Proteins with Glycosylphosphatidylinositol (GPI) Signal Sequences Have Divergent Fates during a GPI Deficiency

GPIs ARE ESSENTIAL FOR NUCLEAR DIVISION IN Trypanosoma cruzi

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Glycosylphosphatidylinositol (GPI) anchors for cell surface proteins of several major protozoan parasites of humans, including Trypanosoma cruzi, the causative agent of Chagas’ disease. To investigate the general role of GPIs in T. cruzi, we generated GPI-deficient parasites by heterologous expression of T. brucei GPI-phospholipase C. Putative protein-GPI intermediates were depleted, causing the biochemical equivalent of a dominant-negative loss of function mutation in the GPI pathway. Cell surface expression of major GPI-anchored proteins was diminished in GPI-deficient T. cruzi. Four proteins that are normally GPI-anchored in T. cruzi exhibited different fates during the GPI shortage; Ssp-4 and p75 were secreted prematurely, while protease gp50/55 and p60 were degraded intracellularly. These observations demonstrate that secretion and intracellular degradation of GPI-anchored proteins may occur in the same genetic background during a GPI deficiency. We postulate that the interaction between a protein-GPI transamidase and the COOH-terminal GPI signal sequence plays a pivotal role in determining the fate of these proteins.

At a nonpermissive GPI deficiency, T. cruzi amastigotes inside mammalian cells replicated their single kinetoplast but failed at mitosis. Hence, in these protozoans, GPIs appear to be essential for nuclear division, but not for mitochondrial duplication.

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The abbreviations used are: GPI, glycosylphosphatidylinositol; AHA, 2,5-anhydromannitol; EtN, ethanolamine; GPI-PLC, glycosylphosphatidylinositol phospholipase C; GlcN, glucosamine; HPTLC, high performance thin layer chromatography; Mann, mannose; Manα, GlcN-PI, Manα1–2Manα1–6Manα1–4GlcNAc1–6inositol-1-phosphoglycerolipid; PGTase, protein-GPI transamidase; PI, phosphatidylinositol; PL-PLC, phosphatidylinositol-specific phospholipase C from Bacillus cereus; PAGE, polyacrylamide gel electrophoresis; VSG, variant surface glycoprotein of T. brucei; FBS, fetal bovine serum; JBAM, jack bean α-mannosidase; PBS, phosphate-buffered saline; GlcN, glucosamine; HPTLC, high performance thin layer chromatography; PBS, phosphate-buffered saline; ER, endoplasmic reticulum; PLAG, phospholipase A2; SAPA, shed acute phase antigen.

the biological function of the majority of GPI-anchored proteins and protein-free GPIs (i.e. unattached to protein or carbohydrate) is unknown. Addressing the physiological functions of GPIs requires the construction of mutants categorically deficient in the GPI pathway; in most biological systems, such cells are not available. In murine T lymphomas, however, several viable mutants exist (3), some of whose GPI-negative phenotype has been reversed with cloned genes (4). In contrast, in the yeast Saccharomyces cerevisiae, some temperature-sensitive GPI mutants are nonviable (5, 6).

Protozoan parasites, which as a group cause about 5 million human deaths a year worldwide, have developmental stages in which proteins on their plasma membrane are overwhelmingly GPI-anchored. Examples include variant surface glycoprotein (VSG) of Trypanosoma brucei, gp63 of Leishmania spp., circumporozoite protein of Plasmodium spp., and Ssp-4 of Trypanosoma cruzi (1, 2). Several hypotheses accounting for the overproduction of GPI-anchored proteins in these parasites have been advanced but none tested (7). Indirect evidence that GPI anchors might be critical for the success of these protozoans as parasites lies in the fact that viable mutants in the “conserved core” of protein-GPIs (EtN-phospho-Manα1–6GlcN-PI) are unknown.

Trypanosoma cruzi is the causative agent of Chagas’ disease, which afflicts an estimated 18 million people in south and central America. Metacyclic trypomastigotes transmitted to humans by Reduviid bugs enter mammalian cells where they differentiate into and replicate as amastigotes. Proliferation of amastigotes is critical to propagation of an infection because trypomastigotes cannot replicate.

We sought to delineate possible functions of GPIs in T. cruzi. Since genes for targeted mutagenesis of the GPI pathway in these parasites are not available, we engineered a phenotypic GPI mutant of T. cruzi by heterologous expression of a GPI-phospholipase C (GPI-PLC) from the related kinetoplastid T. brucei (8–10). GPI-PLC cleaves GPI anchor intermediates in vivo, causing the biochemical equivalent of a dominant-negative loss of function mutation in the GPI pathway (11).

