Accessibility to Proteases of the Cytoplasmic G Protein Domain of Vesicular Stomatitis Virus Is Increased during Intracellular Transport

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Abstract. G_1 and G_2 are two forms of the membrane-integrated G protein of vesicular stomatitis virus that migrate differently in gel electrophoresis because G_1 is modified by high-mannose and G_2 by complex-type oligosaccharide side chains. The cytoplasmic domain in G_1 is less exposed to cleavage by several proteases than in G_2 molecules. Acylation by palmitic acid as well as inhibition of carbohydrate processing by swainsonine and deoxynojirimycin resulted in the same pattern of proteolytic sensitivity of both glycoproteins as in untreated cells. In contrast, accessibility of the cytoplasmic domain to proteases did not change when the intracellular transport of the G protein was blocked in carbonyl cyanide m-chlorophenylhydrazone- or monensin-treated BHK-21 cells, respectively. The results suggest that the increase in accessibility of the cytoplasmic tail of the G protein occurs after the monensin block in the *trans*-Golgi and might reflect a conformational change of functional significance—i.e., making the cytoplasmic domain of the viral spike protein competent for its interaction with the viral core, inducing thereby the formation of the budding virus particle.

THE biosynthesis and modification of membrane glycoproteins are biochemically quite well understood (Sabatini et al., 1982; Kornfeld and Kornfeld, 1985; Burgess and Kelly, 1987), but the mechanisms that specifically guide membrane proteins from their site of synthesis in the endoplasmic reticulum via the Golgi apparatus to their final destination are yet to be elucidated. With the exception of the mannose-6-phosphate receptor for lysosomal enzymes, receptors have not been described that could target different membrane proteins to their specific subcellular compartment. Therefore, it has been postulated that the formation of a correct tertiary and quaternary conformation is essential for the intracellular transport and correct targeting of membrane proteins to their specific destination (Copeland et al., 1986).

The G protein of vesicular stomatitis virus (VSV)[1] is integrated into the viral envelope as a transmembrane protein. The G protein sequence which was derived from a cDNA clone is characterized by four major domains: the NH_2-terminal signal sequence, the main body of the protein containing the two glycosylation sites, the hydrophobic transmembrane domain, and the cytoplasmic COOH-terminal region which is exposed to the cytoplasm (Rose and Gallione, 1981). There are two cell-associated forms of the G protein, termed G_1 and G_2 (Knipe et al., 1977). G_1 has a higher electrophoretic mobility in SDS-PAGE and is the kinetic precursor of G_2. Palmitic acid is covalently attached to the single cysteine residue of the cytoplasmic domain of the G protein in the *cis*-Golgi cisternae or late endoplasmic reticulum (Rose et al., 1984; Mack and Kruppa, 1988; Dunphy et al., 1981; Berger and Schmidt, 1985). It is the G_1 species that is first acylated by palmitic acid (Mack et al., 1987).

Recently, evidence has been obtained that the G protein is assembled into homotrimers that may be essential for exit of the G protein from the endoplasmic reticulum (Kreis and Lodish, 1986; Doms et al., 1987). Mutants of the cytoplasmic and transmembrane domain of the G protein generated by deletions of these domains are greatly inhibited by the efficient transport of the G protein to the plasma membrane (Rose and Bergmann, 1982; Adams and Rose, 1985). In addition, the COOH-terminal tail of the G protein seems to be essential for budding of virus particles at the plasma membrane which is presumably driven by a COOH-terminal tail–nucleocapsid interaction (Metsikkö and Simons, 1986).

We report here about an increase in the accessibility of the cytoplasmic domain of the G protein contained in membrane preparations of VSV-infected baby hamster kidney (BHK) cells to proteolytic digestion during maturation of the G protein. The role of fatty acid acylation and of terminal modification of the oligosaccharides for the alteration of the protease sensitivity of the cytoplasmic domain of the G protein was investigated. Kinetic analysis and the use of inhibitors

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1. *Abbreviations used in this paper*: BHK, baby hamster kidney; CCCP, carbonyl cyanide m-chlorophenylhydrazone; endo H, endoglycosidase H; VSV, vesicular stomatitis virus.

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of intracellular transport established that the change of the accessibility for proteolytic cleavage of the cytoplasmic domain occurred in a late Golgi compartment. Therefore, this change does not reflect an alteration due to trimerization of the G protein but may be essential for the competence of the G protein in virus budding.

Materials and Methods

Virus Growth and Metabolic Labeling of Viral Proteins

The San Juan strain of VSV (serotype Indiana) was grown on BHK cells and purified as described previously (Graeve et al., 1986). Confluent monolayers of BHK cells were infected with a multiplicity of infection of 40 plaque-forming units per cell. The inoculum was removed after 30 min and the infection was continued for 3.5 h at 37°C if not stated otherwise. The monolayers were washed with prewarmed PBS, labeled for 10 min at 37°C in 3 ml methionine-free medium supplemented with 100 μCi [^{35}S]methionine, and chased in serum-free medium containing an excess of unlabeled methionine for the times indicated in the figure legends. Cells were pulse labeled in 3 ml of serum-free medium containing 400 μCi [^{3}H]palmitic acid for the times indicated in the figure legends.

Labeling was terminated by washing the cells with ice-cold PBS. Cells were scraped off, collected by low-speed centrifugation, and resuspended in hypotonic buffer (15 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.5). The cell suspension was adjusted to 1% Triton X-100, nuclei were removed by centrifugation at 800 *g* for 5 min, and the cytoplasmic extracts were stored at −20°C (Garreis-Wabnitz and Kruppa, 1984).

Incubation of VSV-infected BHK Cells with Inhibitors

The glycosylation inhibitors deoxynojirimycin (1 mM) or swainsonine (500 ng/ml) were added 1 h after infection to VSV-infected BHK cells that were labeled as described above. Inhibitors were also present in the chase media.

VSV-infected BHK cells were labeled with [^{35}S]methionine for 10 min at 37°C. The medium was then replaced by 10 ml of ice-cold PBS containing 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 5 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Fries and Rothman, 1980; Morrison and Ward, 1984). After 5 min at 4°C, the ice-cold PBS was replaced by prewarmed PBS containing Ca^{2+}, Mg^{2+}, and 5 μM CCCP and the cells were chased at 37°C for 10 or 40 min. Infected cells were incubated with monensin (10 μM) 1 h after infection before radioactive labeling. Monensin was also present in the chase media.

Preparation and Proteolytic Treatment of Membrane Fractions

VSV-infected cells were incubated on ice for 15 min with hypotonic buffer and then homogenized by 20–30 strokes with a tight-fitting homogenizer (Dounce; Kontes Glass Co., Vineland, NJ). Nuclei were removed by centrifugation at 800 *g* for 5 min. Membranes were sedimented from the supernatant by centrifugation at 18,000 *g* for 15 min at 4°C, resuspended in isotonic buffer (0.2 M sucrose, 20 mM Hepes, pH 7.5, 50 mM KCl, 2 mM Mg-acetate, 1 mM DTT) at a concentration of 300–400 μg of protein/ml, and stored in small aliquots at −70°C.

