Covalent modification with lipid can target cytosolic proteins to biological membranes. With intrinsic membrane proteins, the role of acylation can be elusive. Herein, we describe covalent lipid modification of an integral membrane glycosylphosphatidylinositol-specific phospholipase C (GPI-PLC) from the kinetoplastid Trypanosoma brucei. Myristic acid was detected on cysteine residue(s) (i.e. thiomyristoylation). Thiomyristoylation occurred both co- and post-translationally. Acylated GPI-PLC was active against variant surface glycoprotein (VSG). The half-life of fatty acid on GPI-PLC was 45 min, signifying the dynamic nature of the modification. Deacylation in vitro decreased activity of GPI-PLC 18–30-fold. Thioacylation, from kinetic analysis, activated GPI-PLC by accelerating the conversion of a GPI-PLC-VSG complex to product. Reversible thioacylation is a novel mechanism for regulating the activity of a phospholipase C.

Lipid modification of proteins appears to occur in most eukaryotes. Three general classes of covalent modification have been documented (i.e. N-acylation, isoprenylation, and thioacylation) (reviewed in Ref. 1). Fatty acids, depending on their length, are linked to proteins by one of two bonds; long chain fatty acids (e.g. palmitate) are esterified to cysteines (i.e. thio-palmitoylation) in varying positions along the polypeptide (see Refs. 2 and 3 for reviews). (On occasion, long chain fatty acids occur in amide linkage to lysine (4).) On the contrary, shorter chain fatty acids (e.g. myristate) have been found exclusively in amide linkage to N-terminal glycine (N-acylation) (5).

Trypanosoma brucei, the causative agent of African trypanosomiasis (sleeping sickness), is a protozoan parasite, which in the bloodstream of a mammal is covered with about 10^7 molecules of VSG. With a turnover number (k_{cat}) of 144 min^{-1} (9), there appears to be sufficient enzyme intracellularly to cleave all of the VSGs within a few minutes. However, cleavage of VSG in living T. brucei is not a major catabolic pathway, although purified GPI-PLC cleaves VSG efficiently in vitro (9). These observations suggest, among other possibilities, that (i) GPI-PLC may not have access to VSG in a living cell and/or (ii) GPI-PLC is not enzymatically active in vivo. GPI-PLC has been immunolocalized to the cytoplasmic leaflet of intracellular vesicles (10), apparently sequestered, topologically, from VSG which is attached to the outer (exoplasmic) leaflet of the plasma membrane. Such localization studies support the former hypothesis, although the latter cannot be ruled out completely. Biosynthesis of GPIs is initiated on the cytoplasmic side of the endoplasmic reticulum (11), implying that GPI intermediates probably co-localize with GPI-PLC on the cytoplasmic side of cellular membranes in T. brucei. This assertion is supported by two studies involving the stable transfection of a cDNA encoding T. brucei GPI-PLC into the related protozoan parasites Leishmania major and Trypanosoma cruzi. First, L. major expressing GPI-PLC acquires a GPI-negative phenotype resulting apparently from cleavage of protein-GPI intermediates by the enzyme (12). Second, in T. cruzi GPI-PLC causes a GPI deficiency that is associated with failure of the parasites to sustain division of the cell nucleus (13). Given these observations, it is imperative to examine why GPI-PLC in its native environment (i.e. in T. brucei) does not cause a GPI deficiency.

As part of efforts aimed at unraveling the mechanisms that regulate activity of GPI-PLC in T. brucei, we investigated the possibility that the enzyme, itself an integral membrane protein, had a lipid modification. Herein, we demonstrate that GPI-PLC is covalently modified with myristic acid in a thioester linkage. The observation is the first example of acylation of a GPI-PLC in vivo. The esterified fatty acids modulated activity of this PLC, proving that phospholipases may be regulated by covalently attached lipids.

**EXPERIMENTAL PROCEDURES**

**Cell Types/Strain**

*T. brucei* ILTat 1.3 was harvested from the blood of infected rats by cardiac puncture. Buffy coats were prepared and parasites purified by DE52 chromatography (14).

**Materials**

[9,10-3H]Myristate and Hyperfilm-MP were from Amersham Pharmacia Biotech. [9,10-3H]Palmitate and [11,12-3H]laurate were from American Radiolabeled Chemicals. Hydroxylamine was obtained from Sigma. Cycloheximide was from Calbiochem (San Diego, CA). Protease inhibitors were obtained from Boehringer Mannheim. Entensify and ENHANCE were from DuPont. MeltiLex was from Amersham Pharmacia Biotech, and Uniplate RPS-F silica gel (20 × 20 cm) was from Analtech (Newark, DE). Fatty acid-free bovine serum albumin was from Life Technologies, Inc., and DE52 was from Whatman (Hillsboro, OR). p-Nitro blue tetrazolium and 5-bromo-4-chloroindolyl phosphate were purchased from Bio-Rad. All other reagents were from Sigma.
Metabolic Labeling

A pellet of $2 \times 10^8$ purified T. brucei was resuspended in 20 ml of pre-warmed labeling media (RPMI 1640 containing 20 mM HEPES, pH 7.4). Metabolic labeling was initiated by addition of 400 $\mu$Ci of [9,10-3H]myristic acid (51.0 Ci/mmol) or [9,10-3H]palmitic acid (60 Ci/mmol) or [11,12-2H]arachidic acid (60 Ci/mmol) complexed with fatty acid-free bovine serum albumin (1 mg) in 40 $\mu$l of PBS (140 mM NaCl, 3 mM KCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, pH 7.4). Cells were incubated at 37°C for 1 h, harvested by centrifugation at 14,000 $\times$ g for 10 min, and washed by resuspension in fresh labeling medium and re-centrifugation.

