Biosynthesis of Trypanosoma brucei Variant Surface Glycoproteins

N-GLYCOSYLATION AND ADDITION OF A PHOSPHATIDYLINOSITOL MEMBRANE ANCHOR

(Received for publication, July 26, 1985)

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The variant surface glycoproteins (VSGs) of Trypanosoma brucei are synthesized with a hydrophobic COOH-terminal peptide that is cleaved and replaced by a glycosphospholipid, which anchors VSG to the surface membrane. The hydrophobicity of VSG precursors were studied by metabolic labeling with [35S]methionine and [3H]myristic acid. The COOH-terminal oligosaccharide-containing structure remaining after phospholipase removal of dimyristyl glycerol from membrane-form VSG could be detected serologically within 1 min of polypeptide synthesis in two T. brucei variants studied. Addition of the oligosaccharide-containing structure was resistant to tunicamycin. VSGs synthesized in the presence of tunicamycin displayed lower apparent molecular weights, consistent with the complete inhibition of N-glycosylation at one (variant 117), two (variant 221), or at least three (variant 118) internal asparagine sites. In most experiments, N-glycosylation appeared to occur during or immediately after polypeptide synthesis but in a few cases N-glycosylation was delayed or incomplete. In all cases, addition of the COOH-terminal oligosaccharide-containing structure occurred normally. In dual-labeling studies, cycloheximide caused rapid inhibition of both [35S]methionine and [3H]myristic acid incorporation, suggesting that myristic acid addition also occurs immediately after polypeptide synthesis. Our data suggest that the complex ethanolamine-glycosyl-dimyristylphosphatidylinositol structure of membrane-form VSG is added en bloc within 1 min of completion of the polypeptide.

The parasitic protozoan Trypanosoma brucei expresses a variant surface glycoprotein (VSG1) coat, which protects it from lytic serum factors in the mammalian host. A single trypansosome expresses only one VSG at a time but possesses a repertoire of several hundred distinct VSG genes (Van der Ploeg et al., 1982). The differential expression of different VSG genes allows a trypansome population to evade the host's immune response. The molecular basis of antigenic variation has been recently reviewed (Cross, 1984a; Turner, 1984). The VSG from T. brucei variant 117 is the best characterized. Comparison of the complete amino acid sequence (Allen et al., 1982) with the sequence predicted from cDNA analysis (Boothroyd et al., 1982) showed that the primary mRNA translation product contains hydrophobic peptide extensions at both the NH2 and COOH termini that are absent from the mature polypeptide. The former appears, despite its large size (33 amino acids), to be a conventional NH2-terminal signal peptide, which directs the nascent polypeptide through the rough endoplasmic reticulum membrane and is removed as a co-translational event (McConnell et al., 1981). The COOH-terminal extension (23 amino acids) is sufficiently hydrophobic to be a membrane insertion site. These features appear to be conserved for all VSGs so far analyzed and, although the NH2-terminal signal sequences are diverse, there is striking homology in the COOH-terminal peptide tails and in the mature VSG COOH-terminal amino acid sequences (Cross, 1984a). VSGs can be placed into three subgroups defined by the COOH-terminal amino acid of the mature polypeptide (Asp, Ser, or Asn). Only one example of the Asn subgroup has been described (VSG 118). Members of the Ser subgroup have peptide tails of only 17 amino acids. In all cases the mature VSG COOH-terminal amino acid is linked to a complex oligosaccharide-containing moiety that is the site of an immunologically cross-reacting determinant (CRD), the only ubiquitous epitope in VSGs (Holder and Cross, 1981; Holder, 1983b). The linkage between the COOH-terminal amino acid and the oligosaccharide involves a novel ethanolamine bridge, whereby ethanolamine is amide linked to the amino acid ω-carboxyl group (Holder, 1983a). This linkage requires removal of the COOH-terminal peptide tail prior to addition of the COOH-terminal oligosaccharide moiety. The substitution, as judged by appearance of the CRD, can be deduced from previously published work (McConnell et al., 1983) to occur within 9 min. The mode of membrane attachment of mature VSG remained unclear for some time as the hydrophobic peptide tails were clearly absent from the majority of mature VSG molecules in the cell (Holder, 1983a), and there was no evidence for significant hydrophobic domains in the remainder of the polypeptide (Allen et al., 1982). In addition, VSG was conventionally isolated in a soluble form (sVSG) following cell disruption by mechanical means or by osmotic shock (Cross, 1975, 1984b). A major breakthrough was made by Cardoso de Almeida and Turner (1983) who showed that lysis of trypansomes in boiling detergent solutions or in the presence of 10 mM Zn2+ resulted in the recovery of an amphiphilic...
membrane form of the VSG (mVSG). When isolated in the membrane form, the CKD appeared to be masked. The conversion of mVSG to sVSG during osmotic shock was shown to be due to an endogenous membrane-associated enzyme (Cardosa de Almeida et al., 1984) and to correlate with the presence of a genetically determined myristic acid (Ferguson and Cross, 1984). Recently, the myristic acid of mVSG has been shown to be linked to the COOH-terminal oligosaccharide as sn-1,2-dimyristyl glycerol, and the endogenous mVSG- to sVSG-converting enzyme was identified as a phospholipase C (Ferguson et al., 1985a). The COOH-terminal oligosaccharide is glycosidically linked to a dimyristoylphosphatidylcholine lipid core (Ferguson et al., 1985b). The existence of a phosphodies- ter bridge between the fatty acid-bearing moiety and the rest of the structure has been independently demonstrated (Jackson and Voorheis, 1985).

