Lipid remodelling of glycosylphosphatidylinositol (GPI) glycoconjugates in procyclic-form trypanosomes: biosynthesis and processing of GPs revisited

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INTRODUCTION

During their life cycles, which alternate between the mammalian bloodstream and the tsetse fly vector, African trypanosomes, Trypanosoma brucei, change the composition of their GPI (glycosylphosphatidylinositol)-anchored surface coats to adapt to new environments [1–3]. Interestingly, this change in surface coat during differentiation is accompanied by a switch in the types of GPI anchors they express. In T. brucei bloodstream forms, the GPI glycan core structure of VSGs (variant surface glycoproteins), consisting of ethanolaminephosphate-6-mannose-α1,2-mannose-α1,6-mannose-α1,4-glucosamine-α1,6-inositol-1-phospholipid, is replaced for a short galactose branch [4,5]. In contrast, in the procyclic stage, the GPI core of the procyclins is decorated with the largest and most complex GPI carbohydrate structure known to date, consisting of a series of sialylated, branched N-acetyl-lactosamine/N-lactobiose repeats [6–8]. Moreover, while the lipid moiety of T. brucei bloodstream-form GPI anchors consists exclusively of dimyristoyl-PI (phosphatidylinositol) [4,9], that of procyclic trypanosomes is composed of lyso-(acyl)PI (inositol-acylated acyl-lyso-phosphatidylinositol) [6,10,11]. In T. brucei bloodstream forms, acyl chain remodelling of the GPI precursor involves deacylation and reacylation reactions in the ER (endoplasmic reticulum), leading to replacement of the two fatty acyl chains by myristic acid [15,16]. A further remodelling reaction occurring on protein-bound GPs, known as myristate exchange, ensures that myristic acid remains the only fatty acid attached to the VSG anchor [17]. In contrast, no lipid remodelling reactions on GPI precursors or protein-bound GPI anchors have been described in T. brucei procyclic forms [12].

GPI lipid remodelling has also been reported in other organisms, such as yeast [18], Trypanosoma cruzi [19,20] and mammalian cells [21,22], and some of the enzymes involved in the exchange reactions have been identified [23–26]. Interestingly, addition of the GPs to their respective proteins [12]. In both life-cycle stages, GPI synthesis proceeds by stepwise addition of individual core components to PI. Additionally, in both bloodstream- and procyclic-form trypanosomes, acylation of the inositol ring occurs after the transfer of the first mannose residue to GlcN-PI and the resulting inositol-acylated and non-acylated GPI precursors remain in dynamic equilibrium until the addition of the terminal phosphoethanolamine. However, before being transferred to protein, the GPI precursor in bloodstream forms is remodelled and inositol-deacylated to form glycolipid A [13] or P2 [14], whereas in procyclic forms it is deacylated at the sn-2 position of the glycerol to yield PI, a GPI precursor containing a lyso-(acyl)PI lipid [6,10,11]. In T. brucei bloodstream forms, acyl chain remodelling of the GPI precursor involves deacylation and reacylation reactions in the ER (endoplasmic reticulum), leading to replacement of the two fatty acyl chains by myristic acid [15,16]. A further remodelling reaction occurring on protein-bound GPs, known as myristate exchange, ensures that myristic acid remains the only fatty acid attached to the VSG anchor [17]. In contrast, no lipid remodelling reactions on GPI precursors or protein-bound GPI anchors have been described in T. brucei procyclic forms [12].

GPI lipid remodelling has also been reported in other organisms, such as yeast [18], Trypanosoma cruzi [19,20] and mammalian cells [21,22], and some of the enzymes involved in the exchange reactions have been identified [23–26]. Interestingly,
the observation that GPI anchors are often composed of two identical fatty acyl or alcohol chains and thus distinctly differ from the lipid composition of bulk PI [21,27–31] implies that GPI lipid remodelling reactions are a widespread phenomenon.

Trypanosoma congolense is the main causative agent of Nagana, a devastating disease that affects cattle raised in most parts of sub-Saharan Africa. In the tsetse fly host, this parasite is covered by a dense layer of GPI-anchored molecules (Figure 1). These include GARP (glutamic acid- and alanine-rich protein) [32,33], EPGENGT (Glu-Pro-Gly-Glu-Asn-Gly-Thr) procyclins [34], PRS (protease-resistant surface) molecule (presumably of polysaccharidic nature) [35] and CESP (congolense epimastigote-specific protein) [36]. Unlike in T. brucei procyclic forms, very little is known about the composition and biosynthesis of GPI-anchored glycoconjugates in procyclic-form T. congolense. Previous mass spectrometric analyses have shown that the GPI anchors in T. congolense procyclic forms consist mainly of lyso-(acyl)PI [34,35,37] (see Figure 1). However, it has also been noted that up to 20% of radioactivity released from [3H]myristate-labelled GPI molecules after treatment with GPI-PLD (GPI-specific phospholipase D) was not lyso-PA (lyso-phosphatidic acid), suggesting that a different lipid moiety may be attached to a subpopulation of GPI molecules [35]. In addition, significant amounts of the same labelled material were released by PI-PLC (PI-hydrolysing phospholipase C), indicating that some of the GPI anchors were not inositol-acylated [35]. Moreover, recent analysis of the lipid portion of purified PRS indicated that it is composed of tri-acylated PIs [38]. Taken together, these findings suggest that some GPI anchors in T. congolense consist of lipids other than the previously described lyso-(acyl)PI species.

In the present study we show that T. congolense procyclic forms express GPI-anchored surface molecules with different types of PI moieties. The reason for such heterogeneity is that, unlike T. brucei procyclic forms, T. congolense procyclic trypanosomes use tri-acylated GPI precursors, which then undergo a series of de-acylation and re-acylation reactions after they have been transferred to protein. To our knowledge, this is the first demonstration of lipid remodelling reactions occurring on protein- or polysaccharide-bound GPs in procyclic trypanosomes.

EXPERIMENTAL

Unless otherwise specified, all reagents were of analytical grade and from Merck, Sigma–Aldrich or MP Biomedicals. [9,10(n)-3H]myristic acid ([3H]myristate, 60 Ci/mmol), GDP-[3H]mannose (GDP-[3H]Man, 20–30 Ci/mmol) were purchased from American Radiolabelled Chemicals. Kodak MBX films were from Kodak, and BioMax MS films were from GE Healthcare.

Trypanosomes and culture conditions

Procyclic forms of T. congolense Kilifi STIB745 and T. congolense Savannah TREU1457 were provided by the Swiss Tropical Institute (Basel, Switzerland) and cultured at 27°C in a 1:1 mixture of SM and SDM-79 medium, containing 15% heat-inactivated FBS (foetal bovine serum; Gibco BRL) [34].
Metabolic labelling and isolation of GPI-anchored molecules

Metabolic labelling of Trypanosoma with [3H]myristate was described previously [41]. Briefly, labelled precursors were added to procyclic-form trypanosomes at a density of (0.7–1.0) × 10^7 cells/ml and incubations were continued for various times (2–48 h). Cells were spun down, washed with ice-cold buffer [10 mM Tris/HC1 and 144 mM NaCl (pH 7.4)], and sequentially extracted with 2 × 10 ml of CM [chloroform/methanol; 2:1 (v/v)] to extract bulk phospholipids, followed by 3 × 5 ml of CMW [chloroform/methanol/water; 10:10:3 (by vol.)] to solubilize GPI precursors and free GIPs. The resulting pellet was further extracted with 2 × 0.5 ml of 9% (v/v) butan-1-ol to extract GPI-anchored proteins [6,41]. CMW fractions were pooled, dried under nitrogen and partitioned between butan-1-ol (CMWbut) and water (CMWwaq) to separate GPI lipid precursors and free GIPs respectively [39]. Incorporation of [3H]myristate into the GPI-glycoconjugates extracted with 9% butan-1-ol, proved to be 10-fold more efficient than [3H]palmitate (P. Büttikofer, unpublished work).

