Vesicular Stomatitis Virus Glycoprotein, Albumin, and Transferrin Are Transported to the Cell Surface via the Same Golgi Vesicles

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ABSTRACT   Human hepatoma cells, infected by vesicular stomatitis virus, offer a good system to study simultaneously the intracellular localization of a well defined transmembrane glycoprotein (VSV-G), a secretory glycoprotein (transferrin), and a nonglycosylated secretory protein (albumin). We used monospecific antibodies in combination with 5- and 8-nm colloidal gold particles complexed with protein A to immunolabel these proteins simultaneously in thin frozen sections of hepatoma cells. VSV-G, transferrin, and albumin are present in the same rough endoplasmic reticulum cisternae, the same Golgi compartments, and the same secretory vesicles. In the presence of the ionophore monensin intracellular transport is blocked at the trans cisternae of the Golgi complex, and VSV-G, transferrin, and albumin accumulate in dilated cisternae, which are apparently derived from the trans-Golgi elements. Glycoproteins, synthesized and secreted in the presence of monensin, are less acidic than those in control cultures. This is probably caused by a less efficient contact between the soluble secretory proteins and the membrane-bound glycosyltransferases that are present in the most monensin-affected (trans) Golgi cisternae.

Secretory proteins and plasma membrane glycoproteins in eucaryotic cells are synthesized in the rough endoplasmic reticulum (ER),[1] and are transported in a series of membrane vesicles to the cell surface (1, 2). A number of lines of evidence suggests that both classes of proteins migrate through the Golgi complex, but it is not known whether exactly the same intracellular pathway is employed. In favorable cases, a combination of cell fractionation and immunoelectron microscopic techniques were used to show the transient accumulation of proteins such as vesicular stomatitis virus glycoprotein (VSV-G), histocompatibility proteins, and predominant secreted proteins in the Golgi cisternae (2–4). Additionally, the high-mannose oligosaccharides added to nascent secretory and plasma membrane glycoproteins undergo the same intracellular modifications; many of the modifying enzymes have been localized either to the rough ER or to the Golgi complex (5, 6).

Hepatoma cells infected with VSV offer a good system to study the intracellular transport of secretory proteins (albumin and transferrin) and an integral membrane protein (VSV-G). We have described the kinetics of the intracellular transport of transferrin and VSV-G from the rough ER to the Golgi complex and from the Golgi complex to the plasma membrane, using acquisition of resistance to the endo-glycosidase endo-β-N-acetylglucosaminidase H (Endo H) as a probe for the localization of the proteins to the Golgi complex (7). In these rat hepatoma cells, transferrin travels through the cell much slower than does albumin or VSV-G. Importantly, most of the cell-associated VSV-G and transferrin was sensitive to Endo H, and we concluded that the rate-limiting step in maturation was movement from the rough ER to a region of the Golgi, wherein certain key oligosaccharide processing enzymes are localized. Subcellular fractionation studies combined with characterization of the Endo H-sensitive oligosaccharide on intracellular transferrin confirmed that the rate-limiting and distinctive step in maturation of secretory proteins is movement from the rough ER to the Golgi complex (8).

[1] *Abbreviations used in this paper:* Endo-H, endo-β-N-acetylglucosaminidase H; ER, endoplasmic reticulum, PBS, phosphate-buffered saline; VSV-G vesicular stomatitis virus glycoprotein.

We showed that monensin, a Na⁺-ionophore that blocks intracellular transport within the Golgi complex, did inhibit the transport of albumin, transferrin, and VSV-G to the same extent. However, it blocked transferrin in a stage in which the molecule is still sensitive to Endo H, while VSV-G reached a stage where it was resistant to the enzyme. This result led us to the conclusion that part of the intracellular path (near the Golgi complex) may be different for membrane and secretory proteins (7).

In this study we used immunoelectron microscopy to investigate the exact intracellular localization of these proteins at the highest possible resolution. We applied to ultrathin sections our recently developed immuno-double-labeling technique with two sizes of colloidal gold particles complexed to protein A (9, 10). We describe here the exact localization of VSV-G together with albumin and transferrin in the human hepatoma cell line HepG2. These cells synthesize and secrete albumin and transferrin in high quantities and the kinetics for intracellular transport are similar to the kinetics in the rat hepatoma cells (7).

Our principal conclusion is that the membrane protein VSV-G and the two secretory proteins are present in the same Golgi compartments and secretory vesicles, both in the presence and absence of monensin.

MATERIALS AND METHODS

Cells and Viruses: The human hepatoma cell line HepG2 was cultured in monolayer in Eagle's minimal essential medium containing 10% fetal bovine serum (11). The Indiana serotype of VSV was originally obtained from C. Pringle (Institute of Virology, Glasgow, U.K.). 60-mm diameter dishes were infected with 10 plaque-forming units of VSV per cell. After 60 min, the nonabsorbed virus was removed and the cells were incubated for 3.5 h at 37°C in minimal essential medium containing 10% rabbit serum. The rabbit serum was used to avoid immunolabeling on the cryosections caused by cross-reacting antigens from the serum taken up by the cells. Immunofluorescent labeling of the cells after this procedure using anti-G antibodies showed that all cells were infected, albeit not to the same degree.