Aliquots (60 μl) of [^{35}S]methionine- or [^{3}H]palmitic acid–labeled membrane preparations were incubated for 30 min at 37°C with bromelain (50 μg) or proteinase K (10 μg). PMSF (40 μg/ml) was added to proteinase K digestions and the samples were kept for 10 min on ice. Proteins were precipitated with 1 vol of 20% TCA and collected by centrifugation for 10 min in a centrifuge (Eppendorf, Hamburg, FRG) at 4°C. Pellets were washed in cold acetone and solubilized in gel sample buffer.

PAGE

Proteins and G protein fragments obtained after chemical cleavage at asparagine–glycine bonds by hydroxylamine (Saris et al., 1983) were analyzed on 10 or 12.5% polyacrylamide gels, respectively, using the discontinuous buffer system of Laemmli (1970). Electrophoretic separation was carried out under reducing conditions at 15 mA (constant current) for 11 h. ^{14}C-methylated protein markers (Amersham Buchler GmbH, Braunschweig, FRG) were used as molecular weight standards. Gels were fixed, impregnated with 1 M sodium salicylate, vacuum dried, and exposed on x-ray film (Cronex 4; DuPont de Nemours GmbH, Bad Homburg, FRG) at −70°C (Chamberlain, 1979).

Materials

Triton X-100 and swainsonine were from Sigma Chemical GmbH (Munich, FRG). Bromelain, monensin, tunicamycin, and CCCP were from Calbiochem-Behring GmbH (Frankfurt, FRG). Proteinase K, deoxynojirimycin, and PMSF were purchased from Boehringer-Mannheim GmbH (Mannheim, FRG). Hydroxylamine and guanidinium hydrochloride were from Fluka GmbH (Neu-Ulm, FRG). [^{35}S]Methionine (specific activity 1,190 Ci/mmol) was purchased from Amersham Buchler GmbH. [9,10-^{3}H]Palmitic acid (specific activity 30 Ci/mmol) was obtained from New England Nuclear (Dreieich, FRG).

Results

Influence of the Oligosaccharide Structure on the Electrophoretic Mobility of the G_1 and G_2 Species

Since we planned to study the proteolytic cleavage of membrane-integrated G_1 and G_2 protein molecules, we had to define their structural difference. Chemical cleavage of purified, [^{35}S]methionine-labeled G protein at asparagine–glycine bonds by hydroxylamine (Saris et al., 1983) was used to localize the structural feature responsible for the difference in electrophoretic mobility of the G_1 and G_2 species. G protein has two potential cleavage sites at asparagine^{166} and asparagine^{387} (Rose and Gallione, 1981). Five fragments in addition to uncleaved G protein (Fig. 1 *B*) appeared after hydroxylamine cleavage on the gel. Three fragments with the highest mobilities in SDS-PAGE represent final cleavage products. They can be aligned to the COOH-terminal region containing the cytoplasmic and transmembrane domain and 75 amino acids of the ectodomain (15,900 M_r), to the NH_2-terminal region (19,700 M_r), and to the central part of the G sequence joining these two other fragments (34,700 M_r), which includes the two glycosylation sites of the G protein (Rose and Gallione, 1981). The apparent molecular weight of the NH_2-terminal and COOH-terminal fragments of G_1 and G_2 seem to be identical since they comigrated in SDS-PAGE (Fig. 1 *B*). In contrast, a shift in electrophoretic mobility, similar in size as for the corresponding uncleaved molecules, was observed for the glycosylated central fragments of G_1 and G_2 (Fig. 1 *B*). The difference in the oligosaccharide side chains of the G_1 and the G_2 species was also demonstrated by endoglycosidase H (endo H) cleavage. The G_1 species of the G protein was sensitive whereas the G_2 species was resistant to digestion by endo H (Fig. 1 *A*, lanes *1–4*). Therefore, the structural differences between G_1 and G_2 that influence the electrophoretic mobility are localized in the differentially processed oligosaccharide moieties of these molecules.

This interpretation is consistent with previous results. G_1 represents the core glycosylated form of the G protein. Processing of the initially transferred $GlcNAc_2Man_9Glc_3$ residues of the G_1 species occurs during the passage of the G protein from the endoplasmic reticulum via the Golgi apparatus to the plasma membrane (Kornfeld and Kornfeld, 1985). Conversion of the G_1 to the G_2 species, giving rise to $GlcNAc_5Man_3Gal_3NeuAc_3$ oligosaccharide side chains, is finished before the appearance of the G_2 form on the cell surface where this species is integrated into budding virus particles (Reading et al., 1978; Knipe et al., 1977).

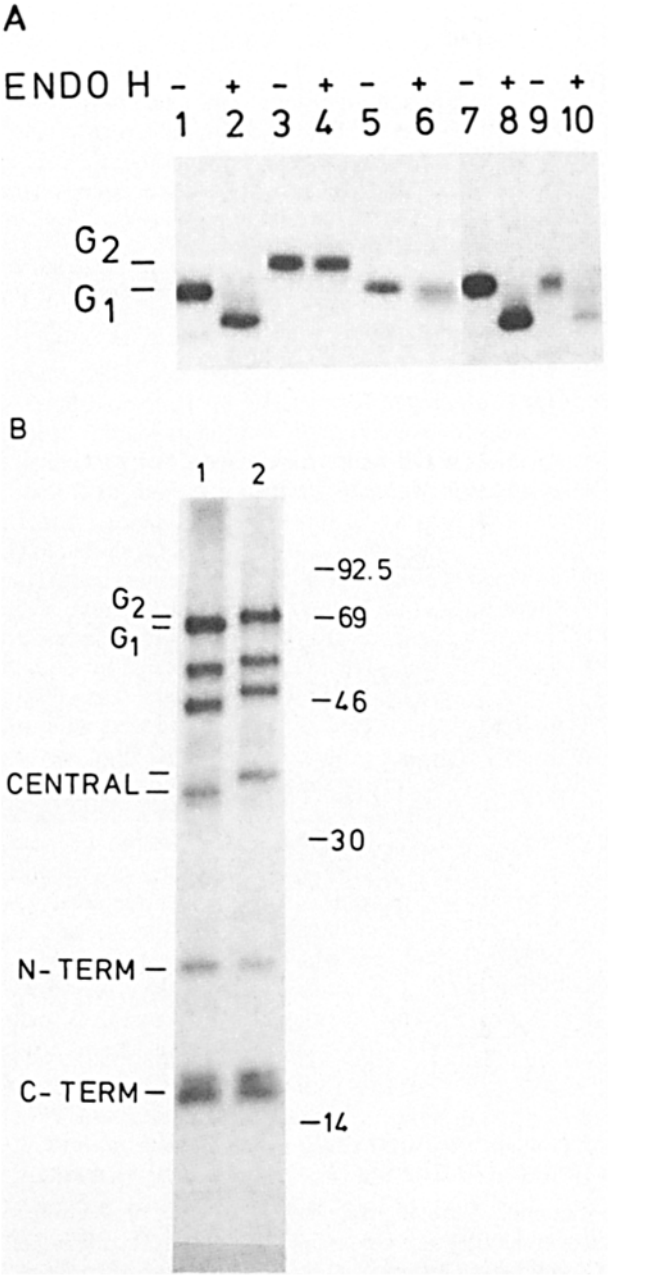

Figure 1. endo H and hydroxylamine cleavage of G_1 and G_2. (*A*) VSV-infected BHK cells were treated with monensin (lanes *5* and *6*), swainsonine (lanes *7* and *8*), and deoxynojirimycin (lanes *9* and *10*) or served as controls (lanes *1–4*). Cells were labeled with [^{35}S]methionine for 10 min and were chased for 10 (lanes *1* and *2*), 40 (lanes *3* and *4*), and 90 min (lanes *5–10*) in the presence of unlabeled methionine. Cytoplasmic extracts were prepared and digested with endo H for 18 h at 37°C (Graeve et al., 1986); endo H was omitted in the controls. Proteins were separated on a 10% SDS-polyacrylamide gel. (*B*) The G_1 (lane *1*) and G_2 species (lane *2*) were excised from the gel and cleaved by hydroxylamine. Fragments were separated on a 12.5% SDS-polyacrylamide gel. Fluorograms of the gels are shown.

