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

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Short title: GPI lipid remodelling in procyclic form trypanosomes

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SYNOPSIS

The African trypanosome, *Trypanosoma brucei*, has been used as a model to study the biosynthesis of glycosylphosphatidylinositol (GPI) anchors. In mammalian (bloodstream) form parasites, diacyl-type GPI precursors are remodelled in their lipid moieties before attachment to variant surface glycoproteins. In contrast, the GPI precursors of insect (procyclic) form parasites, consisting of inositol-acylated acyl-*lyso*-phosphatidylinositol (*lyso*-(acyl)PI) species, remain unaltered before protein attachment. By using a combination of metabolic labelling, cell-free assays and complementary mass spectrometry analyses, we now show that GPI-anchored glycoconjugates in *T. congolense* procyclic forms initially receive tri-acylated GPI precursors, which are subsequently de-acylated either at the glycerol backbone or on the inositol ring. Chemical and enzymatic treatments of [³H]myristate-labelled lipids in combination with ESI-MS/MS and MALDI-QIT-TOF-MS³ analyses indicate that the structure of the lipid moieties of steady-state GPI lipids from *T. congolense* procyclic forms consist of a mixture of *lyso*-(acyl)PI, diacyl-PI and diacyl-(acyl)PI species. Interestingly, some of these species are myristoylated at the *sn*-2 position. To our knowledge, this is the first demonstration of lipid remodelling at the level of protein- or polysaccharide-linked GPI anchors in procyclic form trypanosomes.

Keywords: Glycosylphosphatidylinositol, Trypanosome, Lipid Biosynthesis, Lipid Remodelling, Posttranslational Modification, Mass Spectrometry.

Abbreviations used: GPI, glycosylphosphatidylinositol; ESI-MS, electrospray ionization-mass spectrometry; ESI-MS-CID-MS, collision induced dissociation and tandem mass spectrometry; CESP, congolense epimastigote-specific protein; PRS, protease resistant surface molecule; PI, phosphatidylinositol; GARP, glutamic acid repetitive protein; JBAM, Jack bean α -mannosidase; PI-PLC, phosphatidylinositol-specific phospholipase C; GPI-PLD, GPI-specific phospholipase D; MALDI-QIT-TOF-MS, matrix-assisted laser-desorption/ionization-quadrupole ion trap-time-of-flight mass spectrometry; PA, phosphatidic acid; TLC, thin layer chromatography.

INTRODUCTION

During their life cycles, which alternate between the mammalian bloodstream and the tsetse fly vector, African trypanosomes change the composition of their glycosylphosphatidylinositol (GPI)-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 variant surface glycoproteins (VSG), consisting of ethanolamine-phosphate-6-mannose- α 1,2-mannose- α 1,6-mannose- α 1,4-glucosamine- α 1,6-*myo*-inositol-1-phospholipid, is substituted with 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 today, consisting of a series of sialylated, branched *N*-acetyllactosamine/*N*-lactobiose repeats [6-8]. Moreover, while the lipid moiety of *T. brucei* bloodstream form GPI anchors consists exclusively of dimyristoyl phosphatidylinositol (PI) [4, 9], that of procyclic trypanosomes is composed of inositol-acylated acyl-*lyso*-PI (*lyso*-(acyl)PI) species with mostly stearic acid (C_{18:0}) in the *sn*-1 position and a heterogeneous fatty acyl chain bound to the C2 of the inositol ring [7, 10, 11].

Structural differences in GPI lipid composition between bloodstream and procyclic stages of *T. brucei* are the result of different lipid remodelling reactions that take place before the addition of the GPIs 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 Man 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 PP1, 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 endoplasmic reticulum, leading to replacement of the two fatty acyl chains by myristic acid [15, 16]. A further remodelling reaction occurring on protein-bound GPIs, 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 recently 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 (Fig. 1). These include glutamic acid- and alanine-rich protein (GARP) [32, 33], EPGENG T procyclins [34], protease-resistant surface molecule (PRS; presumably of polysaccharidic nature) [35], and congolense epimastigote-specific protein (CESP) [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 Fig 1). However, it has also been noted that up to 20% of radioactivity released from [³H]myristate-labelled GPI molecules after treatment with GPI-specific phospholipase D (GPI-PLD) was not *lyso*-phosphatidic acid (*lyso*-PA), 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-hydrolyzing phospholipase C (PI-PLC), 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]. Together, these findings suggest that some GPI anchors in *T. congolense* consist of lipids other than the previously described *lyso*-(acyl)PI species.

