Trypanosoma cruzi Surface Mucins with Exposed Variant Epitopes*

Guido D. Pollevick‡§§, Javier M. Di Noia§§§, Maria L. Salto**,§§, Carlos Lima** §§§, M. Susana Leguizamón† ††, Rosa M. de Lederkremer† ††, and Alberto C. C. Frasch‡‡‡

From the ††Instituto de Investigaciones Biotecnológicas, Instituto Tecnológico de Chascomús (CONICET), Universidad Nacional de General San Martín, Av. Gral. Paz s/n, INTI, Edificio 24, 1650, San Martín, Pcia. de Buenos Aires, **CIHIDECAR (CONICET) Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, and the ‡‡‡Departamento de Microbiología, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina

Received for publication, January 12, 2000, and in revised form, June 7, 2000
Published, JBC Papers in Press, June 7, 2000, DOI 10.1074/jbc.M000253200

The protozoan parasite Trypanosoma cruzi, the agent of Chagas disease, has a large number of mucin molecules on its surface, whose expression is regulated during the life cycle. These mucins are the main acceptors of sialic acid, a monosaccharide that is required by the parasite to infect and survive in the mammalian host. A large mucin-like gene family named TcMUC containing about 500 members has been identified previously in T. cruzi. TcMUC can be divided into two subfamilies according to the presence or absence of tandem repeats in the central region of the genes. In this work, T. cruzi parasites were transfected with one tagged member of each subfamily. Only the product from the gene with repeats was highly O-glycosylated in vivo. The O-linked oligosaccharides consisted mainly of β-3-Galp(1→4)-GlcNac and β-3-Galp(1→4)(β-3-Galp(1→6))d-GlcNac. The same glycosyl moieties were found in endogenous mucins. The mature product was anchored by glycosylphosphatidylinositol to the plasma membrane and exposed to the medium. Sera from infected mice recognized the recombinant product of one repeats-containing gene thus showing that they are expressed during the infection. TcMUC genes encode a hypervariable region at the N terminus. We now show that the hypervariable region is indeed present in the exposed mature N termini of the mucins because sera from infected hosts recognized peptides having sequences from this region. The results are discussed in comparison with the mucins from the insect stages of the parasite (Di Noia, J. M., D’Orso, I., Sánchez, D. O., and Frasch, A. C. C. (2000) J. Biol. Chem. 275, 10218–10227) which do not have variable regions.

Mucins in vertebrate cells are highly O-glycosylated proteins having relevant roles in protection and in cell-cell interactions.

The former function is accomplished mainly by high molecular weight epithelial mucins and the latter by the much smaller endothelial and leukocyte mucins, all of them encoded by a few dozen genes (1, 2). In the lower eukaryote Trypanosoma cruzi, the protozoan agent of Chagas disease, mucins seem to have essential functions and unusual characteristics. The parasite mucins, located on the surface membrane, are the main acceptors of sialic acid transferred from host’s glycoconjugates by the trypanosomatid-specific enzyme trans-sialidase (3). Surface mucins seem to be involved, after acquiring sialic acid, in the invasion of host cells by the parasite and in the protection against the alternative complement pathway (4–7). Both steps are essential for parasite replication and survival in mammals.

Mucins from T. cruzi were first isolated with aqueous-phenol and called bands A, B, and C (8, 9). These glycoproteins were strongly labeled after intact parasites were treated with galactose oxidase/NaB3H4, thus proving their surface location (10). More recently, the structure of the O-linked sugars (11–13) was described. The oligosaccharides are linked through GlcNAc, rather than the GalNac commonly found in vertebrate mucins. Most of the O-linked GlcNAc residues are substituted with 1–5 galactosyl units. The structures of the O-linked oligosaccharides are conserved between epimastigotes (the form of the parasite present in the insect vector midgut) and metacyclic trypomastigotes (the infective form in the feces of the insect). However, there are some polymorphisms among the strains, the most important one being the presence of galactofuranosan in the G strain (12, 13), whereas in the Y strain the O-linked oligosaccharides contain only galactopyranosan (11). The mucins present in these forms of the parasite are glycoproteins of about 35–50 kDa, whereas those in cell culture-derived trypomastigotes are considerably larger (70–200 kDa). The latter molecules have O-linked oligosaccharides terminating with α-Galp residues, epitopes that elicit a lytic antibody response in the human (14).

The largest gene family encoding mucin-like genes described so far is present in T. cruzi, showing diversity among and within strains of the parasite (15, 16). 500 mucin-like genes per haploid genome have been estimated to be present in this parasite (16), all members of a family named TcMUC because their overall structure resembled that of mucin genes in higher eukaryotic cells (17). They all have conserved 5’- and 3’-regions, encoding a predicted signal peptide at the N terminus, and a C-terminal region that includes a putative sequence for a GPI anchor. Between the conserved ends, there is a variable

* The work was supported in part by grants from the Agencia Nacional de Promoción Científica y Tecnológica, Argentina, the Swedish Agency for Research Cooperation with Developing Countries (SAREC-SIDA), the International Atomic Energy Agency, the Consejo Nacional de Investigaciones Científicas y Técnicas, and the University of Buenos Aires. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†† CONICET researchers.
‡‡‡ CONICET fellow.
§§ University of Buenos Aires fellow.
§§§ CONICET principal professional.
¶ Supported in part by an International Research Scholar grant from the Howard Hughes Medical Institute. To whom correspondence should be addressed. Tel.: 54-11-4580-7255; Fax: 54-11-4752-9639; E-mail: cfprasch@iib.unsam.edu.ar.

