

# A Role for the Dynamic Acylation of a Cluster of Cysteine Residues in Regulating the Activity of the Glycosylphosphatidylinositol-specific Phospholipase C of *Trypanosoma brucei*\*

(Received for publication, November 29, 1999, and in revised form, December 22, 1999)

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**The glycosylphosphatidylinositol-specific phospholipase C or VSG lipase is the enzyme responsible for the cleavage of the glycosylphosphatidylinositol anchor of the variant surface glycoprotein (VSG) and concomitant release of the surface coat in *Trypanosoma brucei* during osmotic shock or extracellular acidic stress. In *Xenopus laevis* oocytes the VSG lipase was expressed as a nonacylated and a thioacylated form. This thioacylation occurred within a cluster of three cysteine residues but was not essential for catalytic activity *per se*. These two forms were also detected in trypanosomes and appeared to be present at roughly equivalent amounts. A reversible shift to the acylated form occurred when cells were triggered to release the VSG by either nonlytic acid stress or osmotic lysis. A wild type VSG lipase or a gene mutated in the three codons for the acylated cysteines were reinserted in the genome of a trypanosome null mutant for this gene. A comparative analysis of these revertant trypanosomes indicated that thioacylation might be involved in regulating enzyme access to the VSG substrate.**

African trypanosomes such as *Trypanosoma brucei* are parasitic protozoans responsible for sleeping sickness in humans and related diseases in other mammals. These cells are covered by a predominant surface antigen known as the variant surface glycoprotein (VSG),<sup>1</sup> and antigenic variation of this protein allows these parasites to escape the humoral immune defenses

of the mammalian host (for a recent review see Ref. 1). The VSG is attached to the plasma membrane through a glycosylphosphatidylinositol (GPI) anchor (2) but can be released from the cellular surface by cleavage of the GPI anchor during cellular lysis or exposure to nonlytic stress conditions (3, 4). Cleavage of the GPI anchor of the membrane form of VSG (mfVSG) generates a soluble form (sVSG) of the protein that displays a neo-epitope termed the cross-reacting determinant (CRD) that is dependent on the presence of an inositol-1,2-cyclic phosphate that remains associated with the residue of the anchor left attached to the released VSG (5). The enzyme responsible for this cleavage is a GPI-specific phospholipase C, termed VSG lipase (6–8), that is encoded by a single copy gene that is only expressed in bloodstream forms of the parasite (9, 10). This lipase possesses several rather curious features that deserve further comment. First, VSG lipase behaves as an integral membrane protein during purification and subcellular fractionation (6–8, 11), even though the primary sequence does not contain any obvious hydrophobic stretches to account for this physical property. Second, the apparent subcellular localization of the protein to the cytoplasmic face of small intracellular vesicles (12) presents a topological problem: how does the enzyme gain access to the GPI substrate of the VSG, which is thought to be present on the outer leaflet of the plasma membrane? Third, despite considerable effort the physiological role of the VSG lipase remains elusive (13). At present there is a consensus that this enzyme is not obligatorily required during any stage of the life cycle principally because null mutants that lack the gene were capable of cyclical transmission between host and vector (14). Interestingly, these null mutants consistently exhibited lower parasitaemias in mice (14) and appear to be compromised in their ability to undergo stress-induced differentiation from the bloodstream to the insect stage of procyclic form *in vitro* (15).

If the biological function of the VSG lipase in *T. brucei* remains equivocal, what is certain is that this enzyme must be regulated, at least in terms of VSG release. This view is predicated on the relative abundance of VSG lipase in bloodstream trypomastigotes, approximately  $3 \times 10^4$  molecules/cell, which allied to an estimated turnover rate of 100–700 mfVSG molecules/min is more than sufficient to release the entire VSG coat ( $10^7$  molecules/cell) within a few minutes (7, 16). However, large scale release of VSG does not occur under physiological

\* This work was supported by research contracts with the Communauté Française de Belgique (Actions de Recherche Concertées), by the Belgian Fonds de la Recherche Scientifique (Fonds de la Recherche Scientifique Médicale and Crédit aux Chercheurs), and by the Interuniversity Poles of Attraction Programme - Belgian State, Prime Minister's Office, Federal Office for Scientific, Technical and Cultural Affairs. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Reasearch associates of the Belgian Fonds National de la Recherche Scientifique.

¶ Supported by CNPq Brazil, St-John's College, Cambridge, and the Franqui Foundation.

\*\* Supported by Fonds voor Wetenschappelijk Onderzoek.

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<sup>1</sup> The abbreviations used are: VSG, variant surface glycoprotein; GPI, glycosylphosphatidylinositol; n-OG, *n*-octylglucoside; PLC (PLC), phospholipase C (gene); sVSG, soluble form VSG; mfVSG, membrane form VSG; CRD, cross-reacting determinant; PAGE, polyacrylamide gel elec-

trophoresis; Tes, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid.

conditions (4), and the focus of this study was to identify possible regulatory features responsible for this behavior. Heterologous expression of the VSG lipase mRNA in *Xenopus laevis* oocytes revealed equivalent expression of two forms of the enzyme: a nonmodified form as well as a form post-translationally acylated on two or three clustered cysteine residues. Similar results were obtained with *T. brucei*. Although this thioacylation was not absolutely essential for the enzymatic activity, the data supported the view that acylation may be involved in regulating access to the VSG substrate. During the course of this work another group also reported that the VSG lipase was modified by *S*-myristoylation (17). Although our results are similar in some respects to those reported in this study, we report additional data concerning the site of acylation and also present evidence that reversible thioacylation might modulate access of the enzyme to the GPI anchor of the VSG in trypanosomes.

#### EXPERIMENTAL PROCEDURES

**Trypanosomes**—*T. brucei* AnTat 1.1A and 1.3A clones from the EATRO1125 stock were grown in mice and purified by the method of Lanham (18) with certain modifications, e.g. supplementing the isolation buffer with sucrose (80 mM), KCl (5 mM), and adenosine (0.2 mM), designed to avoid nonphysiological conditions during the isolation step that have been shown to cause biochemical changes in the parasite (19). Procytic forms used for transfection were from the null VSG lipase mutant (14). Cultivation, electroporation, and fly transmission of these transformants were performed according to Ref. 20, except that selection was made with 2  $\mu$ g/ml phleomycin. The bloodstream form transformants were analyzed after passaging in immunosuppressed mice.

**Xenopus Oocytes**—Oocyte dissection, maintenance, and microinjection were performed as described elsewhere (21). In all experiments, 30 ng of VSG lipase mRNA were injected in a volume of 15 nl.

**Plasmids**—The VSG lipase transcription vector was constructed by polymerase chain reaction amplification and subcloning of the *T. brucei* GPI-PLC cDNA (9) in pCR2.1 (Invitrogen). The polymerase chain reaction product was released by *Asp718I* digestion and ligated in the same site in the pBSDH1400H-A65 transcription vector (22). This construct was mutagenized using in some cases the Chameleon double-stranded site-directed mutagenesis kit and in other cases the Quick-Change site-directed mutagenesis kit, both from Stratagene. Cysteines 24, 80, 184, 269, 270, 273, 332, and 347 of the VSG lipase were individually substituted with serine, isoleucine, serine, leucine, alanine, arginine, serine, and serine, respectively. In the double cysteine (269 + 270) and triple cysteine (269 + 270 + 273) mutants, cysteines were replaced by serine + arginine and serine + arginine + arginine, respectively. A triple substitution by serine gave rise to unstable protein (data not shown).

To insert the VSG lipase (wild type or mutant) in the pPT vector (23), we took advantage of the pPT construct containing the AnTat 11.17 VSG cDNA sequence flanked with *SacI* and *XbaI* sites at the 5'- and 3'-end, respectively. The wild type and the triple mutant version of the VSG lipase were removed from the pBSDH1400H-A65 constructs by *SacI* + *XbaI* digestion and ligated with the *SacI/XbaI*-cleaved pPT11.17 vector. The final pPT constructs were targeted to the tubulin locus of procytic forms of the null VSG lipase mutant by linearization with *SpeI* and electroporation.

