Immunoelectron Microscopic Studies of the Intracellular Transport of the Membrane Glycoprotein (G) of Vesicular Stomatitis Virus in Infected Chinese Hamster Ovary Cells

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ABSTRACT   An immunoelectron microscopic study was undertaken to survey the intracellular
pathway taken by the integral membrane protein (G-protein) of vesicular stomatitis virus from
its site of synthesis in the rough endoplasmic reticulum to the plasma membrane of virus-
infected Chinese hamster ovary cells. Intracellular transport of the G-protein was synchronized
by using a temperature-sensitive mutant of the virus (0-45). At the nonpermissive temperature
(39.8°C), the G-protein is synthesized in the cell infected with 0-45, but does not leave the
rough endoplasmic reticulum. Upon shifting the temperature to 32°C, the G-protein moves
by stages to the plasma membrane. Ultrathin frozen sections of 0-45-infected cells were
prepared and indirectly immunolabeled for the G-protein at different times after the temper-
ature shift. By 3 min, the G-protein was seen at high density in saccules at one face of the
Golgi apparatus. No large accumulation of G-protein–containing vesicles were observed near
this entry face, but a few 50–70-mm electron-dense vesicular structures labeled for G-protein
were observed that might be transfer vesicles between the rough endoplasmic reticulum and
the Golgi complex. At blebbed sites on the nuclear envelope at these early times there was a
suggestion that the G-protein was concentrated, these sites perhaps serving as some of the
transitional elements for subsequent transfer of the G-protein from the rough endoplasmic
reticulum to the Golgi complex. By 3 min after its initial asymmetric entry into the Golgi
complex, the G-protein was uniformly distributed throughout all the saccules of the complex.
At later times, after the G-protein left the Golgi complex and was on its way to the plasma
membrane, a new class of G-protein–containing vesicles of ~200-nm diameter was observed
that are probably involved in this stage of the transport process. These data are discussed,
and the further prospects of this experimental approach are assessed.

Integral proteins of the plasma membrane are of great impor-
tance in cellular physiology. They play key roles in cell-cell
interactions, intercellular communication, hormone recep-
tion and signal transmission, ion and metabolite transport,
and many other functions. Despite the importance of these
proteins, little is known yet about their biogenesis and the
molecular details of their intracellular transport to the cell
surface. While the presence of several integral protein enzymes
and receptors of the plasma membrane in the Golgi apparatus
(1–4) has shown that organelle to be involved in the intracel-
lular traffic of these proteins, the traffic from within the cell
to the outside has not been clearly differentiated from the

traffic in the reverse direction, and other elements of the
pathway to the cell exterior are unknown. In delineating the
details of these intracellular pathways there are limits to the
information that can be obtained by classical cell fractionation
studies. Many of the details will have to be observed in intact
cells, and for these and other ultrastructural studies, tech-
niques have been developed in this laboratory (5–7) for the
immunolabeling of ultrathin frozen sections of ultrastruc-
turally intact cells and tissues for electron microscopy. To
apply these techniques to the study of the intracellular trans-
port of membrane proteins, an experimental system is re-
quired in which the intracellular traffic of large quantities of

a single membrane protein can be synchronized.

As a model system that is suitable for these studies, we have been investigating the biogenesis in situ of the integral membrane glycoprotein (the G-protein)[1] of vesicular stomatitis virus (VSV) in VSV-infected cells. VSV is such a simple virus (its genome specifies only five polypeptides) that it relies almost exclusively on the host cell's machinery for the synthesis, posttranslational processing, and intracellular transport of its G-glycoprotein to the plasma membrane (for a review, see reference 8). As nearly one-fifth of the translational capacity of infected cells is devoted to the synthesis of this protein, it has proved considerably easier to study its biogenesis than that of other integral plasma membrane glycoproteins. In order to synchronize the passage of the G-protein through the cell, we have been able to take advantage of a temperature-sensitive strain of VSV virus, Orsay-45(0-45), which codes for the synthesis of a G-protein that is unable to leave the rough endoplasmic reticulum (RER) at the nonpermissive temperature (9, 10). However, when the incubation temperature of the infected cells is shifted down to 32°C, G-protein moves from the RER to the cell surface (9, 10). By infecting cells for 3.5 h at the nonpermissive temperature and shifting them to the permissive temperature, we have been able to synchronize the intracellular transport of this protein from the RER. Fixing the cells at various times after the temperature shift, we directly demonstrated that it passes through the Golgi apparatus en route to the plasma membrane (10). In this paper we report on preliminary studies in which we have surveyed some of the structures containing the G-protein at different stages in the intracellular pathway followed by this protein as it moved from the RER to the Golgi apparatus and also from the Golgi apparatus to the cell surface.

MATERIALS AND METHODS

Antibody Preparation and Testing: Rabbit antibody to VSV was prepared and purified on a G-protein affinity column as previously described (10). The specificity of the purified antibody was determined by the method of Towbin et al. (11). Briefly, antigen preparations were separated by electrophoresis on SDS-10% polyacrylamide gels and were then electrophoretically transferred onto nitrocellulose paper. Antigen preparations included 0-45–infected and uninfected Chinese hamster ovary (CHO) cells (3 × 10⁵ cells per lane) or purified vesicular stomatitis virions (20 µg/lane) purified from the medium of cells infected at 32°C in the presence or absence of tunicamycin (0.5 µg/ml). After the nitrocellulose paper was stained with Coomassie Blue in order to locate the different protein bands, the paper was rinsed and cut into strips that corresponded to the electrophoretic lanes. Some strips were incubated for 1 h in a fixative (2% glutaraldehyde, 3% formaldehyde, 150 mM sodium chloride, 10 mM sodium phosphate, pH 7.4) used to prepare cell specimens for immunolabeling in electron microscopy. All strips were then washed in Tris-buffered saline (150 mM sodium chloride, 10 mM Tris, pH 7.5) and soaked in a solution of 3% bovine serum albumin in Tris-buffered saline for 1 h at 37°C. Subsequent treatment of the nitrocellulose strips was carried out as described (11). Affinity-purified antibody to the G-protein was applied at 0.02 µg/ml, and antiserum to VSV was diluted 100-fold.

Cell Culture and Virus Infection: CHO cells were grown in Joklik's modified minimal essential medium with 10% fetal calf serum. Cultures were diluted daily to a concentration of 2 × 10⁵ cells/ml with fresh medium. For infection, 100 ml of CHO cells at 4 × 10⁵/ml were harvested by centrifugation at 1,000 rpm for 5 min. The supernatant medium was discarded and the cell pellet was resuspended in 1 ml of the VSV mutant 0-45. The virus was allowed to adsorb to the cells at room temperature for 30 min after which the cells were diluted in 100 ml of fresh medium. The cells were then incubated at

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[1] *Abbreviations used in this paper:* CHO, Chinese hamster ovary; G protein, the integral membrane glycoprotein of VSV; 0-45, Orsay-45 mutant of VSV; RER, rough endoplasmic reticulum; VSV, vesicular stomatitis virus.