Pulse-Chase

Parasites ($7 \times 10^6$) were labeled with [9,10-3H]myristate (1.4 mCi). The cells were washed and resuspended in 14 ml of fresh labeling medium and incubated for 60 min at 37°C. Two ml aliquots (106 cells) were harvested every 10 min, washed with PBS, and frozen at $-80^\circ$C until use.

Cycloheximide Treatment

After metabolic labeling of $10^6$ cells with 200 $\mu$Ci of [9,10-3H]myristate, the parasites were washed, resuspended in 5 ml of fresh labeling medium containing 1.4 mM cycloheximide, and incubated for 15 min at 37°C. Cells were then harvested, washed with PBS, and frozen at $-80^\circ$C until use. In another set of experiments, $10^6$ cells were pre-treated with 1.4 mM cycloheximide in labeling medium for 15 min at 37°C prior to addition of 200 $\mu$Ci of [9,10-3H]myristate-bovine serum albumin complex. Labeling was continued for 1 h. Control cells were labeled with [9,10-3H]myristate for 1 h, washed, and incubated at 37°C for 15 min. Parasites were washed with PBS and the resulting pellets stored at $-80^\circ$C until use.

Immunoprecipitation of GPI-PLC

Cells ($10^6$) were resuspended in 1 ml of hypotonic lysis buffer (10 mM sodium phosphate, 1 mM EDTA, pH 8) containing a protease inhibitor mixture (PIC). [PIC consisted of leupeptin (2.1 $\mu$g), N-tosyl-l-lysine chloromethyl ketone (0.1 mM), antipain dichloride (20 $\mu$g), aprotinin, and PIC). The lysate was incubated on ice for 30 min. One microliter of mc2A6-6 ascites fluid was added to the detergent-solubilized pellet (107 cell eq), and the sample was then added to monoclonal antibody 2A6-6, which was in turn added to protein A-Sepharose (in 100 $\mu$l aliquot of 1:1000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG). The mixture was subjected to centrifugation, followed by centrifugation at 14,000 $\times$ g for 10 min, and washed by resuspension in fresh labeling medium and re-centrifugation.

Deacylation of GPI-PLC

Immunopurified [3H]GPI-PLC or proteins from [3H]myristate-labeled T. brucei were resolved by SDS-PAGE and transferred to Immobilon P. The membrane was soaked in 200 ml of one of the following solutions: 1 M NH$_4$OH (adjusted to pH 7 with 10 n NaOH), 0.2 M KOH, and 1 M Tris-HCl, pH 7. Each solution was incubated at 30°C with shaking. These solutions were replaced three times (every 30 min), after which the membranes were incubated overnight in 200 ml of the same solution. The membranes were air-dried, coated with MeltiLex wax, and radioabeled proteins detected by fluorography.

In Gel Deacylation of GPI-PLC—Immunopurified [3H]GPI-PLC was electrophoresed on an SDS-PAGE. Gel slices containing the radiolabeled bovine serum albumin complex were excised, sliced, and placed in a 1:5 ml microcentrifuge tube (approximate volume of gel was 400 $\mu$l). After rinsing with 30 ml PBS, the gel slice was deacylated by adding 1 ml of neutral hydroxylamine by incubation at 30°C. At 20-h intervals, the gel suspension was centrifuged at 14,000 $\times$ g for 1 min, and the supernatant was discarded. The incubation and centrifugation steps were repeated five more times, and the supernatants were pooled and dried in vacuo. Released [3H]fatty acids were identified as described below.

Decaylation of Native [3H]Acylated GPI-PLC and [3H]Myristoylated mVSG—A complex of [3H]acylated GPI-PLC (from 4 $\times 10^6$ cells) adsorbed to monoclonal antibody 2A6-6, which was in turn bound to protein A-Sepharose (in 100 $\mu$l) was resuspended in 250 $\mu$l of 1 M NH$_4$OH, pH 7.0. The suspension was incubated at 30°C with shaking, followed by centrifugation at 14,000 $\times$ g for 1 min, and with-drawal of the supernatant. Two more deacylation treatments were performed. Eluates were pooled, and 54 $\mu$l of concentrated HCl added, to acidify the solution. [3H]mVSG was deacylated to provide cell-derived [3H]mVSG as a control in the TLC analysis. Approximately 300,000 dpm of purified protein was incubated at 30°C for 1 h with 900 $\mu$l of 0.2 M KOH, and the solution was acidified with 20 $\mu$l of concentrated HCl.

