Procyclin Null Mutants of *Trypanosoma brucei* Express Free Glycosylphosphatidylinositol on Their Surface

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**Procyclins are abundant, glycosylphosphatidylinositol (GPI)-anchored proteins on the surface of procyclic (insect) form trypanosomes. To investigate whether trypanosomes are able to survive without a procyclin coat, all four procyclin genes were deleted sequentially. Bloodstream forms of the null mutant exhibited no detectable phenotype and were able to differentiate to procyclic forms. Initially, differentiated null mutant cells were barely able to grow, but after an adaptation period of 2 mo in culture they proliferated at the same rate as wild-type trypanosomes. Analysis of these culture-adapted null mutants revealed that they were covered by free GPIs. These were closely related to the mature procyclin anchor in structure and were expressed on the surface in numbers comparable with that of procyclin in wild-type cells. However, free GPIs were smaller than the procyclin anchor, indicative of a lower number of poly-N-acetyllactosamine repeats, and a proportion contained diacylphosphatidic acid. Free GPIs are also expressed by wild-type cells, although to a lesser extent. These have been overlooked in the past because they partition in a solvent fraction (chloroform/water/methanol) that is normally discarded when GPI-anchored proteins are purified.**

**INTRODUCTION**

Glycosylphosphatidylinositol (GPI)-anchored proteins, lipophosphoglycans, and glycosylinositol phospholipids (GIPLs) are abundant components of the surface coat of many protozoan parasites (McConville and Ferguson, 1993; Ferguson, 1999; Ilgoutz and McConville, 2001). A variety of important functions have been attributed to these molecules, including protection against the host’s adaptive immune responses (Cross, 1996), stimulation of a proinflammatory response (Almeida et al., 2000; Ropert and Gazzinelli, 2000), binding to host tissues (Lekutis et al., 2001; Sacks, 2001), and protection against proteolysis (Acosta-Serrano et al., 2001; Sacks, 2001). In addition, the lipid moieties of GPI anchors can modulate host-signaling pathways (Tachado et al., 1997).

**Throughout its life cycle, the protozoan parasite *Trypanosoma brucei* is coated by several million copies of GPI-anchored proteins. The metacyclic form (the infective stage that is transmitted by the tsetse fly) and the bloodstream form in the mammalian host are covered by a coat of variant surface glycoproteins (VSGs). In the mammal, the parasite evades the immune system by periodically replacing the existing VSG coat by a different variant, a phenomenon known as antigenic variation (Cross, 1996). The VSG coat forms a physical barrier that prevents the binding of antibodies to invariant molecules that are embedded in the plasma membrane (Ziegelbauer and Overath, 1993; Salmon et al., 1994; Nolan et al., 2000). When bloodstream forms are ingested by the tsetse fly, they differentiate to procyclic forms in the insect midgut. During this process the VSG coat is progressively replaced by a new set of GPI-anchored glycoproteins known as procyclins. These are characterized by internal dipeptide (EP) or pentapeptide (GPEET) repeats, which confer an extended structure to the polypeptide backbone (Roditi et al., 1989; Treumann et al., 1997). *T. brucei* encodes three EP isoforms (EP1, EP2, and EP3) and a single copy of GPEET (reviewed in Ruepp et al., 1997). We have recently shown that the composition of the procyclin coat changes during development, both in culture and in the tsetse fly. Several hours after initiating synchronous differ-
entiation of bloodstream forms to procyclic forms, all species of procyclin can be detected at similar levels (Vassella et al., 2001). Between 1 and 3 days after triggering differentiation, GPEET is the major form on the surface (Acosta-Serrano et al., 2001; Vassella et al., 2001) but is replaced by the glyco-sylated isoforms EP1 and EP3 within a few days (Acosta-Serrano et al., 2001; Vassella et al., 2001).