The abbreviations used are: GPI, glycosphingolipidinositol; HV, hypervariable; GST, glutathione S-transferase; CRD, cross-reacting determinant; dpi, days postinfection; PAGE, polyacrylamide gel electrophoresis; HPAGE-PAD, high pH anion exchange chromatography with pulse amperometric detection; PBS, phosphate-buffered saline; PI, phosphatidylinositol.
The central domain that characterizes two subfamilies within the mucin-like gene family. In one of these subfamilies, the central region encodes repetitive motifs with the consensus sequence TnKP2. In the second subfamily, the central domain codes for a region lacking amino acid repeats, which is rich in codons for Thr, Ser, and Pro residues, but which is variant in sequence among the members (16). Because of its organization and amino acid composition, these central regions were predicted to contain the target sites for O-glycosylation (17). This assumption is supported by the facts that a repeats-containing member became highly glycosylated when transfected in vero cells (15) and that the synthetic peptide KPnTnKP2 is a good substrate for the enzyme UDP-N-acetylglucosamine:peptide N-acetylglucosaminyl transferase, which starts O-glycosylation in T. cruzi (18). However, a direct demonstration that these genes indeed code for the core protein of parasite mucins is still lacking. The small sequence (8–16 amino acids) between the signal peptide and the repeats, likely to code for the mature N terminus of mucins, is highly variable among members of the family (16). Comparison of this hypervariable (HV) region from 32 cDNA clones showed 22 different variants (16).

A second gene family encoding mucin-type polypeptides (TcSMUG) was recently reported in T. cruzi (17). This family contains members that code G418 resistance using 500 μg/ml Geneticin (Sigma) and used as template for both RA strain and of CL-Brener clone of T. cruzi. A similar result was obtained when each gene was expressed in both strains of the parasite, although the levels of product expression were very different (results not shown). Transcription of the recombinant genes was analyzed by Northern blot at day 6–7 postinfection (19).

## MATERIALS AND METHODS

### Parasites

Epimastigotes of the T. cruzi RA strain (20) and the CL-Brener cloned stock (21) were grown axenically with shaking at 28 °C in BHT medium (22) supplemented with 10% fetal calf serum (Life Technologies, Inc.). Cell-derived trypomastigotes, equivalent to the bloodstream forms of the parasite, were obtained by centrifugation from the supernatant of infected vero cells at day 6–7 postinfection (19).

### Construction of Epitope-tagged Mucin Genes and T. cruzi Transformation

Because sugars might hinder the access of antibodies to protein epitopes of molecules expected to be heavily O-glycosylated like TcMUC products in T. cruzi, a tag made up of the amino acid residues 21–44 of the HCV core protein (23), which lacks N- and O-glycosylation sites, was inserted within the sequence predicted to encode the mature N-terminal region of MUC-CA3 and MUC-RA2 (17). Gene fusions were generated by polymerase chain reaction following standard protocols (24) and using the oligonucleotides indicated in Table I. The region spanning the repeats and C-terminal domains of MUC-CA3 was amplified using primers MREcoRI and P2 and cloned into pBlueScript KS II (Stratagene, San Diego, CA). Primers MNREcoRI and P2 were used to do the same with the central and C-terminal domains of MUC-RA2. Oligonucleotides C21–44A and C21–44B were annealed to create a double-strand insert encoding the HCV core epitope that was cloned into MUC-RA2. Insertion of the tag into the upstream sequences of MUC-RA2 was assessed by restriction analyses, and the transfectants were selected using pRIBOTEX plasmids, using a slight modification of described procedures (25). The transfected parasites were selected by the pRIBOTEX expression vector pRIBOTEX (25), kindly provided by Dr. R. Hernández (Universidad Nacional Autónoma de México, Mexico), and the pET-25b(+) (Novagen, Madison, WI) Escherichia coli expression vector.

Epimastigotes of T. cruzi were transformed by electroporation with the Qiagen-purified (QIAGEN Inc, Chatworth, CA) recombinant pRlBOTEX plasmids, using a slight modification of described procedures (26). The transfected parasites were selected by the plRlBOTEX-encoded G418 resistance using 500 μg/ml Geneticin (Sigma) and used as populations after 40 days of selection. MUC-R and MUC-NR were transfected into epimastigotes of both RA strain and of CL-Brener clone of T. cruzi. A similar result was obtained when each gene was expressed in both strains of the parasite, although the levels of product expression were very different (results not shown). Transcription of the recombinant genes was analyzed by Northern blot washed at 65 °C in 0.1 × SSC, 0.1% SDS as described (19).