Western Blots

The Immobilon P membrane was first soaked in blocking solution (1% (v/v) Tween 20, 10% (v/v) newborn calf serum, 13% (v/v) glycerol, 18% (v/v) As-g-glucose in PBS) for 1 h at room temperature with shaking. This was followed by 1 h incubation in blocking solution containing a rabbit polyclonal antibody raised against GPI-PLC polypeptide (R1823), at a 1:3000 dilution. The membrane was then washed three times (10 min each) with PBS followed by incubation for another hour with 1:1000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG (100 $\mu$l). After repeated, the membranes were incubated twice (10 min each) in alkaline phosphatase reaction buffer (75 mM NaCl, 75 mM Tris-HCl, pH 9.5, and 3.8 mM MgCl$_2$). Antigens were visualized in alkaline phosphatase reaction buffer, using 5-bromo-4-chloroindolyl phosphate and p-nitro blue tetrazolium chloride as substrates.

SDS-PAGE and Fluorography

Immunoadsorbed [3H]acylated GPI-PLC (approximately 50 $\mu$l) was eluted by heating the protein A-Sepharose-antibody complex in 25 $\mu$l of a modified 2.5 $\times$ SDS-PAGE sample buffer. A SDS-PAGE buffer lacking Tris was used. We found that presence of Tris base in SDS-PAGE sample buffer deacylated GPI-PLC when heat (90°C, 5 min) was applied. Eluted samples were centrifuged for 3 min at 14,000 $\times$ g (room temperature). A 20-$\mu$l aliquot of the supernatant was analyzed by SDS-PAGE (14% minigel) (Bio-Rad). The gel was soaked in Entensify (DuPont), dried, and exposed to pre-flashed Hyperfilm-MP at $-80^\circ$C. Autoradiograms were transferred to Immobilon P with a Trans-Blot semi-dry cell (Bio-Rad). It was operated for 2 h at 20 V at 25°C and 0.5 A in 48 ml Tris, 39 ml glycerin, 20% (v/v) methanol, pH 9.2, containing 1.3 mM SDS. The Immobilon P membrane was coated with molten MultiLex wax, air-dried, and exposed to pre-flashed Hyperfilm-MP at $-80^\circ$C.
Identification of Fatty Acids by Reverse-phase High Performance Thin Layer Chromatography

Uniplate RPS-F silica gel (20 x 20 cm) were used with chloroform: methanol:water (15:45:3, v/v/v) as the mobile phase (16). The developed HPTLC plate was air-dried, sprayed with EN'HANCE, and FAMES detected by fluorography. Standard FAMES of [3H]laurate, [3H]myristate, [3H]palmitate, and [3H]stearate were prepared from the corresponding free fatty acids as described above.

Decylation of GPI-PLC and Determination of Phospholipase C Activity

GPI-PLC (from 2 x 10^6 cells of a hypotonic lystate of T. brucei) was adsorbed to monoclonal antibody 2A6-6 (mc2A6-6) and washed as described above. The immunoadsorbent was incubated in 5 ml of 1 mM neutral hydroxylamine at 30°C for 40 min with shaking. One-ml aliquots (approximately 2 μg of GPI-PLC) were withdrawn after 0, 10, 20, 30, and 40 min and centrifuged at 14,000 x g for 1 min. Recovered beads were washed three times in 3 ml of PBS. GPI-PLC was eluted from the column with 100 μl of 50 mM Tris-HCl, pH 12, containing 0.1% Nonidet P-40, into an equal volume of chilled (0°C) 1 mM Tris-HCl, pH 6.0, containing 1.0% Nonidet P-40 to neutralize the elution buffer.

Enzyme activity in the eluate was assayed as follows. A reaction mixture containing 2 μg of [3H]myristate-labeled VSG in 25 μl of AB (50 mM Tris-HCl, 5 mM EDTA, 1% Nonidet P-40) was assembled on ice (in a 1.5-ml microcentrifuge tube). To this mixture, several dilutions (in AB) of the eluate from the mc2A6-6 protein A-Sepharose column was added (the objective being to obtain values within the linear range of the assay (9)). This reaction mixture was incubated at 37°C for 30 min and terminated by vortex mixing with 500 μl of water-saturated n-butanol (at room temperature). Phases were separated by centrifugation (12,000 x g, 1 min, 25°C), and enzyme activity was quantified by measuring the amount of [3H]dimyristoylglycerol released into the upper butanol phase (9).

Kinetic Analysis of Acylated and Decylated GPI-PLC

To obtain GPI-PLC for kinetic analysis, the immunoadsorption protocol was modified to reduce contamination of the purified enzyme with antibody leaking from the monoclonal antibody column. GPI-PLC (from 2.5 x 10^6 parasites) was adsorbed to a 100-μl suspension of mc2A6-6 that had been chemically cross-linked to protein A-Sepharose (9). The complex was treated with 1 mM neutral hydroxylamine or PBS (see above, “Decylation of Native [3H]-Acylated GPI-PLC and [3H]-Myristoylated mVSG”) for 40 min. GPI-PLC was eluted from the immune complex as described above. Amount of GPI-PLC that cleaved approximately 20% of [3H]myristate-labeled VSG was determined empirically and used in the kinetic analysis.

