The protozoan parasite Trypanosoma brucei is coated by glycosylphosphatidylinositol (GPI)-anchored proteins. During GPI biosynthesis, inositol in phosphatidylinositol becomes acylated. Inositol is deacylated prior to attachment to variant surface glycoproteins in the bloodstream form, whereas it remains acylated in procyclins in the procyclic form. We have cloned a T. brucei GPI inositol deacylase (GPIdeAc2). In accordance with the acylation/deacylation profile, the level of GPIdeAc2 mRNA was 6-fold higher in the bloodstream form than in the procyclic form. Knockdown of GPIdeAc2 in the bloodstream form caused accumulation of an inositol-acylated GPI, a decreased VSG expression on the cell surface and slower growth, indicating that inositol-deacylation is essential for the growth of the bloodstream form. Overexpression of GPIdeAc2 in the procyclic form caused an accumulation of GPI biosynthetic intermediates lacking inositol-linked acyl chain and decreased cell surface procyclins because of release into the culture medium, indicating that overexpression of GPIdeAc2 is deleterious to the surface coat of the procyclic form. Therefore, the GPI inositol deacylase activity must be tightly regulated in trypanosome life cycle.

African trypanosomes (Trypanosoma brucei) are digenetic protozoan parasites, which have a dense coat of glycosylphosphatidylinositol (GPI)-anchored proteins. The bloodstream form, which causes sleeping sickness in human, is covered by variant surface glycoproteins (VSG), whereas the procyclic form, which resides in the tsetse fly, is covered by procyclins (reviewed in Refs. 1–4). The infectivity to tsetse fly was almost completely lost when the TbGPI8 gene was disrupted (7, 10). These results indicate that procyclins are important during the infection of the tsetse fly. On the surface of procyclic forms, in which all procyclin genes or TbGPI8 gene are disrupted, free GPI molecules are expressed (7, 10, 11). These free GPI molecules may be important for the viability of these knock-out strains in culture (7, 11). Thus, GPI-anchored proteins are important for the infection and survival of trypanosomes in both life cycle stages (5, 12–14).

The structures of GPI anchors are different between the bloodstream and procyclic forms (reviewed in Refs. 15 and 16) (see Fig. 1). One prominent difference between the GPI structures of procyclins and VSG is that the inositol moiety of procyclin GPI is modified by a fatty acid, whereas inositol is not acylated in the VSG GPIs (17–20). Consistent with these structurally different GPIs, the GPI biosynthesis pathways in bloodstream and procyclic forms are different. In both stages, inositol can be acylated during GPI biosynthesis once the first mannose is attached. The inositol acylation is reversible such that the acylated and non-acylated GPI intermediates are in dynamic equilibrium and is a prerequisite for the addition of phosphoethanolamine to the third mannose (19, 21) (see Fig. 1). In the procyclic form, the inositol remains acylated down to PP1, the final precursor. This is in contrast, in the bloodstream form, inositol is acylated up to C1, but the fatty acid is then removed prior to the attachment of GPI to VSG (19) (see Fig. 1). The significance of inositol acylation and deacylation during GPI biosynthesis and the physiological significance of the structural difference between procyclins and VSG are not well understood. A GPI inositol deacylase (GPIdeAc) gene has been cloned, and the enzyme activity of an affinity-purified recombinant GPIdeAc has been studied in various life cycle stages.
demonstrated (22). Furthermore, the GPIdeAc knock-out resulted in accumulation of inositol-acylated GPI biosynthetic intermediates. However, some GPI inositol deacylase activity remained in the knock-out mutant, suggesting that at least one other inositol deacylase is present (22, 23). Here, we have identified a second *T. brucei* GPI inositol deacylase (GPIdeAc2) based on sequence homology to mammalian GPI inositol deacylase (PGAP1) (24) and shown that GPIdeAc2 is a major GPI inositol deacylase. Our data suggested that the regulation of GPIdeAc2 expression is critical in the life cycle of *T. brucei*

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Strain 427 procyclic and bloodstream clone 221a *T. brucei* were used. Single marker bloodstream-form cell (a gift from Dr. G. A. M. Cross, Rockefeller University) (25), co-expressing T7 polymerase, and the Tet repressor, were used for conditional expression of the GPIdeAc2 double-stranded RNA. Bloodstream-form and procyclic-form cells were cultured in HMI-9 medium and SDM-79 medium, respectively, with appropriate drug selection.

