# Newly Synthesized G Protein of Vesicular Stomatitis Virus Is Not Transported to the Cell Surface During Mitosis

GRAHAM WARREN, CAROL FEATHERSTONE, GARETH GRIFFITHS, and BRIAN BURKE
*European Molecular Biology Laboratory, 69 Heidelberg, Federal Republic of Germany*

ABSTRACT Indirect immunofluorescence, immunoelectron microscopy, and digestion by protease were used to study intracellular transport of the G protein of vesicular stomatitis virus in mitotic and interphase cells. Quantitation showed that the appearance of G protein on the surface of mitotic cells was inhibited at least 10-fold when compared with that on interphase cells, even though similar amounts of viral protein were being synthesized. This dramatic inhibition, taken together with the simultaneous inhibition of endocytosis (Berlin, R. D., and J. M. Oliver, 1980, *J. Cell Biol.* 85: 660–671), points to a general cessation of membrane traffic in the mitotic cell.

Endocytosis is important for the uptake of many nutritional and regulatory proteins (1). It is exceedingly rapid (2) and occurs at all stages of the cell cycle, with the interesting exception of mitosis (3, 4). The reason for this exception is unclear, but it does suggest that endocytosis is incompatible with other processes involving membranes that take place during mitosis. A study of this inhibition might then lend insight not only into these mitotic processes, but also into the mechanisms involved in endocytosis. As a first step we have sought to generalize this inhibition, since it did not seem likely that the cell could inhibit endocytosis and not also other pathways of vesicular traffic. We chose to look at intracellular transport because it is very well characterized (5), and we have used an enveloped animal virus as probe because, in the past, these viruses have greatly facilitated studies on membrane biogenesis (6). In this study, we have used the G protein of vesicular stomatitis virus (VSV)[1] to determine by several techniques whether it reaches the surface of mitotic cells.

## MATERIALS AND METHODS

*Immunofluorescence Experiments:* Normal rat kidney (NRK) cells were grown on coverslips (7), infected with ts045 VSV using >50 plaque forming units/cell, as previously described for Semliki Forest virus (8). Nocodazole (Sigma Chemical Co., St. Louis, MO) was used at 0.04 μg/ml (9). Cells were fixed, labeled (7, 10) with rabbit anti-G protein antibody (11), affinity purified on VSV coupled to agarose beads (12), and with an affinity-purified, rhodamine-conjugated, sheep anti-rabbit IgG (12, 13), and then stained with Hoechst dye 33258 (3).

*Experiments Using Isolated, Mitotic Cells:* The shake-off procedure described by Klevecz (14) was used for Chinese hamster ovary (CHO) cells grown as a monolayer in α minimal essential medium containing 10% fetal calf serum, penicillin, and streptomycin. The cells were infected with VSV and treated with nocodazole as described in the text. The isolated, mitotic cells, and interphase cells were incubated in the presence or absence of nocodazole and were used either to determine the amount of viral protein synthesized, by quantitative immunoblotting (15) using purified VSV (11) as standard, or for quantitative immunoelectron microscopy as follows. Thin, frozen sections were labeled (16) with anti-G protein antibody and protein A gold (5 nm diam) using solutions containing, instead of gelatin, 10% new-born calf serum, which reduced nonspecific labeling to negligible levels. Relative surface labeling was quantitated by standard stereological procedures using a transparent overlay containing a lattice test system in order to relate the number of gold particles to the number of intersections of the plasma membrane with the test lines (17). Isolated, mitotic cells ($5 \times 10^5$ cells) were also labeled with 200 μCi [$^{35}$S]-methionine for 30 min at 37°C, washed, and incubated in medium containing 0.1 mM cold methionine for 90 min (8) in the presence or absence of nocodazole. Half the sample was treated with chymotrypsin (18), and the G protein was extracted using Triton X-114 (19). Samples were fractionated by SDS PAGE, and labeled proteins were visualized by fluorography (20).

