

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

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Abstract. Oligonucleotide-directed mutagenesis was used to construct chimeric cDNAs that encode the extracellular and transmembrane domains of the vesicular stomatitis virus glycoprotein (G) linked to the cytoplasmic domain of either the immunoglobulin μ membrane heavy chain, the hemagglutinin glycoprotein of influenza virus, or the small glycoprotein (p23) of infectious bronchitis virus. Biochemical analyses and immunofluorescence microscopy demonstrated that these hybrid genes were correctly expressed in eukaryotic cells and that the hybrid proteins were transported to the plasma membrane. The rate of transport to the Golgi complex of G protein with an immunoglobulin μ membrane cytoplasmic domain was approximately sixfold slower than G protein with its normal cytoplasmic domain. However, this rate was virtually identical to the rate of transport of μ_m

heavy chain molecules measured in the B cell line WEHI 231. The rate of transport of G protein with a hemagglutinin cytoplasmic domain was threefold slower than wild type G protein and G protein with a p23 cytoplasmic domain, which were transported at similar rates. The combined results underscore the importance of the amino acid sequence in the cytoplasmic domain for efficient transport of G protein to the cell surface. Also, normal cytoplasmic domains from other transmembrane glycoproteins can substitute for the G protein cytoplasmic domain in transport of G protein to the plasma membrane. The method of constructing precise hybrid proteins described here will be useful in defining functions of specific domains of viral and cellular integral membrane proteins.

INTEGRAL membrane proteins and secretory proteins are synthesized on membrane-bound ribosomes of the endoplasmic reticulum (ER)¹. Signal peptides, required to initiate transfer of nascent polypeptides across the ER during synthesis, have been identified for most eukaryotic secretory and integral membrane proteins thus far examined (7, 51). Addition of high mannose core oligosaccharides occurs on the cisternal surface of the rough ER during translocation of the nascent chain (49). Hydrophobic regions of 20–30 amino acids serve to anchor glycoproteins in the membrane. Secretory proteins lack these transmembrane hydrophobic regions and are completely translocated across the ER membrane. Also, complete translocation of the extracytoplasmic domain

of proteins occurs when the transmembrane and cytoplasmic domains are artificially deleted from cDNAs encoding integral membrane proteins (19, 48, 57). From their site of synthesis in the rough ER, integral membrane and secretory proteins are transferred to their site of function, which can include the ER, Golgi complex, lysosomes, or plasma membrane. The mechanisms that direct proteins to these specific destinations in the cell are not well understood, with the exception of mannose 6-phosphate which targets some enzymes to lysosomes (10, 13).

The pathway of biogenesis of the vesicular stomatitis virus (VSV) glycoprotein (G) has been well characterized and serves as a model for many viral and cellular integral membrane proteins targeted to the plasma membrane. In VSV-infected cells, G protein is transported from its site of synthesis in rough ER (24, 38) and nuclear membranes (41) to a site in the Golgi complex where its oligosaccharides are modified (58). G protein is finally transported to the plasma membrane where it is incorporated into budding virions (26). The same pathway of G protein biogenesis is observed in the absence of

1. *Abbreviations used in this paper.* δm , immunoglobulin δ membrane heavy chain; DME, Dulbecco's modified Eagle's medium; endo H, endoglycosidase H; ER, endoplasmic reticulum; FCS, fetal calf serum; G*, G protein with a shortened cytoplasmic domain; G μ , G protein with an immunoglobulin μ membrane heavy chain cytoplasmic domain; HA, hemagglutinin; IBV, infectious bronchitis virus; μ_m , immunoglobulin μ membrane heavy chain; μ_s , immunoglobulin μ secreted heavy chain; SV40, simian virus 40; VSV, vesicular stomatitis virus.

other VSV proteins when a cDNA clone encoding G protein is expressed under control of the simian virus 40 (SV40) late promoter in transfected cells (48).