**RNAi and Transfection**—To generate the plasmid p2T7TAblue/GPIdeAc2, a 536-bp fragment, corresponding to the C-terminal one-fifth of the GPIdeAc2 gene was PCR-amplified using the primers 5'-GGATAATACCGGTCACTGTGTTTGG-3' and 5'-CAGCACCCAAACAATACGAAAAGAC-3' and cloned into Eam1105I site of the tet-racycline-inducible RNAi vector p2T7TAblue (26) (a gift from Dr. D. Horn, London School of Hygiene and Tropical Medicine). The sequence of the 536-bp region had no significant homology with other sequences in *T. brucei* genome data base. For transfection into the bloodstream form via integration into an rDNA spacer region, 2 x 10⁷ log phase cells were electroporated with 50 μg of NotI-linearized p2T7TAblue/GPIdeAc2 plasmid in a total volume of 0.4 ml of cytomix buffer (27). The transfected cells were then selected in a medium containing hygromycin (2.5 μg/ml) exactly following a published procedure (26). RNAi was induced by incubation with doxycycline (1 μg/ml) for 48 h. To express GPIdeAc2 in the procyclic form, we cloned GPIdeAc2 into the HindIII- and BamHI-cut expression vector pPPMCS, which was constructed from pHDS90 (28) by replacing its promoter and luciferase gene with the normal procyclic acidic repetitive protein promoter and the multiple cloning sites (10). After transfection, transfectant cell line was obtained by serial dilution after selection with hygromycin (50 μg/ml).

**Northern Blot Analysis and Real Time PCR**—For the Northern blot analysis, total RNA was isolated using an RNAsasy kit (Qiagen). Filters were hybridized with a probe corresponding to the 536-bp fragment used for the GPIdeAc2 RNAi construct. The α-tubulin gene was used as a loading control. For real time PCR analysis, total RNA was isolated using TRIzol (Invitrogen), reverse-transcribed using SuperScript III RT (Invitrogen), and analyzed using a QuantiTect SYBR Green PCR kit (Qiagen) and ABI sequence detection systems (ABI PRISM 7000).

**GPI Biosynthesis Analysis**—Hypotonic cell lysate was prepared as described previously, except that the tunicamycin treatment was omitted (29). Frozen cell lysate was thawed and washed twice with 10 volumes of HKMTL buffer (50 mM HEPES (pH 7.4), 25 mM KCl, 5 mM MgCl₂, 0.1 mM Na-p-tosyl-L-lysine chloromethyl ketone, and 1.0 μg/ml leupeptin) by centrifugation (6,000 x g, 10 min, 4°C). The membrane pellet was suspended at 10⁹ cell equivalents/ml with HKMTL buffer.
supplemented with 5 mM MnCl₂, 1 mM dithiothreitol, and 0.8 μg/ml tunicamycin. To radiolabel GPI mannose residues, the membranes were incubated with GDP-[3,4,5-³³H]Man (DuPont, 20 μCi/ml) and 1 mM UDP-GlcNAc for 60 min at 37 °C (bloodstream form) or 27 °C (procyclic form). The reaction was terminated by adding CHCl₃/CH₃OH (1:1, v/v) to give a final ratio of CHCl₃/CH₃OH/H₂O (10:10:3, v/v/v). The lipids were extracted for 1 h, and insoluble debris was removed by centrifugation. The supernatants were dried under a stream of nitrogen and were resuspended by adding 150 μl of each of n-butanol-saturated water and water-saturated n-butanol. After centrifugation, the organic upper phase was saved, and the lower aqueous phase was re-extracted with 100 μl of water-saturated n-butanol. The combined butanol phase was back-extracted with 100 μl of n-butanol-saturated water, and the final butanol phase was then dried in a SpeedVac concentrator. Dried lipids were reconstituted in 20 μl of CHCl₃/CH₃OH/H₂O (10:10:3, v/v/v) for application to TLC on high performance TLC silica gel 60 (Merck). TLC plates were developed with the same solvent. For autoradiography, the plates were exposed to an image plate and developed by an image analyzer (BAS-2500, Fuji).

