

Intracellular Transport of A Variant Surface Glycoprotein in *Trypanosoma brucei*

Michael Duszenko,* Ivan Emanuilov Ivanov,† Michael A. J. Ferguson,* H. Plesken,‡
and George A. M. Cross*

*The Rockefeller University, New York, New York 10021; and Department of Cell Biology,
New York University Medical Center, New York, New York 10016.

Abstract. Trypanosome variant surface glycoproteins (VSGs) have a novel glycan-phosphatidylinositol membrane anchor, which is cleavable by a phosphatidylinositol-specific phospholipase C. A similar structure serves to anchor some membrane proteins in mammalian cells. Using kinetic and ultrastructural approaches, we have addressed the question of whether this structure directs the protein to the cell surface by a different pathway from the classical one described in other cell types for plasma membrane and secreted glycoproteins. By immunogold labeling on thin cryosections we were able to show that, intracellularly, VSG is associated with the rough endoplasmic reticulum, all Golgi cisternae, and tubulovesicular elements and flattened cisternae, which form a network in the area adjacent to the *trans* side of the Golgi apparatus.

Our data suggest that, although the glycan-phosphatidylinositol anchor is added in the endoplasmic reticulum, VSG is nevertheless subsequently transported along the classical intracellular route for glycoproteins, and is delivered to the flagellar pocket, where it is integrated into the surface coat. Treatment of trypanosomes with 1 μ M monensin had no effect on VSG transport, although dilation of the *trans*-Golgi stacks and lysosomes occurred immediately. Incubation of trypanosomes at 20°C, a treatment that arrests intracellular transport from the *trans*-Golgi region to the cell surface in mammalian cells, caused the accumulation of VSG molecules in structures of the *trans*-Golgi network, and retarded the incorporation of newly synthesized VSG into the surface coat.

MOST cell-surface membrane proteins are anchored via a hydrophobic peptide spanning the lipid bilayer (41). Recently, for some otherwise unrelated membrane proteins, a new type of membrane anchor, glycan-phosphatidylinositol, has been described (reviewed in 10, 26). Structurally, the variant surface glycoproteins (VSGs)¹ of *Trypanosoma brucei* are the best characterized examples of this new class of membrane proteins. *T. brucei* is covered by a densely packed surface coat consisting of $\sim 10^7$ essentially identical glycoprotein molecules, accounting for $\sim 10\%$ of total cellular protein synthesis (8, 9, 38). Each trypanosome contains the genetic information to sequentially express hundreds of VSGs, enabling the parasite population to evade the host's immune response. The physiological role of this novel membrane anchor is unknown, but trypanosome VSGs are a major substrate for an endogenous glycan-

phosphatidylinositol-specific phospholipase C (GPI-PLC; 6, 16, 21). An enzyme of similar specificity has recently been purified from rat liver membranes (17). Specific endogenous phospholipases could release such membrane bound proteins in a soluble form either continuously, or in response to certain stimuli, or at certain stages during the cell cycle.

Biosynthesis and intracellular transport of cell membrane and secretory proteins have been extensively investigated, and a common scheme has been elaborated, which is widely accepted (11, 33, 40). Usually, an amino-terminal leader sequence (signal peptide) targets the nascent polypeptide to the outer surface of the endoplasmic reticulum (ER) where it is translocated through the ER membrane. Signal peptide cleavage and the initial transfer of core oligosaccharides to asparagine residues occur cotranslationally. After trimming of peripheral glucose and mannose residues, which is effected by ER and Golgi enzymes, further processing steps, including incorporation of galactose, sialic acid, and fucose, O-glycosylation, sulfation or phosphorylation, occur within the Golgi apparatus (12). From here, glycoproteins are directed by vesicular transport to their destined location inside or outside the cell.

Like membrane proteins with a single transmembrane disposition, VSGs are synthesized with a transient amino-

Michael Duszenko's current address is Physiologisch Chemisches Institut der Universität, Tübingen, D-7400 Tübingen, Federal Republic of Germany. Michael A. J. Ferguson's current address is Department of Biochemistry, Oxford University, Oxford OX1 3QU, United Kingdom.

1. *Abbreviations used in this paper:* ER, endoplasmic reticulum; GPI-PLC, glycan-phosphatidylinositol phospholipase C; VSG, variant surface glycoprotein.

terminal signal peptide (5). The completed polypeptide is presumed to be transiently anchored in the ER membrane by the hydrophobic carboxy-terminal amino acid sequence, which is rapidly replaced by a glycopospholipid with a core structure containing ethanolamine, mannose, glucosamine and sn-1,2-dimyristyl phosphatidyl-inositol (13-15). This replacement reaction is insensitive to tunicamycin, whereas asparagine glycosylation, which occurs in different VSGs at one or more positions within the molecule, is completely inhibited. Tunicamycin has no effect on the kinetics of VSG transport to the cell surface, which has a $t_{1/2}$ of ~ 15 min, suggesting that *N*-glycosylation does not serve as a sorting signal (3, 15, 36).

The early posttranslational glypiation (10) and the fact that trypanosome N-linked oligosaccharides are generally of a high mannose type, lacking the terminal sugars normally added in the Golgi apparatus (22, 28, 30, 31), raised the possibility that VSGs might be delivered to the cell surface by a novel route bypassing this organelle. In preliminary experiments (15) we found that monensin, an ionophore for monovalent cations that in many cases inhibited transport of glycoproteins from the Golgi apparatus to the cell surface (reviewed in 11), had no effect on VSG transport. The same result was reported elsewhere (4). One earlier report (18) suggested that VSG not only passed through the Golgi apparatus, but that the carboxy-terminal peptide tail was cleaved in this organelle, rather than in the ER. This conclusion is clearly at variance with more recent reports (3, 4, 15).

We have investigated the intracellular transport of VSG in pulse-chase experiments and by immunogold labeling of thin cryosections. For these studies we used *T. brucei* variant clone 117a. The mature 117a VSG contains seven methionine residues and one N-linked glycan that lacks galactose and is linked to an asparagine residue at position 420 in the 470-residue mature VSG polypeptide (1).

Materials and Methods

Reagents

[³⁵S]Methionine (1,050 Ci/mmol) was purchased from Amersham Corp., (Arlington Heights, IL). Monensin was purchased from Sigma Chemical Co. (St. Louis, MO). Polyclonal antibodies were isolated from rabbit antisera, raised against purified VSG, by affinity chromatography (15).

Preparation of Trypanosomes

T. brucei strain 427, Molteno Institute (Cambridge, U.K.) antigenic type MITat 1.4 (clone 117a), was used for all the experiments. Trypanosomes were grown in rats and isolated as described (8).