## RESULTS

### *Immunofluorescence Studies Using NRK Cells Infected with ts045 VSV*

To get infected mitotic cells that are perturbed as little as possible by the consequences of viral infection, it is important to realise that mitosis cannot be completed if protein synthesis is inhibited more than 1 h before its onset (21). VSV shuts off host protein synthesis 2–3 h after infection, depending on the cell type, and maximal G protein synthesis occurs a little later. We found, in preliminary experiments using NRK cells, that by limiting the length of any experiment to 3–4 h we were able to get infected mitotic cells that would complete mitosis as rapidly as mock-infected cells (Fig. 1) and express reasonable amounts of G protein.

The transport pathway of G protein has been examined in detail in CHO cells infected with ts045 VSV (22). We were not able to use CHO cells in these immunofluorescence

[1] *Abbreviations used in this paper:* CHO, Chinese hamster ovary, NRK, normal rat kidney; VSV, vesicular stomatitis virus.

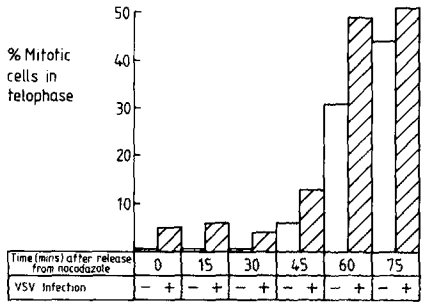
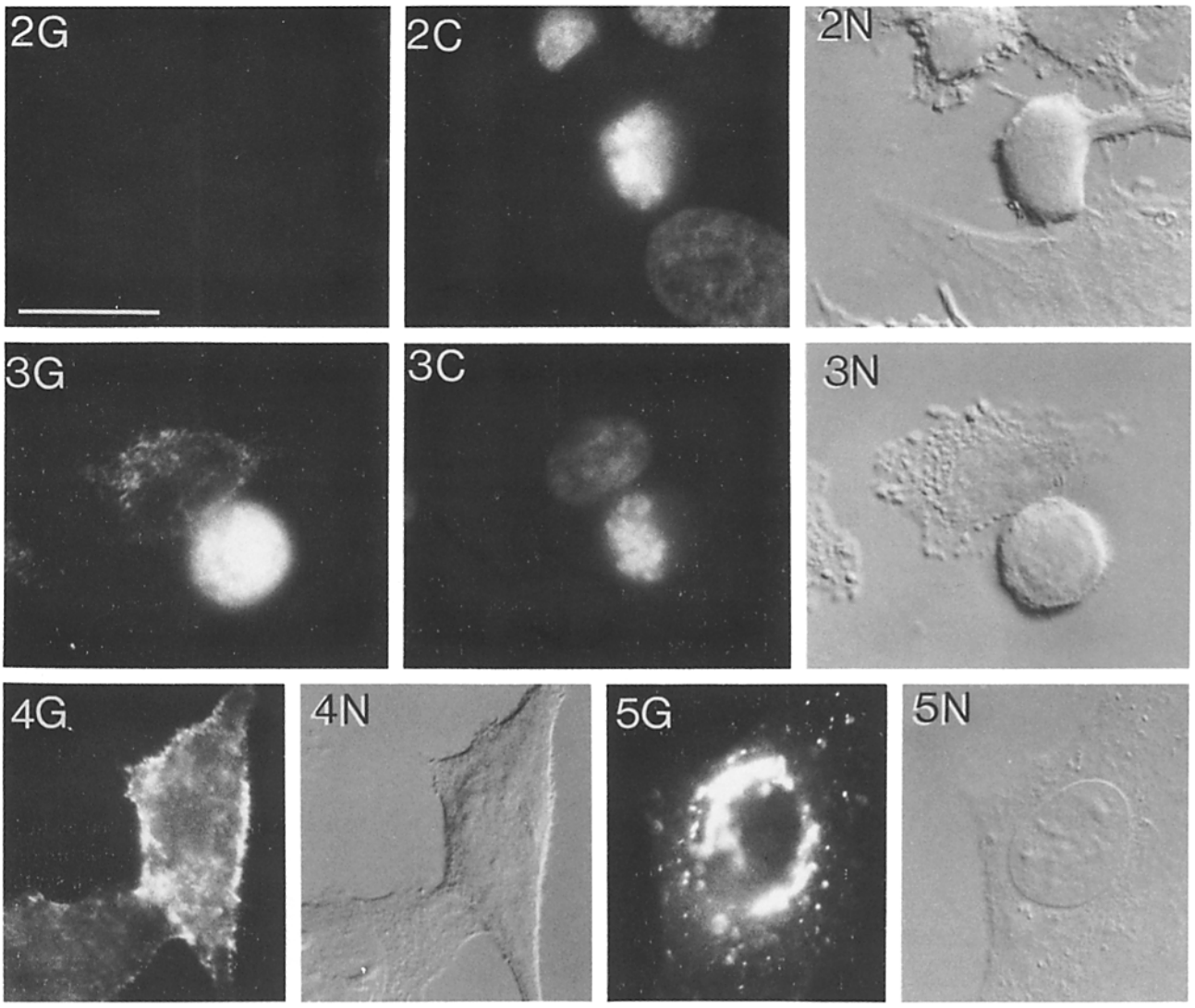


