Newly Synthesized G Protein of Vesicular Stomatitis Virus Is Not Transported to the Golgi Complex in Mitotic Cells

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ABSTRACT Newly synthesized G protein of vesicular stomatitis virus is not transported to the surface of cultured mammalian cells during mitosis (Warren et al., 1983, J. Cell Biol. 97:1623-1628). To determine where intracellular transport is inhibited, we have examined the post-translational modifications of G protein, which are indicators of specific compartments on the transport pathway. G protein in mitotic cells had only endo H-sensitive oligosaccharides containing seven or eight mannose residues, but no terminal glucose, and was not fatty acylated. These modifications were indicative of processing only by enzymes of the endoplasmic reticulum (ER). Quantitative immunocytochemistry was used as an independent method to confirm that transport of G protein out of the ER was inhibited. The density of G protein in the ER cisternae was 2.5 times greater than in infected G1 cells treated similarly. Incubation of infected mitotic cells with cycloheximide, which inhibits protein synthesis without affecting transport, did not result in a decrease in the density of G protein in the ER cisternae, demonstrating that G protein cannot be chased out of the ER. These results suggest that intracellular transport stops at or before the first vesicle-mediated step on the pathway.

Transport of proteins between the membrane-bound organelles of the cell appears to be mediated by budding and fusion of carrier vesicles. Vesicular traffic occurs continuously in all animal cells, except for cells undergoing division. During mitosis a number of specific examples of membrane transport have been shown to be inhibited. These include endocytosis (Fawcett, 1965; Berlin et al., 1978; Berlin and Oliver, 1980), receptor recycling (Warren et al., 1984; Sager et al., 1984), and stimulated secretion (Hesketh et al., 1984).

The examples outlined above all concern transport to or from the plasma membrane. In a previous study, we showed that a model plasma membrane protein, the glycoprotein of vesicular stomatitis virus (VSV), is not transported to the surface of mitotic cells (Warren et al., 1983), but in this case it was not clear whether transport was inhibited at the level of the plasma membrane, or earlier in the transport pathway.

Proteins destined for the plasma membrane must pass through a series of compartments from their site of synthesis in the rough endoplasmic reticulum (ER), through the cisternae of the Golgi complex, to the cell surface (Bergmann et al., 1981; Green et al., 1981). The first vesicle-mediated step on this pathway is probably the step between the transitional elements of the ER and the Golgi complex (Jamieson and Palade, 1968). If inhibition of membrane transport acts on a common feature of all vesicle-mediated transport, then one would expect that all vesicular traffic, not only that to and from the plasma membrane, would be inhibited in mitotic cells. A prediction from this hypothesis is that a newly synthesized plasma membrane protein would not be transported out of the ER. In this study we tested this prediction by attempting to identify the first step in transport to the plasma membrane that is inhibited during mitosis.

We have studied mitotic Chinese hamster ovary (CHO) cells, infected with VSV. This virus directs the synthesis of five viral proteins, one of which, the glycoprotein (G protein), is an integral membrane protein. This protein undergoes a well-defined temporal sequence of post-translational modifi-
lations, including oligosaccharide processing (Hubbard and Ivatt, 1981) and fatty acylation (Schmidt and Schlesinger, 1980), which provide biochemical markers for the compartments through which G protein must pass en route to the plasma membrane. Furthermore, since G protein is synthesized in large amounts in infected cells, it can be easily detected by quantitative immunocytochemical techniques (Bergmann et al., 1981). The results reported here support the notion that G protein does not leave the ER in the mitotic phase, because we found it to be a simpler, more rapid and reliable method.

**MATERIALS AND METHODS**

**Preparation of Mitotic CHO Cells Infected with VSV:** The CHO cell line, obtained from Dr. Stuart Kornfeld (Washington University, St. Louis, MO), was grown and maintained as monolayers on 75-cm² plastic flasks in growth medium (GM) comprising minimal essential medium (MEM) (Gibco Europe, Glasgow) supplemented with 10% (vol/vol) fetal calf serum and 100 U/ml of penicillin and streptomycin, in an atmosphere of 5% CO₂/95% air.

Mitotic cell populations were isolated from monolayer cultures grown in 850-cm² plastic roller bottles (Falcon Labware, Oxnard, CA). Each bottle was seeded with half of the cells from a confluent 75-cm² flask in 100 ml GM. The bottles were gassed with 5% CO₂/95% air for 3 min and then rotated at 0.2-0.3 rpm at 37°C. They were used to prepare mitotic cells 2 d later when the monolayers were ~50% confluent.

