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GPI-anchored Proteins and Free GPI Glycolipids of Procyclic Form *Trypanosoma brucei* Are Nonessential for Growth, Are Required for Colonization of the Tsetse Fly, and Are Not the Only Components of the Surface Coat

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The procyclic form of *Trypanosoma brucei* exists in the midgut of the tsetse fly. The current model of its surface glycocalyx is an array of rod-like procyclin glycoproteins with glycosylphosphatidylinositol (GPI) anchors carrying sialylated poly-*N*-acetyllactosamine side chains interspersed with smaller sialylated poly-*N*-acetyllactosamine–containing free GPI glycolipids. Mutants for *TbGPI12*, deficient in the second step of GPI biosynthesis, were devoid of cell surface procyclins and poly-*N*-acetyllactosamine–containing free GPI glycolipids. This major disruption to their surface architecture severely impaired their ability to colonize tsetse fly midguts but, surprisingly, had no effect on their morphology and growth characteristics in vitro. Transmission electron microscopy showed that the mutants retained a cell surface glycocalyx. This structure, and the viability of the mutants in vitro, prompted us to look for non-GPI–anchored parasite molecules and/or the adsorption of serum components. Neither were apparent from cell surface biotinylation experiments but [^{3}H]glucosamine biosynthetic labeling revealed a group of previously unidentified high apparent molecular weight glycoconjugates that might contribute to the surface coat. While characterizing GlcNAc-PI that accumulates in the *TbGPI12* mutant, we observed inositolphosphoceramides for the first time in this organism.

INTRODUCTION

The tsetse fly–transmitted protozoan parasite *Trypanosoma brucei* causes human sleeping sickness and the cattle disease Nagana in sub-Saharan Africa. The organism undergoes a complex life cycle between the mammalian host and the insect, tsetse, vector. The bloodstream (trypomastigote) form of the parasite avoids the host's innate immune system through the expression of a dense monolayer of 5×10^6 glycosylphosphatidylinositol (GPI)-anchored variant surface glycoprotein (VSG) dimers and avoids specific immune responses through antigenic variation (Cross, 1996; Vanhamme *et al.*, 2001). The bloodstream-form parasites exist as dividing "slender" forms and nondividing "stumpy" forms that are preadapted for survival in the tsetse fly. After ingestion in a blood meal, the stumpy trypomastigote-form parasites differentiate into dividing procyclic-form parasites that colonizes the tsetse midgut. The procyclic trypanosomes express a radically different cell surface coat, thought to be made up $\sim 3 \times 10^6$ procyclin glycoproteins (Roditi *et al.*, 1987; Mowatt *et al.*, 1987; Richardson *et al.*, 1988) and a smaller number ($\sim 1 \times 10^6$) of poly-*N*-acetyllactosamine–containing free GPIs (Lillico *et al.*, 2003; Vassella *et al.*, 2003; Nagamune *et al.*, 2004; Roper *et al.*, 2005). The procyclins are polyanionic, rod-like (Roditi *et al.*, 1989; Treumann *et al.*, 1997), proteins encoded by procyclin genes (Roditi and Clayton, 1999). In *T. brucei* strain 427, used in this study, the parasites contain (per diploid genome) two copies of the *GPEET1* gene encoding 6 Gly-Pro-Glu-Glu-Thr repeats, one copy each of the *EP1-1* and *EP1-2* genes, encoding EP1 procyclins with 30 and 25 Glu-Pro repeats, respectively, two copies of the *EP2-1* gene, encoding EP2 procyclin with 25 Glu-Pro repeats and two copies of the *EP3-1* gene, encoding EP3 procyclin with 22 Glu-Pro repeats (Acosta-Serrano *et al.*, 1999). The EP1 and EP3 procyclins contain a single N-glycosylation site, occupied exclusively by a conventional $Man_5GlcNAc_2$ oligosaccharide, at the N-terminal side of the Glu-Pro repeat domain (Treumann *et al.*, 1997; Acosta-Serrano *et al.*, 1999). Whereas neither EP2 nor GPEET procyclin is N-glycosylated, GPEET1 procyclin is phosphorylated on six of seven Thr residues (Butikofer *et al.*, 1999; Mehlert *et al.*, 1999; Schlaeppi *et al.*, 2003). In culture, the procyclin expression profile depends on the carbon source (Vassella *et al.*, 2000) and metabolic state of the cells (Morris *et al.*, 2002; Vassella *et al.*, 2004) and in the tsetse fly there appears to be a program of procyclin expression such that GPEET procyclin is expressed early, giving way to EP1 and EP3 procyclin expression (Acosta-Serrano *et al.*, 2001; Vassella *et al.*, 2001). GPEET and EP procyclins contain similar GPI membrane anchors. These are based on the ubiquitous ethanolamine-*P*-6Manα1–2Manα1-6Manα1-4GlcNα1-6PI core (where, in this

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case, the phosphatidylinositol [PI] lipid is a 2-*O*-acyl-*lyso*-PI structure; Treumann *et al.*, 1997), but they also contain the largest and most complex known GPI side chains. These side chains are large poly-disperse branched poly-*N*-acetyllactosamine structures (with an average of ~8–12 repeats, depending on the preparation) that can terminate with α2-3–linked sialic acid residues (Ferguson *et al.*, 1993; Treumann *et al.*, 1997). Sialic acid is transferred from serum sialoglycoconjugates to terminal βGal residues by the action of a cell surface GPI-anchored trans-sialidase enzyme (Engstler *et al.*, 1993; Pontes de Carvalho *et al.*, 1993; Montagna *et al.*, 2002). Trans-sialylation of surface components plays a role in the successful colonization of the tsetse fly (Nagamune *et al.*, 2004). In vivo, the N-termini of the procyclins are removed by tsetse fly gut proteases (Acosta-Serrano *et al.*, 2001), though the role of this event is unclear (Liniger *et al.*, 2004), and it is thought that the underlying (protease resistant) anionic repeat units and associated GPI anchor side chains might protect the parasite from the approach tsetse fly gut hydrolases (Acosta-Serrano *et al.*, 2001).

In previous studies, the cell surface architecture of procyclic trypanosomes has been manipulated by gene knockout of the procyclin genes themselves (Vassella *et al.*, 2003) or genes encoding enzymes that act in the later parts of the GPI biosynthetic pathway, i.e., *TbGPI10* and *TbGPI8* (Nagamune *et al.*, 2000, 2004; Lillico *et al.*, 2003), or by galactose-starvation (Roper *et al.*, 2005). The procyclin, *TbGPI10* and *TbGPI8* knockouts all resulted in parasites devoid of GPI-anchored procyclins, but this was apparently compensated for by an up-regulation in free GPI expression.

