Cloning of *Trypanosoma brucei* and *Leishmania major* Genes Encoding the GlcNAc-Phosphatidylinositol De-N-acetylase of Glycosylphosphatidylinositol Biosynthesis That Is Essential to the African Sleeping Sickness Parasite

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The second step of glycosylphosphatidylinositol anchor biosynthesis in all eukaryotes is the conversion of D-GlcNAc-6-O-my/o-inositol-1-HP0\(_{4}\)-sn,1,2-diacylglycerol (GlcNAc-PI) to \(\tau\)-GlcNAc-6-O-my/o-inositol-1-HP0\(_{4}\)-sn,1,2-diacylglycerol by GlcNAc-PI de-N-acetylase. The genes encoding this activity are *PIG-L* and *GPI12* in mammals and yeast, respectively. Fragments of putative GlcNAc-PI de-N-acetylase genes from *Trypanosoma brucei* and *Leishmania major* were identified in the respective genome project data bases. The full-length genes *TbGPI12* and *LmGPI12* were subsequently cloned, sequenced, and shown to complement a *PIG-L*-deficient Chinese hamster ovary cell line and restore surface expression of GPI-anchored proteins. A tetracycline-inducible bloodstream form *Trypanosoma brucei* nonpermissive cell line was created and analyzed under nonpermissive conditions. *TbGPI12* mRNA levels were reduced to undetectable levels within 8 h of tetracycline removal, and the cells died after 3–4 days. This demonstrates that *TbGPI12* is an essential gene for the tsetse-transmitted parasite that causes Nagana in cattle and African sleeping sickness in humans. It also validates GlcNAc-PI de-N-acetylase as a potential drug target against these diseases. Washed parasite membranes were prepared from the conditional null mutant cell line and processed and analyzed under nonpermissive conditions. *TbGPI12* mRNA levels were reduced to undetectable levels within 8 h of tetracycline removal, and the cells died after 3–4 days. This demonstrates that *TbGPI12* is an essential gene for the tsetse-transmitted parasite that causes Nagana in cattle and African sleeping sickness in humans. It also validates GlcNAc-PI de-N-acetylase as a potential drug target against these diseases. Washed parasite membranes were prepared from the conditional null mutant parasites after 48 h without tetracycline. These membranes were shown to be greatly reduced in GlcNAc-PI de-N-acetylase activity, but they retained their ability to make GlcNAc-PI and to process D-GlcNAc-6-O-my/o-inositol-1-HP0\(_{4}\)-sn,1,2-diacylglycerol to later glycosylphosphatidylinositol intermediates. These results suggest that the stabilities of other glycosylphosphatidylinositol pathway enzymes are not dependent on GlcNAc-PI de-N-acetylase levels.

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This paper is dedicated to Professor John S. Brimacombe on the occasion of his retirement from the Roscoe Chair of Chemistry at The University of Dundee.

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been studied in T. brucei (15–20), Trypanosoma cruzi (21), Toxoplasma gondii (22), Plasmodium falciparum (23), Leishman-ia sp. (24–26), Saccharomyces cerevisiae (27, 28), and mammalian cells (29–31), and references therein. In all cases, GPI biosynthesis involves the addition of GlcNAc to phosphati-dylinositol (PI) to give GlcNAc-PI, which is then de-N-acetylated by N-acetyl-o-glucosaminylphosphatidylinositol deacety-lase (EC 3.1.1.69), referred to here as GlcNAc-PI de-N-acety-lase, to form Glc-PI (32–35). De-N-acetylation is a prerequisite for the mannosylation of GlcNAc-PI to form later GPI intermediates (34, 36). The GlcNAc-PI de-N-acetylasmes from protozoan and mammalian sources are similar with regard to their specificity for the acyl (R) group removed from GlcNR-PI substrates (36), but differ with regard to their specificity for the myo-inositol residue. Thus, the trypanosomalan enzyme can de-N-acetylate GlcNAc-PI containing either D- or L-myo-inositol and α- or β-D-GlcNAc, whereas the human (HeLa) enzyme strictly requires α-D-GlcNAc (1–6)D-myo-inositol (37, 38). These differences, and the ability of the trypanosomalan enzyme to tolerate a C8 O-alkyl substituent on C2 of the D-myo-inositol residue, were recently exploited in the design and synthesis of two parasite-specific GlcNAc-PI de-N-acetylase suicide sub-strate inhibitors (38).