[³⁵S]Methionine Labeling of *T. brucei*

Isolated trypanosomes were washed twice in MEM containing additional glucose (33 mM final concentration), Hepes (7.14 g/l), adenosine (12 mg/l), and BSA (1 g/l), but lacking sodium bicarbonate and methionine. Trypanosomes were diluted to a cell density of 3×10^7 /ml in the same medium and preincubated for 10 min in a shaking water bath at 37°C or 20°C as required before [³⁵S]methionine was added. For monensin treatment, cells were preincubated for 10 min at 37°C before monensin (1 μ M, final concentration) was added from a 1,000-fold concentrated stock solution in ethanol. Ethanol alone had no effect on trypanosomes at the concentration used, as judged by motility, [³⁵S]methionine incorporation, and ultrastructural observations. After an additional 10 min incubation, [³⁵S]methionine was added. For all continuous labeling experiments, 50- μ l samples were withdrawn at intervals and precipitated immediately in 1 ml 5% (wt/vol) ice-cold TCA. Precipita-

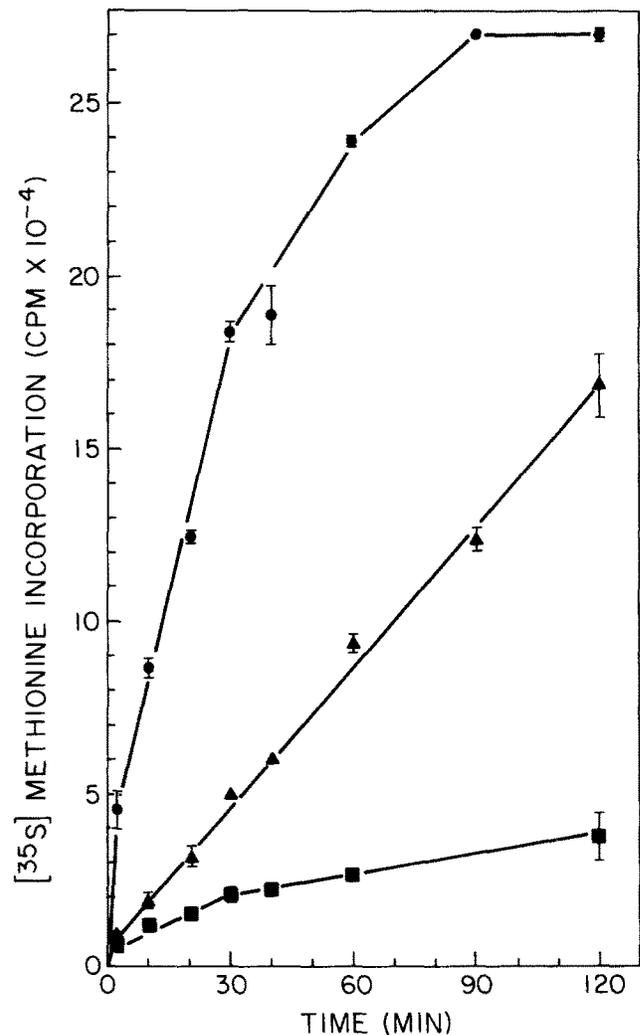


Figure 1. Continuous incorporation of [³⁵S]methionine into total TCA-precipitable protein. Trypanosomes (3×10^7 cells/ml) were incubated with [³⁵S]methionine (10 μ Ci/ml), as described in Materials and Methods. (●) Control cells; (▲) monensin-treated cells; (■) cells incubated at 20°C.

tion was completed overnight in the presence of BSA (50 μ g/ml) as carrier. The samples were filtered (glass microfiber filters GF/C; Whatman Chemical Separation Inc., Clifton, NJ), washed three times with ice-cold TCA (5% wt/vol) and once with ice-cold acetic acid (1% vol/vol), air dried, and counted in a liquid scintillation counter. All samples were taken in duplicate.

For pulse-chase experiments, the procedure was as described above except that [³⁵S]methionine was added for 5 min before being chased by addition of unlabeled methionine to a final concentration of 0.3 mM from a stock solution of 30 mM in MEM. 1-ml samples were withdrawn at intervals, cooled, and centrifuged for 20 s in an Eppendorf microfuge (Brinkmann Instruments, Westbury, NY). Cells were lysed by adding 200 μ l distilled water containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 mM *N*- α -p-tosyl-L-lysine chloromethyl ketone, for 5 min at 20°C, when action of the endogenous GPI-PLC was stopped by addition of 2 μ l 50 mM ZnCl₂ (7). To avoid higher temperatures during handling and centrifugation of the cells, the 20°C water bath and the microfuge were placed in a cold room at 4°C. Samples were analyzed by SDS-PAGE (15).

Electron Microscopy of Epon-embedded Trypanosomes

Trypanosomes were fixed for electron microscopy either directly after isolation from rat blood or at intervals during incubations in vitro. Fixation was performed in 2% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate

buffer, containing 0.12 M sucrose, for 1 h at 4°C. Cells were postfixed in OsO₄ (1.5%, wt/vol) and stained in 0.5% (wt/vol) uranylacetate. Dehydration in ethanol, clearing in propylene oxide and embedding in Epon were performed according to standard procedures. Sections were stained with 5% (wt/vol) uranylacetate and 0.4% (wt/vol) lead citrate.

Immunogold Labeling on Frozen Thin Sections

Untreated and treated trypanosomes (incubated for 60 min at 20°C, or with 1 μM monensin for 60 min at 37°C) were fixed in 2% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer. Cell pellets were infused with 2.3 M sucrose in PBS and cryo-sectioned as described earlier (32, 37). The sections were incubated with whole anti-VSG serum or VSG-affinity purified antibodies diluted 1:10 in PBS to give, in both cases, a final specific antibody concentration of ~50 μg/ml. After washing, the sections were incubated with protein A-10-nm gold particle conjugates. Finally, the sections were embedded in LR White resin (London Resin Co., Ltd., Basingstoke, Hampshire, England) and positively stained according to our modification of the method of Keller et al. (23).

Results

Incorporation of [³⁵S]Methionine into Acid-precipitable Material

To establish appropriate conditions for subsequent experi-

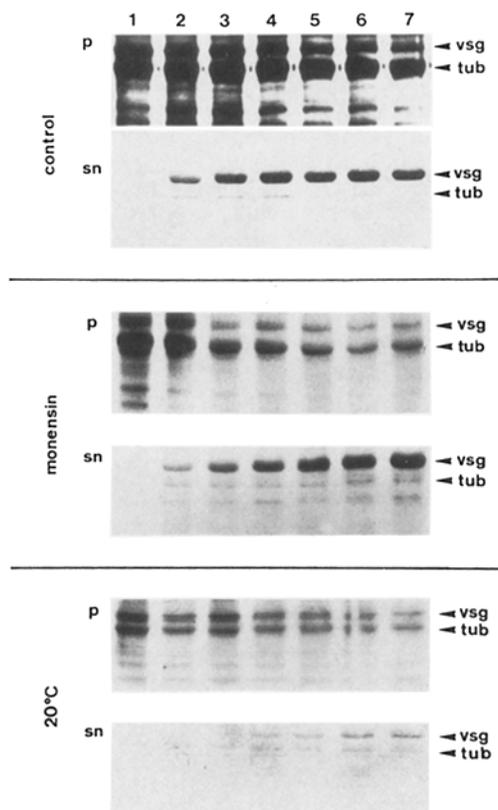


Figure 2. Autoradiographs of proteins from [³⁵S]methionine pulse-labeled cells. Trypanosomes (3×10^7 cells/ml) were incubated with [³⁵S]methionine (17 μCi/ml), as described in Materials and Methods. After SDS-PAGE, the gels were stained in Coomassie Brilliant Blue. Since in all cases an identical cell concentration was used, the protein stains of control, monensin-treated, and 20°C-treated samples were identical (data not shown). Only the relevant part of the autoradiographs is shown; the VSG (*vsg*) and tubulin (*tub*) bands are marked. Lanes 1-7 show the pellet (*p*) and the supernatant (*sn*) fractions of hypotonically lysed cells taken at chase times of 5, 10, 20, 30, 45, 60, and 90 min.