In this article, we describe the phenotype of procyclic trypanosome *TbGPI12* null mutants that cannot synthesize GPI structures beyond GlcNAc-PI. To our surprise, these mutants are viable in culture, though unable to colonize the tsetse midgut. The mutant also revealed that the procyclic trypanosome surface coat contains molecules other than GPI-anchored proteins and free GPIs. Adsorbed serum components were excluded as coat components and [^{3}H]glucosamine labeling revealed a hereto unidentified high-molecular-weight glycoconjugates that may constitute some or all of the residual surface coat.

MATERIALS AND METHODS

Cell Culture and Generation of Procyclic-form T. brucei *TbGPI12 Null and Conditional Null Mutants*

Puromycin (PAC) and blasticydin (BSD) antibiotic resistance genes were cloned into *TbGPI12*-targeted gene replacement plasmids and the tetracycline-inducible expression plasmid (pLew100) containing the *TbGPI12* ORF with a C-terminal *myc*-tag were prepared as described in (Chang *et al.*, 2002). Plasmid maxi-preps were prepared using a Qiagen kit (Qiagen, Crawley, West Sussex, United Kingdom), digested with NotI, heated at 65°C for 30 min, precipitated with ethanol, redissolved in sterile water, and used for electroporation of procyclic forms of *T. brucei* strain 427 clone 29.13, referred in this article as wild-type (WT) cells. These cells were grown in SDM-79 (Brun and Schonenberger, 1979) in the presence of 15% fetal bovine serum (FBS), haemin 7.5 mg/L at 28°C, containing the appropriate antibiotics for selection (G418 at 15 μg/ml to maintain the T7 RNA polymerase; hygromycin at 50 μg/ml to maintain the tetracycline repressor protein; phleomycin at 2.5 μg/ml for pLew100; puromycin at 1 μg/ml; and blasticydin at 10 μg/ml). Cell densities were measured using a hemocytometer and plotted as the product of cell density and dilution.

Southern Blotting

Genomic DNA was prepared using DNAzol (Helena Biosciences, Gateshead, United Kingdom). Probes were amplified by PCR, gel-purified, and dUTP-fluorescein–labeled by random priming (Gene Images Kit, GE Healthcare, Little Chalfont, United Kingdom).

Cell-free System Experiment

Procyclic-form *T. brucei* membranes were prepared (Masterson *et al.*, 1989) from wild-type cells, *TbGPI12* null mutant, and *TbGPI12* conditional null mutant grown with daily addition of 1 μg/ml tetracycline, or without tetracycline for 16 d, in media containing 15% FBS certified tetracycline-free (Clontech, Palo Alto, CA). Trypanosome membranes were washed twice and resuspended at 1×10^{-9}/ml in 2× incorporation buffer (Güther and Ferguson, 1995). Aliquots (2×10^7 cells) were added to equal volume of water containing 2 μCi of UDP[^{3}H]GlcNAc (41.6 Ci/mmol, Perkin Elmer-Cetus) and 2 mM GDP-Man and labeled for 20 min at 30°C. Subsequent chloroform/methanol/water and butan-1-ol extractions were performed as described before (Güther *et al.*, 1994). Samples and glycolipid standards were run on aluminum-backed silica gel-60 HPTLC (Merck, Rahway, NJ) and developed using chloroform/methanol/1 M ammonium acetate/13 M ammonia/water (180:140:9:9:23, vol/vol). Radiolabeled compounds were detected by fluorography at −80°C after spraying with En^{3}Hance (Perkin-Elmer Cetus, Norwalk, CT) using Kodak XAR-5 film (Eastman Kodak, Rochester, NY) and intensifying screens.

Anti-myc Western Blotting

Washed parasites were lysed in 20 mM Tris-HCl, pH 7.2, 2% SDS, boiled and adjusted to 0.3% SDS, and 1% Triton X-100 in 20 mM Tris-HCl, pH 7.2, and aliquots equivalent to 2×10^8 cells were immunoprecipitated with 1 μg anti-*myc* mAb (Upstate Biotechnology, Lake Placid, NY) and protein G agarose (Sigma, Poole, Dorset, United Kingdom). The washed beads were boiled in SDS sample buffer and applied to an Invitrogen gel (Paisley, United Kingdom; 4–12%, NuPage with MOPS buffer). Proteins were transferred to nitrocellulose, and the blocked filter was probed with anti-*myc* mAb (10 ng/ml) and developed with an anti-mouse secondary antibody conjugated to horseradish peroxidase (Sigma; diluted 1:10,000) and ECL reagent (Amersham).

Mass Spectrometry of Lipid Extracts

Wild-type procyclic-form *T. brucei*, *TbGPI12* null mutant cells (5×10^8 total) were washed and resuspended in 0.1 ml phosphate-buffered saline and extracted with chloroform/methanol/water (10:10:3 vol/vol/vol) overnight at 4°C and then sonicated for 15 min in a sonicating water bath. The extract was centrifuged for 5 min at full speed in an Eppendorf microfuge, and the supernatant was transferred to a fresh Eppendorf tube. After drying under a stream of nitrogen, the products were partitioned between butan-1-ol and water (0.2 ml each). The aqueous phase was extracted twice more with 0.2 ml water-saturated butan-1-ol. The combined butan-1-ol phases were back-washed three times with 0.4 ml butanol-saturated water. The washed butan-1-ol phases were dried under nitrogen and dissolved in 200 μl chloroform/methanol/water (10:10:3 vol/vol/vol). Small aliquots were transferred to Waters nanotips (Millipore, Milford, MA; type F) and analyzed by electrospray ionization–mass spectrometry (ES-MS) in negative ion mode on an ABI Q-StarXL mass spectrometer (Surrey, United Kingdom). Individual ions were subjected to collision induced dissociation (CID) and tandem mass spectrometry (ES-MS/MS). The product ion spectra were used to identify phospholipid type and molecular species.

FACS Analysis

For FACS analysis, an aliquot of wild-type and *TbGPI12* null mutant cells were centrifuged, resuspended in 1 ml of fresh SDM-79 media, and incubated with anti-EP mAb 247 (Richardson *et al.*, 1988) diluted 1:100 (30 min, room temperature). Parasites were washed with cold PBS and incubated with FITC anti-mouse (Sigma; diluted 1:10000) for 1 h at room temperature. After incubation, parasites (at the concentration of 5×10^6 cells/ml) were analyzed in a Becton Dickinson FACSCalibur (Cockeysville, MD) using detector FL1-A.

Metabolic Labeling of Live T. brucei *Procyclic Forms*

T. brucei procyclic forms (5 ml at 10^7/ml) were washed twice with SDM-79 media depleted of L-proline, D-mannose, D-glucose, D-glucosamine, L-serine, L-ethanolamine and containing 2% FBS and resuspended in 10 ml of the same media and split into 2-ml aliquots. One aliquot was labeled with 5 μCi/ml [^{14}C]proline for 2 h at 28°C. Another aliquot, after addition of L-proline and L-hydroxy-proline, was labeled either with [^{3}H]ethanolamine (50 μCi/ml) or [^{3}H]glucosamine (200 μCi/ml) for 20 h at 28°C, in the latter case with the further addition of L-serine. The final concentrations of all supplements were as described for SDM-79 (Brun and Schönenberger, 1979). After labeling, cells were washed and resuspended in PBS. An equal volume of 2× SDS-sample buffer containing 0.2 M DTT was added, and the samples were boiled and applied into 4–12% Nupage BisTris polyacrylamide gels and run using MOPS running buffer (Invitrogen). Gels were stained with Coomassie blue, soaked in En^{3}Hance (Perkin Elmer-Cetus), dried, and placed in contact with Kodak XAR-5 film and intensifying screen at −80°C.