An earlier study on VSV G protein addressed the role of the cytoplasmic domain in this transport process. When cDNA clones encoding G proteins in which the carboxyl-terminal half of the normal cytoplasmic domain was replaced with 3–23 extraneous amino acids encoded by SV40 are expressed in transfected cells, G protein is transported to the cell surface at a much slower rate than the wild type G protein (as suggested by rate of acquisition of sugars in the Golgi complex that are resistant to removal by endoglycosidase H [endo H]). When the entire normal 29 amino acid cytoplasmic domain is deleted from G protein and replaced with 3 or 12 vector-encoded amino acids, G protein accumulates in rough ER and nuclear membranes and is not transported to the cell surface (47). A different approach to examining the role of the cytoplasmic domain in transport of G protein to the cell surface yielded similar results. In VSV-infected cells, microinjection of antibodies directed against the 11 carboxyl-terminal amino acids in the cytoplasmic domain of G protein has little effect on the exocytotic transport of G protein, but microinjection of antibodies directed against the 22 carboxyl-terminal amino acids in the cytoplasmic domain completely abolishes G protein transport to the surface (4). These studies, and similar studies examining expression of cDNA clones encoding the Rous sarcoma virus or influenza virus envelope glycoproteins with altered cytoplasmic domains, show that the structure of the cytoplasmic domain is important to a normal rate of glycoprotein transport to the cell surface (15, 62). Such studies suggested that the cytoplasmic domains of at least some proteins might constitute signals that facilitate transport to the cell surface. Deletions have also been constructed in regions of cDNA clones which specify the cytoplasmic domains of the Semliki Forest virus E2 glycoprotein (18) or a murine major histocompatibility antigen (H-2L^d) (39, 65). These mutant glycoproteins are expressed on the cell surface. However, portions of the normal cytoplasmic domains were retained in the constructions (3/31, 7/31, or 11/31 amino acids) and rates of transport through the cell were not measured.

At least two types of cellular transmembrane surface glycoproteins have very short cytoplasmic domains consisting of three amino acid residues, immunoglobulin μ and δ membrane heavy chains (μ_m , δ_m) (11, 25). From previous studies it was determined that biosynthesis and maturation of μ_m or δ_m occurs via a pathway similar to the VSV G protein: from the rough ER through the Golgi complex to the plasma membrane (53, 60). Thus, if the cytoplasmic domain is important for directed transport of these proteins, three amino acids must be sufficient.

In this study, we generated chimeric cDNAs in which nucleotides encoding the cytoplasmic domains of other integral membrane proteins were substituted for nucleotides of the G gene encoding its cytoplasmic domain. Specifically, we were able to construct G genes encoding the extracellular and transmembrane domains of G protein and the cytoplasmic domain of μ_m , the hemagglutinin (HA) glycoprotein of influenza virus, or the small glycoprotein (p23) of infectious bronchitis virus (IBV). When these genes were expressed under control of the SV40 late promoter in eukaryotic cells, the

hybrid glycoproteins were transported to the plasma membrane. Each hybrid protein was transported from the rough ER through the Golgi complex at a different rate, and in one case, that of G protein with the μ_m cytoplasmic domain, this rate was the same as the normal rate of transport of the intact parent μ_m glycoprotein.

Materials and Methods

Oligonucleotide-directed Mutagenesis

Synthetic oligonucleotides used here (34mer, 25mer, and 60mer) were synthesized on a DNA synthesizer (Applied Biosystems, Inc., Foster City, CA or model 1450A, Sys-Tec, Inc., New Brighton, MN) using phosphoramidite chemistry. Deblocked oligonucleotides were purified by electrophoresis on 12% polyacrylamide gels. Each oligonucleotide primer was annealed to purified DNA from an mp8 bacteriophage that contains the negative strand of the G gene (mp8-G4). Extension of primers on the phage template was carried out using the Klenow fragment of DNA polymerase I as described (46). Single-stranded template remaining after the extension was nicked by incubation with a low concentration of S1 nuclease before transformation of *Escherichia coli* strain JM103 (33). 15–25% of plaques screened hybridized (21) with the respective 5'-[³²P]-labeled oligonucleotide. Phage replicative-form DNA was prepared from colonies that hybridized with the oligonucleotide and the fragments containing each of the mutant G genes were excised and ligated into the XhoI site of the SV40-based expression vector pJC119 (54). Correct orientation of the insert was determined by digestion with restriction enzymes. The nucleotide sequence at the site of the mutation was confirmed using the technique of Maxam and Gilbert (35). Initial attempts to use a 28mer to introduce the same mutation in the G gene generated by the 34mer were unsuccessful. This oligonucleotide, which contained only eight complementary terminal nucleotides, appeared to hybridize to, but was not extended on, the mp8-G4 phage template.