39.8°C for 3.5 h and after appropriate times, shifted to 32° C as indicated in the text. At each such time, 10 ml of cell suspension was mixed with 5 ml of shell frozen medium in order to drop the temperature rapidly; the cells were then harvested at 4°C by centrifugation at 1,000 rpm. The supernatant medium was discarded and the cell pellet was fixed for 1 h on ice with a mixture of 2% glutaraldehyde and 3% formaldehyde in phosphate-buffered saline. The fixed cells were stored in that buffer containing 0.05% sodium azide. Minor differences in the way the cells were shifted from the nonpermissive (39.8°C) to the permissive (32°C) temperature affected the rate at which the incubation medium reached 32°C. In different experiments, G-protein was seen entering the Golgi apparatus between 3 and 6 min after transferring the cells from the 39.8°C bath. It was subsequently determined that these differences could be attributed to different rates of cooling of the medium bathing the cells. We have therefore adopted the convention of identifying the different time points by the time after the incubation temperature reached 32°C rather than the time at which the cells were removed from the 39.8°C bath.

Preparation of Cells for Electron Microscopy: The fixed pellets of cells were cut into small blocks and infused with 0.6 molar sucrose, 10% DMSO, 150 mM NaCl, and 10 mM sodium phosphate, pH 7.5. The blocks were rapidly frozen and cryosectioned. The section were then immunolabeled, positively stained with uranyl acetate, and embedded in methyl-cellulose as previously described (10). In some experiments, colloidal gold of 5-nm average particle diameter bound to protein A (12, 13) was used as a secondary reagent; in most experiments, however, a ferritin-conjugate of affinity-purified goat antibodies to rabbit IgG was used (14). Electron microscopy was performed in a Philips Model EM-300 instrument operated at 60 kV.

RESULTS

Specificity of Anti-G-Protein Antibody

As our entire methodology depended upon specific immunolabeling of the G-protein in infected cells, monospecific antibody to the G-protein was required. As can be seen from the immunoblotting experiments in Fig. 1, our affinity-purified antibody bound with high specificity to the G-protein. Very little binding could be detected to the other viral proteins. No binding was detected to host proteins present in either the infected or uninfected cells (lanes *1* and *2*, respectively). In contrast, serum prepared against UV-inactivated VSV reacted with all three of the major viral proteins (lane *7*). In addition, our affinity-purified antibody showed a similar ability to bind to the unglycosylated and the glycosylated G-
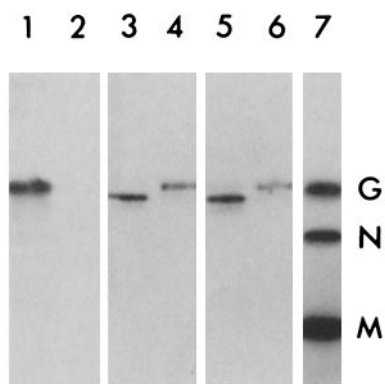

FIGURE 1   Characterization of affinity-purified rabbit antibodies to G-glycoprotein. Antigen preparations were electrophoresed on SDS polyacrylamide gels and transferred to nitrocellulose paper as described in the text. Lanes *1–6* were then labeled with the affinity-purified anti-G-protein antibody, and lane *7* was labeled with whole antiserum raised against UV-inactivated VSV virions; finally ¹²⁵I-protein A was bound, and autoradiography was carried out. The antigen preparations in the several lanes were as follows: *1:* an extract of 3 × 10⁵ VSV-infected CHO cells; *2:* a similar extract from uninfected CHO cells; *3* and *5:* 10 µg of VSV derived from infected cells grown in the presence of 0.5 µg/ml tunicamycin; *4, 6,* and *7:* VSV from cells grown in absence of tunicamycin. The proteins in lanes *1–7* were fixed to the paper with 50% methanol, 10% acetic acid. The proteins in lanes *5* and *6* were additionally reacted with 3% formaldehyde-2% glutaraldehyde before immunolabeling. (G, N, and M, viral proteins).

proteins of virus grown in tunicamycin-treated and control cells, respectively (lanes 3–6). Finally, neither of these forms of the G-protein lost their antigenicity when treated with a mixture of 2% glutaraldehyde, 3% formaldehyde—the fixative used in our subsequent immunolabeling experiments (lanes 5 and 6).

With respect to the specificity of immunolabeling, it was very high. With uninfected cells that were fixed, sectioned, and reacted with antibody to the G-protein in parallel with the infected cells, the level of indirect ferritin labeling on the control sections was very low, <1 ferritin per cell section (not shown). With the indirect gold procedure, the background nonspecific labeling was occasionally somewhat higher than with the ferritin, probably accounting for the staining of the nucleoplasm seen in Fig. 2A (but absent in Fig. 2B).

Entry of G-Protein into the Golgi Apparatus

The object of these experiments was to make a preliminary survey of possible mechanisms involved in the transfer of the G-protein from the RER to the Golgi apparatus as well as through the Golgi complex itself. We had earlier found (10)

that by ~11 min after the shift of the 0–45–infected CHO cells from 39.8°C to 32°C, the G-protein was densely and uniformly distributed throughout the Golgi saccules. Accordingly, to examine earlier stages, we fixed, processed, and examined separate aliquots of the 0–45–infected cells at short intervals after the temperature shift.

