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Glycosylphosphatidylinositols Are Required for the Development of *Trypanosoma cruzi* Amastigotes

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Induction of a glycosylphosphatidylinositol (GPI) deficiency in *Trypanosoma cruzi* by the heterologous expression of *Trypanosoma brucei* GPI-phospholipase C (GPI-PLC) results in decreased expression of major surface proteins (N. Garg, R. L. Tarleton, and K. Mensa-Wilmot, J. Biol. Chem. 272:12482–12491, 1997). To further explore the consequences of a GPI deficiency on replication and differentiation of *T. cruzi*, the in vitro and in vivo behaviors of GPI-PLC-expressing *T. cruzi* were studied. In comparison to wild-type controls, GPI-deficient *T. cruzi* epimastigotes exhibited a slight decrease in overall growth potential in culture. In the stationary phase of in vitro growth, GPI-deficient epimastigotes readily converted to metacyclic trypomastigotes and efficiently infected mammalian cells. However, upon conversion to amastigote forms within these host cells, the GPI-deficient parasites exhibited a limited capacity to replicate and subsequently failed to differentiate into trypomastigotes. Mice infected with GPI-deficient parasites showed a substantially lower rate of mortality, decreased tissue parasite burden, and a moderate tissue inflammatory response in comparison to those of mice infected with wild-type parasites. The decreased virulence exhibited by GPI-deficient parasites suggests that inhibition of GPI biosynthesis is a feasible strategy for chemotherapy of infections by *T. cruzi* and possibly other intracellular protozoan parasites.

Trypanosoma cruzi, the causative agent of Chagas' disease, is an intracellular protozoan parasite which infects an estimated 18 million people in South and Central America (22). Epimastigotes of *T. cruzi* divide in the midgut of reduviid bug vectors and transform into metacyclic trypomastigotes, which are then transmitted to mammalian hosts. Trypomastigotes invade mammalian cells, where they differentiate into and replicate as amastigotes, converting back into trypomastigotes before being released from the intracellular environment (29).

Glycosylphosphatidylinositols (GPIs) are the predominant anchor structures utilized by protozoans for cell surface expression of proteins (10, 19, 27). GPI-anchored molecules like the variant surface glycoproteins of *Trypanosoma brucei* and the surface metallo-protease (gp63) and lipophosphoglycan of *Leishmania* spp. have been implicated in survival and protection of these respective parasites against host defense mechanisms (10, 19, 27).

To better understand the biological role of GPI-anchored molecules in *T. cruzi*, GPI phospholipase C (GPI-PLC) from the related kinetoplastid *T. brucei* (8, 16, 20) was expressed in *T. cruzi*. Biochemical analysis of GPI-PLC-expressing *T. cruzi* revealed that GPI-PLC cleaves GPI intermediates, which results in the depletion of protein GPIs (13). Cell surface expression of GPI-anchored proteins in GPI-deficient *T. cruzi* is reduced compared to that of wild-type cells in all developmental stages. Thus, these transfectants effectively represent a dominant negative loss of function mutation in the GPI-biosynthetic pathway.

In this report, we address the effect of this GPI deficiency on infectivity and replication of *T. cruzi* both in vitro and in vivo. In vitro growth of epimastigotes was not drastically affected by the GPI deficiency, and metacyclic trypomastigotes derived from stationary-phase cultures of GPI-PLC transfectants were infective for host cells. However, proliferation and differentiation of *T. cruzi* amastigotes were severely limited in GPI-PLC transfectants. As expected, GPI-deficient *T. cruzi* also exhibited a significant decrease in virulence in vivo in mice. These studies provide the first demonstration that GPIs are necessary for the transformation and replication of an intracellular protozoan parasite of humans and suggest that inhibition of GPI biosynthesis would be an effective route for chemotherapeutic control of this parasite and perhaps other protozoan parasites.