Cell surface expression of GPI-anchored proteins in GPI-deficient T. cruzi was reduced as compared with wild-type cells. With increased GPI-PLC expression by the parasite, amastigotes inside mammalian cells replicated their single mitochondrion but could not sustain mitosis. These studies establish a role for GPIs in intracellular replication of a protozoan parasite of humans. Since GPI-deficient mammalian cells are viable, drugs targeted at the GPI biosynthetic pathway of trypanosomes are likely to be of chemotherapeutic value.

EXPERIMENTAL PROCEDURES

Parasites

Epimastigotes of T. cruzi (Brazil strain) were cultured at 26 °C in liver infusion tryptose (LIT) medium supplemented with 5% heat-inac-
tivated fetal bovine serum (FBS). Infective metacyclic trypanmastigotes were obtained from 4-week-old stationary phase cultures of epimastigotes (12). Amastigotes were converted extracellularly in LIT medium from Vero (ATCC-CRL-1586)-derived trypanmastigotes (13).

Construction of pTEX GPI-PLC and Transfection of T. cruzi

An EcoRI fragment of T. brucei GPI-PLC cDNA from plasmid pDH4 (8) was cloned into the EcoRI site of a T. cruzi epimastigal expression vector pTEX (14). Epimastigotes were transfected (15) with 25 µg of pTEX GPI-PLC. Following 48 h of incubation at 28 °C, G418 was added to a final concentration of 100 µg/ml for selection of stable transformants, which were eventually subcultured in medium containing 200–800 µg/ml G418. G418-resistant cells were studied 3 months after drug selection.

Cell Lysis, Partial Fractionation, and GPI-PLC Assay

A pellet of 105 cells was lysed on ice in 1 ml of hypotonic buffer (10 mM NaHPO4, 2 mM KH2PO4, 13.7 mM NaCl, 8 mM KCl, pH 7.4) containing a protease inhibitor mixture (11). The cell suspension was incubated on ice for 30 min and centrifuged at 14,000 × g for 15 min at 4 °C. The membranous pellet was washed with PBS (10 mM NaHPO4, 2 mM KH2PO4, 137 mM NaCl, 8 mM KCl, pH 7.4), and extracted with 500 µl of 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1% Nonidet P-40 (1:1 AB). The detergent extract (1 × AB extract) was assayed for GPI-PLC activity using [3H]mysterate-labeled variant surface glycoprotein (VSG) of T. brucei as substrate (16).

Metabolic Labeling, Immunoprecipitation, and “Pulse-Chase” Assay

Amastigotes were washed thrice with PBS and once with methionine-free RPMI 1640 (Life Technologies, Inc.). Parasites (5 × 105) were resuspended in 5 ml of methionine-free RPMI 1640 containing 10% FBS (dialyzed against PBS overnight at 4 °C), 50 mM HEPES, pH 7.0, and incubated for 1 h at 37 °C. Cells were labeled with 250 µCi of [35S]methionine (1322 Ci/mmol; Amersham) for 2 h, washed twice with PBS, and resuspended in RPMI 1640 supplemented with 100 µg/ml nonradioactive methionine (chase medium) at 37 °C. 10% CO2. Resuspended cells (1 ml) were withdrawn at 0, 6, and 20 h into the “chase,” and harvested by centrifugation at 12,000 × g for 5 min at 4 °C. The medium and cell pellet were stored separately at −20 °C until use.

For ethanolamine labeling, washed amastigotes were resuspended in labeling medium (RPMI 1640, 10% FBS, 40 mM HEPES (pH 7.5), 0.2 mM NaOH, 20 mM l-glutamine, and 1 mM nonessential amino acids (Life Technologies, Inc.) and labeled with 100 µCi/ml [2-3H]ethanolamine hydrochloride (50 Ci/mmol, American Radiolabeled Chemicals Inc.) for 16 h at 37 °C (11). Cells were harvested and stored as described above.

