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The *Gup1* homologue of *Trypanosoma brucei* is a GPI glycosylphosphatidylinositol remodelase

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Summary

Glycosylphosphatidylinositol (GPI) lipids of *Trypanosoma brucei* undergo lipid remodelling, whereby longer fatty acids on the glycerol are replaced by myristate (C14:0). A similar process occurs on GPI proteins of *Saccharomyces cerevisiae* where Per1p first deacylates, Gup1p subsequently reacylates the anchor lipid, thus replacing a shorter fatty acid by C26:0. Heterologous expression of the *GUP1* homologue of *T. brucei* in *gup1*Δ yeast cells partially normalizes the *gup1*Δ phenotype and restores the transfer of labelled fatty acids from Coenzyme A to *lyso*-GPI proteins in a newly developed microsomal assay. In this assay, the Gup1p from *T. brucei* (tbGup1p) strongly prefers C14:0 and C12:0 over C16:0 and C18:0, whereas yeast Gup1p strongly prefers C16:0 and C18:0. This acyl specificity of tbGup1p closely matches the reported specificity of the reacylation of free *lyso*-GPI lipids in microsomes of *T. brucei*. Depletion of tbGup1p in trypanosomes by RNAi drastically reduces the rate of myristate incorporation into the *sn*-2 position of *lyso*-GPI lipids. Thus, tbGup1p is involved in the addition of myristate to *sn*-2 during GPI remodelling in *T. brucei* and can account for the fatty acid specificity of this process. tbGup1p can act on GPI proteins as well as on GPI lipids.

Introduction

Trypanosoma brucei is a protozoan parasite causing human sleeping sickness and a related animal disease in Africa. The surface of the bloodstream form of the parasite is coated by variant surface glycoproteins (VSGs), which are linked to the plasma membrane by diacylglycerol-based glycosylphosphatidylinositol (GPI) anchors (Ferguson *et al*., 1988). During biosynthesis, the GPI precursor is first synthesized with longer fatty acids, which subsequently get replaced by two myristates (C14:0). This is achieved through sequential deacylation and reacylation reactions and occurs before the GPI lipid is added to proteins. These so-called GPI lipid remodelling reactions start with the removal of the fatty acid from the *sn*-2 position of the diacylglycerol moiety, followed by the addition of myristate from myristoyl-Coenzyme A (CoA) (Masterson *et al*., 1990). A similar reaction sequence at the *sn*-1 position subsequently leads to the generation of glycolipid A, a dimyristoylated GPI lipid, which is attached to the VSG precursor in the endoplasmic reticulum (ER). Remodelling of GPI lipids in the *sn*-2 position of glycerol takes also place in another protozoan parasite, *Leishmania mexicana* (Ralton and McConville, 1998). Furthermore, GPI lipid remodelling has been described at the level of the protein-linked GPI anchors in mammalian cells (Tashima *et al*., 2006; Maeda *et al*., 2007) and in yeast (Sipos *et al*., 1997; Reggiori *et al*., 1997). Moreover, changes in the fatty acid profile at early stages of mammalian GPI lipid biosynthesis raise the possibility of additional remodelling reactions occurring before GPI lipids are added to proteins (Houjou *et al*., 2007).

As outlined in Fig. 1, mature GPI proteins of *Saccharomyces cerevisiae* contain C26:0 fatty acids, either as a constituent of an inositolphosphoceramide moiety (IPC/B) or in the *sn*-2 position of a phosphatidylinositol moiety (pG1). Yet, the primary GPI lipid added to newly synthesized proteins in the ER contains pG2, a phosphatidylinositol (PI) moiety of similar mobility on thin-layer chromatography (TLC) as the bulk of free PI of cell membranes. pG2 probably contains a diacylglycerol with conventional C16 and C18 fatty acids (Sipos *et al*., 1997; Schneiter *et al*., 1999). The fatty acid in the *sn*-2 position of glycerol is removed from the primordial anchor in a reaction dependent on Per1p, and C26:0 is added instead by an acylation step requiring Gup1p (Fig. 1) (Bosson *et al*., 2006; Fujita *et al*., 2006).

Gup1p is an ER membrane protein with multiple membrane spanning domains harbouring a motif that is characteristic of membrane-bound O-acyl-transferases

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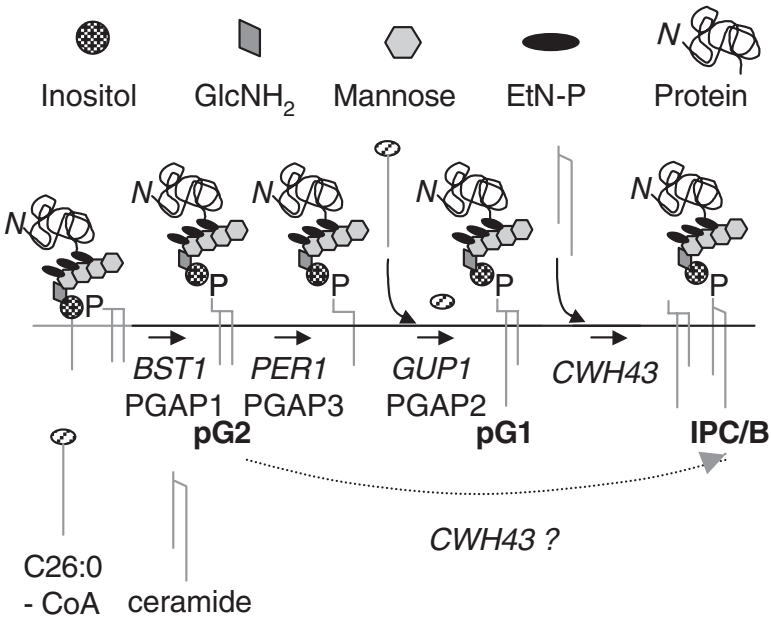

Fig. 1. GPI anchor lipid remodelling in the ER of *Saccharomyces cerevisiae.* Yeast genes implicated in the various steps are indicated in italics, while the corresponding mammalian genes are in non-italics. Anchors are designated according to the lipid moiety they release upon nitrous acid treatment. Bst1p generates pG2 type anchors, which are gradually transformed into pG1 and IPC/B type anchors over about 20–30 min (Sipos *et al*., 1997). Upon arrival in the Golgi apparatus, a small fraction of GPI anchors with a hydroxylated C26:0 fatty acid is generated (= IPC/C-type anchors, not shown). It is not proven that the small amounts of ceramide-containing anchors observed in *per1*Δ and *gup1*Δ mutants are made by Cwh43p (dotted arrow).