In addition to this novel COOH-terminal processing, VSGs are glycosylated at one or more internal sites along the polypeptide. For several VSGs, amino acid sequence data on isolated glycopeptides have revealed that the glycosylated amino acid is Asn present in Asn-X-Ser(Thr) sequences (Holder, 1985). These oligosaccharides contain glucosamine, mannos, and sometimes galactose (Holder and Cross, 1981). Attachment of these oligosaccharides can be inhibited by tunicamycin (Strickler and Patton, 1980; Rovis and Dube, 1981) and catalyzed in vitro by dog pancreatic microsomes (McConnell et al., 1982). These N-linked oligosaccharides show microheterogeneity in some VSG variants (Holder, 1983b; McConnell et al., 1983). Addition of the N-linked oligosaccharides has been reported to occur during (Rovis and Dube, 1981) or following (Strickler and Patton, 1980) polypeptide synthesis.

In this work we have investigated the kinetics of N-glycosylation in VSGs 117, 221, and 118 (from the COOH-terminal Asp. Ser, and Asn VSG subgroups, respectively), the role of N-glycosylation in intracellular transport of VSGs 117 and 118, and the kinetics of CRD (in VSGs 117 and 221) and myristic acid addition (in VSG 117). The results suggest that the VSG COOH terminus is processed rapidly, possibly involving direct replacement of the COOH-terminal peptide tail with a preassembled glycolipid within 1 min of completion of the polypeptide.

**EXPERIMENTAL PROCEDURES**

**Materials**—("$^{[3]}$H)methionine (710 Ci/mmol) and "$^{[3]}$H"glucosamine (30 mCi/mmol) were purchased from Amersham International. [9,10-"$^{3}H"]$\text{myristic}$ acid was purchased from New England Nuclear and complexed to defatted BSA (Ferguson et al., 1985b). Tunicamycin (lot 002689) was purchased from Calbiochem and fixed Staphylococcus aureus (Cowen strain) cells from Miles Laboratories, Inc.

**Preparation of Trypanosomes**—Trypanosome clones of the Mol- teno Institute Trypanozoon antigen types (MITat) 1.2, 1.4, and 1.5 (otherwise known as variants 221, 117, and 118, respectively) of T. brucei strain 427 were purified from infected rat blood as previously described (Cross, 1975). S. aureus was purified as previously described (Cross, 1984).

**Biosynthetic Labeling of Trypanosomes**—A modified Eagle’s mini- nal essential medium was used. The medium was prepared according to the Gibco 320-109 formula minus methionine and vitamins but supplemented with Eagle’s minimal essential medium nonessential amino acids (Gibco, 10 ml/liter of 100 x concentrated), glutamine, 0.3 g/liter, BSA, 1 g/liter, glucose, 10 g/liter, and buffered to pH 7.4 with HEPES, 7.14 g/liter. All labeling experiments were performed at 37°C in a shaking water bath and at cell densities of 3 x 10³ trypanosomes/ml. To measure the incorporation of isotope into tri- chloroacetic acid-precipitable material, duplicate 100 µl aliquots of trypanosome suspensions were rapidly mixed with 1.5 ml of ice-cold 5% w/v trichloroacetic acid containing 2 µCi methionine (for "$^{[3]}$S"methionine experiments) or 2 µCi glucosamine (for "$^{[3]}$H"glucosamine experiments). 10 µl of 5% w/v BSA was simultaneously added to act as protein carrier. After 16 h at 4°C the precipitate was recovered by filtration with Whatman GF/F glass-fiber discs. The precipitates were washed with 15 ml of the respective 5% w/v trichloroacetic acid solution and finally with 5 ml of ice-cold 1% w/v acetic acid. The discs were placed in scintillant and counted.