Enzymatic and chemical treatment of protein- and polysaccharide-bound GPI anchors

[3H]Myristate-labelled extracts were subjected to enzymatic treatment with PI-PLC from Bacillus thuringiensis (Invitrogen) or purified GPI-PLD from bovine serum as described previously [42]. Briefly, dried extracts were incubated in Triton X-100-containing buffer with PI-PLC or GPI-PLD for 2 h at 37°C. The reaction was stopped by extracting the products twice with water-saturated butan-1-ol. Nitrous acid and mild base treatment was an alternative to PI-PLC-released material co-migrated with the solvent front (TLC 2A, right-hand side). Consistent with the known lso-PI anchor of T. congolense GARP [37] and EPGENGT procyclin [34], part of the label migrated as lyso-P A. However, a considerable fraction of radioactivity co-migrated with a PA standard run on the same TLC plate, indicating that a fraction of GPI-anchored molecules consists of diacyl-type PI. In addition, part of the label (30–49%) range over four experiments) was released from T. congolense GPI molecules by treatment with PI-PLC (Figure 2A, right-hand side).

To study whether the relative distribution of label between lso-PA and PA represents a steady-state situation or changes during labelling, T. congolense Savannah and Kilifi procyclin forms were incubated with [3H]myristate for 6, 12 or 23 h and the products released by GPI-PLD were analysed by TLC (Figure 2B). The results showed that the relative amount of radioactivity in PA is maximal after 6 h of labelling and decreases with prolonged incubation time, with a concomitant increase in lyso-PA.

To further analyse the composition and structures of the radioactive GPI lipid moieties, T. congolense Savannah procyclin forms were labelled with [3H]myristate for 6 or 48 h and GPI anchors were analysed by enzymatic and chemical treatment, followed by TLC separation of the products as described above. After 6 h of labelling, most radioactivity released from GPI molecules by GPI-PLD migrated as PA, whereas the PI-PLC-released material co-migrated with the solvent front (Figure 3A; see also Figure 2B). Treatment with nitrous acid (which in GPI molecules specifically cleaves between non-acetylated glucosamine and inositol) released two main products, one of which co-migrated with a PI standard (Figure 3A, left-hand panel), whereas the other migrated closer to the front, consistent with being more hydrophobic and possibly representing.
Figure 2  Identification of [3H]-labelled GPI lipids

(A) T. brucei and T. congolense Kilifi and Savannah procyclic forms (0.5–1.0 × 10^9 cells) were incubated in the presence of [3H]myristate for 40 h and GPI-anchored molecules were extracted into 9% butan-1-ol. [3H]-Labelled lipids were released from labelled butan-1-ol extracts by treatment with GPI-PLD (left-hand side) or PI-PLC (right-hand side), partitioned between butan-1-ol and water, and analysed by TLC using solvent system 1. Radiolabelled products (20–50 000 c.p.m.) were visualized by fluorography. The migration of PA and lyso-PA standards run on the same plate is indicated.

(B) Time course of [3H]-incorporation into GPs. GPI-anchored molecules from T. congolense Savannah and Kilifi procyclic forms were labelled with [3H]myristate for 6–23 h and extracted and treated with GPI-PLD as described in (A). Radiolabelled products (40–60 000 c.p.m.) were analysed by TLC in solvent system 1, visualized by fluorography, and quantified by scanning the lanes with a radioisotope detector. The numbers represent the relative amounts of radioactivity in the PA and lyso-PA spots of each lane; no other labelled products were detected (also see A). T.c., T. congolense.

Figure 3  Characterization of [3H]-labelled GPI lipids

(A) T. congolense Savannah procyclic forms were labelled with [3H]myristate for 6 or 48 h and extracted as described in Figure 2(A), GPI-anchored molecules in the butan-1-ol extracts were treated with GPI-PLD (PLD), PI-PLC (PLC) or nitrous acid (NA) and the released products were analysed by TLC using solvent system 1. The migration of PA, lyso-PA and PI standards run on the same plate is indicated. (B) The products after PI-PLC treatment, migrating with the solvent front in (A), were scraped and re-extracted from the plate and separated by TLC in solvent system 2. The migration of diacylglycerol (DAG) and monoacylglycerol (MAG) standards run on the same plate is indicated.

tri-acylated PI, i.e. diacyl-(acyl)PI. In contrast, after 48 h of labelling, a substantial amount of radioactivity released by GPI-PLD migrated as PA (Figure 4, left-hand side; see also Figures 2B and 3A). In contrast, after 22 h of chase, increasing amounts of label co-migrated with the lyso-PA standard. Interestingly, the 4 h time point showed two spots in the lyso-PA range, one of which co-migrated with the lyso-PA spot after the 22 h chase, whereas the other showed a reduced mobility and co-migrated with the minor spot seen after the pulse (0 h chase). This result suggests that the labelled lyso-PA species released at the end of the pulse consist of less hydrophobic, i.e. shorter, acyl chains compared with the lyso-PA species released after the 4 h chase. A similar experiment with [3H]myristate-labelled T. brucei procyclic forms showed that all radioactivity released from GPI-anchored molecules by GPI-PLD migrated as PA (Figure 4, left-hand side; see also Figures 2B and 3A). In contrast, after 4 and 22 h of chase, increasing amounts of label co-migrated with the lyso-PA standard. Interestingly, the 4 h time point showed two spots in the lyso-PA range, one of which co-migrated with the lyso-PA spot after the 22 h chase, whereas the other showed a reduced mobility and co-migrated with the minor spot seen after the pulse (0 h chase). This result suggests that the labelled lyso-PA species released at the end of the pulse consist of less hydrophobic, i.e. shorter, acyl chains compared with the lyso-PA species released after the 4 h chase. A similar experiment with [3H]myristate-labelled T. congolense procyclic forms showed that all radioactivity co-migrated with lyso-PA during the chase (Figure 4, right-hand side). In summary, the time course and pulse–chase experiments using [3H]myristate-labelled extracts indicate that in T. congolense procyclic forms, candidate GPI molecules receive triacylated, i.e. inositol-acylated diacyl-type, GPI precursors. Subsequently, both the protein- and

Pulse–chase experiments corroborate lipid remodelling of protein-bound GPs in T. congolense procyclic forms

To confirm a putative GPI lipid remodelling, T. congolense Savannah procyclic forms were incubated in the presence of [3H]myristate for a short time only (2 h pulse), and the labelled GPI anchors chased over a period of 22 h in the absence of extracellular label. The results showed that after the pulse, almost all radioactivity released from GPI-anchored molecules by GPI-PLD migrated as PA (Figure 4, left-hand side; see also Figures 2B and 3A). In contrast, after 4 and 22 h of chase, increasing amounts of label co-migrated with the lyso-PA standard. Interestingly, the 4 h time point showed two spots in the lyso-PA range, one of which co-migrated with the lyso-PA spot after the 22 h chase, whereas the other showed a reduced mobility and co-migrated with the minor spot seen after the pulse (0 h chase). This result suggests that the labelled lyso-PA species released at the end of the pulse consist of less hydrophobic, i.e. shorter, acyl chains compared with the lyso-PA species released after the 4 h chase. A similar experiment with [3H]myristate-labelled T. brucei procyclic forms showed that all radioactivity co-migrated with lyso-PA during the chase (Figure 4, right-hand side). In summary, the time course and pulse–chase experiments using [3H]myristate-labelled extracts indicate that in T. congolense procyclic forms, candidate GPI molecules receive triacylated, i.e. inositol-acylated diacyl-type, GPI precursors. Subsequently, both the protein- and
polysaccharide-bound anchors are remodelled by de-acylation on the glycerol or inositol moiety. The presence of PI-PLC-sensitive GPIs further corroborates the idea that some of these molecules are indeed de-acylated on the inositol.