MATERIALS AND METHODS

Parasites. The Brazil strain of *T. cruzi* was used during the course of this study. Epimastigotes of *T. cruzi* were cultured at 26°C in liver infusion tryptose (LIT) medium supplemented with 5% heat-inactivated fetal bovine serum. Metacyclic trypomastigotes were obtained from stationary-phase axenic cultures in LIT medium (9). Culture-derived trypomastigotes were obtained from Vero cells (ATCC CRL-1586; American Type Culture Collection, Rockville, Md.) infected with metacyclic stationary-phase trypomastigotes. Epimastigotes transfected with pTEX or pTEX.GPIPLC (13) were selected and cultured continuously in the presence of 200 to 800 μg of G418 per ml. The GPI-PLC activities in the transfectants were estimated with [^{3}H]myristate-labeled *T. brucei* membrane-form variant surface glycoprotein as the substrate (21).

***T. cruzi* infection of mammalian cells in vitro.** Fibroblasts (Vero, 5A.K^b.α3 [L cells transfected with the murine major histocompatibility H-2K^b gene] or CSWAE1A [C57BL/6 mouse fibroblasts transfected with the adenovirus E1A gene, G418 resistant] cells), cells of the macrophage line IC-21, and bone marrow or peritoneal macrophages from C57BL/6 mice were allowed to adhere to coverslips in 24-well flat-bottomed culture plates and were infected with metacyclic trypomastigotes at a ratio of 20:1 (parasites to host cells) in RPMI 1640–10% fetal bovine serum. After 24 h of incubation, cells were washed with RPMI 1640 to remove extracellular parasites. At 24-h intervals, coverslips were removed, fixed, and stained with LeukoStat (Fisher Scientific, Pittsburgh, Pa.). A minimum of 200 cells in different microscopic fields were examined by light microscopy, and the percentage of infected cells and the average number of parasites per infected cell were determined.

For observation of live infected cells, irradiated (7,500-rad) CSWAE1A cells were infected with metacyclic trypomastigotes of pTEX/*T. cruzi* or pTEX.GPI PLC/*T. cruzi* (20:1, parasites to host cells) and cultured with 400 to 800 μg of G418 per ml on coverslips in 24-well plates as described above. Coverslips were washed after 24 h of incubation to remove extracellular parasites, and then at 24-h intervals, cells were stained with 1 mM SYTO 11 nucleic acid stain (Molecular Probes, Eugene, Oreg.) and visualized with a laser scanning confocal microscope (model MRC-600; Bio-Rad, Hercules, Calif.).

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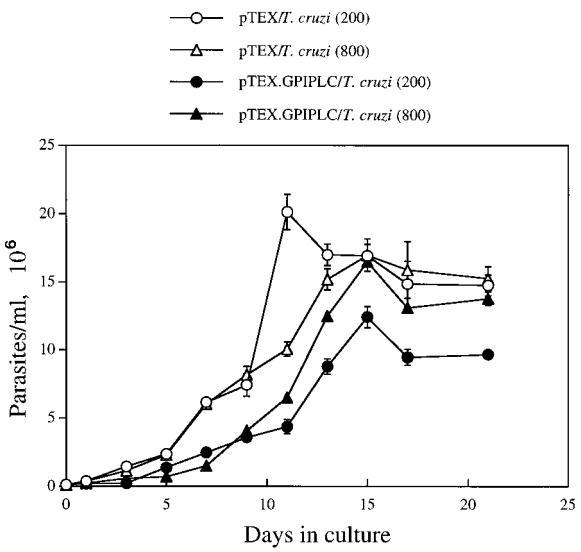

FIG. 1. Growth of *T. cruzi* epimastigotes is largely unaffected by GPI-PLC expression. LIT medium was inoculated with epimastigotes (2×10^5/ml) of pTEX/*T. cruzi* or pTEX.GPIPLC/*T. cruzi* cultured in 200 to 800 μg of G418 per ml. Proliferation was monitored by hemocytometer count on alternate days up to 3 weeks. Results are the means ± standard deviations of results from three separate experiments.

Infection in mice. To observe the course of infection in mice infected with GPI-deficient parasites, C3H/HeSnJ female mice (Jackson Laboratories, Bar Harbor, Maine) injected intraperitoneally with 5×10^4 to 1×10^6 axenic metacyclic trypomastigotes or severe combined immunodeficient (SCID) mice (Jackson Laboratories) infected with 5×10^3 metacyclic trypomastigotes were used. Parasitemias were monitored periodically by examination of tail vein blood, and mortality was recorded daily. Some mice were sacrificed for histological examination of muscle tissue. Heart and skeletal muscles were removed and fixed in 10% buffered formalin for 24 h before routine processing and embedding in paraffin. Sections (5 μm thick) were stained with hematoxylin and eosin and evaluated by light microscopy.