**Preparation of Antibodies**—Anti-sera to purified sVSGs were raised in New Zealand White rabbits as previously described (Cross, 1979). Sepharose 4B-sVSG affinity supports were prepared by coupling purified sVSG (5 mg/ml gel) to CNBr-activated Sepharose 4B (Phar- macia) according to the manufacturer’s instructions. Anti-sVSG sera were applied to 5-ml columns of Sepharose-sVSG and eluted with phosphate-buffered saline until the eluate had an A_{280} < 0.005. Bovine antibody was subsequently eluted with 1 M propionic acid and collected in tubes containing excess solid NaHCO₃ to neutralize the acid. The pooled antibody fractions were dialyzed extensively against phosphate-buffered saline containing 0.02% (w/v) sodium azide and stored at 4°C with a drop of toluene. Anti-sVSG 221 antibody was purified from anti-sVSG 221 serum on Sepharose-sVSG 221; anti-sVSG 117 antibody was purified from anti-sVSG 117 serum on Sepharose-sVSG 117; and anti-CRD antibody was purified from anti-sVSG 055 serum on Sepharose-sVSG 121.

**Quantitative Immune Precipitation of VSG**—In all the experiments described immune precipitations were performed under equivalent to 1.5 x 10³ trypanosomes. For each antibody preparation experiments were conducted that established the maximum precipitation of VSG. Antibody was always used at >4 times the concentration required for maximum precipitation. S. aureus immune absorbent was always in excess of the antibody. The buffers used were as follows: trypanosome lysate buffer, 3% v/v Nonidet P-40, 200 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM N\textsuperscript{6}p-tosyl-L-lysine chloromethyl ketone, and 1 µl/m1 apronin (Sigma); wash buffer A, 0.5% v/v Nonidet P-40, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.5 M NaCl, and 0.1% w/v BSA; wash buffer B, 0.3% v/v Nonidet P-40, 50 mM Tris-HCl, pH 8.0, and 5 mM EDTA. Trypanosome pellets were dissolved in trypanosome lysate buffer (10³ cells/ml), and 15-µl aliquots were made up to 0.5 ml of 0.5% v/v Nonidet P-40, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.2 M NaCl, and 0.05% w/v BSA. Excess antibody was added, and after 30 min 30 µl of 10% v/v fixed S. aureus suspension was added. After a further 30 min the immune complexes were recovered by centrifugation (50 g in an Eppendorf Microfuge). The S. aureus pellet was washed twice in 1 ml of wash buffer A, once in wash buffer B, and finally dissolved in 30 µl of boiling SDS-sample buffer containing 0.1 M dithiothreitol. The immune precipitates were analysed by SDS-PAGE using 10% acrylamide gels. The gels were soaked in ENHANCE (New England Nuclear), dried, and exposed to Kodak XAR film at -70°C. To quantitate the radioactivity in VSG, bands were excised and digested in 0.5 ml of 80% v/v Protocol (New England Nuclear) for 5 h at 55°C. After the addition of scintillant the samples were left for 16 h in the dark before counting. For the dual label experiment, the digested gels were counted in a Packard 4530 counter programmed with dual quench-compensating efficiency curves.

