Intracellular Transport of the Transmembrane Glycoprotein G of Vesicular Stomatitis Virus through the Golgi Apparatus as Visualized by Electron Microscope Radioautography

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ABSTRACT The intracellular migration of G protein in vesicular stomatitis virus-infected cells was visualized by light and electron microscope radioautography after a 2-min pulse with [^{3}H]mannose followed by nonradioactive chase for various intervals. The radioactivity initially (at 5–10 min) appeared predominantly in the endoplasmic reticulum, and the [^{3}H]mannose-labeled G protein produced was sensitive to endoglycosidase H. Silver grains were subsequently (at 30–40 min) observed over the Golgi apparatus, and the [^{3}H]mannose-labeled G protein became resistant to endoglycosidase H digestion. Our data directly demonstrate the intracellular transport of a plasmalemma-destined transmembrane glycoprotein through the Golgi apparatus.

An important aspect of membrane biogenesis is the mechanism by which integral membrane proteins are sorted and transported from the site of synthesis to their final destination in the cell. Studies with exportable proteins have shown that these proteins are transported sequentially through the endoplasmic reticulum (ER), Golgi apparatus, and secretion granules before being discharged (12, 19). Integral transmembrane glycoproteins are generally believed to follow a similar route involving transport from ER to the plasma membrane via the Golgi apparatus.

The transmembrane envelope glycoprotein G of vesicular stomatitis virus (VSV) has provided a model for understanding the sequence of events involved in the biogenesis of integral membrane glycoproteins (6). Studies in vitro from several laboratories have shown that the G protein is synthesized on the membrane-bound ribosome (7, 18, 23, 25) and is inserted into the microsomal membrane vesicle in a cotranslational event (14, 26) that involves the proteolytic cleavage of an NH_2-terminal extension peptide containing 16 amino acids (11, 17). These in vitro studies also demonstrated cotranslational glycosylation involving microsomal membranes and transmembrane asymmetry of the G protein with the NH_2-terminal sequence located intraluminally and a peptide of ~25 amino acids at the COOH terminus exposed to the cytoplasm (14, 25, 26).

Studies of the kinetics of appearance of pulse-labeled G in the various isolated subcellular membrane fraction from VSV-infected cells show that the G protein migrates sequentially from ER to a low-density smooth membrane (presumed to be derived from the Golgi apparatus) and finally to the plasma membrane (10, 15). The core oligosaccharide transferred to G protein in the ER is modified in the smooth membrane by the trimming of mannose residues followed by the addition of terminal sugars (9, 10, 20). These results as well as the localization of terminal glycosyl transferases in the Golgi apparatus (e.g., reference 5) suggested that transmembrane glycoproteins pass through the Golgi apparatus enroute to the plasma membrane. As well, in a recent study using indirected immunofluorescence and immunoelectron microsocpy of VSV-infected cells, Bergmann et al. (4) have demonstrated G protein antigenicity within the Golgi apparatus. A direct approach involving light and electron microscope radioautography was, therefore, carried out to confirm this pathway.

MATERIALS AND METHODS

Viruses and Cells

Plaque-purified VSV (Indian HR-LT) was grown in L cells as described (24). Baby hamster kidney fibroblast (BHK, clone 13) cells were grown in monolayer in Dulbecco's medium containing 10% calf serum at 35°C in an atmosphere at 5% CO_2.