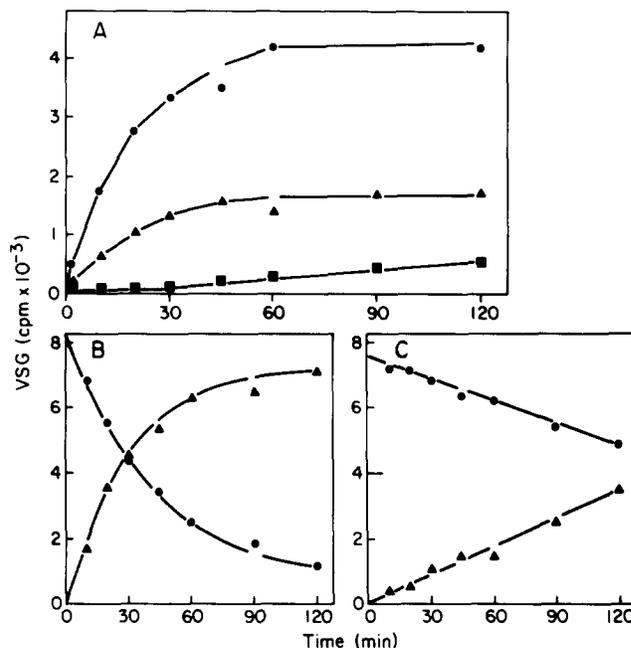


Figure 3. Appearance of newly synthesized VSG in the surface coat. (A) supernatant (surface-released) VSG bands from the SDS-PAGE gels (Fig. 2) were cut out, digested in Protosol and counted in a liquid scintillation counter. (●) Control cells; (▲) monensin-treated cells; (■) cells incubated at 20°C. (B and C) Data from a second experiment where cells were preincubated at 37°C, labeled for 10 min at 37°C, then chased at 37°C (B), or 20°C (C). The VSG bands were cut out from SDS-PAGE as before: (▲) solubilized (surface) VSG and (■) pellet (intracellular) VSG.

ments, total protein synthesis was measured by continuous incorporation of [³⁵S]methionine into TCA-precipitable material. As shown in Fig. 1, protein synthesis decreased significantly when trypanosomes were incubated in the presence of monensin, and dropped to a very low level when cells were incubated at 20°C instead of 37°C. Despite the effect on protein synthesis, cell viability was not significantly affected by monensin nor by incubation at 20°C. Samples checked by light microscopy at regular intervals during the 2-h incubation showed no loss of motility or alteration in shape. Moreover, cell counting by hemocytometer after the 2-h incubation gave live cell values of 3.3×10^7 /ml for the control sample, 3.2×10^7 /ml for the 20°C sample, and 2.7×10^7 for the monensin-treated sample. In the control experiment at 37°C, incorporation of radiolabeled methionine decreased after 40 min and stopped after 90 min, probably due to exhaustion of methionine, initially present at 16 nM.

Kinetics of [³⁵S]Methionine-labeled VSG Secretion

In pulse-chase experiments, we followed the appearance of newly formed VSG on the cell surface, using osmotic shock to activate the endogenous GPI-PLC, which only releases VSG already incorporated into the surface coat (15). Hence, comparison of cleavable and non-cleavable radiolabeled VSG at different chase times indicates the kinetics of intracellular VSG-transport to the cell surface. This interpretation is upheld by the comparable results obtained by using a crosslinking assay to quantitate VSG newly incorporated into the cell surface (4). As judged from protein staining after

SDS-PAGE (data not shown), the bulk of VSG was accessible to the endogenous GPI-PLC. After autoradiography (Fig. 2), the control experiment at 37°C showed the expected increasing intensities of the VSG bands in the supernatant fractions at longer chase times, accompanied by an equivalent decrease in the intensities of the corresponding bands in the pellet fractions. Essentially the same result was obtained in the presence of 1 μ M monensin, although the total incorporation of radiolabeled methionine was less, as expected from the decrease of total protein synthesis. However, when cells were incubated at 20°C, only a very small proportion of VSG was released by endogenous GPI-PLC during osmotic lysis. These observations were quantitated, as shown in Fig. 3 A. Since the appearance of newly formed VSG on

the cell surface showed essentially the same kinetics as in the control experiment, it seemed that monensin, although described as a potential inhibitor of glycoprotein transport from *trans*-Golgi apparatus to the plasma membrane, had no specific effect on intracellular VSG transport in trypanosomes. In contrast, incubation of trypanosomes at 20°C, a temperature that blocks or retards glycoprotein transport from the *trans*-Golgi apparatus to the plasma membrane in other cells (19, 27), showed a different pattern. Virtually no radiolabeled VSG was released during the first 30 min, but, at later time points, a small but steadily increasing amount of labeled VSG reached the cell surface. However, since protein synthesis was drastically inhibited at an incubation temperature of 20°C, the amount of radiolabeled VSG was very