**RESULTS**

The Effect of Tunicamycin on Protein Synthesis and Gly- cosylation—In the preliminary experiments, trypanosomes were preincubated for 30 min in the presence of various concentrations of the antibiotic tunicamycin (Tu) (0.04, 0.08, 0.16, 0.8, and 1.6 µg/ml) prior to addition of "$^{[3]}$S"methionine (10 µCi/ml) or "$^{[3]}$H"glucosamine (2.5 µCi/ml). In these experiments, glucor (3 g/liter) was substituted for glucose in the medium. Following addition of isotope, duplicate samples were taken for trichloroacetic acid precipitation at 10-min intervals up to 1 h. In all cases the incorporation of label into trichloroacetic acid-precipitable material was essentially linear (data not shown). For T. brucei variants 117 and 221, maximum inhibition of "$^{[3]}$H"glucosamine incorporation was observed at an approximate concentration of 0.16 µg/ml or higher. In all subsequent experiments Tu was used at 0.8 µg/ml. Under these conditions the inhibition of "$^{[3]}$S"methionine and "$^{[3]}$H"glucosamine incorporation, respectively, was 10 and 75% for variant 117 and 15 and 82% for variant 221.
Inhibition of VSG N-Glycosylation by Tunicamycin—Trypanosome suspensions were preincubated in the presence or absence of Tu, pulse labeled for 2 min with [35S]methionine, and chased with unlabeled L-methionine for 1–40 min (Fig. 1). In the absence of Tu, a single labeled VSG band was seen co-migrating with an authentic purified sVSG 117 standard of apparent Mr of 57,000. In the presence of Tu this single band migrated with an apparent Mr of 57,000. The result is consistent with the complete Tu inhibition of VSG 117 N-glycosylation, known to occur at asparagine residue 420 (Allen et al., 1982) and to result in the expression of a Man-GlcNAc2 (Mr 1,590) glycan in mature sVSG 117 (Ferguson et al., 1985b).

Rapid Appearance of CRD Is Not Inhibited by Tunicamycin—The CRD resides in the oligosaccharide portion of the glycolipid attached to the VSG COOH-terminal carboxyl group. The CRD is cryptic in mfVSG, and the rapid quantitative enzyme-mediated conversion of mfVSG to sVSG, which occurs during neutral detergent lysis of trypanosomes (Cordosa de Almeida and Turner, 1983; Ferguson and Cross, 1984), allows anti-CRD antibody to precipitate all VSG species that have undergone COOH-terminal processing. Therefore, the kinetics of VSG COOH-terminal processing were investigated by immune precipitation of aliquots of pulse-chased trypanosome lysates, from the experiment illustrated in Fig. 1, with anti-117 and anti-CRD antibodies (Fig. 2). In the absence of Tu (Fig. 2a), labeled VSG was precipitated by both anti-117 and anti-CRD at all time points. When VSG bands were excised for counting, less radioactivity was present in the anti-CRD precipitated material (ranging from 78 to 81% of the corresponding anti-117 precipitates). We attributed this constant difference, irrespective of chase time, to the relatively low affinity of the anti-CRD antibody rather than the presence of VSG species lacking the CRD epitope. In control experiments on the 5-min chase sample we found that about 10% of the VSG label was lost for each washing cycle of the immune precipitates in wash buffer A. Furthermore, sequential precipitation of this sample with anti-CRD (twice) followed by anti-117 resulted in the recovery of 79, 6, and <2%, respectively, of the total VSG label (Fig. 2b). The results suggest that addition of the CRD epitope occurs within 1 min after completion of the VSG polypeptide. Apart from the expected reduction in VSG molecular weight, Tu had no apparent effect on the rapid addition of CRD. Fig. 2c shows a representative immune precipitation on the 1-min chase sample showing the precipitation of VSG by anti-CRD.

All of the results obtained for VSG 117 were also observed for VSG 221 using a slightly modified approach. Trypanosomes (variant 221) were preincubated with or without Tu, labeled with [35S]methionine but not chased. The results are shown in Fig. 3. The difference in apparent Mr of VSG synthesized in the presence of Tu (53,000 instead of 57,000) is consistent with the absence of both N-linked glycans found in mature sVSG 221 (Holder and Cross, 1981). As observed for VSG 117, a constant proportion of total VSG 221 label was precipitated by anti-CRD at all time points. Addition of CRD was apparent within 1 min.

