Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 1178–1183, February 1996
Biochemistry

Myristate exchange on the *Trypanosoma brucei* variant surface glycoprotein

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Communicated by M. Daniel Lane, Johns Hopkins University School of Medicine, Baltimore, MD, October 19, 1995

ABSTRACT **The glycosyl-phosphatidylinositol (GPI) anchor of the *Trypanosoma brucei* variant surface glycoprotein (VSG) is unique in having exclusively myristate as its fatty acid component. We previously demonstrated that the myristate specificity is the result of two independent pathways. First, the newly synthesized free GPI, which is not myristoylated, undergoes fatty acid remodeling to replace both its fatty acids with myristate. Second, the myristoylated precursor, glycolipid A, undergoes a myristate exchange reaction, detected by the replacement of unlabeled myristate by [^{3}H]myristate. Remodeling and exchange have different enzymatic properties and apparently occur in different subcellular compartments. We now demonstrate that the GPI anchor linked to VSG is the major substrate for myristate exchange. VSG can be efficiently labeled with [^{3}H]myristate by exchange in the presence of cycloheximide, an inhibitor that prevents new VSG synthesis and thus anchor addition to protein. Not only is newly synthesized VSG subject to exchange, but mature VSG, possibly recycling from the cell surface, also undergoes myristate exchange.**

African trypanosomes, the protozoan parasites responsible for sleeping sickness, are cloaked with a dense protein coat composed of 10^7 identical variant surface glycoprotein (VSG) molecules. By using a sophisticated system of antigenic variation, these bloodstream parasites can change expression of their VSG to that of another with a different amino acid sequence (1). Since the parasite genome encodes a repertoire of up to 1000 different VSGs, the mammalian immune system, always one step behind, is continually presented with parasites expressing different VSGs. This trypanosome strategy has thwarted the development of a vaccine to prevent trypanosomiasis.

The *Trypanosoma brucei* VSG is attached to the parasite plasma membrane through a glycosyl-phosphatidylinositol (GPI) anchor. A unique feature of this GPI is that it contains exclusively myristate (a 14-carbon, saturated fatty acid) as its lipid moiety (2). The reason for the choice of myristate is not yet understood, but its presence on the GPI led to the discovery that analogs of myristate are selectively toxic to these parasites and therefore are candidate antitrypanosomal agents (3, 4).

The *T. brucei* GPI biosynthetic pathway involves sequential construction of the ethanolamine-*P*-Man_3-GlcN glycan core on phosphatidylinositol to form a GPI known as glycolipid A′ (5). This GPI then acquires its myristate by a specific fatty acid remodeling pathway involving sequential deacylation and reacylation (6). The product, a dimyristoylated GPI known as glycolipid A, is linked to the newly synthesized VSG in the endoplasmic reticulum (ER). We recently discovered that trypanosomes have a second GPI myristoylation pathway known as myristate exchange (7). In this reaction, both myristates of glycolipid A are efficiently replaced with [^{3}H]myristate. Like remodeling, myristate exchange involves deacylation and reacylation. The enzymes responsible for exchange appear to differ from those for remodeling, and the two myristoylation reactions apparently occur in different subcellular compartments. It was surprising that a second myristoylation pathway would exist for glycolipid A, and we therefore examined whether a GPI anchor already on VSG might be the major substrate for myristate exchange. We demonstrated that newly synthesized VSG does undergo exchange, as does mature VSG, which is possibly recycled from the cell surface. Myristate exchange may be a proofreading or a repair mechanism for VSG, ensuring that every VSG molecule is fully myristoylated.

MATERIALS AND METHODS

Metabolic Labeling of Trypanosomes with [^{3}H]Myristate. Cloned ILTat 1.3 trypanosomes were isolated from infected rat blood by DE-52 chromatography (8) or purified from buffy coats (9). Trypanosomes ($1–5 \times 10^7$ cells per ml) were labeled at 37°C with [^{3}H]myristate [30.7 Ci/mmol (1 Ci = 37 GBq; DuPont/NEN) or 54 Ci/mmol (Amersham) prebound to an equimolar quantity of fatty acid-free bovine serum albumin] at 50 μCi/ml in RPMI 1640 supplemented to 25 mM Na Hepes (pH 7.4), 10 mg of glucose per ml, and 1 mg of fatty acid-free bovine serum albumin per ml. At various times, samples (200–500 μl) were diluted in 1 ml of ice-cold BBS/G buffer [50 mM Bicine (pH 8.0), 50 mM NaCl, 5 mM KCl, and 1 mg of glucose per ml] and centrifuged (30 sec; Beckman B Microfuge).