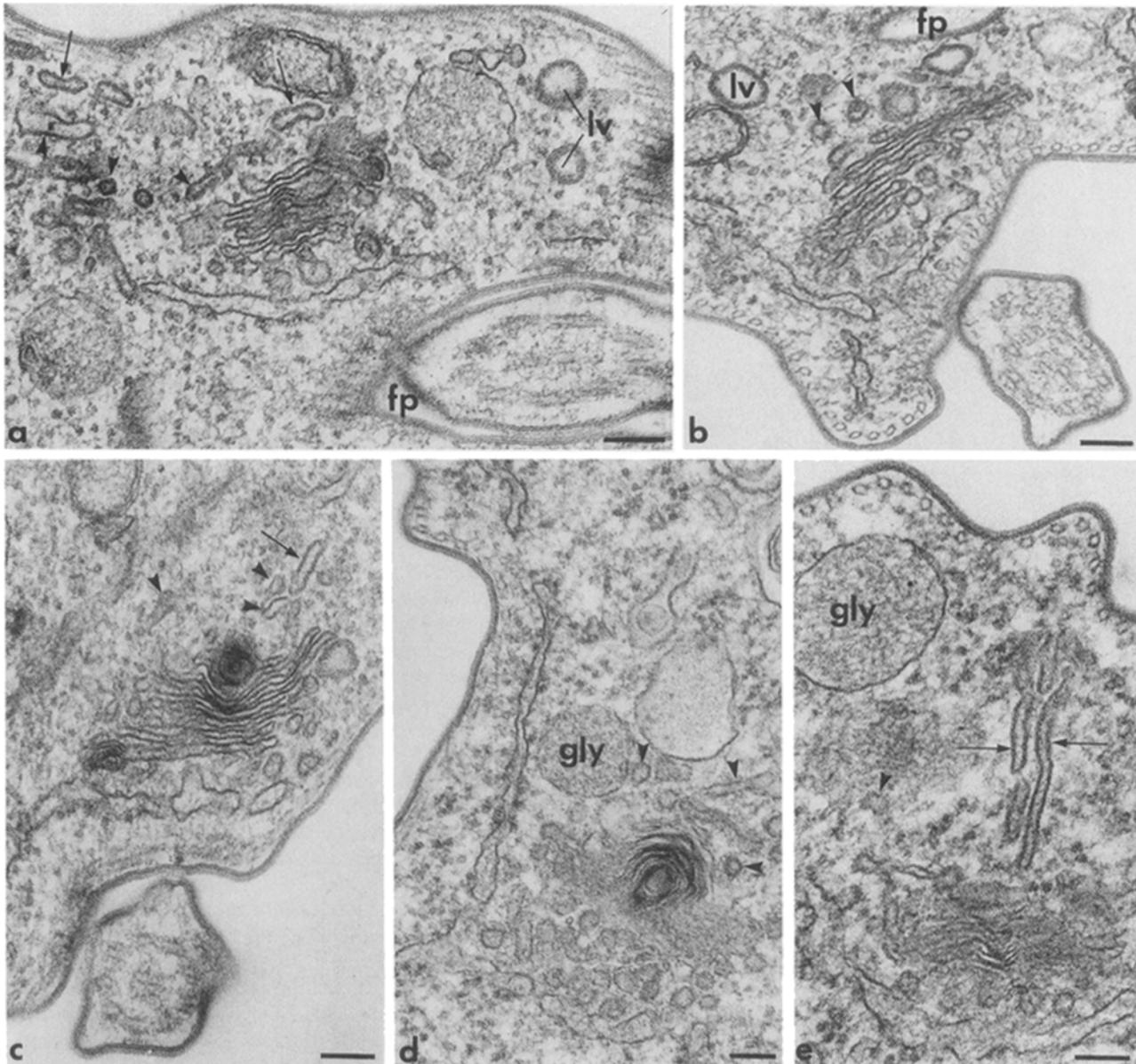


Figure 4. Organization of the Golgi and *trans*-Golgi areas in *T. brucei*. In all figures shown, a transitional cisternae of the ER is clearly visible, which helps to establish the orientation of the *cis* and *trans* faces of the Golgi apparatus. Smooth flattened cisternae (arrows) and tubulovesicular elements (arrowheads) are prominent in the area adjacent to the *trans*-Golgi. Large vesicles (*lv*) that clearly carry a surface coat on the luminal face of their membranes are regularly present near the flagellar pocket (*fp*) (*a* and *b*), but can also be found in the vicinity of the *trans*-Golgi area. Glycosomes (*gly*) can also be found close to the Golgi apparatus (*d* and *e*). Bars, 0.1 μ m.

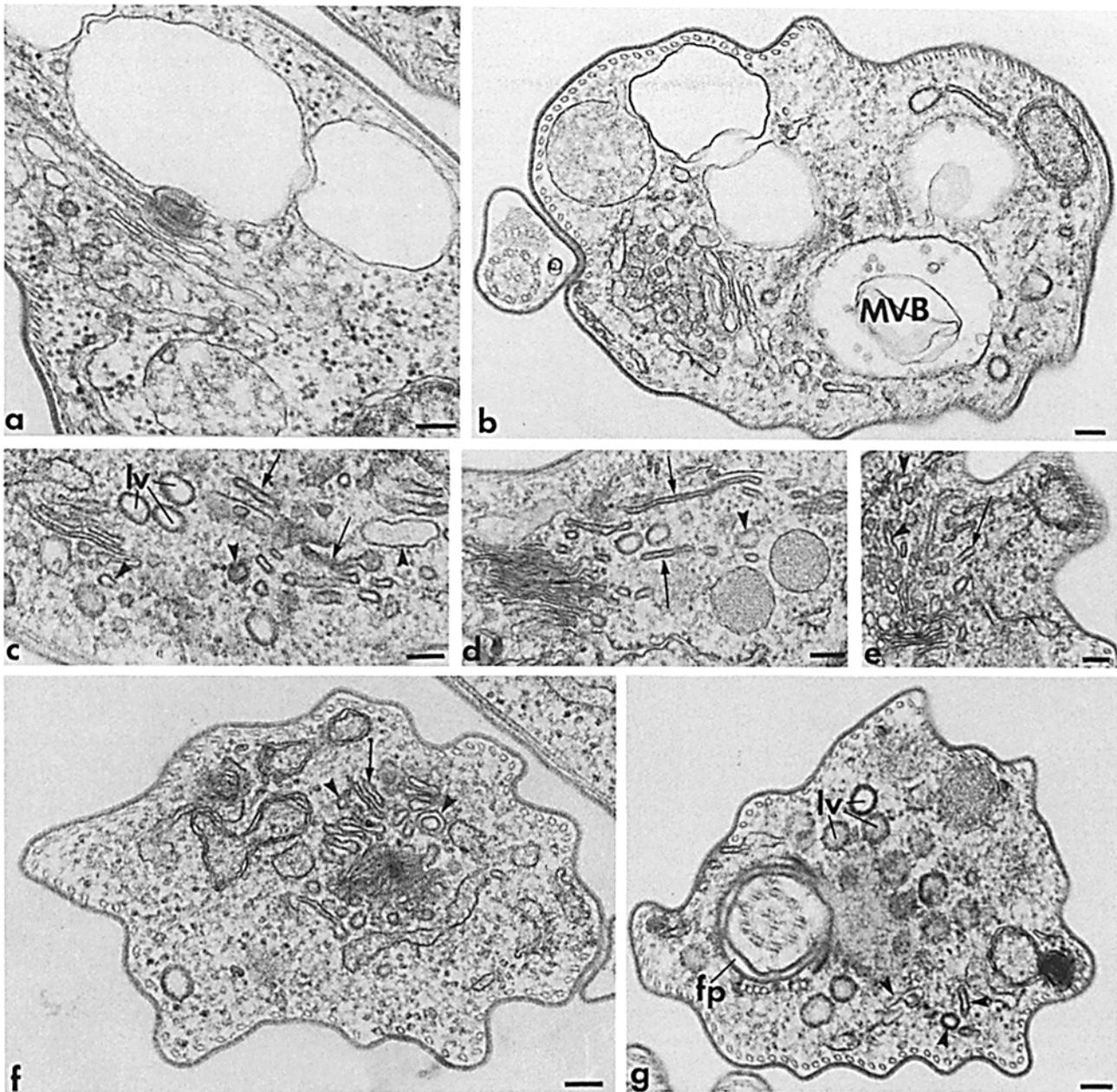


Figure 5. Effect of monensin on the organization of the Golgi apparatus and expansion of the system of cisternae and tubulovesicular elements of the post *trans*-Golgi area in trypanosomes incubated at 20°C. 1 μM monensin for 60 min at 37°C led to dilation of the *trans*-Golgi cisternae, whereas the *cis* Golgi compartment seemed to be unaffected (a). In addition, multivesicular bodies (MVB) were also dilated (b). After incubation at 20°C, trypanosomes showed an increase in the amount of smooth flattened cisternae (arrows) and tubulovesicular elements (arrowheads) present in the area adjacent to the *trans*-Golgi apparatus (c-f). Some cisternae in Golgi stacks were partially misaligned (e-f). A number of large vesicles (lv) carrying a surface coat were also observed in the vicinity of the flagellar pocket (fp) (g). Bars, 0.1 μm.