### Table I

| Name | Sequence | Template |
|------|----------|----------|
| MRCagII | tgcgcgcggagatcATGAAATACCTACATCAGATGAG | MUC-CA3 |
| MRCagII | ggaagTCATACCTTCTGACCAGGCAAGG | MUC-CA3 |
| MREcoRI | ggagatCTAGTCAGAACAATACCTACAG | MUC-CA3 |
| P2 | cccagactCTGTCGCACTACGTTAGGTAAGT | MUC-CA3 |
| MRCaagII | tgcgcgcggagatcATGACAGCTGCGCTGCTG | MUC-RA2 |
| MRCaagII | ggaagTCATACCTTCTGACCAGGCAAGG | MUC-RA2 |
| MREcoRI | ggagatCTGTCGCACTACGTTAGGTAAGT | MUC-RA2 |
| C21–44A | gatcCCGATTGAAATACCTGACGCGGCAAGT | HCV Core |
| C21–44B | ggcgsGCGTGTCTGCGCTGCGCTGCGCTGCGGCTGCTTGGG | HCV Core |
| E13S | gatcCGCTGCGGCAAGGGTGGTGGGTGCAAAAAGCGAATAACG | EMUc-13 |
| E13A | aattctGTATTTCGATTGTTGTTGTTGGTCAAAAAGCGAATAACG | EMUc-13 |
| T15S | gatcCTGCGTGTCTGCGCTGCGCTGCGCTGCGGCTGCTTGGG | EMUc-15 |
| T15A | aattctGTATTTCGATTGTTGTTGTTGGTCAAAAAGCGAATAACG | EMUc-15 |
| T18S | gatcCTGCGTGTCTGCGCTGCGCTGCGCTGCGGCTGCTTGGG | EMUc-18 |
| T18A | aattctGTATTTCGATTGTTGTTGTTGGTCAAAAAGCGAATAACG | EMUc-18 |
| NCA2S | gatcCGCTGCGGCAAGGGTGGTGGGTGCAAAAAGCGAATAACG | MUC-CA2 |
| NCA2A | aattctGTATTTCGATTGTTGTTGTTGGTCAAAAAGCGAATAACG | MUC-CA2 |
Antibodies and Serum Samples

Anti-tag is a polyclonal serum raised in rabbit against the purified recombinant 21–44 amino acid residues of HCV core protein (23) expressed in pGEX-1 (Amersham Pharmacia Biotech) as a fusion with the Schistosoma japonicum glutathione S-transferase (GST). This serum was preadsorbed with GST before use to prevent background signals from T. cruzi homologous enzymes. Anti-cross-reacting determinant (CRD) was obtained from Oxford GlycoSystems, Rosevala, NY. Anti-GST is a polyclonal serum raised in rabbit against purified recombinant GST expressed from the pGEX-1 plasmid vector. Monoclonal antibody 3F5 was a kindly gift from Dr R. Mortara (Escola Paulista de Medicina, Sao Paulo, Brazil).

Sera from 10 rabbits infected with different T. cruzi strains and bled at 15, 30, and 60 days postinfection (dpi) were kindly given by Dr. D. Sánchez (Instituto de Investigaciones Biotecnologicas, San Martín, Argentina). Sera from 12 infected humans in the chronic stage of the disease, as assessed by their differential reactivity against cruzipain and SAPA T. cruzi antigens (27), were used. Mice sera used in Table II were from 42 individuals, each one belonging to any of four different mouse strains, infected with one of seven different T. cruzi strains and bled at postinfection times ranging from 8 to 210 days and so being from the chronic or acute stages of the infection (for details, see the Table II legend). Sera used in Table III were obtained from 13 1-month-old mice sera from different infected animals diluted 1/100 and 125I-protein A.

Preparation of Unlabeled and Labeled Alditols

The corresponding alditols were prepared by reduction of 0.2 mmol of disaccharide or trisaccharide in 9/1 methanol/water (10 ml) with NaBH₄ (2 mM). The mixture was left overnight at room temperature, and the solution was decanted for elution through a column of Bio-Rad AG 50W-X12 (H⁺ form) resin. The solvent was evaporated, and the boric acid was eliminated by five successive coevaporations with methanol. The purity of the alditol was checked by TLC. For the preparation of the labeled alditols, 100 µg of the sugars was reduced with 500 µCi of NaB³H₄ (NEN Life Science Products) in water for 1 h at room temperature, followed by the addition of unlabeled NaBH₄ and processing as above.

Expression and Purification of Recombinant Proteins

Protein M76—The TcMUC repeats-containing gene MUC-M76 (GenBank accession number L20809) was cloned in pGEX-1X-1 vector (Amersham Pharmacia Biotech). The resulting recombinant protein spanned from the HV region to the conserved C terminus fused with GST.

Proteins GST-E13, T15, T18, and NCA—Two complementary oligonucleotides having the sequence encoding the HV region from clones EMUC-E13, EMUC-T15, EMUC-T18 (16), and MUC-C2A (17) were synthesized. Sense and antisense oligonucleotides (Table I) were annealed. All of the GST fusion proteins were purified identically by affinity chromatography on glutathione-Sepharose beads (Sigma) following manufacturer’s instructions. Their purity was assessed by SDS-PAGE and they were quantified using Bradford reagent (Bio-Rad).

GeL Electrophoresis and Western Blots

Gel electrophoresis was performed in 7.5 or 12.5% polyacrylamide in the presence of 0.1% SDS (SDS-PAGE). When needed, gels were prepared for fluorography (29), dried, and exposed to Kodak X-Omat AR-5 films at −70 °C. For Western blot, parasites were resuspended in lysis buffer (150 mM NaCl, 1% Nonidet P-40, 50 mM Tris-HCl, pH 7.5, in the presence of 50 µM E64 and 1 µM phenylmethylsulfonyl fluoride), at 10⁶ parasites/ml, and 5 × 10⁵ epimastigotes were loaded in each lane. After SDS-PAGE, lysates were transferred to nitrocellulose filters (Life Technologies, Inc.), reacted with the appropriate sera, and developed with 125I-protein A (NEN Life Science Products), reacted with the appropriate sera, and developed with 125I-protein A.
Gene Identification and Structure of \( T. \) cruzi Mucins

Fig. 1. A, schematic representation of proteins derived from the transfected repetitive (MUC-R) and nonrepetitive (MUC-NR) MUC genes of \( T. \) cruzi. The structure of the two deduced products is represented with their different regions indicated. The dashed line indicates a gap for best alignment. Degenerate repeats are indicated as black boxes. The boxed sequence of the central and Thr-rich regions is indicated above or below the corresponding representative box. The triangles indicate N-glycosylation consensus sequences. The percentage of similarity is indicated for each region below the scheme. Calculations were done using the program DNASTAR (DNASTAR Inc., Madison, WI). The drawing is not in scale.