**MS analysis of steady-state GPI lipid molecules**

While the above [1H]myristate-labelling experiments (Figures 2–4) provide information on possible lipid remodelling reactions by following trace amounts of radioactivity, they may not reveal the bulk composition of the GPI lipid moiety. Moreover, the changes observed in lipid composition could be accounted for, in part, by differences in cell cycle or age of the cultures. Therefore, to determine the steady-state GPI lipid composition in *T. congolense* procyclic forms, we submitted butan-1-ol extracts containing GPI molecules from unlabelled cells, which were cultured in parallel with the cells used for [3H]myristate-labelling experiments (Figure 3), to nitrous acid deamination. The results showed that the released PI species by negative-ion ESI-MS, ESI-CID-MS, MALDI-QIT-TOF-MS (matrix-assisted laser-desorption ionization–quadrupole ion trap–time-of-flight MS) and MALDI-QIT-TOF-MS3. The collision-induced product spectrum of the major [M–H]− pseudomolecular ion at m/z of 862 (6 h sample), suggests that this species corresponds to 1-stearoyl-2-lyso-sn-glycerol-3-phospho-(2′-O-linoleoyl-1′-myo-inositol) (Supplementary Figure S2 at http://www.BiochemJ.org/bj/428/bj4280409add.htm). This preliminary assignment is based on the typical fragmentation pattern indicative for the presence of an acyl group on the inositol ring, i.e. the low intensity of m/z ions 223, 241 (for inositol 1,2-cyclic phosphate), 279 (C18:2 fatty acid) and the key signature ion at m/z 503 ([C18:2]-inositol 1,2-cyclic phosphate), suggesting that linoleate is attached to inositol. In addition, the high intensities of m/z ions 283 (C18:0 carboxylated fragment), 153 (glycerol-2,3-cyclic phosphate) and 419 (1-stearoyl-sn-glycerol-2,3-cyclic phosphate) indicate the presence of stearate attached to the sn-1 position of the glycerol backbone. Importantly, however, the m/z 577 ion, which in previous reports has been assigned exclusively as the fragment ion missing stearate at the sn-1 position [7,34,37], can also be interpreted as two putative isobaric molecules with the C18:2 fatty acid linked to either the inositol ring (i.e. glycerol-3-phospho-(2′-O-linoleoyl-1′-myo-inositol)) or the glycerol backbone (i.e. 1-stearoyl-2-linoleoyl-sn-glycerol-3-phospho-(1′-myo-inositol) or 1-linoleoyl-2-lyso-sn-glycerol-3-phospho-(1′-myo-inositol), with the former structure being more likely assuming that C18:0 was originally located at the sn-1 position) (see Supplementary Figure S2B).

To further investigate the possible presence of two isobaric structures, we analysed the same samples by MS/MS (tandem MS) using MALDI-QIT-TOF-MS. CID of the ion at m/z 862 produced abundant [M–H]− product ions similar to that obtained by ESI-MS/MS, including the fragment at m/z 577 (results not shown). When the m/z 577 ion was fragmented further, the MALDI-QIT-TOF-MS product ion spectrum clearly generated [M–H]− pseudomolecular ions that corroborate the presence of an inositol-acylated molecule (Supplementary Figure S4A at http://www.BiochemJ.org/bj/428/bj4280409add.htm). This is the case for the signature ion at m/z 503 ([C18:2]-inositol 1,2-cyclic phosphate), which further demonstrates that linoleate is attached to inositol. Moreover, and in agreement with previous reports [7,44], the high intensity of the cyclic glycerophosphate (m/z 153), together with a weak signal for the carboxylated fatty acid ion (m/z 279), strongly suggests the presence of an inositol-acylated PI species containing a weak carboxylated fatty acid (i.e. C18:2) attached to the 2-position of the inositol ring. On the other hand, the product-ion spectrum also supports the presence of a species containing glycerol. In particular, the key ions at m/z 315, 297 and the ion at m/z 153, corresponding to glycerophosphoinositol, glycerophosphoinositol −H2O and glycerol-2,3-cyclic phosphate respectively, suggest that this isobaric species still retains the glycerol backbone [45].

Taken together, these results strongly suggest that the ion at m/z 862 probably represents a mixture of two isobaric species: 1-stearoyl-2-linoleoyl-sn-glycerol-3-phospho-(1′-myo-inositol) and 1-stearoyl-2-lyso-sn-glycerol-3-phospho-(2′-O-linoleoyl-1′-myo-inositol). The relative proportions of the two species cannot be determined from these analyses. Almost identical results were obtained in the ESI-MS/MS and MALDI-QIT-TOF-MS3 collisional spectra of the m/z 862 ion from the 48 h sample (results not shown). In addition, similar product-ion spectra where obtained after fragmentations of the ions with m/z 864 and 866 (results not shown). In both cases, the location of the stearyl chain appears to be conserved at sn-1, but they differ in the type of fatty acids attached to the inositol or the sn-2 position of the glycerol (C18:1 oleoyl and C18:0 stearoyl for m/z 864 and 866 ions respectively). The ESI-MS-CID-MS analysis of the minor ions at m/z 810 (6 h butan-1-ol extract) and m/z 1072 (Supplementary Figures S3 and S5 at http://www.BiochemJ.org/bj/428/bj4280409add.htm). The migration of PA and lyso-PA standards run on the same plate is indicated. T.c. Sav, T. congolense Savannah; T.b., T. brucei.

**Figure 4** Analysis of protein-bound GPIs after pulse–chase labelling with [3H]myristate

*T. congolense* Savannah (left-hand side) and *T. brucei* (right-hand side) procyclic forms were labelled with [3H]myristate for 2 h (pulse). Subsequently, cells were washed to remove unincorporated label and the incubation was continued in the absence of label (chase). After 0, 4 and 22 h, GPI-anchored molecules were extracted as described in Figure 2, and [3H]-labelled products released by GPI-PLD were analysed by TLC using solvent system 1. The migration of PA and lyso-PA standards run on the same plate is indicated. T.c. Sav, T. congolense Savannah; T.b., T. brucei.
Figure 5  Negative-ion ESI-MS-CID-MS analyses of PI moieties of whole butan-1-ol extracts

(A) Negative-ion ESI-mass spectra of PI species obtained after nitrous acid deamination of GPI molecules present in a butan-1-ol extract of parasites cultured for 6 h. A similar spectrum was obtained from a butan-1-ol extract from parasites cultured for 48 h (see Supplementary Figures S1–S3 at http://www.BiochemJ.org/bj/428/bj4280409add.htm). The ions at m/z 956 and 1048 are phospholipid contaminants also present in the last butan-1-ol wash before nitrous acid deamination (not shown). (B) Proposed structures of the most abundant PI moieties.

In summary, these results unequivocally demonstrate that the lipid tails of steady-state GPI anchors in *T. congolense* procyclic forms consist of a mixture of lyso-(acyl)PI, diacyl-PI and diacyl-(acyl)PI species. An overview of the identified PI molecular species is given in Table 1.