Bottles were subjected to two spins at 300 rpm for 5 min at 37°C, separated by an interval of 20 min, to remove debris. The cells were then incubated for 20 min with GM containing 0.04 ug/ml Nocodazole (Sigma Chemie GmbH, Taufkirchen, FRG) (Zieve et al., 1980), before being spun again at 200 rpm for 3 min to remove interphase cells that had become loosely attached as a result of the Nocodazole treatment. The cells that remained were washed twice with medium (MEM, 100 U/ml penicillin, 100 U/ml streptomycin, 0.2% (w/v) bovine serum albumin (BSA), 10 mM HEPES [final pH 6.8]) containing 0.04 ug/ml Nocodazole and infected with 10-20 infecting units (defined below) of VSV per cell (0.85-1.1 x 10⁶ infecting units per bottle) in a total volume of 10 ml for 30 min at 37°C and 0.2-0.3 rpm. The infection medium was carefully removed and replaced with 30 ml GM that contained 0.04 ug/ml Nocodazole. Mitotic cells were selectively detached from the bottle by rotation at 200 rpm (Klevez, 1975) for 3 min at 37°C and recovered by centrifugation at 400 g for 5 min at room temperature, 1-3 x 10⁶ cells were routinely obtained from one bottle by this procedure. Of these, usually >90% were mitotic, as determined by staining with Hoechst dye 33258 (Berlin et al., 1978), and 95% infected, as determined by immunofluorescence microscopy (see below).

**G₁ Cells:** To obtain G₁ cells, infected, mitotic cells were washed once with 50 ml GM to remove Nocodazole, recovered by centrifugation at 400 g for 5 min at room temperature, resuspended in 2 ml GM, and incubated at 37°C for 30 min to allow the cells to complete mitosis.

**Asynchronous Cells:** CHO cells for "interphase" controls were grown on 3.5-cm-diam plastic Petri dishes in GM. They were infected using the same protocol as described for roller bottle cultures.

**Preparation of Virus and Radioactive Virus:** Indiana M4 strain of VSV was grown in baby hamster kidney cells as described by Mathil et al. (1982). ts045 VSV was prepared by essentially the same method, but the cells were grown on 24 x 24 cm plates and infected for 30 h at 37°C. [3H]mannose-labeled virus was prepared as described above and resuspended in 1.5 ml glucose-free MEM containing 10% (wt/vol) and precipitation on ice for 2 h. TCA precipitates were recovered by the addition of trichloroacetic acid (TCA) to a final concentration of 10% (wt/vol) and precipitation on ice for 2 h. TCA precipitates were recovered by centrifugation at ~5,000 g for 5 min at 4°C in a microfuge, dried, and fluorographed at -70°C using preflashed Kodak X-OMAT x-ray film as described by Laskey and Mills (1975).

**Labeling with [35S]Methionine:** VSV-infected, mitotic cells were washed twice with methionine-free MEM containing 100 units/ml of penicillin and streptomycin, 2 mM glutamine, 0.2% (wt/vol) BSA, 10 mM HEPES (pH 7.3), and nonessential amino acids, and 0.5 µg/ml unlabeled methionine. The presence or absence of Nocodazole. Cells were recovered after each wash by centrifugation at 400 g for 5 min at room temperature. The washed cells were resuspended in 2 ml (0.5 x 1.0 x 10⁶ cells/ml) of the same medium containing 100 µCi/L [35S]methionine (7-800 Ci/mmol, Amersham Buchler GmbH, Braunschweig, FRG) and incubated at 37°C for 60 min before chasing with 0.1 ml unlabeled methionine in GM for 60 min. At the end of the chase period, the cells were transferred onto ice, resuspended, and recovered by centrifugation at 400 g and 4°C. The cells were extracted with 250 µl 1% (w/v) Triton X-114 in PBS containing 0.23 mM phenylmethylsulfonyl fluoride (PMSF) and 2.8 µg/ml Trasylol, and extracted on ice for 10 min. Insoluble material was removed by centrifugation at full speed (10,000 g) in a microfuge at 4°C. Pellets were washed twice with PBS, resuspended in 250 µl 1% (w/v) Triton X-114 in PBS, and centrifuged at 12,000 g for 10 min at 4°C. The detergent pellet was washed with ice-cold PBS by three successive rounds of warming to 30°C and resedimentation.

**endo H Digestion and SDS PAGE:** Proteins extracted with Triton X-114 were digested with endo β-N-acetylglucosaminidase H (endo H) from Staphylococcus pilatus (Miles Laboratories Inc., Elkhart, IN) by the addition of an equal weight of Triton X-100 to the detergent pellet on ice, then of 250 in ice for 20 min. 0.2 M sodium citrate, 5.5 x 0.23 mM PMSF, and 100 µg/ml endo H per sample. The mixture was rotated at 37°C for 16-20 h, and the protein was recovered by the addition of 10% (v/v) and precipitation on ice for 2 h. TCA precipitates were recovered by centrifugation at ~5,000 g for 5 min at 4°C in a microfuge, washed twice with ethanol/ether, and dissolved in SDS sample buffer and electrophoresed in a 10% (v/v) polyacrylamide gel as described by Maizel (1969) with the modifications described by Green et al. (1981). Gels were fixed, soaked in ENHANCE (New England Nuclear, Dreieich, FRG), dried, and fluorographed at -70°C using preflashed Kodak X-OMAT x-ray film as described by Laskey and Mills (1975).