B, Northern blot analysis of transfected populations. Total RNA from transfected (MUC-R and MUC-NR transfected with pMUC-R and pMUC-NR respectively) and wild type (wt) \( T. \) cruzi populations were hybridized with an antisense oligonucleotide of the tag.

24 h at 37 °C, acidified with \( \mathrm{m} \) acetic acid, N-acetylated (36), deacetylated by passage through AG 50W-X12 (H\(^{+}\)), and dried in vacuo at room temperature. Boric acid was removed by repeated coevaporations with methanol. The labeled sugar alditols were purified by Bio-Gel P-2 chromatography.

\textbf{Acetylation}

A sample was acetylated with acetic anhydride/pyridine (1/1) for 30 min at 100 °C and dried under a stream of \( \mathrm{N}_2 \). A mixture of acetic anhydride/acetic acid/concentrated \( \mathrm{H}_2\mathrm{SO}_4 \) (10/10/1) was added, and incubation proceeded for 8 h at 37 °C. After the addition of 40 \( \mu \)l of pyridine and evaporation (three times with additions of toluene), partition was performed with 1 ml of water and chloroform. The organic phase was evaporated and deacetylated with sodium methoxide at room temperature for 1 h. The mixture was deacetylated by passage through AG 50W-X12 (H\(^{+}\)).

\textbf{Metabolic Labeling of \( T. \) cruzi}

CL-Brener epimastigotes in the logarithmic growth phase, and cell-derived trypomastigotes from 1-week-infected Vero cells monolayers were harvested, washed twice with PBS, and resuspended at \( 10^9/\text{ml} \) in minimal essential medium/Select-amine (Life Technologies, Inc.) Thr- and fetal calf serum-free, supplemented with 1.5 mg/ml glucose. After a 30-min incubation at 37 °C, a pulse of \( \text{[14C]}\)Thr (NEN Life Science Products) was added and incubated further for 10 min, after which a 2-h chase was done by adding Thr to 0.5 \( \mathrm{mM} \) and 2% fetal calf serum. Parasites were harvested, washed twice in PBS, resuspended in lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate with 1 mM phenylmethylsulfonyl fluoride and 0.5 mM N-\( \text{N\text{-}} \)tosyl-L-lysine chloromethyl ketone) and submitted to aqueous phenol extraction. The aqueous layer was dialyzed, lyophilized, and their mRNA level can be different for each member at a given stage (16). MUC-RA2, the gene used for MUC-NR construction, mRNA level is similar, although very low, in both epimastigotes and trypomastigotes.\(^{2}\) Notwithstanding this, the coding regions cloned into pRIBOTEX, devoid of any extragenic region, are expressed irrespectively of the expression of their endogenous homologous.

Antibodies directed to the tag detected two broad bands of 85 and 90 kDa for the pRMUC-R repetitive gene expressed in \( T. \) cruzi (Fig. 2A, lane 1). The product from this same gene expressed in \( E. \) coli migrated as a band of 35 kDa (Fig. 2A, lane 1), thus suggesting that post-translational modifications were occurring when expressed in \( T. \) cruzi. Moreover, MUC-R products were probed with sera raised against the untagged recombinant protein fused to GST and expressed in \( E. \) coli. None was detected after expression in \( T. \) cruzi, whereas the \( E. \) coli-expressed protein was readily detected (not shown), further supporting the presence of extensive post-translational modifications. The product of pRMUC-NR (nonrepetitive gene) transfected in \( T. \) cruzi showed three to four bands from 35 to 55 kDa after reaction with the anti-tag serum (Fig. 2A, lane 7). The lower molecular mass band migrated in the same position as the product of this construct expressed in \( E. \) coli, but the presence of bands migrating at higher positions suggested that this product has also undergone some post-translational modifications when expressed in \( T. \) cruzi. Wild type epimastigotes used as controls produced no signal when probed with the anti-tag serum (Fig. 2A, lane 4).

Only the product from the repetitive gene MUC-R was detectable in the culture medium from transfected epimastigotes as a 90-kDa band (Fig. 2A, lane 3). Furthermore, although two products were detected in the parasite lysate, only the upper band was observed in conditioned medium from transfected parasites. This behavior is analogous to that reported for soluble variable surface glycoprotein, which lacking the lipid anchor, showed a decreased electrophoretic migration (38).