**In vitro analysis of the GPI biosynthetic pathway in *T. congolense* procyclic forms**

To corroborate that *T. congolense* procyclic forms make triacylated GPI precursors, we analysed the GPI biosynthetic pathway of *T. congolense* procyclic forms by pulse-labelling washed parasite membranes with GDP-[3H]Man and determined the structure of the GPI anchor precursors after chemical and enzymatic cleavage, and TLC analysis of the products. As shown in Supplementary Figures S6 and S7 (at http://www.BiochemJ.org/bj/428/bj4280409add.htm), these experiments strongly suggest that procyclic-form *T. congolense* membranes synthesize GPI precursors (i.e. PP3- and PP1-like) with the same characteristics as previously described in procyclic-form *T. brucei* (see the Supplementary Experimental section at http://www.BiochemJ.org/bj/428/bj4280409add.htm for a detailed explanation of these experiments).

**GPI lipid remodelling in free GPs from *T. brucei* procyclic forms**

It has been reported that free GPs from *T. brucei* procyclic forms consist of a mixture of lyso- and diacyl-type lipids [39]. These results, obtained in mutant parasites lacking the expression of the major GPI-anchored surface proteins EP and GPEET procyclins (EP/GPEET-knockout cells), were in contrast with the previously reported exclusive presence of lyso-(acyl)PI in protein-bound GPI anchors in *T. brucei* procyclic forms [6,7,11]. We re-investigated these findings by incubating EP/GPEET-knockout cells in the presence of [3H]myristate for 1–48 h and analysing the composition of the GPI-PLD-released lipids of free GPs (Figure 6). Our results showed that after 1 h of labelling with [3H]myristate, approximately equal amounts of radioactivity were recovered in lyso-PA and PA. In contrast, after labelling trypanosomes for 48 h, lyso-PA was clearly the dominant product, indicating that the label redistributed from PA to lyso-PA with increasing labelling time. Thus in these *T. brucei* mutant parasites, which express approx. 10-fold more free GPs than wild-type...
Table 1  Suggested identities of the released PI species from GPI-glycoconjugates present in the butan-1-ol extracts from *T. congolense* procyclic forms cultured for 6 and 48 h

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<th>R₂</th>
<th>R₃</th>
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<tr>
<td>810</td>
<td>C₁₈₂₀</td>
<td>C₁₈₂₀</td>
<td>0H⁻</td>
<td>1-stearoyl-2-sn-myristoyl-glycero-3-phospho-(1′-myo-inositol)</td>
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<td>862</td>
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<td>1-stearoyl-2-sn-myristoyl-glycero-3-phospho-(2′-O-hydmoleoyl-1′-myo-inositol)</td>
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* Only present as diacyl-PI species. No evidence was found for the presence of triacyl-PI species. † Only present in lyso-(acyl)PI species. In this case, the sn-2 position of the glycerol backbone is unsubstituted. ‡ Mixture of isobaric PI species.

![PA - lyso-PA -](image)

Figure 6  Incorporation of [³H]myristate into free GPs of *T. brucei* EP/GPEET-knockout cells

EP/GPEET-knockout parasites ([0.2–8] × 10⁶ cells) were incubated in the presence of [³H]myristate for 1–48 h, washed to remove unincorporated label, and delipidated with CM (2:1, v/v). Free GPs were extracted with CMN (10:10:3, by vol.), partitioned into the CMWaq phase, and treated with GPI-PLD. Radiolabelled products were analysed by TLC using solvent system 1 and visualized by fluorography. The lanes contain 4200–8200 c.p.m. of labelled triacylated PI species.

![A novel type of GPI lipid remodelling in *T. congolense* procyclic forms](image)

Our metabolic incorporation and pulse–chase experiments using [³H]myristate suggest that most of the GPI acceptor molecules are likely to be first attached to tri-acylated GPI anchors and subsequently become di-acylated. Our cell-free system experiments using GDP-[³H]Man demonstrated that membranes from *T. congolense* procyclins are indeed capable of synthesizing a tri-acylated GPI precursor, whose migration on TLC and structure resembles that of *T. brucei* PP3. This contrasts with the current model of GPI biosynthesis in *T. brucei* procyclic forms where a lyso-PI lipid (from the GPI precursor PP1) appears to remain unmodified after its transfer to glycoproteins. Thus, unlike *T. brucei*, *T. congolense* procyclic forms have the capability to remodel their GPI lipids after they have been transferred to protein or polysaccharides (i.e. PRS). Evidence for lipid remodelling of protein-bound GPs has previously been reported for *T. cruzi* [19,20] and yeast [48]. In these cases, GPI remodelling involves exchange of the entire lipid moiety, i.e. from PI to inositol phosphorolipid.

Based on the results of the present study, we propose the following model for GPI biosynthesis and processing in *T. congolense* procyclic forms (Figure 7). In a first step, a tri-acylated GPI precursor (PP3-like) is transferred to GPI acceptor molecules. Such a precursor is likely to be heterogeneous in the type of fatty acids (i.e. C₁₈₀, C₁₈₁, and C₁₈₂) linked to the sn-2 position of the glycerol backbone and the inositol ring. Secondly, and possibly during transit between the ER and Golgi, a subpopulation of GPI-anchored molecules, consisting primarily of GARP and EPGENGT procyclin, undergoes de-acylation at the sn-2 position of the glycerol backbone, forming GPI anchors with lyso-(acyl)PI species. In addition, our results suggest that a fraction of these molecules may also undergo de-acylation on the inositol, resulting in the formation of diacyl-type GPI anchors. The presence of these structures was demonstrated by the high amount of PI-PLC-sensitive GPI anchors after short labelling times with [³H]myristate, and by MALDI-QIT-TOF-MS/MS. Clearly, these results are in contrast with the previously published structures of the GARP and EPGENGT procyclin GPI anchors, which showed lyso-(acyl)PI species [34,37]. In these studies, however, the presence of diacyl-PI species may have been missed, probably because of the difficulties in detecting specific fragment ions that would discriminate between lyso-(acyl)PI and diacyl-PI species (many of the MS² fragment ions are generated by both type of lipids). In the present study, these structures could only be demonstrated because of the high sensitivity and precursor-ion-selection resolution of the *Resonance* three-dimensional MALDI-QIT-TOF mass spectrometer, which produced key fragment ions.
that made the identification of isobaric PI species in these samples possible. Unfortunately, we were unable to determine the relative proportions between isobaric species as inositol-acyl bonds are much more stable compared with fatty acids in diacyl-PI lipids [7,44]. However, judging by the intensity of key product ions generated after CID (i.e. especially the MS 3 ion at m/z 315, 297 and 153, which support the presence of a glycerolipid), it appears that the diacyl-PI molecules are abundant species in both samples (i.e. 6 and 48 h). Stronger evidence for the presence of diacyl-GPI molecules in T. congolense samples (i.e. 6 and 48 h) is the MS 2 diagnostic (MS2) product ion at m/z 581 of the putative GPI precursor in T. congolense procyclic forms, thus confirming that the species containing C 18:1 at the sn-2 position, thereby transferred fatty acid remodelling is also operative in the epimastigote form of T. congolense [38].

Inositol de-acylation of protein-bound GPIs in T. congolense may resemble the situation in mammalian cells and yeast, where de-acylation of inositol takes place after transfer of the GPI precursor to protein, a reaction catalysed by PGAP1 (post-GPI attachment to proteins factor 1) in mammalian cells and Bst1p in yeast [49]. In contrast, it clearly differs from T. brucei, where inositol de-acylation occurs during GPI biosynthesis only and is mediated at least by two different enzymes [50,51].