The gene encoding the rat de-N-acetylase (PIG-L) was the first to be cloned (34) and a yeast homologue (GIPI2) has been shown to complement PIG-L-deficient mammalian cells and vice versa (35). Here, we describe the molecular cloning of the T. brucei and L. major homologues TbGPI12 and LmGPI12, demonstrate functional complementation in a PIG-L-deficient mammalian cell line, and describe the creation of a T. brucei TbGPI12 conditional null mutant. We further demonstrate that membranes from the conditional null mutant are deficient in GlcNAc-PI de-N-acetylase activity under non-permissive conditions and that TbGPI12 is an essential gene in blood-stream form of T. brucei.

EXPERIMENTAL PROCEDURES

Cloning of T. brucei and L. major GPI12—The 401-bp end-sequence of an Institute for Genomic Research genome sequence clone sequence (AQ64432), returned from the TBLASTn search with yeast GPI12 (accession number P23797), was used to design a reverse PCR primer (5’-cgcGATCCctagctgatccaatctcactc-3’ (capital letters indicate a BamHI site)) that was used with Pfu polymerase, blood-stream form T. brucei cDNA, and a forward primer based on the 5’ mini-exon (5’-ggcgctattagtatgacgttga-3’) to amplify an ~0.8-kb fragment containing the entire TbGPI12 ORF. Amplification conditions were 95 °C for 45 s, 60 °C for 1 min, and 72 °C for 3 min for 30 cycles. The PCR product was purified from an agarose gel (QIAEX II kit) and fully sequenced. The clone contained the full-length TbGPI12 ORF and the 3’-UTR linked to the 3’ UTR of the TbGPI12 clone was kindly provided by Prof. J. M. Blackwell (Cambridge University) and the 5’-UTR was synthesized. The two PCR products were used together in a further PCR reaction to yield a product containing a silent mutation that removed a HindIII site (capital italic letters indicate the mutation), a myc epitope tag fused to the C terminus of TbGPI12 (underlined letters) and 5’-HindIII and 3’-BamHI restriction sites (capital letters). The purified construct was digested with HindIII and BamHI and ligated into the respective cloning sites of the pcDNA3.1/Hygro (+) (Invitrogen) mammalian expression vector.

The LmGPI12 gene was also PCR-amplified using Pfu in two segments. The 5’-end of the ORF was amplified using forward primer 5’-cgcGATCCctagctgatccaatctcactc-3’ and reverse primer 5’-gacggtgagatctcctctatccttcgtc-3’ and the 3’-end of the ORF was amplified using forward primer 5’-gacggtgagatctcctctatccttcgtc-3’ and reverse primer 5’-cgcGATCCctagctgatccaatctcactc-3’. The two PCR products were used together in a further PCR reaction to yield a product containing a silent mutation that removed a BamHI site (capital italic letters indicate the mutation) and 5’-HindIII and 3’-BamHI restriction sites (capital letters). The purified construct was digested with HindIII and BamHI and ligated into the respective cloning sites of the pcDNA3.1/Hygro (+).