Here we show that *T. congolense* procyclic forms express GPI-anchored surface molecules with different type 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 GPIs in procyclic trypanosomes.

EXPERIMENTAL

Unless otherwise specified, all reagents were of analytical grade and from Merck (Darmstadt, Germany), Sigma-Aldrich (Buchs, Switzerland) or MP Biomedicals (Tägerig, Switzerland). [9,10(n)- ^3H]myristic acid ([^3H]myristate, 60 Ci mmol $^{-1}$), GDP-[^3H]mannose (GDP-[^3H]Man, 20-30 Ci mmol $^{-1}$) were purchased from American Radiolabelled Chemicals Inc. (St. Louis, MO). Kodak MBX films were from Kodak (Lausanne, Switzerland) and BioMax MS films from GE Healthcare (Buckinghamshire, UK).

Trypanosomes and culture condition

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, containing 15% heat-inactivated foetal bovine serum (Gibco BRL, Basel, Switzerland) [34]. Procyclic forms of *T. brucei* 427, Antat 1.1 and EP/GPEET knock out mutants [39] were cultured at 27 °C in SDM-79 [40] containing 15% heat-inactivated foetal bovine serum.

Metabolic labelling and isolation of GPI-anchored molecules

Metabolic labelling of trypanosomes with [^3H]myristate was performed as previously described [41]. Briefly, labelled precursors were added to procyclic form trypanosomes at a density of 0.7-1.0 x 10 7 cells/ml and incubations were continued for various times (2-48 hours). Cells were spun down, washed with ice-cold buffer (10 mM Tris, 144 mM NaCl, pH 7.4), and sequentially extracted with 2 x 10 ml chloroform:methanol (CM; 2:1, v/v) to extract bulk phospholipids, followed by 3 x 5 ml chloroform:methanol:water (CMW; 10:10:3, v/v/v) to solubilize GPI precursors and free GPIs. The resulting pellet was further extracted with 2 x 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 (CMWaqu) to separate GPI lipid precursors and free GPIs, respectively [39]. Incorporation of [^3H]myristate into the GPI-glycoconjugates extracted with 9 % butan-1-ol, proved to be 10 times more efficient than [^3H]palmitate (unpublished).

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 before [42]. Briefly, dried extracts were incubated in Triton X-100-containing buffer with PI-

PLC or GPI-PLD for 2 hours 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 performed as described elsewhere [43]. The cleavage efficiency of each reaction was determined by counting aliquots of the aqueous and butan-1-ol phases. The butan-1-ol phases were pooled and stored at -20 °C.

Thin layer chromatography (TLC)

To analyze the [³H]-labelled reaction products after PI-PLC, GPI-PLD or nitrous acid treatment, extracts were applied to silica gel 60 plates (Merck) and separated by TLC using solvent system 1, composed of chloroform:methanol:acetic acid:water (25:15:4:2, v/v/v/v). [³H]-labelled monoacylglycerol and diacylglycerol were separated using solvent system 2, composed of benzene:diethyl ether:ethanol:acetic acid (50:40:2:0.2, v/v/v/v). Appropriate lipid standards were run alongside the samples on each plate. Radioactivity was detected by scanning the air-dried plates with a radioisotope detector (Berthold Technologies, Regensdorf, Switzerland) and quantified using the Rita Star® software provided by the manufacturer. Alternatively, the plates were sprayed with En³hance (Perkin Elmer) and exposed to MXB film at -70 °C. Lipid spots on TLC plates were visualized by exposure to iodine vapour.

Mass spectrometry analysis

Butan-1-ol extracts containing GARP, EPGENG^T procyclin and PRS (equivalent to ~5 x 10⁸ parasites) from parasites labelled with [³H]myristate for 6 or 48 hours were washed five times with butan-1-ol and water (to eliminate phospholipid contaminants), dried in a speed-vac and submitted to nitrous acid deamination as described [43]. The released PI species were recovered as above, small quantities were then transferred to Water nanoflow tips (Millipore) and analyzed in negative ion electrospray ionization-mass spectrometry (ESI-MS) on a ABI Q-StarXL with tip and declustering potentials of 900 and 60 V, respectively. Collision induced dissociation and tandem mass spectra (ESI-MS-CID-MS) were collected in product ion scanning mode using collision voltages of 25 to 60 V.

