was shown that as with the spontaneous anti-P autoantibodies of MRL mice, the induced anti-P autoantibodies were exclusively directed against the COOH-terminus. The ability of A. salina P proteins to induce anti-P autoantibodies was attributed to a single nonconservative substitution in the COOH-terminal 11 residues (serine in mouse and glutamic acid in A. salina).

We propose that through mechanisms involving molecular mimicry, the ribosomal P proteins participate in the induction of autoreactive antibodies in Chagas' disease. This is made possible because of differences at the COOH terminus of the parasite (particularly TcP0) and host P proteins. Through synergistic mechanisms, the T. cruzi P proteins (P0, P1, and P2) could provide a multivalent epitope(s) which may be necessary for the establishment of an autoimmune process similar to that described for the murine system (14).

We thank Dr. James Burns for advice on the purification of recombinant antigens and the production of rabbit anti-TcP0 serum; Dr. Marvin Fritzler of the University of Calgary, Canada, for providing some of the SLE anti-P sera used in this study; Dr. Nathan Brot of the Roche Institute of Molecular Biology, Nutley, NJ for providing the C22 peptide; and Drs. Julio Voltarelli, University of Sao Paulo and Roberto Badaro, University of Bahia, Brazil, for Chagas' patient sera. We also thank Karen Kinch for assistance with manuscript preparation.

This work was supported in part by grants AI-22726 and AI-16282 (S. G. Reed) and AR-38915, and a research career development award (K. Elkon) from the National Institutes of Health. Dr. Yasir Skeiky is a Fellow of the Medical Research Council of Canada.

Address correspondence to Dr. Steven Reed, Seattle Biomedical Research Institute, 4 Nickerson Street, Seattle, WA 98109-1651.

Received for publication 2 January 1992 and in revised form 23 March 1992.

References
1. Khoury, E.L., V. Ritacco, P.M. Cosio, R.P. Laguens, A. Szarfman, C. Diez, and R.M. Arana. 1979. Circulating antibodies to peripheral nerve in American trypanosomiasis (Chagas' disease). Clin. Exp. Immunol. 36:8.
2. Cosio, P.M., C. Diez, A. Szarfman, E. Kreutzner, B. Candido, and R.M. Arana. 1974. Chagasic cardiopathy. Demonstration of a serum gammaglobulin factor which reacts with endocardium and vascular structures. Circulation. 49:13.
3. Meszi, E.A., G. Levitus, M.H. Joskowicz, G. Dighiero, M.H.V. Regenmortel, and M. Levin. 1990. Major Trypanosoma cruzi antigenic determinant in Chagas' heart disease shares homology with the systemic Lupus erythematosus ribosomal P protein epitope. J. Clin. Microbiol. 28:1219.
4. Szarfman, A., V.P. Terranora, S.L. Renard, J.M. Foidart, F.M.
21. Skeiky, Y.A.W., and K. Iatrou. 1990. Silkmoth chorion antigen of Trypanosoma cruzi that mimics mammalian nervous tissue. J. Exp. Med. 169:641.

22. Feinberg, A.P., and B. Vogelstein. 1984. A technique for radiosilabelling DNA restricted endonuclease fragments to high specific activity. Anal. Biochem. 132:156.

23. Henikoff, S. 1984. Unidirectional digestion with exonuclease III in DNA sequence analysis. Gene. 28:351.

24. Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain termination inhibitors. Proc. Natl. Acad. Sci. USA. 74:5463.

25. Reed, S.G., E.M. Carvalho, C.H. Sherbert, D.P. Sampaio, D.M. Russo, O. Bacela, D.L. Pihl, J.M. Scott, A. Barral, K.H. Grabstein, and W.D. Johnson, Jr. 1990. In vitro responses to Leishmania antigens by lymphocytes from patients with leishmaniasis or Chagas' disease. J. Clin. Invest. 85:690.

26. Burns, J.M., J.M. Scott, D.M. Russo, E.M. Carvalho, C.J. March, K. Van Ness, and S.G. Reed. 1991. Characterization of a membrane antigen of Leishmania amazonensis that stimulates human immune responses. J. Immunol. 146:742.

27. Elkon, K., E. Bonfa, R. Llovet, W. Danho, H. Weissbach, and N. Brot. 1988. Properties of the ribosomal P2 protein autoantigen are similar to those of foreign protein antigens. Proc. Natl. Acad. Sci. USA. 85:5186.

28. Laemmli, U.K. 1970. Clearage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.) 227:680.

29. Towbin, A., T. Stachlin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some application. Proc. Natl. Acad. Sci. USA. 76:4350.

30. Reed, S.G., W.G. Shreffler, J.M. Burns, Jr., J.M. Scott, M. de G. Orge, H.W. Ghali, M. Siddig, and R. Badaro. 1990. An improved serodiagnostic procedure for visceral leishmaniasis. Am. J. Trop. Med. Hyg. 43:632.

31. Milhauzen, M., R.G. Nelson, S. Sather, M. Selkirk, and N. Agabian. 1984. Identification of a small RNA containing the Trypanosoma spliced leader: a donor of shared 5' sequences of Trypanosomatid mRNAs? Cell. 38:721.