**Labeling with [3H]Mannose:** VSV-infected, mitotic cells were isolated as described above and resuspended in 1.5 ml glucose-free MEM containing 2 mM glutamine, 15 mM HEPES (pH 7.3), 100 U/ml penicillin and streptomycin, 0.2% (wt/vol) BSA, nonessential amino acids, 10 mM sodium pyruvate, 100 units/ml of penicillin (Sigma Chemical Co., St. Louis, MO) and incubated at 37°C for 60 min before chasing with 0.1 ml unlabeled mannose and incubating for 60 min more at 37°C. Pulsed and chased cells were recovered by centrifugation at 400 g for 5 min, washed once with ice-cold PBS, and extracted with Triton X-114 as described above. Glycopeptides were prepared from the detergent extracts and analyzed by a Biogel P-4 (200-400 mesh) column as described below. 24 h after infection with VSV, interphase CHO cells were labeled with 1 mcCi [3H]mannose in the presence or absence of Nocodazole, as described for mitotic and G₁ cells. The labeled cells were chased for 60 min and processed in the same way as mitotic and G₁ cells.

**Preparation of Glycopeptides:** Glycopeptides were prepared either in the manner described by Tabas and Kornfeld (1978) or by a simpler and quicker procedure described below which gave essentially the same results.

**[3H]Mannose-labeled cells were extracted with Triton X-114 as described above and the pellet containing the detergent-insoluble and dolichol-linked oligosaccharides was washed to remove the excess. water-soluble [3H]mannose. G protein represented ~50% of the total [3H]mannose-labeled protein in these extracts as determined by SDS gel electrophoresis. Glycopeptides were separated from the dolichol-linked oligosaccharides by the addition of 500 µl of 50 mM HEPES (pH 7.0) containing 10 mg/ml protease K, and rotation at 37°C for 60 min. This released all [3H]mannose-labeled glycopeptides into the supernatant after phase separation, leaving dolichol-linked oligosaccharides in the pellet. The pellets would then be released by digestion with endo H. The glycopeptides were washed by increasing the concentration of Triton X-114 to 2% (w/v) and repeating the phase separation. SDS was added to the supernatant to a final concentration of 2%. SDS PAGE was then run on 10% gels and stained with Coomassie blue.
of 0.1% (wt/vol) and sodium azide to 0.2% (wt/vol), and the mixture was incubated at 56°C for 16–24 h to complete digestion. Protease K was inactivated by heating to 95°C for 3 min, cooling, and adding 0.1 mM PMSF. Debris was removed by centrifugation at 10,000 g in a microfuge for 1 min, and the supernatant was lyophilized.

Digestion of Glycopeptides with endo H and α-Mannosidase: Lyophilized glycopeptides (1–2 × 10⁶ cpm) were either dissolved in 100 mM Tris-HCl (pH 7.0), 0.1% (wt/vol) SDS, and 0.1% (wt/vol) sodium azide and applied directly to a Biogel P4 (200–400 mesh) column equilibrated in the same buffer (Green et al., 1981), or first digested with endo H by the addition of 0.5 ml 200 mM sodium citrate (pH 6.0) containing 0.1 mM PMSF. 2.8 μg/ml Trypsin, 5 μg/ml endo H and incubation for 48 h at 37°C, 0.7-ml fractions were collected. The column was calibrated as described by Rosner et al. (1982) using [14C]-BSA and [14C]mannose as markers for the excluded and included volumes respectively. Markers ranging in size from Man₃GlcNAc to Glc₃ManGlcNAc were provided by Martin Snider, Carnegie Institution of Washington, Baltimore, MD.

The Man₄GlcNAc peak obtained from mitotic cells was desalted on a Biogel P2 column equilibrated in 0.1% (wt/vol) sodium azide, the product was lyophilized and treated with jack bean α-mannosidase as described by Pesonen et al. (1982), and the products (1–2 × 10⁶ cpm) were analyzed using the Biogel P4 column. 0.7-ml fractions were collected.