low, which made it difficult to interpret the data. Therefore the experiment was modified by performing the preincubation and pulse-labeling at 37°C. After addition of cold methionine, the cells were rapidly cooled to and further incubated at 20°C. Again, as compared to the control experiment, the rate of VSG transport to the cell surface was significantly reduced (Fig. 3, B and C). This result is consistent with the assumption that, as in mammalian cells (19, 27, 32, 34), VSG becomes trapped in the *trans*-Golgi apparatus or post *trans*-Golgi compartment at this temperature, retarding its delivery to the cell surface.

Organization of the Golgi Apparatus and the Post *trans*-Golgi Region in *T. brucei* and the Effects of Monensin and Incubation at 20°C

In *T. brucei* the Golgi apparatus is usually located near the flagellar pocket and consists of 4–6 well-aligned stacked cisternae. A transitional ER cisterna, mostly free of ribosomes, is nearly always found near the Golgi apparatus, facilitating the identification of the *cis* face of this organelle. Numerous small vesicles are present in the area between the transitional and Golgi cisternae, and images suggesting their

fusion or budding from the cisternal membranes are frequently seen. Small vesicles are also present in the periphery of the Golgi stacks (Fig. 4, *a-e*). In trypanosomes isolated from very highly infected rats (parasitemia $\sim 1.5 \times 10^9/\text{ml}$), the Golgi apparatus was poorly developed and seldom consisted of more than three stacked cisternae. This probably reflects the greatly diminished growth rate of trypanosomes at this parasitemia, which is equivalent to an in vitro culture approaching the stationary phase.

The area adjacent to the *trans* face of the Golgi apparatus is characterized by the presence of a prominent system of flattened cisternae and tubulovesicular elements. Frequently, the inner faces of the membranes of some of the elements of this system contain an electron-dense material similar to the surface coat (Fig. 4 *e*). Vesicles larger in size (100 nm) than those in the transitional zone and covered by a well formed luminal coat, are regularly found near the flagellar pocket, as well as in the periphery of the *trans*-Golgi region (Fig. 4, *a* and *b*).

Treatment with 1 μM monensin led, within 1 min, to a dramatic swelling of several *trans*-Golgi cisternae (Fig. 5, *a* and *b*), and to the appearance of other large vacuoles, not associated with the Golgi apparatus, containing variable amounts of amorphous material and some small vesicles, and probably representing swollen lysosomes or multivesicular bodies. In spite of these effects observed in the electron micrographs, the shape and motility of the trypanosomes, as judged by light microscopy, were unaffected. This was the case when incubation was prolonged for up to 120 min, but monensin concentrations exceeding 5 μM led to cell death of this clone within 15 min. Interestingly, some other trypanosome clones (e.g., MITat 1.2, variant clone 221a) tolerated monensin concentrations up to 10 μM . Contrasting with the effect of monensin on the *trans*-Golgi cisternae, the flattened cisternae and post *trans*-Golgi tubulovesicular elements were not swollen after monensin treatment.

In trypanosomes incubated at 20°C, the post *trans*-Golgi tubulovesicular system was more highly developed (Fig. 5, *c-g*), and the alignment of Golgi cisternae within the stacks became somewhat looser (Fig. 5, *c*, *e*, and *f*). Large vesicles carrying a luminal coat were very distinct in the region of the flagellar pocket (Fig. 5, *c* and *g*).

Immunolocalization of VSG

The distribution of VSG in normal trypanosomes and in cells treated with monensin or incubated at 20°C was determined by immunogold labeling of thin sections. In all cases, VSG was localized over the entire cell surface, including the flagellum and flagellar pocket. Intracellularly, in normal cells the gold label was found over the rough ER, the Golgi apparatus, and the tubulovesicular elements and flattened cisternae in the post *trans*-Golgi area as well as in the lumenally-coated large (100 nm) vesicles found near the flagellar pocket (Fig. 6). Most importantly, significant concentrations of VSG were found throughout all the cisternae in the Golgi apparatus (Fig. 6, *a-c*). A few gold particles were regularly found over the luminal content of $\sim 50\%$ of the glycosomes (29).

In monensin-treated cells, VSG was present in the large vacuoles derived from the *trans*-Golgi cisternae and in the remaining undilated *cis*-Golgi cisternae and elements of the

post *trans*-Golgi tubulovesicular system (data not shown). In cells incubated at 20°C, the concentration of gold particles increased in the *trans*-Golgi cisternae and in membranes of the overdeveloped system of the tubulovesicular elements, flattened cisternae and large vesicles in the area adjacent to the *trans*-Golgi complex (Fig. 7).

Discussion

The parasitic-flagellated protozoa comprising the family *Trypanosomatidae* represent one of the best characterized lower eukaryotes at the cellular, biochemical and molecular levels. Their ancient divergence in the eukaryotic evolutionary lineage is exemplified by the unique peculiarities of each of the basic cellular processes that have been studied in trypanosomatids. At the start of the work presented in this paper, VSGs were the only proteins for which definitive chemical evidence existed for posttranslational processing by carboxy-terminal hydrophobic peptide cleavage and addition of a glycan phosphatidylinositol moiety (10). Thus, we felt it appropriate to evaluate whether VSG was transported to the trypanosome surface via the Golgi apparatus, especially since published data (22) suggested that terminal modification of the VSG N-linked glycan core structure that is added in the ER was not obligatory, and did not occur in the variant used in the present studies. Our immunocytochemical data revealed VSG in the rough ER and throughout the cisternae of the Golgi apparatus, as well as in flattened cisternae and tubulovesicular elements adjacent to the *trans*-Golgi area. We therefore conclude that the classical transport route for the delivery of integral membrane proteins to the cell surface in higher eukaryotes, via the Golgi apparatus, is also used for VSG in trypanosomes. Within the limits of resolution of the present studies, the addition of glycolipid does not appear to override or modify normal sorting mechanisms. Immunolabeling strongly suggests that, after it traverses the Golgi stacks, VSG enters a post *trans*-Golgi tubulovesicular network that effects its delivery to the cell surface, most likely at the region of the flagellar pocket, which is also thought to be the point of secretion and endocytosis in trypanosomes (25, 35). Although we did not distinguish between VSG in biosynthetic or endocytic pathways, VSG turnover only occurs at a low rate under the experimental conditions used (Duszenko, M., unpublished observations). Thus, it seems likely that the majority of immunolabeled VSG in the Golgi apparatus and ER can be attributed to the biosynthetic pathway.