Ruthenium Red Stain and Ultramicroscopy

Parasites in culture medium were washed twice in cold PBS and fixed at 4°C for 1 h in 2.5% glutaraldehyde, 0.1 M cacodylate buffer containing 0.15%

ruthenium red, 5 mM $CaCl_2$, and 5% sucrose and processed according to Zufferey *et al.*, (2003) through 1% osmium tetroxide/ruthenium red. Further processing included 30-min en bloc staining of the cells with 1% aqueous uranyl acetate before a graded ethanol dehydration, rinsing with propylene oxide (2 × 5 min), and embedding in Epon-Araldite resin. Ultrathin sections were prepared on 200-mesh grids, stained in Reynold's lead citrate for 5 min, and viewed at 120 kV on a Zeiss 912 Omega transmission electron microscope (Thornwood, NY), recording zero-loss images on a 2K Proscan digital camera system (Proscan, Lagerlechfeld, Germany).

Surface Biotinylation of Procyclic Form T. brucei

Wild-type and *TbGPI12* null mutant procyclic cells (10 ml cultures at 3 × 10^7/ml for each) were washed three times with 20 ml PBS and resuspended in 20 ml of EZ-link sulfo-NHS-biotin (Pinpoint Cell Surface Protein Isolation Kit, Pierce, Rockford, IL) in PBS and rotated at 4°C for 30 min. After quenching, lysate preparation, and centrifugation, the supernatant was purified using Neutravidin beads. Proteins were eluted by boiling the beads with SDS-sample buffer containing DTT and were applied into 4–12% Nupage BisTris polyacrylamide gels run with MOPS buffer (Invitrogen). The gel was stained with Coomassie blue, and the more abundant bands in the null mutant lane were cut out of the gel, alkylated, digested with trypsin, and processed for mass-fingerprinting. (Fingerprints Proteomic Facility, University of Dundee, Scotland). Identical samples were applied into a duplicate gel to develop with silver to visualize procyclins.

Tsetse Fly Infections

Pupae of *Glossina morsitans morsitants* were obtained from the Institute of Zoology, Slovak Academy of Science (Bratislava, Slovakia). Newly hatched (teneral) flies were fed with an infected bloodmeal, which consisted of 10^7 parasites mixed with washed defribinated horse blood (containing 10% FBS). In the case of flies infected with *TbGPI12* conditional null mutant cells, the bloodmeal also contained 25 μg/ml tetracycline. The latter was always included, at the same concentration, in successive bloodmeals until flies were dissected. Tetracycline is not harmful for tsetse flies, and it has been successfully used in the past to induce trypanosome gene expression within tsetse compartments (Peacock *et al.*, 2005). Infected flies were fed with bloodmeals every 2–3 d. After 2 wk, midguts were isolated from infected flies and disrupted by mechanical force in cold SDM-79 containing 10% FBS. Isolated parasites from individual midguts were kept on ice until counted on a hemocytometer.

RESULTS

Creation and Growth Phenotype of TbGPI12 Null Mutant Procyclic T. brucei

TbGPI12 is present as a single copy per haploid genome (Chang *et al.*, 2002). We replaced one allele with puromycin acetyltransferase (PAC) and, after antibiotic selection, we replaced the second allele with blasticydin deaminase (BSD). After dual antibiotic selection, a Southern blot with a *TbGPI12* ORF probe showed that both alleles were replaced and that we had created a Δ*TbGPI12::PAC*/Δ*TbGPI12::BSD* null mutant (Figure 1B). A tetracycline-inducible *TbGPI12* conditional null mutant was also created by introducing an ectopic C-terminally *myc*-tagged version of *TbGPI12*, targeted to the ribosomal DNA locus of the null mutant using the pLew100 vector (Wirtz *et al.*, 1999). A Southern blot with a *TbGPI12* ORF probe showed the presence of the ectopic gene and the absence of endogenous copies, i.e., a genotype of ^{Ti}*TbGPI12myc*/Δ*TbGPI12::PAC*/Δ*TbGPI12::BSD* (Figure 1C).

The *TbGPI12* null mutant and the conditional null mutant (with and without tetracycline induction) had identical growth phenotypes in culture to the wild-type cells (Figure 2), demonstrating that, unlike the situation in bloodstream-form trypanosomes (Chang *et al.*, 2002), *TbGPI12* is a nonessential gene in cultured procyclic *T. brucei*. The mutant cells also had normal morphology, as judged by light and scanning electron microscopy (data not shown).

TbGPI12 Null Mutants Lack GlcNAc-PI de-N-Acetylase Activity and Accumulate GlcNAc-PI

We were able to confirm that we had deleted all GlcNAc-PI de-*N*-acetylase activity in the null mutants in two ways.

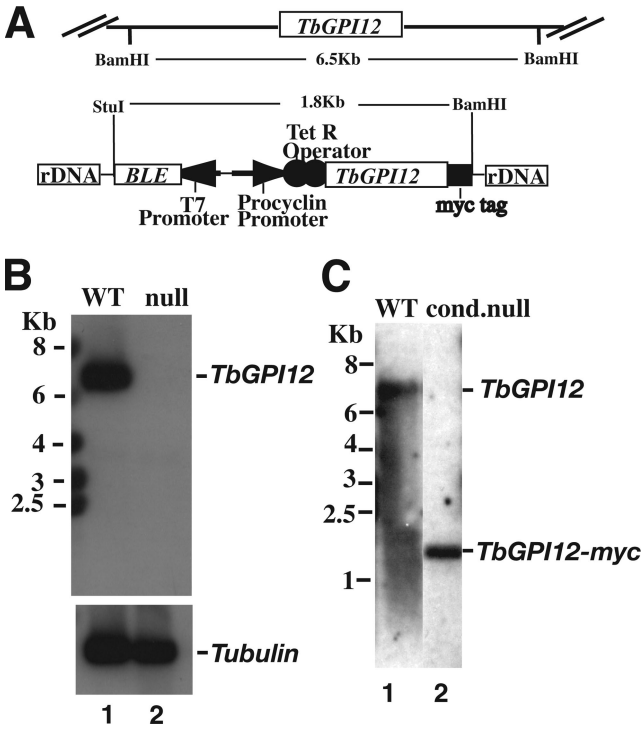

Figure 1. Creation of *TbGPI12* null and conditional null procyclic trypanosomes. (A) Schematic of the endogenous *TbGPI12* locus and the ectopic *TbGPI12myc* construct incorporated into the rDNA locus by homologous recombination, indicating the positions of BamHI and StuI restriction sites and the sizes of the *TbGPI12*-containing fragments. (B) Southern blot of genomic DNA from wild-type (lane 1) and *TbGPI12* null (lane 2) cells digested with BamHI and StuI and probed with the *TbGPI12* ORF. The same blot was reprobed with a β-tubulin probe (bottom) to confirm equal loadings of DNA. (C) Southern blot of genomic DNA from wild-type (lane 1) and the *TbGPI12* conditional null (cond. null) mutant clone used in this study (lane 2) digested with BamHI and StuI and probed with the *TbGPI12* ORF.

