The Interplay between Folding-facilitating Mechanisms in *Trypanosoma cruzi* Endoplasmic Reticulum

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Submitted April 14, 2003; Revised May 15, 2003; Accepted May 22, 2003
Monitoring Editor: Reid Gilmore

Lectin (calreticulin [CRT])-*N*-glycan-mediated quality control of glycoprotein folding is operative in trypanosomatid protozoa but protein-linked monoglucosylated *N*-glycans are exclusively formed in these microorganisms by UDP-Glc:glycoprotein glucosyltransferase (GT)-dependent glucosylation. The gene coding for this enzyme in the human pathogen *Trypanosoma cruzi* was identified and sequenced. Even though several of this parasite glycoproteins have been identified as essential components of differentiation and mammalian cell invasion processes, disruption of both GT-encoding alleles did not affect cell growth rate of epimastigote form parasites and only partially affected differentiation and mammalian cell invasion. The cellular content of one of the already identified *T. cruzi* glycoprotein virulence factors (cruzipain, a lysosomal proteinase) only showed a partial (5–20%) decrease in GT null mutants in spite of the fact that >90% of all cruzipain molecules interacted with CRT during their folding process in wild-type cells. Although extremely mild cell lysis and immunoprecipitation procedures were used, no CRT-cruzipain interaction was detected in GT null mutants but secretion of the proteinase was nevertheless delayed because of a lengthened interaction with Grp78/BiP probably caused by the detected induction of this chaperone in GT null mutants. This result provides a rationale for the absence of a more drastic consequence of GT absence. It was concluded that *T. cruzi* endoplasmic reticulum folding machinery presents an exquisite plasticity that allows the parasite to surmount the absence of the glycoprotein-specific folding facilitation mechanism.

INTRODUCTION

Most proteins following the secretory pathway in eukaryotic cells are *N*-glycosylated in the endoplasmic reticulum (ER). A glycan (Glc₃Man₉GlcNAc₂ in most cells; see below for exceptions) is transferred to Asn residues in growing polypeptides. Glucoses are then trimmed by the action of glucosidase I (GI), which removes the external Glc unit, followed by glucosidase

II (GII), which excises both residues remaining in the glycan. Monoglucosylated *N*-glycans may be formed by partial deglucosylation of the transferred oligosaccharide or by reglucosylation of Glc-free glycans by the UDP-Glc:glycoprotein glucosyltransferase (GT; for reviews see Parodi, 2000 and Trombetta and Parodi, 2002). This enzyme is a sensor of glycoprotein conformations because it exclusively glucosylates *N*-glycans in not properly folded conformers. One or two ER-resident α -mannosidases may degrade Man₉GlcNAc₂ to Man₈GlcNAc₂ and Man₇GlcNAc₂, which may also be reglucosylated by GT. Folding glycoproteins oscillate then between monoglucosylated and unglucosylated forms catalyzed by the opposing activities of GT and GII. The monoglucosylated forms are recognized by two ER-resident lectins, calnexin (CNX), and/or calreticulin (CRT). On reaching the proper tertiary structures, glycoproteins become substrates of GII but not of GT. Properly folded molecules, thus liberated from the lectins, are then free to continue their transit to the Golgi. Proteins that fail to properly fold are retained in the ER and eventually

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E03-04-0228. Article and publication date are available at www.molbiolcell.org/cgi/doi/10.1091/mbc.E03-04-0228.

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Abbreviations used: CNX, calnexin; CRT, calreticulin; CZP, cruzipain; DNJ, deoxynojirimycin; ER, endoplasmic reticulum; GI, glucosidase I; GII, glucosidase II; Grp78/BiP, glucose regulated protein 78/immunoglobulin binding protein; GT, UDP-Glc:glycoprotein glucosyltransferase; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

transported to the cytosol, where they are degraded in the proteasomes. Interaction of monoglucosylated glycans with ER lectins not only retains misfolded glycoproteins in the ER, but also facilitates glycoprotein folding by preventing aggregation, incorrect disulfide bond formation, and premature oligomerization. GT is, therefore, the key constituent of the ER quality control of glycoprotein folding, because it is the only element in such process that discriminates between glycoprotein conformers.

Trypanosomatid protozoa are microorganisms that according to most commonly used criteria belong to an early branch of evolution. From an evolutionary point of view they are much more distance from mammals than fungi. Several features of the pathway leading to the formation of *N*-glycoproteins in trypanosomatids reveal significant differences with those occurring in mammalian cells (for a review see Parodi, 1993). For instance trypanosomatid protozoa are the only wild-type eukaryotic cells known so far to be unable to synthesize dolichol-P-Glc; as a consequence, unglucosylated (Man₆GlcNAc₂, Man₇GlcNAc₂, or Man₈GlcNAc₂, depending on the species) instead of Glc₃Man₆GlcNAc₂, is transferred in vivo in trypanosomatid cells. Nevertheless, in vivo GT-mediated transient formation of monoglucosylated oligosaccharides was detected in all trypanosomatid species studied so far. In vitro assays showed that, the same as the higher eukaryotic enzyme, trypanosomatid GTs exclusively glucosylate not properly folded glycoproteins (Trombetta *et al.*, 1989). It is worth stressing the fact that in trypanosomatid protozoa monoglucosylated compounds are exclusively formed through GT-dependent glucosylation.