A question that remains to be investigated is the significance of the production of PP1-like precursor by membranes of T. congolense procyclic trypanosomes. Although our in vivo labelling experiments with [3H]myristate suggest that PP3-like is the putative GPI precursor in T. congolense procyclics, we cannot rule out that minor quantities of PP1-like are also transferred to protein and then quickly acylated on the sn-2 position, thus producing tri-acylated PIs (e.g. like those in PRS). However, our lipid analyses from the metabolic and pulse–chase experiments suggest that very few lyso-PIs are present at early time points of labelling (Figures 4 and 6), although this process could take place quite rapidly and may therefore be difficult to detect. An alternative explanation is that PP1-like GPIs are produced in excess and thus may represent catabolic products of PP3-like precursors, similar to the situation occurring between glycolipid A and C in T. brucei bloodstream forms [12,47].

Lastly, another group of molecules appears to maintain the same lipid type originally present in the GPI precursor (i.e. PRS, containing C 18:1 at the sn-2 position [38]). This interpretation is supported by MS analysis of the total pool of cellular PI, showing that the species containing C 18:1 at the sn-2 position of the glycerol backbone is most abundant (P. Bütikofer, E. Greganova, M. Serricchio, Y.-C. Liu and A. Acosta-Serrano, unpublished work). Whether the same pool of molecules could also undergo fatty acid remodelling at the sn-2 position, thereby forming, for instance, species containing myristate, remains to be demonstrated. In summary, compared with T. brucei, attachment and processing of GPI anchors in T. congolense procyclic forms is more complex and results in the formation of multiple types of GPI lipid moieties. It would be interesting to determine the lipid structure of CESP and also to investigate whether the same post-transfer fatty acid remodelling is also operative in the epimastigote form of T. congolense.

**GPI remodelling in T. brucei procyclic forms**

Although wild-type T. brucei procyclic forms only use lyso-(acyl)PI to anchor EP [6,11] and GPEET procyclins [7,41], we confirmed an earlier report [39] showing the presence of diacyl-type lipids in the anchor of free GPIs in mutant parasites lacking the expression of T. brucei procyclins. As a result of the deficiency of GPI-anchored coat proteins, EP/GPEET-knockout parasites express 10-fold more free GPIs than wild-type trypanosomes [39]. Thus it is possible that the presence of diacyl-type PIs in free GPIs represents an artefact related to overproduction of free, i.e. unused, GPIs in these cells. Nevertheless, our results clearly demonstrate that T. brucei procyclic forms are capable of making diacyl-type GPI structures. Thus we cannot rule out the possibility

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**Figure 7** Model of lipid remodelling reactions in protein- and polysaccharide-bound GPIs in T. congolense procyclic forms

See the Discussion for details.
that remodelling of protein-bound GPs also occurs in wild-type *T. brucei* procyclic forms, but the turnover of intermediates occurs too rapidly to be detected by labelling. Alternatively, *T. brucei* procyclic forms may use two different GPP precursors, i.e. PPI for protein attachment and PP3 for free GPs (Supplementary Figure S8 at http://www.BiochemJ.org/bj/428/bj4280409add.htm).

**AUTHOR CONTRIBUTION**

Peter Bütkofer and Alvaro Acosta-Serrano designed the research. Peter Bütkofer, Eva Greganova, Yuk-Chien Liu, Ian Edwards and Alvaro Acosta-Serrano performed the experiments. Peter Bütkofer, Michael Lehane and Alvaro Acosta-Serrano analysed the data and wrote the manuscript.

**ACKNOWLEDGEMENTS**

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SUPPLEMENTARY ONLINE DATA

Lipid remodelling of glycosylphosphatidylinositol (GPI) glycoconjugates in procyclic-form trypanosomes: biosynthesis and processing of GPIs revisited

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EXPERIMENTAL

MS analysis

To determine the presence of isobaric lipid structures, PI species obtained after nitrous acid deamination of parasite butan-1-ol samples (i.e. 6 and 48 h) were analysed by negative-ion matrix-assisted laser-desorption/ionization-quadrupole ion trap-time-of-flight MS (MALDI-QIT-TOF-MS and MALDI-QIT-TOF-MSn). Briefly, small aliquots (~0.5 μl) of the samples [dissolved in CMW (10:10:3, by vol.)] were co-crystallized 1:1 (v/v) on a standard stainless-steel MALDI plate, with a solution of 10 mg/ml (dissolved in methanol) 2,4,6-trihydroxyacetophenone matrix. MS and MSn spectra were acquired using an AXIMA Resonance™ MALDI-QIT-TOF mass spectrometer (Shimadzu-Biotech, Manchester, U.K.) in negative, mid-mode 750 [external calibration (1026–2463 m/z) with LaunchPad™ software (Shimadzu-Biotech)]. The laser shot rate was set to 5 Hz. Helium was used for ion cooling and argon for CID.

Preparation of cell lysates and labelling of GPI precursors with GDP-[3H]Man

Hypotonic lysates of T. brucei 427 and T. congolense Savannah TREU1457 procyclic forms were obtained as described previously [1], except that parasites were not pre-incubated with tunicamycin. Briefly, lysates (1 × 10⁸ cell equivalents) were washed twice with 1 ml of HKML buffer [50 mM Hepes (pH 7.4), 25 mM KCl, 5 mM MgCl₂, and 1.0 mg/ml leupeptin], resuspended in 30 μl of HKML buffer, and incubated for 2 min at 27°C with 6.25 mM MnCl₂, 1.25 mM dithiothreitol and 0.8 μg/ml tunicamycin (to prevent formation of N-glycan biosynthetic precursors). GPIs were then pulse-labelled by transferring the membranes into another tube containing 2.0 μCi of GDP-[3H]Man and 2 mM UDP-GlcNAc for 5 min and chased with 1 mM GDP-Man for 30 min at 27°C. Reactions were terminated by adding CM (1:1, v/v) to obtain a final ratio of CMW of 10:10:3 (by vol.) and lipids were extracted for 10 min in a bath sonicator. After centrifugation (3 min at 14000 g), the organic phase was dried under nitrogen stream and lipids were extracted by adding 100 μl of butan-1-ol and 100 μl of water. The butan-1-ol phase was then re-extracted twice with 9 % butan-1-ol, dried in a Speed-Vac and resuspended in 20 μl of CMW (10:10:3, by vol.).

Enzymatic and chemical treatment of GPI precursors

For the assignment of trypanosome GPI precursors synthesized in vitro, dried lipid extracts from cell-free experiments (see above)
were incubated with PI-PLC and GPI-PLD under conditions similar to those described in [2]. For the treatment with phospholipase A₂ from bee venom (Sigma), dried lipids were resuspended in 40 μl of buffer [20 mM Tris/HCl (pH 7.4), 2 mM CaCl₂ and 0.02% Triton-X-100] and incubated for 18 h at 37°C with 2.0 units of enzyme. To release terminal α-Man residues, dried glycolipid extracts were resuspended in 20 μl of buffer [0.1 M sodium acetate (pH 5.0) and 0.1% sodium taurodeoxycholate] and incubated for 18 h at 37°C with 2.5 units of JBAM (jack bean α-mannosidase; Sigma). For the degradation of dolichol-linked glycan intermediates and other contaminants, dried samples were incubated for 10 min at 100°C with 100 μl of 40 mM trifluoroacetic acid. The reactions were stopped by placing the tubes in ice-water and the hydrolysed material was dried in the Speed-Vac.

The products of all enzymatic and chemical treatments were recovered after partitioning between water and butan-1-ol and analysed by high-performance TLC as described below.

Figure S2  Negative-ion ESI-MS-CID-MS of the PI species of m/z 862 from GPI-glycoconjugates present in the butan-1-ol sample from parasites grown for 6 h

(A) Collisional-induced product ion spectrum (see Table 1 of the main paper for assignments). Fragmentation of the same ion of m/z 862, from the butan-1-ol extract from 48 h, generated the same product ion spectrum (not shown). (B) Fragmentation patterns of the two isobaric PI species with a m/z of 862. The positions of the double bonds and the configurations of the unsaturated fatty acids are speculative.