The pcDNA3.1/Hygro (+) plasmids (empty and containing TbGPI12-myc or LmGPI12) were purified (Qiagen Maxi-Prep), precipitated, washed with ethanol, resuspended in sterile water, and used for transient transfections. Trypsin-treated PIG-L-deficient and CD59- and decay-accelerating factor (DAF)-transgenic CHO-K1 (clone M282) cells (34) were washed twice and resuspended at 1–2 x 10^7/ml in ice-cold phosphate-buffered saline. Aliquots of 1 ml were mixed with 50–70 µg of plasmid DNA, incubated for 20 min on ice, and then resuspended in a 4 µl 0.4-cm cuvette for a Bio-Rad gene-pulse. The cells were immediately transferred to 20 ml of Dulbecco’s modified Eagle’s medium with glutamine, 10% fetal calf serum, 100 units/ml penicillin/streptomycin, and 0.3 mg/ml G418 (to maintain the CD59- and DAF-containing plasmid), and cultured at 37 °C. Two days after transient transfection, the cells were incubated with anti-CD59 (B229; 10 µg/ml) and anti-DAF (183.8; 10 µg/ml) mouse monoclonal antibodies, followed by fluorescein isothiocyanate-conjugated secondary antibody (Dako F0313) and visualized by fluores-cence microscopy using an MRC-600 laser scanning confocal imaging system (Nikon Microphot-SA).

Southern Blots—T. brucei genomic DNA (5 µg/lane) was digested with various restriction enzymes and the products were resolved on a 0.7% agarose gel. After transfer to nitrocellulose and UV-cross-linking, the blot was hybridized with fluorescein-labeled TbGPI12 probe (16 h, 60 °C) and washed twice with 1 x SSC, 0.1% SDS for 15 min and twice with 0.5 x SSC, 0.1% SDS for 15 min. Blots were developed with horseradish peroxidase-conjugated anti-fluorescein antibody according to the manufacturers’ instructions (gene images CDP-Star kit; Amer-sham Biosciences).

Generation of a Bloodstream Form T. brucei TbGPI12 Conditional Null Mutant—A T. brucei strain 427 BAC library filter (CHORI RPCI-102), representing 46-fold genome coverage, was probed with a 2 µP-labeling TbGPI12 probe under the same conditions described for the S. cerevisiae library filters. The libraries were hybridized separately and the TbGPI12-containing BAC plasmids were purified from 3-ml cultures of four clones, using the “DNA isolation from BAC & PAC clones” protocol recommended by CHORI BACPAC Resources (www.chori.org/ bapac). The presence of the TbGPI12 gene was confirmed by PCR using 5’-gacggtcctatcatatatgactttgtcaagggct-3’ and 5’-cgcGATCCctagctgatccaatctcactc-3’ forward and reverse primers. One clone was selected and the purified BAC plasmid DNA (1 µg) was used as template for DNA sequencing using primers from within the gene, 5’-ctagctgatcctttgtcag-3’ and 5’-atcggccagactctacctggctg-3’. Consequently, 466 bp of 5’-UTR and 648 bp of 3’- UTR sequence were obtained. Based on these data, 426 bp of 5’-UTR immediately upstream of the stop codon were PCR-amplified using Pfu and genomic DNA template with the forward primer 5’-ataagaactGC GCCGCCTcccccggcctgacctgatg-3’ and reverse primer 5’-gtttaactacacctgcagacgagct-3’. Likewise, 478 bp immediately downstream of the stop codon were PCR-amplified using the forward primer 5’-gacggtcctatcatatatgactttgtcaagggct-3’ and reverse primer 5’-cgcGATCCctagctgatccaatctcactc-3’. The two PCR products were used together in a further PCR reaction to yield a product containing the 5’-UTR linked to the 3’-UTR by a short HindIII, PmeI, and BamHI cloning site (underlined letters) and NolI restriction sites at each end (capital letters). Subsequently, the PCR product was cloned into the NolI site of pGem-SZ (+) vector (Promega) and the hygromycin B resistance gene was inserted at the PmeI site. The resulting construct was digested with HindIII and Sall and subjected to nucleotide sequencing. The drug resistance genes were introduced to the targeting vector via the HindIII/BamHI cloning site. The previously described HindIII-silenced, C-terminally myc-tagged TbGPI12 construct was ligated into the HindIII/BamHI cloning site of the pLew10 tetacycline-inducible ex-

Trypanosomatid GlcNAc-PI De-N-acetylase
Trypanosomatid GlcNAc-PI De-N-acetylase

expression vector (39). Plasmids were prepared (Qiagen maxi-prep), digested with XhoI, precipitated with ethanol, redissolved in sterile water, and used for electroporation of bloodstream form T. brucei strain 427 (variant 221), which are stably transfected to express T7 RNA polymerase and tetracycline repressor protein under continuous G418 selection (39). Cell culture, transformation and drug selection conditions were as described previously (39–42). Tet-system approved fetal calf serum (Clontech) was used in experiments on the effects of tetracycline-removal.