Construction of pG23

cDNA-encoding IBV p23 was cloned from viral genomic RNA (55), and the complete nucleotide sequence of a subclone (p57-6) containing the coding region was determined (J. Rose, unpublished results). The sequence predicts a polypeptide of 225 amino acids, and differs in only a few nucleotides from a sequence recently published for the p23 gene of IBV (9). A 780 base-pair fragment containing the complete coding sequence of p23 was subcloned, using XhoI linkers, into the single XhoI site of the expression vector pJC119 (54). An internal BamHI site at nucleotide 338 (equivalent to nucleotide 453 in reference 9) was utilized to generate a fragment containing the 3' half of the coding sequence. This fragment includes the entire sequence encoding the putative cytoplasmic domain of p23 (9, 50). This fragment was ligated to a BamHI fragment containing the sequence encoding the extracellular and transmembrane domains of G protein, as well as the four amino-terminal amino acids derived from the G cytoplasmic domain, and reinserted into pJC119. The G-encoding fragment was obtained from a mutant generated by oligonucleotide-directed mutagenesis of the G gene, with a single nucleotide change at position 1483 (46) to produce a BamHI site (plasmid pTMB). This recombinant results in the in-frame fusion of the coding sequences of G protein and p23, and was expected to encode a hybrid protein of 612 amino acids: the amino-terminal 485 amino acids of G protein, and the carboxyl-terminal 127 amino acids of p23.

Cell Lines and Viruses

COS-1 cells originally established by Gluzman (20) were maintained in Dulbecco-Vogt's modified Eagle's medium (DME) supplemented with 5% fetal calf serum (FCS). A subcloned line of WEHI 231 cells (WEHI 231.2.TG.3) (42) was obtained from William C. Raschke (La Jolla Cancer Research Foundation, La Jolla, CA). These cells were maintained in DME supplemented with 10% heat-inactivated FCS and 50 μ M 2-mercaptoethanol.

Stocks of wild type VSV, San Juan strain of the Indiana serotype, were prepared by infection of baby hamster kidney cells with virus at a multiplicity of 0.01 plaque-forming units/cell for 16 h. Clarified culture supernatants were used as virus stocks for experiments.

Antibodies and Immunofluorescent Staining

Indirect immunofluorescence microscopy of transfected COS-1 cells was carried out essentially as previously described (48). Paraformaldehyde-fixed cells were

incubated in hyperimmune rabbit serum directed against VSV (1:200), followed by rhodamine- or Texas Red-conjugated goat anti-rabbit IgG (1:200) (Cappel Laboratories, Cochranville, PA, or Jackson Immunolabs, Avondale, PA). The affinity-purified secondary antibodies obtained from Jackson Immunolabs used in some of these experiments exhibited lower background fluorescence than the non-affinity-purified conjugated antibodies used in previous studies (47). Thus, our ability to detect low levels of cell-surface fluorescence was significantly greater than previously described. After permeabilization with detergent, cells were incubated in hyperimmune mouse serum directed against VSV (1:200), then fluorescein-conjugated affinity-purified rabbit or goat anti-mouse IgG (1:20) (Cappel Laboratories). The anti-VSV serum prepared in mice was obtained from Douglas S. Lyles (Wake Forest University Medical Center, Winston-Salem, NC).

Transfection and Labeling of COS-1 Cells

Transfection of COS-1 cells with plasmid DNA precipitated with DEAE-dextran was performed as described (1). Approximately 40 h after transfection, cells were depleted of methionine by incubation with methionine-free DME for 15 min. Cells were pulse-labeled for 15 min with [³⁵S]methionine (100 μ Ci/ml, >600 Ci/mmol) in methionine-free medium containing 2% dialyzed FCS, then chased by incubation in medium containing 5% FCS and unlabeled methionine (2 mM) for varying lengths of time. Proteins from cells at each time point were solubilized in detergent solution (10 mM Tris, pH 7.4, 1% Nonidet P-40, 0.4% deoxycholate, 66 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 100 U kallikrein inhibitor/ml) and nuclei removed by centrifugation at 15,000 g for 15 min. Immunoprecipitation of G protein with anti-VSV serum and treatment of immunoprecipitated proteins with endo H (Miles Scientific Div., Miles Laboratories Inc., Naperville, IL) has been previously described (47). The protein composition of each sample was determined by SDS-polyacrylamide gel electrophoresis (27) and fluorography (8) using preexposed Kodak XAR film (29).