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Radioactive Labeling of Infected Cells with [^{3}H]Mannose

BHK cells were cultured in 100-mm petri dishes until nearly confluent (~6 × 10^6 cells). The cells were infected with 50 plaque forming units (pfu) of VSV in 1.5 ml of monolayer medium and incubated at 35°C. After 45 min, 3.5 ml of prewarmed medium was added and the cells were incubated for a further 3.75 h. The medium was removed and cells were washed three times with 5 ml of warm phosphate-buffered saline. The cells present in nine petri dishes were then labeled for 2 min at 35°C in 1.5 ml of medium containing 3 mCi of D-[2-^{3}H]mannose (14.5 Ci/mmol; New England Nuclear, Boston, MA). After a 2-min pulse the cells present in eight plates were washed three times with 3 ml of prewarmed medium containing 10 mM nonradioactive mannose for chase and were incubated in 5 ml of the same medium for a further 3, 8, 13, 18, 28, 38, 48 and 58 min, respectively, at 35°C. At the end of the 2-min pulse for the ninth plate or at the end of the pulse and chase periods for the other eight plates, the cells were washed three times with 5 ml of ice-cold serum-free medium containing 10 mM mannose. The cells were immediately fixed in a dilute Karnovsky fixative containing 2% glutaraldehyde/1% paraformaldehyde/0.05% $CaCl_2$ in 0.1 M sodium cacodylate (pH 7.4) (13).

Light and Electron Microscope Radioautography

After fixation, the cells were postfixed in 1% OsO_4 and then bloc-stained in uranyl acetate (2, 3). The cells were embedded in Epon 812 and sectioned for light and electron microscope radioautography as described previously (2, 3).

Quantification of Radioautographic Data

For light microscope radioautography, grain counts were carried out on longitudinal- and cross-sectioned cells and averaged. The grain densities were expressed as grains per cell. Grain counts were carried out after 6 and 67 d of exposure with a proportional increase observed. A total of 5,198 grains and 803 cells were scored for the slides processed at 6-d exposure and 22,500 grains and 724 cells at 67-d exposure. Scoring of the electron microscope radioautographs was carried out by means of a transparency equivalent to the diameter of Ilford L4 silver crystals (namely 150-nm diameter). The transparency was placed over groups of individual silver deposits as described previously (3). A pinhole was placed at the geometric center, and another transparency equivalent to a resolution boundary circle of 2HD (300-nm diameter) was superimposed. The membranous structure enclosed within this latter transparency was then scored. Grain densities per compartment per cell were calculated by considering the percent of total silver crystals over that compartment and multiplying this value by the grain density per cell as revealed by light microscope radioautography.

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[^{3}H]Mannose-labeled proteins were analyzed by electrophoresis in a 10% polyacrylamide gel containing 0.1% SDS and were identified by fluorography (25).

Digestion of Labeled Proteins with Endo-β-N-Acetylglucosaminidase H

The procedure of Robbins et al. (20) was followed: Labeled cells or proteins were solubilized in 1% SDS/0.15 M Tris (pH 8.8)/1% (vol/vol) 2-mercaptoethanol by boiling for 3 min. 2 vol of 0.3 M sodium citrate (pH 5.5) was added to each sample and 5 μl of the Endo-β-N-acetylglucosaminidase (Endo H) (30 μg/ml) were added. Samples in the presence and in the absence of Endo H were incubated at 37°C for 18 h. The radioactive proteins were recovered by precipitation with 3 vol of acetone at −20°C. The samples were resuspended and heated in sample buffer (5% SDS/0.1 M Tris (pH 6.8)/10% glycerol/1% [vol/vol] 2-mercaptoethanol) and electrophoresed as described earlier.

Separation of Lipid-linked Mono- and Oligosaccharide and Glycoproteins

The extraction procedure of Behrens et al. (1) was followed. Briefly, the cells were extracted with $CHCl_3/CH_3OH$/4 mM $MgCl_2$ (3:3:0.8, vol/vol/vol) and the lower organic phase was washed with $CHCl_3/CH_3OH$/4 mM $MgCl_2$ (1:16:16, vol/vol/vol). The bottom organic phase contained the mannosyl phosphoryl polyisoprenol. The residual material at the interphase between the organic and aqueous phases was further extracted with $CHCl_3/CH_3OH/H_2O$ (10:10:3, vol/vol/vol) to separate oligosaccharide pyrophosphoryl polyisoprenol from the labeled glycoprotein which was insoluble in the above solvents.