The Protection of the Cytoplasmic Domain of the G Protein by Intracellular Membranes against Proteolysis Is Changed during Intracellular Transport

At the site of synthesis, G protein is anchored by its hydrophobic domain in the lipid bilayer of the endoplasmic reticulum in such a way that the COOH-terminal tail remains exposed to the cytoplasm whereas the main body of the protein points into the lumen of the endoplasmic reticulum and is thus completely surrounded by the microsomal membrane. En bloc transfer of preformed high-mannose oligosaccharides gives rise to the G_1 species with an apparent 66,000 M_r, which is exclusively labeled during the first 20 min (Fig. 2). Trimming and processing of the side chains resulted in the formation of the G_2 species with an apparent 69,000 M_r carrying complex-type oligosaccharides, which appeared after 20 min of chase and became the major species after 40–50 min of chase (Fig. 2). When the membrane fractions were incubated with bromelain, part of the cytoplasmic do-

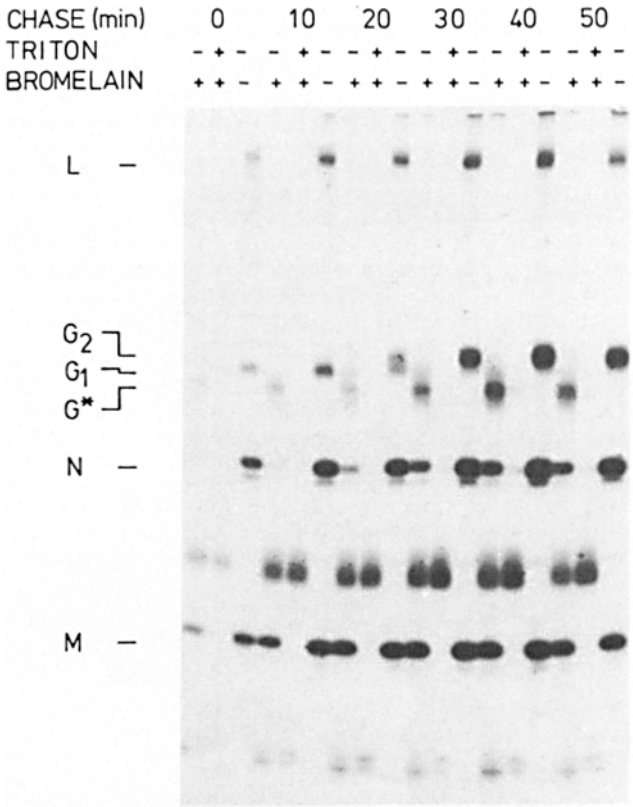

Figure 2. Protease digestion of membrane-integrated G protein at different stages of intracellular transport. VSV-infected BHK cells were labeled with [^{35}S]methionine for 10 min followed by a chase in the presence of unlabeled methionine. Membrane fractions were prepared every 10 min from the beginning of the chase, and aliquots were digested with bromelain in the presence or absence of 0.1% Triton X-100 for 1 h at 37°C. As a control, bromelain was omitted. Proteins were precipitated with TCA and separated by SDS-PAGE. A fluorogram of the gel is shown. (*G**) Proteolytic fragments of G_1 and G_2, respectively. Molecular weights were determined using VSV G_2 (69,000), N (49,500), and M (30,000) proteins as markers. Newly synthesized G protein which is contained in the nuclear envelope and in vesicles of the endoplasmic reticulum that adhere in part to the cell nucleus after mechanical homogenization was partly lost after our cell fractionation procedure (Puddington et al., 1985; Bergmann and Singer, 1983). A better recovery of G protein in smooth vesicles was obtained. Total radioactive incorporation into cellular proteins did not increase during the chase period (data not shown).

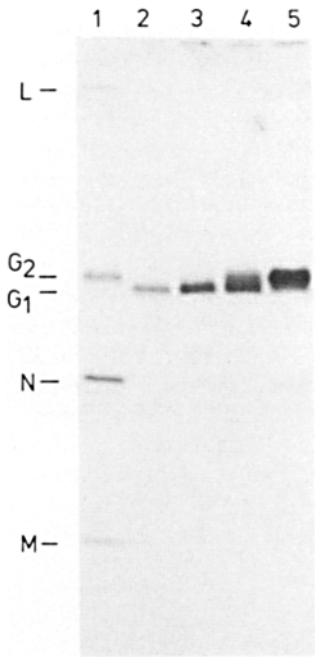

Figure 3. Kinetics of G_1 and G_2 labeling by [^{3}H]palmitic acid. VSV-infected BHK cells were labeled 4.5 h after infection with [^{3}H]palmitic acid for 2 (lane *2*), 5 (lane *3*), 10 (lane *4*), and 30 min (lane *5*). Cytoplasmic extracts were prepared and analyzed by SDS-PAGE. (Lane *1*) [^{35}S]Methionine-labeled VSV proteins as marker. A fluorogram of the gel is shown.

main of both G_1 and G_2 were removed (Fig. 2, lanes *10* and *50*), giving rise to membrane-protected fragments of approximately equal electrophoretic mobility; thus, bromelain removes an apparently larger fragment from G_2 than G_1. The result suggests that in G_2 a proteolytic site closer to the lipid bilayer becomes exposed that seems to be inaccessible to bromelain in G_1. The fragments generated by bromelain

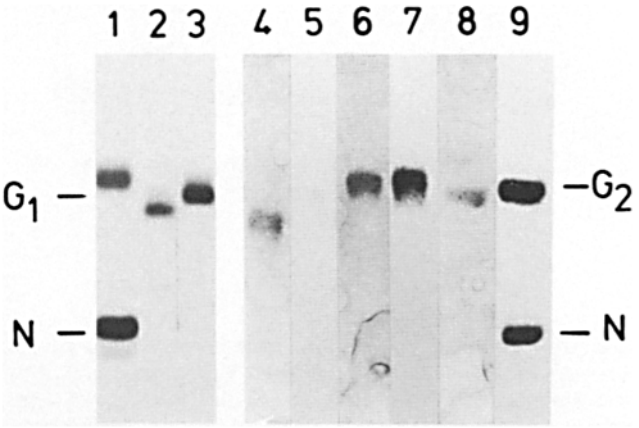

Figure 4. Bromelain digestion of membane-integrated G_1 and G_2 species labeled by [^{3}H]palmitic acid. Membranes of VSV-infected BHK cells labeled with [^{3}H]palmitic acid for 5 (lanes *2* and *3*) and 30 min (lanes *4–7*) were prepared. Aliquots were digested with bromelain for 30 min at 37°C (lanes *2*, *4*, and *5*). Bromelain was omitted in controls (lanes *3*, *6*, and *7*). Proteins were precipitated by TCA and separated on two SDS–polyacrylamide gels. A fluorogram of one gel is shown (lane *1–3*). The second gel was blotted onto nitrocellulose (Mack and Kruppa, 1988). G protein–specific polypeptides were detected with a monospecific rabbit antiserum raised against purified G protein (lanes *4*, *6*, and *8*) (Garreis-Wabnitz and Kruppa, 1984). The blot was then impregnated with 20% diphenyl oxazole in toluene and fluorographed (lanes *5*, *7*, and *9*). A fluorogram of these lanes is shown. [^{35}S]Methionine-labeled VSV served as marker (lanes *1*, *8*, and *9*).

