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Biochemistry

Rapid processing of the carboxyl terminus of a trypanosome variant surface glycoprotein

(cross-reacting determinant/glycolipid)

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ABSTRACT **The variant surface glycoprotein of the parasite *Trypanosoma brucei* contains a glycolipid of unknown structure covalently attached to its COOH terminus. We have shown, by using metabolic labeling with [^{35}S]methionine or [^{3}H]myristic acid, precipitation with specific antibodies, and NaDodSO$_4$/polyacrylamide gel electrophoresis, that this glycolipid is attached to the variant surface glycoprotein polypeptide within 1 min after its translation.**

African trypanosomes are parasitic protozoa that inhabit the bloodstream of their mammalian host. They evade the immune response by a process known as antigenic variation (1–4). A variant surface glycoprotein (VSG) (5) forms a coat that covers the entire cell surface (6). By changing VSGs, through a switch in gene expression, the trypanosome presents a constantly shifting antigenic profile to its host.

VSGs have molecular weights of 50,000–60,000 and comprise ≈10% of the total cellular protein (5). Sequencing studies reveal enormous amino acid sequence differences between different VSGs (7, 8).

VSG is synthesized by membrane-bound polysomes (9) and subsequently undergoes several cotranslational modifications including the cleavage of an NH_2-terminal signal sequence (10, 11) and *N*-linked glycosylation (12, 13). A series of unusual modifications occur at the COOH terminus. A hydrophobic sequence of amino acids is removed (14) and a complex glycolipid structure composed of ethanolamine (15), phosphate (16, 17), sugars (13, 18), glycerol (19), and myristic acid (20) is subsequently attached directly to the α-carboxyl of the new COOH terminus (15). The structure of this glycolipid is not yet known.

The COOH-terminal glycolipid is presumed to anchor the VSG to the plasma membrane by inserting into the lipid bilayer. Osmostic or mechanical lysis of trypanosomes results in the rapid release, catalyzed by an endogenous membrane-bound enzymatic activity, of VSG in a soluble form (sVSG) (5, 17, 21, 22), which lacks the myristyl moiety (20). It is not known if other components of the glycolipid are also removed. In contrast, boiling of trypanosomes in NaDodSO$_4$ preserves an amphiphilic "membrane form" of the protein (mfVSG) (17, 22), which retains the myristic acid (20).

VSG containing the COOH-terminal glycolipid (i.e., mfVSG) can be identified by two criteria. One is the accelerated electrophoretic mobility of mfVSG relative to sVSG (17). The other is the presence of an immunologically cross-reacting determinant (CRD) on VSG polypeptides that are otherwise immunologically distinct (23). This CRD, which is on the glycolipid moiety (18), is cryptic on mfVSG and is detected only on sVSG (17).

Nothing is known about the synthesis of the glycolipid. The CRD has been detected on newly synthesized VSG within 9 min after translation, implying that at least some components of the glycolipid are attached by that time to some of the VSG molecules (24). In another report, based on immunoelectron microscopy, the CRD was not detected on newly synthesized VSG in the endoplasmic reticulum, but it was detectable on VSG in the *trans*-Golgi (25). In a third report, maximum expression of the CRD was not detectable until 1 hr after synthesis of the polypeptide (26).

In this communication, we show that the COOH-terminal glycolipid of the ILTat-1.3 variant of *Trypanosoma brucei brucei* is added to the protein within 1 min after biosynthesis of the polypeptide.

MATERIALS AND METHODS

Trypanosomes. Cloned trypanosomes of variant antigen types ILTat-1.3, ILTat-1.1, and AnTat-1.8 (from R. O. Williams, D. Barry, and S. L. Hajduk, respectively) were grown in Swiss mice or Wistar rats (2- to 3-day passages). Parasites were isolated from infected blood that was diluted 1:4 in Bicine-buffered saline [buffer A: 50 mM Bicine/70 mM glucose/5 mM KCl/50 mM NaCl, pH 8.0 (27)] containing heparin (10 units/ml) at 4°C on DEAE-cellulose columns equilibrated with buffer A (28).

VSG Purification and Production of Anti-VSG Antibodies. sVSG, purified by the methods of Cross (5, 21), was coupled to cyanogen bromide-activated Sepharose (29). Rabbit antisera to VSG were prepared by standard methods. Antibody to ILTat-1.3 sVSG (anti-VSG) was purified by precipitation with 40% saturated ammonium sulfate and affinity chromatography on the ILTat-1.3 VSG Sepharose column. Anti-CRD was generated by the same method by using AnTat-1.8 sVSG as antigen and ILTat-1.1 sVSG Sepharose for affinity purification.