Labeling with [3H]Palmitic Acid and [35S]Methionine: VSV-infected, mitotic CHO cells, isolated from 10 roller bottles (1–3 × 10⁷ cells), were washed once with 100 ml methionine-free MEM containing 1% (vol/vol) fetal calf serum, nonessential amino acids, penicillin and streptomycin, and 0.04 μg/ml Nocodazole. They were centrifuged at 4,000 g for 1 rain at room temperature and resuspended in 4 vol of ice-cold PBS and centrifuged at 400 g for 5 min at room temperature and resuspended in 10 ml of the same medium. 9 ml of the cell suspension was plated onto a 6-cm-diam dish and labeled for 60 min at 37°C with 2.25 μCi [3H]palmitic acid (25-30 Ci/mmol) (New England Nuclear) added dropwise from a 50 μCi/μl solution in 100% ethanol. The remaining 1 ml was plated onto a 3.5-cm-diam dish and labeled with 50 μCi [3H]methionine for 60 min at 37°C. Interphase cell controls were CHO clone 15B cells (obtained from Dr. Stuart Kornfeld and maintained as for the wild type), growing on 3.5-cm-diam dishes. They were washed twice with 2 ml of the medium described above and labeled for 60 min at 37°C with 125 μCi [3H]palmitate acid or with 50 μCi [35S]methionine. After labeling, the cells were chased for 60 min by the addition of an equal volume of MEM containing 20% (vol/vol) fetal calf serum, 2 mM unlabeled methionine, 100 μM unlabeled palmitic acid, and 0.04 μg/ml Nocodazole. At the end of the chase, the dishes were washed three times with PBS and resuspended in 4 vol of ice-cold PBS and centrifuged at 400 g for 5 min at 4°C, while the interphase cells on dishes were washed with 2 ml ice-cold PBS. [3H]Palmitate-labeled mitotic cell pellets were extracted with 2.25 ml 1% (wt/vol) Triton X-114 in PBS, containing 0.25 mM PMSF and 0.28 μg/ml Trypsin. The [3H]methionine-labeled mitotic cell pellet, and cells on dishes, were extracted with 250 μl of the same Triton X-114 solution. The detergent extracts were processed, digested with endo H, and run on an SDS polyacrylamide gel as described above.

Immunofluorescence Microscopy: Normal rat kidney cells were processed for immunofluorescence microscopy following the general procedure of Wang et al. (1982), with modifications described by Warren et al. (1984). The anti-G protein antibody was a rabbit antiserum raised against Trion X-114 extracts of VSV virions. The second antibody was an affinity-purified rabbit anti-mouse IgG conjugated to rhodamine as described by Brandtzaeg (1973).

Immunoelectron Microscopy: VSV-infected cells were fixed for immunoelectron microscopy using formaldehyde because low concentrations of glutaraldehyde were found to abolish the reaction of G protein with the antibody. The cells were centrifuged at 4,000 g for 1 rain at room temperature in a microfuge to form a pellet 1–2 mm thick. The supernatant was discarded and 100 μl of 4% (wt/vol) formaldehyde in 100 mM PIPES (pH 7.0) was carefully layered onto the pellet without disturbing it. At 10-min intervals 8% (wt/vol) formaldehyde, 100 mM PIPES, (pH 7.0) was added to the supernatant to bring the final volume to 1.5 ml after 30–40 min. Small pieces (<1 mm²) of pellet were infiltrated for 15 min with 2.1 M sucrose, fixated in liquid nitrogen, and sectioned at −110°C (Griffiths et al., 1983) using a tungsten-coated glass knife (Griffiths et al., 1984). The sections were thawed and labeled with a rabbit anti-G protein antibody, affinity purified on VSV (a gift from Dr. Kai Simons), and then protein A conjugated to 5-nm gold, as described by Warren et al. (1983). The sections were dried and contrasted as described by Griffiths et al. (1984).

Gold labeling of ER was quantitated by a simple, relative procedure. The volume density of ER was estimated stereologically by point counting (Weibel, 1979) on micrographs at 55,000 magnification. Gold particles on or within 10 nm of the ER membrane were attributed to the ER.

RESULTS

Processing of G Protein Oligosaccharides in Mitotic Cells

G PROTEIN IS SENSITIVE TO DIGESTION WITH ENDO H: VSV-infected cells, arrested in mitosis with Nocodazole, were pulse-labeled for 60 min with [3H]methionine and chased for 60 min with an excess of unlabeled methionine. All of the labeled G protein isolated from these cells was sensitive to digestion by endo H (Fig. 1, lanes a and b), which indicates that the oligosaccharides of the glycoprotein were of the immature, high mannose form. If the mitotic cells were chased in the absence of Nocodazole, the G protein was converted to a resistant form, which shows that oligosaccharide processing was completed when cells were allowed to enter G1 (Fig. 1, lanes c and d).

The failure of G protein to acquire complex oligosaccha-
rides in cells arrested in mitosis was probably not due to a side effect of Nocodazole for the following three reasons:

(a) Complex oligosaccharides were formed on G protein in infected interphase cells, pulse-labeled for 60 minutes, and chased for 60 min in the presence of Nocodazole (Fig. 1, lanes e and f).

(b) The time course of the conversion of endo H-resistant to endo H-sensitive G protein in infected interphase cells was unaffected by the presence of Nocodazole (Fig. 2).