RESULTS

Specific Labeling of the Virus Glycoprotein G in VSV-infected BHK Cells

In agreement with previous reports (9, 10, 15, 20) the glycoprotein G was the only major species observed after pulse-labeling with [^{3}H]mannose. More than 80% of the [^{3}H]mannose-labeled glycoprotein in the VSV-infected cell extract was immunoprecipitated by anti G antisera (data not shown). When the incorporation of [^{3}H]mannose into the oligosaccharide lipid and glycoprotein fractions was followed, the amount of radioactivity in the glycoprotein fraction increased up to 20 min and then showed a decrease (Fig. 1). Similarly, continued incorporation of [^{3}H]mannose into protein for 15–30 min during the chase period was also observed by Robbins et al. (20) in VSV-infected and Sindbis virus-infected cells. Presumably, this continued incorporation of mannose is due to a more stable pool of [^{3}H]mannose-labeled precursors. The lipid-linked oligosaccharide precursor showed continued loss in radioactivity with a half-time of decrease of ~15 min. The monosaccharidal lipid moiety revealed only low labeling at all time intervals studied (~1,200 cpm; data not shown). Both the monosaccharidal and oligosaccharidal glycolipids were hydrolyzed by hot trichloroacetic acid solution and were, therefore, lipid-linked glycoprotein precursors (8) and not structural glycolipids. Electrophoretic analyses confirmed labeled G as the only protein species

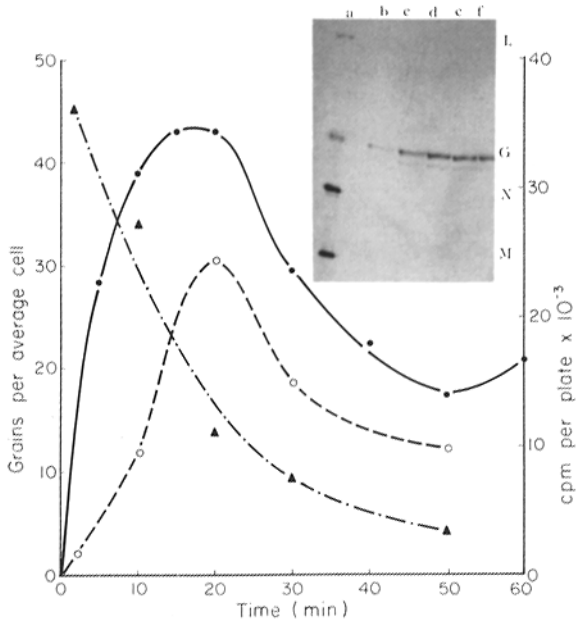

FIGURE 1 Quantification of [^{3}H]mannose-labeled products in the infected cells (●). The grains per average cell were determined by averaging the grain counts over longitudinally and transversally sectioned cells. The efficiency of the light microscope radioautography was 20% and the exposure was for 67 d. The radioactivity present in the oligomannose lipid fraction (▲) and in the glycoprotein fraction (○) was determined in a separate experiment as described in the text. The values are expressed as counts per minute per confluent plate containing 6 × 10^6 fibroblast cells. The counting efficiency was ~10%. The presence of only labeled G protein in the glycoprotein fraction is shown in the fluorogram in the insert. Wells *b*, *c*, *d*, *e*, and *f* correspond to glycoprotein fractions present at 10, 20, 30, 40, and 50 min, respectively. Well *a* contained [^{35}S]methionine-labeled VSV marker. *L*, *N*, and *M* are the nonglycosylated VSV proteins.