One of the more readily detected early stages was the entry of the G-protein into the Golgi apparatus. This process was observable in a majority of the cells by 3 min after the incubation temperature reached 32°C. This is shown in Fig. 2, A and B, in which indirect immunolabeling of the G-protein was carried out using the colloidal gold–protein A procedure. The labeling results using the colloidal gold or ferritin procedures were entirely consistent. Labeling for the G-protein was usually associated preferentially with one or more saccules on one side of the Golgi complex. (We refer to this label distribution as "asymmetric"). In a typical experiment at the 3-min time point, 70% of the Golgi complexes displayed this asymmetric pattern of labeling; 10% showed essentially no labeling, as before the temperature shift (10); 10% displayed slightly elevated labeling that was distributed throughout the Golgi saccules, as in the Golgi apparatus on
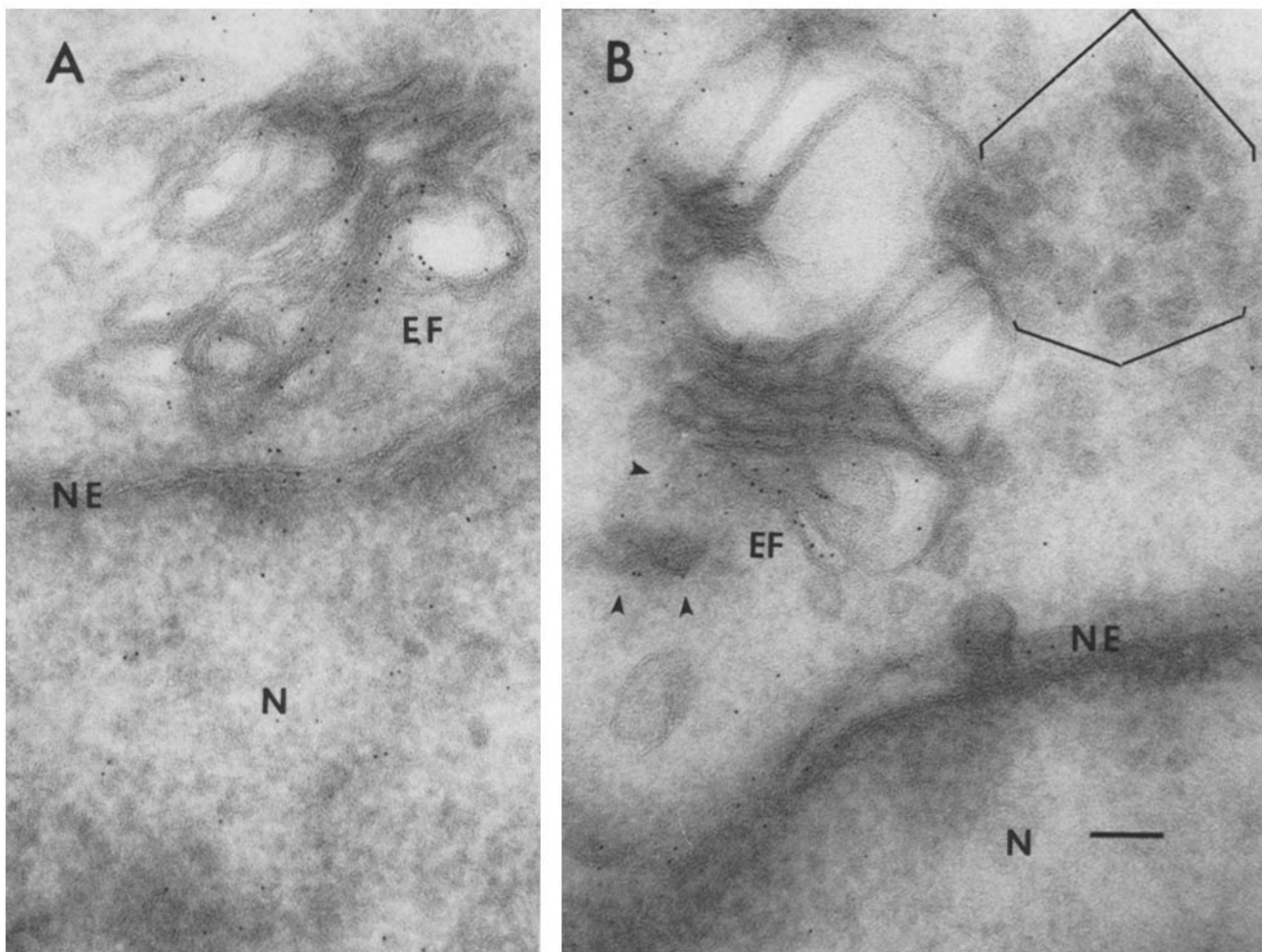

FIGURE 2 Ultrathin frozen sections of 0–45–infected CHO cells fixed 3 min after the temperature shift to 32°C, indirectly immunolabeled for the G-protein using colloidal gold–protein A as the secondary reagent. NE, nuclear envelope; N, nucleus; EF, entry face of the Golgi apparatus. (A) The nucleus-proximal face of the Golgi apparatus is immunolabeled. (B) Oblique section through the Golgi apparatus. The nucleus-proximal face is immunolabeled, as are several 50–70-nm-diam electron-dense vesicular structures (small arrowheads) near the entry face. A cluster of similar-sized and electron-dense vesicles (between brackets) is not immunolabeled. Bar, 100 nm.

the right in Fig. 3*A*; and the remaining 10% were heavily labeled throughout, as in Fig. 3*B*. When a nucleus-proximal face of the Golgi apparatus could be unambiguously identi- fied, it was the face that was labeled (*EF*, Fig. 2, *A* and *B*). However, the Golgi apparatus often appeared randomly ori- ented with respect to the nucleus; in such cases, asymmetric
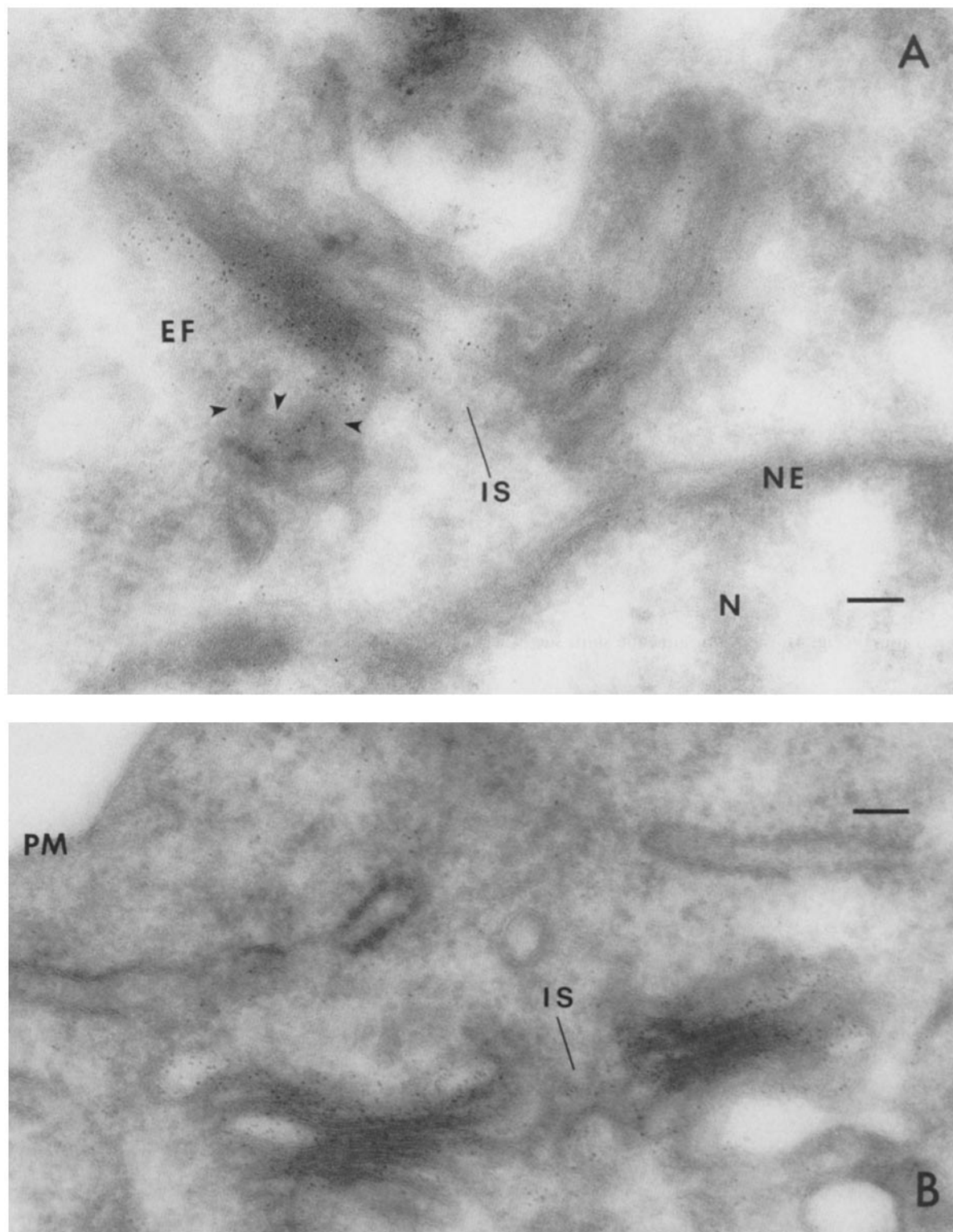
FIGURE 3 Similar specimens as in Fig. 2, except that cells were fixed 4 min after the temperature downshift, and the secondary labeling reagent was ferritin antibody. (*A*) Neighboring stacks of saccules of the Golgi complex show markedly different labeling patterns. The stack on the left side shows dense asymmetric labeling at the nucleus-proximal entry face (*EF*); the stack on the right shows only light labeling. *IS* denotes an intersaccular region between the Golgi stacks. The arrowheads designate labeled vesicular structures similar to those in Fig. 2*B* near the entry face of the stack on the left. (*B*) The Golgi apparatus of a neighboring cell on the same grid shows dense and uniform labeling across all of the saccules. The plasma membrane (*PM*) shows no labeling at this time. Bar, 100 nm.

labeling was also seen (not shown). In the present study, we refer to this side as the entry face (EF) of the Golgi, without necessarily implying any specific mechanism for its biogenesis (see Discussion).