Biosynthetic Labeling of B Lymphoblastoid Cells

WEHI 231 cells were washed with Dulbecco's phosphate-buffered saline (DPBS) and suspended in methionine-free DME for 15 min. Cells (7.5×10^6 /ml) were pulse-labeled for 10 min with [³⁵S]methionine (100 μ Ci/ml) in methionine-free medium containing 2% dialyzed FCS and 50 μ M 2-mercaptoethanol, then chased by incubation in maintenance medium containing an additional 2 mM unlabeled methionine for varying lengths of time. Cells from each time point were washed with DPBS and proteins were solubilized with detergent solution. Proteins present on the plasma membrane were labeled with [¹²⁵I] using a lactoperoxidase-catalyzed technique (33). Nuclei and other cellular debris were removed by centrifugation at 100,000 g for 1 h. Supernatants from an equivalent of 1.2×10^6 cells were precleared with 100 μ l of a 10% suspension of *Staphylococcus aureus* cells. The supernatant was adjusted to 0.5% SDS (52) and subjected to immunoprecipitation with goat anti-mouse IgM (heavy chain-specific, Miles Scientific Div.) or rabbit anti-mouse κ light chain (obtained from Rick Riley, Scripps Clinic and Research Foundation, La Jolla, CA). The extracts were incubated with sufficient antiserum for complete precipitation for 2 h at 4°C, followed by incubation with protein A-Sepharose 4B for 1 h at 4°C. Immunoprecipitates were washed six times with radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris [pH 7.4], 0.15 M NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, and 100 U kallikrein inhibitor/ml) (32). Immunoprecipitated proteins were eluted from Sepharose beads and their disulfide bonds reduced by incubation in 1 M Tris (pH 8.8), 2% SDS, and 10 mM dithiothreitol for 3 min at 100°C. Proteins alkylated by incubation in 30 mM iodoacetamide at room temperature for 1 h (5) were precipitated with acetone at -20°C. Precipitates were washed, boiled with 2% SDS, then treated with endo H (59). The protein composition of each sample was analyzed by electrophoresis on a 12% SDS-polyacrylamide gel and fluorography.

WEHI 231 cells were infected in the presence of 5 μ g/ml actinomycin D with 30 plaque-forming units of VSV/cell. At 5 h postinfection, cells were collected and washed with DPBS. Before labeling, infected cells were depleted of methionine by incubation with methionine-free DME for 15 min. Cells were pulse-labeled for 5 min with [³⁵S]methionine (25 μ Ci/ml) in methionine-free medium containing 2% dialyzed FCS, then chased by incubation in medium containing excess unlabeled methionine (2 mM) for varying lengths of time. Proteins from cells at each time point were solubilized and subjected to immunoprecipitation with anti-VSV serum. As described above for transfected cells, immunoprecipitated proteins were treated with endo H and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography.

Results

Oligonucleotide-directed Domain Replacement

We designed a method to construct hybrid glycoproteins in which the cytoplasmic domain of VSV G protein was replaced with the corresponding domains from other proteins. We define cytoplasmic domains of proteins having similar membrane topology as G protein as that sequence of amino acids from the first basic amino acid residue immediately following the transmembrane domain to the carboxyl-terminus. To avoid problems encountered in previous studies (15, 18, 37, 39, 43, 47, 62, 65) of assessing the contribution of endogenous or extraneous amino acids to functional domains of proteins, we chose to extend the oligonucleotide-directed mutagenesis technique (46) to replace large stretches of nucleotides instead of single nucleotides.

Nucleotides in the G gene that encode the first four amino acids in the cytoplasmic domain of G protein were replaced with nucleotides that encode the three amino acid cytoplasmic domain of IgM or IgD membrane heavy chains and a termination codon. As deduced from sequences of DNA clones, the cytoplasmic domains of μ_m and δ_m are identical and consist of the amino acids Lys-Val-Lys (11, 25). As illustrated in Fig. 1A, an oligonucleotide (34mer) was hybridized to circular template DNA containing the G gene sequence (mp8-G4) and the primer was extended. This primer encodes the sequence Lys-Val-Lys followed by a translation termination codon, and is flanked by 11 nucleotides complementary to the G gene on each side. After transfection of this DNA into *E. coli* we obtained phage carrying the desired replacement of 12 nucleotides. The mutant gene was then subcloned from the double-strand replicative-form DNA into the SV40-based expression vector pJC119 (54). The plasmid was designated pG μ .

