Posttranslational Modification and Intracellular Transport of a Trypanosome Variant Surface Glycoprotein

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Abstract. After synthesis on membrane-bound ribosomes, the variant surface glycoprotein (VSG) of *Trypanosoma brucei* is modified by: (*a*) removal of an N-terminal signal sequence, (*b*) addition of N-linked oligosaccharides, and (*c*) replacement of a C-terminal hydrophobic peptide with a complex glycolipid that serves as a membrane anchor. Based on pulse–chase experiments with the variant ILTat-1.3, we now report the kinetics of three subsequent processing reactions. These are: (*a*) conversion of newly synthesized 56/58-kD polypeptides to mature 59-kD VSG, (*b*) transport to the cell surface, and (*c*) transport to a site where VSG is susceptible to endogenous membrane-bound phospholipase C. We found that the $t_{½}$ of all three of these processes is ~15 min. The comparable kinetics of these processes is compatible with the hypotheses that transport of VSG from the site of maturation to the cell surface is rapid and that VSG may not reach a phospholipase C-containing membrane until it arrives on the cell surface. Neither tunicamycin nor monensin blocks transport of VSG, but monensin completely inhibits conversion of 58-kD VSG to the mature 59-kD form. In the presence of tunicamycin, VSG is synthesized as a 54-kD polypeptide that is subsequently processed to a form with a slightly higher M_r. This tunicamycin-resistant processing suggests that modifications unrelated to N-linked oligosaccharides occur. Surprisingly, the rate of VSG transport is reduced, but not abolished, by dropping the chase temperature to as low as 10°C.

THE variant surface glycoprotein (VSG)[1] of the protozoan *Trypanosoma brucei* forms a coat covering the entire external surface of the cell. By replacing this coat with another, composed of a different VSG, the parasite evades the immune response of its mammalian host. This process of antigenic variation depends on the concerted repression of one VSG gene and expression of another within an individual organism (10, 11, 20, 22, 53).

VSGs have apparent molecular masses of ~60,000 D and ~10^7 of these molecules form the surface coat (17). The antigenic specificity of a given trypanosome variant is a function of the amino acid sequence of its VSG. Sequence data reveal enormous heterogeneity among VSGs in different variants (1, 9, 13, 39, 41, 44).

VSGs are anchored in the plasma membrane by an unusual glycolipid moiety. This glycolipid contains glycerol (21), myristate (23), phosphate (2, 3, 14), and inositol (25) in the form of dimyristyl-phosphatidylinositol (25). Also associated with this structure, in unknown linkages, are mannose, glucosamine, galactose (29, 30, 32), and ethanolamine (31). The glycolipid is attached to the VSG by an amide linkage between the α-carboxyl of the polypeptide and the ethanolamine of the glycolipid (31).

VSG can be isolated in two forms (14, 15). Membrane-form VSG (mfVSG), an amphiphilic protein, contains the intact glycolipid. Soluble VSG (sVSG), a hydrophilic protein, lacks dimyristyl glycerol (24, 34). Upon disruption of trypanosomes by nondenaturing techniques, mfVSG is converted to sVSG by an endogenous membrane-bound (14, 15, 16) phospholipase C (24, 34). Disruption of trypanosomes under conditions that inactivate the lipase preserves VSG in the membrane form.

mfVSG and sVSG can be distinguished immunochemically. There is an immunologically cross-reacting determinant (CRD) found on all VSGs (5, 18) that resides in the carbohydrate portion of the glycolipid (6, 29, 30, 32). Anti-CRD antibodies react only with sVSG (14), presumably because dimyristyl glycerol masks this epitope on mfVSG.

After synthesis on membrane-bound polysomes (38), VSGs undergo several co- and posttranslational modifications. An amino-terminal signal sequence is removed (8, 42) and one or more aspargine-linked oligosaccharides are added (29, 30, 32). In some cases, these oligosaccharides may be subsequently processed (4, 43). Another modification, which occurs immediately after synthesis of VSG (4,

1. *Abbreviations used in this paper*: BBS, Bicine-buffered saline; CRD, cross-reacting determinant; mfVSG, membrane form of variant surface glycoprotein; NP-40, Nonidet P-40; PSG, phosphate/saline/glucose; PNGase F, Peptide-*N*-glycosidase F; sBSA, succinyl bovine serum albumin; Sulfo-SMPB, sulfo succinimidyl 4-(*p*-maleimidophenyl) butryate; sVSG, soluble form of variant surface glycoprotein; TEN buffer, 150 mM NaCl, 5 mM EDTA, and 50 mM Tris-HCl (pH 7.5); VSG, variant surface glycoprotein.

26), involves the removal of a carboxyl-terminal hydrophobic peptide of 17 or 23 amino acid residues (7, 8, 44) and the attachment of the glycolipid to the new carboxyl-terminus of the molecule.

We report here the kinetics of three processes that occur in the posttranslational life of the ILTat-1.3 VSG. First, we determined the kinetics of the changes in M_r of newly synthesized VSG. These changes are attributed mainly to the processing of N-linked oligosaccharides, but are also due, in part, to novel processing event(s) that are unrelated to N-linked sugars. Second, we determined the kinetics of transport of VSG to the cell surface, a process that requires 40–60 min (43, 49). Third, we determined the kinetics of transport to a membrane compartment in which conversion of mfVSG to sVSG by membrane-bound phospholipase C can occur in situ. Finally, we determined the effects of tunicamycin (an inhibitor of N-linked glycosylation [52]), monensin (an inhibitor of some intracellular transport pathways [51]), and low temperature on these three processes.

Materials and Methods

Trypanosomes, VSG Purification, and Production of Antibodies

The source of the cloned ILTat-1.3 variant of *T. brucei*, the growth and isolation of trypanosomes, the purification of VSG, and the production of affinity-purified anti-VSG and anti-CRD antibodies have been described previously (4).

Metabolic Labeling and Lysis of Trypanosomes

Trypanosomes were washed once in Bicine-buffered saline (BBS) (50 mM Bicine, 70 mM glucose, 50 mM NaCl, and 5 mM KCl [pH 8.0]) containing 1 mg/ml bovine serum albumin (BSA) (BBS/BSA). After centrifugation (2,500 rpm, 10 min, 4°C) in a Sorvall HB-4 rotor (E. I. DuPont de Nemours & Co., Inc., Sorvall Instruments Div., Newton, CT), the cells were resuspended at 5×10^7 cells/ml in RPMI-1640 medium without methionine (Gibco Laboratories, Grand Island, NY), supplemented with 10% heat-inactivated fetal calf serum and 25 mM Hepes (pH 7.4). After preincubation (15 min, 37°C), [^{35}S]methionine (Amersham Corp., Arlington Heights, IL; 1,000 Ci/mmol) was added to a final concentration of 100 μCi/ml. Chase was initiated by diluting labeled cells 1:10 into prewarmed medium containing nonradioactive methionine (111 μg/ml). When chases were performed at reduced temperatures, the chase medium was preequilibrated accordingly.