The Repetitive Member of the TcMUC Family Is Expressed in Transfected \( T. \) cruzi Epimastigotes as a Surface Mucin—To analyze if the recombinant proteins behaved as mucins, they

\(^{2}\) G. D. Pollevick, J. M. DiNoia, M. L. Salto, C. Lima, M. S. Leguizamón, R. M. de Lederkremer, and A. C. C. Frasch, unpublished results.
Gene Identification and Structure of T. cruzi Mucins

were isolated from delipidated cells by extraction with water saturated with 1-butanol as reported previously (35). The extract was analyzed by Western blotting, using the anti-tag serum. The product of the repetitive gene (MUC-R), but not the one from the nonrepetitive gene (MUC-NR), was detected in the extract, as expected for a highly O-glycosylated molecule. Nonetheless, the majority remained in the pellet (data not shown). Therefore, a second extraction with 44% phenol/water was conducted. Only the MUC-R product, but not the MUC-NR product, was detected in the aqueous phase, and after probing with the anti-tag serum, revealed with 125I-protein A. C, identical to B for MUC-NR-expressing parasites. D, affinity purification of MUC-R product. The aqueous phase of phenolic extraction was applied to an anti-tag affinity column. The eluted (lane 1) and percolated material (lane 2) was analyzed by Western blot with the anti-tag serum. The calculated molecular mass of standards is indicated on the right. The molecular mass of standards is indicated on the right.

which specifically detects the epimastigote endogenous 35/50-kDa mucins (39). This antibody did not detect the 35–50-kDa mucins in the sample retained by the anti-tag antibody column, indicating that the recombinant mucin was not contaminated with endogenous mucins (data not shown).

Parasite surface mucins were labeled in vivo using galactose oxidase and NaB3H4, in the wild type and pRMUC-R transfected cells, as reported previously (10). Besides the endogenous mucina of 35/50 kDa, a parasite surface glycoprotein of about 85 kDa was slightly and differentially labeled in the transfected parasites (Fig. 3A). Labeling was not improved by previous treatment with neuraminidase. The apparent molecular mass observed corresponded with that of the lower band detected by anti-tag in extracts from pRMUC-R epimastigotes in Fig. 2A. The reason for the different behavior in SDS-PAGE migration of MUC-R and endogenous epimastigote mucins will be discussed later.

Comparison of the Carbohydrate Structure between Endogenous and Recombinant Mucins of T. cruzi—Analysis of the sugars obtained from endogenous and recombinant MUC-R mucins by reductive β-elimination was performed by HPAEC and TLC. The mucins, obtained from wild type cells labeled by the galactose oxidase/NaB3H4 method, were purified on octyl-Sepharose as reported previously (35). The recombinant MUC-R mucin was purified using the affinity column. In this case a low amount of radioactivity was recovered; thus, reductive β-elimination was performed in the presence of NaB3H4. The products obtained were separated from the radioactive contaminants on a Bio-Gel P-2 column (not shown). From the recombinant mucin an included peak was obtained, eluting between maltotriose and maltose (Fig. 4, lane 1). An excluded peak was also eluted. Analysis by paper electrophoresis of the void radioactivity showed that no negatively charged molecules were present, excluding the presence of sialic acid. This fraction was not analyzed further.

On the other hand, it was interesting to investigate the presence of galactofuranose-containing oligosaccharides because this sugar is important in antibody recognition (40). Galactofuranase was detected in the mucins of one parasite strain (12). Analysis by HPAEC (Dionex) allows the easy identification of galactofuranose-containing disaccharitols and trisaccharitols because they elute later than the lactopyranose-containing isomeric oligosaccharitols (35). No galactofuranose-containing disaccharide or trisaccharide was detected (Fig. 5). The presence of β-D-Galp(1→4)GleNAcβ was shown by TLC (Fig. 4, lanes 1 and 2) and HPAEC (Fig. 5), by comparison with an authentic sample of the disaccharitol (Fig. 4, lane 3,
Gene Identification and Structure of T. cruzi Mucins

As three consensus N-glycosylation sites were present in the C terminus of the MUC-R product (Fig. 1), we analyzed if they had an attached oligosaccharide. MUC-R immunoprecipitated with anti-tag from conditioned culture medium was treated with endoglycosidase H and then analyzed by Western blot with anti-tag serum. This treatment increased the electrophoretic mobility of the tag-containing band by 10 kDa (Fig. 6C), suggesting that all of the N-glycosylation sites have attached oligosaccharides.

TcMUC Genes Encode Mucins Present in the Mammalian Stage of the Parasite—From the experiments described above, it can be concluded that the product of the MUC-R-tagged gene encodes the protein core of a mucin glycoprotein in T. cruzi. However, the mobility of the tagged product (about 90 kDa) is different from that of the endogenous mucins observed in the epimastigote stage (35/50 kDa). When mucins extracted by aqueous-phenol from [14C]Thr metabolically labeled T. cruzi CL-Brener were compared, the radioactive bands present in epimastigotes showed the same pattern as those labeled by galactose oxidase on parasites (see Figs. 3, A and B). On the other hand, the Thr-rich mucins from cell-derived trypomastigotes presented a different pattern composed of bands of slower mobility, encompassing the size of the MUC-R product (Fig. 3B). This, along with the level of the mRNA of TcMUC encoding T8KP2 repeats, which is much higher in cell-derived trypomastigotes (37), suggested that MUC-R-encoded mucins are present in this stage.

To analyze further if the natural products of TcMUC repetitive members, like MUC-R, were present in the stages associated with the vertebrate infection we used a second strategy. A recombinant TcMUC product (M76) containing four T8KP2 repeats was expressed in E. coli fused to GST, affinity purified, and probed in dot spots with sera from human and experimental (rabbits and mice) infections. We employed a panel of sera from animals infected with different parasite strains and from a wide range of times postinfection. The results indicated a different response to M76 depending on the species analyzed. It was recognized by almost all sera from infected mice tested (43/45), independently of the parasite strain or the genetic background of mice used. Sera obtained as early as 25 dpi to up to 6 months postinfection recognized M76. Only 3 out of 10 rabbit sera detected M76, and none of the 12 human sera used here did, despite the fact that M76 was isolated by immunological screening of an expression library using human infection sera (42). These results showed that TcMUC repetitive products are present in the stages of the parasite related to the infection (trypomastigote and/or amastigote) as they can elicit an antibody response in mice, although their antigenic properties can vary with the host.