(c) Transport of G protein to the surface of interphase cells in the presence of 100 times more Nocodazole than was used to arrest cells in mitosis had no effect on the appearance of G protein at the cell surface (Fig. 3). This was determined using a temperature-sensitive mutant of VSV, ts045, in which G protein remains in the ER at the nonpermissive temperature (39.5°C) but is transported to the plasma membrane at 31°C. After 4 h of infection with ts045 at the nonpermissive temperature, in the presence or absence of Nocodazole, normal rat kidney cells were either fixed immediately (Fig. 3, A and E) or transferred to the permissive temperature for 60 min (Fig. 3, B–D, F–H). Surface G protein was detected by immunofluorescence microscopy (Fig. 3, A–D). No obvious differences between surface fluorescence intensity of cells treated with 100-fold excess Nocodazole (Fig. 3 B) or 1% (vol/vol) dimethylsulfoxide (Fig. 3 D), and control cells without Nocodazole (Fig. 3 C) could be detected.

The endo H-sensitive form of G protein predominated in cells held in mitosis with Nocodazole for at least 2.25 h after infection with VSV (Fig. 4 b). Between 3 and 5 h after infection this G protein gradually became resistant to digestion by endo H. This correlated with a drop in the mitotic index of the

![Figure 2](image2.png)

**Figure 2** The effect of Nocodazole on the rate at which G protein in infected interphase cells acquires oligosaccharides resistant to endo H. CHO cells were infected with VSV for 30 min and all further treatments were performed in the presence or absence of Nocodazole. After 4 h of incubation at 37°C, the cells were pulse-labeled for 5 min with [35S]methionine and chased for various times with an excess of unlabeled methionine. The cells were extracted with Triton X-114, the detergent pellets were digested with endo H, and the samples were fractionated by SDS PAGE. The endo H-resistant and -sensitive G protein bands, detected by fluorography, were cut out and counted. Closed symbols, with Nocodazole; open symbols, without Nocodazole; squares, endo-H-sensitive G protein; circles, endo H-resistant G protein.

![Figure 3](image3.png)

**Figure 3** Transport of G protein to the surface of interphase cells infected with ts045 VSV, in the presence of Nocodazole. Normal rat kidney cells growing on coverslips were infected at the nonpermissive temperature (39.5°C) in the presence of 4 μg/ml Nocodazole (A, B, E, F), or 1% (vol/vol) dimethylsulfoxide (D, H) to control for the effect of solvent in the stock Nocodazole solution, or in the absence of both (C, G). 4 h after infection the cells were fixed (A, E) or transferred to the permissive temperature (31°C) (B–D, F–H) and incubated for 60 min more before fixation and processing for surface immunofluorescence. (A–D) Surface immunofluorescence. (E and F) Nomarski images of the same field. Bar, 20 μm.

cells from >90% to ~30%. Almost 70% of cells had divided and returned to an interphase condition after 5 h of infection, as judged by staining with Hoechst 33258, despite the continued presence of Nocodazole (Fig. 4a). For this reason the following experiments were completed within 3 h of infection when ~90% of the cells were still in the mitotic state.

**The Oligosaccharides Contain Eight or Nine Sugar Residues:** To determine the structure of the oligosaccharides on G protein, VSV-infected CHO cells, held in
FIGURE 4 The effect of incubation time on the endo H sensitivity of G protein in mitotic cells. Mitotic CHO cells were isolated from roller bottles 30 min after infection and pulse-labeled with [35S]methionine for 60 min at 37°C in the presence of Nocodazole. 2.25 h after infection half of the cells were allowed to divide by washing with Nocodazole-free medium, and the incubation was continued. At various times samples were stained with Hoechst dye 33258 to determine the mitotic index (a), or extracted with Triton X-114 and digested with endo H. The products were separated by SDS PAGE and visualized by fluorography (b). ○, mitotic cells. ○, mitotic cells washed into medium without Nocodazole after 2.25 h of infection. G_{4} and G_{5} as in Fig. 1.

FIGURE 5 Gel filtration of [3H]mannose-labeled glycopeptides and oligosaccharides from infected mitotic and G_{1} cells. Mitotic CHO cells, isolated 30 min after infection with VSV, were pulse-labeled with [3H]mannose for 60 min at 37°C and chased for 60 min with an excess of unlabeled mannose in the presence or absence of Nocodazole. Membrane proteins were extracted with Triton X-114, and proteinase K glycopeptides were prepared and analyzed by gel filtration before and after digestion with endo H. (a) [3H]mannose-labeled glycopeptides from mitotic cells (broken line), and oligosaccharides resulting from endo H digestion of these glycopeptides (solid line). (b) [3H]mannose-labeled glycopeptides and oligosaccharides from G_{1} cells after digestion with endo H (solid line), and glycopeptides from VSV virions labeled with [3H]mannose (broken line). V_{o}, void volume. D, position of elution of Glc_{3}Man_{3}GlcNAC. M_{4}N, M_{6}N, positions of elution of Man_{2}GlcNAc and Man_{3}GlcNAc standards.