The presence, in trypanosomes, of an extensive system of cisternal and tubulovesicular elements in the area adjacent to the *trans* side of the Golgi apparatus has been recognized by other investigators, who suggested that it plays a role in secretion (39), or is involved in endocytosis and intracellular digestion (25). It seems likely that some of the post *trans*-Golgi elements represent the counterpart of the *trans*-Golgi network recently described in mammalian cells, for which a role in the sorting of newly synthesized secretory, lysosomal, and membrane proteins has been suggested (20). In fact we found that, in trypanosomes incubated at 20°C, as is the case with mammalian cells incubated at the same temperature (19, 20, 34), the post-Golgi system was expanded

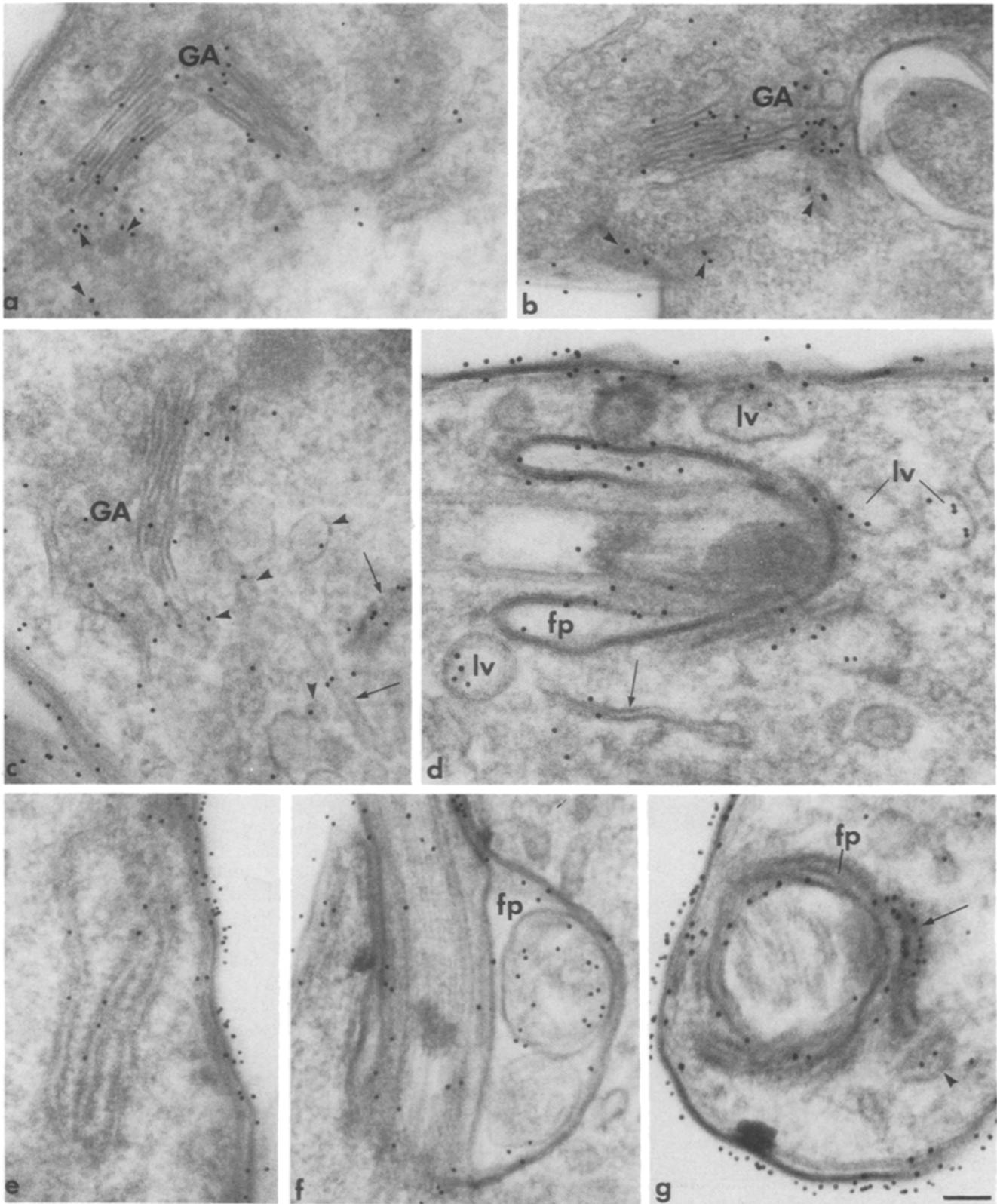


Figure 6. Immunolocalization of VSG in trypanosomes. Thin frozen sections of cells fixed in 2% glutaraldehyde were labeled using anti-VSG antibody and protein A-gold conjugates (10 nm). The cell surface, including the flagellar pocket (*d-e*), was homogeneously labeled. Intracellularly, the membranes of the rough ER cisternae showed low levels of labeling, but it was clear that the gold particles were bound to the luminal face of the membrane (*e*). A prominent amount of the marker was found over the cisternae of the Golgi apparatus (*GA*) (*a-c*). Tubulovesicular elements (*arrowheads*) and flattened cisternae (*arrows*) adjacent to the *trans*-Golgi apparatus and the flagellar pocket (*fp*) were also labeled (*a-d, g*). Large vesicles (*lv*), 100 nm in diameter, usually found close to the flagellar pocket, were systematically labeled on the luminal side of their membranes (*d*). Bar, 0.1 μ m.