RESULTS

MS analysis of steady-state GPI lipid molecules

Characterization of m/z ion 810

The ESI-MS-CID-MS analysis of the ion at m/z 810 (6 h butan-1-ol extract; Supplementary Figure S3) resulted in a rather different fragmentation pattern compared with that of the ion at m/z 862.
strongly suggesting the presence of at least two isobaric diacyl-PI species. For instance, the ions at m/z 363 and 419 suggest that C_{14:0} and C_{18:0} fatty acids respectively, are linked to the glycerolipid backbone. However, the higher intensity of the ion at m/z 419 strongly suggests that C_{18:0} is attached to the sn-1 position, as in other procyclic trypanosome GPI anchors [3–5]. These assignments are supported by the presence of product ions at m/z 525 and 581, resulting from the formation of lyso-PI species after the loss of a stearate and a myristate group respectively. Based on the collisional spectrum, we propose that none of the fatty acids are linked to the inositol ring because (i) the typical companion ions representing fragments of the corresponding acylated inositol-1,2-cyclic phosphate (i.e. m/z 451 and 507, containing myristate or stearate respectively) were not detected in the spectrum, and (ii) the high intensity of the two carboxylated fatty acid ions (i.e. m/z 227 and 283) in conjunction with that corresponding with inositol-1,2-cyclic phosphate (m/z 241), suggest that the majority (if not all) of the hydroxy groups of the inositol ring are unsubstituted.

To further confirm that the ion at m/z 810 represents a diacyl-PI species, the same sample was analysed by MALDI-QIT-TOF-MS and MALDI-QIT-TOF-MS3 (Supplementary Figure S4B). The MS3 spectrum of the ion at m/z 581 did not generate the signature ion at m/z 507 [(C_{18:0})-inositol 1,2-cyclic phosphate], in agreement with the absence of the same species from the ESI-MS-CID-mass spectrum of m/z 810 (Supplementary Figure S3). Instead, it generated m/z ions characteristic of the presence of a species containing glycerol (i.e. m/z 315, 297 and 153; see assignments above), in addition to the fragment at m/z 419 (sn-1-stearoyl glycerol-2,3-cyclic phosphate). Taken together, we assigned the ion at m/z 810 as 1-stearoyl-2-sn-myristoyl-glycerol-3-phospho(1′-myo-inositol). However, although C_{18:0}-stearoyl is highly conserved at the sn-1 position of the glycerol backbone of GPls from procyclic-form trypanosomes and the CID product ion spectra of the ion at m/z 810 strongly supports our assignment, we cannot rule out the presence of an isobaric PI species containing myristate at the sn-1 and stearate at the sn-2 positions.
Characterization of $m/z$ ion 1072

The ESI-MS-CID-MS analysis identified the ion at $m/z$ 1072 (Supplementary Figure S5) as a triacylated PI species, i.e. 1-stearoyl-2-sn-myristoyl-glycero-3-phospho-(2'-O-lynolenoyl-1'-myo-inositol) (Supplementary Figure S5). This assignment is mainly supported by the presence of two highly intense carboxylated fatty acids ions (i.e. $m/z$ 227 and 283), which, paired with their corresponding lyso-cyclic glycerolipid species at $m/z$ 363 and 419, suggest that myristoyl and stearoyl are attached to the sn-2 and sn-1 positions respectively. Furthermore, the low intensity of the carboxylated fatty acid ion at $m/z$ 279, together with the presence of the signature ion at $m/z$ 503 [(C18:2)-inositol 1,2-cyclic phosphate], indicates that linoleic acid is linked to inositol. The same PI species was identified in the GPI anchor of purified PRS [6].

In vitro analysis of the GPI biosynthetic pathway in *T. congolense* procyclic forms

To corroborate that *T. congolense* procyclic forms make triacylated GPI precursors, we analysed the GPI biosynthetic pathway of *T. congolense* procyclic forms by pulse-labelling washed parasite membranes with GDP-[3H]Man. As a reference, these analyses were made alongside membranes from *T. brucei* procyclic forms, in which the GPI biosynthetic pathway is well characterized [7–9].

When *T. brucei* membranes were pulse-labelled for 5 min with GDP-[3H]Man in the presence of excess UDP-GlcNAc and then chased for 30 min in the presence of 1 mM GDP-Man, the major products obtained were the GPI precursors PP3 and PP1 (results not shown; see also [7,10]). Likewise, when membranes from *T. congolense* were pulse-labelled for 5 min, the most
Figure S5  Negative-ion ESI-MS-CID-MS of the PI species of m/z 1072 from GPI-glycoconjugates present in the butan-1-ol sample from parasites grown for 6 h

(A) CID product ion spectrum of m/z ion 1072. (B) Fragmentation pattern and suggested structure. See also Table 1 of the main paper. The positions of the double bonds in the configuration of the C18 fatty acid are speculative.

Figure S6  In vitro synthesis of T. congolense GPI precursors

GPIs were synthesized in a cell-free system containing washed membranes from T. congolense procyclic forms. The membranes were incubated with GDP-[3H]Man and UDP-GlcNAc for 5 min (pulse) and then chased with an excess of non-radioactive GDP-Man for 30, 60 and 120 min as indicated. The reactions were stopped by the addition of organic solvents, and the glycolipids fractionated by high-performance TLC in solvent system 3 were detected by fluorography. The GPI biosynthetic intermediates (i.e. from top to bottom, Man2GlcN-(acyl)PI, Man1GlcN-PI and Man3GlcN-PI) and GPI candidate precursors (PP1-, PP3- and A’-like) are indicated.

The polar product was a band that co-migrated with T. brucei PP3 (results not shown). After the chase with non-radioactive GDP-Man, a T. congolense PP3-like species was the major product and another glycolipid with the same Rf (retention factor) as T. brucei PP1 was also observed (Supplementary Figures S6 and S7). Interestingly, after a longer chase with non-radioactive GDP-Man, we observed a substantial conversion of PP3-like into PP1-like lipids and the formation of glycolipid A’-like, which resembles a non-inositol-acylated diacyl-PI species made by T. brucei procyclic cells [11] (Supplementary Figure S6). The identity of the intermediates and mature GPI precursors from T. congolense procyclic forms was confirmed by treatment with PI-PLC, GPI-PLD, phospholipase A2, JBAM and mild trifluoroacetic acid (Supplementary Figure S7). Taken together, these experiments provide evidence that procyclic form T. congolense membranes synthesize GPI precursors (i.e. PP3- and PP1-like) with the same characteristics as previously described in procyclic form T. brucei.

REFERENCES

Membranes from *T. congolense* procyclic cells were pulse-labelled for 5 min with GDP-[^3H]Man and chased for 120 min with an excess of non-radioactive GDP-Man. Labelled glycolipids were then extracted with organic solvents and equivalent amounts were incubated with GPI-PLD (lane 2), PI-PLC (lane 3), JBAM (lane 4), phospholipase A2 (lane 5) or submitted to mild trifluoroacetic acid (lane 6). All samples were analysed by high-performance TLC using solvent system 3, and detected by fluorography. PP1- and PP3-like lipids were assigned because of (i) their co-migration on TLC with PP3 and PP1 from *T. brucei* (not shown), (ii) their susceptibility to GPI-PLD (lane 2), and (iii) resistance to PI-PLC (lane 3), JBAM (lane 4), phospholipase A2 (lane 5; inositol acylated lipids appear resistant to this enzyme), and mild trifluoroacetic acid (lane 6; only degrades dolichol-linked glycans). Note the disappearance of all GPI intermediates after JBAM treatment (lane 4).