Other components of the lectin-mediated quality control of glycoprotein folding as GII and CRT have also been described in trypanosomatids. These parasitic protozoa apparently lack CNX (Bosch *et al.*, 1988; Labriola *et al.*, 1999). In vitro assays showed that the lectin properties of trypanosomatid CRT did not differ from those of the same protein from other species. Further, in vivo monoglucosylated *N*-glycan-dependent CRT-glycoprotein interaction has been described (Labriola *et al.*, 1999).

The so-called digenetic trypanosomatids, that is, those that have both insect and mammalian hosts have a complex life cycle. For instance, *Trypanosoma cruzi*, the causative agent of American trypanosomiasis or Chagas' disease, occurs in the hematophagous insect vector digestive tract and in its rectum in the proliferative and noninfective epimastigote and in the nonproliferative, infective metacyclic trypomastigote forms, respectively, whereas in the mammalian host the parasite may adopt the infective, nonproliferative bloodstream trypomastigote and the intracellular proliferative amastigote forms. *T. cruzi* plasma membrane glycoproteins are essential components of the mammalian cell-parasite interaction preceding interiorization of the protozoon (Schenkman *et al.*, 1991; Ruiz *et al.*, 1998; Magdesian *et al.*, 2001). Moreover, a lysosomal glycoprotein (cruzipain [CZP], a proteinase) has been identified as one of *T. cruzi* virulence factors, because it is probably involved in proteolytic processes related to differentiation.

Results herein reported show that *T. cruzi* ER folding machinery shows a remarkable plasticity that allows the parasite to surmount a deficiency in the glycoprotein-specific folding facilitation mechanism.

MATERIALS AND METHODS

Cells and Culture Media

Epimastigotes of the *T. cruzi* CL Brener clone were grown in BHT medium as described before (Cazzulo *et al.*, 1985). *Escherichia coli* DH5 α were used in cloning experiments. Bacteria were grown in Luria-Bertani medium, 0.5% NaCl, 1% tryptone (Difco, Detroit, MI), 0.5% yeast extract (Difco), and 100 μ g/ml ampicillin or 50 μ g/ml kanamycin if necessary.

Cloning and Sequencing of *T. cruzi* GT-encoding Gene (*tcgt1*)

An 800-base pair fragment was amplified using *T. cruzi* genomic DNA as template and primers 5'-CTCCTCAGTTAAGACGC-3' and 5'-TCGCACCAGAGCCACTCC-3' designed from the EST TENS2248 of the *T. cruzi* genome project. This EST codes for a protein fragment highly similar to a portion of the C-terminal domains of other species GTs. The fragment was used as probe for screening an ordered *T. cruzi* genomic cosmid library. Three positive cosmids were detected. One of them yielded a 4000-base pair fragment on digestion with *Eco*RI that contained the 800-base pair fragment. The larger fragment was cloned in pBluescript II KS+ (Stratagene, La Jolla, CA) and completely sequenced. Because the 5' end of this fragment lacked the initial ATG sequence, the positive cosmid was digested with *Sac*I. The 5000-base pair fragment thus obtained was also cloned in the same plasmid and sequenced. The gene thus completely sequenced was designated *tcgt1*. It received the EMBL accession number AJ555866.

Constructions Used for *tcgt1* Disruption

Cassettes encoding two antibiotic resistance markers (neomycin phosphotransferase and hygromycin phosphotransferase) were constructed in pBluescript. The first gene was cloned in the plasmid *Hinc*II and *Eco*RI sites. A fragment of 470 base pairs of the 5' UTR of the glyceraldehydephosphate dehydrogenase-encoding gene (GAP), containing the splice acceptor site was cloned in position 5' of the resistance gene. Two *T. cruzi* GT fragments (bases 3178–3698 for the first one and bases 4277–4959 for the second) were amplified using the pBluescript containing the 4000-base pair fragment as template and primers 5'-TACGGTACCGTGTTGAGGCGC-GATGC-3' and 5'-CCAGCTCGAGCTTGCACTGCCGGTGAGG-3' (first fragment) and 5'-CTCCTCAGTTAAGACGC-3' and 5'-ACGGGATCCCTCCAATTCGGTGTCGG-3' (second fragment). The first fragment was cloned in sites *Kpn*I/*Xho*I sites upstream of the 470-base pair fragment mentioned above. The second fragment was cloned in *Sma*I/*Bam*HI sites downstream of the marker gene. The second cassette conferring resistance to hygromycin was obtained by liberating the neomycin resistance marker gene with *Eco*RI and replacing it with the second marker gene in blunt. *Eco*RI treatment abolished the *Bam*HI site present at the 3' end of the GAP gene 5' UTR fragment.

Parasite Transfection and Selection

Epimastigotes of the CL Brener clone (10⁸ cells) in exponential growth phase were harvested, washed with phosphate buffered-saline (PBS), and resuspended in the same solution supplemented with 0.5 mM MgCl₂ and 0.1 mM CaCl₂. Parasites were mixed with 100 μ g of the plasmid containing the Neomycin resistance marker linearized with *Kpn*I/*Not*I and incubated for 1 h in ice. The mixture in a cuvette (0.4-cm electrode GenePulser, Bio-Rad) was subjected to electroporation (one pulse at 0.4 kV and 500 μ F) in an ElectroCell Manipulator (BTX Electroporation System). Parasites were then incubated at room temperature for 10 min, diluted with 8 ml of BHT medium with 10% complete serum, and incubated for 48 h. G418 (Sigma, St. Louis, MO) was then added (500 μ g/ml). Neomycin-resistant parasites (*tcgt1*⁺/*tcgt1*⁻) were cloned in agar-BHT plates.