Northern Blots—Total RNA was prepared (Qiagen RNeasy Protect Midi kit) from 2 × 10⁷ cells. Samples of RNA (5 μg) were run on formaldehyde agarose gel and transferred to Hybond-N nylon membranes (Amersham Biosciences) for hybridization with [α-³²P]CTP labeled TbGPI12 probe (Stratagene Prime-IIT random primer labeling kit). As a loading control, a β-tubulin probe was used on the same blot.

Cell-free System Experiments—Bloodstream form T. brucei membranes (cell-free system) were prepared (15, 19, 43) from wild-type cells and TbGPI12 conditional null mutant cells grown continuously in 1 μg/ml tetracycline and grown tetracycline-free for 48 h. Trypanosomes were washed twice and resuspended at 5 × 10⁸ cell equivalents/ml in 2× incorporation buffer supplemented with 10 mM N-ethylmaleimide or 2 mM dithiothreitol (43, 44). The lysates were briefly sonicated and aliquots of 10⁷ cell equivalents were added to an equal volume of GDP-[²-³H]Man (0.4 μCi), 22 Ci/mmol; PerkinElmer) or UDP-[⁶-³H]GlcNAc (1 μCi, 41 Ci/mmol; PerkinElmer). When 350 pmol of synthetic GlcN-PI or GlcNAc-PI (45) were used, the GDP-[²-³H]Man solution was supplemented 0.3% (w/v) n-octyl-β-D-glucopyranoside. Samples were incubated for 1 h at 30 °C, and glycolipid samples were recovered for analysis by HPTLC before and after enzyme treatments.

HPTLC and Enzyme Digests—Samples were digested with jack bean α-mannosidase and Bacillus thuringiensis phosphatidylinositol-specific phospholipase C (both from Glyko) as described in (46). Samples and a conserved sequence in rat and human were hybridized with [α-³²P]CTP labeled TbGPI12 probe (Stratagene Prime-IIT random primer labeling kit). As a loading control, a β-tubulin probe was used on the same blot.

RESULTS AND DISCUSSION

Cloning of T. brucei and L. major GlcNAc-PI De-N-acetylase Genes (TbGPI12 and LmGPI12)—A partial gene sequence was found in The Institute for Genomic Research T. brucei database with a blastn search, using the Saccharomyces cerevisiae GPI12 protein sequence as the query. The putative TbGPI12 gene fragment contained the 3'-end of the gene (377 bp), including a stop codon, followed by 24 bp of putative 3'-UTR. A cDNA clone was obtained by PCR using bloodstream form T. brucei cDNA as the template, a forward primer based on the 5'-spliced leader (a 35 bp sequence trans-spliced onto all T. brucei mRNA), and a reverse primer based on the 3'-end sequence found in the data base. The 0.8-kb product contained a 759-bp ORF (GenBankTM accession number AY157267). A genomic clone was also obtained by PCR using genomic DNA as the template, a forward primer to the 5'-end of the gene based on the cDNA clone, and a reverse primer based on the 3'-UTR sequence found in the data base. Both the cDNA and genomic DNA ORF sequences were cloned.

A Blast search with the sequence LVIAJHPDEAMMFAP, a conserved sequence in rat and human PIG-L that is largely conserved in yeast GPI12 (34), identified an L. major expressed sequence tag sequence. The corresponding cDNA clone was fully sequenced and found to contain the full-length LmGPI12 gene (GenBankTM accession number AY157268).