Labeling of Trypanosomes with [^{35}S]Methionine. The methionine labeling procedure is based on that of Rovis and Dube (26). ILTat-1.3 trypanosomes, washed in buffer A containing bovine serum albumin (1 mg/ml), were suspended (5×10^6 cells per ml) in RPMI-1640 medium without methionine (GIBCO), supplemented with 10% heat-inactivated fetal calf serum and 25 mM Hepes buffer, pH 7.4. After 15 min at 37°C, [^{35}S]methionine (Amersham, ≈1000 Ci/mmol; final concentration, 50 μCi/ml; 1 Ci = 37 GBq) was added. In experiments with tunicamycin (Calbiochem), the cells were incubated with drug (200 ng/ml) for 1 hr at 37°C before initiation of labeling. In pulse–chase experiments, the cells were labeled with [^{35}S]methionine at a density of 5×10^7 cells per ml. To initiate the chase, they were diluted 1:10 into prewarmed medium containing nonradioactive methionine (100 μg/ml).

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Abbreviations: VSG, variant surface glycoprotein; sVSG, soluble form of VSG; mfVSG, membrane form of VSG; CRD, cross-reacting determinant.

Lysates of labeled cells were prepared by two methods. For mf-lysis (which yields mfVSG) (24), aliquots of cell suspension (200 μl; 5 $\times$ 10^6 cells per ml) were diluted with 1.0 ml of ice-cold buffer A and centrifuged in a Beckman Microfuge B (2 min, 22°C). Cell pellets were resuspended in 200 μl of buffer B (50 mM Tris·HCl/150 mM NaCl/5 mM EDTA, pH 7.5) containing 1% $NaDodSO_4$ and boiled for 10 min. Lysates were then diluted with 0.8 ml of cold buffer B containing 2.5% Triton X-100 and incubated at 0°C for 15 min to allow formation of mixed micelles. s-Lysates (which yield sVSG) were prepared by diluting cell suspensions (200 μl; 5 $\times$ 10^6 cells per ml) in 1.0 ml of ice-cold buffer A and centrifuging. The pellets were resuspended in 20 μl of buffer B containing 1% Nonidet P-40 and incubated at 37°C for 5 min to achieve complete conversion of all mature and immature mfVSG to sVSG; they were then diluted to 1.0 ml with buffer B containing 1% Nonidet P-40. To minimize proteolysis, iodoacetamide (5 mM), leupeptin (1 μg/ml), and *N*-α-tosyllysine chloromethyl ketone (Tos-LysCH_2Cl) (0.1 mM) were added to all lysis buffers.

[^{3}H]Myristate Labeling and Preparation of Crude Membranes. Trypanosomes were labeled with [^{3}H]myristate according to Ferguson and Cross (20). [9, 10-^{3}H]Myristic acid (12.9 Ci/mmol in ethanol; New England Nuclear) was dried, redissolved in 5 μl of 95% ethanol, and mixed with equimolar fatty acid-free bovine serum albumin (200 mg/ml in H_2O). Cells were suspended in RPMI 1640 medium (10^8 per ml) supplemented with 1 mg of fatty acid-free bovine serum albumin per ml (Sigma) and 25 mM Hepes, pH 7.4. The cells were incubated for 15 min at 37°C, and then [^{3}H]myristate–bovine serum albumin complex (300 μCi/ml, final concentration) was added. The labeled trypanosomes (2 $\times$ 10^8) were washed and then lysed hypotonically (5 min, 0°C) in 100 μl of H_2O. Lysates were diluted with 100 μl of cold 2$\times$ concentrated buffer B and incubated 5 min at 37°C to facilitate conversion of mature mfVSG to sVSG. Newly synthesized VSG is not converted under these conditions and is retained in internal membranes (ref. 20; see also Fig. 4 and *Discussion*). Converted lysates were diluted with 2.0 ml of cold buffer B and 1.1-ml aliquots were layed on 100-μl cushions of cold 60% sucrose in buffer B in microfuge tubes. After centrifuging (10 min, 4°C, 10,000 rpm; Sorvall HB-4 rotor), the membranes at the interface and cushions were gently resuspended in 1.2 ml of cold buffer B and centifuged again. Pellets were solubilized by the s- and mf-lysis procedures described above to give crude membrane lysates (10^8 cell equivalents per 500 μl). Solubilization of washed crude membranes by the s-lysis procedure allows conversion of newly synthesized VSG by facilitating mixing of converting enzyme(s) (probably localized predominantly in the plasma membrane) with VSG from internal membranes. Insoluble material was removed by centifugation. All buffers contained 1.0 μg of leupeptin per ml, 0.1 mM Tos-LysCH_2Cl, and 5 mM iodoacetamide.