RESULTS

***In vivo* [³H]myristate-labelling reveals the presence of GPI-anchored molecules with triacylated lipid moieties in *T. congolense* procyclic forms**

Previous reports suggested that at least part of the GPI anchors in *T. congolense* procyclic forms may consist of alternatively acylated PI species. To corroborate these observations, we first pulse-labelled live *T. brucei* (as control parasites) and *T. congolense* procyclic forms with [³H]myristate. The GPI anchors from *T. brucei* procyclic forms have been shown to consist exclusively of *lyso*-(acyl)PI species [7, 11, 34, 37]. In agreement with these reports, we found that in a butan-1-ol extract from *T. brucei* procyclic forms labelled with [³H]myristate for 40 hours, treatment by GPI-PLD released 55-64% (range of 6 independent experiments) of radioactivity from GPI-anchored proteins (mostly procyclins), all of which migrated as *lyso*-PA (Fig. 2A, left). Furthermore, only trace amounts of label (1-3%; range of 3 experiments) were released by PI-PLC (Fig. 2A, right). In contrast, treatment of [³H]myristate-labelled GPI molecules from *T. congolense* Savannah and Kilifi procyclic forms with GPI-PLD released 81-96% (range of 8 independent experiments) of radioactivity, which migrated as two distinct spots on TLC (Fig. 2A, left). Consistent with the known *lyso*-PI anchor of *T. congolense* GARP [37] and EPGENGTP procyclin [34], part of the label migrated as *lyso*-PA. 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 of 4 experiments) was released from *T. congolense* GPI molecules by treatment with PI-PLC (Fig. 2A, right).

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

To further analyze the composition and structures of the radioactive GPI lipid moieties, *T. congolense* Savannah procyclic forms were labelled with [³H]myristate for 6 or 48 hours and GPI anchors were analysed by enzymatic and chemical treatment followed by TLC separation of the products as above. After 6 hours of labelling, most radioactivity released from GPI molecules by GPI-PLD migrated as PA while the PI-PLC-released material co-migrated with the solvent front (Fig. 3A; see also Fig. 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 (Fig. 3A, left panel), whereas the other migrated closer to the front, consistent with being more hydrophobic and possibly representing tri-acylated PI, *i.e.* diacyl-(acyl)PI. In contrast, after 48 hours of labelling, a substantial amount of radioactivity released by GPI-PLD migrated as *lyso*-PA (Fig. 3A, right panel). In addition, a third product is seen after nitrous acid treatment. This product migrated between diacyl-PI and diacyl-(acyl)PI and was resistant to cleavage by phospholipase A₂ and PI-PLC (data not shown) and, therefore, identified as *lyso*-(acyl)PI. Finally, the PI-PLC-released products were analyzed in another TLC system to separate monoacylglycerol and diacylglycerol (Fig. 3B). The results showed that all radioactivity from the 6 and 48 hours time points co-migrated with a diacylglycerol standard. In summary, these analyses indicate that GPI-glycoconjugate lipids in *T. congolense* procyclic forms are remodelled over time, from diacyl-(acyl)PI and diacyl-PI species to *lyso*-(acyl)PI species.

Pulse-chase experiments corroborate lipid remodelling of protein-bound GPIs 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 hour pulse) and the labelled GPI anchors chased over a period of 22 hours 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 (Fig. 4, left; see also Figs. 2B and 3A). In contrast, after 4 and 22 hours 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 hours chase while the other showed a reduced mobility and co-migrated with the minor spot seen after the pulse (0 hour 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 to the *lyso*-PA species released after the 4 hours chase. A similar experiment with [³H]myristate-labelled *T. brucei* procyclic forms showed that all radioactivity co-migrated with *lyso*-PA during the chase (Fig. 4, right). 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 that some of these molecules are indeed de-acylated on the inositol.