(MBOAT) (Hofmann, 2000). The biological function of yeast Gup1p depends on the active site histidine of the MBOAT motif (Hofmann, 2000; Bosson *et al*., 2006). *Gup1*Δ cells make normal amounts of GPI proteins but none of them contains pG1, the PI moiety with C26:0 in *sn*-2 (Fig. 1) and the cells also lack anchors containing IPC/B and IPC/C (Fig. 1). Instead, most mature GPI anchors of *gup1*Δ cells contain abnormal lipid moieties, which either are *lyso*-PI, or PI with conventional long-chain fatty acids, or polar, mild base resistant lipids. *GUP1* is highly conserved among fungi and protozoa and the *gup1*Δ phenotype is partially corrected by *GUP1* homologues of *Aspergillus fumigatus* and *Trypanosoma cruzi* (Bosson *et al*., 2006). Here we further characterize the phenotype of *gup1*Δ cells expressing heterologous Gup1 proteins, report for the first time on the acyltransferase activity of the *GUP1* homologue of *T. brucei* and present evidence that the latter is the first of the two postulated acyltransferases required for remodelling of GPI lipids in *T. brucei* bloodstream forms.

Results

The GUP1 homologue of T. brucei *partially corrects the abnormal phenotype of yeast* gup1Δ *cells*

Trypanosoma brucei contains an open reading frame of 609 amino acids, which over 510 amino acids is homologous to yeast *GUP1* (25% identical residues). This open reading frame, *tbGUP1*, was inserted into a centromeric vector behind the *GAL1,10* promoter for transfection into yeast. Western blot analysis demonstrated that cells harbouring pHAtbGUP1 expressed tbGup1p as a protein of the predicted molecular mass (please refer to Fig. S1A in *Supplementary material*). As shown in Fig. 2A, the expression of *tbGUP1* or a HA-tagged version thereof in *gup1*Δ cells partially corrected their calcofluor white hypersensitivity, as had already been found for the *GUP1* homologues of *A. fumigatus* and *T. cruzi* before (Bosson *et al*., 2006). Previous analysis also had shown that deletion of *GUP1* leads to the accumulation of the GPI protein Gas1p in its immature 105 kDa form in the ER, and that cells at the same time secrete the protein into the medium (Bosson *et al*., 2006; Fujita *et al*., 2006). The underlying delay in transport of Gas1p from ER to Golgi was slightly reduced by the overexpression of *tbGUP1*, because the relative amount of immature Gas1p relative to mature Gas1p was slightly reduced (Fig. S1B and C), but cells still secreted Gas1p into the medium (not shown). Expression of *tbGUP1* also partially corrected the reported loss of the bipolar bud site selection in diploid *gup1*Δ*/gup1*Δ cells (Ni and Snyder, 2001): Normal bipolar budding observed in only 6% of *gup1*Δ*/gup1*Δ cells, rose to 33% in *gup1*Δ*/gup1*Δ cells harbouring *tbGUP1*, but reached 86% in *gup1*Δ*/gup1*Δ cells complemented by yeast *GUP1* (not shown).

To understand the molecular bases of these phenomena, the *gup1*Δ cells expressing *tbGUP1* were labelled with [^{3}H]inositol and the GPI lipid moieties released from proteins were analysed by TLC. As previously reported (Bosson *et al*., 2006; Fujita *et al*., 2006), the non-complemented *gup1*Δ cells exhibit an abnormal pattern of GPI anchor lipids lacking the normal pG1 moiety and showing a severe reduction in the normal anchor inositol-phosphorylceramides IPC/B and IPC/C (Fig. 2B, lanes 2–4). Instead, *gup1*Δ cells have abnormal lipids comigrating with *lyso*-PI (Fig. 2B, lanes 4 and 12) and also contain