digestion were protected by the microsomal membrane because they were degraded after solubilization of the lipid bilayer by 0.1% Triton X-100 (Fig. 2). Similar results were obtained by proteinase K digestions (data not shown). To find the cause for the observed differences in the protease sensitivity of the cytoplasmic domain of the G protein we studied the influence of acylation and carbohydrate processing on the accessibility of the COOH-terminal tail.

The Increase in the Accessibility of the Cytoplasmic Domain Is Independent of Acylation

Palmitic acid is covalently attached to cysteine^{489} in the cytoplasmic domain of the G protein (Rose et al., 1984; Magee et al., 1984; Mack and Kruppa, 1988). Fatty acid binding in the cytoplasmic domain of the G protein could influence the accessibility of this region to proteases. Pulse labeling of VSV-infected BHK cells by [^{3}H]palmitic acid for 2 and 5 min showed exclusively G_1 protein (Fig. 3, lanes *2* and *3*). After 10 min of pulse labeling, a fraction of G_1 shifted to G_2 (lane *4*), which became after 30 min of labeling (lane *5*) the predominant form of the G protein. Two [^{3}H]palmitic acid–labeled membrane preparations containing G_1 protein labeled for 5 min (Fig. 4, lane *3*) and G_2 protein labeled for 30 min (Fig. 4, lane *7*) were incubated with bromelain. The G_1 species completely retained the [^{3}H]palmitic acid label although an amino acid sequence with an apparent 3,000 M_r was removed (Fig. 4, lane *2*). Apparently, the acylated cysteine^{489}, which is located seven amino acids distal to the putative transmembrane domain, and the amino acids nearest to the membrane seemed to be protected against the attack of the endoprotease bromelain. In contrast, the radioactive label was completely removed from the G_2 species (Fig. 4, lane *5*). The resulting G_2 fragment which can be identified by immunoblotting has lost an amino acid sequence of ~5,100 M_r (Fig. 4, lane *4*). This observation implies that in G_2 molecules the accessibility of the acylated cytoplasmic domain is increased to proteolysis.

The Protease Sensitivity of the Cytoplasmic Domain Is Not Changed by Altering Oligosaccharide Processing

The influence of the oligosaccharide structure on the differential accessibility to proteases of the cytoplasmic domain of the G protein was investigated by using the glycosylation inhibitors deoxynojirimycin and swainsonine which block the trimming enzymes glucosidase I and α-mannosidase II, respectively (Romero et al., 1983; Tulsiani et al., 1982). G protein molecules synthesized in cells treated with these inhibitors always retain oligosaccharides that are completely endo H sensitive (Fig. 1 *A*, lanes *7–10*) even after 90 min of chase.

VSV-infected BHK cells were incubated with deoxynojirimycin and swainsonine, respectively, and labeled with [^{35}S]methionine. Membrane fractions containing mainly G_1 and G_2 protein were prepared after 10 and 40 min of chase, respectively, and digested with proteinase K and bromelain. In contrast to control cells (Fig. 5 *C*, lanes *5* and *6*), the G_2 species was not formed in cells treated with these inhibitors (Fig. 5, *A* and *B*, lanes *5* and *6*). However, a similar increase in the protease sensitivity of the cytoplasmic G protein domain was observed in membranes prepared after 40 min of chase from cells treated with deoxynojirimycin (Fig. 5, *A*, lanes

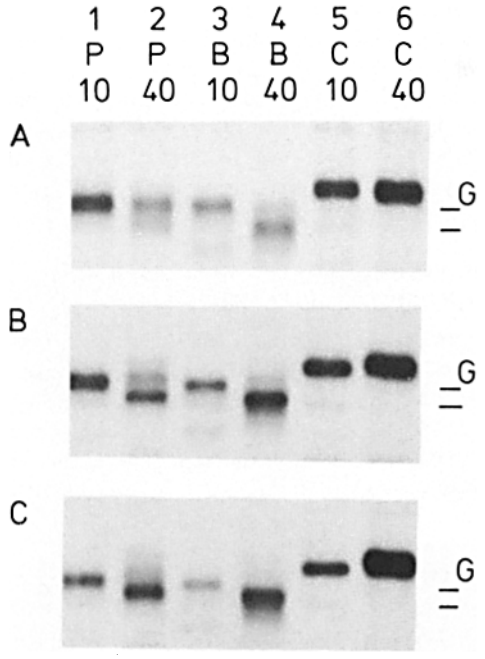

Figure 5. Protease sensitivity of membrane-integrated G protein with altered oligosaccharide processing. VSV-infected BHK cells were treated with deoxynojirimycin (*A*) and swainsonine (*B*) or served as control (*C*). The cells were labeled with [^{35}S]methionine for 10 min and were chased for 10 (lanes *1*, *3*, and *5*) or 40 min (lanes *2*, *4*, and *6*) in the presence of unlabeled methionine. Membrane fractions were prepared, and aliquots were digested with proteinase K (lanes *1* and *2*), and bromelain (lanes *3* and *4*) for 30 min at 37°C. As a control, proteases were omitted (lanes *5* and *6*). Proteins were precipitated with TCA and separated by SDS-PAGE. Fluorograms of the gels are shown. The proteolytic fragments of G_1 and G_2 are marked by lines.

1–4), swainsonine (Fig. 5 *B*, lanes *1–4*), and control cells (Fig. 5 *C*, lanes *1–4*). These results suggested that the increase in protease accessibility of the COOH-terminal tail on the cytoplasmic side of the lipid bilayer of the vesicles is independent of the modification of the carbohydrate side chains to complex-type oligosaccharides that takes place on the other side of the membrane in the lumen of the vesicles. Our observations imply that reduced glycosylation does not prevent the change in accessibility to proteolytic cleavage.

The Accessibility of the Cytoplasmic Domain Is Increased in the trans-Golgi Compartment

Since posttranslational modifications like fatty acid acylation, oligosaccharide processing, and terminal glycosylation were not essential for the differential accessibility to proteases of the cytoplasmic domain, we asked whether the intracellular transport of the G protein to a specific compartment is a prerequisite for the change in protease accessibility of the cytoplasmic domain. This was investigated by blocking intracellular transport at different compartments with CCCP and monensin. CCCP, an inhibitor of oxidative phosphorylation, blocks the exit of membrane proteins from the endoplasmic reticulum (Fries and Rothman, 1980). Monensin blocks the transport from *medial*- to *trans*-Golgi cisternae and leads to an accumulation of membrane proteins in the *medial*-Golgi cisternae (Griffith et al., 1983).

