Cloning of *Trypanosoma brucei* and *Leishmania major* genes encoding the GlcNAc-PI de-N-acetylase of glycosylphosphatidylinositol biosynthesis that is essential to the African sleeping sickness parasite.

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Abstract

The second step of glycosylphosphatidylinositol anchor biosynthesis in all eukaryotes is the conversion of GlcNAc-PI to GlcN-PI 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 databases. The full-length genes, TbGPI12 and LmGPI12, were subsequently cloned, sequenced and shown to complement a PIG-L-deficient CHO cell line and restore surface expression of GPI-anchored proteins. A tetracycline-inducible bloodstream form T. brucei TbGPI12 conditional null mutant cell line was created and analyzed under non-permissive 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 GlcN-PI 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.
Introduction

A significant proportion of eukaryotic cell-surface glycoproteins are attached to the plasma membrane by covalent linkage to a glycosylphosphatidylinositol (GPI) membrane anchor. The structure and biosynthesis of GPI membrane anchors and related molecules have been recently reviewed (1-4). The basic GPI core structure attached to protein comprises $\text{NH}_2\text{CH}_2\text{CH}_2\text{PO}_4\text{H-6Man}\alpha1-2\text{Man}\alpha1-6\text{Man}\alpha1-4\text{GlcN}\alpha1-6\text{Dmyo-inositol-1-HPO}_4$-lipid (EtNP-Man$_3$GlcN-PI), where the lipid can be diacylglycerol, alkylacylglycerol or ceramide. This minimal GPI structure may be embellished with additional ethanolamine phosphate groups and/or carbohydrate side-chains in a species- and tissue-specific manner (5).

Protozoa tend to express significantly higher densities of cell-surface GPI-anchored proteins than do higher eukaryotes (1, 4, 6). For example, *Trypanosoma brucei*, the causative agent of African sleeping sickness, expresses a dense cell-surface coat consisting of approximately $5 \times 10^6$ dimers of a GPI-anchored variant surface glycoprotein that protects the parasite from the alternative complement pathway of the host and, through antigenic variation, from specific immune responses (7). The related kinetoplastid parasite *Leishmania* expresses lower copy numbers of GPI-anchored glycoproteins, such as the promastigote surface protease (Psp or gp63), gp42 and GPI-anchored proteophosphoglycans, but high copy numbers of the GPI-related structures lipophosphoglycan (LPG) and the glycoinositolphospholipids (GIPLs) (4, 6, 8, 9).

It has been suggested by several groups that inhibitors able to arrest the formation of GPI-anchored proteins and/or GPI-related molecules on the plasma membrane of parasitic protozoa might prove useful in the development of anti-parasitic agents. This notion has been validated for *T. brucei*, where disruption of the *TbGPI10* gene encoding the third mannosyltransferase of GPI anchor biosynthesis has been shown to be lethal for the bloodstream form of the parasite (10, 11). The situation is less clear in the *Leishmania* where, for example, *L. mexicana* is infective without LPG, GIPLs and GPI-anchored glycoproteins whereas *L. major* is significantly attenuated in the absence of LPG (12-14).

The sequence of events underlying GPI biosynthesis has been studied in *T. brucei* (15-20), *T. cruzi* (21), *Toxoplasma gondii* (22), *Plasmodium falciparum* (23), *Leishmania* (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 phosphatidylinositol (PI) to give GlcNAc-PI, which is then de-N-acetylated by N-acetyl-D-glucosaminylphosphatidylinositol deacetylase (EC 3.1.1.69), referred to here as GlcNAc-PI de-N-acetylase, to form GlcN-PI (32-35). De-N-acetylation is a prerequisite for the mannosylation of GlcN-PI to form later GPI intermediates (34, 36). The GlcNAc-PI de-N-acetylases from protozoan and mammalian sources are similar with regard to their specificities 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 trypanosomal 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-my-o-inositol (37, 38). These differences, and the ability of the trypanosomal enzyme to tolerate a C8 O-alkyl substituent on C-2 of the D-my-o-inositol residue, were recently exploited in the design and synthesis of two parasite-specific GlcNAc-PI de-N-acetylase suicide substrate inhibitors (38).