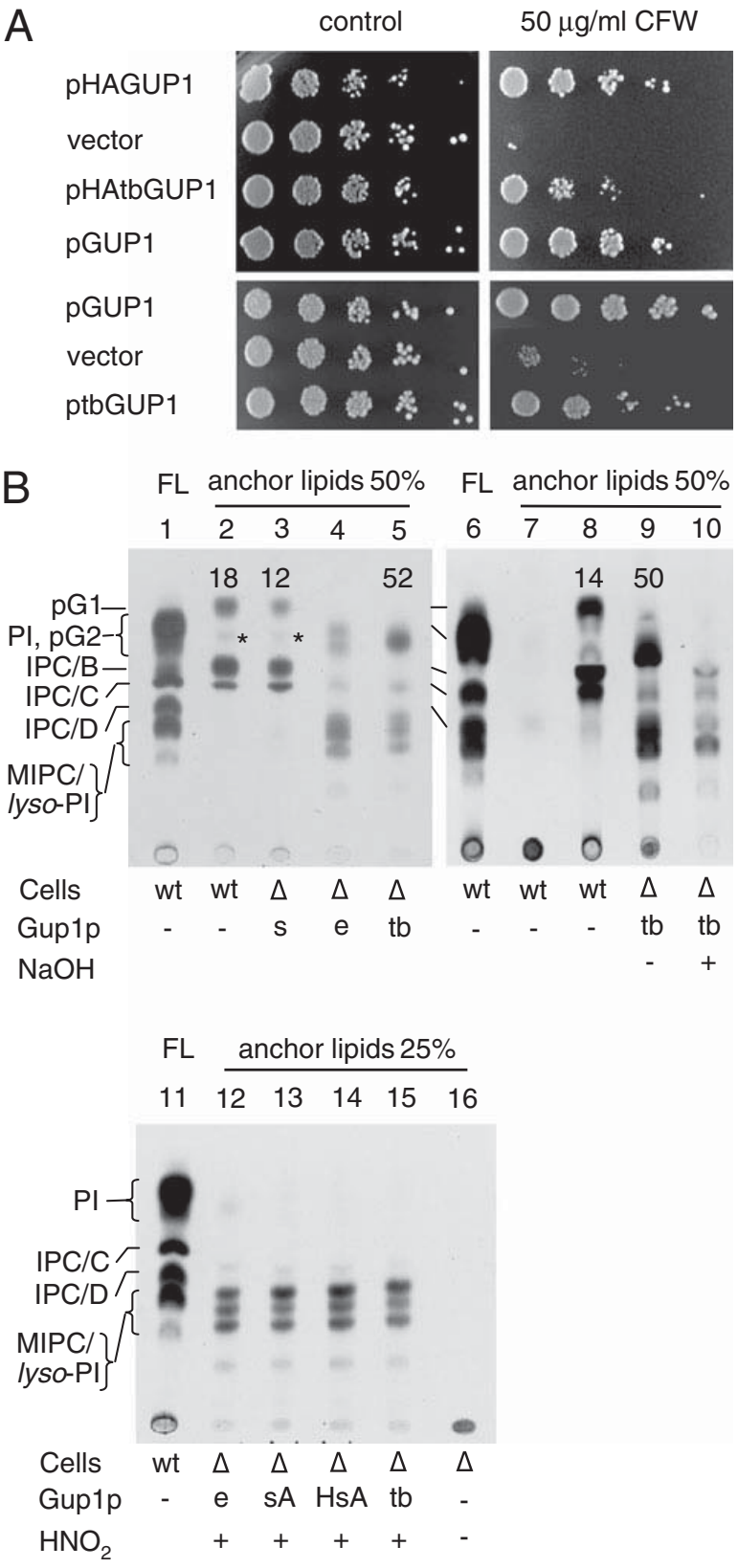

Fig. 2. A. *Gup1*Δ cells harbouring either the empty vector (pGREG505), pGUP1, pHAGUP1, pHAtbGUP1 or ptbGUP1 were plated on SGaa lacking leu and trp with or without 50 µg ml^{-1} of calcofluor white (CFW) and incubated for 3 days at 30°C.
B. BY4742 wild type (wt) or *gup1*Δ (Δ) cells containing either *scGUP1* on plasmid pHAGUP1 (s), or active site mutants thereof on pGUP1^{H447A} (sA) and pHAGUP1^{H447A} (HsA), or empty vector (e), or pHAtbGUP1 (tb) were grown to exponential phase in SGaa. Cells were labelled with [^{3}H]inositol; proteins were delipidated, GPI anchor peptides were prepared and partially purified over octyl-Sepharose eluting first with 25% (lanes 12–16), then 50% propanol (lanes 2–5, 7–10). Labelled lipid moieties were released from the peptides with nitrous acid and analysed by TLC/fluorography. Lanes 1, 6 and 11 contain free lipids (FL) of BY4742 wt cells, lanes 7 and 16 show anchor peptides before HNO_2 treatment. Some lipid anchor lipids were deacylated with mild NaOH treatment (+) or mock incubated (–). The asterisk (*) indicates pG2 (Fig. 1). Based on the radioscans, the radioactivity contained in the remodelled PI species expressed as percentage of total radioactivity present in the lane is indicated above the respective bands. Material from equivalent numbers of cells was deposited in lanes 2–5 and 12–16; lanes 4 and 12 as well as 5 and 15 show consecutive eluates from octyl-Sepharose of the same labelling reaction.

anchor lipids migrating just slightly less than the most abundant form of free PI (Fig. 2B, lane 1) (Bosson *et al.*, 2006). Overexpression of *tbGUP1* in *gup1*Δ cells leads to the appearance of a distinct band in the region of PIs, a band that migrates somewhat differently from the various PI species present in *gup1*Δ (Fig. 2B, lane 5 versus 4). This distinct band is mild base sensitive, as expected for a PI (Fig. 2B, lane 10 versus 9). On the other hand, a large fraction of GPI anchors of *tbGUP1*-complemented *gup1*Δ cells still migrate to the zone of *lyso*-PI (Fig. 2B, lanes 5 and 15).

These data are compatible with the view that, if transferred to yeast, tbGup1p may operate as a *sn*-2 specific acyltransferase adding a fatty acid to the *lyso*-GPI structures. Also, the migration on TLC of the resulting PI moiety suggests that tbGup1p catalyses the incorporation of a shorter fatty acid than scGup1p and that the species origin of the *GUP1* homologue may dictate the fatty acid that is added to anchors. This concept is further supported by the observation that the expression of the *GUP1* homologue from *A. fumigatus* in *gup1*Δ cells leads to the appearance of a mild base sensitive PI species that runs to the same position as the normal C26:0-containing pG1 made by scGup1p (Fig. 3A, lanes 4 and 5). Transfection of the *T. cruzi* homologue of *GUP1* into *gup1*Δ cells, although imparting partial resistance to calcofluor white (Bosson *et al.*, 2006), did not induce a substantial change of the lipid profile on GPI anchors except to slightly increase the ratio of PI/*lyso*-PI forms of the anchor (Fig. 3A, lanes 6 and 7). In another experiment tcGup1p caused the appearance of a distinct band in the region of free PIs that migrates somewhat differently from the PI species present in *gup1*Δ and *tbGUP1*-complemented *gup1*Δ cells (Fig. 3B, lanes 3–6). Importantly, expression of *afGUP1* in *gup1*Δ cells greatly stimulates the biosynthesis of the normal IPC/B and IPC/C anchor lipids (Fig. 3A, lanes 4, 5, 7). Thus, by allowing for the synthesis of pG1, *afGUP1* may create the obligatory precursor for ceramide remodelling. In contrast, the PI species made by tbGup1p allows for the synthesis of only limited amounts of anchors containing the normal IPC/B and IPC/C species (Fig. 2B, lane 10), and small amounts of IPC/B and IPC/C were also observed in many experiments in non-complemented *gup1*Δ cells (not shown). Moreover, the abnormal mild base resistant anchors migrating in the region of *lyso*-PI, which are typical of non-complemented *gup1*Δ cells (Bosson *et al.*, 2006), are no more seen in *gup1*Δ cells harbouring *afGUP1* (Fig. 3A, lanes 4 and 5), while they are still present in *gup1*Δ cells harbouring *tbGUP1* (Fig. 2B, lane 10). Thus, the data suggest that scGup1p and afGup1p generate anchor lipids, which efficiently are further processed towards IPC/B and IPC/C, whereas tbGup1p and tcGup1p fail to do so.

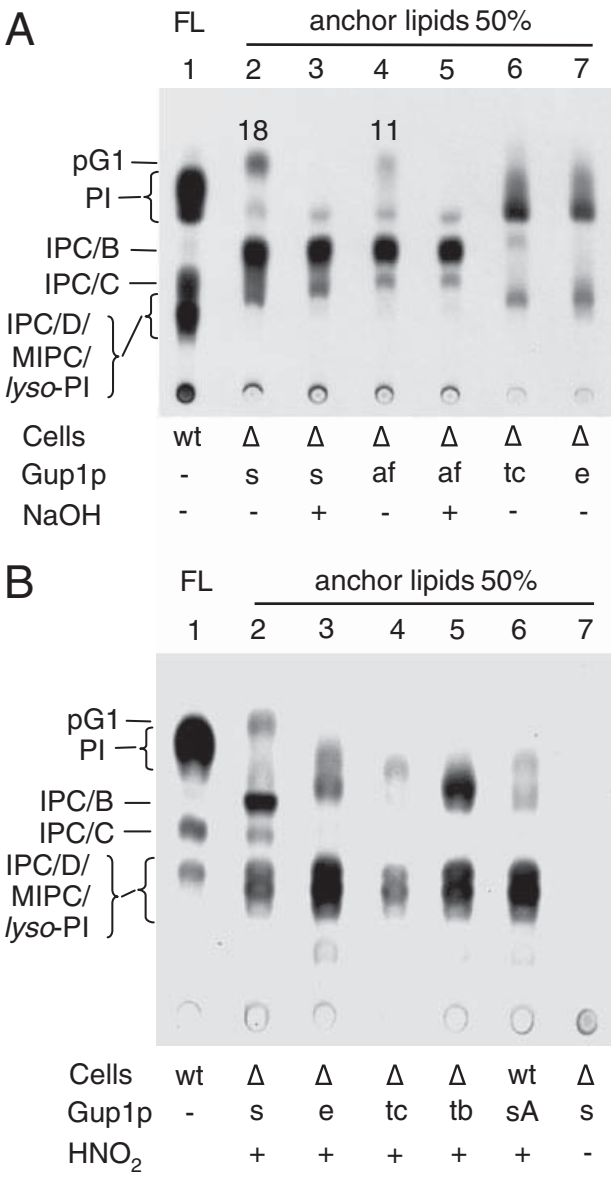

Fig. 3. A and B. BY4742 wild type (wt) or *gup1*Δ (Δ) cells containing Gup1 proteins from yeast on pHAGUP1 (s), pGUP1^{H447A} (sA), from *A. fumigatus* on pHAafGUP1 (af), from *T. cruzi* on pHAtcGUP1 (tc), from *T. brucei* on pHAtbGUP1 (tb) or containing empty vector (e) were grown to exponential phase in SGaa, labelled with [^{3}H]inositol, and anchor lipids were prepared and analysed as described for Fig. 2B.