Cells were treated with the inhibitors, and labeled membrane fractions were prepared and digested with proteinase K and bromelain. CCCP-treated cells contained only G_1 (Fig. 6 *A*, lanes *5* and *6*) and therefore differences in the sensitivity of the cytoplasmic domains to proteolytic digestion could not be detected in membrane preparations from cells chased for 10 or 40 min (Fig. 6 *A*, lanes *1–4*). In monensin-treated cells, G protein was also not processed to the G_2 species (Fig. 6 *B*, lanes *9* and *10*). Using proteinase K and bromelain, no differences in the accessibility of the cytoplasmic domain of G protein could be detected in membrane preparations of cells chased for 10 and 40 min (Fig. 6 *B*, lanes *2*, *4*, *6*, and *8*). The fusiogenic G protein (Florkiewicz and Rose, 1984) apparently did not reach the cell surface in monensin-treated cells because no fusion centers could be detected after lowering the pH to 5.5 (Fig. 7 *C*) as compared with untreated (Fig. 7 *B*), deoxynojirimycin- (Fig. 7 *D*), and

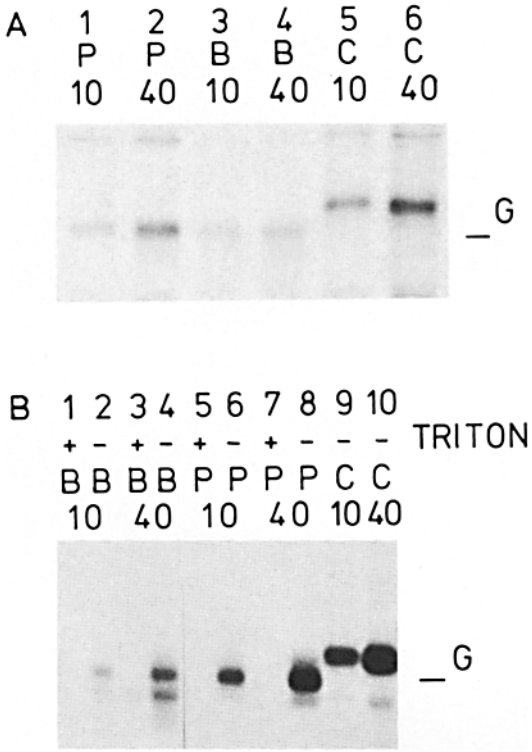

Figure 6. Protease sensitivity of membrane-integrated G protein after treatment of cells with CCCP and monensin. (*A*) VSV-infected BHK cells were labeled with [^{35}S]methionine for 10 min at 37°C. Cells were then treated with CCCP as described in Materials and Methods and chased for 10 (lanes *1*, *3*, and *5*) and 40 min (lanes *2*, *4*, and *6*). Membrane fractions were prepared, and aliquots were digested with proteinase K (lanes *1* and *2*) and bromelain (lanes *3* and *4*) for 30 min at 37°C. As a control, proteases were omitted (lanes *5* and *6*). (*B*) VSV-infected cells were treated with monensin. Cells were labeled with [^{35}S]methionine for 10 min followed by a chase for 10 (lanes *1*, *2*, *5*, *6*, and *9*) and 40 min (lanes *3*, *4*, *7*, *8*, and *10*). Membrane fractions were prepared and digested with bromelain (lanes *1–4*) and proteinase K (lanes *5–8*) in the presence and absence of 0.1% Triton X-100 for 90 min at 37°C. As a control, proteases were omitted (lanes *9* and *10*). Proteins were precipitated with TCA and analyzed by SDS-PAGE. Fluorograms of the gels are shown. The respective proteolytic fragment of G is marked by a line.