The gene encoding the rat de-N-acetylase (PIG-L) was the first to be cloned (34) and a yeast homologue (GPI12) 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 bloodstream form T.brucei.

Experimental Procedures

Cloning of T.brucei and L. major GPI12 – The 401 bp end-sequence of a TIGR genome survey sequence clone (AQ644232), returned from the tBLASTn search with yeast GPI12p (accession P23797), was used to design a reverse PCR primer (5'-cgcGGATCCcatgcgaccccaattccttcacttc-3'; capital letters indicate a BamHI site) which was used with Pfu polymerase, T.brucei bloodstream form cDNA and a forward primer
based on the 5' mini-exon (5'-ggcccgctattattagaacagtttctgta-3') to amplify an approximately 0.8 kb fragment containing the entire \textit{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 ligated into a pUC18 cloning vector using a Sureclone ligation kit (Pharmacia). Twelve representative clones were used for DNA sequencing, revealing a 759 bp ORF. The same PCR product (the \textit{TbGPI12} probe) was fluorescein-labelled by random priming (Gene Images Kit, Amersham) or labelled with \textsuperscript{32}P (Prime-It RmT random primer labelling kit, Stratagene) for use in Southern blotting and for probing a BAC library filter, respectively (see below).

A tBlastn search with the sequence LVIAHPDDEAMFFAP, a strictly conserved sequence in rat and human \textit{PIG-L} and substantially conserved in yeast \textit{GPI12}, identified an \textit{L.major} EST sequence (AA728250) with 85% similarity. The corresponding cDNA clone was kindly provided by Prof. J. M. Blackwell, Cambridge University, and fully sequenced. The clone contained the full-length \textit{LmGPI12} gene.

\textit{Functional complementation} - For expression in mammalian cells, the \textit{TbGPI12} gene was PCR amplified using \textit{Pfu} in two segments. The 5'-end of the ORF was amplified using forward primer 5'-gagAAGCTTCATATGcatggtgctttggcgtttggg-3' and reverse primer 5' catggcggaaaagctGtggaacaatgag-3' and the 3'-end of the ORF was amplified using forward primer 5'-ctcattgttcacGagcttttccgccatg-3' and reverse primer 5'-cgGGATCCtcaCAGGTCTTCTCCGAGATATAGCTTCTGTTCGTTAATTAAtgacgcc ccaattcttt-3'. The two PCR products were used together in a further \textit{Pfu} PCR reaction to yield a product containing a silent mutation that removed a \textit{HindIII} site (capital italic letters indicate the mutation), a \textit{myc} epitope tag fused to the C-terminus of \textit{TbGPI12} (underlined letters) and 5'-\textit{HindIII} and 3'-\textit{BamHI} restriction sites (capital letters). The purified construct was digested with \textit{HindIII} and \textit{BamHI} and ligated into the respective cloning sites of the pcDNA3.1/Hygro (+) (Invitrogen) mammalian expression vector.

The \textit{LmGPI12} gene was also PCR amplified using \textit{Pfu} in two segments. The 5'-end of the ORF was amplified using forward primer 5'- cccAAGCTTGGGatgcacagtatcacagtt-3' and reverse primer 5'-gcaggtgtagGcttgagggcatggtc-3' and the 3'-end of the ORF was amplified using forward
primer 5’-ggacatgcctccaggCatcctccacctgc-3’ and reverse primer 5’-cgcGGATCCgcgctagagctcttcgatctc-3’. The two PCR products were used together in a further Puu 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 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 DAF-transgenic CHO-K1 (clone M2S2) cells (34) were washed twice and resuspended at 1-2 x 10^7/ml in ice-cold PBS. Aliquots of 1 ml were mixed with 50-70 µg plasmid DNA, incubated for 20 min on ice and electroporated in a 0.4 cm cuvette at 260 V, 950 µF, with a Bio-Rad gene-pulser. The cells were immediately transferred to 20 ml Dulbecco Modified Eagle Medium with glutamine, 10% fetal calf serum, 100 µg/ml pen-strep 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 (813.6; 10 µg/ml) mouse monoclonal antibodies, followed by FITC-conjugated secondary antibody (Dako F0313) and visualised by fluorescence 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. Following transfer to nitrocellulose and UV- crosslinking, the blot was hybridised with fluorescein-labelled 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 HRP-conjugated anti-fluorescein antibody according to the manufacturers instructions (Gene Images CDP-Star Kit, Amersham).