[3H]Methionine or [1-3H]ethanolamine-labeled cells (5 × 106) were lysed in 1 ml of ice-cold immunoabsorption buffer (25 mM Tris-HCl, pH 7.5, 5 mM EDTA, pH 7.5, 250 mM NaCl, 1% Triton X-100) and immunoprecipitated as described (17). Each lysate was eluted from the protein A-Sepharose-antibody-antigen complex by heating at 95 °C for 5 min in 50 µl of 2.5 × SDS-PAGE buffer and run on 10% SDS-PAGE/fluorography with preflashed X-Omat AR film (Eastman Kodak Co.). [3H]Methylated proteins (Sigma) were used as molecular weight standards. Quantitation of the fluorograms was performed on an IS-1000 Digital Imaging System (Alpha Innotech Corp.).

Isolation of Glycolipids, and Thin Layer Chromatography

[3H]Ethanolamine-labeled amastigotes (2 × 106 cells) were delipidated and extracted with chloroform/methanol/water (CMW, 10:10:3; Ref. 11). Glycolipids (2 × 106 cell eq) were resolved by thin layer chromatography (TLC) on Silica Gel 60 in CMW, and detected by fluorography.

To isolate GPIs, [3H]EtN-labeled glycolipids were resolved by high performance thin layer chromatography (HPTLC) in CMW on Silica Gel 60 plates. TeAmLP-3 (2 × 106 cell eq) was scraped after the TLC plate and extracted thrice each time with 1 ml of CMW. The eluates were pooled, dried under a stream of nitrogen and redissolved in 100 µl of n-butanol. Samples were stored at −20 °C until use. [HMP50], GlcENPI from T. brucei (ILTat 1.3) bloodstream form were generated in a cell-free system in presence of 0.25 mM phenylmethylsulfonyl fluoride (20).

Partial Structural Analysis of TeAmLP-3

Phospholipase and Mild Base Digestions—[3H]EtN-labeled T. cruzi glycolipids (2 × 106 cell eq), TCL-purified [3H]TeAmLP-3 (40,000 cpm), or standard [3H]Manα1–3GlcN-PI (10,000 cpm) were digested in a final volume of 100 µl as follows. To cleave with recombinant GPI-PLC (16), the sample was resuspended in 100 µl of 1× AB and digested with 100 units (13 ng) of enzyme at 37 °C for 5 h. For digestion with Bacillus cereus PI-PLC (Boehringer Mannheim), substrate was resuspended in 25 mM Etn-HCl (pH 7.5), 0.27 mM sucrose, 1 mg/ml bovine serum albumin, 0.002% sodium azide containing 1 × 105 units of PI-PLC (17 ng), and incubated for 5 h at 37 °C (21). Phospholipase A2 (PLA2, 24 units, from bee venom (Apis melifera), Sigma) was incubated with substrate in 1 × PLA2 buffer (25 mM HEPES, pH 7.4, 1 mM CaCl2) for 5 h at room temperature. For mild base treatment, dried TeAmLP-3 or Manα1–3GlcN-PI resuspended in 100 µl of 40% propan-1-ol, 13% ammonia (1:1) was incubated at 37 °C overnight and then dried under a nitrogen stream at 40 °C. The dried product was resuspended in 15 µl of n-butanol for TLC analysis.

Exoglycosidase Treatment—TeAmLP-3 or Manα1–3GlcN-PI were treated with 2 units of jack bean α-mannosidase (Oxford GlycoSystems, Rosedale, NY) according to manufacturer’s instructions at 37 °C overnight, in 200 µl of sodium acetate, pH 5.0, 2 mM zinc acetate containing 0.3% sodium taurodeoxycholate (22).

Products of cleavage were extracted with 300 µl of water-saturated n-butanol, followed by two back extractions of the butanol phase with 300 µl of water. Butanol-soluble products were dried under nitrogen and resuspended in 10 µl of n-butanol. Products from various digestions were resolved by TLC on Silica Gel 60 in CMW (10:10:3) or CHCl3:MeOH:0.25% KCl (11:9:2) as indicated in figure legends and detected by fluorography.

Microsequencing of GPI-glycans

Purification of Neutral Glycans—[3H]EtN-labeled GPIs (from 1–3 × 106 parasites) were scraped after HPTLC in CMW from Silica Gel 60 plates (EM Separations, Gibbstown, NJ). Following dephosphorylation with hydrofluoric acid, the resulting neutral glycan was deaminated with nitric acid and radiolabeled with sodium [3H]borohydride (DuPont NEN) (23). The [3H]Glycans were separated from impurities present in the sodium [3H]borohydride by HPTLC with propan-2-ol/methanol/water (PAW, 9:6:5) and elution of the appropriate carbohydrates with PAW. The eluates were dried down, resuspended in 100 µl of 40% propanol, and stored at −20 °C until use. Along with the TeAmLP-3, GPI-glycans Manα1–3GlcN and Manα1–3GlcN were isolated from T. brucei (24, 25) and Leishmania mexicana (2), respectively, for use as standards.