A positive clone that displayed the construction correctly integrated as shown by restriction enzyme followed by Southern blotting analysis was amplified and used for transfection of the cassette harboring the Hygromycin resistance gene marker. Parasites resistant to 500 mg/ml Hygromycin B (Sigma) and G418 (*tcgt1⁻/tcgt1⁻*) were used for further characterization.

Infectivity Assays

Rat myoblast cell (L₆E₉) monolayers were grown at 37°C in DMEM medium supplemented with 2% fetal calf serum, streptomycin (100 µg/ml), and penicillin (100 U/ml) in a humidified 5% CO₂ atmosphere. Cells (5 × 10⁴) were infected with pretreated stationary phase wild-type (*tcgt1⁺/tcgt1⁺*), *tcgt1* heterozygous (*tcgt1⁺/tcgt1⁻*, Neomycin resistant) and GT null (*tcgt1⁻/tcgt1⁻*, Neomycin and Hygromycin resistant) epimastigotes at 5:1 and 10:1 parasite/cell ratios. Cells were then washed every 48 h with Hanks' medium to remove nonadherent parasites and DMEM medium plus 2% fetal serum was added after each wash. Cells were finally washed with PBS 16 d after infection and stained with Giemsa. Infected cells were quantified by microscopic observation. Four independent experiments for each parasite/cell ratio were conducted. Each experiment consisted of three plates, each one containing 50,000 cells. The same procedure was used for Vero cells except that untreated epimastigotes were used and two rounds of infection were performed. Pretreatment of stationary phase epimastigotes was performed as follows: parasites were washed twice with PBS and incubated for 30 min at 37°C with a 1:20 dilution of Guinea pig serum (Sigma). Parasites were then washed twice again with PBS and resuspended in the same solution.

T. cruzi Cell Pulse-chase Labeling

T. cruzi epimastigotes (2 g wet weight, exponential growth phase) were twice washed with DME/F-12 Base medium (Met, Gln, Leu, and Lys free, Sigma) supplemented with 365 mg/l Gln, 59.05 mg/l Leu, 91.25 mg/l Lys, 61.2 mg/l MgCl₂, 154.5 mg/l CaCl₂, and 1.2 g/l NaHCO₃. Parasites were resuspended in 8 ml of the same medium and divided into two equal aliquots. Deoxynojirimycin (DNJ, Sigma) was added to one of them at a 6 mM final concentration. Both aliquots were then incubated at 28°C, for 20 min. [³⁵S]Met and [³⁵S]Cys (1 mCi, >1000 Ci/mmol; EasyTag protein labeling mix; New England Nuclear) were added, and both aliquots were incubated for 2 or 15 min at 28°C for immunoprecipitation or ER-lysosome CZP transit experiments, respectively. Suspensions were then subjected to low-speed centrifugation, and the pellets were resuspended in 4 ml of T. cruzi growth medium supplemented with 3 mM Met and 3 mM Cys. DNJ (6 mM) was added to the medium containing cells previously treated with the drug. Aliquots (0.4 ml) were withdrawn after indicated times at 28°C. For immunoprecipitation experiments suspensions were centrifuged and cells were lysed on addition of 350 µl of 50 mM HEPES buffer, pH 7.5 containing 0.2 M NaCl, the indicated Nonidet P-40 concentrations, 0.3 M iodoacetamide, 1 mM phenylmethylsulfonylfluoride (PMSF, Sigma), and 100 µM trans-epoxysuccinyl-1-leucylamido(4-guanidino)butane (E64, Sigma; this compound irreversibly inhibits CZP activity). The supernatants obtained upon centrifugation were subjected to immunoprecipitation. For ER-lysosome transit experiments, pellets obtained by low-speed centrifugation of 0.4-ml aliquots were frozen at -20°C to allow plasma and lysosomal but not ER membrane breakage. Immunoprecipitations with CRT antiserum and purification of lysosomal CZP were performed as already described (Labriola *et al.*, 1995, 1999).

Grp78/BiP-CZP Interaction

For studying Grp78/BiP-CZP interaction epimastigotes (2 g, wet weight, exponential growth phase in 8 ml of growth medium) were treated with 1 mM final concentration of cycloheximide. Aliquots

(0.8 ml) were withdrawn after indicated times, and cells in pellets obtained upon low-speed centrifugations were lysed on addition of 0.3 ml of 50 mM HEPES buffer, pH 7.5, 0.15 M NaCl, 0.1 M iodoacetamide, and 0.5% Nonidet P-40. After 30 min at 0°C, suspensions were centrifuged at 14,000 rpm for 10 min and the supernatants were subjected to overnight immunoprecipitation with CZP antiserum (1:50) at 4°C. The immunocomplexes were isolated with protein A-Sepharose, run on 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose. Western blots were developed with *Trypanosoma brucei* Grp78/BiP antiserum.