**Antibodies**—Anti-VSG lipase antibodies were generated in rabbits by immunization with a purified C-terminal His-tagged VSG lipase expressed in *Escherichia coli* (24). The anti-CRD antibodies were a kind gift of Dr. Paul Englund (Baltimore, MD) and purified according to Ref. 3. The anti-AnTat 1.1 antibodies were a hyperimmune serum from the Institute for Tropical Medicine (Antwerp, Belgium).

**Electrophoresis and Western Blotting**—Electrophoresis was conducted in SDS-10% polyacrylamide gels according to Laemmli except that the protein samples were usually incubated for 15 min at 37 °C in the loading buffer instead of being boiled (25). However, immunoprecipitates were incubated for 5 min at 100 °C to elute proteins bound to the protein A-Sepharose. Electrophoresis was followed by either fluorography (26) or Western blotting (27). Visualization was performed with either anti-rabbit IgG alkaline phosphatase or with the ECL chemiluminescence system (Amersham Pharmacia Biotech). Fluorography was used for <sup>35</sup>S detection in both *Xenopus* and trypanosome extracts, as well as for <sup>3</sup>H-labeled oocyte samples. Analysis of [<sup>3</sup>H]palmitic acid incorporation in trypanosome extracts was performed

after blotting of the gels onto nitrocellulose.

**In Vitro Transcription and Translation**—The *EcoRV*-linearized transcription vectors were used as templates for mRNA synthesis, and the mRNAs were translated in rabbit reticulocyte lysate as described (21). The fate of the [<sup>35</sup>S]methionine-labeled VSG lipase in the oocyte was followed after submitting the reticulocyte reaction mixture to a spun Biogel P-6 DG (Bio-Rad) column (500  $\mu$ l of packed resin) to eliminate the unincorporated radiolabeled methionine. Iodoacetamide treatment of radiolabeled reticulocyte mixture was performed by adding 1/5 volumes of 0.25 M iodoacetamide in 0.25 M Tris-HCl (pH 8.0) to one-half of the sample, whereas the second half received only the buffer. After 15 min of incubation at room temperature, Biogel P-6 DG filtration was performed for both samples as described above.

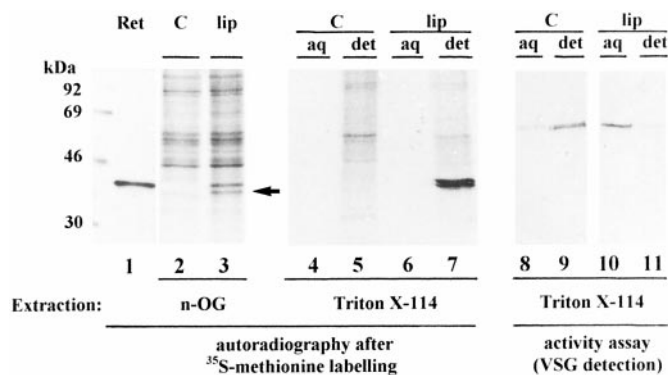
**Xenopus Oocyte Metabolic Labeling and Extract Preparation**—After overnight incubation at 18 °C in saline medium in the presence of 50  $\mu$ g/ml gentamycin (21), the injected oocytes were carefully inspected before being incubated for another 20–24 h in the same medium containing 1 mCi/ml L-[<sup>35</sup>S]methionine (1000 Ci/mM, Amersham Pharmacia Biotech) at a ratio of 300  $\mu$ l for 50 oocytes. Fatty acid labeling was performed by firstly evaporating either [9,10(*n*-<sup>3</sup>H)]palmitic acid or myristic acid (530 Ci/mM in both cases) and then coupling them onto defatted bovine serum albumin to reach the concentration of 200  $\mu$ Ci/ml in the final 300- $\mu$ l incubation volume. Metabolic labeling was then performed as described elsewhere (28). Total oocyte extracts were prepared by a first extensive washing of the labeled oocytes, followed by homogenization at 0 °C in a volume of 20  $\mu$ l/oocyte of NaCl (150 mM), n-OG (1.5%, w/v), 4-(2-aminoethyl)benzenesulfonyl-fluoride (50  $\mu$ M), trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (10  $\mu$ M), pepstatin (1  $\mu$ M), EDTA (1 mM), and Tris-HCl (50 mM, pH 7.5). After centrifugation for 15 min at 500  $\times$  g to pellet the yolk platelets, the supernatants were aliquoted and kept at -70 °C. In all experiments, 10  $\mu$ l of extract, equivalent to half an oocyte, was loaded per lane.

Partition of the VSG lipase by phase separation in Triton X-114 was performed by washing and homogenization at 0 °C of 20 oocytes in 1.1 ml of NaCl (150 mM), Triton X-100 (0.05%, w/v), and Tris-HCl (50 mM, pH 7.5) supplemented with the same mixture of protease inhibitors as above. After centrifugation for 15 min at 500  $\times$  g, 100  $\mu$ l of precondensed Triton X-114 (12%, w/v) were added to the supernatant, and the mixture was incubated at 0 °C for about 30 min. The supernatant of the next 10-min centrifugation at 10,000  $\times$  g was submitted to phase separation (29). Aqueous and detergent phases were each re-extracted once. Both types of samples were precipitated for 30 min at -20 °C with 5 volumes of ice-cold acetone, in the presence of 100  $\mu$ g of RNase as a carrier for the detergent extract only. The dried acetonetic powders were dissolved in Laemmli buffer for SDS-PAGE.

**Activity Assay of VSG Lipase Synthesized in Xenopus Oocytes**—AnTat 1.1 mf-VSG was prepared by homogenizing bloodstream forms (AnTat 1.1; 10<sup>8</sup> cells) in 500  $\mu$ l of NaCl (150 mM), Triton X-100 (0.05%), and Tris-HCl (50 mM, pH 7.5) together with the usual mixture of protease inhibitors and *p*-chloromercuriphenylsulfonic acid (10 mM) to inactivate the endogenous VSG lipase. After addition of 1/2 volume of precondensed Triton X-114, phase partitioning was performed (29). The detergent phase was re-extracted once and then frozen in aliquots. AnTat 1.1 mf-VSG was added to Triton X-114 extracts from [<sup>35</sup>S]methionine-labeled oocytes, injected or not with the VSG lipase mRNA. Both were diluted 30 times in the Tris-NaCl-Triton X-100 buffer to decrease the Triton X-114 concentration to a maximum of 0.05%. In all assays, the mf-VSG was mixed with the Triton phase from four oocytes, and the incubation was performed in a final volume of 550  $\mu$ l for 30 min at 30 °C. After addition of 50  $\mu$ l of Triton X-114, the reaction mixture was submitted to phase separation again. Both phases were re-extracted once, electrophoresed, and blotted. The presence of the VSG lipase was monitored by the <sup>35</sup>S labeling, whereas the VSG was detected by Western blot analysis using anti-AnTat 1.1 antibodies (100,000-fold dilution).

**Metabolic Labeling of Trypanosome**—Trypanosomes (2  $\times$  10<sup>7</sup> cells/ml) were incubated in cysteine/methionine-free medium (Life Technologies, Inc.) supplemented with 30 mM Hepes (pH 7.4), catalase (5  $\mu$ g/ml), adenosine (0.4 mM) and phosphate-buffered saline-dialyzed fetal calf serum (10%, v/v) containing [<sup>35</sup>S]methionine/cysteine (200  $\mu$ Ci/ml, Amersham Pharmacia Biotech PRO-MIX™) as described previously (30). Metabolic labeling with <sup>3</sup>H-labeled fatty acids (200  $\mu$ Ci/ml) was performed as described by Field *et al.* (31).

**Immunoprecipitation**—Trypanosomes were resuspended at a final concentration of 10<sup>9</sup> cells/ml in NaCl (137 mM), KCl (2.7 mM), glucose (5 mM), Na<sub>2</sub>HPO<sub>4</sub> (8 mM), and KH<sub>2</sub>PO<sub>4</sub> (1.5 mM) final pH 7.5, and then an equal volume of phosphate buffer containing 4% SDS (w/v) was added followed by immediate incubation of the cell lysates at 100 °C for 3 min.