For SDS/PAGE analysis of VSG, cell pellets were resuspended directly in 50 μl of 2× SDS sample buffer [10 mM Tris·HCl (pH 6.8), 8% (vol/vol) glycerol, 0.8% SDS, 2% (vol/vol) 2-mercaptoethanol, and 50 μg of bromophenol blue per ml] and run on 17-cm 7.5–15% linear gradient SDS/PAGE gels. Gels were fixed in destain [20% (vol/vol) methanol and 7% (vol/vol) acetic acid in water] and then impregnated with En^{3}Hance (DuPont/NEN) for fluorography. Alternatively, after centrifugation in BBS/G, cell pellets were washed again in 1 ml of BBS/G and precipitated/extracted in 1.2 ml of chloroform/methanol (2:1, vol/vol) for 30 min at room temperature. Methanol (400 μl) was added to lower the fluid density, and samples were centrifuged for 3 min in a Beckman B Microfuge. After air drying, the pellets were resuspended by boiling for 10 min in 50 μl of 2% SDS. Precipitation in chloroform/methanol and boiling in SDS were repeated, and radioactivity in the SDS solution was measured by liquid scintillation counting in Formula-963 fluid (DuPont/NEN). Virtually all radioactivity precipitated by this method is in VSG (10).

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Abbreviations: VSG, variant surface glycoprotein; GPI, glycosylphosphatidylinositol; ER, endoplasmic reticulum; GPI-PLC, GPI-specific phospholipase C.

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For analysis of glycolipids, cell pellets were extracted in 200 μl of chloroform/methanol/water (10:10:3, vol/vol) and treated as described for cell-free system labelings (7).

Labeling of the Cell-Free System with [^{3}H]Myristoyl-CoA. The cell-free system was prepared and labeled as described (7). Protein samples (5×10^7 cell equivalents) were handled as above with chloroform/methanol (2:1, vol/vol) precipitation/extraction except that a solution of 2% SDS, 0.5 mM dithiothreitol, and 15 mM $MnCl_2$ was substituted for 2% SDS for the first resuspension to hydrolyze [^{3}H]myristoyl-CoA during the boiling step.

RESULTS

Labeling VSG by Myristate Exchange. We first tested whether VSG protein, already containing a myristoylated GPI anchor, undergoes myristate exchange. Our approach was to determine whether [^{3}H]myristate labeling of VSG occurred in trypanosomes preincubated with the protein synthesis inhibitor cycloheximide. Since VSG protein receives its myristoylated GPI anchor within 30 sec after synthesis of the polypeptide (11), [^{3}H]myristate labeling occurring in the presence of cycloheximide must occur by exchange on protein that was synthesized prior to the addition of the inhibitor and that already contains a myristoylated anchor. In the experiment in Fig. 1*A*, the cycloheximide was added 2 min prior to the addition of [^{3}H]myristate. Nevertheless, there was significant labeling of VSG in the presence of cycloheximide, roughly half as much at 60 min as in the absence of the inhibitor. [^{3}H]Myristate labeling in the presence of cycloheximide increases rapidly and then levels off after about 20 min. The dashed curve, which is the difference between the curves in the absence and presence of cycloheximide, must represent *de novo* incorporation of [^{3}H]myristate-labeled anchors into newly synthesized VSG.

We conducted three control experiments. First, we demonstrated by SDS/PAGE (Fig. 1*B*) that cycloheximide efficiently blocks incorporation of [^{35}S]methionine into trypanosome proteins including VSG. Second, we demonstrated that in the presence of tunicamycin (to block N-linked glycosylation), cycloheximide blocks [^{3}H]mannose incorporation into VSG, thereby proving that no new GPI anchors are added to VSG in the presence of cycloheximide (Fig. 1*C*). Finally, we used *T. brucei* GPI-specific phospholipase C (GPI-PLC) (13) to prove that the [^{3}H]myristate was incorporated into the GPI anchor. The radioactivity was released from the protein by this enzyme, and it comigrated with dimyristoylglycerol on reverse-phase TLC [a technique that separates diacylglycerols according to fatty acid chain length (6)]. The ^{3}H-labeled fatty acid present in the dimyristoylglycerol, when converted to a fatty acid methyl ester, comigrated on reverse-phase TLC with authentic methyl myristate (14) (data not shown).