A significant feature of this asymmetric labeling was its higher density compared with that of elements of RER (such as the nuclear envelope [NE] in Fig. 2, A and B) in the same specimens, or that of the RER just before the temperature shift (10). This increase in density was about threefold (Table I). Another feature that appears in Fig. 2, A and B is a fairly abrupt drop-off in labeling density beyond the first saccules at the EF face. This feature, however, requires more extensive study to assess its significance.

By 4 min after the temperature shift, a broader distribution of label over a greater part of the Golgi apparatus was observed. It was usually still at higher density on one side of the complex than the other (Fig. 3A). A more detailed study would be required to obtain a reasonably accurate estimate of the gradient of labeling across the Golgi complex during this stage. One of the problems was that the synchrony of transport was imperfect. 1 min after G-protein was first seen entering the Golgi apparatus, Golgi structures that were poorly labeled could be seen adjacent to those in the same cell showing a higher level as well as a gradient of labeling (Fig. 3A), while in other cells on the same grid intense labeling throughout the Golgi apparatus was observed (Fig. 3B). We found no evidence for any increased density of labeling at the dilated rims of the saccules.

By 5 min after the temperature shift, uniform labeling of the Golgi complex was most frequently seen. However, occasionally, a gradient of labeling across the stack of Golgi saccules was found (Fig. 4). By 6 min after the shift, such a gradient of labeling was no longer observed (not shown). We conclude that the movement of the G-protein across the stack of Golgi saccules was completed by 2–3 min after the G-protein first entered the Golgi apparatus.

Distribution of the G-Protein in the Nuclear Envelope

Another set of observations made at early times after the temperature shift dealt with G-protein labeling of the nuclear envelope. At various locations along the nuclear envelope, the outer and inner nuclear membranes were more widely separated than usual (Fig. 5). We refer to these sites as "blebs." One interesting feature of these blebs was that labeling for the

G-protein could clearly be associated with both the inner and outer nuclear membranes (Fig. 5, brackets). Another feature was that the density of labeling was on the average about twice as great on the membranes in the blebbed regions than in regions where the two nuclear membranes were more closely apposed (column 5, Table II). Comparable G-protein labeling measurements of blebbed and apposed regions of the nuclear envelope in cells just before the temperature shift (Table II), however, gave no evidence of a different density of labeling of the two regions at the nonpermissive temperature. There also appeared to be some increase in the relative amount of blebbed to nonblebbed regions of the nuclear membrane by 3 min after the temperature shift (column 4, Table II). These observations require much more intensive study, but they raise the possibility that the blebs are membrane sites on the nuclear envelope where a concentration of G-protein occurs as a stage in the transfer of G-protein from the RER to the entry face of the Golgi apparatus.

Vesicular Elements between the RER and the Golgi Apparatus

In a variety of secretory cells, evidence has been obtained that characteristic vesicular elements (the so-called transfer vesicles) mediate the transfer of secretory components from the RER to the Golgi apparatus (15). The membranes of such transfer vesicles might also be involved in the transfer of integral membrane components from the RER to the Golgi complex. Accordingly, with specimens of 0-45–infected CHO cells prepared during the first several minutes after the temperature shift to 32°C, we looked for the appearance of vesicular structures that were labeled for the G-protein that were candidates for transfer vesicles. Very few labeled vesicular structures were seen, however. In the thinnest sections, we occasionally saw small circular structures of ~50–70-nm diameter that were labeled (Figs. 2B and 3A, small arrowheads) and showed a high internal electron density. These were generally seen in close proximity to the labeled entry face of the Golgi apparatus, and might represent transfer vesicles. Clusters of unlabeled vesicles of similar size and internal electron density were also seen in the vicinity of the Golgi complex (between brackets in Fig. 2B). As the large majority of these vesicles were unlabeled, they were most probably not involved in transporting the G-protein to the Golgi apparatus.

Vesicular Elements Between the Golgi Apparatus and the Plasma Membrane

Regarding the stage of G-protein transport corresponding to full occupancy of the Golgi apparatus, we have little to add to the results previously reported (10) obtained 11 min after the temperature shift. At this time, all of the Golgi saccules were labeled uniformly, including the dilated rims, at a density that was similar to the density at the entry face at early times (compare Fig. 3, A and B, for example). When the Golgi apparatus was thus uniformly labeled, the two sides of the complex could not be unambigously distinguished in these experiments, as no auxiliary labeling methods for the cis- and trans-faces were used (see Discussion). We therefore could not with assurance identify elements containing G-protein that might have been in the process of budding off the trans-face.

Not until about 22 min after the temperature shift (10) was G-protein first detectable in significant quantities on the plasma membrane. We therefore made a preliminary search

TABLE I

Labeling of G-Protein at Entry Face of Golgi Apparatus

Structure	No. of micrographs	Total μm of membrane	No. of ferritins per μm of membrane
"First" saccule at entry face of Golgi apparatus	9	7.2*	31.6 ± 7.9*
Nuclear envelope	13	30.4[‡]	11.0 ± 4.5[‡]

With specimens 3 min after the temperature shift, micrographs containing clearly delineated nuclear envelope and Golgi complexes were taken at random.

* Stacks of Golgi saccules showing strong labeling of just the entry face were chosen for analysis. The entire perimeter of each first saccule was measured for this calculation. The number of ferritins per μm of membrane was then averaged over the nine micrographs (column 2) and a standard deviation calculated.