Generation of a T.brucei Bloodstream Form TbGPI12 Conditional Null Mutant - A T.brucei strain 427 BAC library filter (CHORI RPCI-102), representing 46-fold genome coverage, was probed with a 32P-labelled TbGPI12 probe under the same conditions
described for the Southern blot. Fifty positive clones were identified and the corresponding \textit{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 Resourses (http://www.chori.org/bacpac). The presence of the \textit{TbGPI12} gene was confirmed by PCR using 5’-gagAAGCTTCATATGcatggtgctttggcgtttggg-3’ and 5’-gcgcggggctaaatttcacctcg-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’-catgctttcatgctcggtgtgc-3’ and 5’-atccgctgacccaatgctcaca-3’. As a result, 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 start codon were PCR amplified using \textit{Pfu} and genomic DNA template with the forward primer 5’-ataagaatGCGGCCGCcctccccccgcgcctacggatg-3’ and reverse primer 5’-gtttaaacttacggaccctcaagcttgtatgagtggactcccaac-3’. Likewise, 478 bp immediately downstream of the stop codon were PCR amplified using the forward primer 5’-gacggtccgtaagtttaaacggatccatcgaagaatttagccccgc-3’ and reverse primer 5’-ataagtaaGCGGCCGCgactccggcatctgtaaattg-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 \textit{Hind}III, \textit{Pme}I, and \textit{Bam}HI cloning site (underlined letters) and \textit{Not}I restriction sites at each end (capital letters). Subsequently, the PCR product was cloned into the \textit{Not}I site of pGEM-5Zf(+) vector (Promega) and the \textit{HYG} and \textit{PAC} drug resistance genes were introduced into the targeting vector via the \textit{Hind}III/\textit{Bam}HI cloning site. The previously described \textit{Hind}III-silenced, C-terminally \textit{myc}-tagged \textit{TbGPI12} construct was ligated into the \textit{Hind}III/\textit{Bam}HI cloning site of the pLew100 tetracycline-inducible expression vector (39). Plasmids were prepared (Qiagen Maxi-Prep), digested with \textit{Not}I, precipitated with ethanol, redissolved in sterile water and used for electroporation of bloodstream form \textit{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 (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 x 10^8 cells. Samples of RNA (5 µg) were run on formaldehyde agarose gel and transferred to Hybond-N nylon membrane (Amersham Pharmacia) for hybridisation with [α-32P] dCTP labelled TbGPI12 probe (Stratagene Prime-It RmT Random Primer Labelling 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. Trypanosome membranes were washed twice and resuspended at 5 x 10^6 cell-equivalents/ml in 2 x incorporation buffer supplemented with 10 mM N-ethylmaleimide or 2 mM dithiothreitol (43, 44). The lysates were briefly sonicated and aliquots of 10^7 cell equivalents were added to an equal volume of GDP-[2-3H]Man (0.4 µCi, 22 Ci/mmol, NEN) or UDP-[6-3H]GlcNAc (1 µCi, 41.6 Ci/mmol, NEN). When 350 pmol 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 (JBAM) and Bacillus thuringiensis phosphatidylinositol-specific phospholipase C (PI-PLC) (both from Glyko) as described in (46). Samples and glycolipid standards were applied to 10 cm aluminium-backed silica gel-60 HPTLC plates (Merck) and developed with chloroform/methanol/1 M ammonium acetate/13 M ammonia/water (180 : 140 : 9 : 9 : 23 v/v). Radiolabelled components were detected by fluorography at −70°C after spraying with En3Hance (NEN) using Kodak XAR-5 film and an intensifying screen.