Quantitative Comparison of Myristate Exchange Labeling of Glycolipid A and VSG. To compare myristate exchange labeling of glycolipid A and VSG, we labeled both molecules in the cell-free system (consisting of washed membranes from hypotonically lysed trypanosomes) and *in vivo*. Fig. 2*A* shows the kinetics of VSG and glycolipid A labeling in the cell-free system using [^{3}H]myristoyl-CoA as a substrate. The concentration of [^{3}H]myristoyl-CoA (10 μCi/ml, 326 nM) was far above the 6 nM K_m for this substrate that was determined in myristate exchange of glycolipid A (7). The maximal labeling of VSG is 2.3 times the maximal level of glycolipid A, but the two reactions have similar kinetics ($t_{1/2} \approx 8$–9 min). The reduction in rate of myristoylation at later time points is not due to exhaustion of [^{3}H]myristoyl-CoA, as addition of more [^{3}H]myristoyl-CoA does not result in more labeling of glycolipid A (data not shown). As described in the previous paragraph for VSG labeled *in vivo* with [^{3}H]myristate, VSG labeled in the cell-free system was sensitive to *T. brucei* GPI-PLC, and the product was [^{3}H]dimyristoylglycerol (data not shown). Labeling of either glycolipid A or VSG in the cell-free system was not affected by cycloheximide at 200 μg/ml; the absence of effect on VSG proved that the labeled protein was not synthesized during the reaction but must have existed before addition of the [^{3}H]myristate.

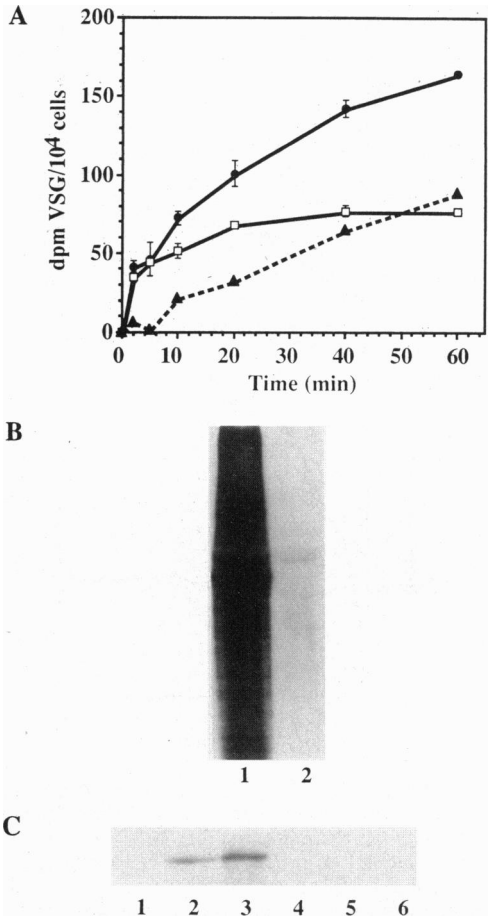

FIG. 1. Metabolic labeling of VSG with [^{3}H]myristate in the presence and absence of cycloheximide. (*A*) Trypanosomes (5×10^7 cells per ml) were preincubated for 2 min with (□) or without (●) 200 μg of cycloheximide per ml (identical results were obtained with 400 μg of cycloheximide per ml) and then labeled with [^{3}H]myristate at 50 μCi/ml. Samples of 2×10^7 cells (400 μl) were precipitated/extracted with chloroform/methanol (2:1, vol/vol), and the pellets were assayed by scintillation counting. The difference between the two curves is plotted (▲). The graph represents two independent cultures with standard error for all time points. (*B*) Trypanosomes (5×10^7/ml) were preincubated without (lane 1) or with (lane 2) 200 μg of cycloheximide per ml for 5 min in methionine-free medium and then labeled for 5 min with [^{35}S]methionine at 50 μCi/ml (>800 Ci/mmol; NEN). Each sample (10^7 cells) was lysed in SDS sample buffer and chromatographed on a 10% SDS/PAGE minigel for fluorography. Identical results were obtained with a 2-min cycloheximide preincubation or with 400 μg of cycloheximide per ml. (*C*) Trypanosomes (5×10^7/ml) were preincubated for 5 min without (lanes 1–3) or with 400 μg of cycloheximide per ml (lanes 4–6) in glucose-free RPMI 1640 supplemented with 25 mM Na Hepes (pH 7.4), 3 mg of glycerol per ml, 10 mg of fatty acid-free bovine serum albumin per ml, 0.2 μg of tunicamycin per ml, 12 μg of adenosine per ml, and 1 μg of catalase per ml (12). The cells were then labeled with 50 μCi of [^{3}H]mannose (34 Ci/mmol; Amersham) per ml for 5, 10.5, or 30 min (lanes 1 and 4, 2 and 5, and 3 and 6, respectively). During the [^{3}H]mannose labeling, the cells were incubated at 37°C with constant shaking at ≈125 rpm. Samples (10^7 cells) were lysed in SDS sample buffer and run on a 17-cm 7.5–15% gradient SDS/PAGE gel for fluorography. In the presence of tunicamycin, the only detectable protein that labels with [^{3}H]mannose is VSG, so only this region of the gel is shown.