Detection of parasites in tissue by reverse transcription-PCR. Tissues removed from heart and skeletal muscles of normal and *T. cruzi*-infected mice were immediately frozen in liquid nitrogen and homogenized, and total RNA was purified with RNAzol solution (Biotecx Labs, Houston, Tex.). First-strand cDNA was synthesized with a SuperScript II kit (Life Technologies, Gaithersburg, Md.) by following the manufacturer's instructions. Amplification of cDNA by PCR was performed with the *T. cruzi* rRNA-based primers 5′-GACTACCCGCC GAACTT-3′ (106 to 122 bp, sense) and 5′-ACACCATGACCTCGTTC-3′ (678 to 684 bp, antisense). The β-actin primers 5′-ATGGATGACGATATCGCT-3′ (81 to 98 bp, forward) and 5′-ATGAGGTAGTCTGTCAGGT-3′ (613 to 631 bp, reverse) were used as internal controls for determining the efficiency of cDNA synthesis and PCR amplification. PCR was performed with 0.4 μM each primer, 200 μM deoxynucleoside triphosphate, 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 0.001% gelatin, 3 mM $MgCl_2$), and 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Foster City, Calif.) in 50-μl reaction mixtures in a model PTC-100 programmable thermal cycler (MJ Research, Watertown, Mass.) for 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 2 min, and extension at 72°C for 40 s.

RESULTS

Expression of *T. brucei* GPI-PLC was achieved in *T. cruzi* by transfection of Brazil strain epimastigotes with pTEX.GPIPLC and selection in G418 at concentrations from 200 to 800 μg/ml (13). Transfectants selected under these conditions maintained the pTEX plasmid extrachromosomally, making it possible to control the level of expression of GPI-PLC by varying the concentration of G418. GPI-PLC activities in transfectants selected in 200, 400, and 800 μg of G418 per ml were, respectively, 370, 560, and 750 U per 10^8 cells (13).

pTEX.GPIPLC/*T. cruzi* epimastigotes showed a lag of 3 to 4 days in achieving log-phase growth in in vitro culture but exhibited a rate of growth similar to that of pTEX/*T. cruzi* transfectants once log-phase growth was initiated (Fig. 1). Stationary phase was reached earlier by pTEX/*T. cruzi* than by pTEX.GPIPLC/*T. cruzi* selected in 200 μg of G418 per ml (10 and 15 days, respectively), though the densities of the two transfectants were similar at day 22 of culture. pTEX.GPIPLC/*T. cruzi* epimastigotes selected in 800 μg of G418 per ml reached the stationary phase within 15 days, but the final cell density was 33% lower than that of comparatively selected pTEX/*T. cruzi* epimastigotes (Fig. 1). Thus, inhibition of GPI biosynthesis in *T. cruzi* has a limited, dose-dependent inhibitory effect on replication of epimastigotes.

Metacyclic trypomastigotes from stationary-phase cultures of pTEX.GPIPLC/*T. cruzi* selected in 200 μg of G418 per ml were able to infect a variety of mammalian cell types (Vero, 5A.K^b.α3, and CSWAE1A cells, IC-21 macrophages, and bone marrow or peritoneal macrophages from C57BL/6 mice). The results of studies performed with the IC-21 mouse macrophage line showed that 75 and 68% of these cells were infected following a 24-h exposure to pTEX/*T. cruzi* and pTEX.GPIPLC/*T. cruzi*, respectively (Fig. 2A and B). Wild-type parasites replicated within IC-21 cells as amastigotes, converted into trypomastigotes, and destroyed the host monolayer upon their release 96 to 120 h postinfection (Fig. 2C). However, intracellular amastigotes of pTEX.GPIPLC/*T. cruzi* replicated at a rate much lower than that of wild-type parasites, rarely reaching an intracellular density of >40 parasites per host cell at any time point postinfection (Fig. 2C). Macrophages containing 1 to 10 amastigotes per cell dominated the monolayers infected with pTEX.GPIPLC/*T. cruzi* as late as 192 h postinfection (Fig. 2C); relatively few pTEX.GPIPLC-expressing parasites were observed to convert into trypomastigote forms and escape from the infected host cell. Apparently, culture of GPI-PLC transfectants in relatively low concentrations of G418 (200 μg/ml) does not inhibit the production of mature GPIs to the level necessary to totally prevent amastigote replication.