32. Pearson, W.R., and D.J. Lipman. 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA. 85:2444.

33. Rich, B.E., and J.A. Steitz. 1987. Human acidic ribosomal phosphoproteins P0, P1, and P2: analysis of cDNA clones, in vitro synthesis, and assembly. Mol. Cell. Biol. 7:4065.

34. Mitsui, K., and K. Tsurugi. 1988. cDNA and deduced amino acid sequence of 38 kDa-type acidic ribosomal protein AO from Saccharomyces cerevisiae. Nucleic Acids. Res. 16:3573.

35. Elkon, K., S. Skelly, A. Parnassa, W. Moller, W. Danho, H. Weissbach, and N. Brot. 1986. Identification and chemical synthesis of a ribosomal protein antigenic determinant in systemic lupus erythematosus. Proc. Natl. Acad. Sci. USA. 83:7419.

36. Mitsui, K., T. Nakagawa, and K. Tsurugi. 1989. The gene and the primary structure of acidic ribosomal protein A0 from yeast Saccharomyces cerevisiae which shows partial homology to bacterial ribosomal protein L10. J. Biochem. 106:223.

37. Maassen, J.A., E.N. Schop, J.H.G.M. Brands, F.J. Van Hemert, J.A. Lenstra, and W. Moller. 1985. Molecular cloning and analysis of cDNA sequences for two ribosomal proteins from Artemia. The coordinate expression of genes for ribosomal proteins and elongation factor I during embryogenesis of Artemia. Eur. J. Biochem. 149:609.

38. Hasler, P., N. Brod, H. Weissbach, A.P. Parnassa, and K.B. Elkon. 1991. Ribosomal proteins P0, P1, and P2 are phosphorylated by casein kinase II at their conserved carboxyl termini. J. Biochem. 226:13815.

39. Sanchez-Madrid, F., R. Reyes, P. Conde, and J.P.G. Ballesta. 1979. Acidic ribosomal proteins from eukaryotic cells: effect on ribosomal functions. Eur. J. Biochem. 98:409.

40. MacConnell, W.P., and N.O. Kaplan. 1982. The activity of the acidic phosphoproteins from the 80s rat liver ribosome.
41. Moller, W., and J.A. Maassen. 1985. On the structure, function and dynamics of L7/L12 from Escherichia coli ribosomes. In Structure, Function and Genetics of Ribosomes. B. Hardesty and G. Kramer, editors. Springer-Verlag New York Inc., New York 310–325.

42. Zinker, S., and J.R. Warner. 1976. The ribosomal proteins of Saccharomyces cerevisiae. J. Biochem. 251:1799.

43. Brot, N., and H. Weissbach. 1981. Chemistry and biology of E. coli ribosomal protein L12. Mol. Cell. Biochem. 36:47.

44. Uchiumi, T., A.J. Wahba, and R.R. Trout. 1987. Topography and stoichiometry of acidic proteins in large ribosomal subunits from Artemia Salina as determined by crosslinking. Proc. Natl. Acad. Sci. USA. 84:5580.

45. Nafziger, D.A., R.F. Recinos, C.A. Hunter, and J.E. Donelson. 1991. Patients infected with Leishmania donovani chagasi can have antibodies that recognize heat shock and acidic ribosomal proteins of Trypanosoma cruzi. Mol. Biochem. Parasitol. 49:325.

46. Levitus, G., M.H. Joskowicz, M.H.V. Van Regenmortel, and M.J. Levin. 1991. Humoral autoimmune response to ribosomal P proteins in chronic Chagas’ heart disease. Clin. Exp. Immunol. 85:413.

47. Krowczynska, A.M., M. Coutts, S. Makrides, and G. Brauerman. 1989. The mouse homologue of the human acidic ribosomal phosphoprotein P0: a highly conserved polypeptide that is under translational control. Nucleic Acids Res. 17:6408.

48. Qian, S., J.Y. Zhang, M.A. Kay, and M.J. Lorena. 1987. Structural analysis of the Drosophila rpA1 gene, a member of the eucaryotic 'A' type ribosomal protein family. Nucleic Acids Res. 15:987.

49. Amos, R., W. Pluijms, and W. Moller. 1979. The primary structure of ribosomal protein eL12/eL12-P from Artemia salina 80s ribosomes. FEBS (Fed. Eur. Biochem. Soc.) Lett. 104:85.

50. Mitsui, K., and K. Tsurugi. 1988. cDNA and deduced amino acid sequence of acidic ribosomal protein A2 from Saccharomyces cerevisiae. Nucleic Acids Res. 16:3575.

51. Mitsui, K., and K. Tsurugi. 1988. cDNA and deduced amino acid sequence of acidic ribosomal protein A1 from Saccharomyces cerevisiae. Nucleic Acids Res. 16:3574.

52. Prieto, J., E. Candel, and A. Coloma. 1991. Nucleotide sequence of a cDNA encoding ribosomal protein P0 in Dictyostelium discoideum. Nucleic Acids Res. 19:1342.