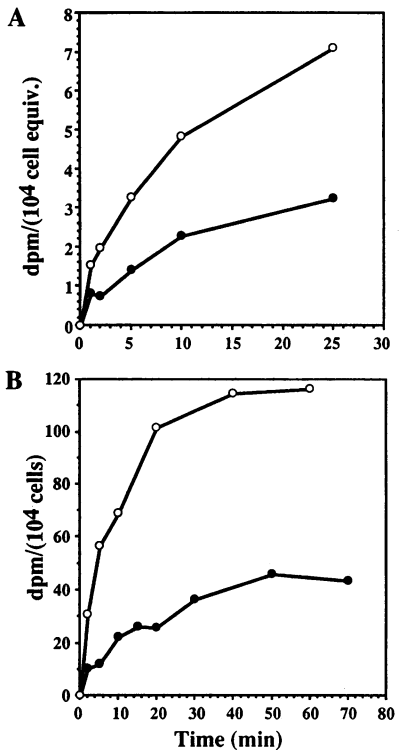

FIG. 2. [^{3}H]Myristate exchange labeling of VSG and glycolipid A. (*A*) The cell-free system was labeled with [^{3}H]myristoyl-CoA (10 μCi/ml, 326 nM; synthesized as in ref. 7). ○, VSG (5×10^7 cell equivalents per sample); ●, glycolipid A (1.5×10^7 cell equivalents per sample). Glycolipid samples were processed as described with absolute dpm calculated from scanner cpm using a standard curve (7). VSG samples were precipitated/extracted with chloroform/methanol (2:1, vol/vol) and assayed by scintillation counting. A $t_{1/2}$ of 8–9 min was extrapolated for both VSG and glycolipid A labeling, using a hyperbolic fit with DELTAGRAPH (Deltapoint Inc.). (*B*) Trypanosomes (5×10^7 cells/ml) were preincubated for 2 min with 200 μg of cycloheximide per ml and then labeled with 50 μCi of [^{3}H]myristate per ml. VSG samples (○; 2×10^7 cells) and glycolipid A samples (●; 1×10^7 cells) were processed as in *A*. The TLC spot of glycolipid A was located by TLC scanning, extracted in chloroform/methanol/water (10:14:3, vol/vol), dried, and scintillation counted.

Fig. 2*B* shows the kinetics of [^{3}H]myristate labeling *in vivo* in the presence of cycloheximide. The concentration of [^{3}H]myristate is saturating at 1.6 μM (50 μCi/ml) (K.A.W. and P.T.E., unpublished data). Similar to the results with the cell-free system, the labeling of VSG is 2.5 times that of glycolipid A. The labeling of each is about 9 times greater *in vivo* than *in vitro*. Note that *in vivo* labeling of glycolipid A includes both myristate exchange and *de novo* incorporation of [^{3}H]myristate by fatty acid remodeling, whereas VSG labeling represents only myristate exchange. For cell-free system labelings, only exchange is measured as nucleotide sugars have been omitted.

Comparison of Inhibition of Myristate Exchange on Glycolipid A and VSG. $CdCl_2$ and $Al_2(SO_4)_3$ inhibit myristate exchange on glycolipid A in the cell-free system but do not affect fatty acid remodeling (7). On the other hand, EDTA and EGTA inhibit the *sn*-2 myristoyl transferase in fatty acid remodeling but do not block myristate exchange (7). We compared the effects of these compounds on myristate exchange on glycolipid A and VSG, and the outcome on both substrates was essentially identical (Fig. 3). Therefore, these results are consistent with the possibility that both substrates are myristoylated by the same enzymatic machinery.