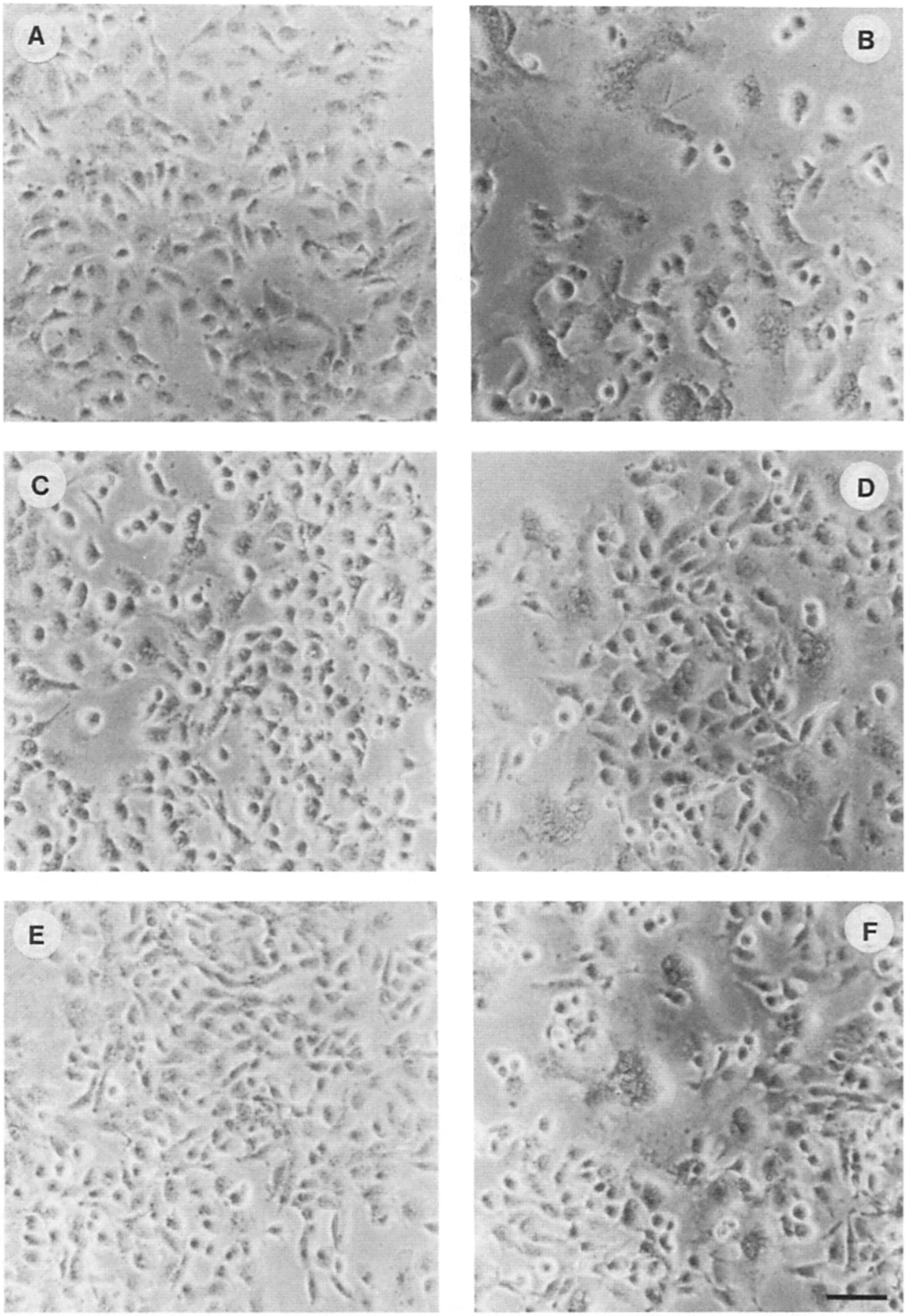

Figure 7. Fusiogenic activity of G protein exported to the cell surface of BHK cells treated with glycosylation and transport inhibitors. VSV-infected BHK cells were treated with monensin (*C*), deoxynojirimycin (*D*), (10 μg/ml) tunicamycin (*E*), and swainsonine (*F*) or were not treated with inhibitors (*B*) and not infected (*A*). 5 h after infection, cells were washed with prewarmed PBS, and prewarmed fusion buffer, pH 5.5, was added for 1 min (Florkiewicz and Rose, 1984). Fusion buffer was removed, and complete medium containing the respective inhibitors was added. After an additional hour at 37°C, cells were washed and fixed with 3% paraformaldehyde for 20 min at room temperature. Representative fields of phase-contrast microphotographs are shown. Bar, 60 μm.

swainsonine-treated (Fig. 7 *F*) cells. Apparently, partially endo H-insensitive G protein molecules accumulated in monensin-treated cells migrating in the G_1 position (Fig. 1 *A*, lanes *5* and *6*). The oligosaccharides of these molecules seem to be trimmed and not completely processed and do not contain neuraminic acid because otherwise they would migrate like G_2 (Knipe et al., 1977). The results suggest that the change in accessibility to proteases of the cytoplasmic domain of the G protein occurs after the monensin block in the *trans*-Golgi cisternae.

In membrane fractions of monensin-treated cells, an additional fragment appeared after bromelain cleavage that had a higher mobility in SDS-PAGE. This fragment was also protected by the lipid bilayer of the vesicle and became degraded when the vesicles were solubilized with Triton X-100 (Fig. 6 *B*, lanes *1–4*). The production of the fragment with the higher mobility may be related to the fragility of swollen Golgi membranes in monensin-treated BHK cells (Griffith et al., 1983).

Discussion

In this report we describe a change in the accessibility to proteases of the cytoplasmic domain of the G_1 and G_2 species of the G protein in membrane preparations of VSV-infected BHK cells. Limited cleavage by hydroxylamine (Fig. 1 *B*) located the structural difference between G_1 and G_2 species on the central glycosylated fragment. Since G_1 and G_2 were sensitive or resistant to cleavage by endo H (Fig. 1 *A*, lanes *1–4*), respectively, we conclude that the structural difference between the G_1 and G_2 species is also located in their oligosaccharide side chains (Knipe et al., 1977) and not due to aberrant electrophoretic behavior of G_1 or G_2. Kinetic analyses demonstrated that this alteration of the protease sensitivity of the cytoplasmic domain of the G protein could be detected as soon as the G_2 species was the predominant labeled species of the G protein (Fig. 2, lanes *30*). The increased accessibility of the cytoplasmic domain of the G_2 species to digestion by two different proteases with differing specificities excluded that the observed alteration was due to a fortuitous property of a single proteolytic enzyme.

During the intracellular transport of the G protein from its site of synthesis in the rough endoplasmic reticulum to the plasma membrane, the G protein is subjected to posttranslational modifications including trimming and terminal glycosylation of its oligosaccharides and covalent attachment of palmitic acid (Kornfeld and Kornfeld, 1985; Schmidt and Schlesinger, 1979).

A [^{35}S]methionine-labeled G_1 protein band in SDS-polyacrylamide gels contains a mixture of acylated and nonacylated G_1 molecules because newly synthesized G_1 molecules need a period of 15–20 min to reach the *cis*-Golgi compartment in which the fatty acid transfer occurs (Schmidt and Schlesinger, 1980). Since bromelain digestion of [^{35}S]methionine-labeled G_1 molecules gives rise to one single band, one has to assume that acylation does not account for the protection of the amino acid sequence that is proximal to the lipid bilayer (Rose et al., 1984; Mack and Kruppa, 1988).

The requirement of different glycoproteins of their oligosaccharide side chains for efficient intracellular transport varies significantly (Kornfeld and Kornfeld, 1985). Efficient intracellular transport of the G protein of the San Juan strain of VSV used in this study is very sensitive to alterations of glycosylation (Leavitt et al., 1977; Gibson et al., 1979, 1981; Schlesinger et al., 1984). The oligosaccharide side chains are thought to contribute to the correct folding of the G polypeptide chain. Since G_1 had endo H-sensitive but G_2 had endo H-resistant oligosaccharides (Fig. 1 *A*, lanes *1–4*), we investigated the effect of inhibitors of glucosidase I and α-mannosidase II, deoxynojirimycin and swainsonine, on the occurrence of the alteration of the accessibility of the cytoplasmic domain of the G protein to protease digestion. Processing of the high-mannose oligosaccharides is inhibited in cells treated with these drugs (Romero et al., 1983; Tulsiani et al., 1982) and therefore the G_2 species of the G protein was not detected (Fig. 5, *A* and *B*, lanes *5* and *6*) and the oligosaccharides remain endo H sensitive even after 90 min of chase (Fig. 1 *A*, lanes *7–10*). However, the change in the protease sensitivity of the cytoplasmic domain occurred identical to the change in untreated control cells (Fig. 5). In contrast, no change of the protease sensitivity of the cytoplasmic domain was observed when N-glycosylation of the G protein was inhibited by tunicamycin (Leavitt et al., 1977; data not shown, but results were similar to those in CCCP-treated cells [see below]). Unglycosylated G protein is not efficiently transported to the plasma membrane in tunicamycin-treated cells (Fig. 7 *E*) but accumulates in the endoplasmic reticulum (Leavitt et al., 1977), whereas the G protein synthesized in deoxynojirimycin- or swainsonine-treated cells reached the cell surface (Fig. 7, *D* and *F*; Kang and Elbein, 1983; Schlesinger et al., 1984), leading to pH-dependent cell fusion. Therefore, inhibition of trimming and terminal glycosylation of the oligosaccharides of the G protein did not influence the alteration in protease accessibility of the cytoplasmic domain as long as the G protein was normally transported to the cell surface.

We used CCCP and monensin, which block the intracellular transport between the endoplasmic reticulum and Golgi apparatus and between *medial*- and *trans*-Golgi cisternae (Fries and Rothman, 1980; Griffiths et al., 1983), to localize the intracellular compartment in which the alteration of the protease accessibility of the cytoplasmic domain of the G protein takes place. Membrane proteins accumulate in the transitional elements of the endoplasmic reticulum or *medial*-Golgi cisternae in cells treated with these drugs. In CCCP-treated cells, no change in protease accessibility of the cytoplasmic domain was detected (Fig. 6 *A*); similarly, there was no change in tunicamycin-treated cells (data not shown).