In inhibitor experiments, cells were preincubated with tunicamycin (Calbiochem-Behring Corp., La Jolla, CA; 200 ng/ml) or monensin (Calbiochem-Behring Corp.; 10^{-7} M). The inhibitors were included in the chase medium at the same concentrations. A tunicamycin stock (20 μg/ml in 25 mM NaOH) and a monensin stock (10^{-3} M in ethanol) were stored at −20°C. On the day of use, monensin was diluted in medium to a 10^{-5} M working stock.

At intervals during the chase period, aliquots of cell suspension (200 or 400 μl) were added to 1.0 ml ice cold BBS/BSA and centrifuged in a microfuge (Beckman Instruments, Inc.; model B) (30 s, 22°C). Pellets were resuspended at 5×10^7 cells/ml in 150 mM NaCl, 5 mM EDTA, and 50 mM Tris-HCl (pH 7.5) (TEN buffer) containing 1% Nonidet P-40 (NP-40). Then, to convert mfVSG to sVSG by the endogenous phospholipase C, the lysates were incubated at 37°C for 5 min. Lysates were then diluted to 2×10^6 cell equivalents/ml with ice cold TEN buffer containing 1% NP-40 and used for immunoprecipitation with anti-VSG. Iodoacetamide (5 mM), leupeptin (1 μg/ml), and *N* α-*p*-tosyllysine chloromethyl ketone (0.1 mM) were included in the lysis buffer to minimize proteolysis.

Cross-linking of Surface VSG

This procedure is a modification of that described by Strickler and Patton (49). At intervals during the chase, 200-μl aliquots of cell suspension were added to 1.0 ml of 57 mM Na_2HPO_4, 3 mM NaH_2PO_4, 44 mM NaCl, and 56 mM glucose (pH 8.0) (37) containing 1.0 mg/ml phosphate/saline/glucose/succinyl-BSA (PSG/sBSA). The BSA, which was included to maintain trypanosome viability, was succinylated to block free amino groups that might react with the cross-linking reagent. Cells were centrifuged in a microfuge (30 s, 22°C), washed once in PSG/sBSA and resuspended in 100 μl PSG/sBSA. The membrane-impermeable cross-linking reagent, Sulfo-SMPB (sulfosuccinimidyl 4-[*p*-maleimidophenyl]butyrate; 2 μl, 20 mM in dimethyl sulfoxide, Pierce Chemical Co., Rockford, IL) was added and the cells were incubated for 15 min at 0°C. After quenching by the addition of 1.0 ml of 50 mM glycylglycine, 70 mM glucose, 50 mM NaCl, and 5 mM KCl (pH 8.0) containing 1.0 mg/ml BSA, the cells were centrifuged and lysed as described in the previous section. All buffers were at 0°C. Mock cross-linkings were done using dimethyl sulfoxide alone. Labeled VSG polypeptides were analyzed by immunoprecipitation with anti-VSG.

sBSA was prepared in a reaction (20 ml) containing 20 mg/ml BSA and 0.5 M $NaHCO_3$ (pH 9.0). Succinic anhydride (400 mg) was added five times, at 10-min intervals, at room temperature. The pH was maintained at 9.0 by the manual addition of 4 M NaOH. The sBSA was dialyzed against 10 mM NH_4HCO_3 and lyophilized.

In Situ Conversion of mfVSG to sVSG

At intervals during the chase, aliquots of cell suspension (400 μl) were added to 1.0 ml ice cold BBS/BSA and centrifuged in a microfuge (30 s, 22°C). The pellets were resuspended in H_2O (180 μl) to lyse the cells. After 5 min at 0°C, 10× TEN buffer (20 μl) was added and the lysates were incubated for 5 min at 37°C. During this incubation, susceptible mfVSG is converted to sVSG. TEN buffer containing 5% SDS was then added (50 μl) and the lysates were boiled for 10 min. Samples were then diluted with 1.0 ml TEN buffer containing 2.5% Triton X-100 and incubated for 15 min at 0°C to allow formation of mixed micelles. Protease inhibitors, as described above, were included in all lysis solutions. Labeled VSG polypeptides were analyzed by immunoprecipitation with anti-CRD.

Immunoprecipitation and SDS Gel Electrophoresis

Lysates of [^{35}S]methionine-labeled trypanosomes (10^6 cell equivalents in 500–625 μl) were treated overnight at 0°C with saturating amounts of anti-VSG or anti-CRD in microfuge tubes. Protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ; 50 μl of an 8% suspension [wt/vol] in TEN buffer containing 0.5% NP-40, 1% BSA, and 0.02% sodium azide) was added and the samples were agitated at 4°C for 1 h. The beads were centrifuged and washed as follows: twice in TEN buffer containing 0.5% NP-40 and 5 mg/ml BSA; twice in TEN buffer containing 0.5% NP-40 and 2.5 M KCl; twice in TEN buffer containing 0.5% NP-40; and once in TEN buffer. 2× SDS sample buffer (30 μl, 2× = 100 mM Tris HCl, [pH 6.8] 2% SDS, 80 mM dithiothreitol, 20% glycerol, and 0.1% bromphenol blue) was added and the samples were boiled for 2 min.

The samples were loaded on 17-cm, 7.5–15% linear gradient, SDS–polyacrylamide gels (36) and were run overnight at a constant voltage of 100 V. Polyacrylamide stacking gels were 3% for samples containing cross-linked VSG and 5% in all other cases. Gels were stained with Coomassie Blue, impregnated with EN^{3}HANCE (New England Nuclear, Boston, MA) and fluorographed using Kodak XAR-5 film. Molecular mass markers (Sigma Chemical Co., St. Louis, MO) were myosin, 205 kD; β-galactosidase, 116 kD; phosphorylase B, 97 kD; BSA, 66 kD; ovalbumin, 45 kD; glyceraldehyde-3-phosphate dehydrogenase, 36 kD; carbonic anhydrase, 29 kD; soybean trypsin inhibitor, 20 kD; and α-lactalbumin, 14 kD.