In order to attain a higher level expression of GPI-PLC, G418-resistant CSWAE1A cells were used as host cells and were infected with transfectant parasites under continuous selection with 400 μg of G418 per ml. The overall pattern of infection and replication of GPI-PLC transfectants selected with 400 μg of G418 per ml were similar to those shown in Fig. 2 for transfectants selected with 200 μg of G418 per ml, with the exception that intracellular replication was more restricted and no CSWAE1A cells which contained >30 parasites were detected (data not shown). GPI-deficient (pTEX.GPIPLC/*T. cruzi*) and wild-type (pTEX/*T. cruzi*) metacyclic trypomastigotes previously adapted to growth in 400 μg of G418 per ml were equally capable of infecting mouse fibroblasts and converting intracellularly into amastigotes (Fig. 3A and B). pTEX/*T. cruzi* amastigotes replicated within the host cell, eventually differentiated into trypomastigotes (Fig. 3C), and lysed the monolayer within 120 h postinfection. However, replication of GPI-deficient amastigotes was slower (Fig. 3C and D) and conversion of the GPI-deficient amastigotes into trypomastigotes could not be detected even 240 h postinfection (Fig. 3E); the irradiated host cells eventually died without releasing any trypomastigotes. Closer examination of the population of cells infected with GPI-deficient parasites revealed that a substantial number of the amastigotes successfully replicated their kinetoplasts but failed to replicate their nuclei (Fig. 3E). Such dikinetoplastid forms were not detected in GPI-PLC-expressing epimastigotes. The significance of this dikinetoplastid phenotype is discussed elsewhere (13).

The decreased capacity of intracellular amastigotes of *T. cruzi* to replicate would be expected to result in decreased