Reaction mixtures were assembled on ice as described above with the exception that the AB buffer contained 0.1% Nonidet P-40. VSG cleavage was allowed to proceed for 15 min. Lineweaver-Burk plots were used to determine the Michaelis constant (K_m) (Table 1). VSG was assumed to be a dimer in the calculation of substrate concentration. For purposes of determining the turnover number (k_cat), protein concentration of eluates from the mc2A6-6 column was determined by quantitative Western blots using known amounts of purified recombinant GPI-PLC as standards.

RESULTS

GPI-PLC Is Esterified with Myristic and Palmitic Acids—Metabolic labeling of T. brucei with [3H]myristate (C_{14}), [3H]palmitate (C_{16}), or [3H]laurate (C_{12}) led to incorporation of radiolabel into GPI-PLC detected after SDS-PAGE (Fig. 1). This observation suggested that a fatty acid might be covalently bound to GPI-PLC. Lipids are covalently attached to proteins by one of three general mechanisms as follows: (i) through an amide bond (e.g. N-acylation), (ii) by esterification, as in fatty acid palmitoylation; or (iii) in an ether linkage (e.g. S-isoprenylation). To define the link between the acyl group and GPI-PLC, attempts were made at decylation after electrophoresis of purified [3H]-acylated GPI-PLC to Immobilon P membrane. Reagents tested were 0.2 M KOH (pH ~ 13), which cleaves both oxysterols and thioesters, 1 M NH₂OH·HCl, pH 7 (neutral hydroxylamine), which can specifically cleave thioesters (17), and 1 M Tris-HCl, pH 7, a control. Cleavage (by de-O-myristoylation) of a GPI anchor (on VSG) was studied as a control for the effectiveness and specificity of these reagents.

Neutral Tris-HCl failed to decylate either VSG (Fig. 2, lane 1) or GPI-PLC (Fig. 2, lane 2). Hydroxylamine, on the other hand, decylated GPI-PLC (Fig. 2, lane 6) but did not decylate VSG (Fig. 2, lane 5). These observations indicate that the acyl moiety on GPI-PLC is present in a thioester link. Consistent with the specificity of hydroxylamine the oxy-esterified [3H]myristate on VSG was left intact, apparently. KOH decylated GPI-PLC (Fig. 2, lane 4) confirming that the fatty acid was indeed esterified to GPI-PLC. VSG was decylated with KOH (Fig. 2, lane 3). In another control experiment, neutral hydroxylamine did not release [3H]myristate from purified [3H]myristate-labeled VSG in aqueous solution (data not presented).

Metabolic labeling of GPI-PLC with three fatty acids (i.e. [9,10-3H]palmitate (C_{16}), [9,10-3H]palmitate (C_{14}), and [11,12,3H]laurate (C_{12})) (Fig. 1) raised the possibility that the enzyme was heterogeneously acylated. To address this issue, the fatty acid on GPI-PLC was characterized. Cells were metabolically labeled with [9,10-3H]myristate; GPI-PLC was immunoadsorbed to protein A-Sepharose and decylated with neutral hydroxylamine. Methyl esters of the released fatty acids were generated and analyzed by reversed phase-high performance thin layer chromatography (RP-HPTLC)/fluorography (Fig. 3, lane 2). Using fatty acid methyl ester standards (Fig. 3, lane 1), myristic and palmitic acids were detected (Fig. 3, lane 2). The ratio of the myristate:palmitate was 3:1, as determined by laser scanning densitometry of the fluorograms. When the parasites were metabolically labeled with [3H]palmitate, myristate and palmitate were detected on GPI-PLC (Fig. 3, lane 1). Although the efficiency of labeling the enzyme was reduced significantly (data not presented). Together these data suggest that T. brucei metabolizes fatty acids before their incorporation into GPI-PLC.
acids on GPI-PLC as myristic acid was in error. For this purpose, purified VSG was deacylated with KOH, and the released fatty acid was converted to the methyl ester and compared with the FAMES from lipids of GPI-PLC. The VSG fatty acids were correctly identified as myristic acid (Fig. 3, lane 3) (18). More importantly, the VSG fatty acid comigrated with the myristate from GPI-PLC (Fig. 3, lane 2). Thus, the assignment of the faster migrating fatty acid species in the GPI-PLC sample as myristate is not an artifact caused by spurious migration of a longer chain fatty acid on the HPTLC plate.