[‡] Both inner and outer membranes were included in the calculation, of both blebbed and nonblebbed regions. The number of ferritins per μm of membrane was then averaged over the 13 micrographs (column 2), and a standard deviation calculated.
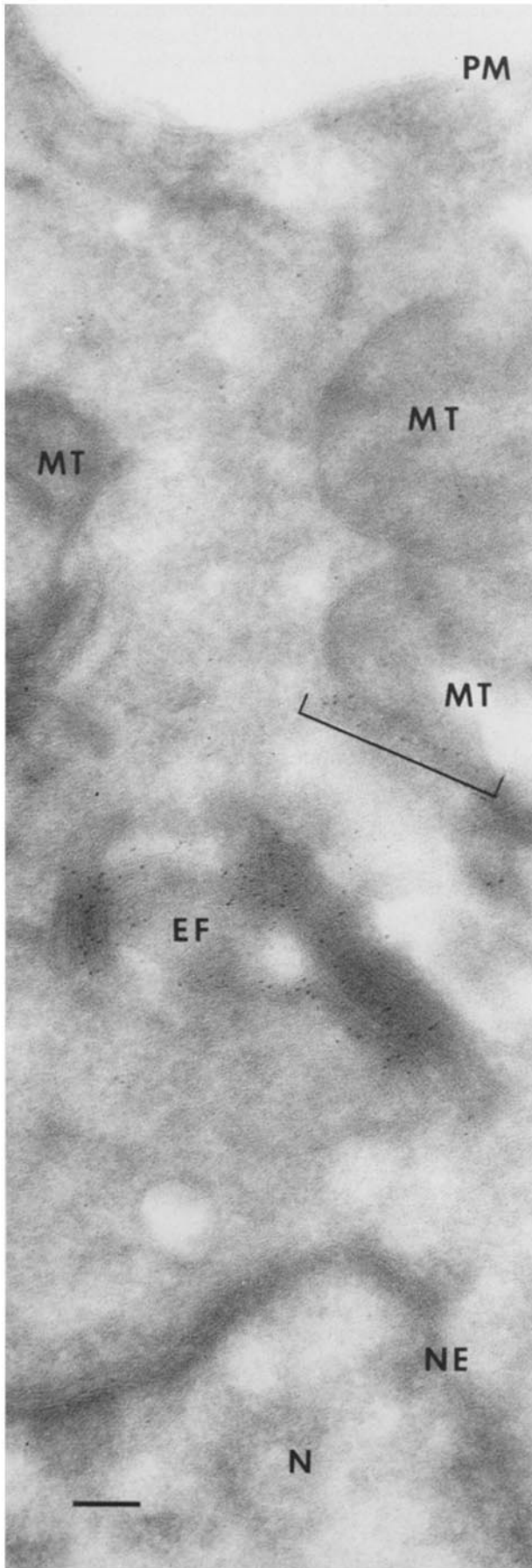
for vesicular elements labeled for G-protein in specimens 11–22 min after the shift, that might be candidates for carriers of the G-protein from the Golgi apparatus to the plasma membrane. During this time interval there was a considerable increase in labeled intracellular vesicles. These included clusters of small electron-dense vesicular structures similar to those previously observed as generally unlabeled at early times after the shift (small arrowheads, Fig. 6A and B, compare with bracket, Fig. 2B). Other labeled vesicles were seen at 22 min after the shift, however, that were not observed at earlier times. These were generally ~150–200 nm in diameter (large arrowheads, Fig. 6C and D). Their content was more electron dense than the cell exterior. These labeled vesicles are very likely involved in the transport of the G-protein from the Golgi apparatus to the plasma membrane. In Fig. 6A (intermediate-sized arrowhead), a labeled vesicle of ~100 nm is also observed; these were extremely rare and were seen even before the temperature shift.

In addition to these labeled vesicles, other unlabeled, very large and electron-transparent vesicles were observed (blunt arrows, Fig. 6B and E). These occasionally contained unlabeled particles of the size and shape of virions (Fig. 6E). Presumably, the particles were formed before the temperature shift and therefore lacked appreciable amounts of G-protein (16). At this time, very little G-protein had reached the cell surface, and the little that was there was mostly in small patches (Fig. 6C) or in budding virus structures (not shown, see reference 10).

DISCUSSION

The evidence summarized in (8) demonstrates that the intracellular transport of the VSV G-protein in VSV-infected cells is a satisfactory model for the pathway followed by integral membrane proteins from their sites of synthesis in the RER to the plasma membrane. The intracellular transport of G-protein was synchronized by the use of a temperature-sensitive mutant of VSV and a temperature shift-down protocol. With this system and the immunoelectron microscopic methodology, we previously showed (10) that the G-protein moves through the Golgi apparatus on its way from the RER to the plasma membrane. In this paper, we have explored some of the further possibilities of this system for detecting and characterizing the pathway between the RER and the Golgi apparatus, within the Golgi complex itself, and between the Golgi and the plasma membrane. This preliminary survey has by no means delineated all the elements in the pathway, but has contributed some firm new information as well as some suggestive observations that require further study. These results will be discussed in the order that corresponds to the sequence of stages in the pathway starting from the RER.

FIGURE 4 Similar specimens as in Fig. 3, except that cells were fixed 5 min after the temperature downshift. This was the latest time after the shift at which an asymmetric distribution of label at the nucleus-proximal face (EF) of the Golgi apparatus was seen. The plasma membrane (PM) shows no labeling at this time. MT, mitochondria. There is fairly intense labeling along the surface of one of the mitochondria (bracket), but not at any of the other mitochondria. We presume that the labeling is of an element of the RER situated close to the mitochondrial surface. Bar, 100 nm.
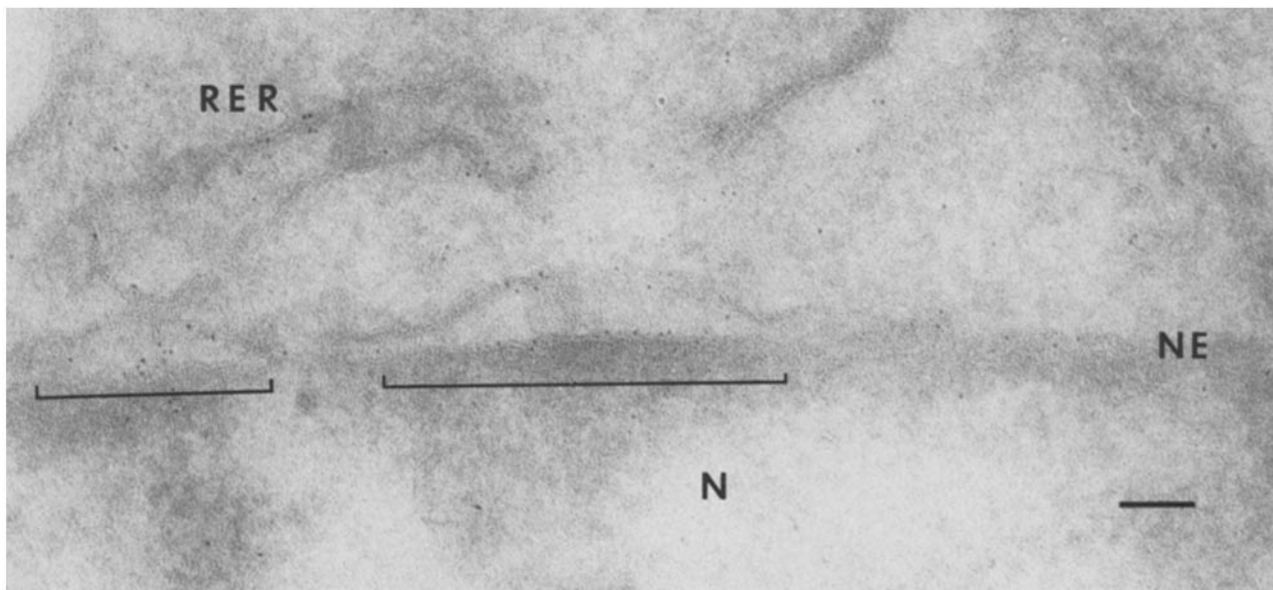
FIGURE 5 Nuclear envelope 3 min after the temperature down-shift. Blebs on the nuclear envelope are designated by the brackets, and show labeling for the G-protein at both the outer and inner membrane and an overall density of labeling that is greater than in nonblebbed regions of the nuclear envelope. Bar, 100 nm.