Radioactive Labeling of HepG2 Cells with [³⁵S]Methionine: Cells grown on 60-mm culture dishes until near confluency were washed with 5 ml of phosphate-buffered saline (PBS) and incubated with 2 ml of medium without methionine and serum at 37°C. After 20 min, 80 μC [³⁵S]methionine (800–1,200 Ci/mmol, The Radiochemical Centre, Amersham, England) was added and the culture was incubated for a further 60 min. Then the medium was aspirated. The cells were washed twice with PBS and 3 ml of minimal essential medium, supplemented with 50 μM unlabeled methionine and 1% rabbit serum was added. At the end of the appropriate chase time the cultures were chilled on ice. The medium was saved and clarified by centrifugation at 10,000 g for 10 min. The cells were washed twice with 5 ml of PBS and lysed in 1.5 ml of 1% Triton X-100 and 0.1 mM phenylmethylsulfonylfluoride in PBS. The lysate was clarified by centrifugation at 10,000 g and used for quantitative immunoprecipitation. Immunoprecipitations contained 100 μl immunomix (12), 100 μl lysate, or 200 μl medium, and 10 μl antiserum raised in rabbit against total human serum proteins (Nordic Immunology, Tilburg, The Netherlands). Immunoprecipitations, gel electrophoresis and fluorography was carried out as previously described (12). Monospecific anti-galactosyltransferase raised in rabbit against human milk enzyme was kindly provided by Dr. E. G. Berger, (University of Bern, Switzerland). Two-dimensional gel electrophoresis was according to the procedure of O'Farrell (13) and employed pH 3.5 to 10 ampholines and 10% (wt/vol) polyacrylamide gels.

Fixation and Cryoultramicrotomy: Cells, attached to the culture dish, were fixed in 1% acrolein in 0.1 M phosphate buffer, pH 7.4, for 30 min and then in 0.5% glutaraldehyde in phosphate buffer for 60 min at 4°C. The cells were then rinsed twice with 0.1 M phosphate, pH 7.4, and scraped off the dish in 1 ml of buffer. The cell suspension was centrifuged in an Eppendorf tube and the pellet resuspended in 10% gelatin (37°C). After 2 min the cells were again centrifuged and the pellet was flattened at 0°C to give a very thin layer of solidified gelatin containing the fixed cells. Cells were stored in 8% paraformaldehyde and 1 M sucrose at 4°C.

Antibodies: For immunocytochemistry we used rabbit antibodies against human albumin and transferrin. These commercial sera (Nordic Im-

munology, Tilburg, The Netherlands) were affinity purified on albumin- and transferrin-AcA-22 Ultrogel columns (LKB Produkter, Stockholm). The purified IgG fractions were tested by Ouchterlony and immunoreplica techniques and were monospecific. For most of the immunocytochemical localization of VSV-G we used a monoclonal antibody (clone c-1, a-7), kindly provided by Dr. Mark Pasternak (Massachusetts Institute of Technology). In this case rabbit anti-mouse IgG was used as an intermediate step. The antibodies were used at a concentration of ~50 μg/ml.

Immunocytochemistry: Cryosectioning and immunolabeling were essential as described before (9). Briefly, ~100-nm cryosections were prepared according to Tokuyasu (14). The sections were immunolabeled with protein A-colloidal gold probes, prepared as described before (15). For single labeling, antibody binding sites were labeled with 8 nm colloidal gold particles. For double labeling, the first antibody was labeled with 4.5 nm protein A-colloidal gold and the second with 8-nm gold probe. To exclude any interference between the first and the second gold probe, we used an intermediate incubation step with 0.05 mg/ml protein A. After immunolabeling, the sections were stained in uranylacetate and embedded in 1.5% methylcellulose. Control sections were treated with affinity purified anti rat pancreas amylase or with protein A-colloidal gold only. Background labeling appeared to be negligible.

RESULTS

HepG-2 cells produce at least 20 known serum proteins (16). When the cells, after infection with VSV, are pulse-labeled with [³⁵S]methionine and chased with unlabeled methionine, different secretory proteins are transported through the cell and are secreted at different rates (7, 8). Pulse-labeled albumin is secreted almost completely in 60 min, while the secretion of labeled transferrin is not yet completed by 150 min (7). These results are similar to those obtained in uninfected HepG-2 cells (8). Monensin impedes transit of secretory and membrane proteins from the Golgi system to the secretory vesicles without interfering with other cellular events as the rate of protein synthesis or transport and discharge of granules distal to the Golgi complex (17). In the presence of 5.10^{-7} M monensin the secretion of both proteins is reduced by a factor of approximately four in HepG-2 cells (data not shown).

Two-dimensional gel analyses of the secretory proteins show that most of the labeled secretory proteins isolated from the cells migrate as single species. This is consistent with studies using Endo H which indicate that "complex" sugars are not present on the majority of intracellular secretory glycoproteins (8). However, secreted proteins exhibit marked charge heterogeneity (Fig. 1). Transferrin and C_3 β-chain migrate as multiple spots, most likely due to the presence of variable amounts of sialic acid. The presence of monensin during pulse-chase labeling affects the charge heterogeneity considerably. Most of the secreted glycoproteins are more basic, which suggests that they contain less sialic acid then normal.