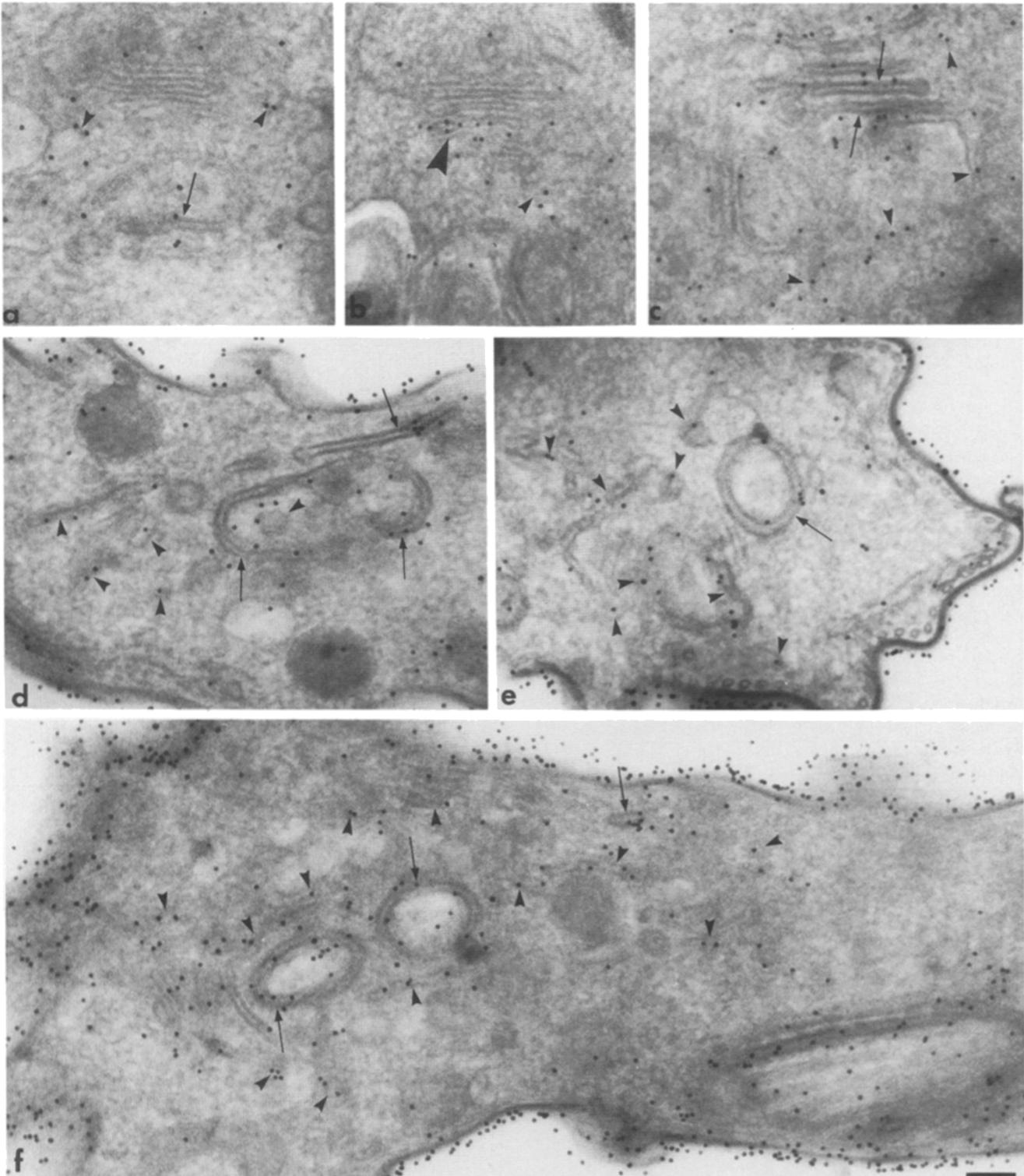


Figure 7. Accumulation of VSG in the expanded system of flattened cisternae and tubulovesicular elements of the post *trans*-Golgi area of trypanosomes incubated at 20°C. After incubation for 60 min at 20°C, cells were processed for immunolabeling as described in the legend for Fig. 6. Intracellularly, the VSG marker was predominantly located on *trans*-Golgi cisternae (b, large arrowhead), and on an increased amount of flattened cisternae (arrows), and tubulovesicular elements (arrowheads) present in the post *trans*-Golgi area, which are characteristic of these experimental conditions (a-f). Bar, 0.1 μ m.

and contained higher concentrations of VSG, and that the delivery of newly synthesized VSG to the cell surface was significantly delayed.

The precise route by which the VSG concentrated in the trypanosomal *trans*-Golgi network reaches the flagellar pocket remains to be elucidated. It is possible that the cisternae of the *trans*-Golgi network intermittently communicate directly with the cell surface or, more likely, that small vesicles budding from them transfer VSG to the plasma membrane. Large vesicles, of ~100 nm diameter, that are found near the flagellar pocket were consistently labeled for VSG. However, these vesicles contained a well-formed surface coat over the luminal aspect of their membranes, and it seems likely that they are endocytic in nature and contain VSG derived from the surface coat. Other authors have shown that similar vesicles can be labeled with endocytosed markers (25).

The lack of effect of monensin on VSG transport is perhaps surprising in view of the clear effects that this ionophore had on Golgi structure. However, cases have been reported of glycoproteins, such as the hemagglutinin of influenza virus synthesized in Madin-Darby canine kidney (MDCK) cells (2), whose transport to the cell surface was not affected by monensin.

Elucidation of the details of modifications occurring in each cellular compartment of the trypanosome secretory pathway will require further studies. Current data (3, 4, 15) are consistent with the hypothesis that glypiation occurs as an immediate posttranslational event in the ER, probably by addition of a preformed glycolipid precursor in a reaction that is closely coupled to cleavage of the polypeptide tail (24). Recent studies have shown that a putative precursor of the protein-bound glycolipid lacks galactose (Menon, A. K., S. Mayor, M. A. J. Ferguson, M. Duszzenko, and G. A. M. Cross, unpublished observations). Thus, further modification of the protein-bound glycolipid may occur in the ER and in the Golgi apparatus.