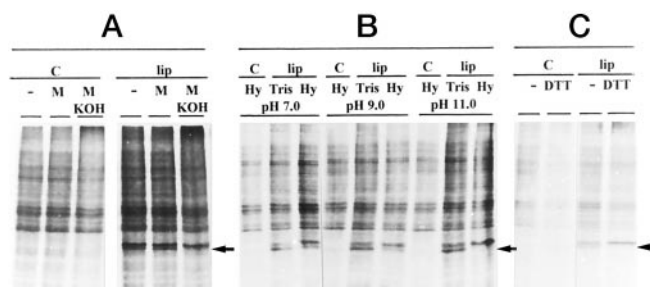
**FIG. 1. Expression of VSG lipase from *T. brucei* in *X. laevis* oocytes.** In lanes 1–7, the translation product of the VSG lipase mRNA in reticulocyte lysates (*Ret*) was aligned with extracts from oocytes injected with VSG lipase mRNA (*lip*) or noninjected oocytes (*C*), treated as indicated. *aq*, aqueous phase; *det*, detergent phase. The arrow points to the 39-kDa component detected in oocytes. Lanes 8–11 demonstrate the VSG lipase activity in the control (*C*) and injected (*lip*) oocytes, as determined by Western blot detection of VSG in the aqueous phase after Triton X-114 partition of the oocyte extracts as described under “Experimental Procedures.”

The boiled extracts were diluted by the addition of 4 volumes of phosphate buffer containing Triton X-100 (2.5%, w/v), leupeptin (20  $\mu$ g/ml), EDTA (1 mM), and phenylmethylsulfonyl fluoride (0.1 mM), and then they were placed on ice for 1 h and centrifuged at  $9000 \times g$  for 5 min. Either 100- $\mu$ l aliquots of the supernatants, which corresponded to the detergent extracts from  $10^7$  trypanosomes, or oocyte samples (10  $\mu$ l of n-OG extract, corresponding to half an oocyte) were mixed with varying amounts of anti-VSG lipase antibodies, ranging from 10 to 90  $\mu$ l. The samples were adjusted to a constant volume of 500  $\mu$ l and incubated overnight at 4  $^{\circ}$ C with gentle inversion. To each sample was added 3 mg of protein A-Sepharose CL 4B (Amersham Pharmacia Biotech), previously swollen and washed two times in Tris-Triton buffer (100 mM NaCl, 1 mM EDTA, 0.1% (w/v) Triton X-100 and 50 mM Tris-HCl, pH 7.5). The mixtures were submitted to a 2-h incubation at 4  $^{\circ}$ C and then centrifuged at  $10,000 \times g$  for 30 s. The resin-bound complexes were washed once in the Tris-Triton buffer, once in the same buffer containing NaCl (500 mM), and, finally, once in the buffer without Triton. The immunoprecipitates were eluted by boiling for 5 min in SDS-PAGE sample buffer.

**Chemical Treatments**—All treatments were performed on the n-OG oocyte extracts essentially as described elsewhere (25, 32). After treatment the proteins were precipitated in acetone in the presence of 10  $\mu$ g of RNase. The alkaline methanol treatment was performed in 0.1 M KOH in anhydrous methanol (obtained by adsorption of methanol on a 0.3-nm molecular sieve from Merck) on acetone-precipitated samples. The control treatment consisted of neutral methanol. Both samples were incubated for 30 min at 30  $^{\circ}$ C. Hydroxylamine treatments were performed by adding to one volume of sample 10 volumes of 1 M hydroxylamine brought to pH 7.0, 9.0, and 11.0 with NaOH. Controls were respectively 1 M Tris solutions at the same pHs, and samples were incubated for 4 h at room temperature. Dithiothreitol treatment was performed by incubating each sample with 1.4 volumes of 2.5 M dithiothreitol for 1 h at room temperature.

## RESULTS

**Identification of a Covalent Modification of VSG Lipase in the Oocyte System**—Translation of the VSG lipase mRNA in a reticulocyte lysate in the presence of [ $^{35}$ S]methionine produced a single polypeptide with an apparent molecular mass of 42 kDa (Fig. 1, lane 1) that was able to convert mfVSG to sVSG *in vitro* (data not shown). However, injection of the same mRNA into *X. laevis* oocytes resulted in the roughly equivalent expression of two polypeptides: one with a size similar to the *in vitro* translation product (~42 kDa) and an additional protein with an apparent molecular mass of 39 kDa (Fig. 1, compare lanes 2 and 3). Both proteins were recognized by anti-VSG lipase antibodies (see below). These polypeptides were termed forms 1 (42 kDa) and 2 (39 kDa). In partition experiments using Triton X-114 (26), both forms were recovered in the detergent phase



**FIG. 2. Evidence for acylation of the VSG lipase in *Xenopus* oocytes.** Oocytes were injected with VSG lipase mRNA and subjected to metabolic labeling (15 h) with [ $^{35}$ S]methionine as described under “Experimental Procedures.” The panels present the autoradiographic analysis of [ $^{35}$ S]methionine-labeled extracts from injected oocytes (*lip*) and from a parallel incubation of noninjected oocytes (*C*) after various treatments. A, the extracts were either untreated (–) or incubated for 30 min at 37  $^{\circ}$ C in methanol (*M*) or methanol/0.1 M KOH (*M KOH*). B, the extracts were treated for 4 h at room temperature with 1 M hydroxylamine (*Hy*) or 1 M Tris buffer (*Tris*) at the various different pH values as indicated. C, the extracts were either untreated (–) or incubated for 1 h at room temperature with 0.5 M dithiothreitol (*DTT*) before precipitation in acetone. The arrows point to form 2 of the VSG lipase.

(Fig. 1, lanes 4–7). It was noted that the relative degree of separation of the two bands on SDS-PAGE gels varied to some extent depending on the precise experimental conditions and choice of detergent employed, e.g. n-OG or Triton. The activity of the enzyme expressed in *Xenopus* oocytes was assayed by mixing mfVSG with the detergent phase from either control or VSG lipase mRNA-injected oocytes. The sVSG was detected, in the aqueous phase, only using extracts from oocytes injected with the VSG lipase mRNA (Fig. 1, lanes 8–11).

**The VSG Lipase Is Thioacylated**—The presence of an additional band with a higher electrophoretic mobility was consistent with acylation of the VSG lipase. Therefore, the oocyte extracts were subjected to treatments known to affect different covalent linkages of fatty acids to polypeptides (32). First, treatment with alkaline but not neutral methanol resulted in the selective loss of form 2 (39-kDa form; Fig. 2A, arrow). Ester but not amide linkages are cleaved under these conditions that eliminated the involvement of relatively stable amide linkages such as those that occur between myristic acid and N-terminal glycine residues. Second, form 2 disappeared progressively in the presence of hydroxylamine as the  $H^+$  concentration was decreased, an effect that was not due to the concentration of  $H^+$  (Fig. 2B). This behavior was consistent with the presence of a thioester rather than oxyester linkage of fatty acid. Finally, treatment with high concentrations of the reducing agent dithiothreitol, which affects thio- but not oxyesters, also resulted in the loss of form 2 (Fig. 2C). The effects of these chemical treatments supported the view that the 39-kDa band represented a form of VSG lipase that was covalently modified by thioacylation.