tbGup1p operates the transfer of fatty acids onto lyso-*GPI proteins in an yeast microsomal remodelase assay*

In order to study the fatty acid specificity of heterologously expressed *tbGUP1*, we developed a yeast microsomal *in vitro* system allowing to assess the acylation of *lyso*-GPI proteins by Gup1 proteins. As can be seen in Fig. 4A, microsomes from *gup1*Δ cells expressing *tbGUP1* incorporated [^{3}H]myristate into proteins. In all experiments the

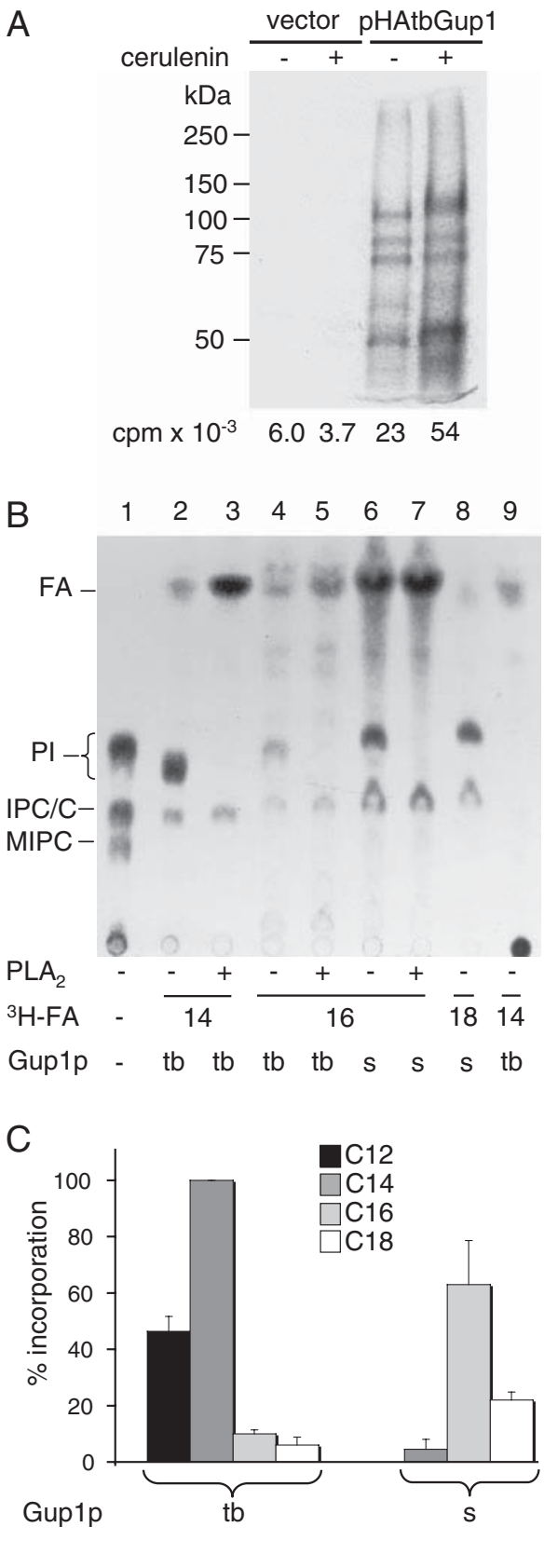

Fig. 4. A. *gup1*Δ cells harbouring empty vector or pHAtbGUP1 were grown overnight in galactose, supplemented with (+) or without (–) cerulenin during the last 3.5 h of culture, and were used for preparation of microsomes. Microsomes equivalent to 100 μg of microsomal proteins were incubated for 60 min with [^{3}H]C14:0, CoA and ATP. Labelled proteins were delipidated and purified by Concanavalin A-Sepharose affinity chromatography. Equivalent amounts representing 90% of each reaction were analysed by SDS-PAGE and fluorography. Ten per cent of each sample was used to determine the radioactivity in delipidated proteins as indicated at the bottom of each lane.
B. Microsomes from *gup1*Δ cells harbouring pHAtbGUP1 (tb) or pHAGUP1 (s) were labelled with [^{3}H]C14:0, [^{3}H]C16:0 or [^{3}H]C18:0 fatty acids (FA) as described for panel A. Proteins were delipidated, GPI anchor peptides were prepared and treated with nitrous acid to release labelled lipid moieties. Samples were further incubated with (+) or without (–) phospholipase A2 (PLA_2), and analysed thereafter by TLC in solvent 2 followed by fluorography. Aliquots of 10^5 c.p.m. of anchor peptides were processed for all lanes except lanes 4, 5 and 8, for which counts were to low. Lane 1 contains the free lipids of wt cells, lane 9 contains anchor peptides not treated with nitrous acid. Free fatty acids in lanes not treated with PLA_2 are not derived from GPI anchors but are non-incorporated free fatty acids, which were not removed during anchor peptide preparation.
C. The incorporation of label into proteins in two independent experiments of the same type as shown in panels A and B and using fatty acids of different length (C12:0 to C18:0) was quantified by counting the radioactivity in delipidated GPI proteins after Concanavalin A-Sepharose affinity purification (Guillas *et al*., 2000). The incorporation of [^{3}H]C14:0 by pHAtbGUP1-complemented microsomes was set as 100%, and results expressed as fraction thereof.

incorporation was enhanced two- to threefold when cells had been pre-incubated with cerulenin, a drug which blocks fatty acid biosynthesis because it inhibits the β-ketoacyl-ACP synthases of type I and type II (Awaya *et al*., 1975; Magnuson *et al*., 1993). In contrast, no incorporation of radioactivity into proteins occurred in microsomes from *gup1*Δ cells, whether or not pre-incubated with cerulenin (Fig. 4A, lanes 1 and 2). Microsomes from *gup1*Δ cells complemented with *scGUP1* incorporated fatty acids into the same proteins, whereby scGup1p-complemented microsomes only incorporated [^{3}H]C16:0 and [^{3}H]C18:0 fatty acids, but not [^{3}H]C14:0 into proteins (Fig. S2). The molecular masses of labelled bands in Fig. 4A correspond to those of immature GPI proteins in the ER, and not of GPI proteins residing in the Golgi or at the cell surface (Reggiori *et al*., 1997). To decide whether the labelled proteins indeed were GPI proteins, we tried to release the labelled lipid component using a protocol for the specific isolation of protein bound GPI lipids (Guillas *et al*., 2000). Indeed, 60% of counts present in the delipidated microsomal proteins were routinely recovered in the 'GPI anchor peptide' fraction, the radiolabel of this fraction could be recovered in the organic solvent phase after nitrous acid treatment, and could be analysed by TLC. As can be seen in Fig. 4B, lane 2, most of the labelled lipids generated by tbGup1p in the presence of [^{3}H]C14:0 migrated to a position corresponding to a relatively polar PI