Peptide-N-Glycosidase Treatment

Immunoprecipitated VSG was eluted from Protein A–Sepharose beads with boiling 1% SDS (200 μl) and precipitated with acetone (1.2 ml, −20°C, 16 h) using 15 μg cytochrome *c* as carrier. The samples were collected by centrifugation (10,000 rpm, 20 min, 4°C, Sorvall HB-4 rotor) and dried under vacuum. After boiling 3 min in TEN buffer (pH 8.6) containing 0.3% SDS and 2.0% 2-mercaptoethanol (15 μl) the samples were diluted with TEN buffer (pH 8.6) containing 3% NP-40 (30 μl). The following protease inhibitors were included in both buffers: leupeptin (2 μg/ml), antipain (2 μg/ml), chymostatin (1 μg/ml), pepstatin (1 μg/ml), *N* α-*p*-tosyllysine chloromethyl ketone (0.1 mM), trasylol (10 U/ml), benzamidine (10 μg/ml), and 1,10 phenanthroline (5 mM). Peptide-*N*-glycosidase F (PNGase F; 1 μl in 2.5 mM EDTA [pH 7.4] containing 50% glycerol; prepared according to Tarentino et al. [50] and generously donated by Dr. Nancy Dahms, Washington University, St. Louis, MO) was added. This amount of enzyme will deglycosylate 30 μg α_1-acid glycoprotein or 75 μg ovalbumin in an

18-h incubation (Dahms, N., unpublished observations). After incubation overnight at 37°C, H_2O (155 μl) was added and the samples were acetone precipitated and centrifuged as described above. The precipitates were solubilized in 1X SDS sample buffer (40 μl).

Results

Posttranslational Processing of VSG

In the few minutes after biosynthesis of the VSG polypeptide, several different forms appear that can be distinguished on SDS gels. These forms probably differ in N-linked glycosylation (4, also see Discussion in this paper). As shown in Fig. 1, a doublet of polypeptides (56 and 58 kD) was present immediately after a pulse-labeling with [^{35}S]methionine (Fig. 1 *A*, lane *1*). After initiation of a chase, the 56-kD species disappeared, leaving the 58-kD polypeptide as the predominant form (lane *2*). Thereafter, the 58-kD species was processed to the mature 59-kD protein (lanes *3–8*).

We made two quantitative analyses of these data. First, densitometry of the fluorograph allowed calculation of the fraction of the total immunoprecipitable polypeptide in the form of mature 59-kD VSG. The apparent $t_{½}$ for processing to the mature form was ~15 min (Fig. 1 *B*). Second, excision of the VSG bands from the gel and measurement of the incorporated radioactivity revealed that the total VSG-specific radioactivity decreased slowly during the chase period (Fig. 1 *B*). Typically, a 10–30% decrease was observed in 45–60-min pulse–chase experiments. Trypanosomes remained fully viable during the culture period, suggesting that this decrease could represent some form of VSG turnover.

We investigated the effects of tunicamycin on posttranslational processing, as previous studies have indicated that this drug blocks N-linked glycosylation of VSG (4, 26, 43, 45, 48). In the presence of this inhibitor, newly synthesized VSG appeared on SDS gels as a single 54-kD species (Fig. 2, lanes *2* and *7*). During the chase period, this polypeptide shifted to a slightly higher M_r form (Fig. 2 *A*, lanes *3–6*). This small shift in mobility was reproducible in separate experiments (e.g., Fig. 3, lanes *3* and *4*). Although the kinetics

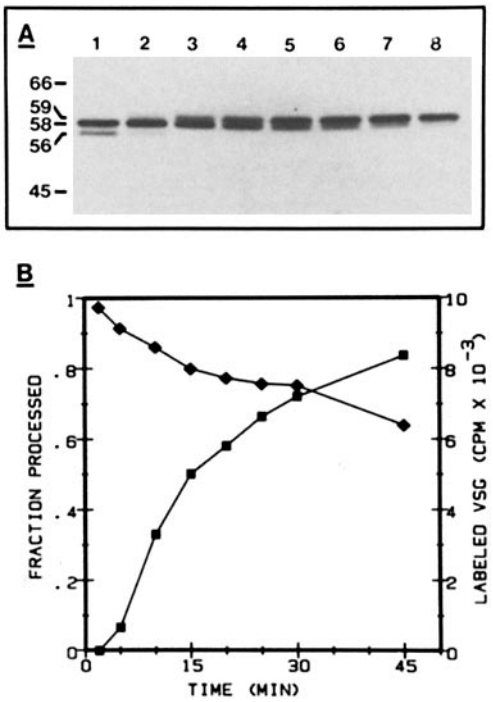

Figure 1. Processing of ILTat-1.3 VSG. Trypanosomes were pulse-labeled 2 min with [^{35}S]methionine and then chased. At intervals after initiation of the chase, samples were lysed and treated with anti-VSG. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. (*A*) Fluorograph of a gel containing immunoprecipitated VSG. Lanes *1–8* show labeled VSG polypeptides from cells sampled at 2, 5, 10, 15, 20, 25, 30, and 45 min. Sample times are relative to the time of initiation of labeling and all lanes contain 10^6 cell equivalents. The scale on the left shows apparent M_r in kilodaltons. (*B*) Kinetics of VSG processing. The absorbance of the 56-, 58-, and 59-kD VSG bands in each lane of the fluorograph shown in *A* was quantitated using a Loats Associates, Inc. (Westminster, MD) video densitometry system. The fluorograph was taken using preflashed Kodak XAR-5 film. The data are presented as the fraction of the total absorbance present in the mature 59-kD VSG band (■). The total radioactivity in VSG at each time was determined by solubilizing the excised bands in 600 μl perchloric acid (23%) and hydrogen peroxide (20%) at 90°C, and counting in 20 ml of Liquiscint (National Diagnostics, Somerville, NJ). Each measurement was corrected for background radioactivity by subtracting the cpm detected in an equivalent unlabeled portion of each lane. The data are presented as cpm/VSG band (◆).