First, cell-free systems of washed parasite membranes made from wild-type and *TbGPI12* null and conditional null

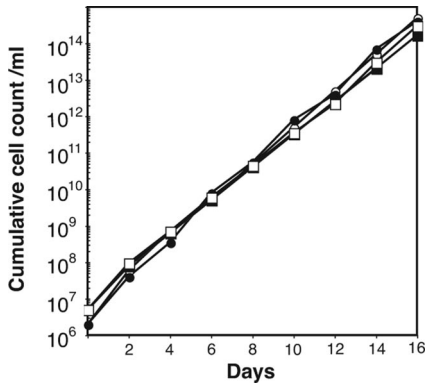

Figure 2. Growth phenotypes of the wild-type and *TbGPI12* null and conditional null mutants. Cells were inoculated into culture, counted and diluted fivefold with fresh media every 2 d. The cumulative cell counts allow for the fivefold dilutions. The growth characteristics for wild-type (●), *TbGPI12* null (○) and *TbGPI12* conditional null cells under permissive (plus tetracycline, closed squares) and nonpermissive (minus tetracycline, □) conditions were indistinguishable.

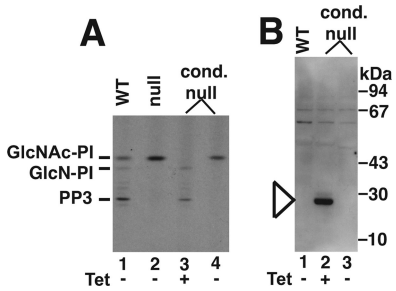

Figure 3. Cell-free system GlcNAc-PI de-*N*-acetylase assays and anti-*myc* Western blots for wild-type and *TbGPI12* mutants. (A) Cell-free systems were prepared from wild-type cells (lane 1), *TbGPI12* null cells (lane 2), and *TbGPI12* conditional null cells grown under permissive conditions (+Tet; lane 3) and nonpermissive conditions (−Tet; lane 4). Each was labeled with UDP-[^{3}H]GlcNAc in the presence of GDP-Man, and extracted glycolipids were analyzed by HPTLC and fluorography. The positions of authentic standards of GlcNAc-PI, its de-N-acetylation product GlcN-PI and its major metabolite Et*N*P-Man$_3$GlcN-(acyl)PI (PP3) are indicated. (B) Whole cell lysates from wild-type (lane 1) and *TbGPI12* conditional null cells grown under permissive (+Tet; lane 2); and nonpermissive (−Tet) conditions were immunoprecipitated with anti-*myc* antibody, subjected to SDS-PAGE, transferred to nitrocellulose, and probed with anti-*myc* antibody. A band with an apparent molecular weight consistent with TbGPI12-*myc* can be seen in lane 2, marked by the arrowhead. The absence of any enzyme activity (A) and of any protein band (B) shows that tetracycline regulation of gene expression is very tight in this mutant.

mutants (grown with and without tetracycline) were labeled with UDP-[^{3}H]GlcNAc in the presence of excess unlabeled GDP-Man. Under these conditions, the wild-type cell-free system incorporates [^{3}H]GlcNAc into [^{3}H]GlcNAc-PI and downstream de-N-acetylated products such as GlcN-PI, with label accumulating in the GPI intermediate PP3, i.e., Et*N*P-Man$_3$GlcN-(acyl)PI (Field *et al.*, 1992; Güther and Ferguson, 1995 and Figure 3A, lane 1). In the *TbGPI12* null mutant, the label accumulated only in GlcNAc-PI (Figure 3A, lane 2), suggesting that these membranes do not contain any residual GlcNAc-PI de-*N*-acetylase activity. The de-N-acetylation of GlcNAc-PI to GlcN-PI is an essential step in GPI biosynthesis, and no further reactions of GlcNAc-PI are expected without de-N-acetylation (Sharma *et al.*, 1997). The same result was observed with the *TbGPI12* conditional null mutant in the absence of tetracycline (Figure 3A, lane 4), indicating the complete absence of detectable *TbGPI12* expression under nonpermissive conditions. On the other hand, the same mutant grown under permissive (plus tetracycline) conditions yielded a cell-free system that did not accumulate any GlcNAc-PI (Figure 3A, lane 3), suggesting some overexpression of GlcNAc-PI de-*N*-acetylase activity in these cells compared with wild type. The expression of the *myc*-tagged GlcNAc-PI de-*N*-acetylase protein under permissive conditions, but not under nonpermissive conditions, is shown by anti-*myc* Western blot (Figure 3B). The band with an apparent molecular weight of 28 kDa (Figure 3B, lane 2) is consistent with the predicted molecular weight of the *TbGPI12-myc* translation product (29.5 kDa). It is worth noting that the level of protein expression under permissive conditions was insufficient for direct Western blotting of whole cell lysates with anti-*myc* antibody and that immunoprecipitation of whole cell lysates from 2×10^8 cells with anti-*myc* was necessary to preconcentrate the target protein before Western blotting.

Second, negative ion ES-MS and ES-MS/MS of lipid extracts from wild-type and null mutant parasites showed the absence of detectable steady state levels of GlcNAc-PI in wild-type cells (Figure 4A), consistent with a very low steady state level of this biosynthetic intermediate, but the accumulation of considerable amounts of GlcNAc-PI in the null mutants (Figure 4, B and C).

From these data we may conclude that *TbGPI12* encodes all detectable GlcNAc-PI de-*N*-acetylase activity of procyclic trypanosomes and that, in the absence of this enzyme, procyclic trypanosomes can accumulate substantial amounts of the GlcNAc-PI precursor.