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.
(TIGR) *T. brucei* database with a tBLASTn 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 *T. brucei* bloodstream form 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 database. The 0.8 kb product contained a 759 bp ORF (accession number: AY157627). 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 database. Both the cDNA and genomic DNA ORF sequences were identical.

A tBlastn search with the sequence LVIAHPDDEAMFFAP, a conserved sequence in rat and human *PIG-L* that is largely conserved in yeast *GPI12* (34), identified an *L.major* EST sequence. The corresponding cDNA clone was fully sequenced and found to contain the full-length *LmGPI12* gene (accession number: AY157628).

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 pfam02585 family of prokaryote sequences that includes Rv1170, a GlcNAcα1-1-β-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 decay-accelerating factor (DAF). These proteins fail to receive a GPI anchor, due to 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 (eg. 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. 2C, D), demonstrating that \textit{TbGPI12} and \textit{LmGPI12} encode functional GlcNAc-PI de-N-acetylases.

\textit{TbGPI12} is an essential gene in bloodstream form \textit{T.brucei} – Southern blot analysis revealed that the \textit{TbGPI12} gene was present as a single copy per haploid genome (Fig. 3). Replacement of both alleles of the \textit{TbGPI12} gene from the diploid genome of bloodstream from \textit{T.brucei} parasites was attempted. Gene replacement by homologous recombination of a single \textit{TbGPI12} allele with the drug-resistance gene puromycin acetyl transferase (\textit{PAC}) or hygromycin phosphotransferase (\textit{HYG}), and selection for transformants with the appropriate antibiotic, was successful. However, attempts to replace the second \textit{TbGPI12} allele in a \textit{\Delta TbGPI12::PAC} clone with \textit{HYG} failed, suggesting that \textit{TbGPI12} may be essential. To test this, we created a conditional, tetracycline-inducible (Ti) 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 ectopic \textit{myc}-tagged \textit{TbGPI12} gene was introduced into the trypanosome ribosomal DNA locus (using the pLew100 expression vector) downstream of a trypanosome (procyclin) promoter and two tetracycline operator (TetOp) sequences (Fig. 4A). Several \textit{TbGPI12-myc\textsuperscript{\textsc{Ti}} \Delta TbGPI12::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 \textit{TbGPI12} allele with \textit{HYG}. Five \textit{TbGPI12-myc\textsuperscript{\textsc{Ti}} \Delta TbGPI12::PAC/\Delta TbGPI12::HYG} conditional null mutant clones were obtained. A Southern blot of one of these clones, and its \textit{TbGPI12-myc\textsuperscript{\textsc{Ti}} \Delta TbGPI12::PAC}, \textit{\Delta TbGPI12::PAC} and ‘wild type’ parent cell-lines, confirmed the loss of both chromosomal \textit{TbGPI12} alleles and the introduction of an ectopic gene copy (Fig. 4B).

The \textit{TbGPI12} conditional null mutant cells grew continuously in culture in the presence of 1 \textmu g/ml tetracycline. Cells were counted daily and cultures were split when densities approached 2 x 10\textsuperscript{6} 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. 5B). The spontaneous recovery of conditional null
mutant cultures in the absence of tetracycline has been reported previously (40, 42, 48)
and is due to the loss 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 (due to dilution
by cell division and protein turnover) to reach unsustainable levels.

Taken together, these data demonstrate that expression of the TbGPI12
gene is essential for parasite growth.