The predicted amino acid sequences of the two parasite putative GlcNAc-PI de-N-acetylases, aligned with related sequences, are shown in Fig. 1. All of the sequences predict proteins with the majority of their sequence in the cytoplasm and anchored to the endoplasmic reticulum via a single N-terminal transmembrane domain, an arrangement that has been demonstrated experimentally for rat PIG-L (34). The PIG-L/GPI12 sequences have similarity to the pfam02555 family of prokaryote sequences that include Rv1170, a GlcNAc-1-O-myo-inositol de-N-acetylase involved in mycothiol synthesis in Mycobacterium tuberculosis (47).

Complementation of a PIG-L-deficient CHO-K1 Reporter Cell Line—the LmGPI12 and TbGPI12 genes, the latter fused to a C-terminal myc-tag, were cloned into the pcDNA3.1 mammalian expression vector. The recombinant plasmids were used to transiently transfect a previously established CHO-K1 reporter cell line (34) deficient in PIG-L. This reporter cell line is stably transfected to express two GPI-anchored proteins: CD59 and DAF. These proteins fail to receive a GPI anchor because of the PIG-L deficiency and cannot be detected on the cell surface by immunofluorescence microscopy (Fig. 2A) unless the cells are complemented by transfection with a gene (e.g. rat PIG-L) encoding a functional GlcNAc-PI de-N-acetylase (Fig. 2B).

Transient transfection with both parasite putative GlcNAc-PI de-N-acetylase genes produced similar results (Fig. 2, C and D), demonstrating that TbGPI12 and LmGPI12 encode functional GlcNAc-PI de-N-acetylases.

TbGPI12 Is an Essential Gene in Bloodstream Form T. brucei—Southern blot analysis revealed that the TbGPI12 gene was present as a single copy per haploid genome (Fig. 3). Replacement of both alleles of the TbGPI12 gene from the diploid genome of bloodstream from T. brucei parasites was attempted. Gene replacement by homologous recombination of a single TbGPI12 allele with the drug-resistance gene PAC or HYG, and selection for transformants with the appropriate antibiotic, was successful. However, attempts to replace the second TbGPI12 allele in a ΔTbGPI12::PAC clone with HYG failed, suggesting that TbGPI12 may be essential. To test this, we created a conditional, tetracycline-inducible null mutant. The “wild-type” trypanosome cell line used in this study is a transgenic parasite that constitutively expresses T7 RNA polymerase and the tetracycline repressor (TETR) protein under G418-selection (39). Thus, a tetracycline-inducible (Ti) ectopic myc-tagged TbGPI12 gene was introduced into the trypanosome ribosomal DNA locus (using the pLew100 expression vector) downstream of a tetransposon (procyclin) promoter and two tetracycline operator sequences (Fig. 4A). Several TbGPI12-myc™ ΔTbGPI12::PAC clones were isolated, and one of these was induced with tetracycline and used for a second round of homologous recombination to replace the second endogenous TbGPI12 allele with HYG. Five TbGPI12-myc™ ΔTbGPI12::PAC/ΔTbGPI12::HYG conditional null mutant clones were obtained. A Southern blot of one of these clones, and its TbGPI12-myc™ ΔTbGPI12::PAC, ΔTbGPI12::PAC, and wild-type parent cell-lines, confirmed the loss of both chromosomal TbGPI12 alleles and the introduction of an ectopic gene copy (Fig. 4B).