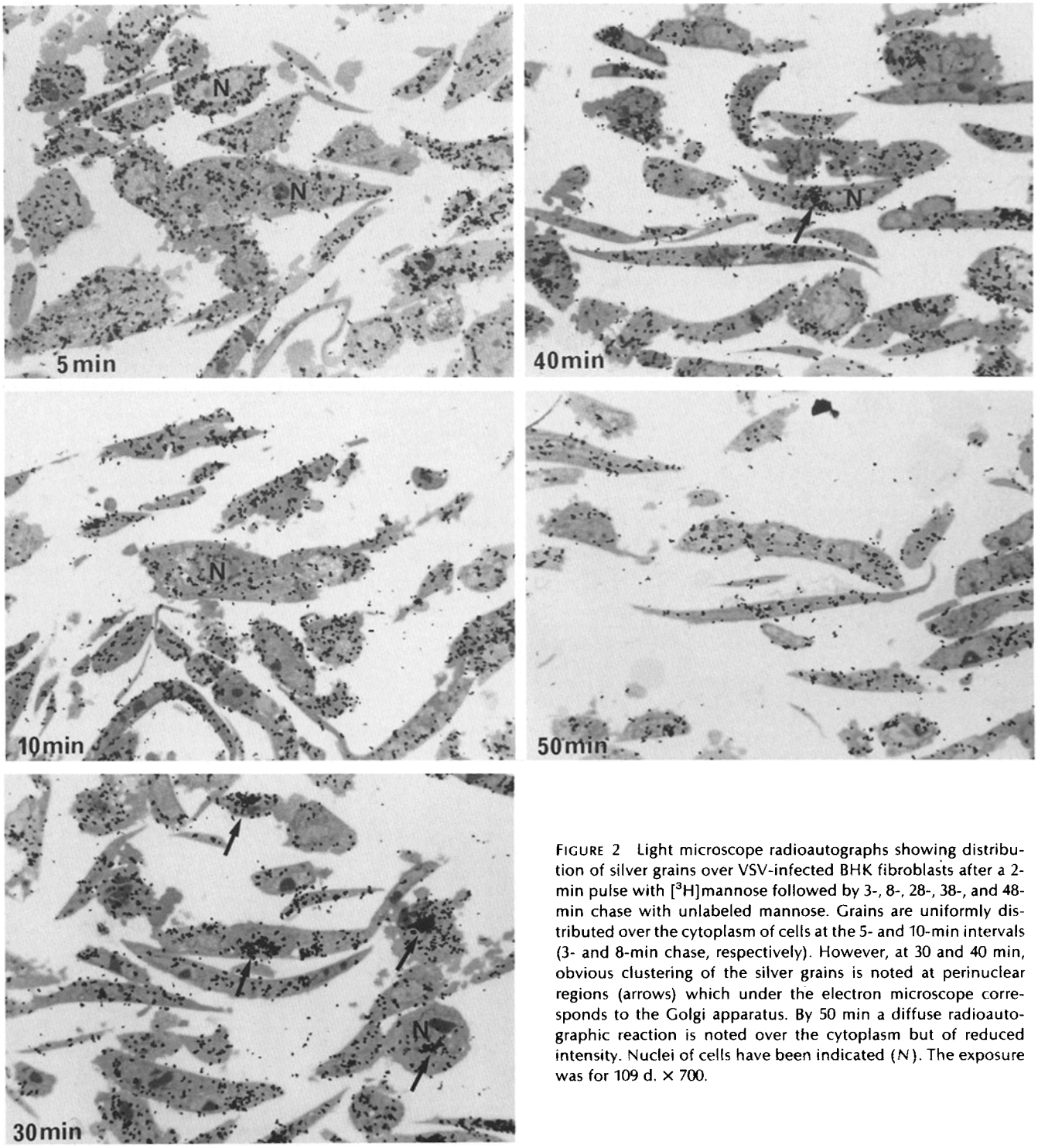

FIGURE 2 Light microscope radioautographs showing distribution of silver grains over VSV-infected BHK fibroblasts after a 2-min pulse with [^{3}H]mannose followed by 3-, 8-, 28-, 38-, and 48-min chase with unlabeled mannose. Grains are uniformly distributed over the cytoplasm of cells at the 5- and 10-min intervals (3- and 8-min chase, respectively). However, at 30 and 40 min, obvious clustering of the silver grains is noted at perinuclear regions (arrows) which under the electron microscope corresponds to the Golgi apparatus. By 50 min a diffuse radioautographic reaction is noted over the cytoplasm but of reduced intensity. Nuclei of cells have been indicated (*N*). The exposure was for 109 d. × 700.