The change from the VSG to the procyclin coat is accompanied by an alteration in the structure of the GPI anchor. Both anchors consist of the common core structure ethanolamine-P-O-Mana1-2Manα1-6Manα1-4GlCN-phosphatiydylinositol, but the lipid moiety is diacylglycerol in the VSG anchor (Ferguson et al., 1985, 1988) and acyl-2-lyso phosphatic acid in the procyclin anchor (Field et al., 1991). In addition, the procyclin anchor contains a fatty acid linked to the myo-inositol ring, rendering the anchor resistant to phosphatidylinositol-specific phospholipase C (PI-PLC) (Roberts et al., 1985, 1988) and, unlike the VSG anchor, is extensively decorated with branched poly-N-acetylactosamine repeats that are capped by sialic acid residues (Treumann et al., 1985, 1988) and acyl-2-lyso phosphatic acid in the procyclin anchor (Field et al., 1991). In addition, the procyclin anchor contains a fatty acid linked to the myo-inositol ring, rendering the anchor resistant to phosphatidylinositol-specific phospholipase C (PI-PLC) (Roberts et al., 1985, 1988) and, unlike the VSG anchor, is extensively decorated with branched poly-N-acetylactosamine repeats that are capped by sialic acid residues (Treumann et al., 1985, 1988). It has been postulated that the branched side chains of the anchor form a dense glycocalyx that contributes to the protective function of the coat against digestive enzymes in the fly midgut (McConvile and Ferguson, 1993).

Two different approaches have been taken to removing the procyclin coat to investigate its function. We previously deleted all EP genes from procyclic culture forms, but we were unable to eliminate the final GPEET gene (Ruepp et al., 1997), suggesting that trypanosomes without a procyclin coat might not be viable under these conditions. The EP null mutant exhibited no phenotype in culture, but it was considerably less effective than the wild-type at establishing heavy infections in the tsetse fly (Ruepp et al., 1997). By disrupting GPI10, a key gene in the GPI anchor biosynthetic pathway, Nagamune et al. (2000) recently generated mutant procyclin forms that were incapable of attaching GPI anchors to proteins. These cells were unable to grow unless cultured in nonadherent flasks, but it is not clear whether this phenotype was due to a lack of procyclins or to some other GPI-anchored protein(s).

In this article, we describe the construction of a procyclin null mutant in bloodstream form trypanosomes. When triggered to differentiate, this mutant was able to shed the VSG coat and express procyclin-specific markers. Freshly differentiated cells were very fragile and barely able to grow, but they became more robust as they were passaged, finally proliferating at the same rate as wild-type trypanosomes. An analysis of these culture-adapted cells revealed that the null mutant had compensated for the lack of a procyclin coat by expressing free GPIs on its surface.

MATERIALS AND METHODS

Trypanosomes

Monomorphic bloodstream forms of T. brucei 427 (Cross and Manning, 1973) were cultured in HMI 9 medium (Hirumi and Hirumi, 1989) supplemented with 10% heat-inactivated fetal bovine serum at 37°C/5% CO2. Procyclic forms were cultured in modified DTM (Vassella and Boshart, 1996) supplemented with 15% heat-inactivated fetal bovine serum at 27°C. Bloodstream form trypanosomes were triggered to differentiate to the procyclic form by the addition of 6 mM cis-aconitate to the culture medium and lowering the incubation temperature to 27°C as described previously (Brun and Schönberger, 1981).

Constructs and Stable Transformation of Trypanosomes

Two constructs, pCorleone-hyg and pKOP, that were designed to delete tandemly linked procyclin genes from the GPEET/PAG3 or EP/PAG1 and EP/PAG2 loci, respectively, are described previously (Ruepp et al., 1997; Vassella et al., 2000). For construction of pCorleone-neo, the neomycin-resistance gene was excised from pKON (Ruepp et al., 1997) and cloned between the HindIII and BamHI sites of pCorleone-hyg, thereby replacing the hygromycin-resistance gene. For construction of pKOPAC, the neomycin-resistance gene in pKON was replaced by the puromycin-resistance gene (PAC) as follows: pKON was linearized by cleavage with HindIII, the ends were repaired by treatment with Klenow, and the neomycin-resistance gene was released by cleavage with BamHI. The PAC gene was excised from pBlueScript(KS+) (Ruepp et al., 1997) by cleavage with AgeI and BamHI and ligated to the linearized vector.

Stable transformation of bloodstream forms was performed as described previously (Li and Gottesdiener, 1996) by using 10 µg of plasmid DNA digested with KpnI and NotI (pKOP and pKOPAC) or with SalI and XhoI (pCorleone-hyg and pCorleone-neo) to release the insert. Individual clones were selected in microtiter plates with 0.1 µg/ml puromycin, 1.5 µg/ml phleomycin, 1.0 µg/ml hygromycin, or 1.0 µg/ml neomycin.

Southern Blot Analysis

Genomic DNA was extracted from individual clones as described previously (Young et al., 1982). One microgram of DNA was digested with PstI and separated on 0.8% agarose gels. Southern blot analysis was performed using standard procedures (Sambrook et al., 1989). A multiprimer-labeled probe (Amersham, Dübendorf, Switzerland) used for hybridization was generated from the coding region of GPEET.