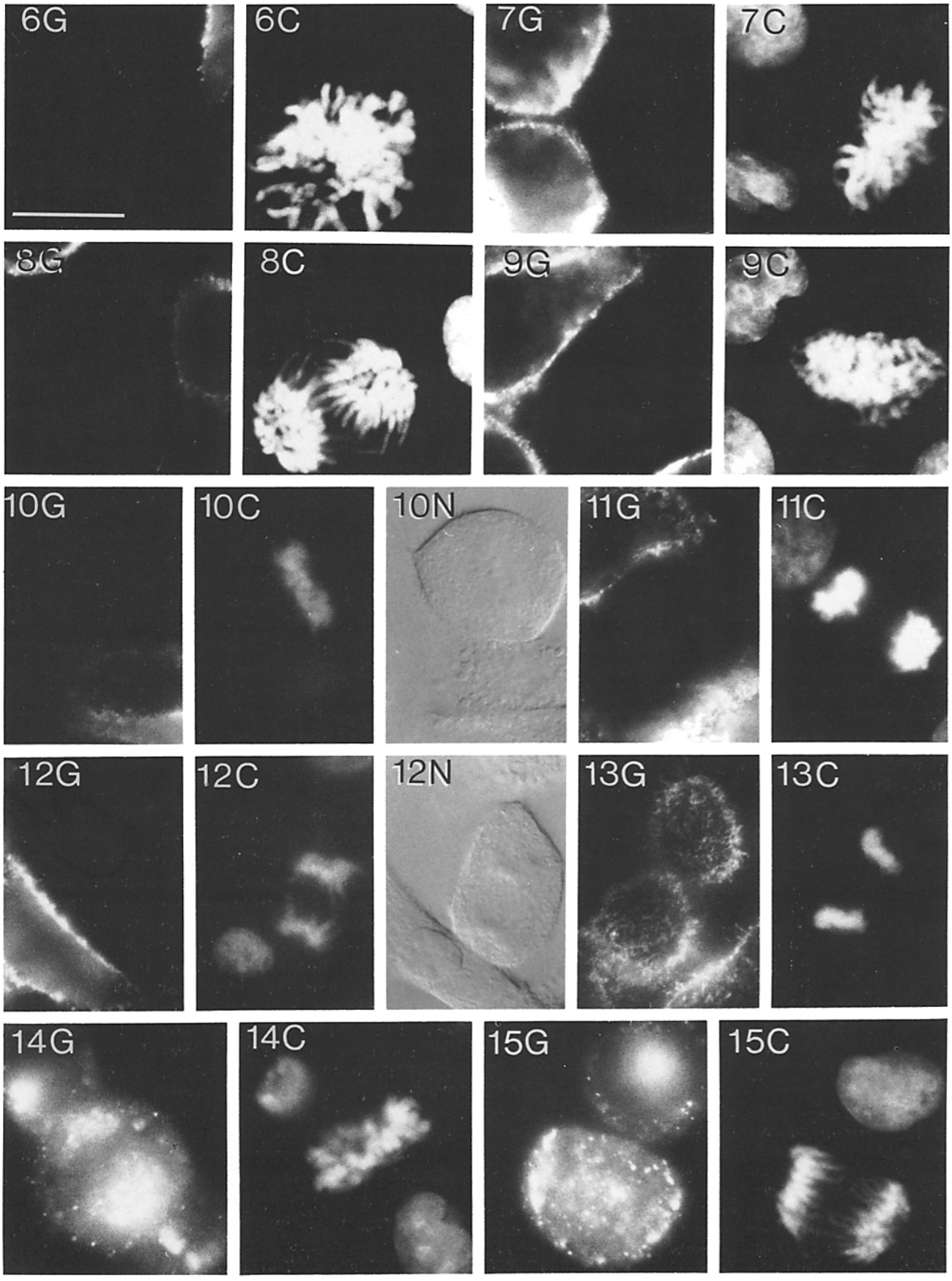

FIGURE 1 Duration of mitosis in NRK cells, mock-infected or infected with ts045 VSV. After 2 h at 39.5°C and 1 h with nocodazole, the latter was removed and the temperature was shifted to 31°C. At different times, cells were fixed and labeled with Hoechst dye 33258 to facilitate the identification of mitotic cells, and the percentage of those in telophase was determined.

studies because, in the mitotic state, they were too easily removed from the coverslip during the antibody labeling procedures. ts045 VSV behaved similarly in NRK cells (23) as shown in Figs. 2–5. The G protein was not transported to the cell surface (Fig. 2*G*) at the nonpermissive temperature of 39.5°C, but remained in the endoplasmic reticulum (Fig. 3*G*). After being shifted to the permissive temperature of 31°C, the G protein appeared in a perinuclear region containing the Golgi complex (Fig. 5*G*) and on the surface of interphase cells (Fig. 4*G*), where it could be observed as early as 15 min after the temperature shift.

To study G protein transport in mitotic cells, nocodazole was added to prevent spindle assembly (24) with the result that cells entering mitosis were held at an early stage with their chromosomes resembling those of prometaphase cells. These mitotic cells had G protein inside (Fig. 3*G*) but not on




FIGURES 2–15 G protein transport in interphase and mitotic NRK cells visualized by indirect immunofluorescence. After infection with ts045 VSV, cells were incubated at 39.5°C either for 3 h (Figs. 10–13) or for 2 h and then 1 h in the presence of nocodazole (Figs. 2–9, 14, 15). After being transferred to medium free of nocodazole at 31°C, cells were fixed either immediately (Figs. 2 