Control labelings with [^{35}S]methionine (50 μCi/ml, final concentration) were performed under identical conditions in the same medium lacking methionine. [^{35}S]Methionine-labeled cells (10^6) were mixed with 2 $\times$ 10^8 mock-labeled cells, and crude membranes were prepared exactly as described above.

Immunoprecipitation and Gel Electrophoresis. Lysates were treated with saturating levels of affinity-purified immunoglobulin and the immune complexes were adsorbed to protein A-Sepharose. After washing, the VSG was eluted from the beads with 2$\times$ electrophoresis sample buffer and electrophoresed on 7.5–15% linear gradient $NaDodSO_4$/polyacrylamide gels (30). Gels were stained with Coomassie blue and fluorographed with EN^{3}HANCE (New England Nuclear).

RESULTS

Electrophoretic and Immunochemical Properties of Newly Synthesized VSG. Fig. 1 shows the kinetics of the metabolic incorporation of [^{35}S]methionine into VSG. From this experiment, we made the following observations. First, radioactivity is incorporated linearly into hot trichloroacetic acid-insoluble material for 60 min; $\approx$7% of this radioactivity is incorporated into VSG (Fig. 1*B*). Second, VSG heterogeneity is apparent during the 60-min labeling period (Fig. 1 *A* and *C*). Anti-VSG precipitates two components from s-lysates of cells labeled 2 min (apparent M_r, 58,000 and 56,000; Fig. 1*C*, lane 1). A third component (apparent M_r, 59,000, identical to mature sVSG, Fig. 1*A*, lanes 4–6) is detected at $\approx$20 min and

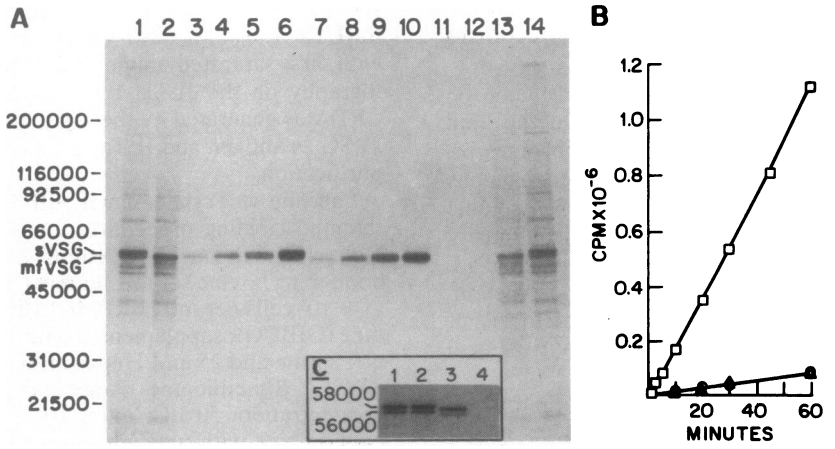

FIG. 1. Kinetics of labeling VSG with [^{35}S]methionine. ILTat-1.3 trypanosomes were labeled *in vitro* and subjected to s- and mf-lysis. (*A*) Fluorograph of $NaDodSO_4$/polyacrylamide gel. Lanes 1 and 14, whole s-lysates of cells (2.5 $\times$ 10^5 cell equivalents) labeled 1 hr and chased 1 hr. Lanes 2 and 13, same as lanes 1 and 14 but mf-lysates. Lanes 3–6, s-lysates of 5 $\times$ 10^5 cells labeled 10, 20, 30, and 60 min, respectively, immunoprecipitated by anti-VSG. Lanes 7–10, same as lanes 3–6, but mf-lysates were used. Lanes 11 and 12, samples identical to those in lanes 6 and 10, respectively, but precipitated by nonimmune IgG. Scale, indicating molecular weights, was determined by Coomassie blue staining of reference proteins. (*B*) Incorporation of [^{35}S]methionine into hot (90°C) 5% trichloroacetic acid-insoluble material (□) and into sVSG (○) and mfVSG (△). Radioactivity refers to 10^6 cells. Radioactivity in VSG was measured by solubilizing gel fragments in 300 μl of perchloric acid (23%), hydrogen peroxide (20%) at 65°C, and counting in 4.0 ml of Liquiscint (National Diagnostics, Somerville, NJ). (*C*) Immunoprecipitates of trypanosomes labeled 2 min. s-Lysates (lanes 1 and 2) and mf-lysates (lanes 3 and 4) treated with anti-VSG (lanes 1 and 3) or anti-CRD (lanes 2 and 4). Fluorograph of $NaDodSO_4$/polyacrylamide gel containing 10^6 cell equivalents per lane.