Enzymatic Digestions—For GPI-specific phospholipase D (GPI-PLD) digestion, glycolipids were incubated overnight with 20 μl of human serum as the enzyme source, mixed with 150 μl of a buffer containing 50 mM Tris-HCl (pH 7.4), 3 mM CaCl₂, and 0.1% Triton X-100. For the PL₂A treatment, incubation was in 50 μl of 50 mM Tris-HCl (pH 7.8), 2.5 mM CaCl₂, and 0.1% sodium deoxycholate containing 250 units/ml of Crotalus adamanteus PL₂A (Sigma) for 4 h at 37 °C. Phosphatidylinositol-specific phospholipase C (PI-PLC) treatment was conducted in 25 μl of 100 mM Tris-HCl (pH 7.4) and 0.2% Triton X-100 containing 2 units/ml of Bacillus cereus PI-PLC (Molecular Probes) for 4 h at 37 °C. For α-mannosidase treatment, incubations were performed in 25 μl of 100 mM sodium acetate (pH 5.0) and 0.1% taurodeoxycholate with 50 units/ml jack bean α-mannosidase (Bom) (Sigma) overnight at 37 °C. After each treatment, lipids were extracted by n-butanol-water partition.

Microscopy and Flow Cytometry—For Hoechst staining, 1 × 10⁶ cells were fixed overnight in methanol at −20 °C. Cells were washed with phosphate-buffered saline and resuspended with 1 ml of phosphate-buffered saline containing 0.5% bovine serum albumin and 1 μg/ml Hoechst 33258 (Molecular Probes). After centrifugation, cells were washed again with phosphate-buffered saline, placed on glass slides, and mounted with PermaFluor solution (Immuno, Pittsburg, PA). For cell cycle analysis using propidium iodide staining and FACS, we followed exactly the published method (30). FACS analysis of EP-procyclins was also performed as described previously (5).

Pulse-chase Analysis of Procyclins—Procyclins were washed in phosphate-buffered saline and were resuspended at 10⁶ cells/ml in SDM-79 without proline supplemented with 10% (v/v) dialyzed fetal bovine serum. [³⁵S]Proline (Amersham Biosciences) was added (10 μCi/ml) and incubated for 30 min at 27 °C. The chase was initiated by 10-fold dilution with prewarmed complete SDM-79 medium containing 10% (v/v) fetal bovine serum and continued for 19 h. At each time point, 1.0 ml of culture was centrifuged, and the supernatant and the cell pellet were separated. Supernatants were filtered through a 0.2-μm pore membrane to eliminate residual parasites. Cell pellets were solubilized in 1.0 ml of buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and protease inhibitor mixture (31). These samples were precleared by mixing with 20 μl of 50% suspension of protein G-Sepharose and gently rotating for 1 h at 4 °C. After centrifugation at 5,000 × g for 10 min, EP procyclins were immunoprecipitated by incubating with 10 μg of anti-EP procyclin monoclonal antibody (Cedarlane Laboratories, Ontario, Canada) or an isotype-matched control monoclonal antibody for 1 h at 4 °C, followed by mixing with 20 μl of a 50% suspension of protein G-Sepharose and gently rotating for 1 h at 4 °C. EP procyclins were analyzed by 10–20% gradient gel SDS-PAGE and autoradiography.

Trans-sialidase Assay—Trans-sialidase activity was assessed by measuring hydrolysis of a fluorogenic substrate, 2’-(4-methylumbelliferyl)-d-N-acetylneuramic acid (Sigma) (32). In brief, the enzyme was incubated with 0.5 mM substrate in 20 mM HEPES (pH 7.2) at 28 °C in a final volume of 20 μl. The reaction was terminated by adding 200 μl of 0.2 M Tris-HCl (pH 9.5), and the fluorescence was measured with a Fluoroskan II microplate reader (Labsystems, Flow Laboratories Inc.). One unit of enzyme is defined as the activity to hydrolyze 1 μmol of the substrate in 1 min.