Rapid Addition of Myristic Acid to VSG—To establish the temporal relationship between VSG polypeptide synthesis and the addition of myristic acid, dual-labeling studies were performed with [35S]methionine and [3H]myristic acid. The incorporation of both isotopes was essentially linear in the absence of cycloheximide. Following addition of cycloheximide, the incorporation of both isotopes stopped almost im-
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VSG, (57K)

**FIG. 3.** Immune precipitation of VSG 221 following incorporation of [35S]methionine in the presence or absence of Tu. Incorporation was terminated after 30 s, 1, 2, and 4 min (lanes 1–4, respectively) by rapid cooling on methanol-dry ice, and centrifugation was carried out for 20 s in an Eppendorf Microfuge followed by addition of trypanosome lysis buffer. Aliquots of the lysates were immune precipitated with anti-221 and anti-CRD.

mediately (Fig. 4), but trypanosomes remained motile in 60 μg/ml cycloheximide for at least 45 min. The data suggest that addition of myristic acid to VSG is tightly coupled to VSG polypeptide synthesis. There is no discernible lag between the shutdown of [35S]methionine and [3H]myristic acid incorporation following cycloheximide addition.

**Release of VSG from Trypanosomes by Osmotic Shock**—Lysis of trypanosomes by osmotic shock in a hypotonic buffer at 37°C results in the rapid release of sVSG (Cross, 1975, 1984b). A small proportion (5–10%) of the VSG remains associated with the trypanosome ghosts as unreleasable mfVSG (Ferguson and Cross, 1984). This residual mfVSG is presumed to be physically segregated from the probably membrane-bound phospholipase C that is responsible for mfVSG to sVSG conversion, since addition of detergent to the trypanosome lysates leads to conversion of the residual mfVSG (Ferguson and Cross, 1984). To investigate the relationship of this unreleasable mfVSG to newly synthesized and processed VSG, pulse-chased cells (variant 117) were subjected to osmotic shock. The results are shown in Fig. 5. Both in the presence and absence of Tu, there was a clear time dependence of the ability to solubilize VSG by osmotic shock. Within 10 min of chase, most of the pulse-labeled VSG remained with the ghosts whereas by 40 min most of the

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**Fig. 4.** Effect of cycloheximide on VSG synthesis and incorporation of myristic acid. A trypanosome suspension (variant 117) was preincubated for 20 min at 37°C. [35S]Methionine (10 μCi/ml) and [3H]myristic acid/BSA complex (150 μCi/ml) were simultaneously added. The suspension was split into two flasks and incubated at 37°C for 30 min. To one flask cycloheximide (60 μg/ml) was added at 12 min. Samples of 400 μl were withdrawn at different times, rapidly mixed with 800 μl of ice-cold phosphate-buffered saline, centrifuged (20 s in an Eppendorf Microfuge), aspirated, and the trypanosome pellets dissolved in 120 μl of boiling SDS-sample buffer. The samples were heated at 95°C for 5 min and made up to 200 μl (by weighing). Aliquots equivalent to 2.5 × 10^6 trypanosomes were analyzed by SDS-PAGE/fluorography. The labeled VSG bands were excised, digested with Protosol, and taken for dual-label counting. The broken lines represent the incorporation of [3H]myristic acid (C) and [35S]methionine (M) into VSG bands before and after cycloheximide addition.

**SUPERNATANTS** | **PELLETS**
---|---
-Tu | 1 2 3 4 5 | 1 2 3 4 5

**Fig. 5.** Kinetics of pulse-labeled VSG 117 release by osmotic shock in the presence and absence of Tu. A trypanosome suspension was pulse labeled with [35S]methionine and chased as described in the legend to Fig. 1. After 5, 10, 20, 40, and 60 min of chase (lanes 1–5, respectively), 0.5-ml samples were withdrawn, cooled, centrifuged, and osmotically lysed in 200 μl of prewarmed 1 mM phenylmethylsulfonyl fluoride, 0.1 mM N-p-tosyl-L-lysine chloromethyl ketone, 1 μl/ml aprotinin, and incubated at 37°C for 5 min. The samples were cooled and centrifuged for 1 h at 50,000 × g and 4°C. The supernatants and pellets (equivalent to 1.5 × 10^6 cells) were analyzed by SDS-PAGE/fluorography. The VSG bands were cut out, digested with Protosol, and counted. An identical experiment was performed in the presence of Tu (0.8 μg/μl).
pulse-labeled VSG was recovered in the supernatant fraction. The kinetics of VSG release were almost identical both in the presence and absence of Tu. In both cases the \( T_u \) for VSG to be releasable by osmotic shock was about 15 min. In a similar experiment with variant 118 (Fig. 6), a similar time dependence of the ability to solubilize VSG 118 by osmotic shock was observed in the absence of Tu. However, in the presence of Tu, VSG solubilization was significantly delayed, with less than half the pulse-labeled VSG being solubilized after a 60-min chase.