**Biochemical phenotype of the TbGPI12 conditional null mutant** - The TbGPI12
conditional null mutant was grown with and without tetracycline for 48 h, harvested and
made into cell-free system (washed membrane preparations) (15) to analyse aspects of
GPI biosynthesis. Incubation of the cell-free systems prepared from tetracycline-induced
(+Tet) cells with UDP-[\(^{3}H\)]GlcNAc, followed by glycoplipid extraction and analysis by
HPTLC and fluorography, revealed the formation of \([^{3}H]\)GlcN-PI (15, 32) and some
labelled downstream GPI products formed from \([^{3}H]\)GlcN-PI at the expense of limiting
amounts of dolichol-P-Man in the washed membranes (19) (Fig. 7A, lane 1). However,
labelling of the cell-free system prepared from non-induced (-Tet) cells revealed a build
up of \([^{3}H]\)GlcNAc-PI (Fig. 7A, lane 2), consistent with significantly lower GlcNAc-PI
de-N-acetylase activity in those membranes. The presence of some \([^{3}H]\)GlcN-PI and
labelled downstream GPI products in (lane 2) shows that there is still some GlcNAc-PI
de-N-acetylase activity in these cells, explaining the viability of the cells at the point of
harvest.

The same cell-free systems, and one prepared from ‘wild-type’ cells, were
labelled with GDP-[\(^{3}H\)]Man in the presence and absence of UDP-GlcNAc (in the
presence of DTT to stimulate UDP-GlcNAc : PI \(\alpha\)-GlcNAc-transferase activity) or
synthetic GlcNAc-PI or GlcN-PI (in the presence of NEM to inhibit UDP-GlcNAc : PI \(\alpha\)-
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 [\(^3\)H]mannosylated-GPI products, from Man,GlcN-PI to glycolipid A’, whereas more dolichol-P-[\(^3\)H]Man and less [\(^3\)H]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. Since 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. However, we observed that, although the level of labelling 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 GlcNAc-PI with the addition of UDP-GlcNAc and with no additions (Fig. 7C). As expected, in the absence of GlcNAc-PI or UDP-GlcNAc, the ‘wild-type’ and induced (+Tet) cell-free systems produced few [\(^3\)H]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 [\(^3\)H]mannosylated GPI intermediates, up to and including glycolipid A’ and glycolipid \(\theta\) Fig. 7C, ... 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 [\(^3\)H]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, due to significantly reduced GlcNAc-PI de-N-acetylase activity, such that cell-free system prepared from these cells harbours a significant pool of endogenous GlcNAc-PI. Thus, when GDP-[\(^3\)H]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 [\(^3\)H]mannosylated GPI intermediates. These [\(^3\)H]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 \(Rf\)
values than those [³H]mannosylated GPI intermediates made from exogenous synthetic sn-1,2-diplamitoylglycerol-containing GlcN-PI. Thus, it is the combination of [³H]mannosylated GPI intermediates made from endogenous and exogenous GlcN-PI that 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 synthesising GlcNAc-PI and of processing GlcN-PI to later GPI intermediates. This suggests that neither the multi-component 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. Since 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-mannosyltransferase (43) suggests some spatial proximity of these enzymes. Further experiments are required to see if 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 Leishmaniases and Chagas’ disease. The genetic validation of GlcNAc-PI de-N-acetylase as an anti-trypanosome target is particularly significant because there has been considerable biochemical characterisation of this enzyme (32-38). Attractive features of this potential drug target include: (i) Relatively low identity and similarity (36% and
54%) between the *T.brucei* and human enzyme peptide sequences. (ii) Significant differences between the substrate specificities of the *T.brucei* and human enzymes (37, 38). (iii) The recent synthesis of two potent (IC$_{50}$ 8 nM) parasite-specific suicide substrate inhibitors of the enzyme (38).

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**References**


Footnotes

1Abbreviations: GIPL, glycoinositolphospholipid; GPI, glycosylphosphatidylinositol; GPI-PLD, GPI-specific phospholipase D; GlcN-PI, D-GlcNα1-6D-myoinositol-1-HPO4-sn-1,2-diacylglycerol; GlcNAc-PI, D-GlcNAcα1-6D-myoinositol-1-HPO4-sn-1,2-diacylglycerol; JBAM, jack bean α-mannosidase; LPG, lipophosphoglycan; PI-PLC, phosphatidylinositol-specific phospholipase C.
Figure legends.