and 3), or after 25 min (Figs. 4, 5, 10–15) or 45 min (Figs. 6–9), and then labeled with anti-G protein antibody before (Figs. 2, 4, 6–13) or after (Figs. 3, 5, 14, 15) being permeabilized by Triton X-100. Labeling with a rhodamine-conjugated, sheep anti-rabbit IgG was followed by staining with Hoechst dye 33258. Mitotic cells shown here are in prometaphase (Figs. 2, 3, 6), metaphase (Figs. 7, 10, 14), early (Fig. 9) and late (Figs. 8, 12, 15) anaphase, and early (Fig. 11) and late (Fig. 13) telophase. *G*: G protein, *C*; chromosomes, *N*; Nomarski optics. Bar, 20 μm. × 1,000.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their surface (Fig. 2*G*). Removal of the nocodazole allowed the arrested cells to continue through mitosis, and they took ~60 min to reach telophase (Fig. 1). This was significantly longer than the 15 min needed for G protein transport to the surface of interphase cells. If nocodazole removal was accompanied by a shift to 31°C, the permissive temperature for G protein transport, we wondered whether G protein would appear on the surface of mitotic cells. Forty min after the temperature shift, prometaphase, metaphase, and anaphase cells had very little or no surface G protein despite the heavy surface labeling of neighboring interphase cells (Figs. 6–9) and the fact that, in parallel experiments, mitotic cells were found to have high levels of internal G protein (Figs. 14 and 15). The inhibition of transport in mitotic cells was not a side effect of this drug because experiments performed in its absence gave identical results (Figs. 10–12). Transport to the cell surface resumed in telophase (Fig. 13) though it was not too uncommon to find early telophase cells with no surface label (Fig. 11).