present in the glycoprotein fractions (Fig. 1, *insert*). As in the infected cell extract, >80% of the radioactivity in the glycoprotein fraction was immunoprecipitated with anti G antisera. The immunoprecipitate contained, in addition to G protein, a minor protein migrating faster than G. The protein in the faster migrating band was derived from G, presumably by proteolysis.

Light Microscope Radioautography

Light microscope radioautography (Fig. 2) revealed a diffuse radioautographic reaction at early intervals of 5 and 10 min, with a focal concentration of silver grains over characteristic Golgi apparatus regions of the cell at 30 and 40 min and a diffuse reaction reappearing at 50 min.

Quantificative analysis of the light microscope radioautographs revealed (Fig. 1) an increase in radioactivity until 20 min (18-min postpulse), then a rapid fall in cellular radioactivity until 50 min and finally a slight increase in radiolabeling by 60 min.

Electron Microscope Radioautography

The electron microscope radioautographs (Figs. 3 and 4) revealed grains over the rough ER at an early interval of 10

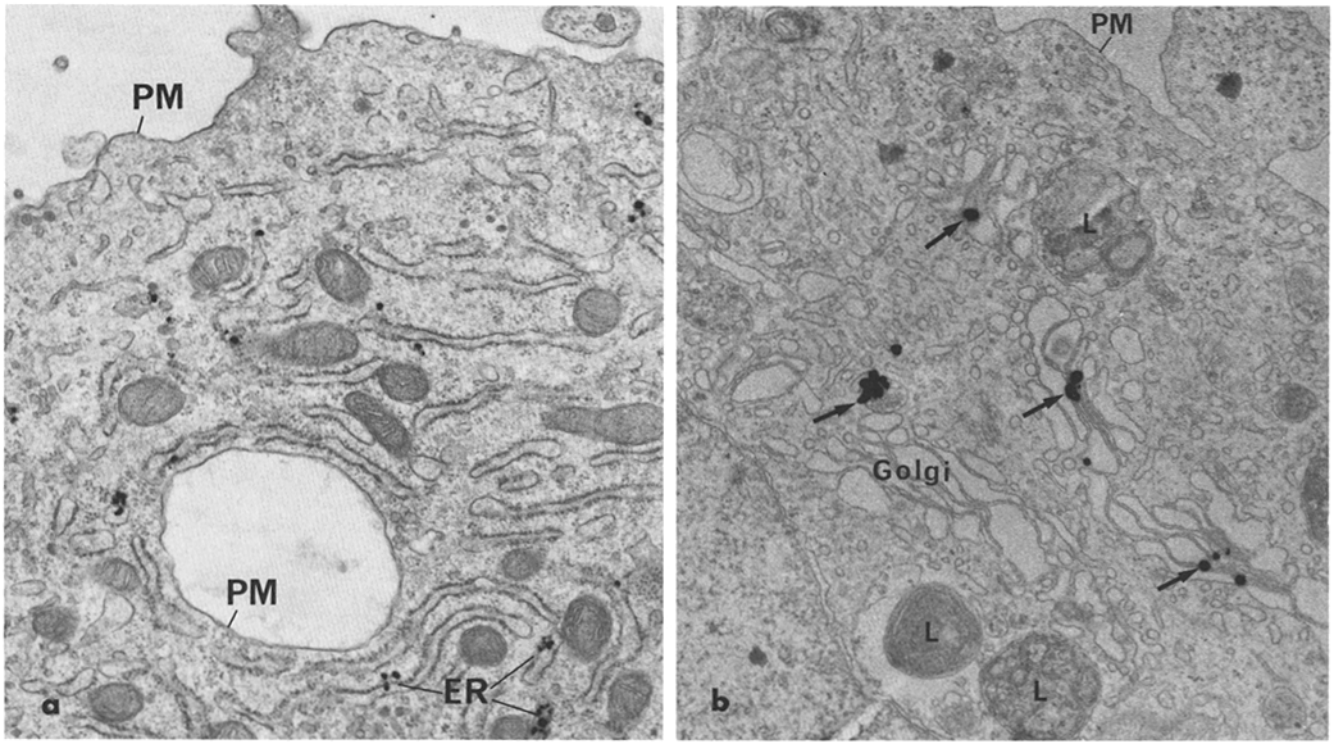

FIGURE 3 Electron microscope radioautographs of thin sections of VSV-infected BHK cells revealing small punctate dense black silver grains (*a*) over the ER at the 10-min interval. In *b* the silver grains (arrows) are over vesicles and saccules of the Golgi apparatus at 30 min. Secondary lysosomes (*L*) and the plasmalemma (*PM*) are indicated. The exposure was for 99 d. × 25,000.