Additional Materials and Methods

T. cruzi genomic DNA was prepared as already described for T. brucei (Borst *et al.*, 1980). Southern blots were performed as described in Sambrook *et al.* (1989). T. cruzi cell microsomes were prepared as described by Bontempi *et al.* (1989). Rat liver GT was purified to homogeneity according to Trombetta and Parodi (1992). Oligosaccharyltransferase, pyruvate kinase, CZP, α-mannosidase, and GII activities were assayed according to Bosch *et al.* (1988), Cazzulo *et al.* (1989), Cazzulo *et al.* (1990), Li and Li, (1972), and Trombetta *et al.* (1996), respectively. Cells were lysed, and immunoprecipitations and Western blotting analysis were performed as described before except where indicated (Labriola *et al.*, 1999). CZP antiserum was that used previously (Labriola *et al.*, 1999), whereas T. brucei Grp78/BiP antiserum was a generous gift of Dr. J. D. Bangs (University of Wisconsin-Madison Medical School). It cross-reacts with the T. cruzi chaperone. In vitro GT assays were performed as before (Trombetta *et al.*, 1989), whereas in vivo assays T. cruzi cells were incubated with 0.5 mM [¹⁴C]Glc (300 Ci/mol, New England Nuclear) for 30 min and further chased with 0.1 M Glc for 30 min, and endo-β-N-acetylglucosaminidase H (Sigma)-sensitive N-glycans from whole cell glycoproteins were isolated (for further details on cell labeling and N-glycan isolation, see Engel and Parodi, 1985). DNJ (5 mM final concentration) was added 30 min before the label. Samples were run on Whatman 1 paper chromatography with solvents A, 1-propanol/nitromethane/water (5:2:4) and B, 1-butanol/pyridine/water (10:3:3).

RESULTS

Sequence Analysis of T. cruzi GT

T. cruzi GT-encoding gene (*tcgt1*) was cloned and sequenced as described in MATERIALS AND METHODS. As almost all trypanosomatid genes sequenced so far, *tcgt1* lacked introns. The conceptual translation of the gene yielded a 1657-amino acid, 188-kDa protein that displayed all amino acid residues known so far to be required for activity (Cs 1518, 1607, 1611, and 1625; Ds 1488, 1490, 1584, and 1586; Q1585; N1589, and L1587). The parasite enzyme appeared to be at least 100 amino acids larger than similar enzymes from yeast, fly, worm, or mammalian cells. GTs are formed by at least two domains: the N-terminal that comprises 80% of the molecule has no homology to other known proteins, is required for proper folding of the C-terminal portion, and has been proposed to be involved in nonnative conformer recognition; and the C-terminal or catalytic domain that binds [β-³²P]5N₃UDP-Glc and displays a similar size and significant similarity to members of glycosyltransferase family 8 (Tessier *et al.*, 2000; Guerin and Parodi, 2003). All GT C-terminal domains from different species share a significant similarity (60–70%), but much lower values occur between N-terminal domains. Similarities of C- and N-terminal domains of several species GTs are depicted in Table 1. It is worth remarking that the high eukaryote GT N-terminal

Table 1. Percent similarities of GT C- and N-terminal domains^{a,b}

Species	<i>T. cruzi</i>	<i>S. pombe</i>	<i>D. melanogaster</i>	<i>R. norvegicus</i>	<i>H. sapiens</i>
<i>T. cruzi</i>	—	55.5–26.5	53.8–32.1	56.6–32.5	56.4–25.3
<i>S. pombe</i>			62.0–18.3	65.2–21.8	64.0–19.0
<i>D. melanogaster</i>				74.1–35.1	75.5–31.6
<i>R. norvegicus</i>					82.5–47.6

^a Values with underscore correspond to similarity values of C-terminal domains.

^b Similarity indexes were obtained by pairing alignment using the Lippman-Pearson method (Align, DNASTar program).

domains showed a higher similarity to the corresponding portion of the *T. cruzi* enzyme than to the *Schizosaccharomyces pombe* one. This result was rather surprising, because the fission yeast is much closer in evolution to the multicellular organisms than the protozoan parasite.

Unlike all known GTs from different species, *T. cruzi* GT lacks an ER retrieval sequence characteristic of luminal soluble ER proteins at its C termini. Because the only possible bona fide transmembrane region corresponds to that of an internal signal sequence (amino acids 35–52), the possibility that *T. cruzi* GT could be a type II membrane protein was further studied. The enzyme was released from microsomes prepared in an isotonic medium to a 100,000 × *g* supernatant on addition of Triton X-100 concentrations that solubilized another luminal soluble protein (GII) but not an ER membrane integral protein as the oligosaccharyltransferase (Figure 1), thus confirming the soluble status of the protozoan enzyme. It is worth mentioning that at least two other *T. cruzi* luminal soluble ER proteins (Grp78/BiP and CRT) are known to display ER retrieval sequences at their C-termini (MDDL and KEDL, respectively; Tibbetts *et al.*, 1994; Labriola *et al.*, 1999). Soluble ER proteins devoid of ER retrieval sequences at their C-termini are known to occur not only in yeast and mammalian cells but also in another trypanosomatid protozoan as is the case of protein disulfide isomerase from *Leishmania donovani* (Trombetta *et al.*, 1996; D'Alessio *et al.*, 1999; Padilla *et al.*, 2003). How is *T. cruzi* GT retained in the ER lumen remains an open question.

Characterization of *T. cruzi* GT Null Mutants

As a step previous to gene disruption, it was necessary to determine the copy number of *tcgt1* in the entire genome as *T. cruzi* is characterized for displaying several (in some cases hundreds) copies of the same gene, a fact that precludes or makes extremely difficult the production of null mutants for those genes. Genomic *T. cruzi* DNA was cleaved with three restriction nucleases, one of them cutting 5' upstream of the gene (*Xho*I) and two cleaving within the gene (*Mlu*I and *Bam*HI; Figure 2A). Fragments generated were subjected to Southern blotting analysis using probes derived from the N-terminus (bases 34–580) and C termini (bases 4277–4805). Results obtained indicated that *tcgt1* was present in a single copy (Figure 2B) as was suggested by detection of only three positive cosmids harboring *tcgt1* in a library in which the entire genome was represented 25 times. In addition, a time course digestion of *T. cruzi* DNA with *Bam*HI followed by Southern blotting analysis with the C termini-derived probe did not show the regular distribution of increasing size bands characteristic of tandemly repeated genes in this parasite genome (our unpublished results).