Western Blot Analysis and Immunofluorescence

Total cell lysates were separated on 12% polyacrylamide gels and transferred to Immobilon-F membranes (Millipore, Bedford, MA). Rabbit anti-VSG 221 antiserum (obtained from M.L. Cardoso de Almeida, Department of Microbiology, Universidade Federal de São Paulo, São Paulo, Brazil) was used at a dilution of 1:2000 and rat anti-CAP 5.5 antiserum (Hertz-Fowler et al., 2001), provided by K. Gull (School of Biological Sciences, University of Manchester, Manchester, United Kingdom) at 1:30.

For immunofluorescence, cells were fixed with 2% formaldehyde for 4°C (Vassella et al., 1997). The anti-EP monoclonal antibody TRBP1/247 (Richardson et al., 1986, 1988) was used at a dilution of 1:500 and rabbit polyclonal anti-GPEET K1 antiserum (Ruepp et al., 1997) at 1:500. Tetramethylrhodamine B isothiocyanate-conjugated anti-mouse antibody (Sigma) was used at 1:400 and fluorescein isothiocyanate-conjugated anti-rabbit antibody (Sigma) at 1:2000.

Labeling of Trypanosomes, Extraction, and SDS-PAGE

Metabolic labeling of procyclic form trypanosomes with [3H]ethanolamine or [3H]myristic acid (Butkóková et al., 1997) was performed for 16–18 h. Surface labeling with [3H]borohydride was performed as described previously (Pollevick et al., 2000).

For extraction of GPI-anchored molecules, 2–4 × 106 radioactively labeled trypanosomes were harvested by centrifugation and washed with phosphate-buffered saline. The wet pellet was extracted twice with 10 ml of chloroform/methanol (used at 2:1 volume ratios) and three times with 5 ml of chloroform/methanol/water (CMW) used at 10:10:3 volume ratios. The pooled CMW-soluble fractions were dried under nitrogen, partitioned between 1
RESULTS

Construction of a Procyclin Null Mutant in Bloodstream Form Trypanosomes

Procyclins are the major surface glycoproteins of procyclic form trypanosomes. They are not detectably expressed in the bloodstream form, however, and therefore unlikely to be essential for this stage. To obtain viable procyclic null mutant clones, we adopted the strategy of deleting all procyclin genes from bloodstream form trypanosomes. Bloodstream forms can be triggered to differentiate to procyclic forms by the addition of cis-aconitate to the culture medium and lowering the incubation temperature from 37 to 27°C (Brun and Schönenberger, 1981), allowing us to investigate whether these cells are able to develop to the procyclic form and to survive without a procyclin coat.

Procyclin genes occur in tandem repeats on chromosomes VI and X (Roditi and Clayton, 1999). To delete pairs of procyclin genes, locus-specific targeting constructs were made that contained antibiotic resistance genes cloned between sequences flanking the procyclin genes (Figure 1A). The constructs pCorleone-hyg (Vassella et al., 2000) and pCorleone-neo were designed to delete the GPEET and EP3 genes from the GPEET/EP3 locus. Two further constructs, pKOP (Buepp et al., 1997) and pKOPAC, containing flanking sequences from the EP/PAG1 or EP/PAG2 loci, respectively, were used to delete the EP1 and EP2 genes. Bloodstream form trypanosomes of the stock 427 were first subjected to two consecutive rounds of electroporation and selection by using the constructs pCorleone-hyg and pCorleone-neo (Figure 1A). Southern blot analysis of a hygromycin and neomycin double-resistant clone, ΔGPEET/ΔEP3, revealed that both copies of GPEET and EP3 genes were deleted (Figure 1B). This clone was stably transformed with the construct pKOP, and two independent phleomycin-resistant clones were selected and subjected to a fourth round of transformation with the construct pKOPAC and selection with puromycin. In two clones, Δprocyclin 1 and 2, which were derived from independent third and fourth rounds of transformation, all procyclin genes were deleted (Figure 1B). These bloodstream form clones had a population doubling time indistinguishable from that of the wild type and no detectable alterations in cell morphology (our unpublished data).