and had similar mobility as the [^{3}H]inositol-labelled PI moiety present in GPI anchors generated by tbGup1p *in vivo* (Fig. 2B, lanes 5 and 9). Labelled lipids were only observed when peptides were treated with nitrous acid, which cleaves GPI structures with high specificity (Fig. 4B, lane 9 versus 2). The label of this polar PI could quantitatively be removed in the form of free fatty acids by PLA_2 treatment (Fig. 4B, lane 3), indicating that proteins had specifically been labelled in *sn*-2 of a phosphoglyceride. The polar PI was also completely eliminated by mild base treatment (not shown). These data indicate that tbGup1p enables *gup1*Δ microsomes to transfer [^{3}H]myristate to the *sn*-2 position of yeast *lyso*-GPI proteins. On the other hand, we were unable to demonstrate the acylation of free GPI lipids in microsomes from *gup1*Δ cells expressing *tbGUP1* (not shown), possibly, because all free GPI lipids of yeast carry a fatty acid on the inositol, which may prevent the release of fatty acids from the *sn*-2 position of the glycerol by Per1p.

tbGUP1 *shows a similar fatty acid specificity in yeast and trypanosomal microsomes*

The acylation of the *lyso*-GPI lipid θ in microsomes from *T. brucei* bloodstream forms has been reported to work best with C12:0-CoA or C14:0-CoA as substrates, whereas C16:0-CoA and C18:0-CoA are not utilized for *sn*-2 remodelling (Morita and Englund, 2001). We examined the fatty acid specificity of the reaction mediated by tbGup1p in yeast microsomes by adding labelled fatty acids of different length to the reaction. As shown in Fig. 4C, in microsomes from *gup1*Δ cells overexpressing tbGup1p, the acylation of proteins had a strong preference for myristate over C12:0, C16:0 or C18:0 fatty acids. In contrast, in microsomes from *gup1*Δ cells overexpressing yeast *GUP1*, the highest incorporation was achieved with [^{3}H]C16:0 and [^{3}H]C18:0 fatty acids as a substrate, whereby in some experiments C16:0 and C18:0 were incorporated equally well. Anchor lipids from *gup1*Δ microsomes labelled with [^{3}H]C16:0 and [^{3}H]C18:0 also yielded lipids migrating in the region of PI, from which the label could be released by PLA_2 (Fig. 4B, lanes 4–8). Microsomes from *gup1*Δ cells complemented with *afGUP1* or *tcGUP1* did not show any reproducible incorporation of fatty acids into *lyso*-GPI proteins (not shown). Also, [^{3}H]C22:0 (behenic acid) or [^{3}H]C22:0-CoA were not incorporated into proteins by microsomes containing any type of Gup1p (*scGUP1*, *tbGUP*, *afGUP1* or *tcGUP1*; not shown). This was surprising because the chemical analysis of anchor lipids (Fankhauser *et al*., 1993) and their migration in TLC (Fig. 3A) make it highly likely that scGup1p and afGup1p have a preference for very long chain fatty acids. A possible reason for the lack of incorporation of [^{3}H]C22:0 may be that very long chain fatty acids are difficult to solubilize in the absence of detergent. Another problem may derive from the fact that our *in vitro* system relies on the endogenous microsomal acyl-CoA synthase Faa1p, which has no activity with fatty acids longer than C18 (Knoll *et al*., 1994), and on Fat1p, the *in vitro* activity of which is extremely low in comparison with other acyl-CoA synthases (Zou *et al*., 2002). Thus, tritiated C22:0-CoA may be rapidly hydrolysed and the microsomal acyl-CoA synthases may not be able to reactivate the C22:0. On the other hand, the data indicate that at least scGup1p can use C16:0-CoA or C18:0-CoA as a substrate. Overall, the different fatty acid specificity of GPI protein acylation occurring in cells and in microsomes harbouring different Gup1 proteins indicates that the specificity of the process is not dictated by the lipid environment of yeast microsomes, but rather by the specific properties of the transfected Gup1p.

Downregulation of tbGUP1 *in* T. brucei *bloodstream forms by RNAi inhibits the attachment of myristic acid to* sn*-2 of the lipid θ intermediate*

To investigate the role of tbGup1p further, a vector harbouring a tetracycline-inducible RNAi for *tbGUP1* was transfected into *T. brucei* bloodstream forms (Wirtz *et al*., 1999). Even when RNAi was not induced, the *tbGUP1* mRNA could not be detected in Northern blots. We therefore used semiquantitative RT-PCR to assess *tbGUP1* mRNA levels. Quantification of the reaction products by agarose electrophoresis showed that the addition of doxycycline clearly decreased the amount of *tbGUP1* mRNA (Fig. S3). In addition, RNAi induced a small increase in the cell doubling time from 7.3 ± 0.3 h to 8.2 ± 0.3 h (mean values ± standard deviations using four different RNAi clones). Interestingly, membranes isolated from trypanosomes cultured in the presence of tetracycline for 144 h showed a pronounced defect in GPI lipid remodelling compared with membranes from control cells, as measured by the classical microsomal remodelling assay (Masterson *et al*., 1990). For this, membranes from thawed trypanosomes were pulse labelled with GDP-[^{3}H]Man and chased with non-radioactive GDP-Man. This procedure generates substantial amounts of GPI lipids C′, A′ and θ (Fig. 5A), but acylation of θ is inefficient, as long as microsomes are not supplemented with acyl-CoA or allowed to make acyl-CoA (Fig. S4). However, upon addition of a mixture of myristoyl-CoA, free CoA and ATP, lipid θ is rapidly acylated to form A″, A and C (Fig. S4; Fig. 5A). As can be seen in Fig. 5B, most of accumulated lipid θ is rapidly metabolized in membranes from cells not incubated with tetracycline (lanes 1–3), but this process is much slower in membranes from cells treated with tetracycline (lanes 4–6). In our hands substantial amounts of lipid θ were lost during butanol-water partitioning of labelled lipids, and back extraction of the butanol phase was therefore omitted in many