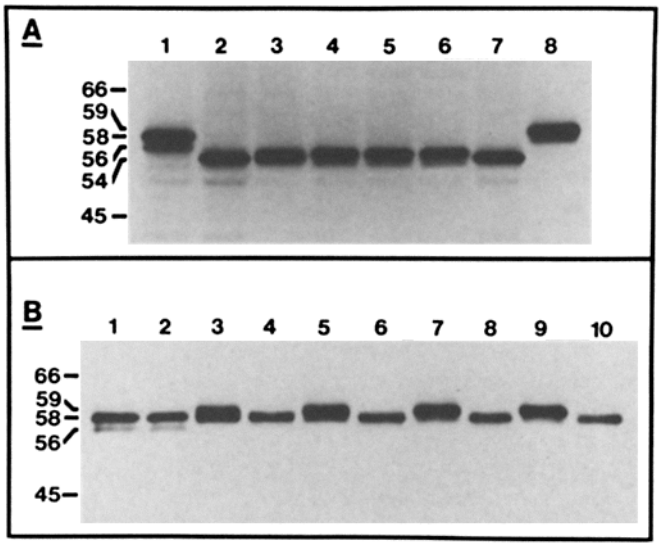

Figure 2. Effects of tunicamycin and monensin on VSG processing. Trypanosomes, treated with either tunicamycin or monensin, were pulse-labeled 2 min with [^{35}S]methionine and then chased. Aliquots were analyzed as in Fig. 1. (*A*) Fluorograph of a gel containing labeled VSG from tunicamycin-treated cells. Lanes *1* and *8*, immunoprecipitated VSG from untreated trypanosomes that were pulse-labeled for 2 min, chased, and sampled at 2 and 60 min, respectively. Lanes *2* and *7*, immunoprecipitated VSG from drug-treated cells sampled at 2 min. Lanes *3–6*, immunoprecipitated VSG from drug-treated cells sampled at 15, 30, 45, and 60 min, respectively. The bands below 54 kD were not detected in other experiments and are probably due to minor proteolysis. (*B*) Fluorograph of a gel containing labeled VSG from monensin-treated trypanosomes. Cells were sampled from pulse–chase mixtures at 2 min (lanes *1* and *2*), 15 min (lanes *3* and *4*), 30 min (lanes *5* and *6*), 45 min (lanes *7* and *8*), and 60 min (lanes *9* and *10*). The gel contains immunoprecipitated VSG from drug-treated (lanes *2*, *4*, *6*, *8*, and *10*) and untreated (lanes *1*, *3*, *5*, *7*, and *9*) trypanosomes. Sample times in both panels are relative to the initiation of the 2-min labeling period and all lanes contain 10^6 cell equivalents. Scales refer to apparent M_r.

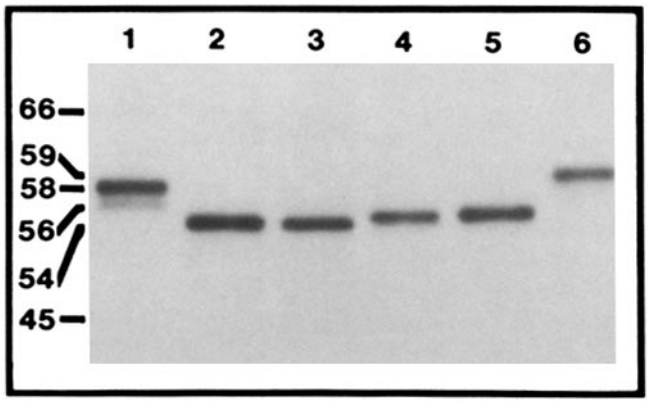

Figure 3. PNGase treatment of VSG. ^{35}S-labeled VSG polypeptides were immunoprecipitated with anti-VSG from cells that were labeled for 2 min (lanes *1–3*) and chased for 58 min (lanes *4–6*) in the presence (lanes *3* and *4*) or absence (lanes *1*, *2*, *5*, and *6*) of tunicamycin. Labeled VSG polypeptides were eluted from Protein A-beads and treated with PNGase F as described in Materials and Methods (lanes *2* and *5*). Eluted controls were either mock-treated (lanes *1* and *6*) or untreated (lanes *3* and *4*). A fluorograph of an SDS gel containing 10^6 cell equivalents/lane is shown. Scale refers to apparent M_r.

of this increase could not be accurately quantitated, this tunicamycin-resistant processing appeared to occur with roughly the same kinetics as the conversion of 58-kD VSG to 59-kD VSG shown in Fig. 1 *A*.

We wished to determine whether the tunicamycin-resistant processing of VSG occurred in the absence of drug or whether it was an artifact of inhibition. We treated immunoprecipitated VSG polypeptides from a pulse–chase culture with PNGase F, an enzyme that removes N-linked oligosaccharides (50). As shown in Fig. 3, PNGase F converted newly synthesized 56- and 58-kD VSG (lane *1*) to a lower M_r form (54 kD, lane *2*) that comigrated with newly synthesized VSG from tunicamycin-treated cells (lane *3*). Similarly, PNGase F converted mature 59-kD VSG (lane *6*) to a lower M_r form (lane *5*) that comigrated with VSG from cells chased 58 min in the presence of tunicamycin (lane *4*). These results suggest that the tunicamycin-resistant processing occurs in normal cells and contributes, at least in part, to the conversion of 58-kD VSG to 59-kD VSG.

We have also examined the effect of monensin, a monovalent cationophore, on VSG processing. We detected the doublet of newly synthesized VSG polypeptides (56 kD and 58 kD) in the presence or absence of 10^{-7} M monensin (Fig. 2 *B*, compare lanes *1* and *2*) and the drug had no effect on the disappearance of the 56-kD form (Fig. 2 *B*, compare lanes *3* and *4*). However, during the subsequent chase period, VSG was not processed to the mature 59-kD species in the presence of 10^{-7} M monensin (Fig. 2 *B*, lanes *5–10*). This concentration of monensin had minimal effects on the viability, morphology, and motility of trypanosomes, even in 2-h cultures, and had little effect on the incorporation of [^{35}S]-methionine into hot TCA-insoluble material (80–90% of controls). Higher concentrations of drug (5×10^{-7}–10^{-6} M) had deleterious effects on the physical characteristics of trypanosomes and lowered incorporation of [^{35}S]methionine to 10–20% of the control levels.