Immunocytochemistry of VSV-infected hepatoma cells makes it possible to compare the biochemical data concerning intracellular transport to the actual localization of the abundant membrane protein VSV-G as well as of the two major secretory proteins, albumin and transferrin. We used colloidal gold particles complexed with protein A to visualize the binding sites of antibodies specific for albumin, transferrin, and VSV-G. After 4.5-h infection by VSV, cells were prepared for immunostaining. Double label immunocytochemistry using 5-nm colloidal gold particles as tags for albumin and 8-nm gold particles for VSV-G is shown in Fig. 2. Both labels are present in the same compartments: the rough ER, small vesicles at the cis-Golgi side, the Golgi stack and larger vesicles in the trans-Golgi area and close to the plasma membrane (Fig. 2). Because of this localization and their secretory protein content, these vesicles are most likely secretory vesicles. The
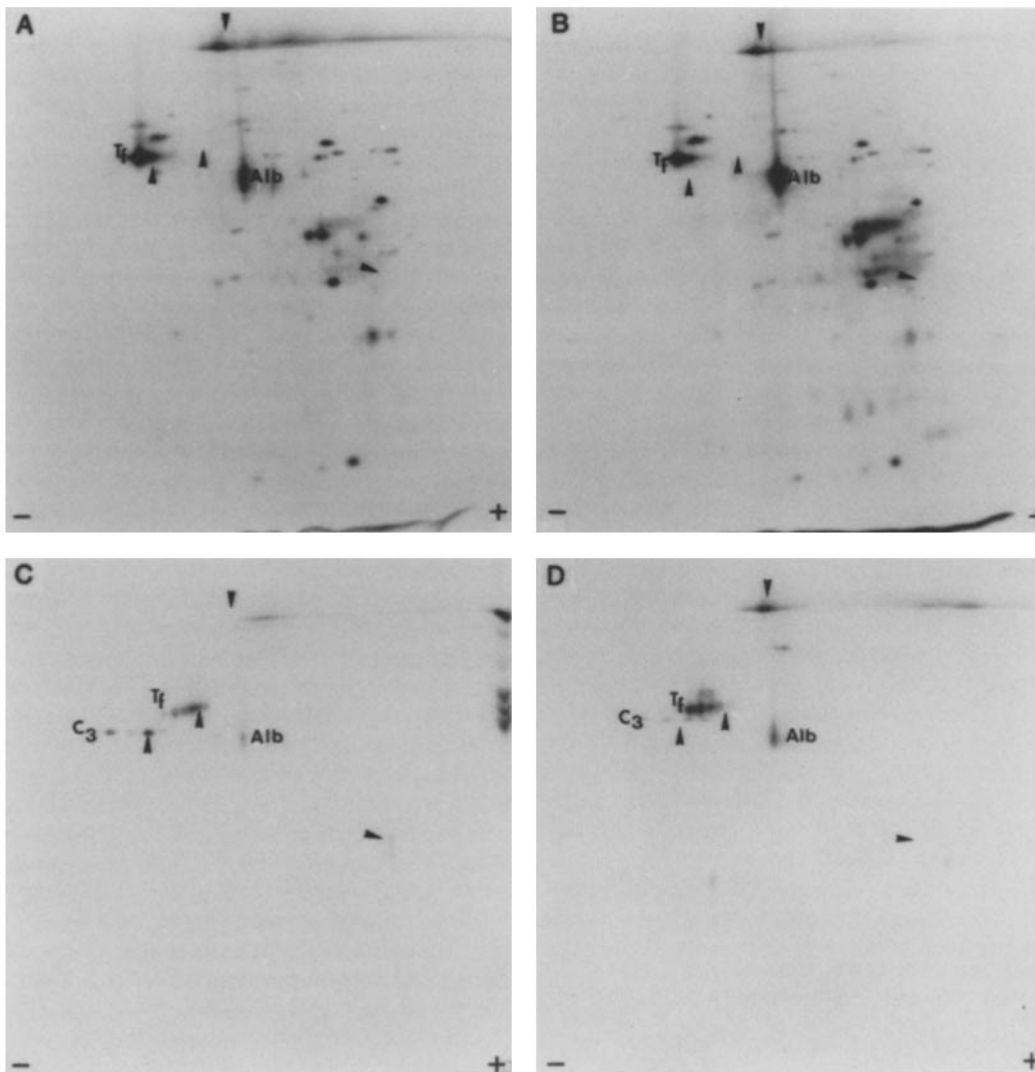
FIGURE 1 Two-dimensional gel electrophoresis of [^{35}S]methionine-labeled immunoprecipitated secretory proteins. Cells, infected with VSV for 4.5 h, were labeled for 60 min and then chased with unlabeled methionine for 3 h. The cells were lysed and the media were clarified by centrifugation. Secretory proteins were isolated by immunoprecipitation. The intracellular form of secretory proteins is seen to be single spots (A). After monensin treatment the pattern is not changed (B). Secreted glycoproteins appear as families of spots, more acidic as compared to the intracellular species (C). When monensin is present, secreted glycoproteins are less acidic (D). In the isofocussing dimension the basic end is to the left. Alb, albumin; Tf, transferrin; C$_3$, β-chain of complement factor 3. Arrowheads point to identical positions in the two-dimensional gels. The right side of C shows total proteins separated only in the SDS gel.

8-nm gold particles are present at the luminal side of vesicles and cisternae (see also Fig. 4), indicating that the (monoclonal) antibodies recognize a region of G localized at the extracyto-plasmic surface of cisternae and vesicles. Vesicles at the trans-side of the Golgi stack are clearly devoid of label. G labeling at the cell surface is rare and background label over nuclei and mitochondria is low.

In a section in which VSV-G and transferrin are double stained, G is present over the Golgi region (Fig. 3). There is almost no transferrin label in the Golgi stack, but transferrin label is clearly present in rough ER cisternae (Fig. 3, inset). In some cells, abundant G protein is present in the rough ER, while albumin or transferrin is practically absent. These cells are probably so well infected that the host cell protein synthesis has been inhibited considerably (Fig. 4).