In monensin-treated cells, G protein did not reach the cell surface (Fig. 7 *C*), was not terminally glycosylated (Fig. 1 *A*, lane *5*), and was partially endo H insensitive (Fig. 1 *A*, lane *6*), and the change in the accessibility of the cytoplasmic domain of the G protein to proteases did not occur (Fig. 6 *B*). Taken together, these data and the observation that the increase in the accessibility of the cytoplasmic domain to proteolytic cleavage occurs as soon as the G_2 species is the predominantly labeled form of the G protein (Fig. 2, lanes *30*) that already had acquired endo H-resistant and terminally modified oligosaccharides (Fig. 1 *A*, lanes *1–4*; Knipe et al., 1977) strongly suggest that the change in the accessibility to proteases of the cytoplasmic domain of the G protein takes place after the monensin block in the *trans*-Golgi cisternae.

Our results show that intracellular transport of the G pro-

tein leads to a change in accessibility of the cytoplasmic domain to proteases but the data do not allow us to conclude that this change is required for efficient transport of the G protein. Studies of deletion mutants of the cytoplasmic and transmembrane domains of the G protein and of hybrid proteins containing the ecto- and transmembrane domains of the G protein fused in phase to the cytoplasmic domains of the hemagglutinin of influenza virus and of an immunoglobulin μ membrane heavy chain demonstrated that the cytoplasmic domain of the G protein is essential for efficient transport to the cell surface since many of these mutant G proteins accumulated in the endoplasmic reticulum or Golgi apparatus or were transported to the cell surface with slow kinetics (Rose and Bergmann, 1983; Adams and Rose, 1985; Puddington et al., 1986). Indirect evidence has been obtained for an oligomerization of the G protein during intracellular transport (Kreis and Lodish, 1986). Since the oligosaccharides of oligomerized G protein were reported to still be endo H sensitive, oligomerization would be assumed to occur before the alteration of the protease sensitivity of the cytoplasmic domain. Recently it has been elegantly demonstrated that trimerization of G protein takes place in the endoplasmic reticulum. Formation of trimers is essential for the subsequent transport of G protein via the Golgi complex to the cell surface (Doms et al., 1987). The proper folding into the correct quaternary structure seems to be one general prerequisite for the exit from the endoplasmic reticulum. This hypothesis has been suggested from studies of monomeric or incompletely assembled subunits of immunoglobulins (Mains and Sibley, 1983; Bole et al., 1986), retinal-binding protein (Ronne et al., 1983), influenza virus hemagglutinin (Gething et al., 1986), and the major histocompatibility complex I antigens (Severinsson and Peterson, 1984), which are not transported.

Since trimerization of G protein takes place in the endoplasmic reticulum, it cannot be directly related to the change in the proteolytic accessibility of the cytoplasmic domain. However, trimerization of the hemagglutinin of influenza virus is also an early event, but stabilization of the trimer to resist detergent extraction and gradient centrifugation takes place only in the *trans*-Golgi cisternae and may involve a change in the base of the trimer or in the transmembrane anchors (Copeland et al., 1986). A similar stabilization event of the G protein trimer could result in the observed increase in the protease accessibility of the cytoplasmic domain. Therefore, it has been postulated that additional information in the cytoplasmic domain may also be required for transport (Rose and Bergmann, 1982; Doyle et al., 1985; Doms et al., 1987).

The increase in accessibility of the cytoplasmic domain in the late Golgi compartment may also be a result of the lower pH of these cisternae (Anderson and Pathak, 1985). The conformational change of the cytoplasmic domain may serve the purpose of making the cytoplasmic tail of the G protein competent for the interaction with the virus core. This speculation is compatible with studies of Metsikkö and Simons (1986) that have shown that the tail is essential for viral particle formation.

On the other hand, the increase of the proteolytic accessibility of the cytoplasmic domain of the G protein could result from other effects than a conformational change. First, proteins of viral or cellular origin could specifically bind to the cytoplasmic domain of the G protein when it reaches the *trans*-Golgi cisternae and may alter the accessibility to proteolytic digestion. Second, different surroundings with respect to lipid and protein composition in different intracellular compartments could result in an altered exposure of the cytoplasmic domain of the G protein. Although the significance of the hydrophobic amino acids of the transmembrane domain of the G protein for membrane anchorage has been clearly established (Rose and Bergmann, 1982; Guan and Rose, 1984), the exact boundary between the transmembrane and cytoplasmic domains is not well defined. Exposure of these boundaries could vary in different compartments and lead to an altered accessibility of the cytoplasmic domain to proteolytic digestion.

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References

Adams, G. A., and J. K. Rose. 1985. Structural requirements of a membrane-spanning domain for protein anchoring and cell surface transport. *Cell.* 41:1007–1015.

Anderson, R. G. W., and R. K. Pathak. 1985. Vesicles and cisternae in the trans Golgi apparatus of human fibroblasts are acidic compartments. *Cell.* 40:635–643.

Berger, M., and M. F. G. Schmidt. 1985. Protein fatty acyltransferase is located in the rough endoplasmic reticulum. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 187:289–294.

Bergmann, J. E., and S. J. Singer. 1983. Immunoelectron microscopic studies of the intracellular transport of the membrane glycoprotein (G) of vesicular stomatitis virus in infected Chinese hamster ovary cells. *J. Cell Biol.* 97:1777–1787.

Bole, D. G., L. M. Hendershot, and J. F. Kearney. 1986. Posttranslational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. *J. Cell Biol.* 102:1558–1566.

Burgess, T. L., and R. B. Kelly. 1987. Constitutive and regulated secretion of proteins. *Annu. Rev. Cell Biol.* 3:243–293.

Chamberlain, J. P. 1979. Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. *Anal. Biochem.* 98:132–135.

Copeland, C. S., R. W. Doms, E. M. Bolzau, R. G. Webster, and A. Helenius. 1986. Assembly of influenza hemagglutinin trimers and its role in intracellular transport. *J. Cell Biol.* 103:1179–1191.

Doms, R. W., D. S. Keller, A. Helenius, and W. E. Balch. 1987. Role for adenosine triphosphate in regulating the assembly and transport of vesicular stomatitis virus G protein trimers. *J. Cell Biol.* 105:1957–1969.

Doyle, C. M., M. G. Roth, J. Sambrook, and M. -J. Gething. 1985. Mutations in the cytoplasmic domain of influenza virus hemagglutinin affect different stages of intracellular transport. *J. Cell Biol.* 100:704–714.

Dunphy, W. G., E. Fries, L. J. Urbani, and J. E. Rothman. 1981. Early and late functions associated with the Golgi apparatus reside in distinct compartments. *Proc. Natl. Acad. Sci. USA.* 78:7453–7457.

Florkiewicz, R. Z., and J. K. Rose. 1984. A cell line expressing vesicular stomatitis virus glycoprotein fuses at low pH. *Science (Wash. DC).* 225:721–723.

Fries, E., and J. E. Rothman. 1980. Transport of vesicular stomatitis virus glycoprotein in a cell-free extract. *Proc. Natl. Acad. Sci. USA.* 77:3870–3874.

Garreis-Wabnitz, C., and J. Kruppa. 1984. Intracellular appearance of a glycoprotein in VSV-infected BHK cells lacking the membrane-anchoring oligopeptide of the viral G-protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:1469–1476.

Gething, M. -J., K. McCammon, and J. Sambrook. 1986. Expression of wild-type and mutant forms of influenza hemagglutinin: the role of folding in intracellular transport. *Cell.* 46:939–950.