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**Figure S7** Enzymatic and chemical treatments of GPI molecules from *T. congolense* procyclics synthesized in vitro

Membranes from *T. congolense* procyclics were pulse-labelled for 5 min with GDP-[^3H]Man and chased for 120 min with an excess of non-radioactive GDP-Man. Labelled glycolipids were then extracted with organic solvents and equivalent amounts were incubated with GPI-PLD (lane 2), PI-PLC (lane 3), JBAM (lane 4), phospholipase A2 (lane 5) or submitted to mild trifluoroacetic acid (lane 6). All samples were analysed by high-performance TLC using solvent system 3, and detected by fluorography. PP1- and PP3-like lipids were assigned because of (i) their co-migration on TLC with PP3 and PP1 from *T. brucei* (not shown), (ii) their susceptibility to GPI-PLD (lane 2), and (iii) resistance to PI-PLC (lane 3), JBAM (lane 4), phospholipase A2 (lane 5; inositol acylated lipids appear resistant to this enzyme), and mild trifluoroacetic acid (lane 6; only degrades dolichol-linked glycans). Note the disappearance of all GPI intermediates after JBAM treatment (lane 4).

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**Figure S8** Model describing the potential fates of the two GPI precursors from procyclic form *T. brucei*

This model is based on previous studies on the biosynthesis and structure of GPI molecules in procyclic form *T. brucei*, in addition to the evidence presented herein suggesting that *T. brucei* free GPIs undergo lipid remodelling. See the Discussion of the main paper for more details.

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SUPPLEMENTARY ONLINE DATA

Lipid remodelling of glycosylphosphatidylinositol (GPI) glycoconjugates in procyclic-form trypanosomes: biosynthesis and processing of GPs revisited

Peter BÜTIKOFER*, Eva GREGANOVA*, Yuk-Chien LIU†, Ian J. EDWARDS‡, Michael J. LEHANE§ and Alvaro ACOSTA-SERRANO†, 2

*Institute of Biochemistry and Molecular Medicine, University of Bern, Bühlstrasse 28, 3012 Bern, Switzerland, †Wellcome Trust Centre for Molecular Parasitology, University of Glasgow, Glasgow G12 8TA, Scotland, U.K., ‡Shimadzu Biotech, Wharfside, Trafford Wharf Road, Manchester M17 1GP, U.K., and §Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, U.K.

EXPERIMENTAL

MS analysis

To determine the presence of isobaric lipid structures, PI species obtained after nitrous acid deamination of parasite butan-1-ol samples (i.e. 6 and 48 h) were analysed by negative-ion matrix-assisted laser-desorption/ionization-quadrupole ion trap-time-of-flight MS (MALDI-QIT-TOF-MS and MALDI-QIT-TOF-MS3). Briefly, small aliquots (~0.5 µl) of the samples [dissolved in CMW (10:10:3, by vol.)] were co-crystallized 1:1 (v/v) on a standard stainless-steel MALDI plate, with a solution of 10 mg/ml (dissolved in methanol) 2,4,6-trihydroxyacetophenone matrix. MS and MSn spectra were acquired using an AXIMA Resonance™ MALDI-QIT-TOF mass spectrometer (Shimadzu-Biotech, Manchester, U.K.) in negative, mid-mode 750 [external calibration (1026–2463 m/z) with LaunchPad™ software (Shimadzu-Biotech)]. The laser shot rate was set to 5 Hz. Helium was used for ion cooling and argon for CID.

Preparation of cell lysates and labelling of GPI precursors with GDP-[3H]Man

Hypotonic lysates of T. brucei 427 and T. congolense Savannah TREU1457 procyclic forms were obtained as described previously [1], except that parasites were not pre-incubated with tunicamycin. Briefly, lysates (1 × 10⁸ cell equivalents) were washed twice with 1 ml of HKML buffer [50 mM Hepes (pH 7.4), 25 mM KCl, 5 mM MgCl₂ and 1.0 mg/ml leupeptin], resuspended in 30 µl of HKML buffer, and incubated for 2 min at 27°C with 6.25 mM MnCl₂, 1.25 mM dithiothreitol and 0.8 µg/ml tunicamycin (to prevent formation of N-glycan biosynthetic precursors). GPIs were then pulse-labelled by transferring the membranes into another tube containing 2.0 µCi of GDP-[3H]Man and 2 mM UDP-GlcNAc for 5 min and chased with 1 mM GDP-Man for 30 min at 27°C. Reactions were terminated by adding CM (1:1, v/v) to obtain a final ratio of CMW of 10:10:3 (by vol.) and lipids were extracted for 10 min in a bath sonicator. After centrifugation (3 min at 14000 g), the organic phase was dried under nitrogen stream and lipids were extracted by adding 100 µl of butan-1-ol and 100 µl of water. The butan-1-ol phase was then re-extracted twice with 9 % butan-1-ol, dried in a Speed-Vac and resuspended in 20 µl of CMW (10:10:3, by vol.).

Enzymatic and chemical treatment of GPI precursors

For the assignment of trypanosome GPI precursors synthesized in vitro, dried lipid extracts from cell-free experiments (see above)
were incubated with PI-PLC and GPI-PLD under conditions similar to those described in [2]. For the treatment with phospholipase A₂ from bee venom (Sigma), dried lipids were resuspended in 40 μl of buffer [20 mM Tris/HCl (pH 7.4), 2 mM CaCl₂ and 0.02% Triton-X-100] and incubated for 18 h at 37°C with 2.0 units of enzyme. To release terminal α-Man residues, dried glycolipid extracts were resuspended in 20 μl of buffer [0.1 M sodium acetate (pH 5.0) and 0.1% sodium taurodeoxycholate] and incubated for 18 h at 37°C with 2.5 units of JBAM (jack bean α-mannosidase; Sigma). For the degradation of dolichol-linked glycan intermediates and other contaminants, dried samples were incubated for 10 min at 100°C with 100 μl of 40 mM trifluoroacetic acid. The reactions were stopped by placing the tubes in ice-water and the hydrolysed material was dried in the Speed-Vac. The products of all enzymatic and chemical treatments were recovered after partitioning between water and butan-1-ol and analysed by high-performance TLC as described below.

**TLC**

To analyse the [³H]Man-labelled GPI molecules, extracts were applied to silica-gel high-performance TLC plates (Merck) and run in solvent system 3, composed of CMW (10:10:3, by vol.). The plates were sprayed with En'Hance and exposed to MXB film at −70°C.

**RESULTS**

**MS analysis of steady-state GPI lipid molecules**

**Characterization of m/z ion 810**

The ESI-MS-CID-MS analysis of the ion at m/z 810 (6 h butan-1-ol extract; Supplementary Figure S3) resulted in a rather different fragmentation pattern compared with that of the ion at m/z 862,
GPI lipid remodelling in procyclic-form trypanosomes

Figure S3 Negative-ion ESI-MS-CID-MS of the PI species of m/z 810 from GPI-glycoconjugates present in the butan-1-ol sample from parasites grown for 6 h

The inset shows the fragmentation pattern and the suggested structure. See the text (Supplementary section) and Table 1 of the main paper for details.

strongly suggesting the presence of at least two isobaric diacyl-PI species. For instance, the ions at m/z 363 and 419 suggest that C_{14:0} and C_{18:0} fatty acids respectively, are linked to the glycerolipid backbone. However, the higher intensity of the ion at m/z 419 strongly suggests that C_{18:0} is attached to the sn-1 position, as in other procyclic trypanosome GPI anchors [3–5]. These assignments are supported by the presence of product ions at m/z 525 and 581, resulting from the formation of lyso-PI species after the loss of a stearate and a myristate group respectively. Based on the collisional spectrum, we propose that none of the fatty acids are linked to the inositol ring because (i) the typical companion ions representing fragments of the corresponding acylated inositol-1,2-cyclic phosphate (i.e. m/z 451 and 507, containing myristate or stearate respectively) were not detected in the spectrum, and (ii) the high intensity of the two carboxylated fatty acid ions (i.e. m/z 227 and 283) in conjunction with that corresponding with inositol-1,2-cyclic phosphate (m/z 241), suggest that the majority (if not all) of the hydroxy groups of the inositol ring are unsubstituted.