Both GT-encoding alleles were disrupted by insertion of genes conferring resistance to Neomycin and Hygromycin as described in MATERIALS AND METHODS (Figure 2A). Cleavage of mutant cell DNA with above mentioned restriction enzymes followed by Southern blotting analysis of the resulting fragments using the same probes indicated that both *tcgt1* alleles had been effectively disrupted (Figure 2C). The absence of GT activity in mutant cells was confirmed by

Figure 1. *T. cruzi* GT is a soluble protein. Microsomes isolated from wild-type *T. cruzi* cells were treated with indicated Triton X-100 concentrations and indicated enzymatic activities assayed in whole extracts or in pellets, and supernatants obtained upon 100,000 × *g* for 60 min centrifugations. Activities in the former were taken as 100%. GII, GT, and OT stand for glucosidase II, UDP-Glc:glycoprotein glucosyltransferase, and oligosaccharyltransferase, respectively.

A **B** **C**

Figure 2. Characterization of *tcgt1* copy number and *tcgt1*^{-/-} (GT null) mutants. Restriction enzyme cleavage sites in *tcgt1* and in the disrupted gene as well as coding regions of probes used are indicated in A. The *Bam*HI site in GAP disappears upon cleaving the Neomycin-resistant construction with *Eco*RI. Wild-type (B) and *tcgt1*^{-/-} (GT null) mutant (C) cell DNAs were subjected to restriction enzyme cleavage followed by Southern blotting analysis using indicated probes. Restriction enzymes used were *Xho*I (lanes 1 and 4), *Bam*HI (lanes 2 and 5), and *Mlu*I (lanes 3 and 6). GAP, Neo, and Hygro stand for a 470-base pair fragment of the 5' UTR of the glyceraldehyde-phosphate dehydrogenase encoding gene (GAP), containing the splice acceptor site, and genes conferring resistance to Neomycin and Hygromycin, respectively. For further details, see MATERIALS AND METHODS.

Table 2. In vitro GT activity of wild type and GT null *T. cruzi* mutants^a

Enzyme	Thyroglobulin	cpm
Rat liver	—	45
Rat liver	+	2424
<i>T. cruzi</i> wild type	—	146
<i>T. cruzi</i> wild type	+	798
<i>T. cruzi</i> GT null	—	44
<i>T. cruzi</i> GT null	+	42

^a Pure rat liver GT or *T. cruzi* microsomes were used as enzyme source.

both in vitro and in vivo assays. Microsomes prepared from wild-type but not from mutant cells displayed GT activity in the presence of denatured thyroglobulin (Table 2). Incubation of live cells with [¹⁴C]Glc in the presence of the GII inhibitor DNJ led to formation of both glucosylated (Glc₁Man₇₋₉GlcNAc₂) and unglucosylated (Man₇₋₉GlcNAc₂) *N*-glycans in the case of wild-type cells, but of only the latter species in the case of the GT null mutants (Figure 3, A and B). Strong acid hydrolysis confirmed the exclusive presence

of Glc units in glycans derived from wild-type cells (Figure 3, C and D). The absolute absence of GT activity as revealed by above tests confirmed that although *tcgt1*⁻/*tcgt1*⁻ cells had not been cloned, they were not contaminated with heterozygotes (*tcgt1*⁺/*tcgt1*⁻).

T. cruzi GT Null Mutants Are Not Affected in Cell Growth Rate and Viability and Only Partially Affected in Differentiation and Infectivity

No differences were observed in the cell growth rate of wild-type (*tcgt1*⁺/*tcgt1*⁺), *tcgt1* heterozygous (*tcgt1*⁺/*tcgt1*⁻), and GT null (*tcgt1*⁻/*tcgt1*⁻) epimastigotes in BHT medium (our unpublished results). The GT-encoding gene has been disrupted in only other organism (*S. pombe*) in which the absence of the enzyme also did not affect cell viability and morphology under normal growth conditions (Fernandez *et al.*, 1996). It is worth mentioning that CNX, the only monoglucosylated *N*-glycan-specific lectin present in this yeast, appeared to be essential for viability, thus indicating that CNX had additional roles besides facilitating glycoprotein folding (Jannatipour and Rokeach, 1995). Two procedures were followed to study differentiation and infectivity of *T. cruzi* GT null mutants. In the first one rat myoblast cell (L₆E₆) monolayers were infected with wild-type,

Figure 3. In vivo characterization of GT null (*tcgt1*⁻/*tcgt1*⁻) mutants. Wild-type (A and C) and GT null mutants (B and D) were pulse-chased with [¹⁴C]Glc in the presence of 5 mM DNJ. Whole cell endo- β -*N*-acetylglucosaminidase H-sensitive oligosaccharides were run on paper chromatography with solvent A (A and B). *N*-glycans were subjected to strong acid hydrolysis and run on paper chromatography with solvent B (C and D). Standards: 1, Glc₁Man₉GlcNAc₂; 2, Man₉GlcNAc₂; 3, Glc₁Man₈GlcNAc₂; 4, Man₈GlcNAc₂; 5, Glc₁Man₇GlcNAc₂; 6, Man₇GlcNAc₂; 7, Glc and 8, Man. For further details, see MATERIALS AND METHODS.