Variability in the Kinetics and Extent of N-Glycosylation of VSG—In most experiments, addition of the Tu-sensitive N-linked oligosaccharides occurred during or immediately after polypeptide synthesis (see Figs. 1 and 3 for examples). However, in some experiments we observed either delayed or incomplete N-glycosylation. The data shown in Fig. 7 illustrate this point. In the first experiment (Fig. 7a), trypanosomes (variant 117) were pulse labeled with \([35S]\)methionine for 2 min and chased with L-methionine in the presence and absence of Tu. At short chase times (1, 5, and 10 min, lanes 1, 2, and 3), in addition to the \( M, 59,000 \) VSG band, a band of \( M, 57,000 \) was seen in the pellet fractions. This lower band has the same mobility as the single VSG synthesized in the presence of Tu. Our interpretation of this result is that the \( M, 57,000 \) band observed in the absence of Tu represents VSG prior to N-glycosylation. In this case N-glycosylation appears to be virtually complete after 15 min of chase.

In a second experiment, the \( M, 57,000 \) band persisted throughout the chase period (up to 60 min). The first of these experiments is shown in Fig. 7b. The faster migrating (\( M, 57,000 \)) VSG band remained essentially unaltered in intensity throughout the chase period. No correspondingly lower \( M, \) band was seen in the Tu-treated samples, suggesting that the VSG heterogeneity observed in the absence of Tu represents incomplete N-glycosylation of the VSG. In the second experiment (Fig. 7c), trypanosomes were pulse labeled for 4 min with \([35S]\)methionine and chased as previously described. After 1, 5, 10, 15, and 60 min of chase \( (lanes 1–5, \) respectively), trypanosomes were recovered and osmotically lysed as described (Fig. 5). The pelleted ghosts were dissolved in trypanosome lysis buffer. The supernatant fractions were immune precipitated with anti-117 antibody. b, trypanosomes were pulse labeled with \([35S]\)methionine for 5 min and chased as previously described in the presence or absence of Tu. After 10, 20, 40, and 60 min of chase, trypanosomes were recovered and aliquots of the lysates precipitated with anti-117 antibody. c, trypanosomes were pulse labeled with \([35S]\)methionine for 4 min and chased as previously described. After 1, 5, 10, 15, 20, and 40 min of chase \( (lanes 1–5) \), trypanosomes were recovered and the lysates precipitated directly with anti-117 or sequentially precipitated with anti-CRD once \( (CRD_1) \), twice \( (CRD_2) \), and finally by anti-117 \( (117b) \).

The reasons for the observed differences in N-glycosylation are unclear. Certain batches of media seemed to predispose the trypanosomes to partial N-glycosylation. However, the main factor may be the metabolic state of the trypanosomes following their purification. These results represent the exception rather than the rule, for the kinetics of VSG N-glycosylation. They are presented to emphasize the need to perform replicate experiments to assess the most likely in vivo processing kinetics and should be borne in mind when
Tunicamycin is a potent and specific inhibitor of protein N-glycosylation, acting at the first step in the biosynthesis of the dolichyl pyrophosphate-oligosaccharide precursor (Schwarz and Datema, 1982). The pathway for biosynthesis of these lipid-linked oligosaccharides and their transfer to protein appears to be ubiquitous in eukaryotes (Hubbard and Patton, 1982) and compelling evidence for the operation of a dolichol pathway of these lipid-linked oligosaccharides and their transfer to and Trypanosoma cruzi (Parodi et al., 1981). The location of glycans at Asn-X-Ser(Thr) sequences (Holder and Cross, 1984) provide compelling evidence for the operation of a dolichol pathway of N-glycosylation in T. brucei, and dolichol oligosaccharides have been directly characterized in T. cruzi (Parodi and Quesada-Allue, 1982; Parodi and Cazzulo, 1982) and C. fasciculata (Parodi et al., 1981; Quesada-Allue and Parodi, 1983). The reduction in VSG apparent M, when synthesized in the presence of Tu can, therefore, be confidently assigned to the inhibition of N-glycosylation. The pulse-chase studies described here show that the kinetics of N-glycosylation in VSG can vary in isolated trypanosomes.