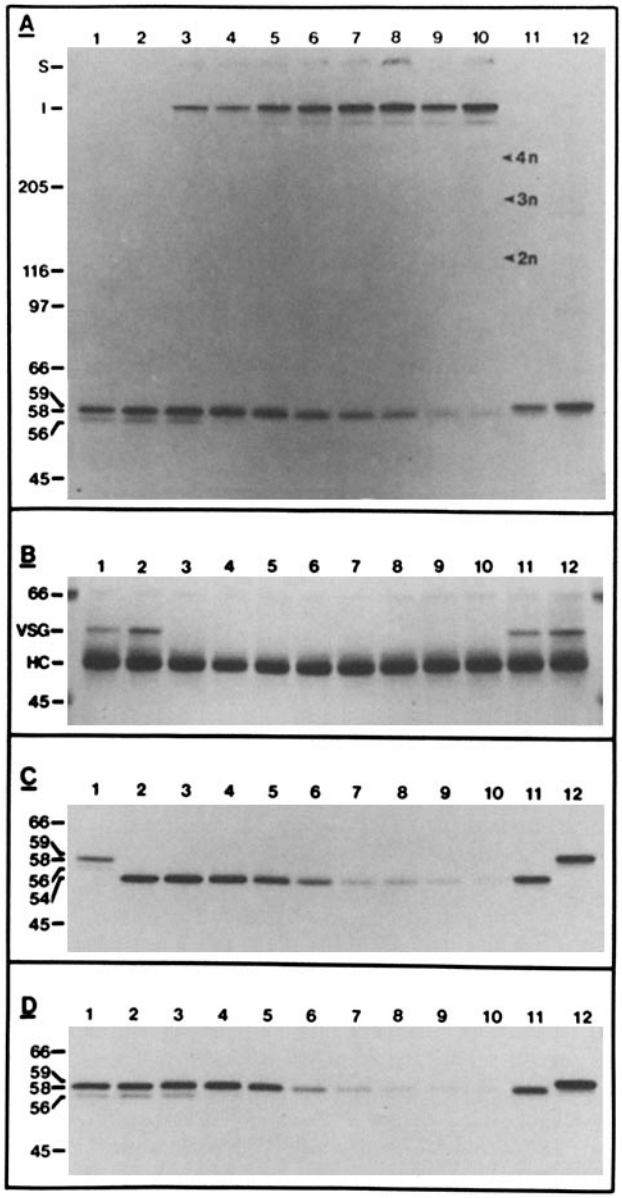

Figure 4. Transport of VSG to the cell surface. Trypanosomes were pulse-labeled 2 min with [^{35}S]methionine and chased. Aliquots were removed and exposed to Sulfo-SMPB. Cross-linked cells were lysed and treated with anti-VSG. (*A*) Fluorograph of a gel containing immunoprecipitated VSG from a cross-linking experiment. Lanes *1* and *12*, immunoprecipitated VSG from untreated trypanosomes sampled at 2 and 60 min, respectively. Lanes *2* and *11*, immunoprecipitated VSG from mock-treated trypanosomes sampled at 2 and 45 min, respectively. Lanes *3–10*, immunoprecipitated VSG from cross-linked trypanosomes sampled at 2, 5, 10, 15, 20, 25, 30, and 45 min, respectively. *S*, location of the gel slot. *I*, interface of the stacking and running gels. (*B*) Coomassie Blue stain of the gel shown in *A*. Only the region of the gel containing VSG monomer is presented. *HC*, immunoglobulin heavy chain. (*C*) identical to *A* except that lanes *2–11* contain immunoprecipitated VSG from cells that were pulsed and chased in the presence of tunicamycin. Only the region of the gel containing monomer VSG is shown. (*D*) Identical to *C* except that the cells were pulsed and chased in the presence of monensin. Sample times in all panels are relative to the initiation of labeling and all lanes contain 10^6 cell equivalents. Scales at left indicate M_r. Scale at right (*A*) indicates positions expected for oligomers of VSG.

Transport of VSG to the Cell Surface

To interpret the kinetics of VSG processing it was necessary to know the time of VSG transit to the cell surface. Therefore, we developed an assay to distinguish internal VSG from external VSG based on the accessibility of surface VSG to Sulfo-SMPB, a membrane-impermeant protein cross-linker. We then determined the time of transit to the surface in a pulse–chase experiment with [^{35}S]methionine. After treating aliquots of labeled cell suspension with Sulfo-SMPB under conditions (0°C, 15 min) where further processing or transport is inhibited, we lysed the cells and analyzed anti-VSG immunoprecipitates by SDS-PAGE.

Control experiments are presented in Fig. 4, *A* and *B*. Internal [^{35}S]VSG, present after a 2-min pulse, was resistant to cross-linking and was detected predominantly as monomer in the fluorograph (Fig. 4 *A*, lane *3*). External [^{35}S]-VSG, present at the end of the chase period, was sensitive to cross-linking and was detected predominantly as high M_r oligomers (Fig. 4 *A*, lane *10*). Two observations indicate that cross-linking of surface VSG is very efficient. First, no monomer VSG was detected by Coomassie Blue staining in the lanes containing cross-linked samples (Fig. 4 *B*, compare lanes *3–8* with lanes *2* and *11*). Second, all cross-linked VSG was detected as oligomers of six or greater (Fig. 4 *A*, lane *10*) and most was retained at the top of the running or stacking gels. Greater than 90% of the trypanosomes remained viable, as assessed by motility, after the cross-linking procedure. However, cross-linked cells appeared constrained in flagellar motion, as if cross-linking added rigidity to the cell surface.

Newly synthesized VSG, which is resistant to cross-linking (Fig. 4 *A*, lane *3*), was rapidly transported to the surface, as indicated by the disappearance of monomer VSG during the chase period (Fig. 4 *A*, lanes *4–10*). The internal VSG population contained predominantly precursor VSG species (56 and 58 kD). Very little mature VSG (59 kD) was detected as monomer. The rate of transport was determined by excising the monomer VSG band(s) and quantitating the decrease in radiolabeled monomer as a function of time (Fig. 5). The apparent $t_{1/2}$ for transport to the surface was ~14 min.

We also studied the effects of tunicamycin and monensin on transport. Fig. 4 *C* shows that VSG was transported to the cell surface efficiently in the presence of tunicamycin; the apparent $t_{1/2}$ for transport was the same as untreated controls (Fig. 5). Interestingly, no increase in M_r (compare with Fig. 2 *A*) was detected in the internal monomer VSG during the chase period, suggesting that the tunicamycin-insensitive processing occurs at about the same time as arrival at the plasma membrane. Fig. 4 *D* shows a similar experiment with monensin. This drug also had no effect on the rate of transport of VSG to the cell surface (Fig. 5).