Co- and Post-translational Acylation of GPI-PLC—To investigate the temporal order of GPI-PLC acylation with regard to protein synthesis, cycloheximide (CHX), an inhibitor of T. brucei protein synthesis, was used in experiments in which metabolic labeling with [3H]myristate was attempted before or after addition of CHX. When the trypanosomes were labeled with [3H]myristate prior to CHX treatment (CHX-II), the inhibitor appeared to have no effect on acylation of GPI-PLC within that time frame studied (Fig. 4A, compare lane 1 to lane 3). However, if the parasites were treated with cycloheximide (CHX-I) prior to the addition of [3H]myristate, acylation of GPI-PLC was inhibited partially (Fig. 4A, lane 2), compared with the control (Fig. 4A, lane 1). Approximately 50% of the lipid modification

![Image](image1.png)

**Fig. 2.** GPI-PLC is thioacylated. GPI-PLC immunoprecipitated from [3H]myristate-labeled cells (5 × 10⁸) and total acylated membrane proteins (10⁷ cell eq) from T. brucei were resolved by SDS-PAGE (14% minigel). The proteins were electro-transferred to Immobilon P. The membranes were treated with 1 M Tris-HCl, pH 7 (lanes 1 and 2), 0.2 M KOH (lanes 3 and 4), or 1 M NH₄OH, pH 7 (lanes 5 and 6). Lanes 1, 3, and 5 contain total acylated membrane proteins, and lanes 2, 4, and 6 contain GPI-PLC immunoprecipitates. Hyperfilm (Amersham Pharmacia Biotech) was developed after gels were exposed for either 18 h (lanes 1 and 5) or 7 days (lanes 2–4, and 6).

![Image](image2.png)

**Fig. 3.** GPI-PLC is modified by both myristate and palmitate. GPI-PLC was immunopurified from [3H]myristate-labeled 4 × 10⁹ T. brucei and deacylated with neutral hydroxylamine (see "Deacylation of Native [3H]-Acylated GPI-PLC and [3H]-Myristoylated mVSG" under "Experimental Procedures"). Purified [3H]mVSG was deacylated with KOH. Lane 1, fatty acid methyl ester standards; lane 2, FAMES of fatty acids released from [3H]GPI-PLC; lane 3, from [3H]mVSG. Hyperfilm (Amersham Pharmacia Biotech) was developed after plate was exposed for 2 weeks. The experiment described in lane 2 has been repeated 15 times.

![Image](image3.png)

**Fig. 4.** Acylation of GPI-PLC occurs co- and post-translationally. Cells were preincubated with cycloheximide (CHX-I) (lane 2) for 15 min prior to addition of [3H]myristate (A and C) or [35S]methionine (B). The parasites were metabolically labeled for 1 h. Alternatively, cells were metabolically labeled for 1 h, followed by addition of CHX (CHX-II) (lane 3) for 15 min (37 °C). Control cells (lane 1) were labeled for 1 h, washed, and incubated in fresh labeling medium for 15 min (37 °C). Each lane represents GPI-PLC immunoadsorbed from a lysate of 5 × 10⁹ T. brucei. Dried gels were exposed to Hyperfilm (Amersham Pharmacia Biotech) for either 5 h (B) or 5 days (A). C, GPI-PLC was detected by Western blotting with R18B3.
on GPI-PLC (as estimated by laser scanning densitometry of fluorographs) occurs in the absence of new protein synthesis. We infer that 50% of the acylation occurred on nascent GPI-PLC, possibly on ribosomes. These observations indicate that acylation of GPI-PLC occurs post-translationally.

In control experiments, the effect of CHX on the GPI-PLC polypeptide backbone was studied in [35S]methionine-labeled T. brucei (Fig. 4B). Here, CHX blocked synthesis of the enzyme if pre-incubated with the cells (Fig. 4B, lane 2), but no effect on GPI-PLC was apparent when added after metabolic labeling (Fig. 4B, lane 3). These observations confirm our previous conclusion that a significant proportion of the S-acylation of GPI-PLC occurs post-translationally. When a gel identical to that depicted in Fig. 4A was analyzed by Western blotting with an anti-GPI-PLC antibody, similar amounts of GPI-PLC were detected in all three lanes (Fig. 4C). We conclude that loss of 3H-lipid (Fig. 4A, lane 2) was due to a decrease in fatty acid addition instead of protein degradation.

To investigate whether myristate or palmitate was preferentially added to GPI-PLC post-translationally, trypanosomes were pretreated with CHX (CHX-1) prior to metabolic labeling with [3H]myristate. [3H]Acyl-GPI-PLC was immunoadsorbed and deacylated (see “Experimental Procedures”). The released fatty acid(s) were analyzed by RP-HPTLC/fluorography (see “Experimental Procedures”) (Fig. 5). Both myristate and palmitate were added to GPI-PLC post-translationally, but myristate addition dropped about 50% (Fig. 5, compare lanes 2 and 3). The ratio of myristate:palmitate (recovered from GPI-PLC) decreased to 2:1 (i.e. from 3:1). As compared with the control lane (Fig. 5, lane 2), the amount of palmitate added post-translationally to GPI-PLC did not change. Apparently, most of the post-translational acylation of GPI-PLC is due to myristate incorporation. These experiments also ruled out the possibility that the palmitate and myristate detected on GPI-PLC when the enzyme was adsorbed to a mc2A6-6-protein A-Sepharose column (Fig. 3) originated from contaminating phospholipid or VSG. Since immunopurified [3H]GPI-PLC isolated from polyacrylamide gel slices contained [3H]palmitate and [3H]myristate (Fig. 5, lane 2), it is clear that both fatty acids are covalently attached to GPI-PLC. The ratio of [3H]myristate: [3H]palmitate on GPI-PLC was again 3:1, as determined by laser scanning densitometry of fluorographs from the HPTLC plates.