In eukaryotic cells the principal pathways for protein acylation involve attachment of myristic acid or palmitic acid and differ by the timing of the modification. Myristic acid is generally added co-translationally, whereas palmitoylation usually occurs post-translationally and is reversible. To determine when the modification of the VSG lipase occurred, the protein was first translated *in vitro* in the presence of  $^{35}$ S-labeled methionine, separated from the unincorporated methionine, and then injected into oocytes. Approximately 4 h after injection form 2 (39 kDa) began to appear at the expense of form 1, and 15 h after injection both forms were present in approximately equal amounts (Fig. 3A) as observed in oocytes injected with VSG lipase mRNA (Fig. 1). As expected, form 2 was lost after alkaline methanol treatment (Fig. 3B). Finally, if prior to

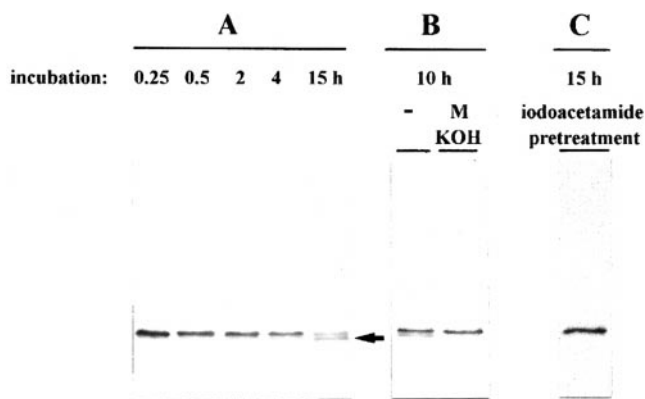


FIG. 3. Post-translational acylation of the VSG lipase. A shows an autoradiographic analysis of oocyte extracts prepared at different time intervals after injection of [<sup>35</sup>S]methionine-labeled VSG lipase that had been synthesized in a reticulocyte lysate. B, the oocyte extracts were either untreated or incubated in methanol/0.1 M KOH (M KOH). C, presents the VSG lipase pattern in an oocyte extract prepared 15 h after injection of the enzyme pretreated with 0.05 M iodoacetamide.

the injection into the oocyte, the *in vitro* translated protein was first treated with iodoacetamide, an alkylating reagent that blocks thiol groups, form 2 was not detected (Fig. 3C). Taken together, these results supported the view that the appearance of a faster migrating species, form 2, was not due to nonspecific degradation of the injected polypeptide but reflected post-translational thioacylation of the VSG lipase. Although the kinetic data (Fig. 3A) suggested that thioacylation of the injected protein occurred rather slowly, whether or not this was a genuine feature of the thioacylation pathway in oocytes or a secondary effect because of problems in processing the injected polypeptide remains uncertain.

Direct evidence for acylation of VSG lipase was obtained by metabolically labeling oocytes injected with VSG lipase mRNA with <sup>3</sup>H-labeled palmitic or myristic acid (Fig. 4A). Both fatty acids were incorporated but only into form 2, and overall the incorporation of palmitic acid was far higher than myristic acid (compare lanes 2 and 4). In both cases the acylated form comigrated with the 39-kDa form observed in [<sup>35</sup>S]methionine metabolic labeling experiments (lane 1), and the label was lost when the extracts were treated with alkaline methanol (Fig. 4B) or hydroxylamine (Fig. 4C).

**Identification of the Site of Thioacylation in VSG Lipase**—To identify the site of thioacylation each of the 8 cysteines present in the VSG lipase were individually mutagenized (Scheme 1). Mutations at c1, c2, c3, c7, and c8 did not lead to loss of the doublet pattern when expressed in oocytes, but each of the mutations in the clustered cysteines 4, 5, and 6 (residues 269, 270, and 273) converted the enzyme to a single form (Fig. 5A). In all cases the protein was recovered in the detergent phase. Because each of the c4, c5, and c6 mutants were labeled by [<sup>3</sup>H]palmitic acid, albeit to a lesser than in the other mutants (Fig. 5B), double and triple mutants of these cysteines were generated. The double c4 + c5 mutant showed only a very faint labeling, whereas the triple c4 + c5 + c6 mutant was no longer labeled (Fig. 5B). These data suggested that in the oocytes, about half the VSG lipase molecules were acylated on at least two of the three cysteines 269, 270, and 273. Significantly, the triple c4/c5/c6 mutant was active because mfVSG was converted to sVSG, which was recovered in the aqueous phase, when mfVSG was incubated with extracts of oocytes that expressed the triple mutant form of the enzyme (Fig. 5C). These data indicated that neither these cysteines nor their acylation were essential for lipase activity when mfVSG was the substrate, which was in agreement with the results from detailed

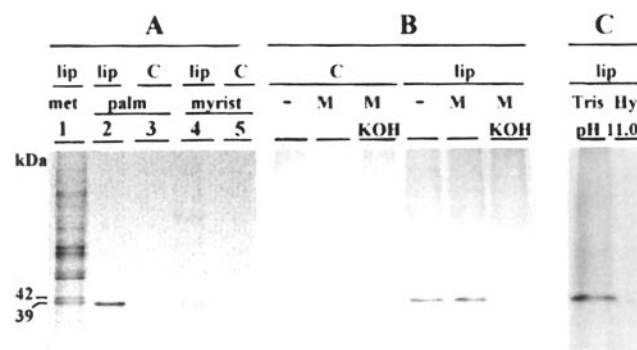


FIG. 4. Incorporation of fatty acids in the VSG lipase expressed in *Xenopus* oocytes. Oocytes that had been injected with VSG lipase mRNA were incubated with either [<sup>3</sup>H]palmitic acid or [<sup>3</sup>H]myristic acid as described under "Experimental Procedures." A shows the autoradiographic analysis of extracts after metabolic labeling with [<sup>3</sup>H]palmitic acid (lanes 2 and 3) or [<sup>3</sup>H]myristic acid (lanes 4 and 5) of oocytes injected with the VSG lipase mRNA (lanes 2 and 4) or noninjected (lanes 3 and 5). The pattern of [<sup>35</sup>S]methionine labeling observed in a 50-fold diluted extract of injected oocytes (lane 1) is included to show the alignment of the 39-kDa component with the <sup>3</sup>H-labeled band. B shows the effect of methanol (M) and alkaline methanol (M KOH) on the [<sup>3</sup>H]palmitic acid labeling in control (C) and injected (lip) oocytes. C shows the effect of either 1 M hydroxylamine (Hy) or 1 M Tris (Tris) at pH 11.0 on the [<sup>3</sup>H]palmitic acid labeling in oocytes injected with the VSG lipase mRNA.

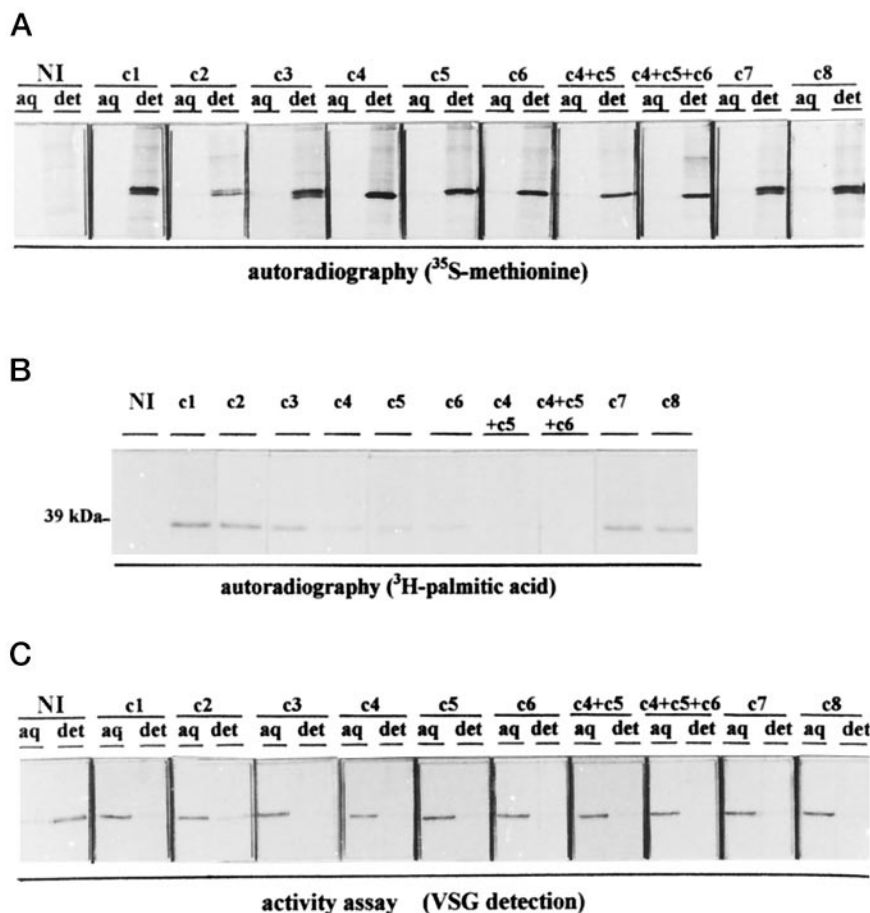
Cys number	Position	Substitution
1	24	Ser
2	80	Ile
3	184	Ser
4	269	Leu
5	270	Ala
6	273	Arg
7	332	Ser
8	347	Ser
4 + 5	269/270	Ser/Arg
4 + 5 + 6	269/270/273	Ser/Arg/Arg

SCHEME 1. A representation of the VSG lipase indicating the positions of the cysteine residues. Each of the cysteine residues were mutagenized as detailed above. Substitution of the cysteine residues in the triple mutant with three serines produced an unstable polypeptide.

studies on the enzyme expressed in *E. coli* (24). These data also implied that the presence of an acyl chain was not solely responsible for the hydrophobic properties of the protein because the nonacylated mutant enzyme still partitioned into the Triton X-114 detergent phase (Fig. 5B).