The TbGPI12 conditional null mutant cells grew continuously in culture in the presence of 1 μg/ml tetracycline. Cells were counted daily and cultures were split when densities approached 2 × 10⁸ cells/ml (Fig. 5A). However, cells washed three times and cultured in medium without tetracycline grew for about 3–4 days and then died (Fig. 5B). The few surviving cells (below the limits of detection by light microscopy) failed to divide unless tetracycline was reintroduced at day 6, whereupon they resumed sustained growth (Fig. 5C), or until around day 14, when some cultures spontaneously started to grow once more at normal rates (Fig. 5D). The spontaneous recovery of conditional null mutant cultures in the absence of tetracycline has been reported previously (40, 42, 48) and is caused by the loss of tetracycline control through, for example, deletion of the TETR gene (42).
Northern blot analysis showed that TbGPI12 mRNA levels were undetectable within 8 h of tetracycline removal (Fig. 6). However, the cells continued to divide for 2–3 days, suggesting that it takes this length of time for de-N-acetylase loss (because of dilution by cell division and protein turnover) to reach unsustainable levels. Taken together, these data demonstrate
cells, were labeled with GDP-[3H]Man in the presence and absence of UDP-GlcNAc (in the presence of DTT to stimulate UDP-GlcNAc:PI α-GlcNAc-transferase activity) or synthetic GlcNAc-PI or GlcN-PI (in the presence of N-ethylmaleimide to inhibit UDP-GlcNAc:PI α-GlcNAc-transferase activity (49)). In the first set of experiments (Fig. 7B), the addition of synthetic GlcNAc-PI to the cell-free systems from wild-type and tetracycline-induced (+Tet) conditional null mutant cells lead to similar levels of 3H-mannosylated GPI products, from Man,GlcN-PI to glycolipid A', whereas more dolichol-P-[3H]Man and less 3H-mannosylated Man,GlcN-PI to glycolipid A' were observed with the cell-free systems from the non-induced (−Tet) conditional null mutant cells. These results are also consistent with lower GlcNAc-PI de-N-acetylase activity in the non-induced membranes. The control experiment, using GlcN-PI instead of GlcNAc-PI, produced an interesting result. Because the processing of GlcN-PI does not require prior de-N-acetylation, we expected the products of the wild-type and induced (+Tet) and non-induced (−Tet) cell-free systems to be similar. We observed, however, that although the level of labeling was indeed comparable, the non-induced cell-free system produced a more complex band pattern than the wild-type and induced cell-free systems. To investigate this further, additional experiments were performed comparing the addition of GlcN-PI with the addition of UDP-GlcNAc and with no additions (Fig. 7C). As expected, in the absence of GlcN-PI and UDP-GlcNAc, the wild-type and induced (+Tet) cell-free systems produced few 3H-mannosylated products from the processing of the limiting amounts of GPI intermediates in these membranes (Fig. 7C, lanes 3 and 4). However, the non-induced (−Tet) cell-free system produced abundant 3H-mannosylated GPI intermediates, up to and including glycolipid A' and glycolipid θ (Fig. 7C, lane 9). Furthermore, the levels of these products were not increased by the addition of UDP-GlcNAc (Fig. 7C, lane 8), whereas the addition of UDP-GlcNAc greatly stimulated the formation of 3H-mannosylated GPI intermediates in the wild-type and induced (+Tet) cell-free systems (Fig. 7C, lanes 2 and 5). We interpret these results to mean that the non-induced (−Tet) cells accumulate significant amounts of endogenous GlcNAc-PI in their endoplasmic reticulum membranes because of significantly reduced GlcNAc-PI de-N-acetylase activity, such that cell-free system prepared from these cells harbors a significant pool of endogenous GlcNAc-PI. Thus, when GDP-[3H]Man is added to the cell-free system, endogenous GlcN-PI generated by the action of residual de-N-acetylase activity on this pool provides the necessary substrate for the formation of 3H-mannosylated GPI intermediates. These 3H-mannosylated GPI intermediates are based on endogenous GlcN-PI that contain predominantly stearic acid at the sn-1 position and a mixture of C18-C22 fatty acids at sn-2 (49) and, therefore, have slightly higher Rₚ values than those 3H-mannosylated GPI intermediates made from exogenous synthetic sn-1,2-dipalmitoylglycerol-containing GlcN-PI. Thus, the combination of 3H-mannosylated GPI intermediates made from endogenous and exogenous GlcN-PI leads to the complex band pattern in Fig. 7B (lane 6 compared with lanes 4 and 5) and Fig. 7C (lane 7 compared with lanes 1 and 6).