Partial Acid Hydrolysis and Exoglycosidase Digestion—The reduced neutral [3H]Glycan (~20,000 cpm) was resuspended in 100 µl of 100 mM trifluoroacetic acid and heated at 100 °C for 4 h. When needed, complete hydrolysis was carried out in 300 mM trifluoroacetic acid for 8 h. Additionally, the sealed reaction tube was monitored so that liquid which evaporated on to the cap was recovered repeatedly by microcentrifugation. After drying in a SpeedVac, the products were analyzed by HPTLC/fluorography.

Reduced neutral [3H]Glycans were digested with jack bean α-mannosidase (J1BM, 2.5 units/ml, Boehringer Mannheim) in 100 µl of 100 mM NaOAc, pH 5.0, 1.5 mM ZnSO4 for 16 h at 37 °C. The reaction was terminated and analyzed as described previously (23).

Partial Aminoargarid—Dried, deaminated, reduced [3H]Glycans were subjected to acetolysis and processed for HPTLC as described (23).

HPTLC of Neutral Glycans—Glycans were resolved on aluminum-backed Silica Gel 60 HPTLC plates, which were developed sequentially in (i) 1-propanol/acetonewater (9:6:5, v/v/v), (ii) 1-propanol/acetonewater (5:4:1, v/v/v), and (iii) 1-propanol/acetonewater (9:6:5, v/v/v) (23).

Flow Cytometric Analysis

T. cruzi cells were washed in PBS containing 0.1% bovine serum albumin (Sigma) and 0.1% sodium azide (PAB). For each assay, 1 × 106

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parasites were resuspended in 50 μl of PAB containing purified antibody (100 μg/ml) at either 1:1000 dilution or in undiluted hybridoma supernatants and kept for 30 min at 4 °C. The parasites were washed with 1 ml of PAB and incubated for 30 min at 4 °C in the dark in 50 μl of PAB containing a 1:50 dilution of fluorescein isothiocyanate-labeled affinity-purified goat F(ab′)2 anti-mouse or anti-rabbit Ig (IgG-IgM) antibody (Southern Biotechnology Associates, Birmingham, AL). Parasites were washed, resuspended in 1 ml of PAB, and analyzed by flow cytometry on an EPICS 753 Elite cytometer (Coulter Electronics, Hialeah, FL). In some experiments, before staining, parasites (2 × 105) were washed with PBS and then treated with 2 × 104 units of B. cereus PI-PLC.

Growth of T. cruzi Amastigotes in Mammalian Cells

CSWAE1A cells (a neomycin-resistant mouse fibroblast cell transfected with EIA gene of adenovirus) were irradiated with 7500 Rad to stop their division, and allowed to attach to coverslips in 24-well flat-bottomed culture plates for 24 h at 37 °C, 5% CO2 in RPMI, 5% FBS containing 400 μg/ml G418. The fibroblasts were infected with metacyclic trypomastigotes (20:1, parasites:CSWAE1A cells) of pTEX/GPI-PLC expressing amastigotes were deficient in an anionic lipid transfer activity/108 cells. Adapting the cells to grow in higher concentrations of G418 (400 or 800 μg/ml) raised the level of GPI-PLC expression to 500 and 750 units (per 108 cells), respectively. The background level of VSG cleavage activity in control pTEX/tracy/ or pTEX/GPI-PLC/ transfected cells was blocked: Under identical conditions, the mannosyl residues of the control GPIs (Manα1–2GlcN-PI) were cleaved by JBAM (Fig. 1D, lane 12). Treatment with phospholipase A2 did not affect the mobility of TcAmLP-3 (Fig. 1D, lane 4). As a positive control, [3H]mannose-labeled Manα1–2GlcN-PI from T. brucei was cleaved by JBAM (Fig. 1D, lane 12). The lysosomal mannosyl residues of the control GPIs (Manα1–2GlcN-PI) were cleaved by JBAM (Fig. 1D, lanes 6 and 11, respectively). Thus, the inability of PLA2 to cleave TcAmLP-3 suggests it contains a fatty acid only in the sn-1 position of a glycerolipid, or that the alkyl group is derived from ceramide. The latter possibility is excluded by the mild base sensitivity of TcAmLP-3. We infer from these properties that TcAmLP-3 is a GPI containing a phosphoglycerol backbone, which is acetylated at the sn-1 position.