persists in the sample labeled for 60 min. Similar heterogeneity is detectable in the mf-lysates (Fig. 1 *A* and *C*), but the resolution of the bands present at 20–60 min is poor. This heterogeneity will be discussed again in relation to Fig. 3. Third, ^{35}S-labeled VSG polypeptides are precipitated by anti-CRD from s-lysates of cells labeled for only 2 min (Fig. 1*C*, lane 2). As found previously with mature VSG (17), anti-CRD does not precipitate VSG polypeptides from mf-lysates of these cells (Fig. 1*C*, lane 4) because the immunological cross-reactivity is not expressed by mfVSG (17). Fourth, the electrophoretic mobility of all VSG polypeptides detected at all time points with either anti-VSG or anti-CRD is dependent on the method of lysis. VSG polypeptides immunoprecipitated from mf-lysates always have a slightly faster mobility than the corresponding polypeptide in s-lysates (compare lanes 6 and 10 in Fig. 1*A* or lanes 1 and 3 in Fig. 1*C*). Results identical to those in Fig. 1*C* were obtained from cells labeled only 1 min (data not shown).

The observations that VSG labeled for 1 or 2 min is precipitable by anti-CRD (from s-lysates) and behaves as mfVSG (after mf-lysis) in gel electrophoresis indicated that the glycolipid is attached to the polypeptide within 1 min after its synthesis. Furthermore, the amounts of labeled VSG polypeptides detected with anti-VSG and anti-CRD in s-lysates in short labelings are roughly equivalent (Fig. 1*C*, compare lanes 1 and 2), indicating that all newly synthesized VSG molecules may contain the CRD. To prove this point and to rule out the possibility that the anti-VSG and anti-CRD used in the experiment in Fig. 1*C* precipitated different populations of VSG polypeptides, we treated s-lysates sequentially with anti-VSG and anti-CRD (Fig. 2). Regardless of which antibody is used in the primary immunoprecipitation (lanes 1, 3, 5, and 7), no VSG polypeptides were detectable in the secondary immunoprecipitate (lanes 2, 4, 6, and 8). Therefore, anti-VSG and anti-CRD must recognize the same molecules. Incorporation of CRD onto VSG must occur rapidly, and it must occur rapidly to all of the molecules.

Identification of Early Intermediates in VSG Biosynthesis. We wanted to obtain direct chemical evidence for rapid incorporation of glycolipid into VSG. Ferguson and Cross have already shown that this moiety in mature VSG can be efficiently labeled with [^{3}H]myristate (20). Therefore, if glycolipid is rapidly incorporated, it should be possible to detect [^{3}H]myristate on a newly synthesized form of this polypeptide. Possible candidates for newly synthesized forms are the low M_r VSG polypeptides detected in the 2-min labelings in Fig. 1*C*.

To provide evidence that these forms are indeed early biosynthetic intermediates, we conducted a pulse–chase experiment with [^{35}S]methionine (Fig. 3*A*). Gel electrophoresis of immunoprecipitates revealed a doublet VSG band (lane 3), identical to that observed in Fig. 1*C* (lane 1) in s-lysates prepared immediately after a 2-min pulse. During the chase (lanes 4–7), the lower band quickly disappeared and by 15 min, the third band, identical in electrophoretic mobility to mature VSG, appeared. By the end of the 58-min chase, virtually all VSG was in this M_r 59,000 form. Analysis of this experiment by mf-lysis showed essentially the same result (lanes 8–12), although there was poor resolution of the forms appearing after 15 min. This experiment strongly indicates the lower M_r forms of VSG detected at early time points are early intermediates in the biosynthesis of mature VSG.