The results were presented in a semi-quantitative form by scoring cells for surface label; the inhibition of transport during metaphase and anaphase is clearly seen in Fig. 16. Cycloheximide had no effect on this pattern of results, showing that G protein synthesized at the nonpermissive temperature was available for intracellular transport. The degree of inhibition can be calculated by determining the latest time after the temperature shift at which anaphase cells are commonly found (45 min) and dividing this by the earliest time at which G protein appears on the surface of interphase cells (15 min). This threefold inhibition is of course a lower estimate limited by the duration of mitosis and of intracellular transport.

## *Studies on mitotic CHO Cells Infected with VSV*

To determine whether the rate of intracellular transport was inhibited by more than the threefold suggested by the immunofluorescence experiments, it was necessary to arrest

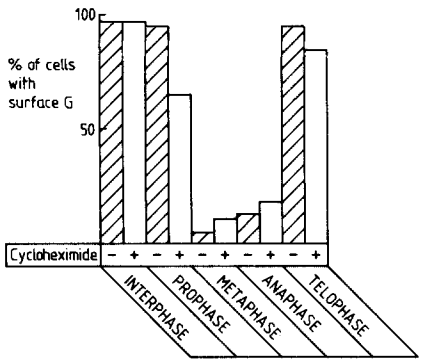


FIGURE 16 Appearance of G protein on the surface of interphase and mitotic NRK cells under conditions described in the legend to Figs. 10–13, except that incubation at 31°C was carried out in the presence or absence of 10 μg/ml cycloheximide. For each stage of the cell cycle, 50-100 cells were counted and scored as 0 (no surface labeling) or 1 (detectable surface labeling) and the results were presented as a percentage of the maximum possible score. Labeling of internal G protein in a parallel experiment showed that >98% of the interphase cells and >95% of those in mitosis were infected. Note that the occasional labeling of metaphase and anaphase cells was always very faint.

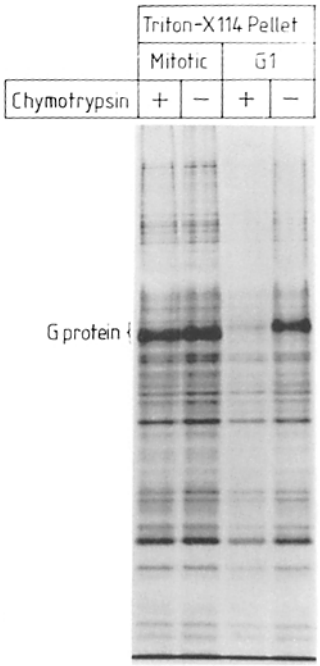


FIGURE 17 Accessibility of G protein to digestion by chymotrypsin in mitotic and $G_1$ cells. The G protein was labeled and chased in the presence or absence of nocodazole and the cells were then treated with chymotrypsin. Samples were fractionated by SDS PAGE after extraction with Triton X-114 and the labeled proteins were visualized by fluorography.

cells in mitosis. CHO cells can be arrested early in mitosis for up to 5 h using nocodazole, and >90% of them will still complete mitosis upon removal of this drug (9). However, we found that after infection by VSV this time was reduced to 3 h. To maximize use of this time window we infected the CHO cells in the roller bottle in the presence of nocodazole (0.04 μg/ml), and isolated the arrested mitotic cells 30 min later by rotation at 200 rpm for 3 min. We routinely obtained 1–3 × $10^6$ cells/roller bottle, of which >95% were infected and >90% were mitotic. Continued incubation in the presence of nocodazole caused a slight drop in the mitotic index to 80–85%, whereas incubation in the absence of this drug allowed >95% of the cells to enter $G_1$. The following experiments were carried out.

ACCESSIBILITY OF G PROTEIN TO CHYMOTRYPSIN DIGESTION: Infected mitotic cells, released from nocodazole so that they could progress through to $G_1$, were labeled using [$^{35}$S]methionine and then incubated for sufficient time to allow transport of the G protein to the cell surface. As shown in Fig. 17, the G protein in these $G_1$ cells was accessible to chymotrypsin, and ~70% of it was removed. A number of host cell proteins were still being synthesized at these early times after infection, so we confirmed the identity of the G protein by immunoprecipitation using specific antibodies (data not shown). Comparable experiments using mitotic cells kept in nocodazole showed that most of the G protein was resistant to digestion by chymotrypsin. In the experiment shown in Fig. 17, only 20% of the G protein was removed. The molecular weight of the G protein in these mitotic cells was also lower than that in the $G_1$ cells, suggesting that it had not yet acquired complex oligosaccharides (18) and had not therefore reached the Golgi complex. The inhibition of transport was not a side effect of nocodazole because transport to the cell surface of interphase cells was not affected by this drug, though we routinely found that only 40–55% of the G protein was accessible to chymotrypsin compared with the 70% found for $G_1$ cells (Fig. 17).