Regardless of the complexities of the results described above, it is clear that the GlcNAc-PI de-N-acetylase deficient membranes of the conditional null mutant grown for 48 h without tetracycline are perfectly capable of synthesizing GlcNAc-PI and of processing GlcN-PI to later GPI intermediates. This suggests that neither the multicomponent UDP-GlcNAc:PI α1–6 GlcNAc transferase (50) nor the downstream mannosyltransferases and ethanolamine phosphate transferases are significantly affected by a reduction in GlcNAc-PI de-N-acetylase...
level. Because the expression levels of tightly associated subunits of GPI biosynthesis complexes do affect each other (50, 51), this result may suggest that the *T. brucei* GlcNAc-PI de-N-acetylase does not tightly associate with the upstream GlcNAc-transferase or the downstream α-mannosyltransferase. On the other hand, the apparent substrate channelling between the de-N-acetylase and the downstream α-/β-mannosyltransferase (43) suggests some spatial proximity of these enzymes. Further experiments are required to determine whether the *T. brucei* de-N-acetylase is associated with other components of GPI biosynthesis or whether, like the mammalian enzyme (34), it behaves as a free-standing protein.

**CONCLUSIONS**

Two kinetoplastid parasite GlcNAc-PI de-N-acetylase gene homologues were readily identified and cloned, thanks to the ongoing *T. brucei* and *L. major* genome sequencing projects, and their functionality was confirmed by complementation. The TbGPI12 gene has been shown here to be a single-copy gene that is essential for the disease-causing bloodstream form of *T. brucei*, thus validating this particular enzyme as a potential drug target for the development of therapeutic agents against African sleeping sickness and, possibly, against related parasitic diseases, such as the leishmaniasis and Chagas’ disease. The genetic validation of GlcNAc-PI de-N-acetylase as an antitrypanosomal target is particularly significant because there has been considerable biochemical characterization of this enzyme (32–38). Attractive features of this potential drug

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**FIG. 4.** Construction and characterization of a conditional TbGPI12-null mutant. *A*, scheme of the targeted replacement of one TbGPI12 allele with PAC, the introduction of an ectopic tetracycline-inducible copy of myc-tagged TbGPI12 into the rDNA locus, and replacement of the second TbGPI12 allele with HYG. The cells used for the aforementioned transformations stably express T7 polymerase and tetracycline repressor protein (TetR) under G418 selection (39). *B*, aliquots of DNA (5 μg) were digested with *Pst*I (which gives conveniently sized TbGPI12-containing fragments) and Southern blotted with the TbGPI12 probe. The DNA was from wild-type TbGPI12+/+ cells (lane 1), ΔTbGPI12::PAC cells (lane 2), TbGPI12-myc+/ΔTbGPI12::PAC cells (lane 3), and conditional TbGPI12-myc+ ΔTbGPI12::PAC/ΔTbGPI12::HYG null mutant clones (lane 4). The probe reveals allelic (TbGPI12) copies at 2.7 kb and ectopic tetracycline-inducible (TbGPI12-myc+) copies at 5 kb.

**FIG. 5.** Growth of the *T. brucei* conditional TbGPI12 null mutant. Cells were grown in the continuous presence of tetracycline and subcultured as the cell density approached 2 × 10^6 cells/ml (A). Cells harvested from the culture in A (asterisk) were washed in tetracycline-free medium and used to inoculate tetracycline-free cultures (B and C). Cells were subcultured as they approached 2 × 10^6 cells/ml (B, closed squares, and C) or left without sub-culturing (B, open squares). Tetracycline was added back to one of the cultures after 6 days (C, arrow), leading to rapid recovery of the culture.

**FIG. 6.** Northern blot of the *T. brucei* conditional TbGPI12-null mutant. Northern blot of total RNA prepared from wild-type *T. brucei* (lane 1) and conditional TbGPI12 null mutant parasites grown without tetracycline for 0, 2, 4, 8, 24, and 48 h (lanes 2–7) and probed with TbGPI12 probe (top) and a β-tubulin probe (bottom).
target include: (i) relatively low identity and similarity (36 and 54%) between the T. brucei and human enzyme sequences; (ii) significant differences between the substrate specificities of the T. brucei and human enzymes (37, 38); and (iii) the recent synthesis of two potent (IC$_{50}$, 8 nm) parasite-specific suicide substrate inhibitors of the enzyme (38).

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