Myristate Exchange Occurs on Mature as Well as Newly Synthesized VSG. We next studied whether myristate exchange occurs on mature VSG as well as on newly synthesized VSG. To distinguish between these forms, we pretreated the cells for 90 min with tunicamycin, a drug that blocks N-linked glycosylation. We had shown previously that VSG synthesized after this treatment does not become N-glycosylated (as assayed by SDS/PAGE) and that tunicamycin does not change the kinetics of VSG transport through the secretory pathway (11, 15). The newly synthesized VSG appears smaller (55 kDa) than mature VSG (57 kDa). When we added cycloheximide together with [^{3}H]myristate, we observed exchange labeling of both 55- and 57-kDa VSG (Fig. 4*A Bottom*), indicating that mature as well as newly synthesized VSG undergoes exchange. When we added cycloheximide earlier (−15 or −30 min), there was a gradual decrease in labeling of the 55-kDa protein, but there was little or no effect on labeling of the 57-kDa VSG (Fig. 4*A, middle*). The decrease in labeling of 55-kDa VSG must be due to depletion of VSG in the secretory pathway by cycloheximide treatment. When we added cycloheximide at −45 min, labeling of 55-kDa VSG was abolished, but again there was no effect on labeling of mature 57-kDa VSG (Fig. 4*A, Top*). Densitometry of the fluorographs shown in Fig. 4*A* and from similar experiments confirmed that the amount of 57-kDa VSG labeled is independent of the time cycloheximide is added.

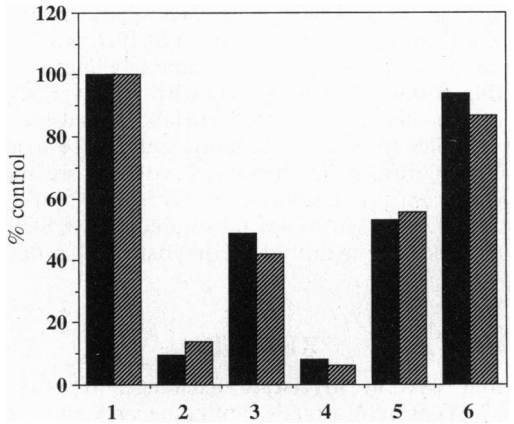

FIG. 3. Inhibition characteristics of myristate exchange on VSG and glycolipid A in the cell-free system. The cell-free system was labeled with [^{3}H]myristoyl-CoA as in Fig. 2*A* but in the presence of inhibitors. Glycolipid A (■; 1.5×10^7 cell equivalents per sample) was analyzed by TLC and scanning. VSG (▩; 5×10^7 cell equivalents per sample) was analyzed by SDS/PAGE (10% minigel). After fluorography, the VSG bands were cut out of the gel. These dried gel slices were rehydrated with 400 μl of water and then shaken at 37°C overnight with 325 μl of Protosol (DuPont/NEN) and 4 ml of Formula-963 scintillation fluid (DuPont/NEN). Radioactivity was quantitated by scintillation spectrometry. It should be noted that the concentration dependence of inhibition varies slightly with different lysates, and so all the conditions in this experiment were performed with the same lysate at the same time. Bars: 1, control; 2, 2 mM $CdCl_2$; 3, 1 mM $CdCl_2$; 4, 2 mM $Al_2(SO_4)_3$; 5, 0.5 mM $Al_2(SO_4)_3$; 6, 10 mM EDTA and 10 mM EGTA.

In a control experiment, we evaluated whether the 55-kDa species is in fact unglycosylated VSG. We used peptide-*N*-glycosidase F, an enzyme that removes N-linked glycans, to digest a sample from cells that had been preincubated for 45 min with tunicamycin and then labeled for 40 min with [^{3}H]myristate in the presence of cycloheximide. We found that peptide-*N*-glycosidase F reduced the molecular mass of the 57-kDa VSG to 55 kDa (Fig. 4*B*). If cells are labeled with [^{3}H]myristate in the absence of tunicamycin, all ^{3}H-labeled VSG is 57 kDa. Furthermore, VSG in untreated cells is 57 kDa as judged by Coomassie blue staining (data not shown).