RESULTS

Cloning the GPIdeAc2 Gene Homologous to Mammalian GPI Inositol Deacylase—We used the amino acid sequence of rat PGAP1 to identify a gene encoding a GPI inositol decalysase in the T. brucei genome database (BLAST). We found an open reading frame Tb927.3.2610 with BLAST search and amplified its homologue with 99% nucleotide sequence identity from T. brucei 427 genomic DNA. We named this gene GPIdeAc2 (DBDL/GenBank/EMBL accession number AB219559). The GPIdeAc2 open reading frame encoded a protein of 815 amino acids, which had an approximately 50-amino-acid extension at the N-terminus compared with PGAP1 (supplemental Fig. 1). The yeast PGAP1 homologue Bst1p had a similar N-terminal extension compared with PGAP1 (24). There is a second methionine at position 35 within the extension in GPIdeAc2. We chose the first methionine rather than the second as the initiation site because the sequences of the extensions in GPIdeAc2 and Bst1p had some homology (sequence not shown). GPIdeAc2 had 20.3% amino acid identity to rat PGAP1 (supplemental Fig. 1) and 20.6% to Bst1p. An alignment of the PGAP1 and GPIdeAc2 sequences showed a conserved esterase/lipase/thioesterase motif with a catalytic serine (InterPro IPR00379) (supplemental Fig. 1). Hydropathy profiles of GPIdeAc2 and PGAP1 were similar, having a large hydrophilic region with the catalytic serine followed by multiple transmembrane regions (data not shown).

Transcript Levels of GPIdeAc2 in Procyclic and Bloodstream Forms—We compared the mRNA levels of GPIdeAc2 in procyclic and bloodstream forms using Northern blots and real time PCR. A 2.4-kb transcript, which corresponded to the length of the GPIdeAc2 gene, was expressed at a six times higher level in the bloodstream form than in the procyclic form (Fig. 2A). Consistent with this result, the mRNA level of GPIdeAc2 determined using real time PCR was six times higher in the bloodstream form than in the procyclic form (Fig. 2B). The high expression of GPIdeAc2 mRNA in the bloodstream form coincides with the deacylated structure of VSG GPI in the bloodstream form, whereas the low expression of GPIdeAc2 mRNA in the procyclic form coincides with the usage of acylated inositol in procyclin GPI. Thus, the transcript profile of GPIdeAc2 is consistent with the inositol acylation and deacylation status of GPI.

Acylated GPI Intermediates Accumulated in the Presence of GPIdeAc2 RNAi in the Bloodstream Form—To determine whether GPIdeAc2 mediates GPI inositol decalysation in the bloodstream form, we used RNAi to reduce the expression of GPIdeAc2. After induction of RNAi by doxycycline for 48 h, we examined the mRNA levels of GPIdeAc2 and α-tubulin (a loading control) by Northern blot analysis. The induction of RNAi caused a ~70% decrease in the GPIdeAc2 mRNA level (Fig. 3A).
Then, we examined whether the suppression of GPIdeAc2 affected the synthesis of GPI precursors. After induction of RNAi for 48 h, cell lysates were prepared and incubated with GDP-[3H]mannose to label GPI biosynthetic intermediates. Lipid extracts were treated with GPI-PLD and PI-PLC prior to TLC. GPI-PLD cleaves all GPI intermediates (data not shown). PI-PLC cleaves GPI intermediates without inositol acylation, whereas it does not cleave inositol-acylated GPI. One distinctive band was seen in GPIdeAc2 knockdown cells (Fig. 3B, lane 7, arrow) and its RF value was consistent with glycolipid C'. This species was sensitive to GPI-PLD (lane 10) but resistant to PI-PLC (lane 8), indicating that it was inositol-acylated GPI. It was also resistant to JBαM digestion, indicating that the non-reducing terminal mannose was protected by an ethanolamine phosphate group (Fig. 3C, lanes 7 and 8, arrow). Based on these results, we concluded that the accumulated GPI was indeed glycolipid C' (EtNP-Man₃GlcN-(acyl)-PI) (see Fig. 1). Comitant with glycolipid C' accumulation, the amount of glycolipid A' (EtNP-Man₃GlcN-PI) decreased to ~50% of control in the GPIdeAc2 knockdown cells (Fig. 3B, compare lane 5 with 7). Furthermore, the amount of glycolipid θ was also decreased, and the inositol-acylated form of θ (termed lyso-C') (19, 33) accumulated (Fig. 3B, lanes 7 and 8, arrowhead). These results suggested that knockdown of GPIdeAc2 resulted in slower conversion of glycolipid C' and lyso-C' to A' and θ, demonstrating that GPIdeAc2 is involved in GPI inositol decacylation in the bloodstream form.