Table 3. The effect of GT null mutation on *T. cruzi* infectivity^a

Parasite/cell ratio	Wild type	Heterozygote	GT null
5:1	45 ± 6	50 ± 5	27 ± 4
10:1	93 ± 8	106 ± 11	60 ± 2

^a Values correspond to the average number of infected cells found in four independent experiments, each one conducted with three plates containing 50,000 cells each.

GT heterozygote (*tcgt1*⁺/*tcgt1*⁻), and GT null (*tcgt1*⁻/*tcgt1*⁻) stationary phase epimastigotes previously treated with guinea pig serum to enrich the population in infective forms (see MATERIALS AND METHODS). Two parasite/cell ratios were used (5:1 and 10:1). Cells were stained with Giemsa, and infected cells were counted. Values in Table 3 correspond to the average of four experiments. In the second procedure, Vero cells were infected with untreated stationary phase epimastigotes. In a first-infection experiment only the wild-type and heterozygote cells yielded infected cells, as judged from direct observation of unstained cells. However, when supernatants were collected and cells were subjected to a secondary-infection experiment, observation of Giemsa-stained cells gave results similar to those shown in Table 3. It may be concluded that absence of GT, and thence of CRT-mediated glycoprotein folding facilitation, only partially affected *T. cruzi* differentiation and infectivity.

The Effect of GT Absence on Glycoprotein Content

The folding process of a known virulence factor in GT null mutant cells was studied to better understand how the parasite cell ER reacted to the absence of one of the known folding facilitating mechanisms. CZP is a lysosomal proteinase that constitutes ~5% of total soluble cellular protein. As will be further discussed below, there is evidence indicating that >90% of all CZP molecules interact with CRT during the folding process occurring in the wild-type cell ER. Nevertheless, the total lysosomal CZP level was scarcely affected (5–20% decrease) in GT null mutants (Figure 4). On the other hand, a more pronounced decrease was observed in the level of another lysosomal glycoprotein (α -mannosidase). The level of a cytosolic nonglycoprotein enzyme (pyruvate kinase) was not modified (Figure 4). These results confirm that lectin-monomonosylated glycoprotein interaction may not be absolutely required for proper folding and that the absence of such interaction may have differential effects on the folding efficiency of different glycoproteins. The same conclusions have been reached before using GII-deficient mammalian or yeast cell mutants (Reitman *et al.*, 1982; D'Alessio *et al.*, 1999). The model system used here (GT null trypanosomatid cells) obviates any effect that may have been produced by inhibition of normal *N*-glycan processing because of the continued presence of Glc residues.

The Effect of GT Absence on CRT-Glycoprotein Interaction

ER folding and oligomerization is the rate-limiting step in the eukaryotic cell secretion process. We have previously

Figure 4. Total glycoprotein content in GT null mutant cells. Cell pellets were freeze-thawed and resuspended in 50 mM Tris-HCl, pH 7.6, 0.15 M NaCl and centrifuged for 20 min at 14,000 rpm. Indicated enzymatic activities were assayed in the supernatants (cytosolic and lysosomal contents). Values in wild-type cells were taken as 100%. PK, CZP, and α -MAN stand for pyruvate kinase, cruzipain, and α -mannosidase, respectively. Assays were performed with cells withdrawn from cultures at the three indicated cell densities

determined that addition of DNJ lengthened CRT-CZP interaction and thence delayed arrival of the proteinase to lysosomes Labriola *et al.*, 1995, 1999). Contrary to what happens in other eukaryotic cells, in which addition of GI and GII inhibitors hinder lectin-glycoprotein interaction by promoting accumulation of tri- and diglycosylated *N*-glycans, in trypanosomatid cell the inhibitors favor the temporal persistence of monoglycosylated species. The same effect of DNJ addition on the arrival of CZP to lysosomes observed previously in Tulahuen 2 strain wild-type cells was now reproduced in CL Brener clone cells (Figure 5, A and B). As expected, no effect of DNJ was detected when GT null mutants were used, thus confirming that the effect observed in wild-type cells was dependent on creation of monoglycosylated *N*-glycans (Figure 5, C and D). Quite unexpectedly, however, kinetics of CZP arrival to lysosomes in the presence or absence of the drug were similar not to those observed in wild-type cells in the absence of the drug but to those observed in its presence. This constitutes a firm indication that in GT null mutants CZP molecules were being retained in the ER by an alternative mechanisms not involving a lectin-*N*-glycan type of interaction. Two possible protein-protein interaction mechanisms were investigated, involving either CRT or Grp78/BiP.

CRT Does Not Interact with Unglycosylated CZP

It has been claimed that either CNX or CRT may weakly associate with unglycosylated glycoproteins or even with unglycosylated proteins and that such interaction has remained mostly undetected as it may be disrupted by the relatively high detergent concentrations usually used for cell lysis previous to coimmunoprecipitation. As already described, addition of DNJ to wild-type live cells pulse-labeled with [³⁵S]Met plus [³⁵S]Cys greatly enhanced the amount of CZP that coimmunoprecipitated with CRT on addition of CRT antiserum to cells lysed with 1% Nonidet P-40. No immunoprecipitation was observed either in the presence or absence of the drug when GT null mutant cells were used (Figure 6A). We were unable to detect CZP coimmunopre-

Figure 5. Kinetics of CZP arrival to lysosomes. Wild-type (A and B) and GT null mutant (C and D) cells were pulse-chased with [35 S]Met and [35 S]Cys, and CZP in lysosomes were isolated, run on 10% SDS-PAGE, and subjected to autoradiography. Intensity after a 300-min chase was taken as 100%. Cells in B and D were pulse-chased in the presence of 6 mM DNJ. For further details, see MATERIALS AND METHODS.

cipitating with CRT antiserum when labeled mutant cells were lysed with Nonidet P-40 concentrations as low as 0.1% (Figure 6B). The same result was observed with similar ($\leq 1\%$) digitonin concentrations (our unpublished results).