The negative ion ES-MS and ES-MS/MS analyses also identified the major phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and PI components of procyclic-form *T. brucei* (Figure 4, A and B), which appear to present in comparable relative amounts in the two samples. Of particular note is a cluster of ions at *m/z* 750, 752, 778, and 780. These produced intense inositol-1,2-cyclic phosphate and inositol-monophosphate product ions at (*m/z* 241 and 259) in ES-MS/MS (data not shown), characteristic of inositolphosphoceramide (IPC) ions. These are discussed later. Negative product ion spectra of IPCs do not provide fine detail on the long-chain base and fatty acid compositions of the ceramide portions of IPCs, other than total carbon numbers and degrees of unsaturation (which are C32:1, C32:0, C34:1, and C34:0 for the *T. brucei* IPCs). These are, on average, smaller than those found in *T. cruzi* epimastigotes IPCs, which are mainly C34:1, C34:0, C36:1, and C36:0 (Bertello *et al.*, 1995), and *Leishmania* promastigote IPCs, which are mainly C34:1 and C36:1 (Zufferey *et al.*, 2003, Denny *et al.*, 2004). The ES-MS/MS product ion spectra of the two major PE ions at *m/z* 726 and 728 (data not shown) showed these to be alkenylacyl-PE species (also known as plasmenylethanolamines) that appear to be common in kinetoplastid organisms (Villas Boas *et al.*, 1999; Zufferey *et al.*, 2003).

TbGPI12 Null Mutants Lack Cell Surface Procyclin and Free GPIs

In the absence of a functional GPI anchor biosynthetic pathway, we would not expect the expression of cell surface procyclins, as has been described for TbGPI10 and TbGPI8 knockout procyclic trypanosomes (Nagamune *et al.*, 2000, 2004; Lillico et al., 2003). Analysis of cells by flow cytometry using anti-procyclin antibodies and FITC-labeled secondary antibody revealed a strong signal for wild-type cells (Figure 5A) and only background labeling for the *TbGPI12* conditional null mutant grown under nonpermissive conditions (Figure 5B). Strong labeling was restored in the *TbGPI12* conditional null mutant cells under permissive (plus tetracycline) conditions (Figure 5C).

The absence of cell surface procyclin indicated by cytometry was not due to a lack of procyclin protein synthesis. SDS-PAGE and fluorography of anti-procyclin immunoprecipitated cell extracts labeled for 2 h with [^{14}C]proline revealed low-molecular-weight procyclin precursor in the *TbGPI12* mutants (Figure 6A, lane 2), whereas wild-type cells produced fully processed GPI-anchored procyclin (Figure 6A, lane 1). In pulse-chase experiments (2-h pulse and 18-h chase) the majority of [^{14}C]proline-labeled procyclin made in the *TbGPI12* mutants was found secreted into the medium as multiple degradation products (data not shown).

Unlike the *TbGPI10* and *TbGPI8* null mutants, that can make poly-*N*-acetyllactosamine modified Man$_2$GlcN-(acyl)lyso-PI and Et*N*P-Man$_3$GlcN-(acyl)lyso-PI free GPIs, respectively (Lillico *et al.*, 2003; Nagamune *et al.*, 2004), *TbGPI12* null mutants should not be able to synthesize any kind of GPI structure

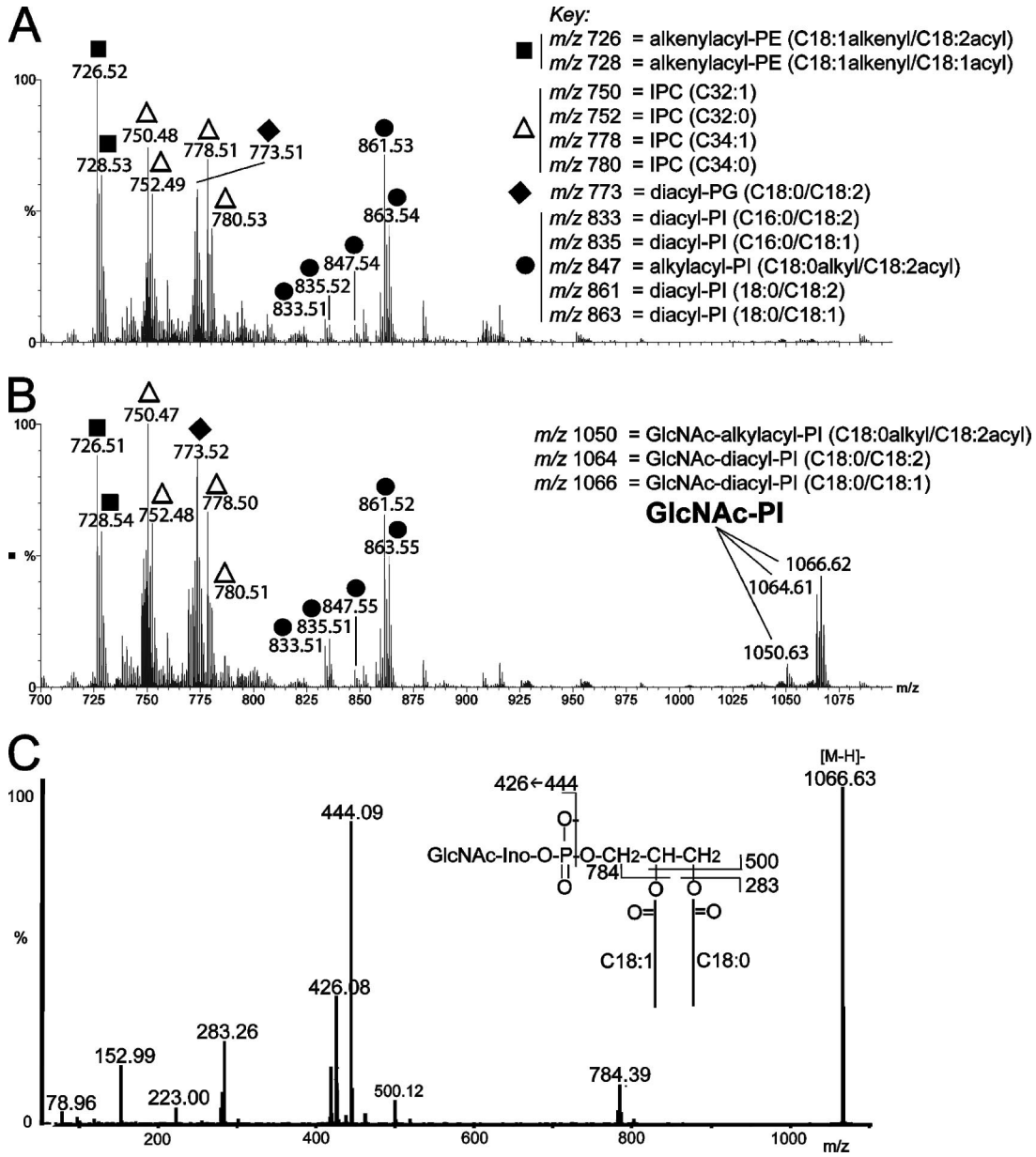

Figure 4. Negative ion electrospray mass spectrometry of lipid extracts from wild-type and *TbGPI12* null mutant cells. Mass spectrum of lipid species from wild-type (A) and *TbGPI12* null mutant cells (B) and a tandem (MS/MS) spectrum of the major GlcNAc-PI ion at *m/z* 1066.6 (C). The identities of the major ions are indicated. PE, phosphatidylethanolamine; IPC, inositolphosphoceramide; PG, phosphatidylglycerol; PI, phosphatidylinositol. The figures in brackets describe the carbon chain length and degree if unsaturation of the acyl, alkenyl, or alky chains or, for the IPCs, the ceramide moiety. The inset in C shows the product ion assignments for GlcNAc-PI. The ion at *m/z* 444 is [GlcNAc-*myo*-inositol-1,2-cyclic phosphate]$^-$, and its dehydration product at *m/z* 426, are characteristic of negative ion GlcNAc-PI product ion spectra. The ions at *m/z* 79, 153, 223 and 283 are $[PO_3]^-$, [glycerol-cyclic phosphate]$^-$, [inositol-1,2-cyclic phosphate–H_2O]$^-$, and $[CH_3(CH_2)_{16}CO_2]^-$, respectively.