TABLE II

Labeling of G-Protein at the Nuclear Envelope

Time after temperature shift	Structures*	No. of micrographs	Total μm of membrane	No. of ferritins per μm of membrane‡
min				
0	Blebs	6	10.6	7.7 ± 4.6
	Nonblebs	8	44.2	6.5 ± 2.6
3	Blebs	10	26.6	15.0 ± 7.0§
	Nonblebs	12	36.4	7.0 ± 2.8§

Electron micrographs were taken at random of those regions where both inner and outer nuclear membranes were clearly distinguishable, and the total membrane in those regions was analyzed for ferritin labeling density.
* Blebs were defined as regions where the separation between the inner and outer nuclear envelope was >60 nm and included the adjoining 130 nm to either side of such separations. Nonblebs were defined as those remaining regions of the nuclear envelope where the separation between the inner and outer nuclear envelope did not exceed 60 nm.
‡ The number of ferritins per μm of membrane (see Table I) was calculated for the blebbed and nonblebbed regions of each micrograph. These values were then averaged over the number of micrographs indicated in column 3, and a standard deviation calculated.
§ A Student's *t* test of these two values indicates that the difference was significant at a 99% confidence level.

Events in the Rough Endoplasmic Reticulum

At the nonpermissive temperature, we previously showed (10) that the G-protein was labeled at a low density in the RER, including the nuclear envelope. In order to observe any changes in this distribution as the G-protein began to move out of the RER on its way to the Golgi apparatus at early times after the temperature shift, we paid particular attention to the nuclear envelope, since it was morphologically the best-defined part of the RER in these cell sections. Blebbed regions of the nuclear envelope, where the inner and outer membranes were widely separated, could be recognized both before and after the temperature shift, but appeared to increase somewhat relative to nonblebbed regions after the shift (column 4, Table II). Furthermore, the average density of labeling

for the G-protein in the blebbed regions was about twice the density in nonblebbed regions of the same nuclear envelope 3 min after the temperature shift (Fig. 5), or on the membranes of blebs in cells kept at the nonpermissive temperature (column 5, Table II). Although this increased density of labeling in the blebs is small, it is statistically significant. Such blebs might correspond to structures that have been observed morphologically at the nuclear envelope (17–19) and at the RER (20), which have been postulated to represent so-called "transitional elements" on the pathway from the RER to the Golgi complex. Such transitional elements might then pinch off to form vesicles to deliver the G-protein to the Golgi complex. On this view of the possible function of the blebs, the small average increase and the large variability of the density of labeling of the blebs after the temperature shift (column 5, Table II) could be ascribed to different functional states of the blebs, some of them perhaps not yet filled and others already emptied of the G-protein. More experiments with this system, however, as well as with other systems, would be necessary to confirm the significance of these proposals.

Contiguity of the Inner and Outer Nuclear Membranes

One of the by-products of this study has been information regarding physical relationships between the inner and outer membranes of the nucleus. The outer membrane of the nuclear envelope has long been known to be continuous with elements of the RER, and to be studded with ribosomes. The inner membrane lacks ribosomes, but has a unique peripheral protein skeleton attached to it (21). The inner and outer membranes of the nuclear envelope are separated by nuclear pores that contain "pore complexes." Little is known about the ability of these pore complexes to restrict the passage of integral membrane proteins from the outer to the inner nuclear membrane. Artifacts have plagued both immunocyto-
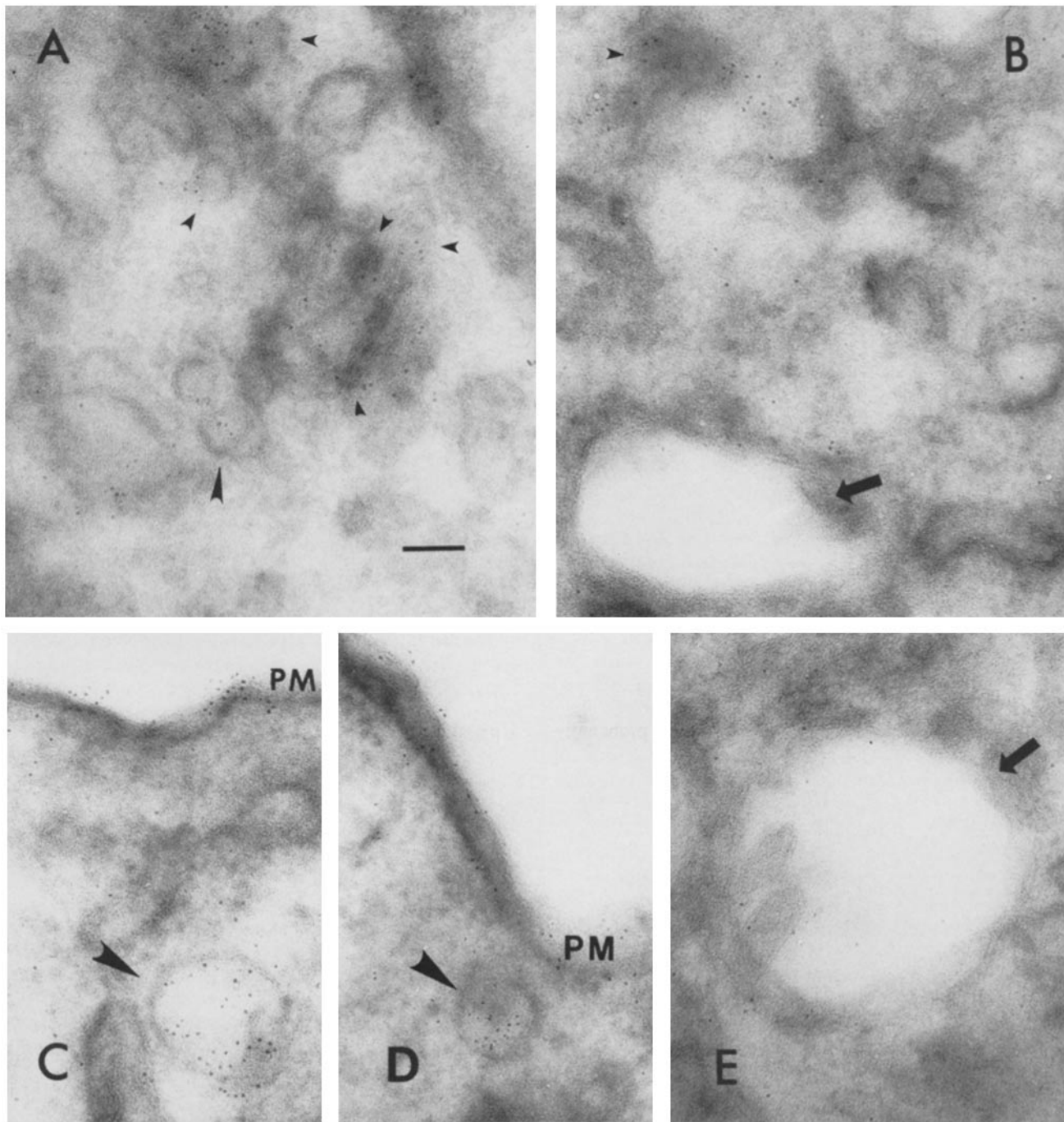
FIGURE 6 Vesicular structures labeled for G-protein seen at a time when the G-protein is first detectable at the plasma membrane (*PM*). Similar specimens as in Fig. 3, except that cells were fixed 22 min after the temperature shift. Three types of labeled vesicular structures were seen at this time. Clusters of small vesicles 50–70 nm diam (small arrowheads, *A* and *B*); 80–100-nm vesicles (intermediate-sized arrowhead, *A*); and large vesicles of 150–200 nm diam (large arrowheads, *C* and *D*). The G-protein labeling of these large vesicles appears to be internal, especially in *D*, rather than apposed to the vesicle membrane as expected. This could be due to the inclusion of the top or bottom vesicle surface within the section. In addition, unlabeled large and electron-lucent vesicles were seen (arrow, *B*). Some of these vesicles contain unlabeled virions (arrow, *E*). The G-protein that had reached the plasma membrane (*PM*) at this time was found in patches (*C*) or on budding virions (10). Bar, 100 nm.