To obtain more insight in the transport route, we used the ionophore monensin; its effect on the morphology and the

distribution of G protein, albumin, and transferrin is shown in Fig. 5. After 30 min in the presence of 5.10^{-7} M monensin, the Golgi cisternae become dilated. Albumin and VSV-G (Fig. 5a) are present in all Golgi elements with the highest concentrations in the most dilated parts of the Golgi complex. Fig. 5b shows transferrin present in the same locations as G. Although the amounts of antitransferrin label are less than that of albumin, there is a significant increase in the amount of transferrin present in the Golgi complex, compared to the situation without monensin (Fig. 3). Monensin causes accumulation of all three antigens in the most dilated vesicles of the Golgi complex. The latter part of the Golgi complex are derived from the trans-side of the stack as can be concluded from immunolabeling with antigalactosyltransferase antibodies (Fig. 6). Galactosyltransferase is normally present in one or two trans-cisternae of the Golgi stack (18). Double-labeling with anti-G shows that, after monensin treatment,
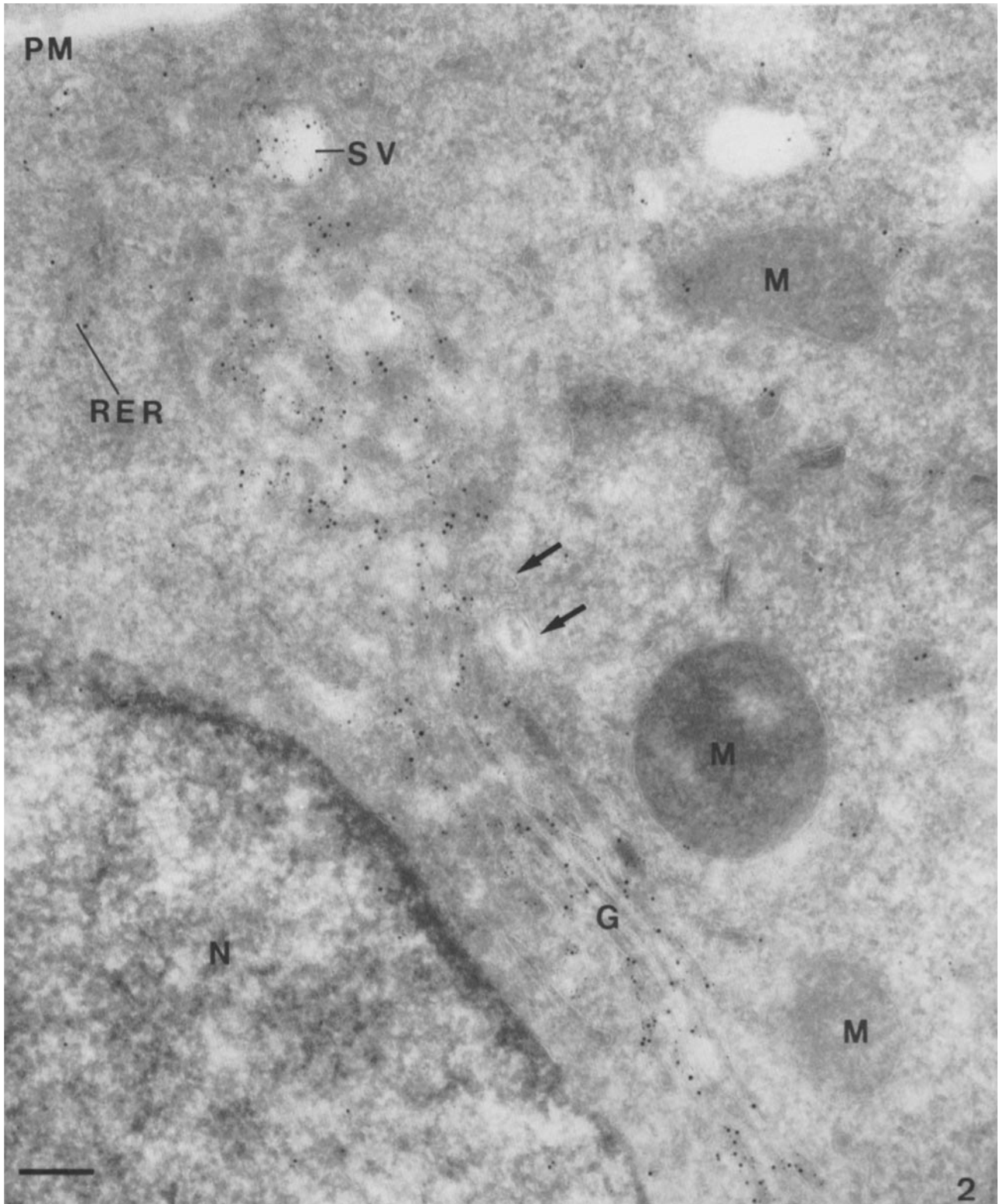