Gibson, R., S. Schlesinger, and S. Kornfeld. 1979. The non-glycosylated glycoprotein of vesicular stomatitis virus is temperature-sensitive and undergoes intracellular aggregation at elevated temperatures. *J. Biol. Chem.* 254:3600–3607.

Gibson, R., S. Schlesinger, and S. Kornfeld. 1981. The effect of oligosaccharide chains of different sizes on the maturation and physical properties of the G protein of vesicular stomatitis virus. *J. Biol. Chem.* 256:456–462.

Graeve, L., C. Garreis-Wabnitz, M. Zauke, M. Breindl, and J. Kruppa. 1986.

The soluble glycoprotein of vesicular stomatitis virus is formed during or shortly after the translation process. *J. Virol.* 57:968–975.

Griffiths, G., P. Quinn, and G. Warren. 1983. Dissection of the Golgi complex. I. Monensin inhibits the transport of viral membrane proteins from *medial* to *trans* Golgi cisternae in baby hamster kidney cells infected with semliki forest virus. *J. Cell Biol.* 96:835–850.

Guan, J. -L., and J. K. Rose. 1984. Conversion of a secretory protein into a transmembrane protein results in its transport to the Golgi complex but not to the cell surface. *Cell.* 37:779–787.

Kang, M. S., and A. D. Elbein. 1983. Alterations in the structure of the oligosaccharide of vesicular stomatitis virus G protein by swainsonine. *J. Virol.* 46:60–69.

Knipe, D. M., H. F. Lodish, and D. Baltimore. 1977. Localization of two cellular forms of the vesicular stomatitis viral glycoprotein. *J. Virol.* 21:1121–1127.

Kornfeld, R., and S. Kornfeld. 1985. Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* 54:631–646.

Kreis, T. E., and H. F. Lodish. 1986. Oligomerization is essential for transport of vesicular stomatitis viral glycoprotein to the cell surface. *Cell.* 46:929–937.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Leavitt, R., S. Schlesinger, and S. Kornfeld. 1977. Impaired intracellular migration and altered solubility of non-glycosylated glycoproteins of vesicular stomatitis virus and Sindbis virus. *J. Biol. Chem.* 252:9018–9023.

Mack, D., and J. Kruppa. 1988. Fatty acid acylation at the single cysteine residue in the cytoplasmic domain of the G protein of vesicular stomatitis virus. *Biochem. J.* 256:1021–1027.

Mack, D., M. Berger, M. F. G. Schmidt, and J. Kruppa. 1987. Cell-free fatty acylation of microsomal integrated and detergent-solubilized glycoprotein of vesicular stomatitis virus. *J. Biol. Chem.* 262:4297–4302.

Magee, A. I., A. H. Koyama, C. Malfer, D. Wen, and M. Schlesinger. 1984. Release of fatty acids from viral glycoproteins. *Biochim. Biophys. Acta.* 798:156–166.

Mains, P. E., and C. H. Sibley. 1983. The requirement of the light chain for the surface deposition of the heavy chain of immunoglobulin M. *J. Biol. Chem.* 258:5027–5033.

Metsikkö, K., and K. Simons. 1986. The budding mechanism of spikeless vesicular stomatitis virus particles. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:1913–1920.

Morrison, T. G., and L. J. Ward. 1984. Intracellular processing of the vesicular stomatitis virus glycoprotein and the Newcastle disease virus hemagglutinin-neuraminidase glycoprotein. *Virus Res.* 1:225–239.

Puddington, L., M. O. Lively, and D. S. Lyles. 1985. Role of the nuclear envelope in synthesis, processing, and transport of membrane proteins. *J. Biol. Chem.* 260:5641–5647.

Puddington, L., C. E. Machamer, and J. K. Rose. 1986. Cytoplasmic domains of cellular and viral integral membrane proteins substitute for the cytoplasmic domain of the vesicular stomatitis virus glycoprotein in transport to the plasma membrane. *J. Cell Biol.* 102:2147–2157.

Reading, C. L., E. E. Penhoet, and C. E. Ballou. 1978. Carbohydrate structure of VSV glycoprotein. *J. Biol. Chem.* 253:5600–5607.

Romero, P. A., R. Datema, and R. T. Schwartz. 1983. *N*-methyl-1-deoxynojirimycin, a novel inhibitor of glycoprotein processing, and its effect on fowl plague virus maturation. *Virology.* 130:238–242.

Ronne, H., C. Ocklind, K. Wiman, L. Rask, B. Obrink, and P. A. Peterson. 1983. Ligand dependent regulation of intracellular protein transport: effect of vitamin A on secretion of retinol-binding protein. *J. Cell Biol.* 96:907–910.

Rose, J. K., and J. E. Bergman. 1982. Expression from cloned cDNA of cell surface and secreted forms of the glycoprotein of vesicular stomatitis virus in eucaryotic cells. *Cell.* 30:753–762.

Rose, J. K., and J. E. Bergmann. 1983. Altered cytoplasmic domains affect intracellular transport of the vesicular stomatitis virus glycoprotein. *Cell.* 34:513–524.

Rose, J. K., and C. J. Gallione. 1981. Nucleotide sequences of the mRNA's encoding the vesicular stomatitis virus G and M proteins determined from cDNA clones containing the complete coding regions. *J. Virol.* 39:519–528.

Rose, J. K., G. A. Adams, and C. J. Gallione. 1984. The presence of cysteine in the cytoplasmic domain of the vesicular stomatitis virus glycoprotein is required for palmitate addition. *Proc. Natl. Acad. Sci. USA.* 81:2050–2054.

Sabatini, D. D., G. Kreibich, T. Morimoto, and M. Adesnik. 1982. Mechanisms for the incorporation of proteins in membranes and organelles. *J. Cell Biol.* 92:1–22.

Saris, C. J. M., J. van Eenbergen, B. G. Jenks, and H. P. J. Bloemers. 1983. Hydroxylamine cleavage of proteins in polyacrylamide gels. *Anal. Biochem.* 132:54–67.

Schlesinger, S., C. Malfer, and M. J. Schlesinger. 1984. The formation of vesicular stomatitis virus (San Juan strain) becomes temperature-sensitive when glucose residues are retained on the oligosaccharides of the glycoprotein. *J. Biol. Chem.* 259:7597–7601.

Schmidt, M. F. G., and M. J. Schlesinger. 1979. Fatty acid binding to vesicular stomatitis virus glycoprotein: a new type of post-translational modification of the viral glycoprotein. *Cell.* 17:813–819.

Schmidt, M. F. G., and M. J. Schlesinger. 1980. Relation of fatty acid attachment to the translation and maturation of vesicular stomatitis and Sindbis virus membrane glycoproteins. *J. Biol. Chem.* 255:3334–3339.

Severinsson, L., and P. A. Peterson. 1984. β_2-Microglobulin induces intracellular transport of human class I transplantation antigen heavy chains in *Xenopus laevis* oocytes. *J. Cell Biol.* 99:226–232.

Tulsiani, D. R. P., S. C. Hubbard, P. W. Robbins, and O. Touster. 1982. α-Mannosidases of rat liver Golgi membranes: mannosidase II is the GlcNAcMan$_5$-cleaving enzyme in glycoprotein biosynthesis and mannosidase 1A and 1B are enzymes converting Man$_9$ precursors to Man$_5$ intermediate. *J. Biol. Chem.* 257:3660–3668.