Mass spectrometry analysis of steady state GPI lipid molecules

While the above [^3H]myristate-labelling experiments (Figs. 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 to the cells used for [^3H]myristate labelling experiments (Fig. 3), to nitrous acid deamination and analyzed the released PI species by negative-ion ESI-MS, ESI-CID-MS, MALDI-QIT-TOF-MS and MALDI-QIT-TOF-MS³. The results showed that the ESI mass spectra of the released PI species are identical in both butan-1-ol samples and consist mainly of species with m/z 810, 862, 864, 866 and 1072 (Fig. 5 and Fig. S1). The collision-induced product spectrum of the major $[\text{M} - \text{H}]^-$ pseudomolecular ion at m/z of 862 (6 hours sample), suggests that this species corresponds to 1-stearoyl-2-lyso-*sn*-glycero-3-phospho-(2'-*O*-linoleoyl-1'-*myo*-inositol) (Fig. S2). 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 ($\text{C}_{18:2}$ fatty acid), and the key signature ion at m/z 503 (($\text{C}_{18:2}$)-inositol 1,2-cyclic phosphate), suggesting that linoleate is attached to inositol. In addition, the high intensities of m/z ions 283 ($\text{C}_{18:0}$ 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 $\text{C}_{18:2}$ 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-lyso-2-linoleoyl-*sn*-glycero-3-phospho-(1'-*myo*-inositol) or 1-linoleoyl-2-lyso-*sn*-glycero-3-phospho-(1'-*myo*-inositol), with the former structure being more likely assuming that $\text{C}_{18:0}$ was originally located at the *sn*-1 position) (see Fig. S2B).

To further investigate the possible presence of two isobaric structures, we analyzed the same samples by tandem mass spectrometry using MALDI-QIT-TOF-MS. CID of the ion at m/z 862 produced abundant $[\text{M} - \text{H}]^-$ product ions similar to that obtained by ESI-MS/MS,

including the fragment at m/z 577 (not shown). When the m/z 577 ion was further fragmented, the MALDI-QIT-TOF-MS³ product ion spectrum clearly generated $[M - H]^-$ pseudomolecular ions that corroborate the presence of an inositol-acylated molecule (supplemental Fig. S4A). This is the case of the signature ion at m/z 503 ((C_{18:2})-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.* C_{18:2}) 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 – 1 H₂O and glycerol-2,3-cyclic phosphate, respectively, suggest that this isobaric species still retains the glycerol backbone [45].

Together, these results strongly suggest that the ion at m/z 862 likely represents a mixture of two isobaric species: 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phospho-(1'-*myo*-inositol) and 1-stearoyl-2-*lyso*-*sn*-glycero-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 hour sample (not shown). In addition, similar product ion spectra were obtained after fragmentations of the ions with m/z 864 and 866 (not shown). In both cases, the location of the stearoyl chain appears 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 (C_{18:1} oleoyl and C_{18:0} stearoyl for m/z 864 and 866 ions, respectively). The ESI-MS-CID-MS analysis of the minor ions at m/z 810 (6 hours butan-1-ol extract) and m/z 1072 (Figs. S3 and S5), suggest that both species contain myristate at the *sn*-2 position.

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 on 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 analyzed 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 Figs. S6 and S7, 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 section for a detailed explanation of these experiments).

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

It has been reported that free GPIs 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 knock out cells), were in contrast to 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 knock out cells in the presence of [³H]myristate for 1-48 hours and analyzing the composition of the GPI-PLD-released lipids of free GPIs (Fig. 6). Our results showed that after 1 hour of labelling with [³H]myristate, roughly equal amounts of radioactivity were recovered in *lyso*-PA and PA. In contrast, after labelling trypanosomes for 48 hours, *lyso*-PA was clearly the dominant product, indicating that the label re-distributed from PA to *lyso*-PA with increasing labelling time. Thus, in these *T. brucei* mutant parasites, which express approximately 10-fold more free GPIs than wild-type trypanosomes [39], similar lipid remodelling reactions may be active as in *T. congolense* procyclic forms.

DISCUSSION

The GPI lipid moieties of surface glycoconjugates from *T. brucei* and *T. congolense* procyclic forms have been known to consist exclusively of *lyso*-(acyl)PI species [6, 7, 11, 34, 35, 37, 41]. These results, which are based on chemical and mass spectrometric analyses, are consistent with the current scheme for GPI biosynthesis in procyclic form *T. brucei*, where *lyso*-(acyl)PI precursors are attached to protein ([11, 13, 46], reviewed by [12, 47]). However, prompted by our observations that in *T. brucei* procyclin null parasites [39] and *T. congolense* procyclic forms [35] at least a subpopulation of GPI anchors contain different lipid moieties, we re-investigated the lipid structures and biosynthesis of GPIs in procyclic form *T. congolense*.