Procyclin Null Mutant Clones Require an Adaptation Period to Establish Proliferating Procyclic Form Cultures

To investigate whether null mutants were able to differentiate to procyclic forms, they were exposed to cis-aconitate and a temperature shift. To monitor differentiation, expression of cytoskeletal-associated protein CAP5.5, which is considered to be a late marker of differentiation (Matthews and Gull, 1994), was analyzed by immunoblotting with a specific antiserum. As shown in Figure 2A, procyclin null mutant cells were able to express high levels of CAP5.5, but the kinetics of appearance of this protein were delayed relative to the wild type and the ΔGPEET/ΔEP3 mutant. CAP5.5 occurred with similar kinetics in the two independent Δprocyclin clones 1 and 2 (our unpublished data), suggesting that the delay in differentiation of these cells was not due to...
DNA was digested with Southern blot analysis of procyclin knockout (ko) mutants. Genomic loci from the two copies of EP/PAG2 form trypanosomes (Schü new truncated, giving rise to high levels of expression in bloodstream UTR downstream of the resistance gene is METHODs). The 3ľUTR and downstream intergenic region of the second procyclin gene of the same locus. Each construct is ľank-ank-ľank-ľank-ľank-ľank-ľank-ľank-ľank-

Figure 1. Deletion of procyclin genes. (A) Schematic depiction of the procyclin loci together with the constructs used to delete tandemly linked procyclin genes by homologous recombination. Each construct contains the promoter and 5ľ untranslated region (UTR) of the first procyclin gene from the GPEET/PAG3 or EP/PAG1 locus, respectively, followed by an antibiotic-resistance gene. The 3ľflanking region in each construct consists of the last 19 base pairs of the 3ľUTR and downstream intergenic region of the second procyclin gene of the same locus. Each construct is flanked on both sides by locus-specific sequences for site-directed integration into the GPEET/PAG3 loci or the EP/PAG2 loci, respectively (Ruepp et al., 1997; Vassella et al., 2000; see MATERIALS AND METHODS). The 3ľUTR downstream of the resistance gene is truncated, giving rise to high levels of expression in bloodstream form trypanosomes (Schü et al., 1997; Vassella et al., 2000). (B) Southern blot analysis of procyclin knockout (ko) mutants. Genomic DNA was digested with PstI, which separates the EP/PAG1 and the EP/PAG2 loci from the two copies of GPEET/PAG3. A GPEET-specific probe was used for hybridization under conditions that allow cross-hybridization to the EP genes.

clonal variability. In keeping with the expression profile of CAP5.5, release of the VSG also occurred with delayed kinetics in the two null mutant clones (Figure 2A). Immunofluorescence analysis revealed that 97% of the wild-type cells had shed the VSG coat within 48 h. In contrast, >90% of the null mutant cells were still positive for VSG after 48 h, decreasing to ~60% by days 4–6 and 14% by day 8. Δprocyclin cells which expressed CAP5.5 and had lost VSG had the morphology of procyclin forms and had repositioned the kinetoplast correctly (Figure 2B). EP and GPEET procyclins were not detected in these cells by immunofluorescence microscopy (Figure 2B) or immunoblotting (our unpublished data), confirming that all procyclin genes were deleted.

Analysis of the different cultures revealed that, in contrast to the wild-type and the ΔGPEET/ΔEP3 clone, freshly differentiated Δprocyclin cells were unable to proliferate, but remained at a constant cell density for a period of up to 2 mo (Figure 3A). To our surprise, however, the null mutant cells resumed growth after this period and exhibited the same population doubling time as the wild type when cultured under the same conditions (Figure 3B). The two independent null mutant clones showed similar growth properties during the adaptation period. In summary, these results indicate that the procyclin coat is not required for the survival of procyclic form trypanosomes in vitro, but null mutant cells require an adaptation/seleclon period to be able to grow exponentially.

Are Procyclin Null Mutants Naked?

To investigate whether culture-adapted procyclic forms of the null mutant express an alternative glycoprotein coat, cells were metabolically labeled with [3H]ethanolamine or [3H]myristate, which are incorporated into GPI-anchored proteins (Field and Menon, 1992). After extensive delipidation, the insoluble cell pellet was extracted sequentially with 9% butan-1-ol, 0.1% Triton X-100, and 1% SDS, and aliquots of the different fractions were analyzed by SDS-PAGE followed by autoradiography. In [3H]ethanolamine-labeled wild-type cells, most radioactivity was incorporated into GPEET and EP procyclins. As shown previously (Bütikofer et al., 1997), these were soluble in 9% butan-1-ol (Figure 4A, left). The only additional product in wild-type cells was a faint band of ~40 kDa in butan-1-ol and SDS fractions,
which constitutes a convenient internal marker for the labeling reaction. It has been suggested that this protein is most likely to be elongation factor EF-1α (Rosenberry et al., 1989), which is posttranslationally modified with ethanolamine residues. Apart from the 40-kDa protein, no further radio-labeled products were detected in the different extracts of the null mutant (Figure 4A, right). Consistent with these results, incubating null mutant cells with [3H]myristic acid did not yield any labeled proteins in the butanol or Triton X-100 extracts (Figure 4B).