Transport of VSG to a Phospholipase C–containing Membrane Compartment

The glycolipid on newly synthesized VSG is resistant to hydrolysis by the endogenous membrane-bound phospholipase C when trypanosomes are lysed hypotonically (4, 26). These molecules are not converted from mfVSG to sVSG and remain membrane-bound, whereas mature VSG on the surface

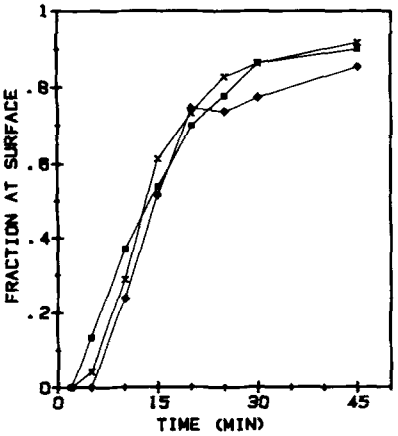

Figure 5. Kinetics of VSG transport to the cell surface. The radioactivity in the monomer VSG bands in the gels shown in Fig. 4, *A*, *C*, and *D* was quantitated as described in the legend to Fig. 1. The radioactivity detected in the 2- and 45-min mock-treated samples (lanes *2* and *11*) were averaged and taken as the value for total labeled VSG. The radioactivity detected in the cross-linked samples (lanes *3–10*) was normalized as a fraction of average total labeled VSG. The data are presented as the fraction of the total VSG at the cell surface as a function of time for untreated (■), tunicamycin-treated (◆), and monensin-treated (×) trypanosomes.

of the same cells is rapidly converted and released (19). The resistance to hydrolysis of the newly synthesized molecules is not due to an altered glycolipid structure, as this moiety is readily hydrolyzed if trypanosomes are solubilized in nonionic detergent. These facts imply that newly synthesized VSG resides in a membrane compartment that lacks the phospholipase C activity (4). We therefore determined how long it takes VSG to reach a membrane compartment where conversion of mfVSG to sVSG can occur in situ during hypotonic lysis. In situ conversion implies that the lipase and VSG are colocalized in the same membrane, although it is possible that VSG resides in a distinct membrane site and only becomes accessible to enzyme during hypotonic lysis.

Our assay for this process takes advantage of the specific reactivity of anti-CRD antibodies with sVSG but not mfVSG (14). Cells from pulse–chase cultures were lysed hypotonically and incubated at 37°C to facilitate conversion of susceptible mfVSG molecules to sVSG. After boiling in SDS, Triton X-100 was added to form mixed micelles and the sVSG was specifically immunoprecipitated with anti-CRD.

As Fig. 6 *A* shows, no labeled VSG was immunoprecipitated from cells that have been pulse-labeled with [^{35}S]methionine for 2 min, confirming that newly synthesized VSG is indeed resistant to in situ conversion (lane *2*). During the chase period, the labeled VSG rapidly became susceptible to in situ conversion, as indicated by the increasing amount of immunoprecipitated VSG detected in lanes *3–9*. It is important to note that only mature 59-kD VSG was detected in the converted VSG population. We measured the radioactivity in the labeled VSG bands and Fig. 7 shows the kinetics of transport to a membrane compartment where in situ conversion can occur. The apparent $t_{1/2}$ for this process was ~14 min.

We also investigated the effects of tunicamycin and monensin on in situ conversion. Neither inhibitor had any detectable effects on this process (Figs. 6, *B* and *C*, and 7). It should

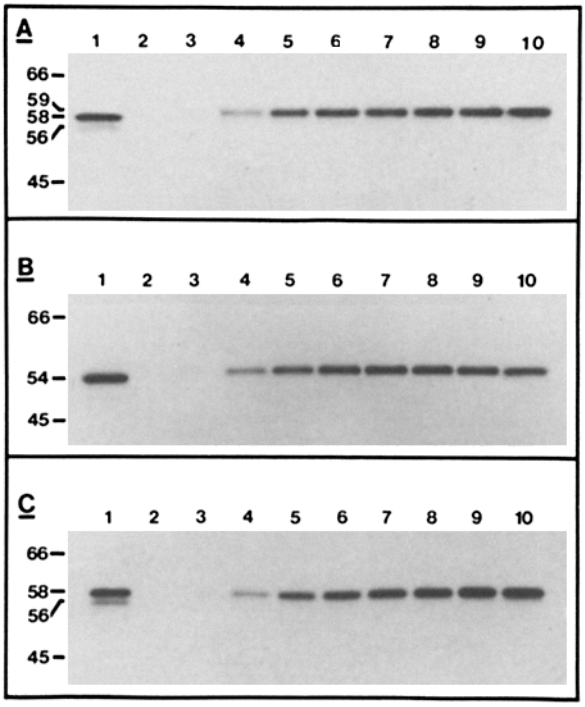

Figure 6. Transport of VSG to a phospholipase C–containing compartment. Trypanosomes were pulse-labeled 2 min with [^{35}S]methionine and chased. Aliquots of cells were sampled, lysed hypotonically, and treated to allow in situ conversion of mfVSG to sVSG. (*A*) Fluorograph of a gel showing immunoprecipitated VSG from an in situ conversion experiment. Lanes *1* and *10* contain VSG precipitated with anti-VSG from control NP-40 lysates of trypanosomes that were sampled at 2 and 45 min, respectively. Lanes *2–9* contain VSG precipitated with anti-CRD from hypotonic lysates of trypanosomes sampled at 2, 5, 10, 15, 20, 25, 30, and 45 min, respectively. (*B*) Identical to *A* except that the cells were pulsed and chased in the presence of tunicamycin. (*C*) Identical to *A* except that the cells were pulsed and chased in the presence of monensin. Sample times in all panels are relative to the initiation of labeling and all lanes contain 10^6 cell equivalents. Scales indicate apparent M_r in kD.

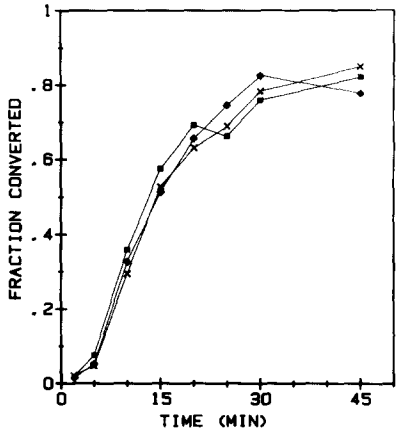

Figure 7. Kinetics of transport of VSG to a phospholipase C–containing compartment. The radioactivity in the VSG bands in the gels shown in Fig. 6, *A–C*, was quantitated as described in the legend to Fig. 1. The radioactivities detected in the 2- and 45-min control samples (lanes *1* and *10*) were averaged and taken as the value for total labeled VSG. The radioactivity detected in the hypotonically lysed samples (lanes *2–9*) was normalized as a fraction of the average total labeled VSG. The data are presented as the fraction of VSG converted from mfVSG to sVSG as a function of time for untreated (■), tunicamycin-treated (◆), and monensin-treated (×) trypanosomes.

be noted, however, that only the higher M_r form of VSG synthesized in the presence of tunicamycin was detected in the converted fraction (Fig. 6 *B*, compare lanes *4–9* with lane *10*).