beyond GlcNAc-PI. Labeling of cells with [^{3}H]ethanolamine confirmed this supposition. Thus, in wild-type cells, [^{3}H]ethanolamine labeled procyclin and free GPIs (Figure 6B, lane 1), whereas these were absent in the *TbGPI12* null mutants (Figure 6B, lane 2). The labeled band running above procyclin in both wild-type and mutant cells is most likely protein synthesis elongation factor 1a (Dever *et al.*, 1989).

Attempts To Find Molecules That Might Replace Procyclin and Free GPIs in TbGPI12 Null Mutants

Transmission electron microscopy (TEM) of ultrathin sections of wild-type and *TbGPI12* null procyclic trypanosomes stained with ruthenium red, a stain used to preserve prokaryote and eukaryote glycocalyx structure (Luft, 1966, 1971; Szubinska and Luft, 1971; Zufferey *et al.*, 2003), revealed that the cell surface coats of these preparations were indistinguishable (Figure 7). This was a surprising finding because procyclins have been assumed to be the major component of the procyclic trypanosome surface coat (Roditi *et al.*, 1989; Mehlert *et al.*, 1998).

We considered that procyclins and free GPIs might have been replaced by adsorbed serum components from the medium, so we labeled the cell surface with a cleavable sulfo-

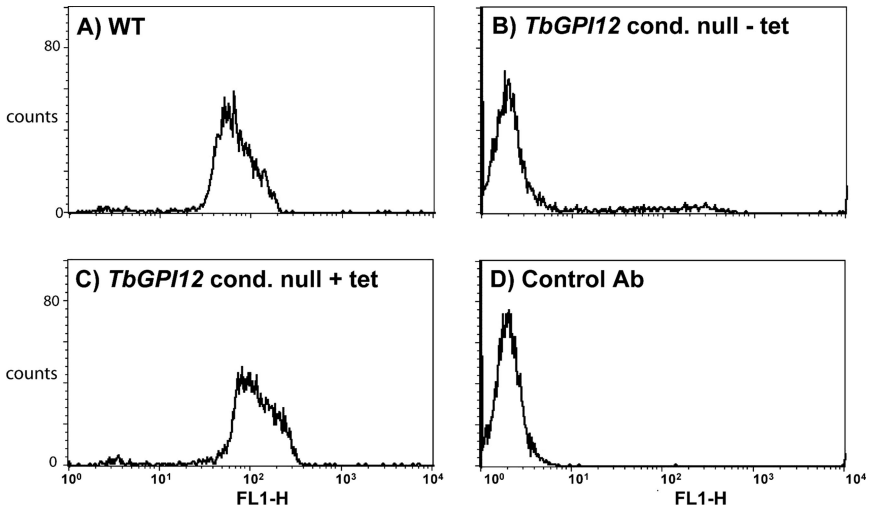

Figure 5. Flow cytometric (FACS) analysis for surface procyclin in the wild-type and *TbGPI12* conditional null mutants cells. (A) Wild-type cells stained with anti-procyclin antibody. (B) *TbGPI12* conditional null mutants cells grown under nonpermissive (−tet) conditions. (C) *TbGPI12* conditional null mutants cells grown under permissive (+tet) conditions. (D) No primary anti-procyclin antibody control.

NHS-biotin reagent. However, analysis of the neutravidin-purified surface-biotinylated components by SDS-PAGE and Coomassie blue staining did not reveal any obvious components unique to the null mutants (Figure 8A). Even those proteins that appeared more intense in the mutant compared with the wild-type cells proved to be intracellular *T. brucei* proteins, and not bovine serum proteins, by peptide mass fingerprinting. These same preparations were stained with silver, which then revealed the non-Coomassie–stainable cell-surface procyclins of the wild-type cells, as expected (Figure 8B, lane 1). However, no new biotin-labeled components were obvious in the *TbGPI12* null cell extracts (Figure 8B, lane 2).

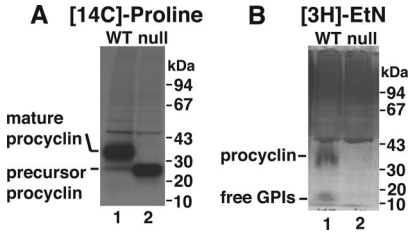

Figure 6. Biosynthetic labeling of wild-type and *TbGPI12* null cells with [^{14}C]proline and [^{3}H]ethanolamine. (A) Wild-type (lane 1) and *TbGPI12* null cells (lane 2) were labeled with [^{14}C]proline and lysed and anti-procyclin immunoprecipitates were subjected to SDS-PAGE and fluorography. (B) Wild-type (lane 1) and *TbGPI12* null cells (lane 2) were labeled with [^{3}H]ethanoamine and subjected to SDS-PAGE and fluorography.

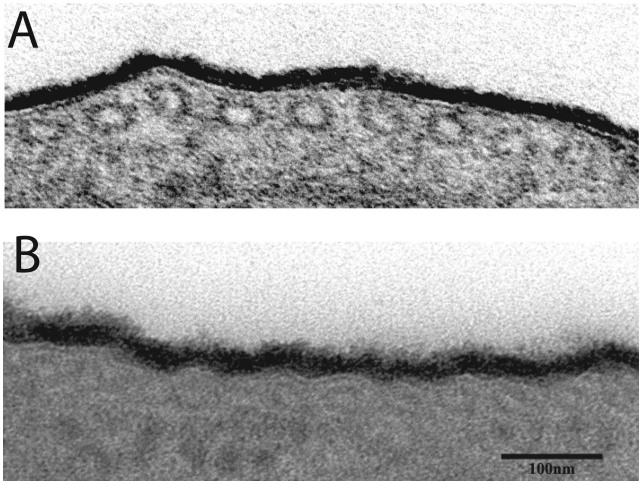

Figure 7. Transmission electron microscopy of the surface glycocalyx of wild-type and *TbGPI12* null cells. Ruthenium red–stained ultrathin sections of (A) wild-type and (B) *TbGPI12* null cells. Bar, 100 nm.