**Thioacylation of the VSG Lipase Also Occurs in Bloodstream Forms of T. brucei**—A Western blot analysis of different clonal variants of freshly isolated bloodstream forms of *T. brucei* using anti-VSG lipase antibodies revealed a doublet of bands (with molecular masses of ~42 and 39 kDa) similar to that observed in *X. laevis* oocytes (Fig. 6A). The lower band of this doublet also disappeared upon alkaline methanol treatment, consistent with its modification by acylation in trypanosomes (Fig. 6A). The results from metabolic labeling experiments were also consistent with acylation of the enzyme in trypanosomes. A doublet of 39/42 kDa was immunoprecipitated using

**FIG. 5. Characterization of the cysteine mutants of the VSG lipase.** The codons for each of the 8 cysteines of the VSG lipase mRNA were individually mutagenized (c1–c8, from the N to the C terminus as detailed in the legend to Fig. 1), and double/triple mutants were also generated (c4 + c5 and c4 + c5 + c6, respectively). These mRNAs were injected into *Xenopus* oocytes, and the catalytic activity and electrophoretic pattern of the translation products were compared. Extracts from noninjected oocytes were analyzed as controls (NI). **A** presents an autoradiographic analysis of the Western blot. **B** shows the fluorographic analysis of n-OG extracts from oocytes injected with these mRNAs and subjected to metabolic labeling with [<sup>3</sup>H]palmitic acid. **C** shows the activity of [<sup>35</sup>S]methionine-labeled VSG lipase using mfVSG as substrate and assayed by phase partition as described under “Experimental Procedures.”



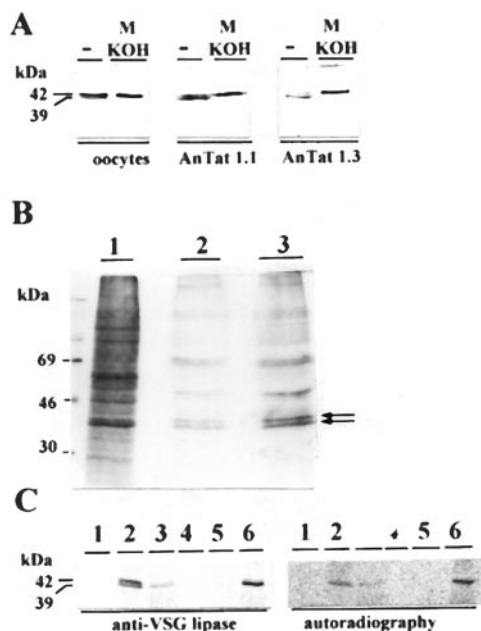
anti-VSG lipase antibodies from lysates of [<sup>35</sup>S]methionine-labeled trypanosomes (Fig. 6B, arrows), but only the 39-kDa component of this doublet was labeled by [<sup>3</sup>H]palmitic acid (Fig. 6C, lanes 3 and 6). This component co-migrated with a similarly labeled band immunoprecipitated from extracts of *Xenopus* oocytes injected with the VSG lipase mRNA and subjected to metabolic labeling with [<sup>3</sup>H]palmitic acid (Fig. 6, lane 2). These data clearly supported the view that the VSG lipase was also thioacylated in trypanosomes.

**Thioacylation of the VSG Lipase Is Dynamic in Trypanosomes**—The nonacylated and acylated forms of the VSG lipase appeared to be present in roughly equal amounts in freshly isolated trypanosomes (Fig. 6A), but when these cells were lysed by Nonidet P-40 extraction or osmotic shock, both of which lead to release of the VSG by activation of the VSG lipase (3, 4, 14), this doublet of bands was not detected, and the protein migrated as a single species with a size similar to that of the acylated form, *i.e.* 39 kDa (Fig. 7A, lanes 1 and 3). This finding suggested that treatments that lead to VSG lipase-mediated release of VSG in trypanosomes also resulted in the conversion of all of the enzyme to an acylated form. Moreover, this conversion was observed in trypanosomes but not in oocytes (lane 4), nor did it occur when the trypanosomes were lysed under denaturing conditions, *e.g.* by boiling in SDS (lane 2). Both of these features suggested the presence of an additional post translational acylation step in trypanosomes.

The nature of this acylation step was investigated further by exposing the cells to a short term nonlytic stress. For example, a short preincubation in a mildly acidic medium (pH 5.5) at 4 °C followed by incubation at a physiological pH (pH 7.5) has been shown to induce a transient but reversible activation of the VSG lipase as judged by the release of CRD-positive VSG (4). After a preincubation at pH 7.5, there was a small amount

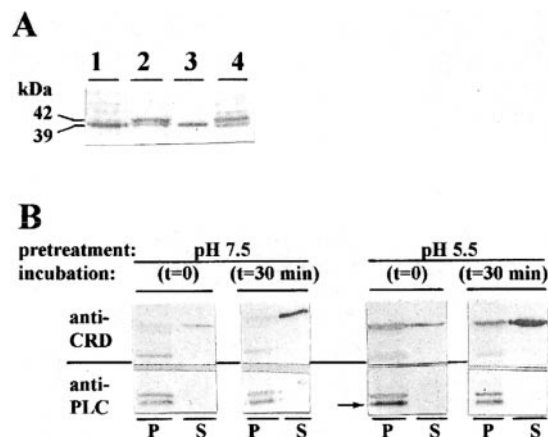
of CRD-positive VSG released (shown at time 0), but this release was greater when the cells were preincubated at pH 5.5 (Fig. 7B). When the cells were transferred to pH 7.5 buffer (37 °C) for 30 min, further release of CRD-positive VSG was detected in the supernatant fraction (S), but this release was significantly greater for cells pretreated at pH 5.5 than at pH 7.5 (compare last lane of each panel). Interestingly, the extent of VSG release in these experiments, as judged by the anti-CRD antibodies, appeared to correlate with the initial enrichment of the lower band (form 2) of the VSG lipase in the case of cells subjected to the pH 5.5 pretreatment (shown by the arrow). However, at the end of the 30-min incubation at pH 7.5 this enrichment of form 2 was no longer visible, and a resting 1:1 ratio of acylated to nonacylated forms appeared to be restored (compare cellular pellets at 0 and 30 min). Together these data suggested that acylation of the VSG lipase in whole cells was dynamic and reversible and that a shift occurred in the ratio of the nonacylated to acylated form of lipase under conditions that lead to cleavage of the GPI anchor of the VSG.