Processing and Transport of VSG at Low Temperature

Since the kinetics of the three posttranslational processing reactions are essentially identical (all have a $t_{1/2}$ of 14–15 min), no conclusions can be made concerning the order in which these events occur. In other systems, low temperature has been used to block transport of membrane glycoproteins in a pre-Golgi compartment (15°C, [33, 47]) and in the trans-Golgi (20°C; [40, 47]). Therefore, in an attempt to separate these processes on a temporal basis we performed experiments in which cells were pulsed-labeled at 37°C and chased at 10–20°C. We then used our standard assays for transport to the surface and to a phospholipase C–containing membrane.

Surprisingly, transport to the surface still occurred at temperatures as low as 10°C (Fig. 8 *C*). At 15°C, the rate for transport was reduced fourfold relative to transport at 37°C ($t_{1/2}$ = 60 min, Fig. 8 *C*). The amount of mature 59-kD VSG that was detected in the internal VSG pool, at 15°C, was increased slightly relative to the 37°C chase temperature (compare Figs. 4 *A* and 8 *A*). These data would be consistent with an internal site for processing of 58-kD VSG to 59-kD VSG. At the end of a 2-h chase period at 15°C, ~80% of the VSG was on the surface (Fig. 8 *C*), but only slightly more than half was in the mature form (Fig. 8 *A*, lane *10*). This result suggests that at 15°C some 58-kD VSG was transported to the surface.

The rate of transport of VSG to a phospholipase C–containing membrane at 15°C was essentially the same as that for transport to the surface (Fig. 8 *C*). Compared with the 37°C chase temperature (Fig. 6 *A*), an increased amount of 58-kD VSG was sensitive to in situ conversion (Fig. 8 *B*).

Discussion

Newly synthesized VSG molecules of the ILTat-1.3 variant are detected as a 56-kD and 58-kD doublet (Fig. 1 *A*). The 56-kD species disappears rapidly (<5 min) and probably is converted to the 58-kD species. It is likely, for several reasons, that these polypeptides represent singly and doubly N-glycosylated species, respectively. First, this variant has two Asn-X-Ser/Thr glycosylation sites (44). Second, in the presence of tunicamycin this VSG is synthesized as a 54-kD species (Fig. 2 *A*). Third, treatment of newly synthesized VSG with PNGase F yields a single species of the same electrophoretic mobility as VSG synthesized in the presence of tunicamycin (Fig. 3). Finally, proteolytic peptide maps of [^{3}H]mannose-labeled 59-kD VSG yield three labeled peptides, one of which corresponds to the C-terminal glycolipid (data not shown). Since the 56- and 58-kD VSG molecules both have the carboxyl-terminal glycolipid (4) these data suggest that at least some N-linked glycosylation occurs