Acylated GPI-PLC Is Enzymatically Active—Lipid modification has a potential for regulating the activity of GPI-PLC. For example, acylation of the protein could inactivate the enzymatic activity. Alternatively, deacylation might then be required to impart catalytic activity to the polypeptide. These ideas were tested as described below.

GPI-PLC cleaves the [3H]myristate-labeled GPI of VSG during hypotonic lysis of T. brucei, releasing the radioactive lipid as di-[3H]myristoylglycerol (19). If deacylation of GPI-PLC was necessary for enzyme activity, loss of the fatty acid from GPI-PLC might be detectable under conditions where cleavage of VSG is documented, that is loss of [3H]acyl group from GPI-PLC might precede or be coincident with the loss of [3H]myristate from the VSG GPI.

We attempted to determine whether any changes in the extent of GPI-PLC acylation occurred during VSG cleavage. For this purpose, [3H]myristate-labeled trypanosomes were lysed hypotonically and incubated either at 4 °C (Fig. 6B, lanes 1–4) or 37 °C (Fig. 6B, lanes 5–8) in an attempt to initiate hydrolysis of the VSG GPI. GPI-PLC cleaves approximately 70% of [3H]myristate-labeled VSG during hypotonic lysis of T. brucei (20). Residual VSG that is not cleaved initially is the substrate of interest in these experiments because it is lumenal (21, 22) and therefore inaccessible to GPI-PLC which is cytoplasmically oriented (10, 12). Detergent is needed to promote vesicle mixing which brings the enzyme and substrate together.

Cleavage of VSG was monitored by loss of [3H]myristate from the protein. Nonidet P-40 was added (to 1% final concentration) in some experiments (Fig. 6B, lanes 3, 4, 7, and 8; see Fig. 6A for outline of protocol) to facilitate mixing of membrane vesicles. Immunoprecipitated [3H]-acylated GPI-PLC (Fig. 6B, lanes 1, 3, 5, and 7) and [3H]myristate-labeled VSG (Fig. 6B, lanes 2, 4, 6, and 8) were monitored by SDS-PAGE/fluorography.

Cleavage of VSG occurred at both 4 and 37 °C in the presence of detergent (Fig. 6B, lanes 4 and 8). However, there was no discernible loss of the [3H]acyl group from GPI-PLC (Fig. 6B, lanes 3 and 7). That loss of the [3H]myristate from VSG (i.e., GPI cleavage) was not the result of degradation of VSG was confirmed by Western blotting; full-length VSG was present in the lysate after loss of the [3H]myristate (data not presented).

We conclude that deacylation is not required for GPI-PLC activity. In support of this assertion, acylated GPI-PLC is catalytically more proficient than the deacylated form of the enzyme (Table 1). Thus, the possibility that a minute fraction of unacylated GPI-PLC is responsible for cleavage of VSG in these lysates is very remote.

Acylation of GPI-PLC Is Reversible in Vivo—GPI-PLC in T. brucei and recombinant GPI-PLC expressed in Escherichia coli...
are both integral membrane proteins (6, 9), suggesting that membrane binding is not the result of an eukaryote-specific chemical modification(s). Therefore, thioacylation (which has been demonstrated only in eukaryotes) seemed unlikely to be responsible for the ability of the enzyme to bind to membranes. Given these circumstances, the possibility that thioacylation played a regulatory role on the activity of the enzyme was entertained. Regulatory modifications on proteins (e.g. phosphorylation) are frequently reversible. Accordingly, we tested the possibility that acylation of GPI-PLC was dynamic.

Acylation of *T. brucei* GPI-PLC was reversible. When trypanosomes were labeled with [3H]myristate and then "chased," lipid detected on the polypeptide decreased with a half-life of 45 min (Fig. 7). After a 60-min "chase" only 30% of the acylation signal was detectable on GPI-PLC. Reversibility of the acylation reaction and the ability of *T. brucei* to add the lipids post-translationally indicate that thioacylation of GPI-PLC in *T. brucei* is dynamic.

Deacylation Reduces Activity of GPI-PLC—An hypothesis that thioacylation activated GPI-PLC was next tested, by determining whether removal of the fatty acid from GPI-PLC would affect the kinetics of GPI cleavage. For this purpose, a complex consisting of GPI-PLC immunoadsorbed to protein A-Sepharose (i.e. a GPI-PLC mcAb2A6-6-protein A-Sepharose) (see "Experimental Procedures") was treated either with neutral NH2OH or PBS (as a control) for varying lengths of time. GPI-PLC was then eluted from the column and its enzymatic activity assayed (9). Deacylation of GPI-PLC under these conditions reduced activity 4-fold (Fig. 8A). This decrease in activity was not due to loss of GPI-PLC, since the full-length polypeptide was detectable by Western blotting following the hydroxylamine treatment (Fig. 8B). By following the loss of the [3H]acyl signal from 3H-acylated GPI-PLC during a similar deacylation experiment, a 4-fold decrease in signal was obtained after 40 min of neutral hydroxylamine treatment (Fig. 8C). There is a correlation between decrease in acylation (Fig. 8C) and loss of enzyme activity (Fig. 8A), suggesting that 100% deacylation might lead to complete loss of GPI-PLC activity. Exposure of GPI-PLC to phosphate-buffered saline neither deacylated nor inhibited the enzyme (data not presented). Finally, when recombinant GPI-PLC expressed in *E. coli* was subjected to the above deacylation conditions, no inhibition of enzyme activity was observed (data not presented). This result