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References

- Allen, G., L. P. Gurnett, and G. A. M. Cross. 1982. Complete amino acid sequence of a variant surface glycoprotein (VSG 117) from *Trypanosoma brucei*. *J. Mol. Biol.* 157:527-546.
- Alonso, F. V., and R. W. Compans. 1981. Differential effect of monensin on enveloped viruses that form at distinct plasma membrane domains. *J. Cell Biol.* 89:700-705.
- Bangs, J. D., D. Hereld, J. L. Krakow, G. W. Hart, and P. T. Englund. 1985. Rapid processing of the carboxyl terminus of a trypanosome variant surface glycoprotein. *Proc. Natl. Acad. Sci. USA.* 82:3207-3211.
- Bangs, J. D., N. W. Andrews, G. W. Hart, and P. T. Englund. 1986. Post-translational modification and intracellular transport of a trypanosome variant surface glycoprotein. *J. Cell Biol.* 103:255-263.
- Boothroyd, J. C., C. A. Paynter, G. A. M. Cross, A. Bernards, and P. Borst. 1981. Variant surface glycoproteins of *Trypanosoma brucei* are synthesized with cleavable hydrophobic sequences at the carboxy and amino termini. *Nucl. Acids Res.* 9:4735-4743.
- Bulow, R., and P. Overath. 1986. Purification and characterization of the membrane-form variant surface glycoprotein hydrolase of *Trypanosoma brucei*. *J. Biol. Chem.* 261:11918-11923.
- Cardoso de Almeida, M. L. and M. J. Turner. 1983. The membrane form of variant surface glycoproteins of *Trypanosoma brucei*. *Nature (Lond.)* 302:349-352.
- Cross, G. A. M. 1975. Identification, purification and properties of clone specific glycoprotein antigens constituting the surface coat of *Trypanosoma brucei*. *Parasitology.* 71:393-417.
- Cross, G. A. M. 1984. Structure of the variant glycoproteins and surface coat of *Trypanosoma brucei*. *Phil. Trans. R. Soc. Lond. B.* 370:3-12.
- Cross, G. A. M. 1987. Eukaryotic protein modification and membrane attachment via phosphatidylinositol. *Cell.* 48:179-181.
- Farquhar, M. G. 1985. Progress in unraveling pathways of Golgi traffic. *Annu. Rev. Cell Biol.* 1:477-488.
- Farquhar, M. G., and G. E. Palade. 1981. The Golgi apparatus (complex) (1954-1981)—from artifact to center stage. *J. Cell Biol.* 91:77s-103s.
- Ferguson, M. A. J., and G. A. M. Cross. 1984. Myristylation of the membrane form of a *Trypanosoma brucei* variant surface glycoprotein. *J. Biol. Chem.* 259:3011-3015.
- Ferguson, M. A. J., M. G. Low, and G. A. M. Cross. 1985. Glycosyl-sn-1,2-dimyristyl phosphatidylinositol is covalently linked to *Trypanosoma brucei* variant surface glycoprotein. *J. Biol. Chem.* 260:4963-4968.
- Ferguson, M. A. J., M. Duszzenko, G. S. Lamont, P. Overath, and G. A. M. Cross. 1986. Biosynthesis of *Trypanosoma brucei* variant surface glycoproteins: N-glycosylation and addition of a phosphatidylinositol membrane anchor. *J. Biol. Chem.* 261:356-362.
- Fox, J. A., M. Duszzenko, M. A. J. Ferguson, M. G. Low, and G. A. M. Cross. 1986. Purification and characterization of a novel glycan-phosphoinositide-specific phospholipase C from *Trypanosoma brucei*. *J. Biol. Chem.* 261:15767-15771.
- Fox, J. A., N. M. Soliz, and A. R. Saltiel. 1987. Purification and characterization of a phosphatidylinositol glycan specific phospholipase C from hepatic plasma membranes. *Proc. Natl. Acad. Sci. USA.* 84:2663-2668.
- Grab, D. J., P. Webster, and Y. Verjee. 1984. The intracellular pathway and assembly of newly formed variable surface glycoprotein of *Trypanosoma brucei*. *Proc. Natl. Acad. Sci. USA.* 81:7703-7707.
- Griffiths, G., S. Pfeiffer, K. Simons, and K. Matlin. 1985. Exit of newly synthesized membrane proteins from the trans cisterna of the Golgi complex to the plasma membrane. *J. Cell Biol.* 101:949-964.
- Griffiths, G., and K. Simons. 1986. The trans-Golgi network: sorting at the exit site of the Golgi complex. *Science (Wash. DC).* 234:438-443.
- Hereld, D., J. L. Krakow, J. D. Bangs, G. W. Hart, and P. T. Englund. 1986. A phospholipase C from *Trypanosoma brucei* which specifically cleaves the glycolipid on the variant surface glycoprotein. *J. Biol. Chem.* 261:12813-12819.
- Holder, A. A., and G. A. M. Cross. 1981. Glycopeptides from variant surface glycoproteins of *Trypanosoma brucei*: C-terminal location of antigenically cross-reacting carbohydrate moieties. *Mol. Biochem. Parasitol.* 2:135-150.
- Keller, G. A., K. T. Tokuyasu, A. H. Dutton, and S. J. Singer. 1984. An improved procedure for immunoelectron microscopy: ultrathin plastic embedding of immunolabeled ultrathin frozen sections. *Proc. Natl. Acad. Sci. USA.* 81:5744-5747.
- Krakow, J. L., D. Hereld, J. D. Bangs, G. W. Hart, and P. T. Englund. 1986. Identification of a glycolipid precursor of the *Trypanosoma brucei* variant surface glycoprotein. *J. Biol. Chem.* 261:12147-12153.
- Langreth, S. G., and A. E. Balber. 1975. Protein uptake and digestion in bloodstream and culture forms of *Trypanosoma brucei*. *J. Protozool.* 22:40-53.
- Low, M. G. 1987. Biochemistry of the glycosyl-phosphatidyl-inositol membrane protein anchors. *Biochem. J.* 244:1-13.
- Matlin, K., and K. Simons. 1983. Reduced temperature prevents transfer of a membrane glycoprotein to the cell surface but does not prevent terminal glycosylation. *Cell.* 34:233-243.
- Medelzon, D. H., J. O. Previato, and A. J. Parodi. 1986. Characterization of protein-linked oligosaccharides in trypanosomatid flagellates. *Mol. Biochem. Parasitol.* 18:355-369.
- Opperdoes, F. R., P. Baudhuin, I. Coppens, C. De Roe, S. W. Edwards, P. J. Weijers, and O. Misset. 1985. Purification, morphometric analysis, and characterization of the glycosomes (microbodies) of the protozoan hemoflagellate *Trypanosoma brucei*. *J. Cell Biol.* 98:1178-1184.
- Parodi, A. J., L. A. Q. Allue, and J. J. Cazzulo. 1981. Pathway of protein glycosylation in *Crithidia fasciculata*. *Proc. Natl. Acad. Sci. USA.* 78:6201-6205.
- Previato, J. O., D. H. Mendelzon, and A. J. Parodi. 1986. Characterization of dolichol monophosphate- and dolichol diphosphate-linked saccharides in trypanosomatid flagellates. *Mol. Biochem. Parasitol.* 18:343-355.
- Rindler, M. J., I. E. Ivanov, H. Plesken, and D. D. Sabatini. 1985. Polarized delivery of viral glycoproteins to the apical and basolateral plasma membranes of Madin-Darby canine kidney cells infected with temperature-sensitive viruses. *J. Cell Biol.* 100:136-141.
- Sabatini, D. D., G. Kreibich, T. Morimoto, and M. Adesnik. 1982. Mechanisms for the incorporation of proteins in membranes and organelles. *J. Cell Biol.* 92:1-22.

34. Saraste, J., and E. Kuismanen. 1984. Pre- and post-Golgi-vacuoles operate in the transport of Semliki forest virus membrane glycoproteins to the cell surface. *Cell*. 38:535-549.
35. Steiger, R. 1971. Some aspects of surface coat formation in *Trypanosoma brucei*. *Acta Tropica*. 28:341-346.
36. Strickler, J. E., and C. L. Patton. 1980. *Trypanosoma brucei*: inhibition of glycosylation of the major variable surface coat glycoprotein by tunicamycin. *Proc. Natl. Acad. Sci. USA*. 77:1529-1533.
37. Tokuyasu, K. T. 1980. Immunocytochemistry on ultrathin frozen sections. *Histochem. J.* 12:381-403.
38. Turner, M. J. 1982. Biochemistry of the variant surface glycoproteins of salivarian trypanosomes. *Adv. Parasitol.* 21:69-153.
39. Vickerman, K. 1969. The fine structure of *Trypanosoma congolense* in its bloodstream phase. *J. Protozol.* 16:54-69.
40. Walter, P., R. Gilmore, and W. Blobel. 1984. Protein translocation across the endoplasmic reticulum. *Cell*. 38:5-8.
41. Wickner, W. T., and H. F. Lodish. 1985. Multiple mechanisms of protein insertion into and across membranes. *Science (Wash. DC)*. 230:400-407.