chemical and biochemical approaches to this problem (18). Recently, Gerace et al. (22) have identified a 190,000-dalton transmembrane glycoprotein that is probably a component of the pore complex. Such transmembrane proteins could conceivably prevent mixing of integral membrane proteins between the inner and outer nuclear membranes. In the "blebs" in the nuclear envelope discussed above, the inner and outer

membranes were separated by at least 60 nm (the limit of resolution of our immunolabeling technique is 20–30 nm). In such regions it was clear that the G-protein was associated with both the inner and outer membranes (Fig. 5). It seems most likely that the G-protein, having been synthesized on ribosomes attached to the outer membrane and co-translationally inserted into that membrane, arrived at the inner

membrane by diffusion from the outer. An alternative possibility, that vesicular transport through the cisternal space carried the G-protein from the outer to the inner membrane, seems much less likely; in any event no such vesicles were observed. Thus, our evidence strongly suggests that at least some integral components of the two membranes can undergo diffusional exchange.

Transport of the G-Protein from the RER to the Golgi Apparatus

The participation of so-called "transfer vesicles" in the traffic between the RER and the Golgi complex has long been postulated, particularly in the secretory pathway (15). Accumulations of small vesicles have been observed between the RER and the cis-face of the Golgi apparatus (reference 20, Figs. 32 and 33) that have been presumed to be "transfer vesicles." We therefore searched in specimens shortly after the temperature shift for vesicular components or structures that were labeled for G-protein in the vicinity of the Golgi complex. There was no indication that labeled vesicles accumulated in large numbers near the entry face of the Golgi complex when that face was already densely labeled for G-protein (see below). However, small vesicular-like electron-dense structures containing G-protein were occasionally observed at these early times near the entry face (small arrowheads, Figs. 2A and 4B). These 50–70-nm vesicular structures are the best candidates for transfer vesicles that we found. Such labeled small vesicles are difficult to visualize in frozen sections, as membranes are not always clearly delineated. Also, since the sections are of a thickness often only slightly greater than the vesicle diameter, there is some probability that any one vesicle is not sectioned through to expose its interior membrane face to the antibody reagents. However, even taking these factors into account, only a low frequency of labeled vesicles were seen where they might have been expected. Such vesicles may exist, however, but be difficult to detect in our experiments if they are very short-lived.

It is interesting that clusters of vesicles of similar small size and electron density were seen in the vicinity of the Golgi complex at these early times (brackets, Fig. 2B) but generally showed no labeling for G-protein. While on morphological grounds alone, these vesicles might be considered as candidates for transfer vesicles, the lack of significant amounts of G-protein labeling indicates that they are not likely to be involved in the transport of G-protein from the RER to the Golgi complex.

Entry of the G-Protein into the Golgi Complex

In those CHO cells infected with 0-45 mutant of VSV, the entry of the G-protein into the Golgi complex occurred by 3 min after the temperature was shifted down to 32°C. The G-protein appearing in the Golgi at this time was very likely that previously synthesized and blocked in the RER at the nonpermissive temperature, since 3 min was probably insufficient time for a substantial amount of new G-protein to be synthesized and transported to the Golgi after the temperature shift.

Our results provide the first direct evidence that an integral membrane protein enters the Golgi complex more-or-less uniformly at one face of the complex. We have used the term "entry face" for this structure. This face at which G-protein enters the Golgi complex is probably the same as the cis-face

defined by several other parameters (23), but this was not directly demonstrated in the present study. To establish this relation, some characteristic second staining procedure would have to be used in conjunction with the immunolabeling of the G-protein. One such procedure could be the labeling of the trans-most saccules with appropriate ricin conjugates (24). The cis-face would be defined as the saccules that were not labeled with the ricin conjugates.

This asymmetric addition of the G-protein to the Golgi apparatus did not appear to depend on some unique geometric orientation of the Golgi saccules vis-a-vis the nuclear envelope and plasma membranes. Although the entry face was more frequently seen facing the nuclear envelope than oriented away from it, such asymmetric addition also occurred when the Golgi complex was located within an invagination of the nuclear envelope and when the Golgi saccules appeared to be oriented perpendicularly to the nuclear envelope (Bergmann and Singer, unpublished observation). Although definitive overall geometric orientation of these Golgi apparatuses could not be assessed without three-dimensional reconstructions from serial sections, the individual views strongly suggest that specific molecular mechanisms, rather than considerations of proximity, operate to guide the G-protein to the entry face. In experiments where the Golgi apparatus was removed from its perinuclear location and dispersed throughout the cell by the disruption of the microtubules of the infected cell, the rate of transport of G-protein to the Golgi apparatus and from the Golgi apparatus to the plasma membrane were unaffected (25). These results also suggest that the location and orientation of the Golgi apparatus are not critical factors in G-protein transport from the RER to the Golgi apparatus.

The density of G-protein in the membranes at the entry face was remarkably large (Fig. 2, A and B and Fig. 3A), approximately three times the average density in the RER membranes from which the G-protein was derived (Table I). This suggests that between the RER and the entry face some appreciable concentration of the G-protein must occur. We have no firm evidence about how or where one or more concentration mechanisms might operate. There is a suggestion, discussed above, that a concentration of G-protein might occur in the membrane of the nuclear envelope in conjunction with localized blebbed regions.

Passage of the G-Protein Through the Golgi Complex

After the temperature shift the interval between the time when the G-protein showed an asymmetric distribution at the entry face of the Golgi complex, and the time when G was distributed throughout the Golgi complex, was ~3 min. Thus the average transit time for the G-protein to traverse the entire stack of Golgi saccules was of the order of 2 min in these CHO cells at 32°C. Since each Golgi stack appeared to contain between 5 and 8 saccules, the average transit time for the G-protein between one saccule and the next was of the order of 0.3 min. These are the first direct estimates of these transit times for a membrane protein. The rapidity of these events is remarkable, and must be accommodated in any mechanistic model of Golgi apparatus dynamics.