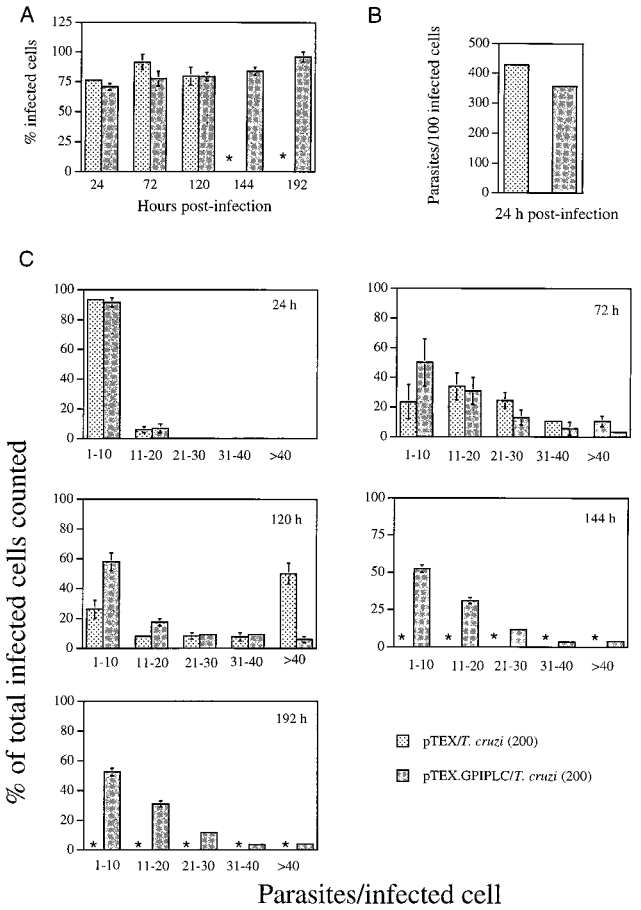

FIG. 2. (A) Infectivity of *T. cruzi* trypomastigotes is not affected by GPI deficiency. Mouse IC-21 cells were infected with trypomastigotes of pTEX/*T. cruzi* or pTEX.GPIPLC/*T. cruzi* selected for growth in 200 μg of G418 per ml. Macrophage monolayers were monitored microscopically for up to 192 h postinfection. (B) Intracellular survival of GPI-deficient metacyclic trypomastigotes is not affected. Mouse IC-21 cells infected with trypomastigotes of pTEX/*T. cruzi* or pTEX.GPIPLC/*T. cruzi* selected for growth in 200 μg of G418 per ml were fixed and stained with Leukostat 24 h postinfection, and the number of parasites/cell was recorded by light microscopy (magnification, ×40). (C) GPIs are required for amastigote replication. Infected macrophages were fixed and stained as described above, and the number of parasites in at least 200 infected cells, reflecting the replication capacity of amastigotes, was recorded. Results are means ± standard deviations of triplicate determinations from separate experiments. ⋆, the macrophage monolayer was destroyed by the release of trypomastigotes.

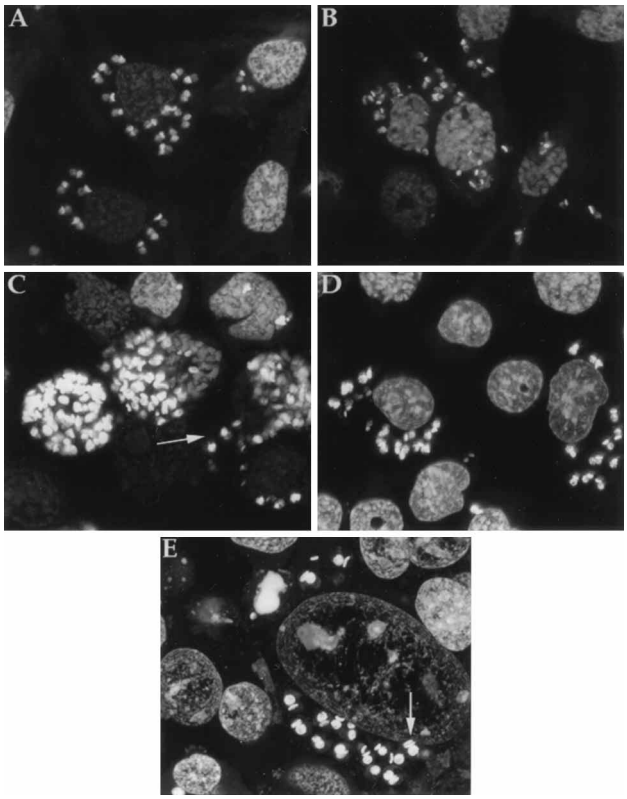

FIG. 3. Development of GPI-deficient amastigotes is blocked at a nonpermissive GPI-deficiency level. Following infection with pTEX/*T. cruzi* (A and C) or pTEX.GPIPLC/*T. cruzi* (B, D, and E) axenic metacyclic trypomastigotes selected for growth in 400 μg of G418 per ml, CSWAE1A cells were stained with SYTO 11 at 48 h (A and B), 120 h (C and D), and 240 h (E) postinfection. Infected cells were analyzed by laser scanning confocal microscopy (magnification, ×60). Amastigotes are characterized by crescent-shaped kinetoplasts positioned close to nuclei (marked with an arrow in panel E); trypomastigotes show greater separation of kinetoplasts from nuclei (marked with an arrow in panel C).

virulence in infected mice. Indeed, highly susceptible C3H/HeSnJ mice infected with 5×10^4 wild-type metacyclic trypomastigotes showed a steady increase in parasitemia and all mice died within 25 days postinfection (Fig. 4A and B). However, mice infected with GPI-deficient *T. cruzi* metacyclics selected with 200 μg of G418 per ml showed at least a 10-fold-lower level of parasitemia (Fig. 4A) and four of six of these mice survived the infection (Fig. 4B). In several additional experiments, only 2 of 26 C3H/HeSnJ mice infected with 5×10^4 to 1×10^6 wild-type parasites survived acute infection, compared to 22 of 26 mice infected with the same numbers of the GPI-deficient *T. cruzi*.

Histopathological analysis of pTEX.GPIPLC/*T. cruzi*-infected C3H/HeSnJ mice 1 year postinfection failed to detect tissue parasites, though the presence of parasites in their hearts and skeletal muscles could still be confirmed by reverse transcription-PCR (data not shown). The presence of very mild inflammation in the skeletal muscles and heart tissues of these animals (data not shown) also suggests that despite the decreased virulence, the GPI-deficient *T. cruzi* parasites selected with 200 μg of G418 per ml were not totally avirulent or perhaps had lost the expression of GPI-PLC during in vivo development in mice.