mitosis with Nocodazole, were pulse-labeled for 60 min with [3H]mannose and chased for 60 min with an excess of unlabeled mannose. G protein was isolated from the cells by Triton X-114 extraction, and glycopeptides were prepared by proteinase K digestion. The glycopeptides were analyzed by gel filtration on Biogel P4 before and after digestion with endo H. Those isolated from cells in mitosis were completely sensitive to endo H, detected as a decrease in molecular weight of [3H]mannose-labeled material on gel filtration after digestion (Fig. 5a). In the experiment shown here the major peak of endo H-sensitive oligosaccharide eluted in the same position as markers containing eight sugar residues, with minor amounts equivalent to those of oligosaccharides containing seven and six sugar residues. In similar experiments a peak containing nine sugar residues was also often observed. When mitotic cells were allowed to enter G_{1} by removal of Nocodazole during the chase period, endo H-resistant glycopeptides were found (Fig. 5b). That these eluted in the same position as glycopeptides prepared from VSV virions confirms that the inhibition of oligosaccharide processing in mitotic cells is reversible upon entry into G_{1}. The lower molecular weight peak that appeared in the G_{1} extracts after digestion with endo H eluted in the same position as an oligosaccharide containing at least nine sugar residues, slightly larger than the major peak found in mitotic cells. This perhaps indicates that some oligosaccharide trimming occurs in mitotic cells during the second hour of mitotic arrest, that is, during the chase period.

THE OLIGOSACCHARIDES LACK GLUCOSE RESIDUES: Oligosaccharides containing eight or nine sugar residues after digestion with endo H would normally result from trimming of the three outer glucose residues plus one or two mannose residues. Alternatively, one could envisage that in mitotic cells aberrant trimming of four or five outer mannose residues might also occur, leaving the outer glucose residues on the oligosaccharides. To distinguish between these two possibilities the endo H-sensitive oligosaccharides were further digested with jack bean α-mannosidase, which can trim outer mannose residues but not those blocked by glucose. The
products of the digestion were again analyzed by P4 column chromatography (Fig. 6). The major peak obtained was coincident with [14C]mannose used to calibrate the column, and the small peak of slightly higher molecular weight eluted at the position calculated for the Man β1-4 GlcNAc disaccharide, which cannot be hydrolyzed by the ER glucosidases. The ratio of counts in these two peaks varied between 1:8.3 and 1:12.3 in three experiments, somewhat lower than the expected ratio of 1:6 or 1:7. The reasons for this are unknown.

The absence of any species of higher molecular weight, for example Glc₂Man₃GlcNAc or Glc₂Man₄GlcNAc (Fig. 6), implies that the structure of the endo H–sensitive oligosaccharides was Manα1-2 GlcNAc, and that they were processed by the ER glucosidases. The oligosaccharides were also trimmed by a mannosidase, probably the ER α1-2 mannosidase that normally trims high mannose oligosaccharides from Man₉ to Man₄ (Bischoff and Kornfeld, 1983; Atkinson and Lee, 1984).

It is most unlikely that Nocodazole itself affects oligosaccharide processing, since the elution patterns of glycopeptides and oligosaccharides isolated from infected interphase cells treated with or without Nocodazole were essentially the same (Fig. 7).

**Fatty Acylation of G Protein in Mitotic Cells**

Fatty acylation of G protein probably occurs in the late ER or early Golgi complex, at a point before the protein becomes resistant to endo H (Schmidt and Schlesinger, 1980). We could detect fatty acylation of G protein in mitotic and G₁ cells only if we used more than 2 mCi [1H]palmitic acid to label cells isolated from 10 roller bottles, but, because of the time required to handle this number of bottles, we could not complete these experiments within the 3 h that guarantees a high mitotic index (see Fig. 4). In experiments in which the mitotic index ranged from 42–84% at the end of the experiment, fatty acylation of G protein was observed, but the [1H]palmitic acid was always incorporated into the endo H–resistant form of G protein. When a preparation of mitotic cells was divided in two, and one-half was labeled with [1H]palmitic acid and the other with [35S]methionine under identical conditions (Fig. 8), 50% of the [35S]-labeled protein was sensitive to endo H (Fig. 8, lane b), whereas the [1H]palmitate was only found in the endo H–resistant fraction of the protein (Fig. 8, lane a). To show that this band was truly resistant to endo H and not the result of incomplete digestion due to the presence of fatty acid on the protein, interphase CHO clone 15B cells, in which oligosaccharides are not processed to the complex form, were labeled similarly and treated in parallel with endo H. The data shown in Fig. 8, lanes c–f, demonstrate that endo H digestion of fatty acylated G protein went to completion and that the distribution of 35S- and 1H-labeled G protein before and after endo H digestion was similar. In interphase CHO cells >90% of [35S]methionine and [1H]palmitic acid were incorporated into endo H–resistant G protein under the conditions of the experiment (data not shown). We know from labeling with [1H]mannose or [35S]methionine that G protein in mitotic cells is >90% endo H sensitive (Figs. 1 and 5); therefore the simplest interpretation of these data is that [1H]palmitic acid is only incorporated into G protein in those cells that are not mitotic, due to division of mitotic cells as they begin to overcome the Nocodazole block.