The reasons for this variability are unclear, but the most frequent results from many such experiments (Ferguson, 1982) are similar to those shown in Figs. 1 and 3, implying complete co-translational or immediately post-translational N-glycosylation. Given the numerous precedents for co-translational N-glycosylation (Lennarz, 1983), we suggest that this is the normal in vivo situation for VSG processing. In all cases, any VSG heterogeneity could be attributed to partial N-glycosylation. VSG synthesized in the presence of Tu produced a single VSG species, as judged by SDS-PAGE, even at very early (1 min) chase times, or after as little as 30 s of continuous [35S]methionine incorporation. All of the pulse-labeled VSG species appeared to quantitatively possess the CRD. These results support our interpretation of the nature of the VSG bands observed on SDS-PAGE and suggest that the COOH-terminal hydrophobic peptide tails of VSG precursors are replaced rapidly by a CRD-containing moiety. The precursor VSG with the peptide tail would appear to be such a transient species as to be undetectable by these methods.

The ability to release pulse-labeled VSG in a soluble form (sVSG) by osmotic shock showed a clear time dependence. The kinetics of this event did not correlate with either N-glycosylation (VSG 117 and 118) or CRD addition (VSG 117). VSG that remains associated with the trypanosome-ghost pellet after osmotic shock is not simply entrapped sVSG but represents intact mVSG with attached myristic acid (Ferguson and Cross, 1984). It is known from cell-surface labeling experiments that essentially all of the VSG present on the plasma membrane is released as sVSG by osmotic shock (Cross, 1984b), suggesting that the ability of osmotic shock to release pulse-labeled VSG reflects some physical translocation step in the transport pathway of newly synthesized VSG from the rough endoplasmic reticulum to the plasma membrane. In support of this view, the kinetics of VSG release by osmotic shock shown in Fig. 5 are similar to those described for the transport of VSG from the rough endoplasmic reticulum to the plasma membrane (McConnell et al., 1983; Strickler and Patton, 1982). These results are consistent with a plasma membrane location for the mVSG to sVSG-converting phospholipase C. Such a location has also been inferred from subcellular fractionation studies (Turner, 1984). It is interesting that inhibition of N-glycosylation by Tu did not affect translocation of VSG 117 (Fig. 5) but significantly retarded VSG 118 translocation (Fig. 6), increasing the T1/2 of osmotic shock solubilization from about 15 min to >60 min. These two VSGs belong to different subgroups. VSG 117 expresses only one high-mannose N-linked glycan (Holder and Cross, 1981) whereas VSG 118, which is the most highly glycosylated VSG so far analyzed (Johnson and Cross, 1977), expresses at least three galactose-containing N-linked glycans (Holder, 1985). These differences in VSG structure and carbohydrate processing might reflect differences in their intracellular sorting that might be reflected in the different effects of Tu on their translocation to the plasma membrane.

One possible criticism of the [35S]methionine pulse-chase studies that show rapid CRD addition to VSG is that this epitope is only detectable following the artificial cleavage of diglyceride from mVSG by the trypanosome phospholipase C when trypanosomes are solubilized by detergent (Cardoso de Almeida et al., 1984; Ferguson and Cross, 1984). It was conceivable that, in addition to activating the phospholipase C, detergent solubilization might also lead to artificial processing of the VSG COOH terminus (peptide tail removal and CRD addition). This possibility was ruled out by Bangs et al. (1985), who showed that VSG synthesized in vitro did not undergo any processing when mixed with trypanosomes during lysis.

In a dual labeling experiment, cycloheximide inhibited both [35S]methionine and [3H]myristic acid incorporation into VSG. If myristic acid addition to VSG occurred several minutes after polypeptide synthesis, at some distal site, [3H]myristic acid incorporation would be expected to continue for this amount of time after the inhibition of protein synthesis, as observed for the O-glycosylation of mammalian glycoproteins (White and Speake, 1980), which is known to occur in the Golgi apparatus (Lennarz, 1983). The simultaneous inhibition of protein synthesis and VSG acylation suggests that myristic acid is added to VSG immediately following protein synthesis.