The neutral glycan present in TcAmLP-3 (NG-TcAmLP-3) was characterized. GPI-glycan standards Manα3–6indohydramninositol (Manα3–6AHM) and Manα2–3AHM from T. brucei (26) and L. mexicana (2), respectively, were used as standards. The HPTLC mobility of NG-TcAmLP-3 indicated that it was smaller than Manα3–6AHM (Fig. 2A, lanes 1–3). By partial acid hydrolysis, a ladder of glycans containing Manα3–6AHM, Manα2–3AHM, and Manα2–6AHM were generated from Manα2–6AHM and Manα2–3AHM (Fig. 2B, lanes 2 and 5, respectively). Comparing the mobility of these glycans with NG-TcAmLP-3 (Fig. 2B, lanes 6 and 7) showed that Manα2–6AHM was the major component of TcAmLP-3.

NG-TcAmLP-3 was subjected to acetylation and JBAM treatment (Fig. 2C, lanes 7–9). After acetylation, no change in the mobility of NG-TcAmLP-3 occurred, indicating the absence of an α1–6 glycosidic bond. Control Manα2–6AHM and Manα2–3AHM were both cleaved with production of Manα2–3AHM, as expected (Fig. 2C, lanes 2 and 5). Digestion of the NG-TcAmLP-3 with JBAM resulted in cleavage only of a minor species, which comigrated with Manα2–3AHM. JBAM was not released from the T. cruzi Manα2–3AHM species (Fig. 2C, lane 8). (AHM was produced from the Manα2–6AHM and Manα4–6AHM standards (Fig. 2C, lanes 3 and 6).) Nevertheless, TcAmLP-3 can be metabolically labeled with [3H]mannose (data not presented) and with [3H]EtN (Fig. 1). In addition, trifluoroacetic acid released [3H]AHM from the neutral [3H]glycan of TcAmLP-3 (Fig. 2B, lane 8), denoting the existence of glycosidic bonds in that molecule. The JBAM resistance suggests that the Man residue is most likely not in an α2, α3, or α6 linkage, since the enzyme's specificity for the terminal mannoses is α2Man, α6Man > α3Man. An α4Man linkage in NG-TcAmLP-3 (see below) might
exhibit some relative resistance to JBAM.

In summary, TcAmLP-3 consists predominantly of EtN-phospho-Man₁-GlcN-Ins-phospho-sn-1-glycerolipid. Although we did not specifically determine the location of the EtN, we presume that it is found on the single mannosyl substituent, since it is the only component of GPI-glycans known to accept the phospho-EtN (reviewed in Ref. 2).

**GPI-deficient T. cruzi Express Less GPI-anchored Protein on Their Plasma Membrane**—The effect of a GPI deficiency (see Fig. 1A) on the expression of cell surface proteins was assessed by staining three developmental stages (epimastigotes, trypomastigotes, and amastigotes) of *T. cruzi* with antibodies raised against GPI-anchored proteins. Flow cytometric analysis showed that in all three stages, the cell surface expression of...
GPI-anchored proteins was decreased in the GPI-deficient strain (pTEX.GPI-PLC/T. cruzi) relative to control wild-type or pTEX/T. cruzi parasites (Fig. 3). In GPI-deficient epimastigotes stained with anti-gp50/55 (Fig. 3A) and in amastigotes stained with anti-Ssp-4 or anti-gp50/55 (Fig. 3, C and D, respectively), the decrease in expression of these GPI-anchored proteins was