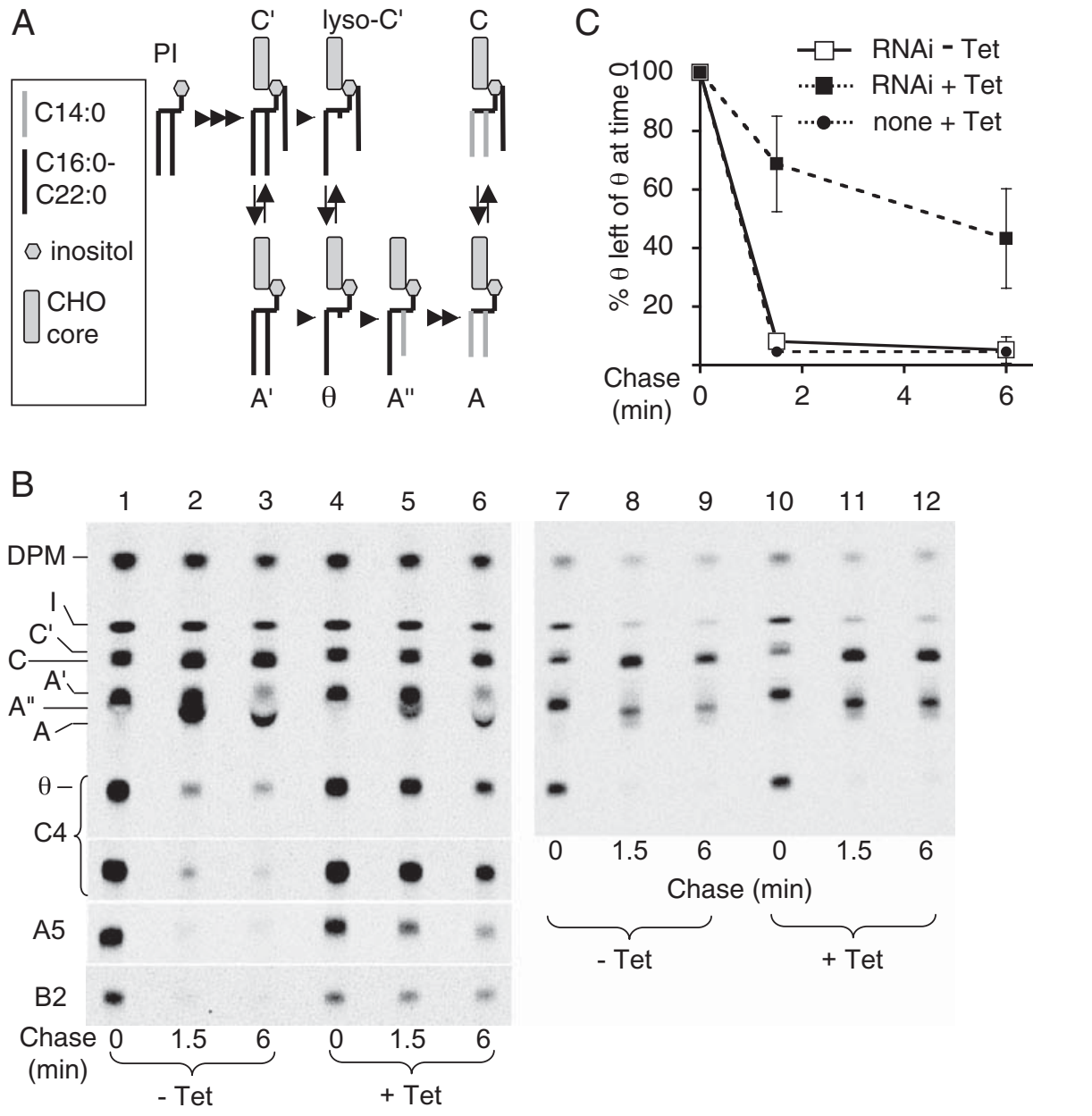

Fig. 5. A. Schematic representation of GPI lipid remodelling pathways in *T. brucei* (Guther and Ferguson, 1995; Morita *et al.*, 2000; Hong *et al.*, 2006). The rectangle denotes the carbohydrate (CHO) core with the bridging ethanolamine-phosphate group. None of the enzymes involved in this pathway have been genetically identified except for two inositol deacylases (Guther *et al.*, 2001; Hong *et al.*, 2006).
B. *T. brucei* clones (C4, A5 and B2) harbouring $RNAi_{tbGUP1}$ (lanes 1–6) and non-transfected cells (lanes 7–12) were grown for 144 h without tetracycline (lanes 1–3, 7–9) or with tetracycline (lanes 4–6, 10–12) in order to decrease the *tbGUP1* mRNA. Membranes were prepared from thawed cells and incubated with GDP-[^{3}H]mannose for 5 min, chased for 2 min with non-radioactive GDP-mannose and further chased for 0, 1.5 and 6 min in the presence of a cocktail of CoA, ATP and myristoyl-CoA. Lipids were extracted, desalted by butanol-water partitioning and run on TLC plates, which were then analysed in a molecular imager. The smaller panels below the top panel of lanes 1–6 show the TLC region containing lipid θ for a second experiment with clone C4, and for analogous experiments with clones A5 and B2. The incorporation of the radiolabel into lipids amounted to about 4% of added radioactivity on average and was not influenced by the presence of tetracycline in the preculture. All lipids except for dolicholphosphomannose (DPM) were mild base sensitive (not shown). I = is an uncharacterized GPI intermediate.
C. Lipids θ of the experiments shown in B as well as independent duplicate experiments for clones A5 and B2 were quantified using a Berthold radioscanner and expressed as percentage of lipid θ present at time 0. Then the percentages of lipid θ from the three clones (six experiments) analysed after culture without tetracycline (RNAi – Tet; panel B, lanes 1–3) or with tetracycline (RNAi + Tet; panel B, lanes 4–6) for each time point were averaged and plotted. Data of cells not harbouring the RNAi vector and grown in the presence of tetracycline were plotted in the same way (none + Tet; panel B, lanes 10–12).

experiments, albeit at the price of loosing the clear separation of lipids A′, A″ and A (Fig. 5B). Quantification of six independent experiments involving three independent clonal cell lines is shown in Fig. 5C. These data and numerous similar experiments performed in slightly different conditions and involving also one additional clone of *T. brucei* all showed that the downregulation of *tbGUP1* resulted in a marked delay of the disappearance of lipid θ, as well as a delay in the appearance of A″/A and C. The delay in processing of lipid θ was not due to any non-specific effect of tetracycline itself, because non-transfected cells cultured for 144 h in tetracycline showed normal remodelling of lipid θ (Fig. 5B, lanes 7–12). This strongly suggests that tbGUP1 is an acyltransferase capable of acylating lipid θ and that its presence is necessary for efficient acylation of lipid θ in this microsomal *in vitro* system.

When tested by immunofluorescence, *T. brucei* cells having been cultured for 6 days with tetracycline were found to express normal amounts of VSG (Fig. S5).