**Post-translational Thioacylation May Regulate the Functional Activity of the Enzyme in Trypanosomes**—The finding that the VSG lipase appeared to be reversibly acylated under condition known to trigger VSG release suggested a possible regulatory role for this modification, perhaps by regulating access to the mfVSG substrate. This idea was investigated by comparing the behavior of the nonacylatable, triple mutant, and wild type form of the enzyme when expressed in a trypanosome VSG lipase null mutant (14). As a first step in these studies the relative activities of these two forms of the enzyme were compared in a semi-quantitative fashion using known amounts of recombinant enzyme or serially diluted extracts of oocytes or trypanosomes that expressed either the wild type or triple mutant form of the enzyme. In these experiments the mfVSG



**FIG. 6. Thioacylation of the VSG lipase in trypanosomes.** A presents a Western blot analysis of the VSG lipase, using specific antibodies, in total extracts from either *Xenopus* oocytes injected with the VSG lipase mRNA or from *T. brucei* variant clones as indicated. The extracts were either untreated (-) or incubated in alkaline methanol as described previously in the legend to Fig. 2 (M KOH). B shows an autoradiographic analysis of immunoprecipitates from AnTat 1.1 trypanosomes metabolically labeled with [<sup>35</sup>S]methionine. Various amounts of anti-VSG lipase antibodies were employed, 30  $\mu$ l (lane 2) and 90  $\mu$ l (lane 3), and the immunoprecipitates were aligned with 10  $\mu$ l of a total n-OG extract from [<sup>35</sup>S]methionine-labeled oocytes injected with the VSG lipase mRNA (lane 1). Arrows point to the doublet of VSG lipase-specific bands. C shows the results of immunoprecipitation of n-OG lysates from AnTat 1.1 trypanosomes metabolically labeled with [<sup>3</sup>H]palmitic acid. In lanes 3 and 6, respectively 30 and 90  $\mu$ l of anti-VSG lipase antibodies were used, whereas in lanes 4 and 5 the same respective amounts of preimmune antibodies were used. Anti-VSG lipase immunoprecipitates of n-OG extracts of VSG lipase mRNA injected (lane 2) or control (lane 1) oocytes after metabolic labeling with [<sup>3</sup>H]palmitic acid are included for comparison. The immunoprecipitates were blotted onto nitrocellulose after SDS-PAGE, probed with anti-VSG lipase antibodies, and subjected to autoradiography (exposure for 15 days).

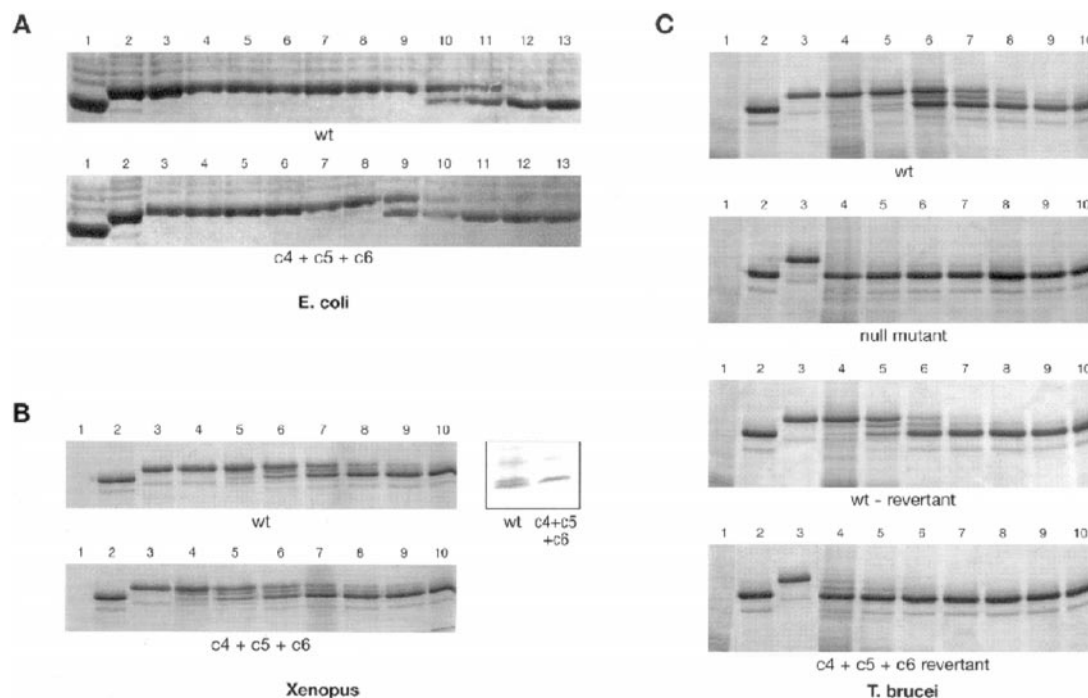
was the substrate, and the basis of the assay was the apparent decrease in the electrophoretic mobility of the VSG on SDS-PAGE gels that occurred when mfVSG was converted to sVSG by the lipase (Fig. 8). When expressed in *E. coli* or *Xenopus* oocytes the wild type enzyme appeared to be approximately twice as active as the triple mutant form of the enzyme because about twice as much triple mutant form as wild type enzyme was required to convert completely a fixed amount of mfVSG to sVSG within the time course of the assay (Fig. 8, A and B). In the case of the revertant trypanosomes, the wild type enzyme appeared to be about 25-fold more active than the triple mutant form (compare tracks 4 and 6, respectively, for the triple mutant and wild type revertant in Fig. 8C). However, this difference in activity in the trypanosome revertants was partly due to an absolute difference in the level of expression of either form of the protein (Fig. 9). Immunoprecipitation experiments suggested a 2-fold difference in the level of expression (Fig. 9A), whereas Western blots indicated at least a 5-fold difference in the level of expression of the wild type compared with the triple mutant form of the protein in these revertants (Fig. 9B). Interestingly, the level of lipase protein in both revertants was significantly lower than that observed in the true wild type trypanosome. When the activity comparisons (Fig. 8C) were corrected for this difference in the actual amount of lipase



**FIG. 7. Dynamic thioacylation of the VSG lipase in trypanosomes.** A, Western blot detection of the VSG lipase in trypanosomes lysed under different conditions. AnTat 1.1 parasites were lysed at  $6.6 \times 10^8$  cells/ml using 0.2% Nonidet P-40/TBS (lane 1), 1% SDS/TBS (lane 2), or water (lane 3). Lane 4 contains an extract of *Xenopus* oocytes injected with the VSG lipase mRNA. The blot was incubated with anti-VSG lipase rabbit antibody and probed with protein A-alkaline phosphatase. B illustrates the reversible shift in the ratio of two forms of the VSG lipase that occurs when trypanosomes are subjected to nonlytic mild acid stress as described by Rolin *et al.* (4). Briefly, AnTat 1.1 parasites were incubated at  $2 \times 10^7$  cells/ml in 125 mM phosphate buffer, pH 5.5 or 7.5, with 1% glucose for 30 min at 4 °C. In the case of both pretreatments, the cells were then harvested and subjected to a short incubation (5 min at 4 °C) under swell dialysis conditions as described in Ref. 4 by resuspension at  $6 \times 10^8$  cells/ml in 55 mM KCl, 1 mM glucose, 1 mM EGTA, 13.3 mM Tes buffer (pH 7.5). The osmolarity of the suspension of parasites was then adjusted to physiological levels by addition of 1/20 volume of 3 M KCl and parasites diluted to  $1.2 \times 10^8$  cells/ml with phosphate buffer (pH 7.5). Aliquots were then removed and microcentrifuged at 14,000 rpm for 5 min, and pellet (P) and supernatant (S) were retained for SDS-PAGE (incubation  $t = 0$ ). The remaining cells were transferred to 37 °C for 30 min and also prepared for SDS-PAGE ( $t = 30$  min). The supernatant and pellets were analyzed by Western blotting, and each lane represents the equivalent of  $4.5 \times 10^6$  cells. The upper and lower parts of the same nitrocellulose sheet were probed with anti-CRD and anti-VSG lipase as indicated. The anti-CRD antibodies employed in these blots demonstrated no cross-reactivity to mfVSG (not shown).

protein expressed in the two revertants (2–5-fold in favor of the wild type; Fig. 9), the data suggested that the triple mutant form (nonacylatable) was between 5- and 12.5-fold less active than the wild type acylated form in trypanosomes when mfVSG was the substrate.