GPI-deficient parasites were also less virulent in SCID mice, exhibiting a 10-fold-lower level of parasitemia than SCID mice infected with wild-type parasites (Fig. 5A). However, SCID mice infected with pTEX.GPIPLC/*T. cruzi* survived only 2 days longer on average than SCID mice infected with wild-type parasites (21 versus 23 days postinfection) (Fig. 5B).

DISCUSSION

We have previously shown that the generation of a GPI deficiency in *T. cruzi* achieved by expression of GPI-PLC from *T. brucei* results in reduced cell surface expression of GPI-anchored proteins (13). This phenotype is the result of cleavage of GPI precursors by GPI-PLC and the subsequent secretion or intracellular degradation of proteins which would normally be GPI anchored. In this study we showed that this GPI deficiency phenotype also results in reduced intracellular growth and decreased in vivo virulence of *T. cruzi*.

GPI deficiency in eukaryotic cells is likely to have multiple

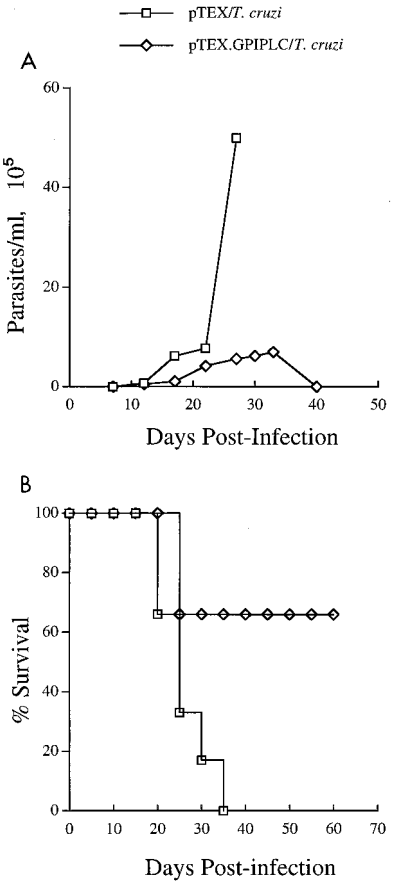

FIG. 4. Decreased virulence of GPI-deficient parasites in vivo. (A) C3H/HeSnJ mice (six per group) were injected intraperitoneally with 5×10^4 metacyclic trypomastigotes selected for growth in 200 μg of G418 per ml, and parasitemia was determined at 5-day intervals. (B) Mortality of C3H/HeSnJ mice infected with pTEX/*T. cruzi* or pTEX.GPIPLC/*T. cruzi*.

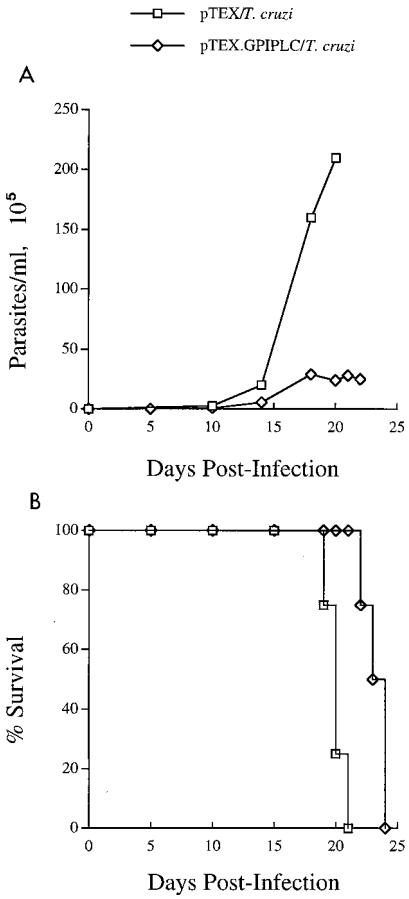

FIG. 5. GPI-deficient parasites are not avirulent in SCID mice. (A) SCID mice (four per group) were injected intraperitoneally with 5×10^3 pTEX/*T. cruzi* or pTEX.GPIPLC/*T. cruzi* metacyclic trypomastigotes selected for growth in 200 μg of G418 per ml, and parasitemia was monitored. (B) Mortality of SCID mice infected with pTEX/*T. cruzi* or pTEX.GPIPLC/*T. cruzi*.