In an attempt to find nonbiotinylatable and/or non-silver–stainable surface molecules, we labeled the wild-type and *TbGPI12* null mutant cells with [^{3}H]glucosamine. We chose this label because glucosamine and/or its metabolite *N*-acetylglucosamine is/are common to all known trypanosomatid glycoconjugates (McConville and Ferguson, 1993; Guha-Niyogi and Turco, 2001; McConville *et al*., 2002; Atrih *et al.*, 2005; Mendonca-Previato *et al.*, 2005). Strong labeling of procyclin was apparent in the wild-type cells (Figure 9, lane 1), as expected, whereas this band was completely absent in the *TbGPI12* null mutant, consistent with the cytometric and [^{14}C]proline-labeling experiments described above (Figure 9, lane 2). Although [^{3}H]glucosamine labeling did not reveal any obviously up-regulated glycoconjugates in the *TbGPI12* null mutant, it did reveal a smear of labeled glycoconjugates, ranging in apparent molecular weight from ~90 to >200 kDa, that were labeled in both the wild-type and *TbGPI12* null mutant parasites.

TbGPI12 Null Mutant Procyclic Trypanosomes Fail To Infect the Tsetse Midgut

The discovery of a non-GPI surface glycocalyx in *TbGPI12* null mutant parasites, which seem to be displayed with a similar thickness to that observed on the surface of WT cells (Figure 7), prompted us to determine its possible functional role in establishing a midgut infection in the tsetse fly. Thus, we infected teneral flies with WT and *TbGPI12* conditional null cells cultured under permissive and nonpermissive (i.e., with and without tetracycline) conditions, and after 2 weeks their midguts were dissected and checked for the presence of parasites. As observed in (Figure 10), although flies infected with either WT or *TbGPI12* conditional null cells grown under permissive conditions and also fed with bloodmeals containing 25 μg/ml tetracycline developed comparable infection indexes, no parasites were detected in flies

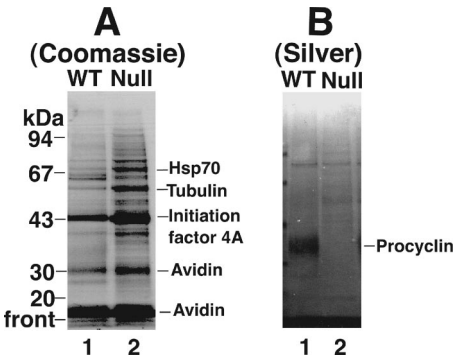

Figure 8. Cell surface biotinylation of wild-type and *TbGPI12* null cells. Wild-type cells (lane 1) and *TbGPI12* null cells (lane 2) were surface-labeled with sulfo-NHS-SS-biotin, and labeled products were purified on neutravidin beads and analyzed by SDS-PAGE and Coomassie blue staining (A) or silver staining (B). The proteins indicated were identified by protein mass fingerprinting.

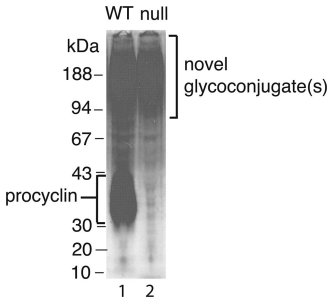

Figure 9. Biosynthetic labeling of wild-type and *TbGPI12* null cells with [^{3}H]glucosamine. Wild-type (lane 1) and *TbGPI12* null cells (lane 2) were labeled with [^{3}H]glucosamine and subjected to SDS-PAGE and fluorography.

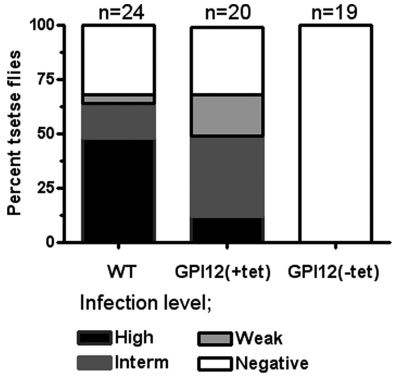

Figure 10. *TbGPI12* null mutant cells are unable to establish midgut infections. Tsetse flies were fed with infected bloodmeals containing wild-type (WT) or *TbGPI12* conditional null mutants under permissive (GPI12(+tet)) and nonpermissive (GPI12(−tet)) conditions. After 15 d, flies were dissected and infections scored as heavy (black; more than 100 parasites per field), intermediate (dark gray; 20–100 parasites), weak (light gray; 1–19 parasites), and negative (no detectable parasites). The number of dissected flies is indicated on top of each group.

infected with *TbGPI12* conditional null cells (previously grown under nonpermissive conditions) and continuously fed with bloodmeals lacking tetracycline, suggesting that *TbGPI12* expression is essential for the colonization of the tsetse fly.

Taken together, although the components and the chemical nature of the second (non-GPI) surface glycocalyx from procyclic trypanosomes remain to be determined, these experiments suggest that GPI-anchored proteins and free GPIs are probably the most functionally important components of the surface glycocalyx of procyclic *T. brucei* with respect to colonization of the tsetse vector.

DISCUSSION

As for bloodstream-form trypanosomes (Chang *et al.*, 2002), the T*bGPI12* gene clearly encodes all the GlcNAc-PI de-*N*-acetylase activity of procyclic-form trypanosome and its deletion prevents GPI synthesis beyond the early intermediate GlcNAc-PI (Figure 3). The accumulation of substantial quantities of GlcNAc-PI in the *TbGPI12* null mutants, detected by ES-MS (Figure 4), indicates that the first step of GPI biosynthesis (i.e., the transfer of GlcNAc from UDP-GlcNAc to PI) is not sensitive to product inhibition. In the cell-free system and living cells, GlcNAc-PI de-*N*-acetylase activity may be a rate-limiting step in GPI biosynthesis and procyclin surface expression, respectively, because both are slightly higher in the *TbGPI12* conditional null mutant when the expression of the ectopic copy of *TbGPI12* is fully induced by tetracycline (Figures 3 and 5).

The molecular species of GlcNAc-PI that accumulate in the *TbGPI12* null mutant mostly contain C18:0/C18:1 and C18:0/C18:2 diacyl-PI lipids, together with a small amount of C18:0/C18:2 alkylacyl-PI (Figure 4B). Interestingly, the relationship of C18:0/C18:2 (*m/z* 861) > C18:0/C18:1 (*m/z* 863) for the major diacyl-PI species is reversed for the GlcNAc-PIs that accumulate, where C18:0/C18:2 (*m/z* 1064) < C18:0/C18:1 (*m/z* 1066). In addition, the C16:0/C18:2 and C16:0/C18:1 diacyl-PI species (*m/z* 833 and 835) do not appear to be converted to GlcNAc-PI species, whereas the less-abundant C18:0/C18:2 alkylacyl-PI is converted to the corresponding GlcNAc-PI at *m/z* 1050. This apparent selection of particular PI species for GlcNAc addition may be consistent with a recent report (Martin and Smith, 2006b) that suggests that, at least in bloodstream-form *T. brucei*, two distinct pools of PI are synthesized, one for bulk membrane lipid and one for GPI anchor biosynthesis.