Discussion

Remodelling of GPI lipids or anchors is described in several species and may more firmly anchor GPI proteins in the lipid bilayer or direct them to the correct cellular compartment or membrane subdomain (Maeda *et al.*, 2007). Several independent lines of evidence indicate that *tbGUP1* is required for the remodelling of GPI lipids in *T. brucei* and that it encodes the *sn*-2 specific acyltransferase that catalyses the acylation of lipid θ to generate lipid A″. First, heterologously expressed *tbGUP1* can partially rescue the defect of yeast cells lacking yeast Gup1p, a *sn*-2 specific acyltransferase necessary for generating the remodelled GPI anchor lipid pG1 in yeast (Bosson *et al.*, 2006; Fujita *et al.*, 2006). Second, the presence of tbGup1p is necessary and sufficient for the *in vitro* transfer of [^{3}H]myristate to the *sn*-2 position of yeast *lyso*-GPI proteins to occur. Third, the specificity of tbGup1p in yeast microsomes closely matches the reported specificity of the acyltransfer reaction transforming lipid θ into A″ in microsomes of *T. brucei* (Morita and Englund, 2001). Finally, the downregulation of *tbGUP1* in *T. brucei* leads to a pronounced delay in the transformation of lipid θ into lipid A″.

Trypanosoma brucei contains two different GPI deacylation/reacylation pathways, one acting on lipid A′ to generate lipid A (Fig. 5A) and another one acting on both GPI proteins and lipid A, this latter pathway exchanging myristate for myristate. The two pathways have been called lipid remodelling and lipid exchange, respectively, and seem to operate in different subcellular compartments and are susceptible to different inhibitors (Masterson *et al.*, 1990; Buxbaum *et al.*, 1994; 1996). The finding that tbGup1p, when expressed in yeast *gup1*Δ cells, adds fatty acids to GPI-anchored proteins was surprising, as at the same time our data show that tbGup1p adds myristic acid to lipid θ in *T. brucei*. It is highly unlikely that in the absence of protein translation and sugar nucleotides our yeast microsomal system would support the biosynthesis of GPI lipids, which then would be added to proteins. Thus, it appears that, depending on the context, tbGup1p adds fatty acids to either lipids or proteins. This finding raises the possibility that *T. brucei* cells use *tbGUP1* for both processes, lipid remodelling and exchange. The fact that tbGup1p adds myristate to *lyso*-GPI anchors in the yeast microsomal remodelase assay is all the more remarkable as the GPI structure of yeast proteins shows significant structural differences with regard to the natural substrate of this enzyme, i.e. lipid θ: The yeast structure contains two additional ethanolaminephosphate groups on the α1,4- and the α1,6-linked mannoses and also carries an additional α1,2-linked mannose. Neither one of these three additional groups nor the presence of a protein on the ethanolaminephosphate of the third mannose seem to prohibit substrate recognition by tbGup1p, but the present data cannot exclude the possibility that the presence of these non-physiological elements would increase the Km for the GPI substrate. On the other hand, it is clear that the bulk of free PI of *T. brucei* does not undergo any tbGup1p-dependent deacylation/reacylation cycle, because free PI contains mostly polyunsaturated C18, C20 or C22 fatty acids in *sn*-2, while in *sn*-1 it contains C18:0, the same fatty acid as was also found in lipid A′ (Masterson *et al.*, 1990; Patnaik *et al.*, 1993; Doering *et al.*, 1994).

In spite of the marked biochemical defect induced by *tbGUP1* RNAi, the growth rate of trypanosomes in culture was not dramatically reduced. When tested by immunofluorescence, *T. brucei* cells having been cultured for 6 days with tetracycline were found to express normal amounts of VSG (Fig. S5). This suggests either that GPI lipid remodelling is not important for the stability and surface expression of VSG in culture or that the residual Gup1p activity of RNAi-suppressed cells was sufficient to allow for GPI proteins still to obtain remodelled GPI anchor lipids. A further possibility is that the lack of GPI lipid remodelling can be compensated *in vivo* by the myristate exchange pathway operating on proteins (Masterson *et al.*, 1990; Buxbaum *et al.*, 1994; 1996). It may be that *tbGUP1* knock-out strains will provide a better understanding of these issues.

Although the mature GPI proteins of *A. fumigatus* have so far been found to contain only ceramides, a cell free microsomal assay system shows that *A. fumigatus* initiates GPI biosynthesis by the addition of N-Acetylglucosamine to PI, not ceramide (Fontaine *et al.*, 2003; 2004). Thus, this pathogenic fungus may need to remodel its GPI anchors and, as *S. cerevisiae*, may require a

primary remodelling step to generate a pG1-like anchor lipid, which could be the preferred substrate for remodelases which introduce the ceramide. The data here support this concept by showing that *afGUP1* supports the generation of a pG1-like anchor lipid in *gup1*Δ cells *in vivo* and at the same time restores biosynthesis of anchors having IPC/B and IPC/C type lipid moieties (Fig. 3A, lane 4). It is conceivable that in certain organisms the fatty acid specificity of the remodelling process is dictated by the availability of acyl-CoAs rather than the acyltransferase itself. Nevertheless, the comparison of tbGup1p, tcGup1p and afGup1p in the very same heterologous environment of *S. cerevisiae* cells and microsomes suggests that at least part of the specificity of the reacylation of *lyso*-GPI anchors is encrypted in the sequence of *GUP1* genes themselves.

Based on short-term pulse chase experiments, pG2 has been proposed to be the primary anchor lipid (Sipos *et al*., 1997). It is interesting to note that the PI-type anchor moieties of *gup1*Δ cells are slightly different from the pG2 (Fig. 2B, lanes 2–4). One explanation to be considered is that in the absence of a dedicated Gup1 protein, the *lyso*-GPI proteins may be reacylated by Slc1p or Slc4p, which recently have been shown to acylate free *lyso*-PI (Benghezal *et al*., 2007; Jain *et al*., 2007; Riekhof *et al*., 2007; Tamaki *et al*., 2007). It appears that transfection of *tbGUP1* into *gup1*Δ cells makes pG2 or possibly the *SLC1*-generated PI forms disappear and generates a new type of PI on the anchors, while a large part of proteins retain the abnormal *lyso*-GPI anchor. It is conceivable that remodelling of *lyso*-GPI proteins by tbGup1p is less efficient than remodelling by scGup1p and afGup1p, not because of a low stability or a low catalytic activity of tbGup1p, but simply because the product of the remodelling reaction is very similar to the primary pG2 anchor so that yeast Per1p may continuously be destroying the product of tbGup1p. Thus, the presence of *tbGUP1* and *tcGUP1* may cause an only modest relative increase of fully acylated GPI anchors at the expense of *lyso*-GPI anchors and a correspondingly small improvement of GPI protein export out of the ER. Indeed, only a minor correction of the transport defect can be expected, as the Gas1p of *per1*Δ cells, probably having C18:1 instead of the normal C26:0 in this *sn*-2 position, strongly accumulates in the ER and as *per1*Δ cells are hypersensitive to calcofluor white (Fujita *et al*., 2006). In normal cells scGup1p will add C26:0 to *lyso*-GPI anchors, which may make them Per1p-resistant or allow for rapid transport to the Golgi, thus allowing them to escape Per1p. Further studies are required to resolve this issue.