To further confirm that the ion at m/z 810 represents a diacyl-PI species, the same sample was analysed by MALDI-QIT-TOF-MS and MALDI-QIT-TOF-MS^3 (Supplementary Figure S4B). The MS^3 spectrum of the ion at m/z 581 did not generate the signature ion at m/z 507 [(C_{18:0})-inositol 1,2-cyclic phosphate], in agreement with the absence of the same species from the ESI-MS-CID-mass spectrum of m/z ion 810 (Supplementary Figure S3). Instead, it generated m/z ions characteristic of the presence of a species containing glycerol (i.e. m/z 315, 297 and 153; see assignments above), in addition to the fragment at m/z 419 (sn-1-stearoyl glycerol-2,3-cyclic phosphate). Taken together, we assigned the ion at m/z 810 as 1-stearoyl-2-sn-myristoyl-glycerol-3-phospho-(1′-myo-inositol). However, although C_{18:0}-stearoyl is highly conserved at the sn-1 position of the glycerol backbone of GPIs from procyclic-form trypanosomes and the CID product ion spectra of the ion at m/z 810 strongly supports our assignment, we cannot rule out the presence of an isobaric PI species containing myristate at the sn-1 and stearate at the sn-2 positions.
Figure S4  Negative-ion MALDI-QIT-TOF-MS$^3$ of PI product ions obtained after CID-MS$^2$ of intact PI species present in the butan-1-ol samples from parasites grown for 6 h

(A) CID-MS$^2$ product ion spectrum of m/z 577, which was produced after CID-MS of m/z 862 parent ion. (B) CID-MS$^2$ product ion spectrum of m/z 581, which was produced after CID-MS of m/z 810 parent ion. (C and D) Fragmentation patterns and putative structures of the product ions with a m/z of 577, which indicate the presence of two isobaric structures. (E) Fragmentation pattern and putative structure of the product ion with a m/z of 581. The positions of the double bonds and the configurations of the unsaturated fatty acids are speculative.

Characterization of m/z ion 1072

The ESI-MS-CID-MS analysis identified the ion at m/z 1072 (Supplementary Figure S5) as a triacylated PI species, i.e. 1-stearoyl-2-sn-myristoyl-glycero-3-phospho-(2′-O-lynolenoyl-1′-myo-inositol) (Supplementary Figure S5). This assignment is mainly supported by the presence of two highly intense carboxylated fatty acids ions (i.e. m/z 227 and 283), which, paired with their corresponding lyso-cyclic glycerolipid species at m/z 363 and 419, suggest that myristoyl and stearoyl are attached to the sn-2 and sn-1 positions respectively. Furthermore, the low intensity of the carboxylated fatty acid ion at m/z 279, together with the presence of the signature ion at m/z 503 [(C$_{18:2}$)-inositol 1,2-cyclic phosphate], indicates that linoleic acid is linked to inositol. The same PI species was identified in the GPI anchor of purified PRS [6].

In vitro analysis of the GPI biosynthetic pathway in T. congoense procyclic forms

To corroborate that T. congoense procyclic forms make triacylated GPI precursors, we analysed the GPI biosynthetic pathway of T. congoense procyclic forms by pulse-labelling washed parasite membranes with GDP-[H]Man. As a reference, these analyses were made alongside membranes from T. brucei procyclic forms, in which the GPI biosynthetic pathway is well characterized [7–9].

When T. brucei membranes were pulse-labelled for 5 min with GDP-[H]Man in the presence of excess UDP-GlcNAc and then chased for 30 min in the presence of 1 mM GDP-Man, the major products obtained were the GPI precursors PP3 and PP1 (results not shown; see also [7,10]). Likewise, when membranes from T. congoense were pulse-labelled for 5 min, the most
Figure S5  Negative-ion ESI-MS-CID-MS of the PI species of m/z 1072 from GPI-glycoconjugates present in the butan-1-ol sample from parasites grown for 6 h.

(A) CID product ion spectrum of m/z ion 1072. (B) Fragmentation pattern and suggested structure. See also Table 1 of the main paper. The positions of the double bonds the configuration of the C18 fatty acid are speculative.

Figure S6  In vitrosynthesis of T. congolense GPI precursors

GPIs were synthesized in a cell-free system containing washed membranes from T. congolense procyclic forms. The membranes were incubated with GDP-[3H]Man and UDP-GlcNAc for 5 min (pulse) and then chased with an excess of non-radioactive GDP-Man for 30, 60 and 120 min as indicated. The reactions were stopped by the addition of organic solvents, and the glycolipids fractionated by high-performance TLC in solvent system 3 were detected by fluorography. The GPI biosynthetic intermediates (i.e. from top to bottom, Man₂GlcN-(acyl)PI, Man₁GlcN-PI and Man₃GlcN-PI) and GPI candidate precursors (PP1-, PP3- and A’-like) are indicated.

polar product was a band that co-migrated with T. brucei PP3 (results not shown). After the chase with non-radioactive GDP-Man, a T. congolense PP3-like species was the major product and another glycolipid with the same RF (retention factor) as T. brucei PP1 was also observed (Supplementary Figures S6 and S7). Interestingly, after a longer chase with non-radioactive GDP-Man, we observed a substantial conversion of PP3-like into PP1-like lipids and the formation of glycolipid A’-like, which resembles a non-inositol-acylated diacyl-PI species made by T. brucei procyclic cells [11] (Supplementary Figure S6). The identity of the intermediates and mature GPI precursors from T. congolense procyclic forms was confirmed by treatment with PI-PLC, GPI-PLD, phospholipase A₂, JBAM and mild trifluoroacetic acid (Supplementary Figure S7). Taken together, these experiments provide evidence that procyclic form T. congolense membranes synthesize GPI precursors (i.e. PP3- and PP1-like) with the same characteristics as previously described in procyclic form T. brucei.

REFERENCES


Membranes from *T. congolense* procyclic cells were pulse-labelled for 5 min with GDP-[3H]Man and chased for 120 min with an excess of non-radioactive GDP-Man. Labelled glycolipids were then extracted with organic solvents and equivalent amounts were incubated with GPI-PLD (lane 2), PI-PLC (lane 3), JBAM (lane 4), phospholipase A2 (lane 5) or submitted to mild trifluoroacetic acid (lane 6). All samples were analysed by high-performance TLC using solvent system 3, and detected by fluorography. PP1- and PP3-like lipids were assigned because of (i) their co-migration on TLC with PP3 and PP1 from *T. brucei* (not shown), (ii) their susceptibility to GPI-PLD (lane 2), and (iii) resistance to PI-PLC (lane 3), JBAM (lane 4), phospholipase A2 (lane 5; inositol acylated lipids appear resistant to this enzyme), and mild trifluoroacetic acid (lane 6; only degrades dolichol-linked glycans). Note the disappearance of all GPI intermediates after JBAM treatment (lane 4).

Figure S8 Model describing the potential fates of the two GPI precursors from procyclic form *T. brucei*

This model is based on previous studies on the biosynthesis and structure of GPI molecules in procyclic form *T. brucei*, in addition to the evidence presented herein suggesting that *T. brucei* free GPIs undergo lipid remodelling. See the Discussion of the main paper for more details.

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