GT Absence Lengthens Grp78/BiP-CZP Interaction

To study the influence of the GT-created monoglucosylated *N*-glycans on Grp78/BiP-CZP interaction cycloheximide was added to wild-type and GT null *T. cruzi* cells to stop protein synthesis. Cells from aliquots withdrawn after several time periods were lysed and immunoprecipitated with CZP antiserum. Immunoprecipitates were then analyzed by Western blotting analysis using *T. brucei* Grp78/BiP antiserum, which cross-reacts with the same *T. cruzi* protein. Results shown in Figure 7, A–C, indicate that Grp78/BiP-interaction persisted for significantly longer time periods in GT null mutants. This result agrees with the higher Grp78/BiP levels detected in these last cells when compared with the wild-type ones (Figure 7D). It may be concluded that absence of CRT-glycoprotein interaction triggered induction of alternative folding facilitation mechanisms.

DISCUSSION

Results presented show that in spite of glycoproteins being essential components of *T. cruzi* complex life cycle, absence of the ER glycoprotein-specific folding facilitation mechanism only had quantitative but not qualitative effects on differentiation and infection processes. Description of the folding process of one of known *T. cruzi* glycoprotein virulence factors (CZP) showed that the parasite reacted to the absence of lectin-*N*-glycan interaction by upregulating alternative mechanisms that enhance ER folding efficiency.

CZP has been identified as one of *T. cruzi* virulence factors (Franke de Cazzulo *et al.*, 1994). In fact, specific CZP inhibitors are currently being tested as candidates for Chagas' disease chemotherapy (Engel *et al.*, 1998). CZP is composed of two domains, the enzyme proper or catalytic domain and the C-terminal extension (McGrath *et al.*, 1995). The former domain has two *N*-glycosylation consensus sequences, at least one of which is occupied, whereas the latter has only one of such sequences, which has been shown to bear an *N*-glycan (Cazzulo *et al.*, 1992; Metzner *et al.*, 1996). We have previously determined that $\sim 65\%$ of glycans in either the C-terminal extension or in the catalytic domain are glucosylated by GT (Labriola *et al.*, 1995). Because it has been determined that GT exclusively glucosylates *N*-glycans in the close vicinity of structural perturbations and both CZP domains are known to fold independently from each other (McGrath *et al.*, 1995; Ritter and Helenius, 2000), it was concluded that $>90\%$ of CZP molecules were glucosylated in at least one of its glycans and thus interacted with CRT during the folding process. If the second *N*-glycosylation consensus sequence in the catalytic domain were occupied and glucosylated, it may be statistically calculated that practically all CZP molecules interacted with CRT. Because CZP has seven disulfide bonds, it should be expected its proper folding to be heavily dependent on interaction with CRT if a protein similar to ERp57 also occurs in the *T. cruzi* cell ER. ERp57 is a member of the protein disulfide isomerase family that in mammalian cells is tightly associated with CNX/CRT and promotes native disulfide bond formation in monoglu-

Figure 6. CRT-CZP interaction in wild-type and GT null mutant cells. Cells were pulsed for 2 min with [35 S]Met plus [35 S]Cys, lysed with 1% Nonidet P-40 (A) or with indicated concentrations of the same detergent (NP-40) (B), and lysates were subjected to immunoprecipitation with CRT antiserum. Immunoprecipitates were run on 10% SDS-PAGE and subjected to autoradiography. Where indicated 6 mM DNJ was added 30 min before the pulse. CZP and CRT stand for cruzipain and calreticulin, respectively. For further details, see MATERIALS AND METHODS.

cosylated glycoprotein folding intermediates (Oliver *et al.*, 1999). Rather surprisingly, however, our results show that the absence of CRT-CZP interaction only slightly affected the total proteinase content. The most plausible explanation for this result is that the absence of GT led to the ER accumulation of misfolded glycoproteins and thence to increased synthesis of other ER chaperones and folding facilitating proteins that replaced CRT in its task of enhancing CZP folding efficiency (unfolded protein response; Parodi, 2000; Trombetta and Parodi, 2002). It has been already observed that inhibition of monoglucosylated *N*-glycan formation in mammalian and *S. pombe* cells either by the addition of GII inhibitors or by the disruption of GII- or GT-encoding genes resulted in the induction of BiP mRNA (Balow *et al.*, 1995; Pahl and Baeuerle, 1995; D'Alessio *et al.*, 1999). In accordance with these findings, we observed higher Grp78/BiP levels in GT null than in wild-type cells.