**Location of G Protein in Mitotic Cells by Immunocytochemistry**

Using quantitative immunocytochemistry that employed a polyclonal antibody to G protein and protein A conjugated to gold, we demonstrated that the surface of mitotic cells, 3 h after infection, had 6% of the G protein found on the surface of G₁ cells treated similarly (Warren et al., 1983). Because similar amounts of G protein were synthesized in mitotic and...
test whether transport between the ER and the Golgi complex could occur during mitosis, we took mitotic CHO cells that had been infected with VSV for 2.5 h at 37°C, and divided them in half. One was fixed immediately, and the other was treated with 10 μg/ml cycloheximide for 30 min at 37°C before fixation and processing for immunocytochemistry as before. The density of G protein in the ER of mitotic cells was found to be similar before and after treatment with cycloheximide (Fig. 9 and Table I, experiment 2), as would be predicted if the transport step between the ER and Golgi complex was inhibited in mitosis. The small proportion of interphase cells in the mitotic preparations served as internal controls for the effective inhibition of protein synthesis. This is seen most clearly for labeling of the Golgi complex in interphase cells. Fig. 10 shows clearly the decrease in G protein in the interphase Golgi stacks after treatment with cycloheximide for 30 min.

**DISCUSSION**

Our observation that transport of the VSV glycoprotein to the surface of CHO cells is quite dramatically inhibited during mitosis (Warren et al., 1983) is the first example of a physiological inhibition of membrane protein transport through the pathway from ER to the plasma membrane. In this paper we have extended this observation by attempting to assess which step in the transport pathway is inhibited during mitosis. This has been possible by making use of the posttranslational modifications of VSV G protein known to occur as the protein passes through the ER and Golgi complex.

All of the posttranslational processing of G protein that we have observed in mitotic cells is consistent with the retention of the protein within the ER. We have shown that [35S]-methionine-labeled G protein, and [3H]mannose-labeled oligosaccharides from VSV-infected, mitotic cells are digested by endo H. These high mannose oligosaccharides were shown to have a Manα1-4GlcNAc2 structure. High mannose oligosaccharides are transferred en bloc from dolichol phosphate-linked precursors to the nascent polypeptide as it is inserted into the membrane of the ER (see Hubbard and Ivatt [1981] for a review). Very soon after, three glucose residues and one outer mannose residue are trimmed from the oligosaccharides. ER glucosidases (Grinna and Robbins, 1979) and an α1,2-mannosidase (Bischoff and Kornfeld, 1983) have been identified that can perform these trimming reactions. Further modifications of G protein oligosaccharides occur in the Golgi complex. These include trimming of mannose residues, the addition of N-acetylgalcosamine, and then further trimming of other mannose residues.

| TABLE I. Quantitation of G Protein in the ER of Mitotic and G1 Cells by Immunocytochemistry |
|---------------------------------|-----------------|-----------------|-----------------|
|                                | Cell            | Nocodazole     | Cycloheximide   | G Protein*      |
| Experiment 1                   | Mitotic         | +               | −               | 52.4 ± 5.8      |
|                                | G1              | −               | −               | 19.0 ± 2.7      |
| Experiment 2                   | Mitotic         | +               | −               | 17.4 ± 1.6      |
|                                | Mitotic         | +               | +               | 25.0 ± 2.2      |

* Gold particles per square micrometer of membrane on the micrograph. Results are expressed as the mean ± SEM of values from 20 micrographs. Background, counted as gold particles over mitochondria, was 1.3 ± 0.4-1.8 ± 0.6 per μm². Gold particles not attributable to ER membranes were 7% of the total number of particles counted.
FIGURE 9 Immunocytochemical location of G protein in infected mitotic cells. VSV-infected mitotic CHO cells were incubated at 37°C until 2 h after infection. The cells were then halved, one-half was fixed immediately, and the second half was incubated for 30 min more in the presence of cycloheximide before fixation. Thin, frozen sections of both were stained with rabbit anti-G protein antibody, and then protein A conjugated to 5 nm gold. Labeling of ER in a mitotic cell is shown before (a) and after (b) cycloheximide treatment. Arrows, gold particles attributed to ER. Arrowheads, gold particles not attributable to membranes. P, plasma membrane. Bar, 100 nm.
FIGURE 10  Clearance of G protein from the Golgi complex in interphase cells after treatment with cycloheximide. The samples described in the legend to Fig. 9 contained contaminating interphase cells that served as internal controls for the inhibition of protein synthesis by cycloheximide. The Golgi stacks in interphase cells in the mitotic cell preparation were heavily labeled before treatment with cycloheximide (a), but no labeling was found over similar stacks in cells after treatment with cycloheximide (b). C, Golgi stack. Arrow, trans Golgi reticulum. Bar, 100 nm.