Effect of Hypotonic Lysis on Myristate Exchange Labeled VSG. Since mature VSG is a substrate for myristate exchange, we tested whether exchange could occur on the cell surface.

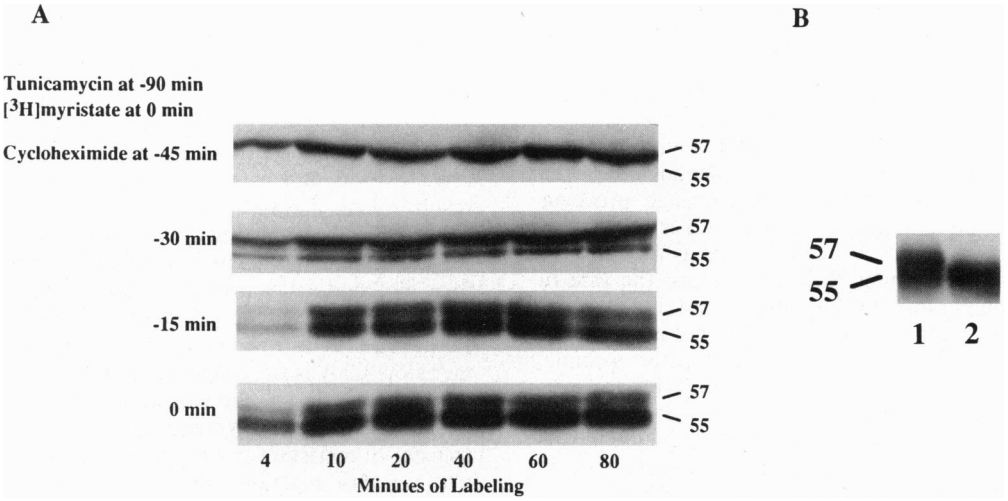

FIG. 4. Labeling of newly synthesized and mature VSG by myristate exchange. The mature form, 57 kDa, contains two N-linked oligosaccharides (16); the newly synthesized form, 55 kDa, is deficient in N-linked glycosylation due to preincubation with tunicamycin. (*A*) Trypanosomes (5×10^7/ml) were preincubated at 37°C with 0.2 μg of tunicamycin per ml for 90 min before labeling. Cycloheximide (400 μg/ml) was added at the indicated times before labeling with [^{3}H]myristate (50 μCi/ml). Samples (10^7 cells) were prepared for 7.5–15% linear gradient SDS/PAGE and fluorography as described in *Materials and Methods*. (*B*) Trypanosomes were preincubated for 45 min with 0.2 μg of tunicamycin per ml and then labeled for 40 min with [^{3}H]myristate (50 μCi/ml) in the presence of 400 μg of cycloheximide per ml. Lane 1, no treatment; lane 2, digested with peptide-*N*-glycosidase F (17). Note that the apparent molecular masses are for the membrane form of VSG (containing the complete GPI anchor) and are therefore lower than those of the soluble form of VSG (lacking dimyristoylglycerol) reported in ref. 15.

For this experiment we exploited the endogenous trypanosome GPI-specific phospholipase C (GPI-PLC), an enzyme that cleaves the dimyristoylglycerol from VSG's GPI anchor and thus removes the [^{3}H]myristate label. Hypotonic lysis of trypanosomes activates this enzyme, resulting in cleavage of all of the surface VSG; however, VSG in the secretory pathway is protected from cleavage (11, 18). We can therefore use hypotonic lysis to determine if myristate exchange into VSG occurs at the plasma membrane. If exchange were to take place on the plasma membrane, then exchange-labeled VSG would be cleaved by GPI-PLC upon hypotonic lysis.

Fig. 5 shows the metabolic labeling of VSG with [^{3}H]myristate in the presence of cycloheximide. Cells were hypotonically lysed on ice and then inhibited for 5 min at 37°C in the presence or absence of *p*-chloromercuriphenylsulfonic acid, an inhibitor of GPI-PLC, prior to precipitation of the VSG. Almost all of the [^{3}H]myristoylated VSG was protected from cleavage by GPI-PLC for up to 10 min. By 10 min, VSG starts reaching the plasma membrane and is cleaved; hence, the small difference between the two curves. This experiment shows that myristate exchange does not take place on the cell surface, because VSG labeled by exchange is protected from GPI-PLC cleavage during hypotonic lysis.