A novel type of GPI lipid remodelling in *T. congolense* procyclic forms

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 de-acylated. Our cell-free system experiments using GDP-[^3H]Man demonstrated that membranes from *T. congolense* procyclics 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 PPI) appears to remain unmodified after its transfer to procyclins. 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 GPIs 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 inositolphosphoryl ceramide.

Based on our results, we propose the following model for GPI biosynthesis and processing in *T. congolense* procyclic forms (Fig. 7). In a first step, a tri-acylated GPI precursor (PP3-like) is transferred to GPI acceptor molecules. Such a precursor is likely heterogeneous in the type of fatty acids (*i.e.* C_{18:0}, C_{18:1} and C_{18:2}) 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 EPGENGTT procyclin, undergoes de-acylation at the *sn*-2 position of the glycerol backbone, forming GPI anchors with *lyso*-(acyl)PI species. In addition, our data suggest that a small 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 tandem mass spectrometry. Clearly, these results are in contrast to the previously published structures of the GARP and EPGENGTT procyclin GPI anchors, which showed *lyso*-(acyl)PI [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 this work, these structures could only be demonstrated because of the high sensitivity and precursor ion selection resolution of the *Resonance* 3D 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 to 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 hours). Stronger evidence for the presence of diacyl-GPI molecules in *T. congolense* procyclic forms came from the CID product ion spectra of the ion at m/z 810: both the EIS-MS/MS and the MALDI-QIT-TOFMS³ analyses of the diagnostic (MS^2) product ion at m/z 581 unambiguously identified the $[M - H]^-$ pseudomolecular ion m/z 810 as 1-stearoyl-2-*sn*-myristoyl-glycero-3-phospho-(1'-*myo*-inositol).

Interestingly, the diacyl-PI species characterized by mass spectrometry contain myristate, presumably at the *sn*-2 position of the glycerol backbone. This reinforces our recent finding that the GPI anchor of PRS is myristoylated and, in addition, suggests that some of the machinery involved in the addition of myristic acid to the VSG anchor in bloodstream form trypanosomes is likely to be active in the procyclic stage of *T. congolense* as well [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 catalyzed by PGAP1 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 by at least 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 using [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 little *lyso*-PIs are present at early time-points of labelling (Figs. 4 and 6), although this process could take place quite rapidly and, therefore, may 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 (Bütikofer, P., Greganova, E., M. Serricchio, Liu, Y-C, and Acosta-Serrano, A., manuscript in preparation). 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 to *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 knock out parasites express 10-fold more free GPIs as 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 data clearly demonstrate that *T. brucei* procyclic forms are capable of making diacyl-type GPI structures. Thus, we cannot rule out the possibility that remodelling of protein-bound GPIs 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 GPI precursors, *i.e.* PP1 for protein attachment and PP3 for free GPIs (Fig. S8).

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Figure legends

Figure 1: Schematic representation of the major GPI-anchored glycoconjugates on the surface of *T. congolense* procyclic and epimastigote forms. The lipid moieties of GARP [37], EPGENGTT procyclin [34] and PRS [38] are shown in detail; the structure of the CESP lipid anchor is unknown. While GARP is heavily modified with phosphoglycans, the presence of this modification in EPGENGTT procyclin is uncertain. Except for GARP, nothing is known about the structures of the GPI glycan cores of these molecules and, thus, the sequences shown are hypothetical. PRS is likely to be a GPI-anchored polysaccharide [35].

Figure 2: Identification of [^3H]-labelled GPI lipids. A, *T. brucei* and *T. congolense* Kilifi and Savannah procyclic forms ($0.5\text{--}1.0 \times 10^9$ 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 panel) or PI-PLC (right panel), partitioned between butan-1-ol and water, and analyzed by TLC using solvent system 1. Radiolabelled products ($20\text{--}50'000$ cpm) 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 GPIs. 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 ($40\text{--}60'000$ cpm) were analyzed 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 (see also A).