The chemical differences between the forms of VSG detected in the pulse–chase experiment are not known. To examine the possibility that these forms differ in *N*-linked glycosylation, we conducted a 2-min labeling in the presence of tunicamycin (Fig. 3*B*), a drug that inhibits incorporation of *N*-linked oligosaccharides (31). In this case, we observed a unique polypeptide of significantly higher mobility than observed in the absence of drug (Fig. 3*B*, compare lane 5 with lane 1). No heterogeneity was observed in a subsequent 60-min chase with nonradioactive methionine (data not shown). However, this form of VSG, which presumably contains no asparagine-linked oligosaccharides, still appears to contain the COOH-terminal glycolipid. It reacts with anti-CRD in s-lysates (lane 6) but not in mf-lysates (lane 8). Furthermore, the form in mf-lysates has the accelerated electrophoretic mobility characteristic of mfVSG (compare lane 7 with lane 5). This experiment confirms previous reports that tunicamycin does not block incorporation of the CRD (24, 26).

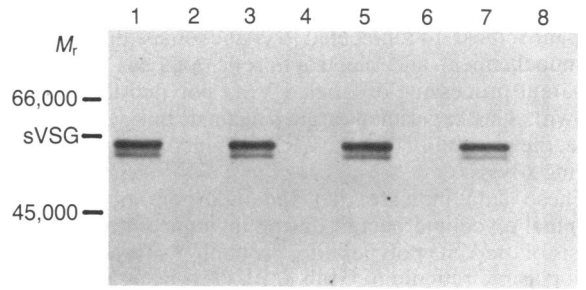

FIG. 2. Sequential immunoprecipitation of VSG with anti-VSG and anti-CRD. ILTat-1.3 trypanosomes were labeled with [^{35}S]methionine for 2 min. s-Lysates (5×10^5 cell equivalents) were immunoprecipitated with either anti-VSG or anti-CRD and treated again with protein A-Sepharose. No additional radiolabeled material was precipitated by this step (data not shown). Supernatants were then precipitated again with one or the other antibody. Primary (lanes 1, 3, 5, and 7) and secondary (lanes 2, 4, 6, and 8) immunoprecipitates were analyzed as described in Fig. 1. Lanes: 1, anti-VSG; 2, anti-VSG, followed by anti-VSG; 3, anti-VSG; 4, anti-VSG, followed by anti-CRD; 5, anti-CRD; 6, anti-CRD, followed by anti-VSG; 7, anti-CRD; 8, anti-CRD, followed by anti-CRD.

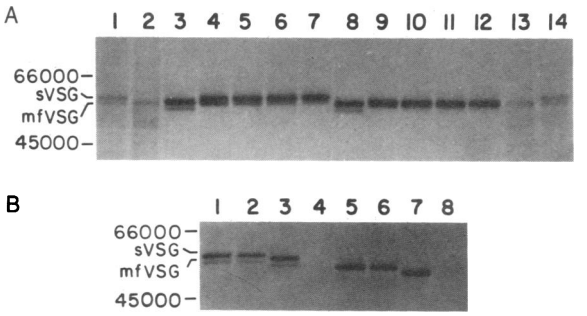

FIG. 3. (*A*) Pulse–chase experiment on trypanosomes labeled with [^{35}S]methionine. ILTat-1.3 trypanosomes were labeled for 2 min and then chased for 58 min. Samples were analyzed as described in Fig. 1. Control experiments showed that the radioactivity incorporated into hot trichloroacetic acid-insoluble material remained constant during the chase period. Lanes 1 and 14 are s-lysates of 5×10^3 cells (identical to that in Fig. 1, lane 1). sVSG is indicated. Lanes 2 and 13 are mf-lysates of 5×10^3 cells (identical to that in Fig. 1, lane 2). mfVSG is indicated. Lanes 3–7 show immunoprecipitates of s-lysates (5×10^5 cell equivalents) sampled at 2.5, 15, 30, 45, and 60 min, respectively. Lanes 8–12, same as lanes 3–7, except the samples were prepared by mf-lysis. All sample times are relative to the time of initiation of labeling. Scale refers to molecular weights of reference proteins. (*B*) Effect of tunicamycin on VSG biosynthesis. ILTat-1.3 trypanosomes were labeled for 2 min with [^{35}S]methionine without tunicamycin (lanes 1–4) or with 200 ng of tunicamycin per ml (lanes 5–8). The cells were subjected to s-lysis (lanes 1, 2, 5, and 6) or mf-lysis (lanes 3, 4, 7 and 8). The lysates (5×10^5 cell equivalents) were treated with anti-VSG (lanes, 1, 3, 5, and 7) or anti-CRD (lanes 2, 4, 6, and 8), and analyzed as described in Fig. 1.