DISCUSSION

We previously identified two distinct myristoylation pathways for glycolipid A, the GPI precursor. The first, fatty acid remodeling, involves a sequential replacement of the longer fatty acids on glycolipid A′ (the unremodeled product of the GPI biosynthetic pathway) with myristate (6). The second pathway, myristate exchange, involves trading one myristate for another at both positions of preexisting glycolipid A (7). Although exchange could be a relatively trivial reaction, if it simply exploited the remodeling machinery, we presented evidence that they are independent pathways (e.g., they apparently occur in different subcellular compartments, and they have different susceptibilities to inhibitors). The subcellular fractionation showed that exchange on glycolipid A does not occur in the ER and therefore must occur downstream of the ER (7). Since it seemed surprising that the trypanosome would have enzymatic machinery whose function was to myristoylate glycolipid A that had escaped the ER, we explored in the current study the possibility that the major substrate for myristate exchange is VSG protein.

Since VSG receives a myristoylated GPI anchor within 30 sec after synthesis of the polypeptide (11), we reasoned that *de novo* GPI anchoring of VSG would be inhibited in cells treated with cycloheximide. Under these conditions, we found that there was substantial [^{3}H]myristate incorporation into VSG (Fig. 1*A*). This incorporation must be by myristate exchange, and the difference between incorporation in the absence and

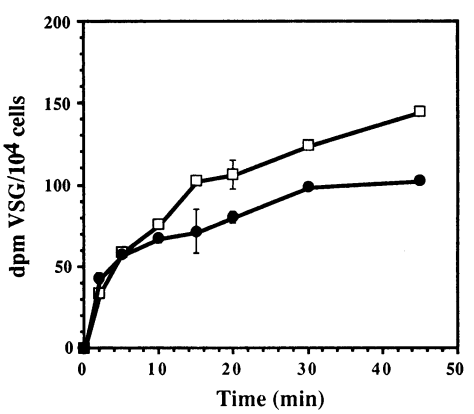

FIG. 5. Sensitivity of [^{3}H]myristate exchange-labeled VSG to endogenous GPI-PLC. Trypanosomes (10^7 cells per ml) were preincubated for 5 min with 400 μg of cycloheximide per ml and then labeled with 50 μCi of [^{3}H]myristate per ml. The culture was sampled (5×10^6 cells) at the indicated times. GPI-PLC was activated by hypotonic lysis in 0.2 mM N^{α}-(*p*-tosyl)-L-lysine chloromethyl ketone and 2 μg of leupeptin per ml for 5 min at 0°C, followed by adjustment to 50 mM Tris·HCl (pH 7.5), 5 mM EDTA, and 150 mM NaCl and incubation for 5 min at 37°C (●). Control samples were hypotonically lysed in the presence of 2 mM *p*-chloromercuriphenylsulfonic acid (a GPI-PLC inhibitor), 0.2 mM N^{α}-(*p*-tosyl)-L-lysine chloromethyl ketone, and 2 μg of leupeptin per ml (□). Then, all samples were precipitated/extracted with chloroform/methanol (2:1, vol/vol) and scintillation counted. The graph shows standard errors for duplicate samples for all points except the 45-min points and the 2-min point with *p*-chloromercuriphenylsulfonic acid, which were single samples.

in the presence of cycloheximide must be due to *de novo* anchor attachment (dashed line in Fig. 1*A*).

It was surprising that exchange leveled off in Fig. 1*A* while *de novo* synthesis continued linearly. One explanation for this difference could be that these reactions depend on separate pools of myristate, although addition of [^{3}H]myristate at 30 min did not result in increased exchange labeling (data not shown). We did find consistently that exchange proceeds linearly for a much longer time if the labeling takes place at 10^7 cells per ml, rather than 5×10^7 cells per ml as used for the experiment in Fig. 1*A*. Under these conditions, the rate of exchange was comparable to the initial rate in Fig. 1*A*, although it was linear for about 40 min (data not shown). The exchange machinery also appears to be labile. For example, storage of purified trypanosomes at 0°C and pH 8 for 6 hr resulted in severalfold reduction of exchange activity, even at 10^7 cells per ml (data not shown). We also conducted experiments with axenically cultured bloodstream *T. brucei* {strain 221, cultured as described previously (19) and labeled at 10^7 cells per ml with [^{3}H]myristate in the presence of cycloheximide} as these are likely to be metabolically more stable and dividing more consistently than trypanosomes transferred from rat blood to culture medium. With these cells, we found that exchange proceeded linearly for 45 min at about 65% of the initial rate shown in Fig. 1*A* and that the rate of *de novo* synthesis was comparable to that of exchange (data not shown). The overall conclusion of many experiments is that exchange is a significant process for overall *in vivo* incorporation of [^{3}H]myristate into VSG and accounts for 50–90% of this incorporation. Furthermore, the finding that the exchange into VSG was severalfold greater than that into glycolipid A indicates that VSG is the most significant substrate for exchange (Fig. 2). The similar sensitivity to inhibitors of exchange labeling of VSG and glycolipid A (Fig. 3) is consistent with the notion that the same enzymatic machinery is responsible for labeling both substrates.