RELATIVE QUANTITATION OF SURFACE G PROTEIN BY IMMUNOELECTRON-MICROSCOPY: At the end of the

2.5-h incubation in the presence or absence of nocodazole, thin, frozen sections of the cells were prepared for labeling with anti-G protein antibody and protein A gold. As shown in Fig. 18, there was very little surface label in mitotic cells compared with $G_1$ cells. The surface labeling was quantitated by standard stereological techniques, with the additional advantage that contaminating interphase cells in the mitotic cell samples were easily recognized and could be ignored. Table I shows the dramatic difference between mitotic and $G_1$ cells, the former having only 6% of the surface label of the latter. To control for possible side effects of nocodazole, interphase cells were treated in a comparable way with VSV and nocodazole, and the presence of this drug was found to reduce the surface label to 70% of the original. Taken together, these results point to at least a 10-fold inhibition of G protein transport in mitotic cells.

RATE OF VIRAL PROTEIN SYNTHESIS: The conclusion just presented is valid only if the mitotic and $G_1$ cells synthesized similar amounts of viral protein. We had to show that the absence of surface G protein in mitotic cells was not simply the result of nocodazole inhibiting viral protein synthesis. Some evidence in support of this has already been provided (Fig. 17) but we also took samples for quantitative immunoblotting to determine the total amount of viral protein synthesized, and the results are presented in Table II. Despite some variation from experiment to experiment in the amount of viral protein synthesized, the ratio of the amount synthesized in the presence and absence of nocodazole in any single experiment showed clearly that, if anything, more protein was synthesized in the presence of nocodazole, not less.

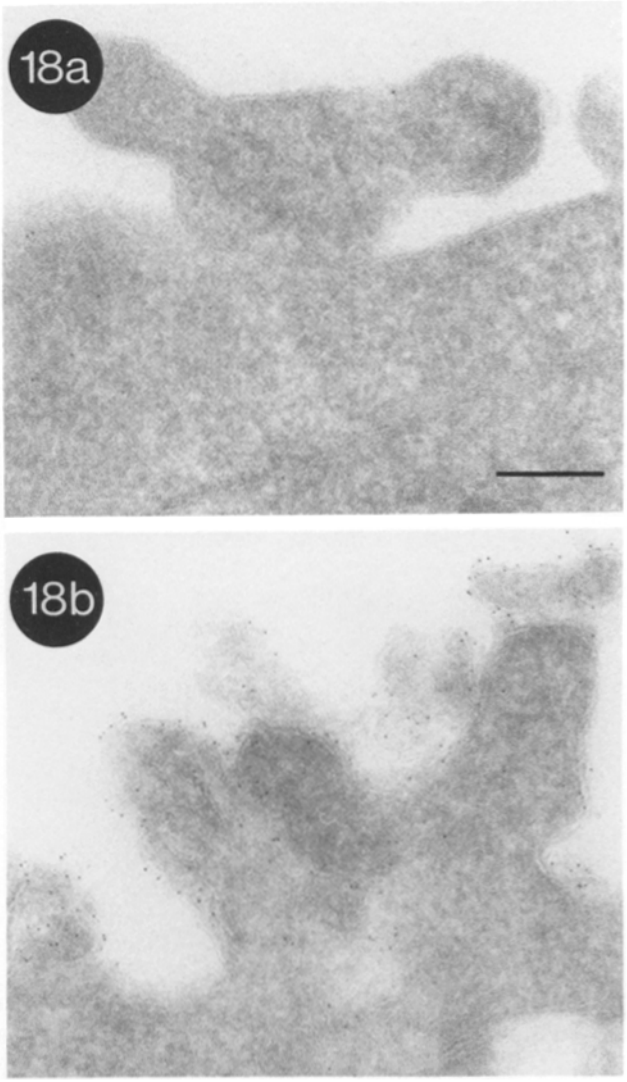


FIGURE 18 Surface G protein in CHO cells infected with VSV for 3 h and either held in mitosis using nocodazole (*a*) or released from nocodazole to give $G_1$ cells (*b*). The thin, frozen sections were labeled with anti-G protein antibodies followed by protein A gold. Bar, 0.2 μm. × 70,000.