effects on cellular physiology, since many different kinds of macromolecules, including polysaccharides, enzymes, receptors, and glycoproteins, are GPI anchored (10, 19). To fully address the physiological function of GPIs, mutants deficient in the GPI-biosynthesis pathway are required. Induction of a GPI deficiency in *T. cruzi* by high-level expression of GPI-PLC resulted in epimastigotes which replicated only slightly less vigorously than wild-type or pTEX-expressing parasites and which converted to infective stationary-phase metacyclic forms.

In contrast to epimastigotes, amastigotes of pTEX.GPI PLC/*T. cruzi* were profoundly affected by the GPI deficiency, as was demonstrated by their limited replication and failure to complete intracellular development by conversion to trypomastigotes. The differential effect of GPI-PLC expression on growth of epimastigotes and amastigotes might be explained in several ways. Epimastigotes appear to have relatively few GPI-anchored surface proteins (gp50/55, a cysteine protease which is expressed in all three developmental stages [11, 17], is the only one reported so far). In contrast, trypomastigote and amastigote stages of *T. cruzi* appear to anchor the majority of their surface proteins by GPIs. If epimastigotes are less dependent on GPIs as a mechanism of protein anchoring, then these insect-stage parasites might be expected to be less affected by a GPI deficiency. It is also possible that GPI precursors in epimastigotes are less sensitive to cleavage by GPI-PLC than those in amastigotes, resulting in a less severe GPI deficiency in epimastigotes. Though a complete characterization of the structure of epimastigote GPIs and a determination that they are susceptible to GPI-PLC are required to support this hypothesis, preliminary experiments failed to detect the depletion of GPI intermediates in GPI-PLC-transfected epimastigotes (data not shown). Also, surface expression of GPI-anchored proteins was not as drastically reduced by GPI-PLC expression in epimastigotes as in amastigotes (13).

The competence of GPI-deficient metacyclic trypomastigotes to invade host cells was somewhat surprising. The GPI-anchored mucin-like proteins and the Ssp-3 molecule that are present on metacyclic trypomastigotes and bloodstream trypomastigotes, respectively (25, 26), are reportedly involved in attachment and invasion of mammalian cells by *T. cruzi* (5). Pretreatment of mammalian host cells with anti-Ssp-3 monoclonal antibodies or purified mucin-like proteins results in reduced efficiency of invasion (14, 23, 26). The substantially reduced cell surface expression of GPI-anchored molecules in pTEX.GPIPLC/*T. cruzi* under conditions of GPI deficiency (13) was therefore expected to significantly decrease the infectivity of these parasites. However, GPI-deficient parasites infected a variety of host cells with an efficiency equal to that of their wild-type counterparts. One explanation for the retention

of infectivity in GPI-deficient *T. cruzi* is that a low but sufficient level of GPI-anchored proteins which are important in invasion is still expressed. Alternatively, it is possible that in the absence of these particular molecules, invasion occurs via as yet uncharacterized, non-GPI-anchored ligands. The report of successful infection by *T. cruzi* of Chinese hamster ovary cells that are 90% deficient in sialylated proteins, the necessary donors for invasion-related sialic acid addition to mucin-like proteins and Ssp-3 (24), is consistent with either of these possibilities.