Hyervariable Regions Present in Mature Mucins Are Antigenic during the Infection—The above mentioned results showed that membrane-located mucins, encoded by TcMUC genes, might be present in the trypomastigote stage, which is exposed to the vertebrate’s immune system. The repetitive TcMUC genes encoding these molecules are all highly similar, except for the region predicted to be nonglycosylated and at the mature N terminus; this is the HV region (16). Two mice infected with parasites expressing MUC-R produced antibodies against the HCV tag as assessed by dot spot (not shown), suggesting that the HV region was present in the mature protein and could be immunogenic during the infection. To determine if the natural HV region was indeed expressed and remained in the mature product during natural and experimental infections and gave rise to an antibody response, GST fusion proteins having a single randomly chosen HV region (proteins E13, T15, T18, and NCA2, see “Materials and Meth-
Gene Identification and Structure of T. cruzi Mucins

To study further the humoral response against these antigens, sera from a follow-up in infected mice were analyzed. Infected mice were bled at 15, 52, and 150 dpi and sera analyzed by dot spot. No reactivity was detected early during the infection, but antibodies against HV regions were detected at 52 dpi and remained at 150 dpi (Table III). Most of the sera (9/11) detected more than one fusion protein by day 150. Signals against HV regions were positive but weaker than those obtained against M76 protein. Taken together, these results confirmed the presence of the TcMUC repetitive products during the infection and showed that the HV region was retained in the mature product in vivo. They also showed that more than one HV region could be expressed in a parasite population during the infection and that they could be targets of specific antibodies.

**DISCUSSION**

There are a few examples in protozoan parasites of large and variable gene families coding for proteins with similar function, as is the case in African trypanosomes and *Plasmodium falciparum* (43, 44). In both, the large number of genes is directly related to a requirement for parasite survival in the host and sequence variability is the naturally selected character. The N-terminal region of these proteins, the one exposed to the host antibodies, differs largely in sequence among members of these families. The finding that *T. cruzi* contains a large family of mucin-type genes with their putative mature N terminus having HV regions (16) raised the question of the reason for this diversity. Tempting possibilities could be antigenic variation or some related immunoevasive role and ligand diversity generation. However, several necessary prerequisites must be demonstrated to sustain any of these hypotheses, including the
mucin nature of TcMUC gene products, the persistence of the HV region at the protein level, and their exposure to the host antibody response. In this paper, structural and serology data were obtained answering these questions.

Two different representative TeMUC products (MUC-R and MUC-NR) were traced in vivo by the use of T. cruzi transfection with tagged genes. The MUC-R gene product behaved as a mucin in phenol/water extractions, and its O-glycosylation was studied, consisting mainly of $\beta$-Galp(1→4)GlcNAc and $\beta$-Galp(1→4)$\beta$-Galp(1→6)-GlcNAc. This post-translational modification, along with the presence of N-glycosylation and GPI anchor and the finding of a secreted form, are indications that the transfected MUC-R yielded a mucin able to traverse the secretory pathway when expressed under these conditions. The MUC-R product is likely to be highly O-glycosylated in the repeats region because the reactivity with a serum recognizing the recombinant protein expressed in E. coli is lost when expressed in T. cruzi. All of the products from TcMUC repetitive genes, like MUC-R, are highly conserved, differing only in the number of repeats and the sequence of the HV region (16). So they would be expected to be processed equally in vivo. Differences in the repeat number may contribute to the heterogeneity in the apparent molecular mass of T. cruzi mucins.

A second transfected gene that lacks repeats (MUC-NR) is translated into a product that, albeit not behaving as a typical mucin in the phenol/water extraction, is its O-glycosylation was studied, consisting mainly of $\beta$-Galp(1→4)GlcNAc and $\beta$-Galp(1→4)$\beta$-Galp(1→6)-GlcNAc. This post-translational modification, along with the presence of N-glycosylation and GPI anchor and the finding of a secreted form, are indications that the transfected MUC-R yielded a mucin able to traverse the secretory pathway when expressed under these conditions. The MUC-R product is likely to be highly O-glycosylated in the repeats region because the reactivity with a serum recognizing the recombinant protein expressed in E. coli is lost when expressed in T. cruzi. All of the products from TcMUC repetitive genes, like MUC-R, are highly conserved, differing only in the number of repeats and the sequence of the HV region (16). So they would be expected to be processed equally in vivo. Differences in the repeat number may contribute to the heterogeneity in the apparent molecular mass of T. cruzi mucins.

A second transfected gene that lacks repeats (MUC-NR) is translated into a product that, albeit not behaving as a typical mucin in the phenol/water extraction, is its O-glycosylation was studied, consisting mainly of $\beta$-Galp(1→4)GlcNAc and $\beta$-Galp(1→4)$\beta$-Galp(1→6)-GlcNAc. This post-translational modification, along with the presence of N-glycosylation and GPI anchor and the finding of a secreted form, are indications that the transfected MUC-R yielded a mucin able to traverse the secretory pathway when expressed under these conditions. The MUC-R product is likely to be highly O-glycosylated in the repeats region because the reactivity with a serum recognizing the recombinant protein expressed in E. coli is lost when expressed in T. cruzi. All of the products from TcMUC repetitive genes, like MUC-R, are highly conserved, differing only in the number of repeats and the sequence of the HV region (16). So they would be expected to be processed equally in vivo. Differences in the repeat number may contribute to the heterogeneity in the apparent molecular mass of T. cruzi mucins.