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**Table I**

| Enzyme               | $K_m$ (Exp. 1) | $K_m$ (Exp. 2) | $k_{cat}$ (Exp. 1) | $k_{cat}$ (Exp. 2) |
|----------------------|----------------|----------------|-------------------|-------------------|
| Acylated GPI-PLC     | 2.7            | 2.6            | 1188              | 1636              |
| De-acylated GPI-PLC  | 1.7            | 2.0            | 39                | 89                |

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**Fig. 6. Acyl-GPI-PLC is active.** A, *T. brucei* (10^9) were metabolically labeled with [3H]myristate and lysed hypotonically. Cell lysates were processed as illustrated. B, a membranous pellet of a lysate from [3H]myristate-labeled cells was resuspended in lysis buffer and incubated for 15 min either at 4°C (lanes 1–4) or 37°C (lanes 5–8). In some experiments, Nonidet P-40 was added to 1% (final concentration) before incubation (lanes 3, 4, 7, and 8). Lanes 1, 3, 5, and 7 depict immunoprecipitates of GPI-PLC (from 5 x 10^6 cell eq), and lanes 2, 4, 6, and 8 contain 10^6 cell eq of total proteins. SDS-PAGE (14% minigel) was used to resolve the proteins, which were then detected by fluorography. The dried gel was exposed to Hyperfilm™ (Amersham Pharmacia Biotech) for 5 days.

**Fig. 7. Acylation of GPI-PLC is dynamic.** [9,10-3H]Myristate-labeled trypanosomes (7 x 10^9 cells) were resuspended in 14 ml of labeling medium and incubated for 60 min (37°C). Two-ml aliquots (10^9 cells) were harvested every 10 min, immunoprecipitated, and the eluted GPI-PLC analyzed by SDS-PAGE/fluorography. Dried gels were exposed to Hyperfilm™ (Amersham Pharmacia Biotech) for 12 days. Images were quantitated using an IS-1000 Digital imaging system. The value obtained at $t = 0$ was assigned a value of 100%.
is consistent with our inability to detect acylation of GPI-PLC in the bacterium. Furthermore, the result indicates that the deacylation conditions per se do not inhibit GPI-PLC. We conclude that deacylation reduces the enzymatic activity of GPI-PLC significantly and infer that thioacylation activates the enzyme.

**Turnover Number of GPI-PLC Is Reduced by Deacylation**—A kinetic basis for the reduced rate of GPI cleavage by deacylated GPI-PLC (Fig. 8A) was sought. Rate of the reaction could be decreased by altering either $k_{\text{cat}}$ or $K_m$; therefore, both parameters were determined for acylated GPI-PLC and the deacylated form of the enzyme (Table I). During enzyme-catalyzed reactions, product formation is preceded by assembly of an enzyme-substrate complex (ES). At steady state, the Michaelis constant ($K_m$) equals $(k_{-1} + k_2)/k_1$, where $k_1$ is the kinetic constant for formation of an $E\S$ complex, $k_{-1}$ is the reaction rate constant for breakdown of the $E\S$ complex into free enzyme ($E$) and substrate (S) (i.e. GPI-PLC and VSG, respectively). $k_2$ is the rate constant for conversion of an $E\S$ complex to product (P) and free enzyme; $k_2$ equals $k_{\text{cat}}$ when $k_{-1} \ll k_2$.

Deacylation of GPI-PLC did not have a profound effect on VSG binding by the enzyme, since $K_m$ was not altered significantly (Table I). However, when the maximal reaction velocity was corrected for amount of enzyme added (to obtain the turnover number, $k_{\text{cat}}$), the two enzymes had strikingly different properties. Turnover number decreased 18–30-fold for deacylated GPI-PLC, as compared with the acylated enzyme (Table I). The properties of deacylated *T. brucei* GPI-PLC appear to be similar to *E. coli*-expressed recombinant GPI-PLC which is not acylated and whose turnover number was 10-fold less than GPI-PLC isolated from *T. brucei* (15).

In essence, acylation alters the properties of the $E\S$ complex. The reduced reaction velocity of the deacylated enzyme is traceable to the diminished rate of converting the GPI-PLC $z\VSG$ complex to products. We surmise that thioacylation of GPI-PLC promotes rapid conversion of an $E\S$ complex to product (P) and free enzyme ($E$).