To investigate whether null mutant cells, which lack the major GPI acceptors, express nonprotein-linked (free) GPIs, the pooled CMW-soluble extracts of [3H]ethanolamine or [3H]myristic acid-labeled cells were dried and subsequently partitioned between butan-1-ol and water (see MATERIALS AND METHODS). When aliquots of the butan-1-ol phase (91% butan-1-ol), containing the GPI precursor lipids (Field and Menon, 1992), and the aqueous phase (9% butan-1-ol) were also analyzed by SDS-PAGE and autoradiography, we detected radiolabeled products in the aqueous phase with an apparent molecular mass of 14–20 kDa. These were labeled with both [3H]ethanolamine and [3H]myristic acid (Figure 5, A and B, right), suggesting the presence of GPI-anchored molecules. The same species were also detected in wild-type trypanosomes, although the amount of labeled products per cell equivalent was approximately fourfold lower in these cells than in the null mutant (Figure 5, A and B, left). To investigate whether these molecules contain protein, they were treated extensively with Pronase. This had no effect on the mobility of the labeled products on SDS-polyacrylamide gels (Figure 6, compare lanes 1 and 2), strongly suggesting that they are free GPIs. The Pronase-treated procyclin anchor and the free GPI differed not only in their electrophoretic mobility (Figure 6, lanes 2 and 4) but also in physicochemical properties. Whereas the free GPI was completely soluble in CMW, the Pronase-digested procyclin anchor was only partially soluble (our unpublished data). It has been shown previously that the C-terminal glycine residue of mature procyclin, to which the GPI anchor is attached, is not removed by Pronase (Büttikofer et al., 1997), but this alone is unlikely to account for the differences in the mobility and solubility of the two molecules. A more probable explanation is that the two GPIs differ in their carbohydrate or lipid moieties.

**Free GPI Is Related to Procyclin Anchor**

To provide further evidence for the GPI structure of the radiolabeled molecule(s), extracts of [3H]myristic acid-labeled trypanosomes were treated with GPI-PLD, PI-PLC, or with nitrous acid. Cleaved lipids were extracted with butan-
1-ol, and the percentage of radioactivity released into the organic phase was quantified by scintillation counting. In agreement with published results (Büttikofer et al., 1997), 58 and 69% of radioactivity of the procyclin anchor was released into the butan-1-ol phase after treatment with GPI-PLD and nitrous acid, respectively (Table 1). The free GPI was also sensitive to cleavage with GPI-PLD (62% release) and nitrous acid (65% release) confirming the identity of a free GPI molecule (Table 1). Procyclins contain a fatty acid residue linked to the inositol ring that renders the GPI anchor insensitive to cleavage with PI-PLC (Roberts et al., 1988). Both the procyclin anchor and the free GPI were resistant to cleavage with this enzyme, whereas the VSG anchor, in which the inositol ring is not acylated (Ferguson et al., 1988), was sensitive (Table 1). Thus, the two GPIs seem to be structurally related.

Acyl-lyso- and -diacyl-phosphatidic Acid Are Major Components of Free GPI

The major product of the procyclin anchor released into the butanol phase after treatment with GPI-PLD is acyl-2-lyso-phosphatidic acid (Field et al., 1991). However, analysis of GPI-PLD–cleaved material of the free GPI by TLC revealed two products, one of which comigrated with acyl-lyso-phosphatidic acid from the procyclin anchor (Figure 7A, compare lanes 1 and 2). No radiolabeled material was recovered in the butanol phase in the absence of GPI-PLD (Figure 7A, lane 3). When the TLC plate was exposed for a longer period, it became apparent that the faster migrating, unknown cleavage product was also present in extracts from the procyclin anchor (Figure 7A, lane 2). Quantification of

Table 1. Enzymatic and chemical treatment of cell extracts of trypanosomes labeled with [3H]myristic acid

| GPI-anchored molecule | Release of radiolabel (%) | | | |
|-----------------------|---------------------------|----------------|----------------|----------------|----------------|
|                       | GPI-PLD | PI-PLC | Nitrous acid | Untreated | |
| Free GPI              | 62 ± 7  | 9 ± 3  | 65 ± 20      | 9 ± 1      | n = 4 |
| Procyclin             | 58 ± 6  | 5      | 69           | 1          | n = 1 |
| VSG 221               | 93      | 86     | 78           | n.d.      | n = 2 |

* Extraction of free GPI or procyclin molecules was performed as described in the legends to Figures 4 and 5, and labeling of bloodstream form trypanosomes (T. brucei 427) and extraction of VSG 221 was performed as described previously (Büttikofer et al., 1996).