A second transfected gene that lacks repeats (MUC-NR) is translated into a product that, albeit not behaving as a typical mucin in the phenol/water extraction, is its O-glycosylation was studied, consisting mainly of $\beta$-Galp(1→4)GlcNAc and $\beta$-Galp(1→4)$\beta$-Galp(1→6)-GlcNAc. This post-translational modification, along with the presence of N-glycosylation and GPI anchor and the finding of a secreted form, are indications that the transfected MUC-R yielded a mucin able to traverse the secretory pathway when expressed under these conditions. The MUC-R product is likely to be highly O-glycosylated in the repeats region because the reactivity with a serum recognizing the recombinant protein expressed in E. coli is lost when expressed in T. cruzi. All of the products from TcMUC repetitive genes, like MUC-R, are highly conserved, differing only in the number of repeats and the sequence of the HV region (16). So they would be expected to be processed equally in vivo. Differences in the repeat number may contribute to the heterogeneity in the apparent molecular mass of T. cruzi mucins.

A second transfected gene that lacks repeats (MUC-NR) is translated into a product that, albeit not behaving as a typical mucin in the phenol/water extraction, is its O-glycosylation was studied, consisting mainly of $\beta$-Galp(1→4)GlcNAc and $\beta$-Galp(1→4)$\beta$-Galp(1→6)-GlcNAc. This post-translational modification, along with the presence of N-glycosylation and GPI anchor and the finding of a secreted form, are indications that the transfected MUC-R yielded a mucin able to traverse the secretory pathway when expressed under these conditions. The MUC-R product is likely to be highly O-glycosylated in the repeats region because the reactivity with a serum recognizing the recombinant protein expressed in E. coli is lost when expressed in T. cruzi. All of the products from TcMUC repetitive genes, like MUC-R, are highly conserved, differing only in the number of repeats and the sequence of the HV region (16). So they would be expected to be processed equally in vivo. Differences in the repeat number may contribute to the heterogeneity in the apparent molecular mass of T. cruzi mucins.
the first attached sugar and independent of the composition of the oligosaccharide, and its length has been measured for many mucins (49–51). An average length of 0.25 nm/amino acid residue was established (45, 48). It is reasonable to assume that O-GlcNAc in T. cruzi would have a structural effect similar to that of O-GalNAc in vertebrate mucins. Therefore, we can estimate the length of a TcMUC repetitive product with the average number of three T8KP2 repeats, plus the 37 amino acids that follow up to the likely GPI addition site and 10 residues an HV region. No secondary structure is predicted for the mucins from the insect stages of the parasite. Mucins are covering the parasite surface would be dangerous for the immune system pressure is causing the appearance of variants as a way of evading the immune response. This was described in Plasmodium spp. (53, 54), Streptococcus (55), the hepatitis C virus (56), and the simian immunodeficiency virus (57), among others. The presence of antibodies against the exposed N-terminal HV region of TcMUC-encoded mucins in sera from infected individual suggests that variation is being selected by the immune system of the vertebrate.

We have recently identified a novel mucin-type gene family in T. cruzi, named TeSMUG, whose deduced products have all of the characteristics of epimastigote apomucins (19). Beside the presence of two groups of genes, all members within each group showed no sequence variability. The sequence of one of the groups of TcSMUG-deduced proteins coincided with peptide sequences obtained from the 35–50-kDa mucins purified from epimastigotes,3 suggesting that this new family encodes the mucins of the insect stages of the parasite. Mucins are also the main surface glycoprotein in epimastigotes (52), but variant sequences would not represent an adaptive advantage in an insect host, which has a nonspecific immune response. On the other hand, a large number of identical N termini in molecules covering the parasite surface would be dangerous for the epimastigote under a high affinity specific immune response, and having to survive in the blood long enough to invade different tissues and for being available to the insect vector. We presented herein evidence that the parasite expresses different mucins in this stage, encoded by TcMUC repeats-containing genes, with variant exposed HV regions probably selected to delay the maturation of the immune response. In relation with this, trypomastigote-specific surface glycoproteins belonging to the trans-sialidase superfamily (58) displayed variant but related epitopes at defined regions of different family members (59). These variants are expressed simultaneously in a single parasite, indicating that a potentially protective CD4+ response becomes anergic (60). So, the presence of variant but related epitopes in the surface of the trypomastigote might be a general strategy of T. cruzi to evade an early immune response and allow the infection to be established.

Acknowledgments—We acknowledge Drs. J. J. Cazzulo, A. Parodi, and O. Campetella for critically reading the manuscript. We thank Dr. D. Sánchez for helpful discussions and suggestions and for sera from infected rabbits, Dr. R. Mortara for providing the SFS antibody, Dr. R. Hernández for pRIBOTEX plasmid, and Dr. I. C. Almeida for letting us include unpublished results. We thank L. Sferco and B. Franke de Cazzulo for technical assistance and I. D’Orso and C. Ascencio for help in some of the electrophoresis assays.