Experimental procedures

Yeast strains, media and materials

Yeast strains *gup1*Δ (*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 gup1::kanMX4*) and BY4742 (*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*) were grown in minimal media supplemented with glucose (SD), raffinose (SR) or galactose (SG) and amino acids (aa) as described (Bosson *et al*., 2006). Budding patterns were evaluated by light microscopy in > 200 cells per strain after staining of cells with calcofluor white (Ni and Snyder, 2001). Cerulenin was from Sigma. Complete protease inhibitor cocktail tablets (11 873580 001) were from Roche Applied Science, Rotkreuz, Switzerland. Tritiated C12:0, C14:0, C16:0 and C18:0 fatty acids (60 Ci mmol^{-1}), [2–^{3}H]*myo*-inositol (20 Ci mmol^{-1}) and GDP-[6-^{3}H]mannose (40–60 Ci mmol^{-1}) were obtained from ANAWA Trading SA, Wangen, Switzerland. Suppliers of other chemicals have been described (Bosson *et al*., 2006).

Construction of plasmids

The construction of plasmids pGUP1, pHAGUP1 and pHAafGUP1, pHAtcGUP1 in pGREG505 and pGREG535, respectively, has been described previously (Bosson *et al*., 2006) and the protocol used to generate those plasmids was used here to amplify by PCR the open reading frame Tb10.61.0380 from genomic DNA of *T. brucei* and to insert it into pGREG505 and pGREG535, thus yielding ptbGUP1 and pHAtbGUP1. The *tbGUP1* insert was verified by sequencing.

Yeast labelling and analysis of GPI anchor lipid moieties

Methods for labelling of yeast cells with [^{3}H]inositol, extraction of proteins, isolation of anchor peptides, nitrous acid treatment and analysis of anchor lipids by TLC as well as enzymatic and chemical hydrolysis of anchor lipids and other methods have been described before (Guillas *et al*., 2000; Bosson *et al*., 2006). Unless indicated otherwise, the GPI anchor lipids eluted from Octyl-Sepharose with 50% propanol were analysed by TLC, whereby the bulk of the abnormal *lyso*-GPI anchor lipids of *gup1*Δ cells elute already with the 25% propanol fraction (Bosson *et al*., 2006). Plates were sprayed with EN3HANCE (PerkinElmer) and exposed to film for fluorography. Alternatively, the TLC plates were exposed to an image plate and developed by the BIORAD molecular Imager FX.

Yeast microsomal remodelase assay

*Gup1*Δ cells harbouring either pHAGUP1 or pHAtbGUP1 were grown in SRaa lacking leu at 30°C, transferred to SGaa for 16 h and further cultured in SGaa for 3.5 h in the presence of cerulenin (10 μg ml^{-1}). The cells were washed with 5 ml of 0.02% NaN_3 and spheroplasts were prepared using zymolyase. Then the spheroplasts were washed with buffer and resuspended in lysis buffer (100 mM sodium phosphate pH 7.4, 0.2 M sorbitol, 5 mM $MgCl_2$, 1 mM EDTA and protease inhibitor cocktail) and lysed by forcing them with a pre-cooled syringe 10 times through a 0.4 mm × 19 mm wide needle. The microsomes were filtered through a 2 μm pore filter (Millipore) to avoid contamination with intact cells, centrifuged at 15 000 *g* for 40 min at 4°C, and resuspended in lysis buffer. For *in vitro* remodelling microsomes equivalent to 100 μg of microsomal proteins were incubated 1 h at 30°C with a mix containing 1 mM ATP, 1 mM GTP, 1 mM CoA, 30 mM creatine phosphate, 1 mg ml^{-1} of creatine kinase and

25 μCi of tritiated fatty acid. GPI-anchored proteins were delipidated and purified by Concanavalin A-Sepharose affinity chromatography as described (Guillas *et al*., 2000). Bound proteins were either released from Concanavalin A-Sepharose by boiling in SDS sample buffer and analysed by SDS-PAGE/fluorography or were released from Concanavalin A-Sepharose using pronase and their radioactivity detected by scintillation counting (Guillas *et al*., 2000).

T. brucei *cell culture, RNAi construct, transfection*

For conditional expression of double-stranded RNAi bloodstream forms of *T. brucei*, we used strain NY-SM, which is derived from the monomorphic strain 427 and containing a vector-born T7 RNA polymerase and tetracycline-inducible repressor (Wirtz *et al*., 1999). Trypanosomes were cultured at 37°C and 5% CO_2 in HMI-9 medium containing 10% heat-inactivated fetal bovine serum (Gibco) (Hirumi and Hirumi, 1989) and 1 μg ml^{-1} G418 to retain the vector born T7 RNA polymerase and tetracycline-inducible repressor. RNA interference was achieved by introducing a stem loop construct on plasmid pALC14 (Bochud-Allemann and Schneider, 2002), which was kindly given to us by A. Schneider, University of Fribourg, Switzerland. Plasmid pALC14 contains a puromycin-resistance marker and represents a modified form of pLEW100, in which the fire fly luciferase gene is replaced by an irrelevant 460 bp stuffer sequence. A 399 bp fragment of *tbGUP1* was amplified using two separate PCR reactions using the following primers: 5′-GCTGTACTATTCCTTAAGC TTCCTTTGGCTG-3′ and 5′-CGTGATTGTAAGTCTAGAAA CACTGACCATTCC-3′ for the upstream fragment, and 5′-GCTGTACTATTCCTTGGGATCCCTTTGGCTGAAGT-3′ and 5′-CGTGATTGTAAGTCCTCGAGCACTGACC-3′ for the downstream fragment. The two fragments were inserted in opposite direction into pALC14 to yield pAS0380, which thus produces a double-stranded RNAi of 399 nucleotides corresponding to nucleotides 1202–1601 of the open reading frame Tb10.61.0380. After stable transfection of pAS0380 into bloodstream form trypanosomes, transfectants were selected in 24 well plates with 0.1 μg ml^{-1} puromycin. For the preparation of membranes, trypanosomes were harvested at a density of 5×10^5–2×10^6 cells ml^{-1}, pelleted, snap-frozen in liquid N_2 and stored at –70°C.

T. brucei *microsomal remodelase assay*

Hypotonic cell lysate was prepared by resuspending frozen cells in H_2O supplemented with 1 μg ml^{-1} leupeptin and 0.1 mM of TLCK. Membranes were then washed twice with 10 vols of HKMTL buffer as described (Hong *et al*., 2006), were washed two more times with HKMTL supplemented with 1 mM dithiothreitol and 0.8 μg ml^{-1} of tunicamycin and resuspended at 10^9 cell equivalents ml^{-1} in the same buffer, without any addition of $MnCl_2$ in most of the experiments. Membranes from 1.5×10^7 cells were incubated with GDP-[6-^{3}H]mannose and 1 mM UDP-GlcNAc for 5 min at 37°C followed by a chase of 2 min with 1 mM GDP-mannose. Remodelling was initiated by adding a mixture of 50 μM C14:0-CoA, 200 μM of ATP and 200 μM of CoA and further incubation for either 0, 1.5 or 6 min as described (Morita *et al*., 2000). The reaction was terminated and products analysed by TLC as described (Hong *et al*., 2006) except that the back extraction of combined butanol phases with water was occasionally omitted to prevent the loss of lipid θ.

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