Incorporation of [^{3}H]Myristic Acid into Early Intermediates of VSG Biosynthesis. The identification of VSG precursors by gel electrophoresis (Fig. 3*A*) allowed us to investigate whether these precursors could be labeled with [^{3}H]myristate. After labeling for 2 min, cells were lysed hypotonically and a crude membrane fraction was isolated as described in *Materials and Methods*. VSG was solubilized from these membranes by the standard s- or mf-lysis procedures followed by immunoprecipitation with anti-VSG and NaDodSO$_4$ gel electrophoresis. The use of crude membranes in these experiments had significant advantages. Hyptotonic lysis selectively releases mature VSG as sVSG but preserves newly synthesized VSG in the mf-form (ref. 20; see also *Discussion*). Since most of the VSG had been released, much greater numbers of cells could be analyzed by our immunoprecipitation and electrophoresis procedures.

A fluorograph of the gel is shown in Fig. 4*A*. Lane 3 shows a control immunoprecipitate of an s-lysate of crude membranes from [^{35}S]methionine-labeled cells. Lane 4 shows a corresponding mf-lysate. The VSG precursors observed in Figs. 1*C* and 4 are easily detectable, demonstrating that newly synthesized VSG is retained in crude membranes. Lane 5 contains an immunoprecipitate of an mf-lysate of crude membranes from [^{3}H]myristate-labeled cells. Radioactivity comigrates with both bands of VSG precursors (compare lanes 5 and 4), although the resolution of the bands is slightly poorer than that of the bands from methionine-labeled cells. This experiment provides further evidence that myristic acid is incorporated into VSG soon after synthesis of its polypeptide. Lane 6 contains an immunoprecipitate of an s-lysate of crude membranes from [^{3}H]myristate-labeled cells. Myristate is removed when mature mfVSG is converted to sVSG (20). The absence of radioactivity in lane 6 confirms that myristate is also removed from newly synthesized VSG during s-lysis.

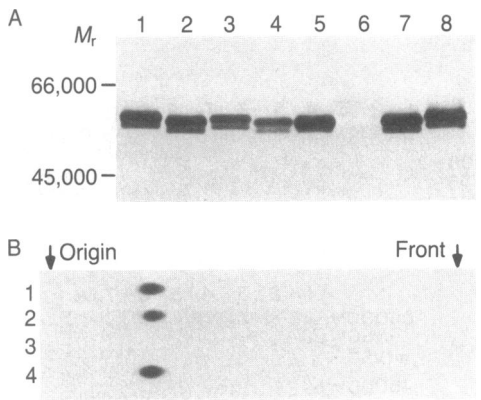

FIG. 4. (*A*) [^{3}H]Myristate labeling of precursor VSG. ILTat-1.3 trypanosomes (10^8 per ml) were labeled for 2 min with [^{3}H]myristic acid or [^{35}S]methionine under the myristate-labeling conditions. Samples were analyzed as described in Fig. 1. Lanes 1 and 8, s-lysates of 5×10^5 [^{35}S]methionine-labeled whole trypanosomes. Lanes 2 and 7, same as lanes 1 and 8 but mf-lysates. Lane 3, s-lysate of crude membranes from 5×10^5 [^{35}S]methionine-labeled and 10^8 mock-labeled cells. Lane 4, same as lane 3 but mf-lysis was used. Lane 5, mf-lysate of crude membranes from 10^8 [^{3}H]myristate-labeled cells. Lane 6, same as lane 5 but s-lysis was used. (*B*) Hydrolysis of [^{3}H]myristate-labeled VSG. Crude membranes from 10^8 trypanosomes labeled 3 min with [^{3}H]myristate were solubilized by the s- or mf-lysis procedures. Anti-VSG immunoprecipitates were hydrolyzed in 6 M HCl for 5 hr at 100°C. The hydrolysates were analyzed by silica gel 60 thin-layer chromatography (hexane/diethyl ether/acetic acid; 70:30:1) and fluorography with EN^{3}HANCE Spray (New England Nuclear). Lanes 1 and 4, authentic [^{3}H]myristic acid (1100 cpm); lane 2, hydrolysate of mfVSG; lane 3, hydrolysate of sVSG.