of mannose residues, which renders the structure resistant to digestion by endo H. The structure of the oligosaccharides isolated from mitotic cells implies that the G protein was processed by the ER glucosidases I and II as well as an α1-2 mannosidase, probably the ER enzyme. Although usually only one outer mannose residue is trimmed in the ER (Atkinson and Lee, 1984), it has been shown that the α1-2 mannosidase of rat liver ER has activity in vitro towards high mannose oligosaccharides containing from six to nine mannose residues, with preference towards Man9 (Bischoff and Kornfeld, 1983). In contrast, the Golgi α1-2 mannosidase is known to trim high mannose oligosaccharides efficiently to Man5. Therefore, G protein in mitotic cells probably had access only to the ER mannosidase. Similar trimming has been observed in other instances in which a glycoprotein was retained within the ER; for example, bovine thyroglobulin (Godelaine et al., 1981) and the Z variant of human plasma α1-antitrypsin (Hercz and Harpaz, 1980) accumulate in the rough ER of
liver with from five to seven mannose residues per oligosaccharide chain.

Although fatty acylation of G protein was always observed in mitotic cell preparations, this label was incorporated into an endo H-resistant form of G protein. We attribute this labeling to contamination of the mitotic cell preparations with interphase cells, which could not be avoided for technical reasons. This suggests that the G protein that accumulates in an endo H-sensitive form in mitotic cells does not become fatty acylated, indicating that transport of G protein is inhibited at a step before the compartment in which fatty acylation occurs. The proposed location of this enzyme is based on the kinetic relationship of fatty acylation to complex oligosaccharide formation. Endo H-resistant oligosaccharides are constructed in the trans Golgi cisternae (Roth and Berger, 1982), and G protein becomes labeled with [3H]palmitic acid 3–6 min before it acquires resistance to endo H (Schmidt and Schlesinger, 1980). This time is very similar to the time taken for G protein to move from the cis to the trans side of the Golgi stack (Bergmann and Singer, 1983). Therefore, fatty acylation appears from this evidence to be a cis Golgi function. This is supported by work on Semliki Forest virus-infected cells treated with monensin, where the spike glycoproteins become labeled with [3H]palmitic acid in or before transport to the medial Golgi cisternae (Quinn et al., 1983). If fatty acylation occurs in the cis part of the Golgi stack, our data would suggest that the first vesicle-mediated step of intracellular transport is inhibited during mitosis. However, as the rates of transport through different compartments on the pathway are unknown, such a precise morphological interpretation of the kinetic evidence may be unjustified.

Genetic evidence suggests that fatty acylation occurs in the ER in yeast secretory mutants blocked in transport between the ER and Golgi complex (Wen and Schlesinger, 1984). This would be consistent with the kinetic evidence only if the rate of transport from the ER to the Golgi complex was relatively fast, and if fatty acylation occurred at a late stage after protein synthesis, perhaps in the transitional elements of the ER. This is supported by the evidence that G protein of the ts045 mutant of VSV, which accumulates throughout the ER at the nonpermissive temperature, is not fatty acylated (Zilberstein et al., 1980), which suggests that acylation is not a function of the rough ER. If this interpretation is correct, the absence of endo H-sensitive fatty acylated G protein in mitotic cells suggests that the rough ER and transitional elements are functionally, and possibly also physically, separated during mitosis. In this instance, we cannot draw any conclusions about inhibition of vesicular transport between the ER and Golgi complex, and will only be able to do so when the fatty acylation enzyme can be located by subcellular fractionation using a direct assay such as that described by Berger and Schmidt (1984).

Neither complex oligosaccharide formation (Schlesinger et al., 1976; Robertson et al., 1978), trimming by mannnosidase (Burke et al., 1984), nor fatty acylation (Rose et al., 1984) is essential for G protein transport. Our observations therefore, could be explained if some processing enzymes performing posttranslational modifications were inactivated during mitosis but transport continued as usual. This seemed unlikely since the data presented here showed that the ER glucosidases and 1,2-mannosidase were active in mitotic cells. Also, Quinnart et al. (1979) showed galactosyl transferase activity in mitotic cell extracts. Because we could not detect mannnosidase activity in homogenates of interphase or mitotic CHO cells, we used quantitative immunocytochemistry as an independent method to confirm that G protein accumulated in the ER in mitotic cells. The observation that G protein could not be chased out of the mitotic ER during incubation with cycloheximide provides additional evidence that the inhibited step of transport occurs before, rather than within, the Golgi complex at a point before mannnosidase action and endo H resistance are conferred. G protein was probably not retained in the ER during cycloheximide treatment due to the limited capacity of the Golgi cisternae for viral glycoproteins, since viral glycoproteins have been shown to accumulate in the Golgi of cells infected with a temperature-sensitive mutant of Semliki Forest virus, ts7 (Saraste et al., 1980) and in Semliki Forest virus-infected cells treated with monensin (Quinn et al., 1983). Given the limitations of morphometry, especially at early times of infection when labeling density is rather low, these results provide good evidence that G protein does not leave the ER in mitotic cells.

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