As expected, both osmotic shock and mild acid stress led to the release of soluble, CRD-positive VSG in the case of the wild type revertant, with the release being more pronounced in the former case (Fig. 10A, lanes 1 and 3). Significantly, neither of these conditions resulted in the release of CRD-positive VSG in the case of the triple mutant revertant (Fig. 10A, lanes 2 and 4). Indeed, there was no significant release of CRD-positive VSG by the triple mutant revertants even when hypotonic lysis was performed at 30 °C for 10 min (Fig. 10B, middle panel, lanes 4 and 5). It was clear that under these conditions the VSG remained associated with the membrane pellet because when the blot was subsequently incubated with recombinant VSG lipase (bottom panel, lanes 4 and 5), most of the CRD-positive VSG was detected in the membrane pellet and not in the supernatant fraction of hypotonically lysed triple mutant revertant cells. These results were identical to those obtained when cells were lysed under denaturing conditions using SDS-PAGE sample buffer (see lane 3) and demonstrated that the absence of VSG lipase-mediated cleavage during osmotic lysis was not due to the presence of a PLC-insensitive VSG anchor in these cells. In contrast to osmotic lysis CRD-positive VSG was readily detected when the triple mutant revertants were lysed using



**FIG. 8. A comparative analysis of a nonacylable triple mutant and wild type form of the VSG lipase.** The activity of the nonacylable triple mutant (c4 + c5 + c6) and wild type (*wt*) form of the VSG lipase was compared in a semi-quantitative fashion by monitoring the decrease in the electrophoretic mobility of VSG on SDS-PAGE gels when mfVSG was converted to sVSG by the enzyme. **A**, decreasing amounts of purified recombinant wild type or triple mutant form of the lipase were incubated with a fixed amount of mfVSG as described in (24). After an incubation at 30 °C for 30 min, the samples were processed for SDS-PAGE. *Lanes 1* and *2* represent, respectively, the mfVSG substrate and sVSG product, whereas *lanes 3–13* represent incubations containing decreasing amounts of purified recombinant VSG lipase starting with 50 ng of enzyme (*lane 3*). The amount of enzyme present in each incubation was decreased by one-half in each of the subsequent *lanes*. **B**, comparison of wild type and triple mutant VSG lipase activities expressed in *Xenopus*. The relative amounts of wild type or triple mutant form lipase expressed in oocytes was estimated by densitometric quantification of the respective [<sup>35</sup>S]methionine-labeled bands of the protein after analysis by SDS-PAGE/fluorography (*inset* on the *right side* of the *panel*). Based on this analysis the lysates were adjusted to contain the same amount of the enzyme and were subsequently serially diluted in Tris-Cl (50 mM, pH 8) containing Nonidet P-40 (0.02%). Estimation of activity was performed by incubating the serially diluted lysates with mfVSG (10 μg) for 30 min at 30 °C. The incubation was terminated by the addition of SDS-PAGE sample buffer followed by electrophoresis and Coomassie Blue staining. *Lane 1* represents an incubation containing only the initial oocyte lysate, *lanes 2* and *3* represent, respectively, the mfVSG substrate and sVSG product, and *lanes 4–10* represent incubations of oocyte lysates serially diluted 1/2. **C**, comparison of wild type and triple mutant VSG lipase activities expressed in *T. brucei*. Lysates of various transgenic trypanosomes were prepared at a concentration of  $5 \times 10^8$  cells/ml in Tris-Cl (50 mM, pH 8.0) containing EDTA (5 mM) and Nonidet P-40 (0.02%) and then serially diluted in the same buffer 1/5. MfVSG (10 μg) was added to 20 μl of each lysate, and the mix was incubated for 30 min at 30 °C. The samples were then subjected to SDS-PAGE followed by staining with Coomassie Blue. *Lane 1* represents a lysate equivalent of  $5 \times 10^6$  cells incubated without VSG, *lanes 2* and *3* represent mf and sVSG, respectively, and *lanes 4–10* represented serial dilutions (1/5) of the cell lysates commencing in *lane 4* with  $5 \times 10^6$  cell equivalents and terminating with  $3.2 \times 10^3$  cell equivalents in *lane 10*.

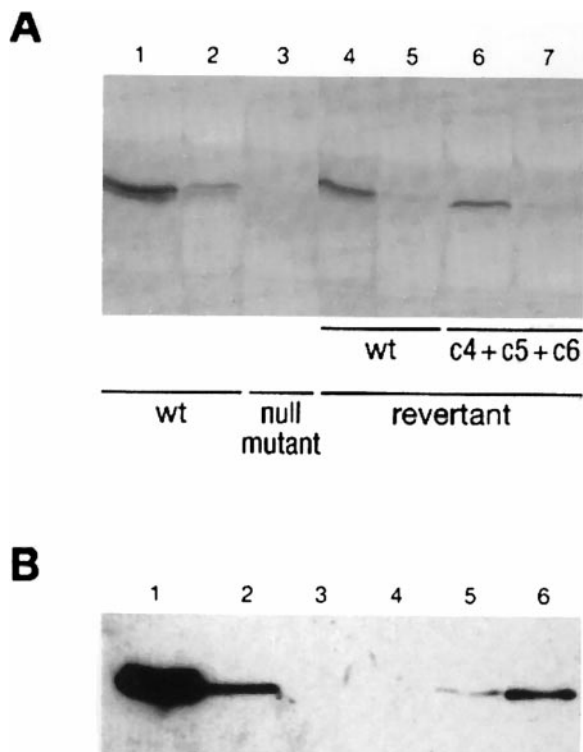
the neutral detergent Nonidet P-40 (*middle panel, lane 6*). Moreover, the amount of CRD-positive material detected in this case was identical to that observed when exogenous recombinant VSG lipase was included in the incubation (Fig. 10B, compare *lanes 6* and *7* in *middle* and *bottom panels*). Taken together these important results demonstrated that although the nonacylable, triple mutant VSG lipase possessed a lower absolute activity to that observed for the wild type enzyme, nevertheless this activity was sufficient to cleave the GPI anchor of all the VSG when the revertants were lysed with neutral detergent but did not do so when the same cells were subjected to osmotic lysis. These findings were consistent with the view that acylation might be involved in regulating the accessibility of the VSG lipase to the mfVSG substrate in trypanosomes.

#### DISCUSSION

A variety of biochemical and molecular approaches were employed in this study to demonstrate that the VSG lipase from *T. brucei* is post-translationally modified by thioacylation. This thioacylation involves a group of three closely clustered cysteine residues located in the C-terminal region of the polypeptide, but the exact number of cysteines actually acylated remains uncertain. Because none of the individual cys-

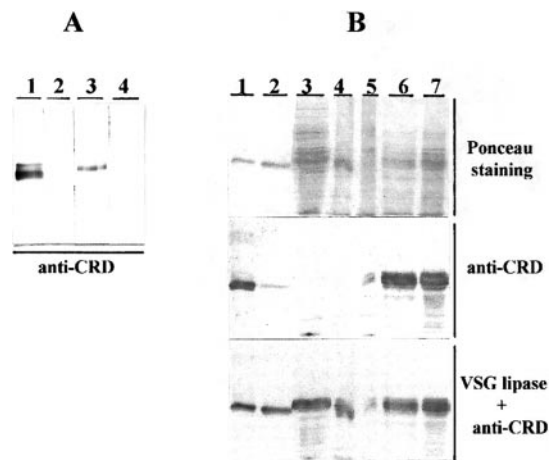
teine mutations abrogated acylation, and only the triple mutant was totally devoid of the acyl label, it seems reasonable to conclude that more than one cysteine must be acylated. Whether all three cysteines are modified is less certain because the possibility that mutation of one of the cysteines indirectly affects the modification of the others cannot be excluded. However, it was interesting to note that the VSG lipase possesses a similar pattern of clustered cysteine residues (CCXXC) to that found at the acylation site of some members of the  $\alpha$ -subunit family of G proteins (33), and double palmitoylation of adjacent cysteines in these proteins has been reported (34, 35). The precise nature of the covalently attached acyl groups also remains equivocal, but the finding that both palmitic and myristic acid were incorporated in metabolic labeling experiments is in agreement with a recent report (17), even though in our studies labeling with palmitic acid was always significantly greater than with myristic acid in trypanosomes and oocytes.