* Percentage of recovery of radiolabel after extraction with water-saturated butanol.

Figure 5. Analysis of the CMW fraction of labeled trypanosomes. Wild-type trypanosomes (lanes 1 and 2) and null mutant cells (lanes 3 and 4) were labeled with [3H]ethanolamine (A) or [3H]myristic acid (B). The CMW-soluble extract was dried under nitrogen and partitioned between butan-1-ol and water. The lower 9% (vol/vol) butan-1-ol phase and the upper 91% (vol/vol) butan-1-ol phase were dried under nitrogen and analyzed by SDS-PAGE and autoradiography. The labeled material at the bottom of the gel in the 91% butan-1-ol fraction most probably consists of GPI precursors or phospholipids.

Figure 6. Protease digestion of the free GPI and the procyclin anchor. Wild-type and procyclin null mutant cells were labeled with [3H]ethanolamine and extracted as described in the legends to Figures 4 and 5. The 9% (vol/vol) butan-1-ol extracts of the CMW-soluble (+) fraction containing the free GPI and the CMW-insoluble (−) fraction containing the procyclins were incubated for 24 h in the presence (+) or absence (−) of Pronase and analyzed by SDS-PAGE and autoradiography.
the GPI-PLD-treated products by scintillation counting of material scraped from TLC plates revealed that 29 ± 10% (n = 4) of the radiolabeled free GPI and 6 ± 1% (n = 3) of the radiolabeled procyclin anchor consisted of the unknown cleavage product. PLB cleaves fatty acids bound in ester linkage to sn-1 and sn-2 positions of the glycerol moiety of a phospholipid. When the products, released from the free GPI by cleavage with GPI-PLD and PLB, were subsequently treated with PLB, or subjected to strong base hydrolysis, the radioactivity was released quantitatively from both GPI-PLD cleavage products and comigrated with fatty acids at the front of TLC plates (Figure 7B, lanes 1 and 2). In addition, the faster migrating compound, but not lyso-phosphatidic acid, was sensitive to cleavage with PLA₂ (Figure 7B, lanes 3 and 4), which specifically releases ester-linked fatty acid residues from the sn-2 positions. Together, these results indicate that the faster migrating lipid is diacyl-phosphatidic acid, whereas the slower migrating form is acyl-2-lyso-phosphatidic acid.