FIGURE 2   Distribution of VSV-G and albumin in the Golgi region of a HepG-2 cell. Binding sites of the first antibody (antialbumin) were labeled with 5-nm gold particles and those of the second antibody (VSV-G) with 8-nm gold particles. Both antigens are equally distributed over all cisternae of the Golgi complex (G) and in smaller vesicles near the Golgi stack. Vesicles at the trans-side of the complex are devoid of label (arrows). Both labels are also present in the numerous small structures between the Golgi complex and the plasma membrane. N, nucleus; RER, rough endoplasmic reticulum; SV, secretory vesicle; PM, plasma membrane; G, Golgi complex. Bar, 0.2 μm. × 65,000.
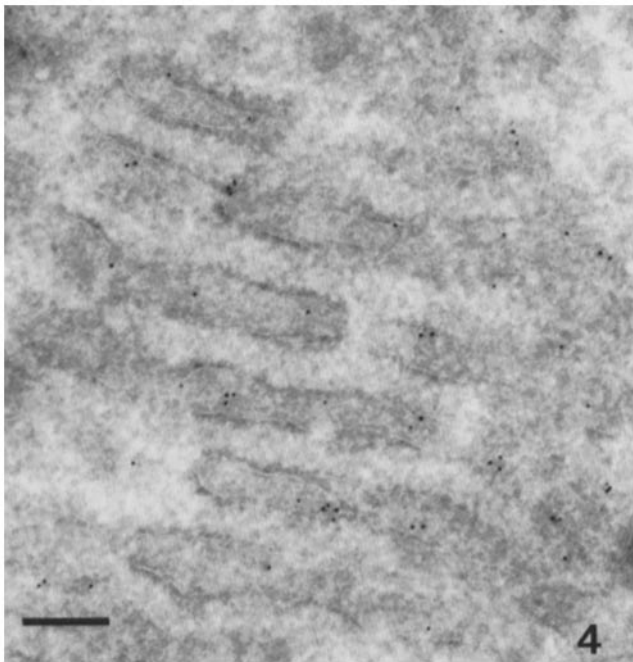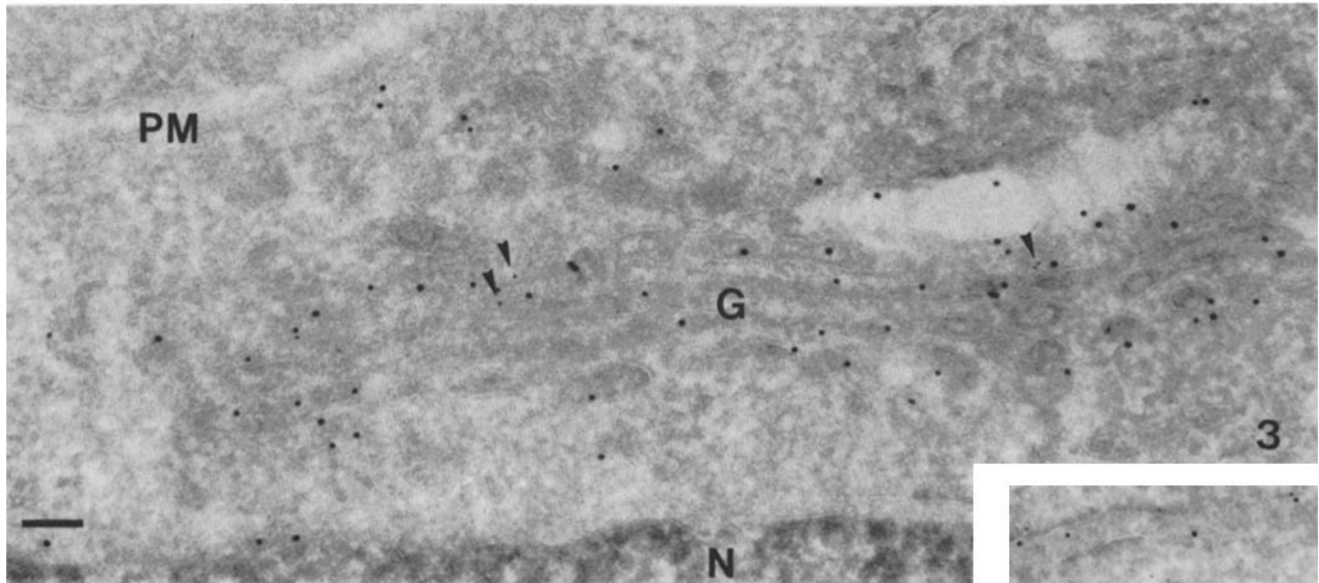
FIGURES 3 and 4   Fig. 3: Simultaneous demonstration of VSV-G (8 nm) and transferrin (5 nm) in the Golgi complex. Transferrin is present in low concentration in the Golgi stack (arrowheads). VSV-G is throughout the whole stack, probably in the highest concentration at the periphery of the Golgi system. (Inset) Single label (8 nm) with antitransferrin in a rough ER cisterna. Transferrin can be visualized in the rough ER in significant amounts. PM, plasma membrane; G, Golgi complex. Bar, 0.1 μm. × 40,000. Fig. 4: Rough endoplasmic reticulum cisternae containing both albumin (5 nm) and VSV-G (8 nm). Bar, 0.2 μm. × 57,500.