**FIG. 2.** Microsequencing of the TcAmLP-3 GPI-glycan. A, the neutral glycan from TcAmLP-3 is smaller than Man$_3$-AHM. Neutral [$^3$H]glycans were resolved by Silica Gel 60 HPTLC (see “Experimental Procedures”) and analyzed by fluorography. Lane 1, Man$_3$-AHM from T. brucei; lane 2, Man$_2$-AHM from L. mexicana; lane 3, TcAmLP-3 neutral glycan (NG-TcAmLP-3). B, NG-TcAmLP-3 is mainly Man$_1$-AHM. Reduced neutral [$^3$H]glycans were hydrolyzed with trifluoroacetic acid for 4–8 h, and the products analyzed by HPTLC/fluorography. Lane 1, Man$_3$-AHM; lane 2, Man$_2$-AHM treated with trifluoroacetic acid for 4 h; lane 3, Man$_2$-AHM treated with trifluoroacetic acid for 8 h; lane 4, Man$_3$-AHM; lane 5, Man$_4$-AHM treated with trifluoroacetic acid for 4 h; lane 6, NG-TcAmLP-3; lane 7, NG-TcAmLP-3 treated with trifluoroacetic acid for 4 h; lane 8, trifluoroacetic acid-treated NG-TcAmLP-3 (8 h). C, partial acetolysis of NG-TcAmLP-3. Man$_3$-AHM (lanes 1–3), Man$_4$-AHM (lanes 4–6), and NG-TcAmLP-3 (lanes 7–9) were subjected to partial acetolysis (lanes 2, 5, and 8, respectively), or digested with jack bean a-mannosidase (lanes 3, 6, and 9, respectively). Products were analyzed by HPTLC/fluorography.
Similar to that obtained when wild-type parasites were treated with an excess of extracellularly added phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus cereus*. Trypomastigotes of pTEX.GPI-PLC/T. cruzi also showed less cell surface expression of a trans-sialidase (evident by anti-SAPA staining (Fig. 3B)). The general decrease in cell surface expression of proteins in pTEX.GPI-PLC/T. cruzi was specific for GPI-anchored molecules, as shown by staining with the anti-amastigote antibody IC10B; expression of the molecule recognized by this monoclonal antibody was similar in wild-type and GPI-deficient amastigotes (Fig. 3E).

A Select Group of Proteins Is Degraded Intracellularly in GPI-deficient *T. cruzi*—To investigate the fate of GPI-anchored proteins, amastigotes were metabolically labeled with [35S]methionine and chased. For both cells and "chase medium," a profile of [35S]methionine-labeled proteins was obtained after SDS-PAGE and fluorography.

Significant differences were observed between the major [35S]methionine-labeled amastigote proteins of pTEX.GPI-PLC/T. cruzi and the wild-type control cells. Two proteins of ~60 kDa (p60 doublet, marked with arrows; Fig. 4A) present in wild-type cells at the beginning of the chase period, remained unchanged relative to other proteins (in the same lysate) during a 20-h chase (Fig. 4A, compare lanes 1, 3 and 5). The p60 doublet was absent from pTEX.GPI-PLC/T. cruzi even at the beginning of the chase (Fig. 4A, compare lanes 2, 4, and 6), and was not detected in amastigotes labeled for 15 min with [35S]methionine (data not shown). Apparently, p60 is either not expressed at all, or is degraded very rapidly in the GPI-deficient cells. A 50-kDa protein, p50 (marked with asterisk, Fig. 4A) was expressed in wild-type *T. cruzi* at levels apparently similar to p60 and remained unchanged during a 20-h chase. In pTEX.GPI-PLC/T. cruzi, p50 was present in slightly higher amount at 0 h than in wild-type cells (Fig. 4A, lane 1 and 2). The level of this protein remained unchanged within the first 6 h (Fig. 3A, lane 4), but only ~30% of the total remained after 20 h (Fig. 4A, compare lanes 2 and 6).

**Fig. 3.** GPI-deficient *T. cruzi* express fewer GPI-anchored proteins on their cell surface. The staining patterns of monoclonal antibodies against GPI-anchored surface proteins of wild-type *T. cruzi* (thin dashed line), pTEX.GPI-PLC/T. cruzi (solid line), and *T. cruzi* treated extracellularly with *Bacillus cereus* PI-PLC (thick dashed line) was determined by flow cytometry (details are given under “Experimental Procedures”). Antibodies and developmental stages analyzed were as follows: A, mAb C10 (anti-gp50/55), epimastigotes; B, anti-SAPA, trypomastigotes; C, mAb 2C2 (anti-Ssp-4), amastigotes; D, mAb C10 (anti-gp50/55), amastigotes; and E, mAb IC10B (against a protein that is not GPI-anchored), amastigotes. Background staining with a control antibody is shown by dotted line.