Figure 3: Characterization of [^3H]-labelled GPI lipids. *T. congolense* Savannah procyclic forms were labelled with [^3H]myristate for 6 or 48 h and extracted as described in Fig. 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 analyzed 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.

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

T. congolense Savannah (left panel) and *T. brucei* (right panel) 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 Fig. 2 and [³H]-labelled products released by GPI-PLD were analyzed by TLC using solvent system 1. The migration of PA and lyso-PA standards run on the same plate is indicated.

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 Figs. S1-S3). The ions at *m/z* 956 and 1048 are phospholipid contaminants also present in the last butanol wash before nitrous acid deamination (not shown). *B*, proposed structures of the most abundant PI moieties.

Figure 6: Incorporation of [³H]myristate into free GPIs of *T. brucei* EP/GPEET KO cells.

EP/GPEET KO parasites ($8-0.2 \times 10^8$ 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 GPIs were extracted with CMW (10:10:3, v/v/v), partitioned into the CMW_{aq} phase, and treated with GPI-PLD. Radiolabelled products were analyzed by TLC using solvent system 1 and visualized by fluorography. The lanes contain 4'200-8'200 cpm of labelled extracts.

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

See Discussion section for details.

Table 1: Suggested identities of the released phosphatidylinositol species from GPI-glycoconjugates present in the butanol extracts from *T. congolense* procyclic forms cultured for 6 and 48 hours.

m/z^a	Suggested fatty acid composition and locations			Suggested PI identity ^d
	R ₁ ^b	R ₂ ^b	R ₃ ^c	
810	C _{18:0}	C _{14:0}	OH ^e	1-stearoyl-2- <i>sn</i> -myristoyl-glycero-3-phospho-(1'- <i>myo</i> -inositol)
862	C _{18:0}	±C _{18:2} ^f	±C _{18:2} ^g	1-stearoyl-2-linoleoyl- <i>sn</i> -glycero-3-phospho-(1'- <i>myo</i> -inositol) and 1-stearoyl-2- <i>lyso</i> - <i>sn</i> -glycero-3-phospho-(2'- <i>O</i> -linoleoyl-1'- <i>myo</i> -inositol) ^h
864	C _{18:0}	±C _{18:1} ^f	±C _{18:1} ^g	1-stearoyl-2-oleoyl- <i>sn</i> -glycero-3-phospho-(1'- <i>myo</i> -inositol) and 1-stearoyl-2- <i>lyso</i> - <i>sn</i> -glycero-3-phospho-(2'- <i>O</i> -oleoyl-1'- <i>myo</i> -inositol) ^h
866	C _{18:0}	±C _{18:0} ^f	±C _{18:0} ^g	1-stearoyl-2-stearoyl- <i>sn</i> -glycero-3-phospho-(1'- <i>myo</i> -inositol) and 1-stearoyl-2- <i>lyso</i> - <i>sn</i> -glycero-3-phospho-(2'- <i>O</i> -stearoyl-1'- <i>myo</i> -inositol) ^h
1072	C _{18:0}	C _{14:0}	C _{18:2}	1-stearoyl-2- <i>sn</i> -myristoyl-glycero-3-phospho-(2'- <i>O</i> -lynolenoyl-1'- <i>myo</i> -inositol)

^a Detected as [M - H]⁻

^b R₁ and R₂ indicate the positions of the fatty acids on the glycerol backbone (*sn*-1 and *sn*-2, respectively).

^c Indicates acylation on the inositol ring.

^d In most cases the structures were deduced after analysis by negative-ion ESI-MS and ESI-MS-CID-MS, and MALDI-QIT-TOF-MS³ (supplementary Figs. S1-S5). PI nomenclature according to LIPID MAPS structure data base (http://www.lipidmaps.org/data/classification/LM_classification_exp.php)

^e Indicates unsubstituted glycerol-phosphate (most likely at the 2-position of the inositol ring). No evidence found for the presence of isobaric PI species.

^f Only present in diacyl-PI species, thus the 2-position of the inositol ring must be unsubstituted.

^g Only present in *lyso*-(acyl)PI species. In this case, the *sn*-2 position of the glycerol backbone is unsubstituted.

^h Mixture of isobaric PI species.

Figure 1

Figure 2

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20100229

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7