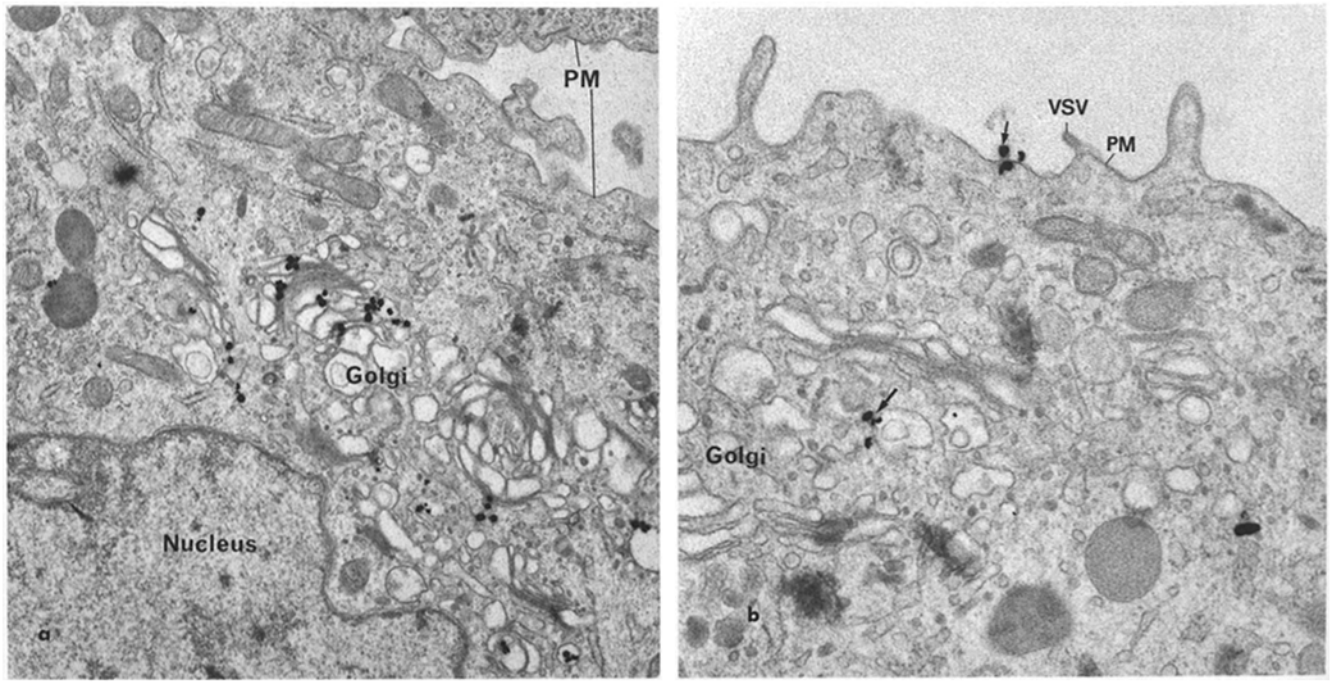

FIGURE 4 Electron microscope radioautograph of thin sections of VSV-infected BHK cells at the 40- (*a*) and 50-min (*b*) intervals. In *a* silver grains are observed only over the Golgi apparatus. The cell nucleus and plasmalemma (*PM*) are indicated. At 50 min (*b*), silver grains are shown over the *PM* and the Golgi apparatus. The budding of an unlabeled VSV is also shown (*VSV*). The exposure time was 99 d. *a*, × 20,000. *b*, × 30,000.

min with progression to the Golgi complex at later intervals of 30 and 40 min with some labeling of plasmalemma observed at 50 min.

A detailed analysis of the electron microscope radioautographs was carried out (Fig. 5). The analysis was based on 2,026 grains in which a flow of radioactivity through the Golgi complex was observed with a peak at 30 min when 48% of the grains were present in the Golgi apparatus. Other compartments of the cell were also analyzed (Table I). Grain densities calculated in each compartment per cell were high over the ER

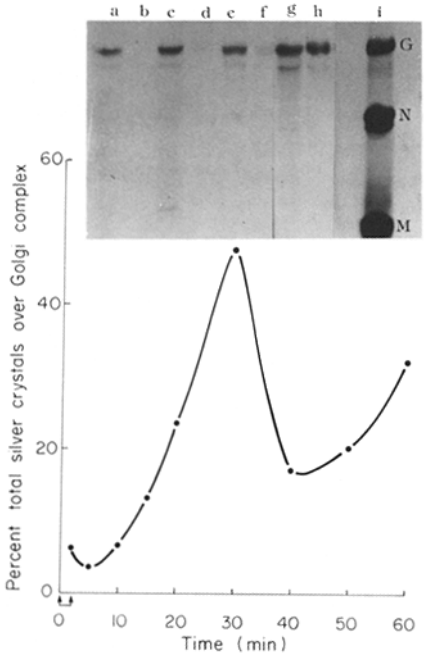

FIGURE 5 Flow of mannose radioactivity through the Golgi apparatus. The proportion of silver grains present over the Golgi apparatus at each of the time intervals was determined (Table I). The peak at 30 min corresponded to a loss of 50% of the radioactivity as observed in the light microscope radioautographs (Fig. 1). The fluorogram in the insert shows the endoglycosidase H sensitivity of the G protein at 10 min (wells *a* and *b*), 20 min (wells *c* and *d*), 30 min (wells *e* and *f*), and 40 min (wells *g* and *h*). Only the samples present in wells *b*, *d*, *f*, and *h* were digested with endoglycosidase H. Well *i* contained [^{35}S] methionine-labeled VSV marker.

at 10 min and decreased with time. Ribosome-free smooth membranes also revealed a considerable accumulation of silver grains with a pattern similar to that observed for ER. In contrast, the Golgi apparatus revealed low grain density at 10 min but a high grain density at 30 min.

Endo H Digestion of [^{3}H]Mannose-labeled G

Digestion of glycoprotein intermediates with Endo H has been used to distinguish between glycoproteins containing core oligomannose and the glycoproteins in which the oligomannose has been processed and modified by addition of terminal sugars (9, 20). It has been suggested that a specific mannose localized in the Golgi membrane is involved in the trimming of the core oligomannose (22). The Endo H assay has, therefore, been used to probe biochemically the transport of the glycoprotein precursors from the ER to the Golgi apparatus (21). Digestion of [^{3}H]mannose-labeled G protein obtained at various time intervals of chase showed that, in agreement with the previous results (9, 20), only the G protein obtained at 5–10 min was sensitive to Endo H digestion while the G protein present after the 30-min period was insensitive (Fig. 5, *insert*). Both the G protein and the protein migrating faster than G were immunoprecipitated by anti G antisera. Together, these two proteins represented ~95% of the input radioactivity.