In summary, it appears from the biosynthetic labeling studies that both the COOH-terminal oligosaccharide (or at least that portion which constitutes the CRD) and myristic acid are added to the VSG polypeptide within 1 min after completion of the protein, in good agreement with a recent report by Bangs et al. (1985). Given the partial structure of the mVSG glycolipid (Ferguson et al., 1985b) and the indistinguishably rapid appearance of the oligosaccharide and lipid portions, it seems likely that the mVSG glycolipid is synthesized as a discrete precursor that is transferred en bloc to the VSG polypeptide. One possible mechanism would involve the direct and concerted replacement of the predicted COOH-terminal hydrophobic peptide tail by the prefabricated glycolipid via a putative membrane-bound transferase enzyme or multienzyme complex. This hypothetical model would allow for efficient VSG COOH-terminal processing as the VSG would remain bound to the endoplasmic reticulum membrane at all times, first by the hydrophobic peptide tail, second as a transferase intermediate, and last via the attached glycolipid. Because of the similar energies of the peptide and ethanolamine-amide bonds, activation of one of the substrates might be required for the transferase reaction.

The variability in galactose content in some sVSG COOH-terminal oligosaccharides (Holder, 1985) could reflect differential processing of the glycolipid either before or after its transfer to VSG. Whatever the sequence of processing events, all COOH-terminal oligosaccharide-containing structures so
The glycolipid from corn consisting of an inositol phosphothosphonate (Ferguson et al., 1982). The carbohydrate composition of this glycoconjugate appears to vary between myristyl phosphatidylinositol.

The biosynthesis of the putative glycolipid precursor in VSG cardiac glycosyl phosphoinositides. These include a series of phosphatidylinositols, mannosylated at the myo-inositol 2 and 6 positions, from Mycobacteria (Lee and Ballou, 1965), a phytoglycolipid from corn consisting of an inositol phosphothosphonate (Ferguson et al., 1982), and a series of related structures from tobacco leaf and fungi (Lester et al., 1978).

In the case of the tobacco leaf, the myo-inositol is substituted with varying amounts of arabinose, galactose, and mannose. Interestingly, in about half of these structures the glucuronic acid residue is not N-acetylated. In the protozoan Acanthamoeba castellani, a lipophosphoglycan (an inositol-,aminophosphonate-, and carbohydrate-containing ceramide) has been described (Dearborn et al., 1976), and the lipopeptidophosphoglycan of Trypanosoma cruzi appears to be an inositol phosphoceramide substituted with mannose, galactose, glucosamine, and mannose. In the tobacco leaf, the myo-inositol is substituted with glucuronic acid, 1,4-glucuronic acid together with varying amounts of arabinose, galactose, and mannose. Interestingly, in about half of these structures the glucuronic acid residue is not N-acetylated. In the protozoan Acanthamoeba castellani, a lipophosphoglycan (an inositol-, aminophosphonate-, and carbohydrate-containing ceramide) has been described (Dearborn et al., 1976), and the lipopeptidophosphoglycan of Trypanosoma cruzi appears to be an inositol phosphoceramide substituted with mannose, galactose, glucosamine, and mannose (De Lederkremer et al., 1978), and small amounts of 2-aminoethylphosphonate (Ferguson et al., 1982).

The carbohydrate composition of this glycoconjugate appears to vary between different Trypanosoma cruzi populations (Ferguson et al., 1985c). A complex phospholipase-sensitive glycoconjugate has been reported in Leishmania major (Handman and Goding, 1985).

The biosynthesis of the putative glycolipid precursor in VSG processing could involve the sequential glycosylation of sn-1,2-dimyristyl-phosphatidylinositol or the transfer of a preassembled oligosaccharide (from another carrier) to sn-1,2-dimyristyl-phosphatidylinositol.

Given the rapid kinetics of processing, the enzymes involved would presumably be pre-Golgi and close to the site of protein synthesis and sequestration, in the rough or smooth endoplasmic reticulum. In preliminary experiments, monosialic (Tartakoff, 1985; Griffiths et al., 1983; Machamer and Cresswell, 1984) did not inhibit trypomastigote motility. VSG 117 processing, or transport (as judged by the ability of osmotic shock to release pulse-labeled VSG as sVSG) over a 60-min period, despite severe disruption of Golgi morphology. Interestingly, in some cells, parts of the Golgi stacks appeared unaffected, even after 60 min in 2 μM monosialic at 37 °C. The intracellular route of VSG from the rough endoplasmic reticulum to the plasma membrane is currently under investigation to determine whether the glycolipid membrane anchor causes VSG to be routed in a different way to proteins anchored by a conventional hydrophobic peptide membrane-spanning domain (Sabatini et al., 1982).