It was also noted that RNAi of GPIdeAc2 caused a nearly complete loss of one glycolipid (double asterisk) and a significant decrease of the other (asterisk) (Fig. 3, B and C, lane 5 versus 7). These fast moving glycolipids may be non-inositol-acylated GPI bearing three or two mannoses.

GPIdeAc2 is Important for the Surface Expression of VSG and Optimal Growth of the Bloodstream Form—We examined the expression of VSG on the surface of the GPIdeAc2 knockdown cells by flow cytometric analysis. Control cells (empty vector transfectants) maintained surface VSG expression after a 48-h induction, whereas GPIdeAc2 knockdown cells showed a 65 ± 15% decrease in the surface expression of VSG, indicating GPIdeAc2 is important for the efficient surface expression of VSG (Fig. 4A).
We then examined the growth of the GPIdeAc2 knockdown cells. Cell growth was monitored in the absence or presence of doxycycline for 5 days. GPIdeAc2 knockdown caused a severe inhibition of cell growth, indicating that GPIdeAc2 was essential for optimal growth of the bloodstream form. Many morphologically abnormal cells were seen in the GPIdeAc2 knockdown population. They had multiple flagella and multiple nuclei.

**FIGURE 4.** GPIdeAc2 knockdown cells have decreased surface expression of VSG and a reduced growth rate. A, surface expression of VSG. Bloodstream-form cells harboring the p2T7T7[496]p/GPIdeAc2 (GPIdeAc2 RNAi) and empty vector (Control) were induced with 1 μg/ml doxycycline (Dox) for 48 h. Each cell was stained with rabbit anti-VSG (shaded lines) or control (dotted line) rabbit preimmune serum and analyzed by FACS. A representative result of three independent experiments is shown. B, growth of control and GPIdeAc2 RNAi cells monitored in the absence or presence of 1 μg/ml of doxycycline. Mean ± S.D. of triplicate counts is shown. C, morphology of GPIdeAc2 RNAi cells before (Dox(--)) and 48 h after addition of doxycycline (Dox(+) ). Size bars: 5 μm. D, cell cycle analysis in the absence (left) and presence (right) of doxycycline for 48 h. Cells in four stages in cell cycle (G1, S, G2/M, and >G2/M as determined by FACS analysis) are indicated in percentage of total. G1, 2n; S, 2n-4n; G2/M, 4n; >G2/M, 6n or more.

Deacylated GPIs Were Produced in the GPIdeAc2 Overexpressing Procyclic Form—As shown in Fig. 2, the mRNA level of GPIdeAc2 was low in the procyclic form consistent with the structural characteristics of procyclin GPI anchors. We next asked whether maintenance of low GPIdeAc2 expression is required for inositol-acylated GPI anchors of procyclins. We stably overexpressed GPIdeAc2 in the procyclic form and examined GPI biosynthesis by incubating cell lysates with GDP-[3H]mannose. If overexpressed GPIdeAc2 mediates GPI inositol deacy-
GPI Inositol Deacylase of Trypanosoma brucei

**A.** PI-PLC  
Vector: deAc2  
- - + +  
PP3, A\(^*\)-like, and \(\theta\)-like  

**B.** PLA2  
Vector: deAc2  
- + - +  
PP3, A\(^*\)-like, and \(\theta\)-like  

**C.** JBaM  
Vector: deAc2  
- - - -  
narrow and arrowhead  

**D.** GPI-PLD  
Vector: deAc2  
- + - +  
PP3, A\(^*\)-like, and \(\theta\)-like  
arrowhead  

![Figure 5. GPI biosynthesis in GPIdeAc2 overexpressing procyclic cells.](image)

FIGURE 5. GPI biosynthesis in GPIdeAc2 overexpressing procyclic cells. Procyclic cells were transformed with an empty vector (Vector) or GPIdeAc2 plasmid (deAc2). The membranes were incubated with GDP-[3H]mannose to label GPI, and aliquots were subjected to TLC after treatment (+) or non-treatment (-) with PI-PLC (A), PLA2 (B), JBaM (C), or GPI-PLD (D). The identities of mannolipids are shown on the left: A\(^*\)-like, an intermediate bearing three mannoses with ethanolamine phosphate on the third mannose; PP3, A\(^*\)-like intermediate with acylation on inositol; PP1, complete GPI precursor (a lyso-form of PP3); \(\theta\)-like, A\(^*\)-like intermediate with a lack of sn-2 fatty acid.