DISCUSSION

The intracellular pathway of migration of secretory protein has been elucidated by quantitative radioautography (12, 16, 19). We have attempted to confirm the predicted pathway (19, 21)

TABLE I

Subcellular Distribution of Silver Grain Density

Compartment	Grains per compartment per cell			
	10 min	20 min	30 min	50 min
	(288)*	(406)	(132)	(109)
Rough ER‖	12.9	11.1	5.8	4.5
Smooth membranes§	10.8	10.9	5.4	3.7
Golgi apparatus	2.6	9.9	14.1	3.5
Secondary lysosomes	0.7	0.6	0.4	0.4
Plasmalemma	3.4	2.7	1.1	2.1
Other structures¶	8.4	6.0	2.7	3.2
Total grains per cell (light microscope)	38.8	42.2	29.5	17.4

The estimation of grains per compartment per cell was carried out by first analyzing the percent distribution of silver grains for each compartment by electron microscope radioautography (see Materials and Methods) then multiplying this value by the cellular grain densities as determined by light microscope radioautography.

* Number of silver crystals scored; a silver crystal is defined as one to three silver deposits which can be encompassed within a transparent circle of diameter 150 nm (3).

‖ Also includes silver grains over the nuclear envelope. The nuclear envelope compartment shows a maximum of 4.6% of the silver grains.

§ The smooth membrane compartment consisted of elements of the smooth ER, in addition to a smooth membrane tubular system observed beneath the plasmalemma and smooth vesicles distributed throughout the cytoplasm.

¶ Includes the cell sol, mitochondria, cell nucleus, and extracellular grains.

of intracellular transport of an integral transmembrane glycoprotein, G protein of VSV by this technique. An overall similarity between light microscope radioautographic grains counted with radioactivity in G protein but not with lipid-linked precursors is observed (Fig. 1). It has previously been suggested that all labeled glycolipids are extracted during sample preparation for radioautography (16). However, the disproportionality between the grain counts at early intervals and the proportion of total mannose radioactivity in G protein indicated some contribution of labeled glycolipid to the radioautographic grain densities. All of the labeled glycolipid was acid labile and, therefore, of the dolichol-linked type (8). Thus, the grains represented G glycoprotein or requisite lipid intermediates but not any other radioactive source. The continued incorporation of [^{3}H]mannose into glycoprotein for 20 min during the chase period was a reflection of the relatively stable pools of labeled mannose precursors including GDP mannose, mannose phosphate dolichol, and the lipid oligosaccharide as previously pointed out by Robbins et al. (20). Thus, despite only a 2-min labeling with [^{3}H]mannose, the actual extent of a labeling is a consequence of the oligosaccharide lipid labeling.

By electron microscope radioautography a maximum of 47.7% of the total silver grains was observed over the morphologically defined Golgi apparatus at 30 min (Fig. 4). At this time interval, 82% of the total cellular radioactivity was in G protein and we therefore feel confident that G protein transport was indeed being observed through this compartment. From 10 to 30 min, 7.1 grains per cell were lost from the rough ER compartment and 5.4 grains per cell from the smooth membrane compartment. As 11.5 grains per cell were gained into the Golgi apparatus compartment during the same time intervals, it was therefore concluded that all grains lost from the rough and smooth membrane compartments were accounted for by the appearance of G protein in the Golgi apparatus. The residual silver grains remaining over the rough ER and smooth membrane compartments at 50 min may represent residual mannose-labeled glycolipid as well as a small amount of non-

transported G protein.

The loss in radioactivity at 30- to 50-min periods when the G protein was concentrated in the Golgi apparatus and the appearance of Endo H resistant G protein suggested that the trimming of the oligomannose residues also occurs in the Golgi apparatus within the same time period as the addition of terminal sugars. The radioautographic data thus support the biochemical evidence (22) suggesting the Golgi apparatus as the site of core oligomannose trimming.

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