TABLE I

*Quantitation of Surface G Protein in Mitotic and Interphase Cells after 3 h of Infection*

| Cell | Nocodazole | Surface G Protein* | % of level without nocodazole |
|---|---|---|---|
| Mitotic | + | 0.25 ± 0.09 | 6 |
| $G_1$ | − | 4.10 ± 0.37 | |
| Interphase | + | 2.44 ± 0.38 | 70 |
| Interphase | − | 3.50 ± 0.35 | |

* Gold particles per intersection. There were ~3 intersections/μm of plasma membrane and the results are expressed as the mean and standard error of the mean. For each condition 15 micrographs were used for counting.

TABLE II

*Effect of Nocodazole on the Rate of Viral Protein Synthesis in Mitotic and $G_1$ CHO Cells*

| Cell | Nocodazole | Viral protein*‡ | Protein synthesized + nocodazole‡/protein synthesized − nocodazole |
|---|---|---|---|
| | | *pg per cell/3 h* | |
| Mitotic | + | 1.4–3.7 | 1.0–1.4 |
| $G_1$ | − | 0.9–2.9 | |

* Amount of G, L, N, NS, and M proteins synthesized after 3 h of infection.
‡ The range of results was obtained from three experiments.

## DISCUSSION

Mitotic cells, infected with VSV, synthesize G protein but cannot transport it to the cell surface, as determined by the lack of surface labeling by specific antibodies and inaccessibility of the G protein to added protease. Nocodazole was used in most of these experiments to arrest cells early in mitosis, but its presence cannot explain the inhibition observed. Not only did this drug have no great effect on the appearance of cell surface G protein in interphase cells (Table 2) but it also was absent from one of the immunofluorescence experiments (Figs. 10–13) and the results were the same. A more serious objection is to the use of mitotic cells infected with a virus; one might ask whether such results can be generalized to host plasma membrane proteins. No definitive answer can yet be given, except that many pioneering studies of membrane biogenesis were made possible by the use of virally infected interphase cells and that, so far, in no single instance is the viral envelope protein known to behave in a manner different from that of a cellular counterpart.

The 10-fold inhibition of intracellular transport is a minimum estimate that can be compared with the 30-fold inhibition of endocytosis observed by Berlin and his colleagues (see reference 4). Endocytosis stops early in prophase and resumes in telophase (4). Intracellular transport also resumes in telophase and it does not occur early in mitosis in cells arrested

by nocodazole. We cannot yet say whether it is inhibited early in prophase, because the earliest time point for observation in our immunofluorescence experiments was dictated by the time taken for G protein to reach the surface of interphase cells. Prophase cells would have progressed through to, say, metaphase in this time, and the observed surface labeling of prophase cells in our experiments (Fig. 16) presumably represented late $G_2$ cells that had had sufficient time to express surface G before entering mitosis.

The inhibition of intracellular transport and endocytosis (4) point to a general cessation of vesicular traffic during mitosis, and it is not unreasonable to think that a common mechanism is responsible for this inhibition. The effect is so dramatic and unexpected that it warrants further study, and our present efforts are aimed at confirming the suggestion (Fig. 17) that the G protein is not transported through the Golgi complex in mitotic cells.

We thank Alix Cockcroft, Ruth Giovanelli, and Sabine Zimmermann for excellent technical assistance; Iris Killisch and Doris Fernholz for help with the stereological analysis; Kai Simons for critical reading of the manuscript; Karl Matlin and Hilkka Virta for the anti-G protein antibody; Nicole Genty (Orsay, France) for the ts045 mutant of VSV; Stuart Kornfeld (St. Louis, MO) for the CHO cells; and Annie Steiner for typing the manuscript.

C. Featherstone was supported by a European Fellowship from the Royal Society.

*Received for publication 13 July 1983.*

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