Fig. 1. **ClustalW alignment of predicted TbGPI12 and LmGPI12 protein sequences with representative PIG-L/GPI12 homologues.** The sequences are from *T. brucei* (Tb), *L. major* (Lm), human (Hu), rat (Ra), *Caenorhabditis elegans* (Ce), *Saccharomyces cerevisiae* (Sc), *Drosophila melanogaster* (Dm).

Fig. 2. **Complementation of PIG-L-deficient CHO reporter cells.** PIG-L-deficient CHO-K1 cells that stably express CD59 and DAF (34) were transiently transfected with empty vector (panel A), rat PIG-L (panel B), *TbGPI12* (panel C) or *LmGPI12* (panel D) and analysed by phase contrast light microscopy (left) and for surface expression of CD59 by confocal immunofluorescence microscopy (right). Note: Results using anti-DAF antibodies were similar.

Fig. 3. **Southern blot of T. brucei DNA with TbGPI12 probe.** *T. brucei* genomic DNA was digested with the restriction enzymes indicated and subjected to Southern blotting with the *TbGPI12* probe (see Experimental Procedures). A restriction map of the *TbGPI12* ORF predicted from the DNA sequence is shown above the blot. The results indicate that *TbGPI12* is a single-copy gene per haploid genome.

Fig. 4. **Construction and characterization of a conditional TbGPI12 null mutant.** Panel 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). Panel B: Aliquots of DNA (5 µg) were digested with *Pst*I (that gives conveniently-sized *TbGPI12*-containing fragments) and Southern blotted with the *TbGPI12* probe. The DNA was from wild type *TbGPI12*⁺⁺ cells (lane 1), ∆*TbGPI12::PAC* cells (lane 2), *TbGPI12-myc⁷* ∆*TbGPI12::PAC* cells (lane 3) and conditional *TbGPI12-myc⁷* ∆*TbGPI12::PAC / ∆*TbGPI12::HYG* null mutant clones (lane 4). The probe reveals allelic (*TbGPI12*) copies at 2.7 kb and ectopic tetracycline-inducible (*TbGPI12-myc⁷*) 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 sub-cultured as the cell density approached 2 x 10^6 cells/ml (panel A). Cells harvested from the culture in panel A (asterisk) were washed in tetracycline-free medium and used to inoculate tetracycline-free cultures (panels B and C). Cells were sub-cultured as they approached 2 x 10^6 cells/ml (panel B, closed squares, and panel C) or left without sub-culturing (panel B, open squares). Tetracycline was added back to one of the cultures after 6 days (panel 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 (upper panel) and a β-tubulin probe (lower panel).

Fig. 7. Cell-free system experiments with *T. brucei* conditional *TbGPI12* null mutant membranes. Panel A: Cell-free systems made from *T. brucei* conditional *TbGPI12* null mutant cells cultivated with (+) or without (-) tetracycline (Tet) for 48 h were incubated with UDP-[^3]H]GlcNAc and the glycolipid products were analysed by HPTLC and fluorography. The positions of authentic GlcN-PI and GlcNAc-PI standards are shown on the left. Panel B: The same cell-free systems, and one prepared from ‘wild-type’ (wt) cells, were labelled with GDP-[^3]H]Man in the presence of NEM and either GlcNAc-PI or GlcN-PI, as indicated. Glycolipid products were analysed by HPTLC and fluorography. The bands were identified as dolichol-P-Man (DPM), Man<sub>1,3</sub>GlcN-PI (M1-3), Man<sub>3</sub>GlcN-(acyl)PI (aM3) and glycolipids A’ and θ by PI-PLC, JBAM and GPI-PLD digestions (data not shown). Panel C: The same cell-free systems were incubated with GDP-[^3]H]Man alone or with UDP-GlcNAc or GlcN-PI, as indicated. Glycolipid products were analysed by HPTLC and fluorography.
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