GPI-anchored Proteins Were Released into the Medium from the GPIdeAc2 Overexpressing Procyclic Form—We examined whether the surface expression of procyclins is affected by overexpression of GPIdeAc2. If the GPI anchors, which contain only a single fatty acid (\(\theta\)-like), are attached to procyclins such procyclins might be unstably anchored to the plasma membrane. If the GPI with only one fatty acid is not attached to procyclins, procyclins cannot be membrane-bound. Consistent with these possibilities, the surface EP-procyclin expression was significantly decreased in the GPIdeAc2 overexpressing procyclic form (Fig. 6A).

To examine the fate of procyclins in the GPIdeAc2-overexpressing cells, EP-procyclins in empty vector or GPIdeAc2 transfecants were pulse-labeled with [3H]proline for 30 min and chased for various times up to 19 h. At each time point, we immunoprecipitated EP-procyclins from the cell lysates and the culture supernatants and analyzed them by SDS-PAGE. In the empty vector transfecant, radiolabeled EP-procyclins were efficiently chased into the mature 50-kDa band, and little was found in the culture supernatants (Fig. 6, B and D). In contrast, in the GPIdeAc2 overexpressing cells, more of the immature ER-form EP-procyclins were found in the cell lysates (Fig. 6C). Furthermore, EP-procyclins were released into the medium and degraded (Fig. 6E). These data together with flow cytometric analysis (Fig. 6A) suggested that the procyclic form lost cell surface EP-procyclins because of release into medium. The release was most likely due to an unstable association of the \(\theta\)-like GPI anchor with the plasma membrane because EP-procyclins in the transfected cells matured at a similar rate to that of the non-transfected cells with only a minor accumulation of immature species. Taken together, overexpression of GPIdeAc2 is deleterious to the surface expression of EP-procyclins.

To see whether the expression of other GPI-anchored proteins was affected by overexpression of GPIdeAc2, we measured the trans-sialidase activity in the cell lysates and the culture supernatants. A higher trans-sialidase activity was found in the culture supernatant of GPIdeAc2-overexpressing cells than in that of wild-type cells (Fig. 6F, right). The secreted level of the enzyme was comparable to that of TbGPI110 knock-out cells, which secrete non-GPI-anchored trans-sialidase because of the hydrolysis of its GPI attachment signal by transamidase (10). TbGPI8 knock-out cells that are defective in GPI transamidase did not secrete trans-sialidase, as expected (10). These results are consistent with the pulse-chase experiments showing that EP-procyclins are unstably associated with the membrane of the procyclic form when GPIdeAc2 is overexpressed.

**DISCUSSION**

In the present study, we have identified a major GPI inositol deacylase, GPIdeAc2, in *T. brucei*. This finding enabled us to determine the roles of inositol acylation and deacetylation in the trypanosome life cycle.

Because the inositol-linked acyl chain is removed before fatty acid remodeling and attachment to VSG in bloodstream form, we knocked down GPIdeAc2 and analyzed its effect on the surface expression of VSG and growth of the parasite. The RNAi-mediated decrease of GPIdeAc2 mRNA by 70% caused accumulation of glycolipid C\(^{-}\) and lyso-C\(^{-}\) and concomitant decreases in glycolipids A\(^*\) and \(\theta\) (deacylated intermediates downstream to C\(^{-}\) and lyso-C\(^{-}\)), indicating that deacetylation of inositol was significantly inhibited (Fig. 3, B and C). Under these conditions, the surface expression of VSG was severely decreased, and growth was nearly completely stopped (Fig. 4). RNAi with knockdown constructs corresponding to two other regions in GPIdeAc2 caused similar accumulation of glycolipid C\(^{-}\) (data not shown). It appeared that the inhibition of inositol deacetylation resulted in decreased fatty acid-remodeled GPI that is competent for attachment to VSG and the