**DISCUSSION**

**Thiomyristoylation, a Novel Modification of a Phospholipase C**—The use of myristate in thioacylation (i.e. $S$-myristoylation) of GPI-PLC is unique. Frequently, palmitate is the fatty acid attached to cysteines on proteins (reviewed in Ref. 1). Myristate is typically linked to polypeptides through an amide bond (N-acylation) (1). In *T. brucei*, myristate is a rare fatty acid (23,

![Fig. 8. Deacylation reduces activity of GPI-PLC. A. GPI-PLC was immunoprecipitated from $2 \times 10^6$ trypanosomes with 100 µl of mc2A6-6 protein A-Sepharose complex, which was then treated with neutral hydroxylamine (see “Deacylation of GPI-PLC and Determination of Phospholipase C Activity” under “Experimental Procedures”) for the indicated periods. GPI-PLC was eluted, and activity of the enzyme was determined. Activity obtained at time 0 was assigned a 100% value. *B*, decrease in activity of GPI-PLC (A) is not due to loss of the polypeptide. Western blot analysis was performed on the deacylated samples using R18B3, a polyclonal anti-GPI-PLC antibody. *Lane 1*, purified GPI-PLC expressed in *E. coli*; *lane 2*, mc2A6-6 monoclonal antibody; lanes 3–6, *T. brucei* GPI-PLC deacylated for 0, 10, 30, and 40 min, respectively. *C*, time course for deacylation of [3H]GPI-PLC. [3H]Myristate-labeled GPI-PLC was deacylated with neutral hydroxylamine for the indicated periods. Protein eluted from the complex was analyzed by SDS-PAGE/fluorography.
24, with most of it found on mature GPIs of VSG (23). S-Myristoylation may confer some unusual properties on GPI-PLC.

Thioacylation in T. brucei has properties that appear to be derived from two distinct types of lipid modifications, namely S-myristoylation and N-acylation, found in animal cells. First, similar to S-myristoylation, cysteine is the acceptor amino acid in GPI-PLC myristoylation. Second, the chemical link between fatty acid and the protein is an ester bond. Third, a significant proportion of the S-myristoylation occurs post-translationally. Fourth, S-myristoylation is reversible. Other features of S-myristoylation mimic N-acylation as follows: 1) a fatty acid of short/medium chain length is used, and 2) a large fraction of the myristate on GPI-PLC is incorporated co-translationally, in sharp contrast to S-myristoylation.

Given that the parasites were originally labeled with myristate, detection of palmitate on GPI-PLC was unexpected. The observation indicates that some myristate was elongated to palmitate prior to incorporation into the enzyme. This conclusion is consistent with the ability of T. brucei to convert myristate into palmitate and stearate (23). Finally, as compared with the extent of myristoylation, the degree of palmitoylation of GPI-PLC could have been underestimated; [3H]palmitoyl-CoA, the presumed activated donor of the fatty acid, could be competing with a large intracellular pool of unlabelled palmitoyl-CoA for incorporation into GPI-PLC. Further work will be needed to resolve this issue, since the relative levels of myristoyl-CoA and palmitoyl-CoA in T. brucei are unknown.

S-Acylation may regulate activity of proteins positively or negatively—Thioacylation has the potential to modulate activity of proteins in a positive or negative manner, much like phosphorylation. Since deacylation reduces activity of GPI-PLC (Fig. 8 and Table I), the fatty acid(s) appear to be positive modulators of the activity of the enzyme. In contrast, S-myristoylation of Gz decreases its interaction with the corresponding GTPase-activating protein (25), and active site cysteine acylation inhibits activity of some mitochondrial enzymes (26). Hence, reversible thioacylation could have a general role of positive and negative control of protein function.

We speculate that GPI-PLC activity is controlled by reversible thioacylation in T. brucei. In this model (Fig. 9), acylated GPI-PLC is presumed to be the state in which most of the enzyme exists. Deacylation of the GPI-PLC by a myristoylthioesterase, possibly in response to extracellular signals, would suppress enzyme activity. Reacylation of GPI-PLC by S-myristoyltransferase would restore optimal activity to the phospholipase C. Endogenous regulators of T. brucei origin are not known yet, although some aminoglycoside antibiotics (e.g. G418) can activate GPI-PLC in vitro (27). In addition, protein kinase C inhibitors promote cleavage of VSG by GPI-PLC (19). Whether these events can be tied into a cycle of acylation/deacylation in vivo awaits exploration.

Phosphatidylinositol-specific phospholipases C (PI-PLCs) act at the interface between a lipid bilayer and the aqueous cytosol. In general, these enzymes are soluble, only making transient contact with membranes to hydrolyze their membrane-bound substrates, usually in response to extracellular signals (reviewed in Ref. 28). Lipid modification could be used by phosphatidylinositol-specific PLCs as a means of gaining access to membranes. However, no acylation of a phosphatidylinositol-specific PLC has been reported yet.

T. brucei GPI-PLC is the only eukaryotic PLC known to be stably integrated into a lipid bilayer (6–8). Could modification with lipid be responsible for the membrane association of this enzyme? Current evidence suggests otherwise. When expressed in E. coli, GPI-PLC remains tightly associated with membranes (15), but attempts to label the enzyme metabolically with fatty acids have yielded negative results. Hence, membrane binding by GPI-PLC does not appear to require an eukaryote-specific modification.

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