**Free GPIs Are Exposed on the Surface of the Null Mutant**

To investigate whether the free GPI is exposed on the surface of the null mutant, living procyclin forms were incubated in serum-free medium, resulting in the removal of sialic acid residues by the endogenous trans-sialidase (Pollevick et al., 2000). The cells were subsequently treated with galactose oxidase and the oxidized terminal β-galactose residues were reduced with [3H]borohydride. Under these conditions, wild-type and null mutant cells incorporated similar amounts of radioactivity (~6 × 10⁵-labeled molecules/cell). Although procyclins were the major tritium-labeled product in the wild type, most of the radioactivity in null mutant cells was incorporated into free GPIs. These were soluble in CMW and migrated with the same apparent molecular mass as the metabolically labeled products in the null mutant (Figure 8). These results indicate that wild-type and mutant cells express similar numbers of GPI-anchored molecules on their surfaces. In contrast to Δprocyclin cells, however, only a minority of free GPIs are exposed on the surface of wild-type cells. Procyclins are the major acceptors of sialic acids, which are transferred from external proteins to the GPI anchor moiety by a cell surface-associated trypanosomal trans-sialidase (Engstler et al., 1993; Pontes de Carvalho et al., 1993). Because free GPI is on the surface of the null mutant, it is possible that this molecule contains sialic acids. Cells were fixed with formaldehyde and incubated with Texas Red-conjugated *M. amurensis* lectin, which binds sialic acids. Wild-type and null mutant cells bound similar numbers of lectin molecules (Table 2). To verify the specificity of this reaction, fixed cells were treated with neuraminidase (which cleaves sialic acids) before incubation with the lectin. Under these conditions, cells showed minimal lectin binding, similar to untreated bloodstream forms, which contain no sialic acids (Engstler et al., 1993). Direct measurements of the sialic acid content by fluorometric reversed phase HPLC confirmed that wild-type and null mutant cells contained similar amounts of sialic acid (Table 2). Removal of sialic acids by neuraminidase exposes terminal β-galactose residues, which are specific ligands of peanut agglutinin. Neuraminidase-treated null mutant or wild-type cells, but not untreated cells, bound Texas Red-conjugated peanut agglutinin, demonstrating that sialic acids are linked to β-galactose residues in both the wild-type and the null mutant. Furthermore, because terminal β-galactose residues of the free GPI in desialylated null mutant cells are the major acceptors for the borohydride-labeling reaction (Figure 8), we conclude...
that the free GPI is also the major acceptor for sialic acids in these cells. The free GPI is up-regulated on the surface of the null mutant, but does this also hold true for other GPI-anchored molecules? To compare the expression levels of trans-sialidase (which has been shown to contain a GPI anchor; Engstler et al., 1993) in wild-type and null mutant cells, the activity of this enzyme per cell equivalent was determined as sialidase activity. As shown in Table 3, both clones exhibited similar enzyme activities, suggesting that trans-sialidase is present at similar levels in wild-type and null mutant cells. Thus, in contrast to the free GPI, trans-sialidase seems not to be up-regulated on the surface of the null mutant.

**DISCUSSION**

To generate procyclin null mutants, four sequential rounds of stable transformation were undertaken in bloodstream form trypanosomes. Null mutants showed no detectable phenotype at this stage of the life cycle and were also capable of differentiating to the procyclic form as indicated by appropriate changes in morphology, repositioning of the kinetoplast, loss of the VSG coat, and expression of CAP 5.5. However, the null mutants released the VSG coat more slowly than the parental line implying that, under normal circumstances, procyclins contribute to this process. These might disrupt the dense packing of the coat, rendering the VSG molecules more susceptible to the proteases involved in shedding (Bülow et al., 1989; Ziegelbauer et al., 1993).

Initially, in contrast to wild-type cells, the Δprocyclin mutants were barely able to proliferate as procyclic forms, but after a period of 2 mo in culture they grew with the same population doubling time as wild-type cells. Because the same phenomenon was observed with two independent clones, this was unlikely to be due to unrelated secondary mutations. In light of these results, we can now explain why we were previously unsuccessful in generating total procyclin knockouts in procyclic form trypanosomes (Ruepp et al., 1997). Because null mutants are considerably more fragile and require a period of adaptation before they can proliferate, they would tend to be overgrown by cells that had integrated the construct incorrectly and retained a procyclin gene.

Our data demonstrate unequivocally that trypanosomes can survive without any form of procyclin. Although GPI10 null mutants, which are unable to attach GPI anchors to proteins, were negative for procyclins by flow cytometry (Nagamune et al., 2000), several proteins could be immunoprecipitated from the cell pellet and culture supernatant with anti-EP antibodies. The simplest explanation is that these represent unanchored forms of the protein, some of which are secreted, although it is surprising that they were much larger than the polypeptide precursors (11–14 kDa) or the mature N-glycosylated form after chemical removal of the anchor (M_, ~15 kDa by SDS-PAGE) (Bütikofer et al., 1997). It also cannot be excluded that, in GPI10 null mutants, some procyclin precursors are attached to the cell surface by the hydrophobic C terminus, as was recently shown to be the case for prions in platelets derived from patients with a defect in GPI synthesis (Holada et al., 2002). These might have been overlooked because several antibodies directed against GPI-anchored molecules, including the commercially available monoclonal antibody against EP procyclin used by Nagamune and colleagues, react very poorly with the protein after removal of the GPI lipid moiety (Bütikofer et al., 2001).

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**Table 2. Comparison of the sialic acid content of the wild type and Δprocyclin (clone 1)**

<table>
<thead>
<tr>
<th>Clone (stage)</th>
<th>Fluorescence intensitya</th>
<th>Sialic acid contentd</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>MAAb</td>
<td>Neurama</td>
</tr>
<tr>
<td>Δprocyclin (procyclic)</td>
<td>2017 ± 241</td>
<td>91 ± 17</td>
</tr>
<tr>
<td>Wild type (procyclic)</td>
<td>2546 ± 189</td>
<td>72 ± 30</td>
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<tr>
<td>Wild type (bloodstream)</td>
<td>67 ± 24</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

MAA, *Maackia amurensis* lectin; n.d., not determined; PNA, peanut agglutinin.