lation, then the presence of normally inositol-acylated GPI species would be decreased, and deacylated GPI species, such as A\(^*\)- and \(\theta\)-like intermediates, which are not usually seen in procyclic cells, would be generated. As expected, the concentration of PP3, which is a major acylated species, was greatly decreased (Fig. 5A, lane 1 versus 3). In addition, the accumulation of two slow migrating glycolipids was seen (Fig. 5A, lane 3, arrow and arrowhead). To characterize these glycolipids, we treated them with PI-PLC (Fig. 5A), PLA2 (Fig. 5B), JBaM (Fig. 5C), and GPI-PLD (Fig. 5D). Both were sensitive to PI-PLC, indicating that these glycolipids were indeed deacylated (Fig. 5A, lane 4). The upper glycolipid (arrow) was sensitive to PLA2 (Fig. 5B, lane 4) and GPI-PLD (Fig. 5D, lane 4) but not to JBaM (Fig. 5C, lane 4), indicating that it is an A\(^*\)-like glycolipid. The other glycolipid (arrowhead) was also sensitive to PI-PLC and GPI-PLD (Fig. 5, A and D, lane 4) but resistant to JBaM (Fig. 5C, lane 4) and PLA2 (Fig. 5B, lanes 3 and 4, arrowhead). Therefore the lower glycolipid is \(\theta\)-like. These results indicate that inositol deacetylation occurred in the procyclic form overexpressing GPIdeAc2 and that maintaining a low expression level of GPIdeAc2 is required for inositol-acylated GPI in the procyclic form.

Upon overexpression of GPIdeAc2, two fast migrating glycolipids also accumulated (Fig. 5A, lane 3, asterisk and double asterisk). They were sensitive to PI-PLC (Fig. 5A, lane 4) and JBaM (Fig. 5C, lane 4), indicating that these glycolipids were deacylated forms of early GPI intermediates, such as Man\(_4\)GlcN-PI and Man\(_5\)GlcN-PI. These results suggest that GPIdeAc2 can be involved in a dynamic equilibrium between inositol-acylated and non-acylated forms of mannosylated intermediates (Man\(_4\)GlcN(acyl)-PI \(\Rightarrow\) Man\(_5\)GlcN-PI) known to occur in the early steps of GPI biosynthesis (19, 34).
decreased surface expression of VSG, which in turn caused cessation of growth. We, therefore, demonstrated that deacylation of inositol is critical for VSG-GPI anchor biosynthesis in the bloodstream form.

The effect of GPIdeAc2 knockdown on cell cycle progression (Fig. 4, C and D) was similar to that of TbGPI8 knockdown (7) in that cells became multinuclear, multikinetoplast, and multiflagellar. These knockdown phenotypes suggest that the defects in GPI biosynthesis result in the inhibition of cytokinesis while allowing cell cycle progression. In a striking contrast, VSG knockdown triggered a specific cell cycle checkpoint at 2N2K stage resulting in blocking of cell division (8).

In cells with defective GPI biosynthesis, transcription and translation of VSG continue and only post-translational modification by GPI is inhibited. It is, therefore, possible that monitoring system for VSG protein or transcript (8) cannot detect the abnormality.

In contrast to the bloodstream form, GPI anchors are inositol-acylated in the procyclic form (17, 18). The mRNA level of GPIdeAc2 in the procyclic form is only 1/15% of that in the bloodstream form (Fig. 2), suggesting that GPIdeAc2 is down-regulated during or after differentiation from the bloodstream form to the procyclic form. We forced the expression of GPIdeAc2 in the procyclic form by transfection and assessed its effect on the surface expression of EP-procyclins. Overexpression of GPIdeAc2 caused a nearly 90% decrease in the surface EP-procyclins because of secretion into culture media.