Previous biochemical and cell fractionation studies of G-protein transit through CHO cells infected with wild-type VSV, carried out by pulse-labeling the G-protein and following the acquisition of sialyl residues on its oligosaccharide

chains (9), provided an estimate of the time interval between the synthesis of its G-protein and its presumed appearance in the *trans*-saccules of the Golgi apparatus (26). This was of the order of 20 min at 39.8°C. Thus, our estimate of a *trans*-Golgi transit time of ~2 min is well within the longer time interval.

Another relevant kinetic measurement was made by Green et al. (4), studying, however a different virus (Semliki Forest virus) infecting a different cell (baby hamster kidney). They used cycloheximide to inhibit further synthesis of the membrane proteins (spike proteins) of Semliki Forest Virus in a steady-state infected cell, and then measured by immunoelectron microscopy of ultrathin frozen sections the time course of the depletion of the spike proteins from the Golgi apparatus. They found the approximate time required for half the protein to leave the Golgi stacks was ~22 min at 37°C. It should be noted, however, that this time is quite different from the transit time across the Golgi stack. It is probably determined by one or more of the following slower steps: of spike-protein loss from the plasma membrane by viral budding; of transfer of the spike proteins from the Golgi apparatus to the plasma membrane; and of recycling of previously synthesized spike proteins in the reverse direction.

After filling the complex, the G-protein was fairly uniformly distributed throughout the saccules at a density comparable to that early seen at the entry face, and the distribution remained uniform at later times (Fig. 3B and reference 10). A similar essentially uniform distribution was observed (4) for the spike proteins of SFV throughout the Golgi apparatus under steady-state conditions. Thus there was no indication that a collection or a concentration of G-protein was produced upon passage through the Golgi complex, or that any piling up occurred at the mature face before exit from the Golgi complex. Such a uniform distribution across the Golgi complex of a protein passing through it is in contrast to the nonuniform distribution of certain endogenous integral proteins of Golgi membranes, such as the *trans*-side concentration of the enzyme galactosyl transferase (27).

These results on the entry of the G-protein into the Golgi apparatus, and its passage across the stack of saccules, bear on some of the basic problems of the biogenesis and dynamics of the Golgi complex. The history and current status of these problems have recently been admirably reviewed (23). Briefly, two major views exist about the mechanisms of transport into and across the Golgi apparatus. One view, which Farquhar and Palade (23) refer to as the "conveyor-belt" model, is that a *cis*-face saccule forms de novo by the fusion of appropriate transfer vesicles derived from the RER, then moves as a unit across the complex. When it becomes the *trans*-most saccule, it is finally dispersed into vesicular elements that leave the Golgi apparatus. The other view supposes that vesicular transfer between successive saccules is the mechanism of transport across the Golgi stack. In this view, the individual saccules retain their positions in the Golgi apparatus, but their membrane and cisternal contents are in continual flux, presumably by way of vesicular elements that selectively transfer components moving through the Golgi in one direction from other components moving in the opposite direction. This *trans*-Golgi traffic is accompanied by vesicle-mediated recycling events between the *trans*-elements of the Golgi and the plasma membrane, and between the *cis*-elements of the Golgi and the RER.

Our present results do not discriminate between the two models, but more detailed studies along related lines might

help to do so. A critical difference between the two models is in their respective predictions about the gradient of the G-protein across the stack during the time interval in which it was filling the stack. On the vesicle transfer model, the filling of successive saccules should occur gradually as more and more G-protein was transferred. On the conveyor-belt model, however, there should always be an all-or-none distribution between successive adjacent saccules as filling progressed. Unfortunately, given the very rapid transport across the Golgi stack and its imperfect synchrony; the diverse geometries of the Golgi saccules in our sections; and the limitations of the immunolabeling technology used in this study; we did not obtain the data that might firmly distinguish between these two possibilities in this investigation.

Transport of the G-Protein from the Golgi Complex to the Plasma Membrane

It is generally believed that the shuttling of components between the Golgi complex and the plasma membrane occurs by way of vesicles (15). By immunolabeling for the G-protein of 0-45–infected cells between the time after the temperature shift when the Golgi apparatus was first filled with G-protein and the time when G-protein could first be detected at the cell surface in significant amounts, any new vesicles containing the G-protein could be identified as outward-bound. During this time interval, clusters of 50–70 nm vesicles similar in size and electron density to those seen in Fig. 2, A and B were found to be labeled with increasing frequency (small arrowheads, Fig. 6, A and B). It is possible that these are transfer vesicles between the Golgi apparatus and the plasma membrane. On the other hand, it is also possible that they represent transfer vesicles between the RER and the Golgi apparatus, such as the similar ones in Fig. 2B, which were transporting a population of G-protein molecules that were newly synthesized after the shift to the permissive temperature. (These two possibilities might be distinguished by appropriate double labeling experiments, see below.)

In addition to these small vesicles, a new class of labeled vesicles 150–200-nm in diameter were observed (Fig. 6C and D, large arrowheads) at a time coincident with the first detectable appearance of G-protein at the plasma membrane. The contents of these vesicles were more electron dense than the medium surrounding the cells, an additional indication that they were not derived by endocytosis of the plasma membrane. This class of vesicles is a strong candidate for a transport function between the Golgi apparatus and the plasma membrane, but whether they were directly derived from the *trans*-face of the Golgi, or were the result, for example, of intervening vesicle fusion events, was not ascertained in these experiments.

Besides the two types of vesicles just discussed, there appears in Fig. 6A (medium-sized arrowheads) an intermediate-sized labeled vesicular structure of ~10-nm diameter. Such structures were very rare, and were seen even before the temperature shift. Their role in G-protein transport is unclear; because they are observed at the nonpermissive temperature, they may be associated with the endoplasmic reticulum. Other vesicular structures were seen at this time that were as electron lucent as the extracellular space, but their boundary membranes were not labeled for the G-protein. The presence of occasional virus particles in these latter vesicular structures (Fig. 6E), but not in the former ones, further suggests that only these

electron-lucent vesicles were inbound from the plasma membrane.

In this survey study, we have characterized certain features of the intracellular pathway of an integral membrane protein on its way from the RER to the plasma membrane, using a particular virus-infected cell system under one set of experimental conditions. Much additional information may be derived extending the same experimental approach. The intracellular transport of the G-protein could be slowed down by a temperature shift to still lower temperatures than 32°C, perhaps allowing highly transient intermediate structures to be trapped and observed. Furthermore, the application of double and perhaps triple electron microscopic immunolabeling procedures on the same ultrathin frozen section (7) should enable more definitive characterization of elements in the pathway to be made. For example, we have already referred to the use of appropriate ricin conjugates (24) as labels to distinguish *trans-* from *cis-*elements of the Golgi apparatus on specimens immunolabeled for the G-protein. The same type of double labeling might distinguish G-protein–containing vesicles bound for the Golgi apparatus from the RER, from similar vesicles bound for the plasma membrane from the Golgi apparatus. Double immunolabeling for the G-protein and clathrin (28) could determine whether coated vesicles were involved at different stages in G-protein transport, as has been suggested might be the case (29). The future possibilities of this approach to the study of intracellular transport are promising.

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