2 M. A. M. Santos and R. L. Tarleton, unpublished data.
Since the identities of p60 (Fig. 4A) and p50 were unknown and we have no antibodies specific to either protein, the possibility that they are GPI-anchored was checked by Triton X-114 phase partition before and after PI-PLC digestion. Membrane fractions from a hypotonic lysate of the parasites were studied for this purpose. The [35S]methionine-labeled p60 doublet, similar to p50, was in the detergent phase prior to PI-PLC digestion (Fig. 4B, lanes 1 and 2) and subjected to phase partition in Triton X-114 (see “Experimental Procedures”). Proteins from detergent and aqueous phases were analyzed by SDS-PAGE/fluorography. Lanes 1 and 3, aqueous phase; lanes 2 and 4, detergent phase. C, protease gp50/55 is degraded intracellularly. T. cruzi amastigotes were labeled with [35S]methionine and chased for 0 h (lanes 1 and 4), 8 h (lanes 2 and 5), and 24 h (lanes 3 and 6). A lysate of 5 × 10⁷ cells was immunoprecipitated with anti-gp50/55 monoclonal antibody (mAb C10). Lanes 1–3, pTEX.GPI-PLC/T. cruzi; lanes 4–6, pTEX/T. cruzi. D, quantitation of intracellular gp50/55. Fluorographic images of intracellular gp50/55 in pTEX/T. cruzi or pTEX.GPI-PLC/T. cruzi from C were quantitated on an IS-1000 Digital Imaging System (Alpha Innotech Corp.). Shaded bars, pTEX/T. cruzi; hatched bars, pTEX.GPI-PLC/T. cruzi.

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The fate of a known GPI-anchored protease, gp50/55, was monitored by immunoprecipitation of total cell lysate and chase medium from [35S]methionine-labeled amastigotes with monoclonal antibody C10 (21). In pTEX/T. cruzi, 55% of gp50/55 was retained in the cell after a 24-h chase (Fig. 4, C, lanes 4–6, and D). In contrast, only 10% of gp50/55 is retained in GPI-deficient amastigotes at 24 h (Fig. 4, C, lanes 1–3, and D). Most likely, gp50/55 is degraded within the cell, since it was never detected in the chase medium of either pTEX/T. cruzi or pTEX.GPI-PLC/T. cruzi. Thus, GPI-deficient amastigotes degrade gp50/55 approximately 5 times faster than wild-type T. cruzi.

Ssp-4 and p75 Are Secreted Rapidly in GPI-deficient T. cruzi—We examined the basis of the decreased cell surface expression of Ssp-4 (Fig. 4) by studying the kinetics of Ssp-4 cell association. Release of Ssp-4 was monitored by immunoprecipitation from chase medium of [35S]methionine-labeled amastigotes with an Ssp-4-specific monoclonal antibody 2C2. In wild-type or pTEX/T. cruzi, an amount of Ssp-4 equivalent to that released in 20 h from wild-type cells is detectable only 6 h into the chase (Fig. 5A, compare lane 3 with lane 5). Furthermore, at the 6-h time point, 5-fold more Ssp-4 (~180 kDa) was released by
increases the rate and extent of Ssp-4 secretion. Amastigotes were

The chased medium was also examined for proteins not detected inside GPI-deficient T. cruzi (i.e. as compared with wild-type cells; Fig. 4A). The predominant radiolabeled proteins in the medium were 50, 56, 75, and 180 kDa (p50, p56, p75, and p180, respectively) (Fig. 5C). Interestingly, the predominant secreted proteins p75 and p180 were not the major polypeptides in the total cell lysate (compare Figs. 4A and 5C). The latter observation indicates that secretion of proteins did not occur en mass. Several other proteins of less than 45 kDa were secreted in the GPI-deficient cells (Fig. 5C, lanes 4 and 6).

In conclusion, two effects of a GPI deficiency are observed on amastigote proteins that are normally GPI-anchored, as exemplified by Ssp-4 and gp50/55: (i) a 5-fold acceleration of secretion (Fig. 5, A and B), and (ii) a 5-fold increase in the rate of intracellular degradation (Fig. 4, B and C).

**GPI Deficiency Is Associated with Inhibition of Nuclear Division**—The GPI deficiency in T. cruzi was associated with striking phenotypic changes. Metacyclic trypomastigotes obtained from epimastigotes that had been cultured in 200 μg/ml G418 infected various mammalian fibroblast cell lines, differentiated into and replicated slowly as amastigotes, but still completed the life cycle. However, transfectants cultured at 400 μg/ml G418, thereby increasing the level of expression of GPI-PLC, infected mammalian cells and differentiated into amastigotes but divided only once.