FIGURE 6. Surface expression of EP-procyclin on GPIdeAc2 transfectants and secretion of GPI-anchored proteins from GPIdeAc2 transfectant. A, the empty vector (Vector) or GPIdeAc2 plasmid (GPIdeAc2) was transfected into procyclic-form cells, and the surface expression of EP-procyclins was assessed by FACS analysis using anti-EP procyclin antibody staining (shaded lines) or isotype control (dotted line). A representative result of three independent experiments is shown. B–E, pulse-chase experiment of EP-procyclins in GPIdeAc2 transfectant. The empty vector (B and D) or GPIdeAc2 (C and E) transfectant was pulse-labeled with [14C]proline for 30 min and chased for 19 h. After the chase, cell pellets (B and C) and supernatants (D and E) were isolated from the aliquot samples and analyzed by SDS-PAGE. Lane 1, chase 0 min; lane 2, 5 min; lane 3, 15 min; lane 4, 30 min; lane 5, 1 h; lane 6, 2 h; lane 7, 4 h; lane 8, 8 h; lane 9, 19 h. F, trans-sialidase activity secreted from GPIdeAc2 transfectant. Wild-type (WT), GPIdeAc2 transfectant (deAc2), TbGPI8 knock-out (8KO) and TbGPI10 knock-out (10KO) cells were cultured for 3 days and trans-sialidase activities in cell lysates, and culture supernatants were measured. Trans-sialidase activity in the supernatant was normalized by cell density and is expressed in microunits/10⁶ cells. The results show the means and S.D. of three independent experiments.
medium (Fig. 6). It was reported that the lipophosphoglycans of *Leishmania major*, containing lyso-alkyl-PIP, are released from the cell surface (35). This result is consistent with our interpretation that the secretion of procyclins into the medium from the GPIdeAc2-overexpressing procyclic cells was because of an unstable association of procyclins bearing a single fatty acid chain. Therefore, down-regulation of GPIdeAc2 is critical for generation of the procyclin coat on the procyclic form. These findings together indicate that GPIdeAc2-mediated inositol decylation must be tightly regulated in the trypanosome life cycle.

It was reported that knock-out of another inositol deacylase GPIdeAc led to reduced inositol deacylase activity (22). GPIdeAc consists of 558 amino acids, which is much smaller than GPIdeAc2 and PGAP1. GPIdeAc has an N-terminal signal peptide but no transmembrane domain, in contrast to GPIdeAc2 and PGAP1, which have multiple transmembrane domains. GPIdeAc is homologous to mammalian acyloxyacyl hydrolase that removes fatty acids from bacterial lipopolysaccharides. GPIdeAc has no significant sequence homology with GPIdeAc2 and PGAP1. GPIdeAc is a non-essential gene and partially accounts for inositol deacylase activity in *T. brucei*. If GPIdeAc also deacylates glycolipid C, its activity alone is not sufficient to generate enough amount of glycolipid A. Alternatively, GPIdeAc may act on other inositol-deacylation reactions. It was reported that there are two pools of glycolipid A, of which only one corresponding to 38% of total glycolipid A is competent for attachment to VSG. Disruption of the GPIdeAc gene resulted in the increase of the VSG-attachment competent pool to 75% of the total, suggesting the presence of complex regulation to VSG-anchor precursor (23).

In the GPI biosynthetic pathway of *T. brucei*, inositol acylation takes place only after the formation of mannosyl-glucosaminyl-phosphatidylinositol. The inositol-linked acyl chain can be removed from and added again to any of the three mannosylated intermediates, that is, GPI intermediates bearing one to three mannosylated domains. Inositol deacylation is achieved by a unidirectional inositol-deacylation reaction (22), which is a key step in the deacylation of GPI intermediates bearing two and three mannoses (Fig. 5, lane C). Overexpression of GPIdeAc2 in the trypanosome life cycle causes accumulation of two non-acylated GPI forms bearing two and three mannosylated intermediates (23). As discussed above, GPIdeAc2 acts on various GPI intermediates, suggesting that the biggest structure it recognizes is mannosyl-glucosaminyl-(acyl)-phosphatidylinositol. Because PGAP1 does not deacylate free GPs, it may additionally recognize the amide linkage between ethanolamine and the C-terminal amino acid. In agreement with this possibility of a structurally larger recognition motif, PGAP1 has a significantly larger luminal catalytic domain than GPIdeAc2. In conclusion, the present study revealed critical roles of GPIdeAc2 in the life cycle of African trypanosomes and demonstrated that expression of GPIdeAc2 must be tightly regulated.

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