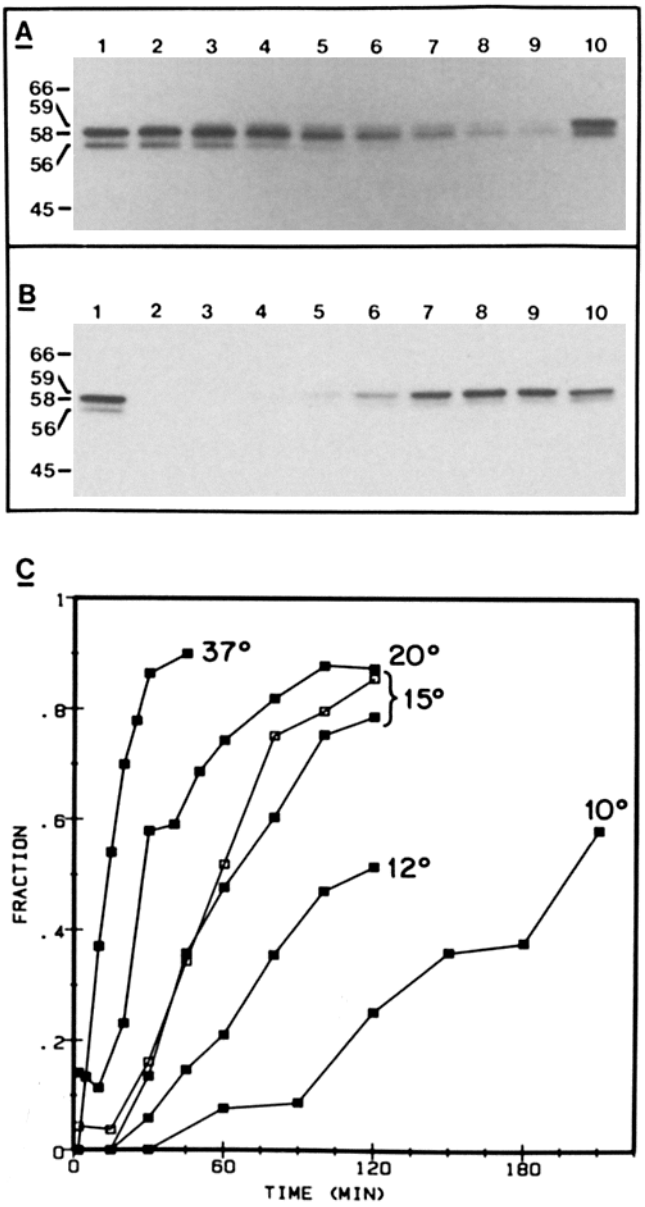

Figure 8. Intracellular transport of VSG at low temperature. Trypanosomes were pulse-labeled for 2 min with [^{35}S]methionine at 37°C and chased at 10–37°C. Aliquots were removed and analyzed for transport to the surface, as in Fig. 4, or for transport to a phospholipase C–containing membrane compartment, as in Fig. 6. (*A*) Fluorograph of a gel containing samples from a cross-linking experiment. Cells were chased at 15°C. Lanes *1* and *10*, immunoprecipitated VSG from mock-treated trypanosomes sampled at 2 and 120 min, respectively. Lanes *2–9*, immunoprecipitated VSG from cross-linked trypanosomes sampled at 2, 15, 30, 45, 60, 80, 100, and 120 min, respectively. (*B*) Fluorograph of a gel containing samples from an in situ conversion experiment. Cells were chased at 15°C. Lanes *1* and *10* contain VSG precipitated with anti-VSG from NP-40 lysates of cells sampled at 2 and 120 min, respectively. Lanes *2–9* contain VSG precipitated with anti-CRD from hypotonic lysates of cells sampled at 2, 15, 30, 45, 60, 80, 100, and 120 min, respectively. (*C*) Graph showing the kinetics, at 10–37°C, of transport to the surface (■) and to a phospholipase C–containing membrane (□). The data for 15°C are from *A* and *B*. Radioactivity was determined as described in the legends to Fig. 5 and 7. The data are presented as the fraction of total VSG at the surface (■) and the fraction of VSG converted from mfVSG to sVSG (□). Sample times in all panels are relative to the initiation of labeling and all lanes contain 10^6 cell equivalents. Scales at the left of *A* and *B* indicate apparent M_r.

posttranslationally in this variant. Similar findings have been reported in other trypanosome variants (26).

After the addition of N-linked oligosaccharides, 58-kD VSG is processed to the mature 59-kD species with an apparent $t_{½}$ of 15 min (Fig. 1). VSG is also subsequently processed to a slightly higher M_r form in tunicamycin-treated cells (Fig. 2 *A*). This tunicamycin-resistant processing is not artifactual since PNGase F treatment of 56/58-kD and 59-kD VSG yield single polypeptides that have the same electrophoretic mobility as the low and high M_r forms, respectively, of VSG synthesized in tunicamycin-treated cells. However, the tunicamycin-resistant increase in M_r does not appear to be large enough to account for the apparent 1-kD increase in M_r observed in untreated cells. Therefore, it is likely that both the tunicamycin-resistant processing and the processing of N-linked oligosaccharides contribute to the conversion of 58-kD to 59-kD VSG.

The nature of the tunicamycin-resistant processing is not known, but one possibility is that the carboxyl-terminal glycolipid is modified. All the components of the glycolipid appear to be in fixed molar ratios except galactose, which varies among the variants that have been examined (32). Perhaps some or all of these residues are added after the glycolipid is attached to the carboxyl-terminus.

In agreement with McConnell et al. (43), most of the VSG synthesized in a 2-min pulse-labeling reaches the cell surface within 45 min, as assessed by reactivity with a membrane-impermeant cross-linker (Fig. 4). Transport of newly synthesized VSG to a phospholipase C–containing membrane compartment, as assessed by conversion from mfVSG to sVSG during hypotonic lysis, also occurs within 45 min of synthesis (Fig. 6). All attempts to separate these transport processes (i.e., tunicamycin, monensin, and low temperature chases) were unsuccessful. Remarkably, the apparent $t_{½}$ for both of these processes (~15 min, Fig. 5 and 7) is essentially the same as that for processing to the mature 59-kD form.

It is not clear at what point in the secretory pathway that VSG enters a phospholipase C–containing membrane. Several observations suggest that this occurs at the plasma membrane. First, in pulse–chase experiments at 37°C only mature 59-kD VSG is detected in the sVSG fraction of hypotonically lysed cells (Fig. 6 *A*). Second, transport to a phospholipase C–containing membrane has the same kinetics as transport to the surface at both 37°C and 15°C. Finally, phospholipase C has been reported to be in the plasma membrane (54). Nevertheless, it is equally possible that colocalization occurs at an internal site and that subsequent transport to the surface is very rapid.

It is likely, however, that oligosaccharide processing occurs internally since the appropriate glycosyltransferases are found in the Golgi apparatus of all eukaryotes (35). This would be consistent with both the increased detection of mature 59-kD VSG in the internal VSG population at lowered chase temperatures (Fig. 8) and the effects of monensin on VSG processing (Fig. 2 *B*). *N*-acetylglucosaminyl- and galactosyltransferase activities have been detected in trypanosome microsomal and Golgi fractions (27, 46). However, glycosyltransferase activity has also been reported in the plasma membrane of trypanosomes (12) and, while it seems

unlikely, this cannot be ruled out as a site of oligosaccharide processing.

We were surprised to find that pulse-labeled VSG is transported to the surface during chases at temperatures as low as 10°C (Fig. 8). Continued transport at low temperature may be a function of the lipid composition of trypanosome membranes. In addition, VSG is anchored in membranes by the acyl chains of the carboxyl-terminal glycolipid and might be expected to have greater diffusional freedom than an embedded membrane protein.

With these results we can propose a model of how ILTat-1.3 VSG is synthesized, processed, and transported to the cell surface. Other VSGs may be expected to fit this model to varying degrees (e.g., one highly glycosylated VSG has been described whose rate of transport is greatly reduced by tunicamycin [26]).

After the removal of the amino-terminal signal sequence, the first event in the processing of VSG is probably the cotranslational addition of N-linked oligosaccharides. However, at least some core glycosylation appears to occur posttranslationally. Removal of the carboxyl-terminal hydrophobic peptide from the initial translation product and its replacement with the glycolipid anchoring group occurs immediately after synthesis of the polypeptide (4, 26) but before the completion of N-linked glycosylation. This step must be posttranslational since the 23-residue carboxyl-terminal peptide is not long enough to span the endoplasmic reticulum membrane and the cleft of the large ribosomal subunit and still be in the form of a peptidyl-tRNA. The speed of carboxyl-terminal processing suggests that this event occurs in the endoplasmic reticulum and that the glycolipid may be attached en bloc in a concerted reaction with removal of the peptide (4).

After these initial events, VSG is transported to an intracellular site where processing of the N-linked oligosaccharides and possibly the glycolipid occurs. Although not certain, it seems likely that this site is in the Golgi complex. This localization would be consistent with the detection of VSG in a putative Golgi fraction of trypanosomes (28).

Thereafter VSG would be rapidly transported to the plasma membrane and incorporated into the surface coat. It seems likely from the kinetic data presented here that VSG first enters a compartment containing phospholipase C when it arrives at the surface. However, the possibility that VSG first colocalizes with phospholipase C in an internal membrane compartment followed by rapid transit to the surface cannot be formally excluded.

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