galactosyltransferase is mainly present in the swollen areas of the Golgi system, while G is present both in these and in intact Golgi cisternae. The more cis-Golgi cisternae, the transitional elements, and the proximal vesicles are not affected by treatment with monensin for 30 min. Accumulation of G, albumin, and transferrin did not occur within these structures.

DISCUSSION

Using double-label immunoelectron microscopy, we have shown that the intracellular localizations of two secretory proteins and the integral plasma membrane protein VSV-G are identical.

Albumin, transferrin, and VSV-G protein are synthesized on membrane-bound polysomes (19, 20). Although immunostaining of the rough ER was low for all three antigens studied, we did not find regions of the rough ER that were especially enriched in any of the antigens tested. This suggests that

polysomes that synthesize these proteins are randomly distributed on the ER membrane, and is consistent with the finding that nascent secretory proteins and VSV-G protein use the same receptors, and are probably sequestrated into the same rough ER vesicles (21).

In a previous study, using rat hepatoma cells we found that transferrin required more time to move from the rough ER to the Golgi complex than albumin and VSV-G (7). We can now conclude that this rate difference is not caused by a difference in pathway, but rather a difference in time needed to reach the Golgi complex. Recent experiments with HepG-2 cells showed that transport of secretory proteins between the rough ER and the Golgi membranes is not a bulk-phase movement of the luminal contents of the endoplasmic reticulum. Each of five proteins studied moved at a different rate. This movement is probably mediated by one or more membrane bound receptor proteins (8, 22).

Albumin, transferrin, and VSV-G are found together in all

5 A

5 B
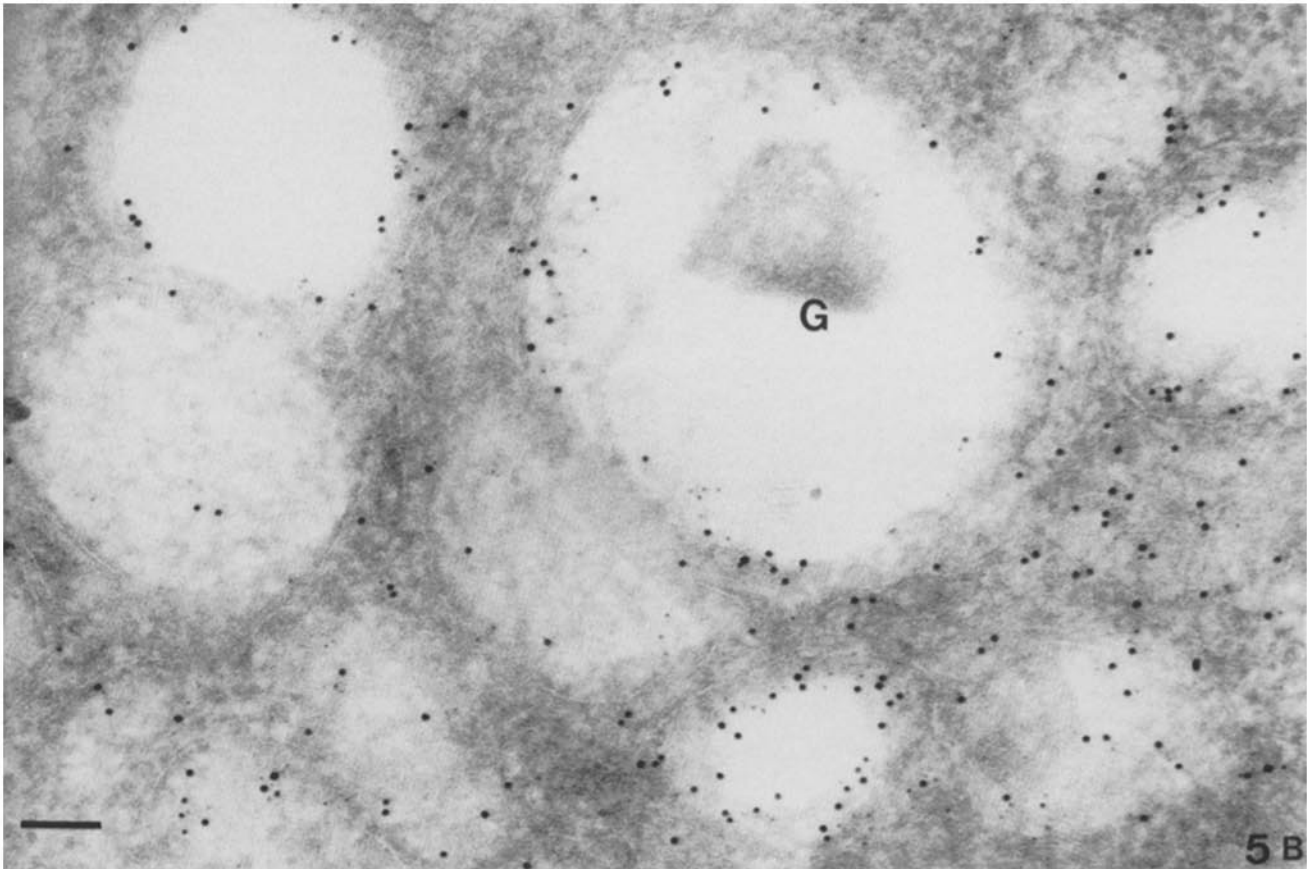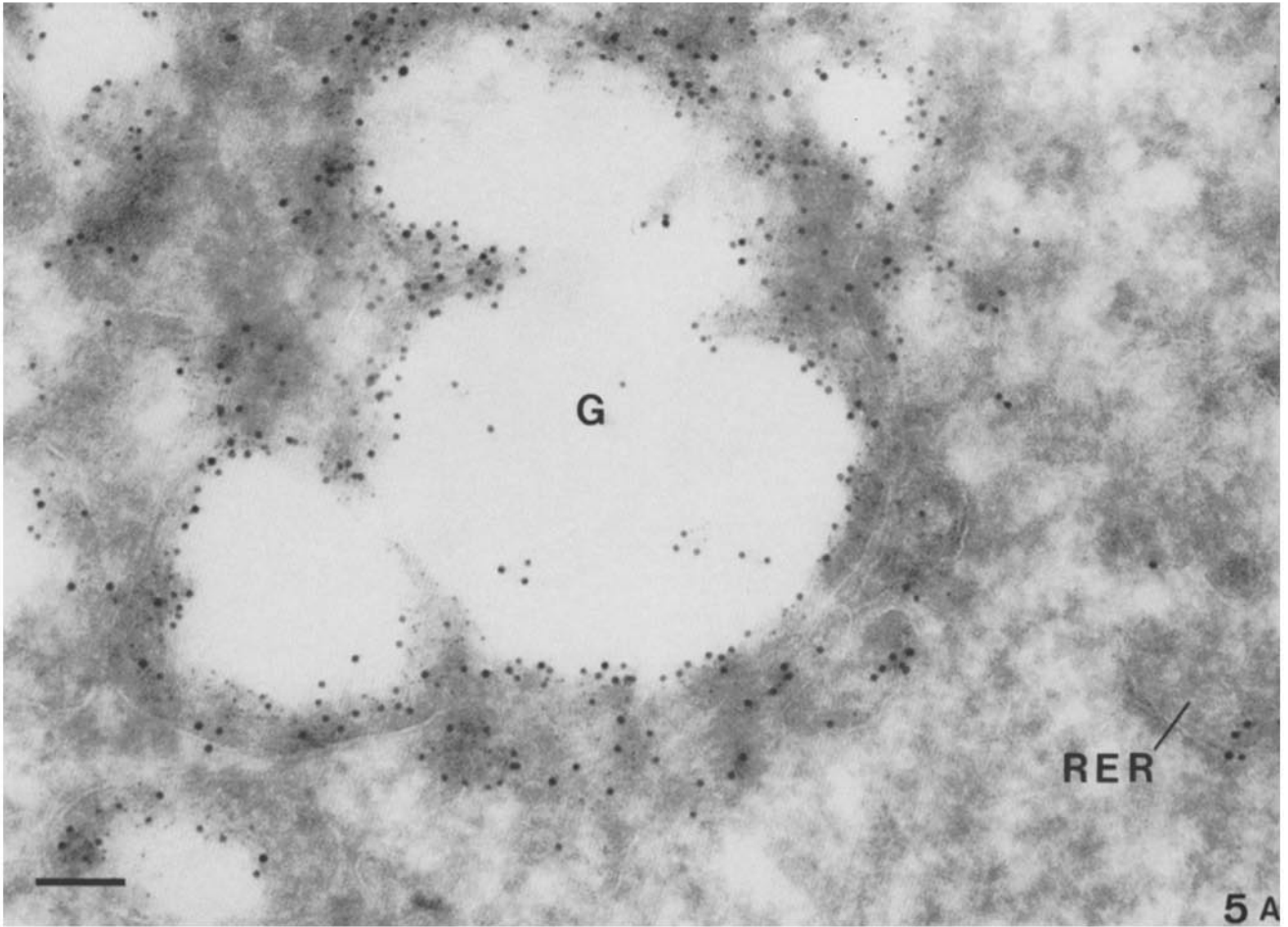
regions of the Golgi complex. This observation is in accordance with studies localizing secretory and membrane proteins in the pancreas (23), IgG in plasma cells (24), and VSV-G in Chinese hamster ovary cells (25, 26). In all cases, each of the proteins were found in all cisternae of the Golgi stack. This suggests that all Golgi cisternae are involved in transport of both secretory and membrane proteins. Secretory proteins apparently move through, or are shuttled from, the cis- to the trans-side of the Golgi complex at the same time as VSV-G moves through the membranes of the same compartments. As the terminal glycosyltransferases and other oligosaccharide processing enzymes are present in the Golgi complex, it is likely that both secretory and membrane proteins are processed and glycosylated by the same sets of enzymes. Why some membrane proteins (like VSV-G) pass through the Golgi complex to the plasma membrane, while others (like the