REFERENCES

1. Van Klafka, B. W., Dekker, J., Buller, H. A., and Einerherd, A. C. (1995) Am. J. Physiol. 269, G613–627
2. Van Klafka, B. W., Einerherd, A. C. W., Buller, H. A., and Dekker, J. (1998) Anal. Biochem. 265, 193–146
3. Schenkm, S., Ferguson, M. A., Heise, N., de Almeida, M. L., Mortara, R. A., and Yoshida, N. (1993) Mol. Biochem. Parasitol. 59, 293–303
4. Tominson, S., and Baper, J. (1998) Parasitol. Today 14, 354–359
5. Schenkm, S., Jiang, M. S., Hart, G. W., and Nussenzweig, V. (1991) Cell 65, 1117–1125
6. Schenkm, S., Kuroski, T., Baretch, J. V., and Nussenzweig, V. (1992) J. Exp. Med. 175, 1635–1641
7. Ruiz, R. d. C., Rigoni, V. L., Gonzalez, J., and Yoshida, N. (1993) Parasite Immunol. 15, 121–125
8. Alves, M. J. M., and Coll, W. (1975) FERS Lett. 52, 188–190
9. De Leukderker, R. M., Alves, M. J., Fonseca, G. C., and Coll, W. (1976) Biochem. Biophys. Acta 444, 85–96
10. Zingales, B. Martin, N. F., de Leukderker, R. M., and Coll, W. (1982) FERS Lett. 148, 232–248
11. Previti, J. O., Jones, C., Xavier, M. T., Wait, R., Travassos, L. R., Parodi, A. J., and Mendonça-Previti, L. (1996) J. Biol. Chem. 271, 7241–7250
12. Previti, J. O., Jones, C., Gencalves, L. P., Wait, R., Travassos, L. R., and Mendonça-Previti, L. (1994) Biochem. J. 301, 151–159
13. Serrano, A. A., Schenkm, S., Yoshida, N., Mehlert, A., Richmond, J. M., and Ferguson, M. A. (1995) J. Biol. Chem. 270, 27244–27253
14. Almeida, I. C., Ferguson, M. A., Schenkm, S., and Travassos, L. R. (1994) Biochem. J. 304, 783–802
15. Di Noia, J. M., Pollevick, G. D., Xavier, M. T., Previti, J. O., Mendonça-Previti, L., Sanchez, D. O., and Frasch, A. C. (1996) J. Biol. Chem. 271, 32078–32083
16. Di Noia, J. M., D’Orso, I., Aslind, L., Sanchez, D. O., and Frasch, A. C. (1998) J. Biol. Chem. 273, 10841–10846
17. Pastini, A. C., Iglesias, S. R., Carricarte, V. C., Guerin, M. E., Sanchez, D. O., and Mortara, R. A. (1999) Mol. Biochem. Parasitol. 104, 239–243
18. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
19. Almeida, I. C., personal communication.

3. 1. Almeida, personal communication.
49. Shogren, R., Gerken, T. A., and Jentoft, N. (1989) Biochemistry 28, 5525–5536
50. Gerken, T. A., Butenhof, K. J., and Shogren, R. (1989) Biochemistry 28, 5536–5543
51. Cyster, J. G., Shotton, D. M., and Williams, A. F. (1991) EMBO J. 10, 893–902
52. Pereira-Chioccola, V. L., Acosta-Serrano, A., Correia de Almeida, I., Ferguson, M. A., Souto-Padron, T., Rodriguez, M. M., Travassos, L. R., and Schenkm an, S. (2000) J. Cell Sci. 113, 1299–1307
53. Fernandez, V., Hommel, M., Chen, Q., Hagblom, P., and Wahlgren, M. (1999) J. Exp. Med. 190, 1395–1403
54. Plehanski, M., Flanagan, K. L., Lee, E. A. M., Reece, W. H. H., Hart, K., Gelder, C., Gillespie, G., Pinder, M., and Hill, A. V. S. (1999) Immunity 10, 651–660
55. Stockhauer, K. E., Grigshy, D., Pan, X., Fu, Y., Perea Mejia, L. M., Cravioto, A., and Musser, J. M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3128–3133
56. Weiner, A. J., Geyser, H. M., Christopherson, C., Hall, J. E., Mason, T. J., Saracco, G., Bonino, H., Crawford, K., Marion, C. D., Crawford, K. A., Brunetto, M., Barr, P. J., Miyamura, T., McHutchinson, J., and Houghton, M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3468–3472
57. Evans, D. T., O'Connor, D. H., Jing, P., Dzuris, J. L., Sidney, J., da Silva, J., Allen, T. A., Horton, H., Venham, J. E., Rudersdorf, R. A., Vogel, T., Pauza, C. D., Bontrop, R. E., DeMars, R., Sette, A., Hughes, A. L., and Watkins, D. I. (1999) Nat. Med. 5, 1270–1276
58. Campetella, O., Sánchez, D. O., Cazculo, J. J., and Frasch, A. C. C. (1992) Parasitol. Today 8, 378–381
59. Kahn, S. J., and Wleklinski, M. (1997) J. Immunol. 159, 4444–4451
60. Millar, A. E., Wleklinski-Lee, M., and Kahn, S. J. (1999) J. Immunol. 162, 6092–6099
Trypanosoma cruzi Surface Mucins with Exposed Variant Epitopes
Guido D. Pollevick, Javier M. Di Noia, Maria L. Salto, Carlos Lima, M. Susana Leguizamón, Rosa M. de Lederkremer and Alberto C. C. Frasch

J. Biol. Chem. 2000, 275:27671-27680.
originally published online September 1, 2000

Access the most updated version of this article at doi:

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 57 references, 21 of which can be accessed free at http://www.jbc.org/content/275/36/27671.full.html#ref-list-1