To rule out the possibility that the [^{3}H]myristate had been metabolized prior to incorporation, we hydrolyzed the [^{3}H]-myristate-labeled mfVSG. All of the radioactivity cochromatographed on thin layer chromatography with authentic [^{3}H]myristic acid (Fig. 4*B*).

DISCUSSION

These experiments led to the unexpected conclusion that the COOH-terminal glycolipid is attached to VSG within 1 or 2 min after synthesis of the polypeptide. Since the structure of this group is not known, we initially used two indirect criteria to detect this modification. One is reactivity of anti-CRD antibodies with sVSG but not with mfVSG (17). The second is convertibility from mfVSG to sVSG during s-lysis as detected by a slight decrease in electrophoretic mobility (17). As shown in Fig. 1*C* and 3, all VSG synthesized in a 2-min pulse-labeling appears to contain the COOH-terminal glycolipid as judged by these criteria.

To obtain further evidence that the glycolipid is attached to VSG soon after its synthesis, we exploited the fact that exogenous [^{3}H]myristic acid can be incorporated into the glycolipid (20). As expected, we found that [^{3}H]myristate is incorporated during a 2-min pulse-labeling into the two low M_r VSG precursors (Fig. 4*A*). The relative labeling of these two precursors differs with [^{3}H]myristate and [^{35}S]methionine, probably because of differential rates of uptake, different pool sizes, or small variations in labeling conditions.

Because of the low level of radioactivity incorporated into VSG during short [^{3}H]myristate labelings, it was necessary to analyze large numbers of cells (10^8). To avoid overloading our immunoprecipitation and electrophoresis systems, we isolated newly synthesized VSG from crude membranes prepared from hypotonic lysates. Hypotonic lysis allows conversion of mature mfVSG to sVSG by a membrane-bound enzyme activity (22). However, newly synthesized VSG remains bound to membranes as mfVSG, as shown in Fig. 4, probably because it is localized in internal membranes, which are deficient in the converting activity (ref. 20, unpublished results). As judged by Coomassie blue staining in several experiments (data not shown), this procedure results in the release of virtually all mature VSG with good retention of VSG synthesized in a 2-min pulse.

We considered the unlikely possibility that VSG synthesized in a short pulse with [^{35}S]methionine does not have the CRD, but that it was added *in vitro* during s-lysis. To test this possibility, we added [^{35}S]methionine-labeled *in vitro* translation product (prepared with total mRNA from ILTat-1.3 trypanosomes) to unlabeled trypanosomes during s-lysis. Immuochemical and electrophoretic analysis revealed no apparent processing of labeled VSG polypeptides (data not shown). This experiment argues against, but does not eliminate, the possibility that the CRD was incorporated into VSG during s-lysis.

These data indicate that the incorporation of COOH-terminal glycolipid occurs during or immediately after synthesis of the VSG polypeptide, probably while it is still in the endoplasmic reticulum. Grab *et al.* (25), using anti-CRD for immunoelectron microscopy, could detect CRD in the *trans*-Golgi, flagellar pocket, and plasma membrane, but not in the endoplasmic reticulum. Detection of VSG in any subcellular compartment with anti-CRD implies that some conversion of mfVSG to sVSG must have occurred during fixation. Thus, the absence of detectable CRD in the endoplasmic reticulum could be explained by the observed resistance of newly synthesized VSG to conversion in the absence of detergent (ref. 20; see above). We have found that newly synthesized VSG, labeled in a 2-min pulse with [^{35}S]methionine, is partially susceptible to conversion in the absence of detergent

after a 13-min chase (unpublished observation); this lag time could be the time taken for the molecules to reach the *trans*-Golgi.

It is possible that the components of the glycolipid are added separately to the protein in an ordered manner. However, the rapidity of modification raises the possibility that the glycolipid is preconstructed and added in one step. Of course, it may be further modified after it is attached to the protein.