oligosaccharide processing enzymes themselves) keep their positions in the Golgi membranes, remains to be elucidated. All proteins move from the Golgi complex to the plasma membrane in only a few minutes. Again, we found that secretory vesicles contained both secretory and VSV-G plasma membrane proteins. Whether secretory vesicles are the only structure carrying the proteins to the cell surface or that additional smaller vesicles are also involved remains to be elucidated.

Our studies with the ionophore monensin showed that the transport of both the G protein as albumin and transferrin is blocked at the trans-side of the Golgi complex, causing accumulation of these proteins in the dilated (trans) Golgi cisternae. The localization of the secretory proteins is mainly along the membranes. One possible explanation for this is that, because of low protein concentration the fixation conditions
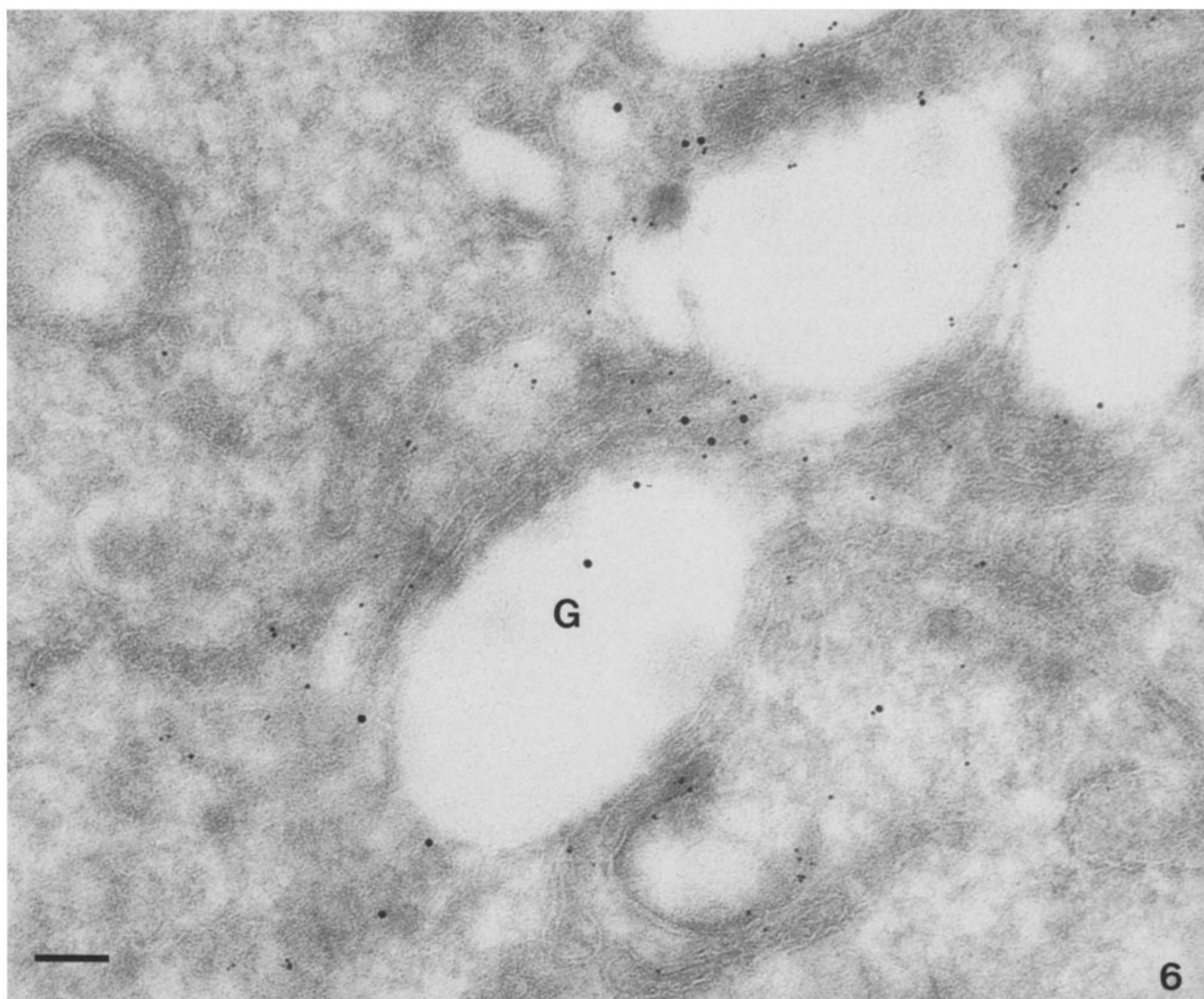

FIGURE 6 Simultaneous demonstration of VSV-G (5 nm) and galactosyltransferase (8 nm) in monensin treated cells. Anti-galactosyltransferase label is mainly present in or close to the swollen areas of the Golgi, while VSV-G is also present in other parts of the complex. G, swollen Golgi cisterna. Bar, 0.1 μm. × 105,000.

FIGURE 5 Simultaneous demonstration of VSV-G, albumin and transferrin in the Golgi complex after 30 min of monensin. AntiVSV-G, stained with 8 nm gold, antialbumin with 5 nm in the upper electron micrograph (a) and antitransferrin with 5 nm gold in the lower micrograph (b). All three antigens are present in high concentrations in the swollen parts of the Golgi complex. Strikingly, most of the albumin and transferrin label is localized close to the membrane in the swollen vesicles. Bar, 0.1 μm.

are poor and the soluble antigens are partly lost during preparation of the sections. However, we showed that albumin is evenly distributed in the luminal space of the vesicles present between the Golgi complex and the plasma membrane (Fig. 2). Another possibility is that the secretory proteins are guided and transported through the rough ER into the Golgi complex by specific transport proteins, embedded in these membranes (8, 22), and that, in monensin treated cells, these proteins remain bound to these receptors.

In a previous study (7) we found that monensin blocks transferrin at a stage where the glycoprotein is still susceptible to Endo H, while VSV-G is blocked at a stage where it is resistant to Endo H. We suggested that membrane and secretory proteins were present in different Golgi or post-Golgi compartments, that are expanded by monensin. The present micrographs show clearly that both albumin, transferrin, and VSV-G accumulate within the same Golgi compartments, whether or not monensin is present. The explanation for different behavior of transferrin and VSV-G with respect to oligosaccharide processing as probed by Endo H treatment remains unclear. Probably monensin affects not only the movement of macromolecules through the Golgi cisternae, but also has a direct effect on oligosaccharide processing of individual proteins (27). As shown by two-dimensional gel electrophoresis, it is clear that glycoproteins, secreted by monensin treated cells, are less negative charged, presumably caused by inefficient addition of sialic acid. The primary effect of monensin is apparently dilation of galactosyltransferase containing (trans)-cisternae of the Golgi complex (Fig. 6). As a consequence, the formation of secretory vesicles is hampered, causing accumulation of secretory and membrane proteins in distended Golgi-like vesicles. A second consequence might be that contact between the membrane-bound glycosyltransferases (like sialyl- and galactosyltransferases) and the soluble glycoproteins is disturbed, thus causing incomplete modification of the oligosaccharides on the secreted proteins (Fig. 1).

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