We were surprised to find that not only newly synthesized VSG but also mature VSG is a substrate for myristate exchange. To distinguish between these two VSG populations, we preincubated the cells with tunicamycin, an inhibitor of N-glycosylation. Mature VSG, which has two N-linked oligosaccharides (16), has an apparent molecular mass of 57 kDa. In contrast, VSG synthesized in the presence of tunicamycin, and therefore missing these oligosaccharides, has an apparent molecular mass of 55 kDa. The unexpected labeling of mature VSG by myristate exchange (Fig. 4) raised the possibility that exchange could take place at or kinetically near the plasma membrane. We tested this hypothesis with a hypotonic lysis experiment, using the fact that cell surface VSG is cleaved by an endogenous GPI-PLC upon hypotonic lysis of the trypanosomes. In contrast, intracellular VSG is protected from this enzyme and thus retains its [^{3}H]myristate label (2, 11, 15). We found that VSG labeled by myristate exchange remains fully protected from cleavage for about 10 min (Fig. 5), thus demonstrating that exchange does not occur at or kinetically near the plasma membrane.

Myristate exchange on mature VSG likely requires recycling of the protein from the surface into an internal cellular compartment where exchange takes place. Published experiments indicate that VSG recycling is a major pathway in trypanosomes (refs. 20 and 21 and reviewed in ref. 22). About 9% of the total surface VSG is internalized per hour, and, since very little is degraded during this process, it must recycle back to the surface (21). It is likely that myristate exchange occurs during this recycling and in fact may be an important function of VSG internalization.

What is the function of myristate exchange? It is likely that exchange is a proofreading mechanism designed to ensure that each newly synthesized VSG molecule has a fully myristoylated GPI. In addition it could be a repair process, designed to correct any surface VSG that had either lost its myristate or that had adventitiously exchanged a myristate for some other fatty acid. It is well known that in mammals, membrane phospholipids undergo enzymatic remodeling of their fatty acids (reviewed in ref. 23); as the VSG anchor constitutes roughly 7% of the plasma membrane outer leaflet phospholipids‡, accidental action by such remodeling systems could result in loss of VSG myristate.

There are unanswered questions concerning myristate exchange. One concerns its intracellular location and the secretory and endocytic processes that bring VSG to this site. Another concerns the enzymatic mechanism of exchange. Based on studies of myristate exchange of glycolipid A (7), myristoyl-CoA is the likely myristate donor and the reaction involves deacylation and reacylation at both the *sn*-1 and *sn*-2 positions of the GPI. Nevertheless, we cannot rule out direct transfer of myristate from a phospholipid to the VSG GPI, in a transacylase reaction either dependent on or independent of coenzyme A (reactions are reviewed in ref. 23). Perhaps the most important unanswered question is why it is so important that the GPI anchor on VSG is myristoylated. It is remarkable that trypanosomes have developed two independent systems to ensure that its GPI anchors have strictly myristate.

‡This figure is based on the cross-sectional area of a VSG molecule [600 Å^2 (24)] and the cross-sectional area of a phospholipid [40 Å^2 (25)].

We thank Jay Bangs, Tamara Doering, Kojo Mensa-Wilmot, and Jayne Raper for valuable suggestions and for reviewing this manuscript. We also thank Jay Bangs for making the first observation that [^{3}H]myristate labels VSG in the presence of cycloheximide. This research was supported by grants from the National Institutes of Health (AI21334) and the MacArthur Foundation (to P.T.E.). L.U.B. was supported by MSTP Grant 5T32GM07329, K.A.W. was supported by National Institutes of Health postdoctoral fellowship AI08917, and K.G.M. was supported by a Prize Traveling Research Fellowship from the Wellcome Trust.

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