The latter model has intriguing implications. Holder showed that the glycolipid is attached to the protein through ethanolamine, which is joined by an amide linkage to the α-carboxyl group of the COOH-terminal amino acid residue (15). Therefore, it is certain that the COOH-terminal hydrophobic sequence of the VSG must be removed prior to attachment of the glycolipid. The maturation of the COOH-terminus of the VSG may involve a transferase reaction in which the COOH-terminal amino acid sequence is displaced from the VSG polypeptide by the ethanolamine moiety of the glycolipid. In this case, the COOH-terminal hydrophobic sequence could act as a transient membrane anchor until transfer of the VSG polypeptide to the glycolipid. Thus, the VSG would never be released into the lumen of the endoplasmic reticulum and the processing reaction would take place in two dimensions rather than three.

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1. Vickerman, K. (1978) *Nature (London)* **273,** 613–617.
2. Englund, P. T., Hajduk, S. L. & Marini, J. C. (1982) *Annu. Rev. Biochem.* **51,** 695–726.
3. Borst, P. & Cross, G. A. M. (1982) *Cell* **29,** 291–303.
4. Turner, M. J. (1982) *Adv. Parasitol.* **21,** 69–153.
5. Cross, G. A. M. (1975) *Parasitology* **71,** 393–417.
6. Vickerman, K. (1969) *J. Cell Sci.* **5,** 163–193.
7. Bridgen, P. J., Cross, G. A. M. & Bridgen, J. (1976) *Nature (London)* **263,** 613–614.
8. Rice-Ficht, A. C., Chen, K. K. & Donelson, J. E. (1981) *Nature (London)* **294,** 53–57.
9. Lheureux, M., Lheureux, M., Vervoort, T., Van Meirvenne, N. & Steinert, M. (1979) *Nucleic Acids Res.* **7,** 595–609.
10. McConnell, J., Grunett, A. M., Cordingly, J. S., Walker, J. E. & Turner, M. J. (1981) *Mol. Biochem. Parasitol.* **4,** 225–242.
11. Boothroyd, J. C., Paynter, C. A., Cross, G. A. M., Bernards, A. & Borst, P. (1981) *Nucleic Acids Res.* **9,** 4735–4743.
12. Strickler, J. E. & Patton, C. L. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 1529–1533.
13. Holder, A. A. & Cross, G. A. M. (1981) *Mol. Biochem. Parasitol.* **2,** 135–150.
14. Boothroyd, J. C., Cross, G. A. M., Hoeijmakers, J. H. J. & Borst, P. (1980) *Nature (London)* **288,** 624–626.
15. Holder, A. A. (1983) *Biochem. J.* **209,** 261–262.
16. Baltz, T., Giroud, Ch., Baltz, D., Duvillier, G., Degand, P., Demaille, J. & Pautrizel, R. (1982) *EMBO J.* **1,** 1393–1398.
17. Cardoso de Almeida, M. L. & Turner, M. J. (1983) *Nature (London)* **302,** 349–352.
18. Holder, A. A. (1983) *Mol. Biochem. Parasitol.* **7,** 331–338.
19. Duvillier, G., Nouvelot, A., Richet, C., Baltz, T. & Degand, P. (1983) *Biochem. Biophys. Res. Commun.* **114,** 119–125.
20. Ferguson, M. A. J. & Cross, G. A. M. (1984) *J. Biol. Chem.* **259,** 3011–3015.
21. Cross, G. A. M. (1984) *J. Cell Biochem.* **24,** 79–90.
22. Cardoso de Almeida, M. L., Allan, L. M. & Turner, M. J. (1984) *J. Protozool.* **31,** 53–60.
23. Barbet, A. F. & McGuire, T. C. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 1989–1993.
24. McConnell, J., Turner, M. & Rovis, L. (1983) *Mol. Biochem. Parasitol.* **8,** 119–135.
25. Grab, D. J., Webster, P. & Verjee, Y. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 7703–7707.
26. Rovis, L. & Dube, D. K. (1981) *Mol. Biochem. Parasitol.* **4,** 77–93.
27. Balber, A. E., Bangs, J. D., Jones, S. M. & Proia, R. L. (1979) *Infect. Immun.* **24,** 617–627.
28. Lanham, S. M. & Godfrey, D. G. (1970) *Exp. Parasitol.* **28,** 521–534.
29. March, S. C., Parikh, I. & Cuatrecasas, P. (1974) *Anal. Biochem.* **60,** 149–152.
30. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
31. Tracz, J. S. & Lampen, J. O. (1975) *Biochem. Biophys. Res. Commun.* **65,** 248–257.