An unexpected finding from the lipid mass spectrometry data are the identification of IPC in *T. brucei*, although these are clearly not converted to GlcNAc-IPC in the *TbGPI12* null mutant. To our knowledge, this is the first evidence for the presence of IPCs in *T. brucei*, but it is consistent with the very recent discovery of novel IPC synthase genes in this and other kinetoplastid organisms (Denny *et al.*, 2006). In *T. brucei*, IPCs appear to be specific to the procyclic form because mass spectrometric analyses of bloodstream-form *T. brucei* lipids show them to be mostly C18:0/C18:2 and C18:0/C22:4 diacyl-PIs (Martin and Smith, 2006a, 2006b).

Thus far, the molecular architecture of procyclic-form *T. brucei* has been manipulated in four ways: 1) Knockout of the multiple procyclin genes themselves (Vassella *et al.*, 2003); 2) Knockout of *TbGPI10*, encoding the third α-mannosyltransferase of GPI biosynthesis (Nagamune *et al.*, 2000, 2004); 3) Knockout of *TbGPI8*, encoding the catalytic subunit of the GPI transamidase complex (Lillico *et al.*, 2003); and 4) Conditional knockout of *TbgalE*, encoding UDP-Glc 4′-epimerase, producing galactose starvation under nonpermissive conditions (Roper *et al.*, 2005). In this article, we describe altering the cell surface in a distinct way by knocking out the *TbGPI12* gene that, uniquely, simultaneously prevents the cell surface expression of procyclins and poly-*N*-acetyllactosamine–containing free GPI glycolipids (Figures 5 and 6).

The procyclin and *TbGPI10* and *TbGPI8* knockouts all result in the complete loss of cell surface procyclins, but this is compensated for by an up-regulation in the expression of wild-type (procyclin and *TbGPI8* knockout) or slightly modified (*TbGPI10* knockout) poly-*N*-acetyllactosamine–containing free GPI glycolipids. In the *TbGPI12* knockout, reported here, poly-*N*-acetyllactosamine–containing free GPI glycolipids cannot be made and, therefore, cannot compensate for the loss of surface procyclins. We were surprised that the *TbGPI12* gene was not essential for the growth of procyclic-form *T. brucei*, because deletion of this gene simultaneously wipes out both surface procyclin and poly-*N*-acetyllactosamine–containing free GPI expression. Currently, these molecules are assumed to represent the majority of the cell surface coat glycocalyx of this lifecycle stage of the organism (Mowatt and Clayton, 1987; Roditi *et al.*, 1987, 1989; Richardson *et al.*, 1988; Mehlert *et al.*, 1998; Lillico *et al.*, 2003; Vassella *et al.*, 2003, Roper *et al.*, 2005). It seems unlikely that the minimal glycolipid GlcNAc-PI, which accumulates in the mutant, could compensate for loss of these much larger surface molecules. Curiously, whereas the *TbGPI10* and *TbGPI8* knockouts have significantly and slightly, respectively, impaired cell culture growth kinetics compared with wild-type cells (Nagamune *et al.*, 2004), the *TbGPI12* knockout cells have perfectly normal growth characteristics (Figure 2). We have no obvious explanation for these differences between the mutants.

Analysis of the cell surface by ruthenium red staining and TEM showed that the density and depth of the cell surface glycocalyx did not appear to be significantly altered in the *TbGPI12* null mutants compared with wild type (Figure 7). This, and the excellent growth characteristics of the *TbGPI12* null mutants, prompted us to look for the possible up-regulation of other parasite surface molecules and/or the adsorption of serum components from the medium to compensate for the loss of procyclins and poly-*N*-acetyllactosamine–containing free GPIs. However, neither of these were apparent from surface biotinylation, followed by isolation on neutravidin and detection with Coomassie blue or silver stain (Figure 8). Because carbohydrate-rich proteoglycan- or mucin-like molecules might contain few primary amine groups for biotinylation, we took a different approach, namely biosynthetic radiolabeling with the ubiquitous trypanosomatid glycoconjugate precursor glucosamine. This revealed polydisperse glycoconjugates with high apparent molecular weights (90 to >200 kDa) on SDS-PAGE that were present in similar levels in *TbGPI12* null and wild-type cells (Figure 9). It remains to be seen whether the majority of the surface coat observed by TEM is constituted by the novel glycoconjugates revealed by [^{3}H]glucosamine labeling. This material is certainly a candidate, but the answer will have to await its isolation and characterization and the generation of mono-specific reagents that recognize its principal component(s).

TbGPI10 and *TbGPI8* knockouts are partially and severely compromised, respectively, with respect to their ability to colonize the tsetse midgut (Nagamune *et al.*, 2004). The difference in tsetse infectivity is thought to correlate with surface sialic acid content, governed by the status of the normally GPI-anchored transialidase (TS) enzyme (Nagamune *et al.*, 2004). Thus, although *TbGPI10* knockout parasites secrete anchorless TS and, therefore, still acquire surface sialic acid, the GPI-transamidase null *TbGPI8* knockout parasites fail to remove the GPI-addition signal peptide from the TS precursor, to degrade it intracellularly, and to sialylate their surface. In the case of the *TbGPI12* knockout cells, we observe normal growth kinetics but severely reduced tsetse infectivity (Figure 10). These cells will be able to secrete TS (like the *TbGPI10* knockouts) but cannot express cell surface procyclin or free GPI sialic acid acceptors. The *TbGPI12* null phenotype with respect to tsetse colonization is, therefore, like that of *TbGPI8* null parasites, supporting the hypothesis that sialylation of procyclin GPI anchor side chains and/or free GPI side chains (that, uniquely, are both are absent in *TbGPI12* mutant) are essential for tsetse midgut infection.

Although deletion of surface free GPIs and/or procyclins does not prevent procyclic trypanosome cell division in culture, galactose starvation is cytostatic (Roper *et al.*, 2005). We previously suggested that galactose starvation might exert these cytostatic effects via inhibition of poly-*N*-acetyllactosamine side chain addition to procyclin GPI anchors and/or free GPIs, an idea that was consistent with the apparently compensatory 10-fold up-regulation in side chain–free procyclins before the cessation of cell division. However, this notion may need to be revised in the light of the healthy growth of the *TbGPI12* knockout because this mutant has no GPI structures that can be modified with poly-*N*-acetyllactosamine side chains. Thus, it is possible that it is the effects of galactose starvation on one or more non-GPI–anchored glycoprotein(s), possibly the novel high apparent molecular weight molecules identified in this article, that cause cell division to cease. Alternatively, one could argue that the accumulation of procyclins without GPI side chains under galactose starvation might be toxic to the cells and that because this cannot occur in the *TbGPI12* knockout cells, the latter grow normally.

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