Thioacylation was not an absolute requirement for catalytic activity, which was not too surprising because several studies have shown that when expressed in *E. coli* (16, 24, 36) or as shown here when translated *in vitro* the enzyme was active. In addition our data clearly demonstrated that acylation was not solely responsible for the hydrophobic behavior of the protein.



**FIG. 9. Quantification of the amount of wild type and triple mutant form VSG lipase expressed in transgenic trypanosomes.** A, VSG lipase was immunoprecipitated from trypanosomal lysates. The immunoprecipitates were subjected to Western blot analysis and probed with anti-VSG lipase antibody followed by protein A coupled to alkaline phosphatase. The precipitations were performed as follows. Lanes 1 and 2, from  $4.5 \times 10^8$  and  $4.5 \times 10^7$  cell equivalents, respectively (true wild type; wt); lane 3, from  $4.5 \times 10^8$  cell equivalents (VSG null mutant trypanosomes); lanes 4 and 5, from  $4.5 \times 10^8$  and  $4.5 \times 10^7$  cell equivalents, respectively (revertant wild type); lanes 6 and 7, from  $4.5 \times 10^8$  and  $4.5 \times 10^7$  cell equivalents, respectively (triple mutant revertant). B, Western blot analysis of VSG lipase expression in true wild type trypanosomes (lanes 1–3), in null mutant trypanosomes (lane 3), in triple mutant revertant trypanosomes (lane 5), and in wild type revertant trypanosomes (lane 6). Lanes 1–3 represent a sequential 5-fold reduction in the number of cells loaded per lane starting with  $2 \times 10^6$  cells in lane 1. In the case of lanes 4–6 the loading was equivalent to  $2 \times 10^6$  cells.

These considerations raise an obvious question: why do trypanosomes thioacylate the enzyme? Several findings point to a possible explanation that involves a regulatory role for thioacylation of the lipase in trypanosomes that is designed to modulate access of the enzyme to the GPI anchor of the VSG. Firstly, there appears to be a 1:1 ratio between the acylated (39 kDa) and nonacylated (42 kDa) form of the enzyme at least in trypanosomes freshly isolated under conditions designed to minimize any deleterious biochemical changes in the cells during the isolation procedure (19). Moreover, this ratio was also observed when the VSG lipase was expressed in *Xenopus* oocytes. Secondly, acylation of the VSG lipase in whole cells appears to be reversible, and a shift occurs in this resting 1:1 ratio in favor of the acylated form under conditions known to lead to the cleavage of the GPI anchor of the VSG, e.g. osmotic shock, detergent lysis, or mild acid stress. Thirdly, even though a comparative analysis demonstrated that the nonacylatable, triple mutant form of the VSG lipase possessed a lower intrinsic catalytic activity than the wild type enzyme, in fact between a 0.5 and 1 order of magnitude lower, which might be expected as a consequence of the amino acid substitutions employed, this activity remained sufficient to cleave the GPI anchor of all of the VSG, as assayed by the extent of release of CRD-positive VSG, when a trypanosome cell line expressing only a nonacy-



**FIG. 10. Evidence that thioacylation is involved in the functional activity of VSG lipase in trypanosomes.** A, Western blot analysis, using anti-CRD antibodies (200-fold dilution), of acetone-precipitated supernatants after osmotic lysis (lanes 1 and 2) or nonlytic acid stress (lanes 3 and 4) of  $10^7$  cells of revertant trypanosome null mutants for VSG lipase expressing either the wild type (lanes 1 and 3) or triple mutant (lanes 2 and 4) form of the enzyme. B illustrates the activity of the triple mutant VSG lipase under different conditions. Triple mutant revertants were lysed in SDS-PAGE sample buffer (lane 3), water (lanes 4 and 5), or Nonidet P-40 (lanes 6 and 7). In the case of water lysis, the extracts were incubated for 10 min at  $30^\circ\text{C}$  and centrifuged at 14,000 rpm for 10 min at  $4^\circ\text{C}$  to separate the pellet (lane 4) and supernatant (lane 5). In the case of Nonidet P-40 lysis, the cells were lysed in 0.2% Nonidet P-40 in 150 mM NaCl, 50 mM Tris-HCl (pH 8) and incubated for 10 min at  $30^\circ\text{C}$  in the absence (lane 6) or presence (lane 7) of exogenous recombinant VSG lipase. In each case an aliquot equivalent to  $5 \times 10^6$  trypanosomes was applied per lane. The blots were stained with Ponceau (top panel), then probed with anti-CRD antibody to reveal the action of endogenous GPI-PLC (middle panel), and then further incubated with recombinant VSG lipase overnight at  $4^\circ\text{C}$  and re-probed with anti-CRD (bottom panel). Both sVSG and mfVSG MITat 1.6 (lanes 1 and 2, respectively) were included as controls for this treatment.

lable form of the lipase was lysed with neutral detergent. However, when these same trypanosomes were subjected to osmotic shock there was no release of CRD-positive VSG. This absence of osmotic shock induced VSG release is precisely the phenotype of null mutant trypanosomes that lack the VSG lipase gene with one important difference: the presence of neutral detergent makes no difference to the result in the case of the null mutant (16). On the basis of these results, it is difficult not to conclude that acylation of the lipase has some function in regulating enzyme access to mfVSG in trypanosomes.

This view is also supported by the finding that acylation appears to involve two separate steps, the second of which only occurs in trypanosomes. The first acylation seems to be stable, because the 39-kDa band was always detected in trypanosomes under resting conditions as well as in *Xenopus* oocytes. The second acylation occurs only in trypanosomes under conditions known to result in release of CRD-positive VSG and involves conversion of the remaining 42-kDa nonacylated VSG lipase polypeptides to the 39-kDa acylated form and appears to be reversible. The reversibility of this second acylation might account for the puzzling observation of Bülow and Overath (6), who noted that immediately after purification of the lipase from detergent lysates of trypanosomes, conditions that should result in the acylation of all of the VSG lipase polypeptides, the protein was recovered as a single 39-kDa species. However, upon storage about 50% of the 39-kDa species was converted to a 42-kDa species, which probably represented the nonacylated form, and this ratio remained stable afterward.

Precisely how only half the lipase polypeptides are acylated



under resting conditions is unclear, but it might involve the formation of quaternary structure. For example, on the basis of the existence of potential coiled-coil regions in this enzyme as well as the fact that the VSG lipase was detected as apparent dimers and oligomers under nonreducing SDS-PAGE,<sup>2</sup> the observed 1:1 ratio between the nonacylated and acylated forms may be due to dimerization with the relevant cysteines of one of the two subunits of the dimer being blocked and is accessible to modification only under appropriate conditions. The possibility that this second thioacylation step was due to uncatalyzed transacylation of the VSG lipase, as reported for the  $\alpha$ -subunits of some G proteins (37–39), seems unlikely because it was not observed in oocytes under any conditions nor when trypanosomes were lysed in SDS. In addition, attempts to detect autoacylation of purified recombinant VSG lipase using palmitoyl CoA as described by other workers (38) were unsuccessful (data not shown).

Overall these findings suggest a tentative hypothesis for the sequence of thioacylation events in *T. brucei*. This model suggests that half the VSG lipase polypeptides are constitutively and stably thioacylated shortly after translation by a mechanism common to trypanosomes and *Xenopus*. This hemi-acylated form is catalytically active but restricted to a cellular compartment or microenvironment which limits or denies access to the mfVSG, e.g. perhaps the small vesicles observed by Bülow *et al.* (12). The equilibrium between acylated and nonacylated forms is maintained unless the cells are triggered to release their surface coat. This stimulus leads to a rapid but unstable acylation of the remaining polypeptides by a trypanosome-specific acyltransferase that confers functional activity on the enzyme by allowing access to the mfVSG substrate. This model is consistent with an emerging consensus that reversible thioacylation is involved in the functional regulation of several plasma membrane proteins and the processes mediated by them (39, 40).

*Acknowledgments*—We are very grateful to D. Franck and S. Lips for assistance in the preparation of the figures and also to Y. Claes (Antwerp) for help in the cyclical transmissions.

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<sup>2</sup> M. L. Cardoso de Almeida, unpublished data.