The profound effect of GPI-PLC expression on the intracellular growth of *T. cruzi* amastigotes is striking. The signals that control conversion of trypomastigotes into amastigotes, the replication of amastigotes, and the eventual conversion of these amastigotes back into trypomastigotes, all of which occur within the host cell cytoplasm, are not known. Nevertheless, we hypothesize that the limited intracellular replication exhibited by GPI-deficient parasites may be due to one of several mechanisms. Some specific GPI-anchored proteins or free GPIs in the parasite membrane may act as mediators or receptors for signals that trigger amastigote development and proliferation. The relative absence of transduction of such signals into the parasite may result in failure to initiate cell division. One may also envision a feedback mechanism of inhibition of replication mediated by the release of parasite molecules into the host cell cytoplasm. This protein release by intracellular parasites has been well-documented (1, 2, 12), and enhanced release into the host cell cytoplasm of molecules which are normally GPI anchored is evident in GPI-PLC-expressing amastigotes of *T. cruzi* (13). A third possibility is that the GPI deficiency in *T. cruzi* is analogous to the situation with the temperature-sensitive *Saccharomyces cerevisiae* mutants, which synthesize GPIs and replicate like wild-type cells when they are cultured at 24°C but are not capable of growth and accumulate GPI intermediates at 37°C (4). Similarly, epimastigotes of pTEX. GPIPLC/*T. cruzi* cultured at 26°C are relatively unaffected by GPI deficiency, but intracellular amastigotes cultured at 37°C exhibit drastic effects of the GPI deficiency on replication and differentiation.

The possibility that the decreased rate of replication of GPI-deficient amastigotes is related to adverse effects of GPI-PLC on the host cells can be largely ruled out because, as with *T. brucei*, GPI-PLC in *T. cruzi* is a cytoplasmic-membrane-associated protein that is not secreted (13a, 21). The presence of GPI-PLC-expressing parasites has no obvious side effect on the host cells; rather, these host cells survive considerably longer than cells infected with pTEX/*T. cruzi* (Fig. 2). Likewise, we also consider it unlikely that GPI-PLC expression decreases amastigote development by altering the endogenous level of phosphatidylinositols (PIs) in *T. cruzi*. Although GPI-PLC can cleave PIs when they are presented in the absence of GPIs (6, 7), GPI is a better substrate by at least 2 orders of magnitude (6). Even under conditions optimized for PI cleavage (7), up to 900-fold more GPI-PLC is needed to cleave a PI than is required to cleave a GPI (22a). GPIs are likely to be present in large excesses over PIs in trypanosomes; therefore, it seems highly unlikely that PIs are cleaved by GPI-PLC in vivo. In fact, there is no evidence that PI is cleaved by GPI-PLC in a living cell, and *Escherichia coli*, which makes PIs but not GPIs, does not exhibit a slow-growth phenotype when it expresses *T. brucei* GPI-PLC.

As expected, the decreased replicative capacity of GPI-deficient *T. cruzi* was accompanied by significantly decreased virulence, although not by absolute avirulence, in mice. The success of the GPI-PLC transfectants in SCID mice, which lack many of the immunological mechanisms necessary for the control of wild-type parasites, is particularly revealing. We attribute the failure of these transfectants to exhibit a total loss of virulence to the fact that the GPI-PLC gene was expressed extrachromosomally in these transfectants and would likely be lost in the absence of drug selection in vivo. Mutational inactivation of the GPI-PLC gene during in vivo infection is also a possibility. Although this loss of GPI-PLC activity in vivo has not been formally proven, there is strong selection for such a loss. Stable expression of GPI-PLC may be achieved by chromosomal integration of this gene; efforts to achieve stable expression are ongoing. Stable, GPI-deficient mutants of *T. cruzi* produced either by heterologous expression of *T. brucei* GPI-PLC or, more efficiently, through deletion of genes involved in GPI biosynthesis are predicted to be useful as live, avirulent vaccines. Based on the results presented herein, we expect these mutants to be viable as epimastigotes and as invasive metacyclics but to lack the ability to replicate as amastigotes and to convert to trypomastigotes.

The inhibition of *T. cruzi* replication and development caused by a GPI deficiency also highlights the importance of GPIs in this intracellular parasite and presents the possibility that specific inhibitors of GPI biosynthesis can be useful chemotherapeutics. In contrast to many protozoan parasites, mammalian cells are far less dependent on GPI biosynthesis for cellular function. Several mammalian cell lines, including T-lymphoma GPI mutants which can grow without surface expression of GPI-anchored proteins (18, 28) and yeast temperature-sensitive mutants defective in GPI biosynthesis which grow at permissive temperatures (3, 4, 15), have been described. However, the critical importance of several of the GPI molecules for the survival of protozoans in insect and mammalian hosts (10, 19, 27) supports the proposal that inhibition of GPI biosynthesis may potentially provide a wide spectrum of control of protozoan parasites with limited side effects.

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