We have detected interaction of Grp78/BiP with CZP in both GT-positive and -negative cells. A longer interaction was observed, nevertheless, with the latter than with the former cells, thus suggesting that Grp78/BiP might be responsible for the similar kinetics of CZP arrival to lysosomes observed in wild-type cells in the presence of the GII inhibitor DNJ and in GT null mutants in the presence or absence of the drug. Although the delay observed in CZP arrival to lysosomes in wild-type cells incubated with DNJ was due to a lengthened CRT-CZP interaction, the delay observed in mutant cells was probably due to a prolonged interaction with Grp78/BiP and other molecular chaperones. It has been reported that Grp78/BiP preferentially recognizes heptapeptides having bulky hydrophobic residues in alternating positions exposed in extended protein conformations, whereas GT showed affinity for hydrophobic amino acid patches in collapsed, molten globule-like structures (Blond-Elguindi *et al.*, 1993; Caramelo *et al.*, 2003). In other words, a change in the affinity of glycoproteins for Grp78/BiP to CNX/CRT would occur once sufficient primary sequence information is present in the ER lumen to allow proteins to collapse. In fact, there are several examples in which a sequential glycoprotein interaction, first with Grp78/BiP and then with CNX/CRT has been observed in intact mammalian cells (Hammond and Helenius, 1994; Kim and Arvan, 1995; Tomita *et al.*, 1999; Molinari and Helenius, 2000).

It may be speculated that a similar sequential interaction occurs in normal *T. cruzi* cells but that in the case of GT null mutants, higher Grp78/BiP levels triggered by the unfolded protein response would prolong the extended conformation to prevent glycoprotein aggregation.

Availability of GT null mutants allowed studying if protein-protein, in addition to protein-glycan, recognition-mediated *T. cruzi* CRT-glycoprotein interaction. There have been several reports in the literature suggesting that CRT and a soluble form of CNX in vitro prevented aggregation not only of denatured monoglucosylated oligosaccharide-bearing glycoproteins as Glc₁Man₆GlcNAc₂-soybean agglutinin but also of denatured proteins lacking *N*-oligosaccharides as deglycosylated soybean agglutinin, malate dehydrogenase, and citrate synthase. They also suppressed the thermal denaturation of above-mentioned nonglycosylated proteins and enhanced their refolding by maintaining the unfolded species in a folding-competent state. Both lectins formed stable complexes with unfolded conformers but not with the native molecules. It was concluded that CRT and truncated CNX behaved in vitro as bona fide classical chaperones (Ihara *et al.*, 1999; Saito *et al.*, 1999; Stronge *et al.*, 2001).

Whether the conclusions reached are applicable to in vivo situations is questionable because CRT was found to display a cooperative denaturation transition with a midpoint at about the same temperature (44°C) at which the above-mentioned experiments were performed (Bouvier and Stafford 2000; Li *et al.*, 2001). Concerning in vivo trials, it was reported that certain glycoproteins synthesized in mammalian cells devoid of either GI or GII activities or in the presence of an inhibitor of both enzymes (castanospermine; i.e., conditions that prevent formation of monoglucosylated glycans) were immunoprecipitated with CNX antibodies if special precautions were taken when cell lysis and immunoprecipitating techniques were used (Danilczyk and Williams, 2001). Results presented here show that even under those special experimental conditions, no CRT-CZP interaction was detected in GT null mutant cells, thus confirming the exclusive lectin nature of *T. cruzi* CRT.

CNX/CRT-glycoprotein interaction was shown not to be essential for mammalian or fungal cell viability under normal growth conditions (Reitman *et al.*, 1982; Ray *et al.*, 1991;

Figure 7. Grp78/BiP-CZP interaction. Protein synthesis in wild-type (A) or GT null mutant (B) cells was stopped on addition of 1 mM cycloheximide, and samples were withdrawn after indicated times. Cells were lysed and subjected to immunoprecipitation with CZP antiserum. Immunoprecipitates were run on 10% SDS-PAGE and subjected to Western blotting analysis with *T. brucei* Grp78/BiP antiserum. In C, intensity of the first aliquot was taken as 100%. In D, 10^7 wild-type or GT null epimastigotes in the exponential growth phase were harvested and heated with cracking buffer followed by SDS-PAGE and Western blotting analysis as above. For further details, see MATERIALS AND METHODS.

Fernandez *et al.*, 1996; D'Alessio *et al.*, 1999). Because folding is an error-prone but essential process, cells have alternative ways for helping protein acquisition of proper tertiary structures in the ER (Parodi, 2000; Trombetta and Parodi, 2002). As a general rule, when one folding facilitation system is absent, an alternative one fills the job. Nevertheless, there are exceptions to the last statement and CNX/CRT-glycoprotein interaction was reported to be necessarily required for production of infective HIV-1 and hepatitis B virus (Gruters *et al.*, 1987; Fischer *et al.*, 1996; Mehta *et al.*, 1997) and for viability of the fission yeast *S. pombe* under severe ER stress conditions (Fanchiotti *et al.*, 1998). In the first case, virus particles produced in the absence of CNX/CRT-glycoprotein interaction were noninfectious due to a misfolded Vi-V2 loop in HIV-1 gp120. Similarly, folding of the M glycoprotein of hepatitis virus B was severely compromised when lectin-glycoprotein interaction was prevented and virus assembly was hindered. In the yeast case, a glycoprotein essential for cell wall assembly necessarily required CNX-glycoprotein interaction for proper folding under conditions of severe ER stress.

Results presented herein show, therefore, that *T. cruzi* ER folding machinery presents an exquisite plasticity that allows the parasite to surmount the absence of the glycoprotein-specific folding facilitation mechanism.

ACKNOWLEDGMENTS

Work reported herein has received financial support from the U. S. Public Health Service (National Institutes of Health), from the Howard Hughes Medical Institute, from the United Nations Development Program/World Bank/World Health Organization Special Program for Research and Training in Tropical Diseases, and from the National Agency for the Promotion of Science and Technology (Argentina).

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