* Measured by quantitative fluorescence microscopy. The mean (±SD) from 200–300 segments is presented.

b Texas Red-conjugated *M. amurensis* lectin used at 1 μg/ml.

c Texas Red-conjugated peanut agglutinin from *A. hypogaea* used at 2 μg/ml.

d Determined by flurometric reversed phase HPLC. The mean (±SD) from four independent measurements is presented.

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**Table 3. Comparison of trans-sialidase activity in Δprocyclin and wt cells**

<table>
<thead>
<tr>
<th>Clone (stage)</th>
<th>trans-Sialidase activitya</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RFU/10^7 cellsb</td>
</tr>
<tr>
<td>Null mutant (procyclic)</td>
<td>1153 ± 89</td>
</tr>
<tr>
<td>Wild type (procyclic)</td>
<td>1263 ± 109</td>
</tr>
<tr>
<td>Wild type (bloodstream)</td>
<td>12 ± 8</td>
</tr>
</tbody>
</table>

* Measured as sialidase activity by using the substrate MU-Neu5Ac.

b The mean (±SD) of the cleaved product determined as relative fluorescence units (RFU).
One possible way of compensating for the lack of procyclins might be to increase the expression of other GPI-anchored proteins. Δprocyclin mutants incorporated radiolabeled GPI precursors to the same extent as wild-type cells, but the radiolabel could not be detected in the butanol and Triton X-100 fractions where GPI-anchored proteins normally partition (Ferguson et al., 1993; Büttikofer et al., 1997). Furthermore, the surface trans-sialidase, which is GPI anchored (Engstler et al., 1993; Pontes de Carvalho et al., 1993), exhibited the same level of activity in wild-type and Δprocyclin cells. The null mutants were not naked, however, because they expressed free GPIs on their surface. These were closely related to the procyclin anchor in structure (same core structure, acylated inositol, phosphatidic acid), and, as judged by [3H]borohydride labeling of living cells, the radiolabel could not be detected in the butanol and Triton X-100 fractions where GPI-anchored proteins nor-

Surface-associated GPIs are abundant, if enigmatic, molecules in other organisms ranging from mammals (van’t Hof et al., 1995; Singh et al., 1996) to protozoan parasites (Lederkremer et al., 1991; Ralton and McConville, 1998). Several recent studies indicate that free GPIs are not essential for the viability of Leishmania mexicana in culture (Garami and Ilg, 2001; Garami et al., 2001). However, the fact that the density of GPI molecules on the surface of Δprocyclin mutants was similar to that of wild-type cells suggests that export of the anchor is required for growth of T. brucei procyclic forms. Consistent with these results, the GPI10 null mutant, which is unable to transfer the third mannose residue to the GPI precursor, and is thus unable to attach GPI anchors to proteins (Nagamune et al., 2000), is now known to express free GPIs (Ferguson, personal communication). This would not have been apparent from the biosynthetic labeling experiments performed previously with this mutant (Nagamune et al., 2000) because the N-acetylactosamine residues prevent the free GPIs from migrating on TLC plates.

GPI lipid moieties might be important for the physicochemical properties of the plasma membrane, whereas the sialic acid residues would provide negative charges on the cell surface that might compensate, in part, for the absence of the highly acidic procyclin polypeptide. It has also been proposed that the elaborate side chains form a glycoalyx that protects underlying proteins from digestion by tsetse fly proteases (McConville and Ferguson, 1993). Although free GPIs are mainly intracellular in wild-type cells (compare Figures 5 and 8), a subpopulation might be exported to the cell surface to tile the spaces between procyclin molecules. In Leishmania, GPI-anchored proteins and free GPIs are transported with different kinetics, suggesting that these molecules are packaged into different transport vesicles (Ralton et al., 2002). Newly differentiated Δprocyclin cells might fail to grow because they require GPIs on their surface, but are unable to export them in sufficient quantities. Alternatively, transport might be necessary to prevent the accumulation of toxic amounts of intracellular GPIs. At present, we can only speculate about the nature of the alterations that occur during the adaptation period. One possibility is that vesicles loaded with free GPIs become capable of bypassing a retention signal, so that they can be transported to the plasma membrane.

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