GPI-deficient amastigotes in most cases replicated the kinetoplast, but failed to sustain replication of the cell nucleus, and were di-kinetoplastid (Fig. 6). Hence, GPI-deficient T. cruzi amastigotes are arrested in the cell cycle apparently in anaphase. In agreement with these observations, replication of GPI-deficient T. cruzi was severely inhibited such that a lawn of CSWAE1A cells, which is normally lysed in 4 days if infected with wild-type T. cruzi, remained intact after 10 days of infection with GPI-deficient parasites.

**DISCUSSION**

**GPI Deficiency in T. cruzi Conferred by Heterologous Expression of a GPI-PLC**—One of our major interests was to examine the effect of a GPI deficiency on T. cruzi. However, studies of GPI biosynthesis in T. cruzi have only recently been initiated (27–29). No genes involved in this pathway have been cloned. In the absence of genes for targeted mutagenesis of the GPI pathway, we generated cells with this desired deficiency by stable expression in T. cruzi of a GPI-PLC gene from the related protozoan parasite T. brucei. This approach was used previously to explore the topography of the protein and polysaccharide-GPI pathways in Leishmania (11).

The GPI-deficient T. cruzi as compared with wild-type parasites (Fig. 5, A, compare lanes 2 and 5, and B).

FIG. 5. Rapid and increased release of Ssp-4. A, GPI deficiency increases the rate and extent of Ssp-4 secretion. Amastigotes were

metabolically labeled with [35S]methionine and chased for 0 h (lanes 1 and 4), 6 h (lanes 2 and 5), and 20 h (lanes 3 and 6). [35S] incorporation into macromolecules was determined by trichloroacetic acid precipitation of a lysate of 10⁶ cells to be 27,696 cpm and 29,323 cpm for wild-type and pTEX.GPI-PLC/T. cruzi, respectively.) Ssp-4 was immuno precipitated from chase medium (5 × 10⁵ cell eq) with mAb 2C2. Lanes 1–3, T. cruzi; lanes 4–6, pTEX.GPI-PLC/T. cruzi. B, quantitation of secreted Ssp-4. Fluorographic images of Ssp-4 secreted into culture media (A) were quantitated on an IS-1000 Digital Imaging System. Open bars, T. cruzi; hatched bars, pTEX.GPI-PLC/T. cruzi. C, other proteins released into the chase medium. [35S]Methionine-labeled amastigotes (2 × 10⁷ parasites) were chased for 0, 6, and 20 h. Proteins in the corresponding culture medium from chases at 0 h (lane 1, T. cruzi; lane 2, pTEX.GPI-PLC/T. cruzi), 6 h (lane 3, T. cruzi; lane 4, pTEX.GPI-PLC/T. cruzi) and 20 h (lane 5, T. cruzi; lane 6, pTEX.GPI-PLC/T. cruzi) were analyzed by SDS-PAGE and fluorography.

A

![Ssp-4](image)

B

![Graph](image)

C

![Graph](image)
Fig. 6. Nuclear division is blocked in GPI-deficient amastigotes. Following infection with metacyclic trypanosomes of pTEX.GPI-PLC/T. cruzi, CSWA1A cells were stained with SYTO11 (Molecular Probes). Infected cells were visualized by laser scanning confocal microscopy (Bio-Rad MRC-600). Amastigotes are characterized by crescent-shaped kinetoplast (k) positioned close to the nucleus (n).

GPI-PLC requires GlcN(α1–6)Ins for efficient substrate recognition (30) and is therefore highly specific for GPs.4 The enzyme cleaves most GPI biosynthetic intermediates in vivo (Fig. 1), thereby conferring on pTEX.GPI-PLC/T. cruzi a phenotype equivalent to a dominant-negative loss of function mutation in the GPI pathway. The severity of the phenotype was dependent upon the amount of GPI-PLC expressed, which was in turn driven by the concentration of G418 used in the culture medium. At a permissive G418 concentration (i.e. in turn driven by the concentration of G418 used in the culture dependent upon the amount of GPI-PLC expressed, which was
tation in the GPI pathway. The severity of the phenotype was notype equivalent to a dominant-negative loss of function mu-

deficiency. The structure of TcAmLP-3 is EtN-P-Man-GlcN-
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Cell Cycle Arrest of GPI-deficient T. cruzi

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Proteins with Glycosylphosphatidylinositol (GPI) Signal Sequences Have Divergent Fates during a GPI Deficiency: GPIs ARE ESSENTIAL FOR NUCLEAR DIVISION IN TRYPANOSOMA CRUZI

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