Using the same technique, we introduced a termination codon following nucleotides encoding the first three amino acids of the normal cytoplasmic domain of G protein (Fig. 1B). Finally, a synthetic 60mer was used to replace 29 nucleotides in the cytoplasmic domain of G protein with nucleotides encoding the cytoplasmic domain of an HA glycoprotein of influenza virus and a termination codon (Fig. 1C). The plasmid DNAs for expression in eukaryotic cells that carry these mutant G genes were designated pG* and pGHA, respectively.

Expression and Transport of G Protein with the μ_m Cytoplasmic Domain

All mutant genes studied here were inserted into the SV40-based vector pJC119 (54) and transfected onto COS-1 cells (20). These cells support extensive replication of the vector, therefore a high level of G protein synthesis is observed in cells that express the transfected genes (1, 33, 47, 48). To determine if the three amino acid cytoplasmic domain of μ_m was sufficient for transport of G protein to the cell surface, we performed double-label indirect immunofluorescence microscopy of COS-1 cells transfected with the plasmid pG μ that encodes G protein with the μ_m cytoplasmic domain (G μ). It should be noted that this and all other mutant proteins were analyzed by SDS-polyacrylamide gel electrophoresis (see below) and were found to have the apparent molecular weights predicted from the DNA sequence. Fig. 2 shows both surface

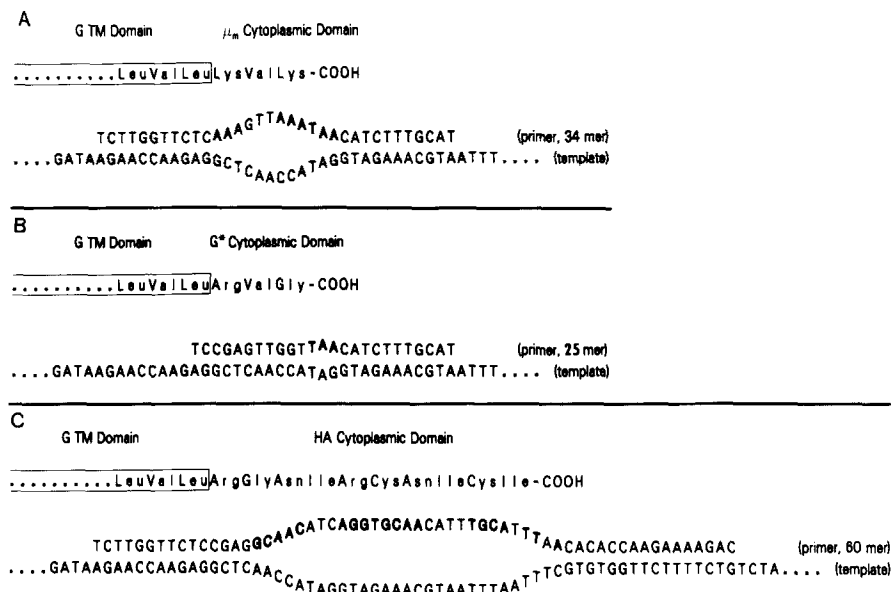


Figure 1. Oligonucleotide-directed domain replacement. The oligonucleotide primers used in this study (34mer, 25mer, and 60mer) are shown hybridized to template DNA from the mp8-G4 bacteriophage. Incorporation of the indicated mismatched bases (bold letters) into the cDNA-cloning G protein will change codons in the region of the G gene encoding the cytoplasmic domain. Such replacement of nucleotides results in precise fusions of the extracellular and transmembrane domain of G protein with the cytoplasmic domains of μ_m (A) or of HA glycoprotein of influenza virus (C; e.g., strain A/Aichi/2/68; 61). A precise termination within the G protein cytoplasmic domain coding region was also generated (B).

and internal fluorescence labeling patterns of the same cells expressing each of the indicated glycoproteins. As expected, the wild type G protein is present on the cell surface. Internal labeling of G protein in the nuclear membrane and a Golgi-like region is detected in some cells expressing the wild type G protein but is frequently difficult to resolve due to the extensive accumulation of G protein on the plasma membrane (Fig. 2, A and B; 48). In most cells (60–90%) expressing G μ or G protein with a shortened cytoplasmic domain (G*), immunofluorescent labeling of the plasma membrane was observed (Fig. 2, C–F), however it appeared less intense than for the wild type G protein. The internal labeling pattern of transfected cells that expressed G proteins with cytoplasmic domains of three amino acids included a reticular ER-like pattern including the nuclear envelope and a perinuclear Golgi-like region. These results suggest that a short cytoplasmic domain derived from a normal cellular glycoprotein μ_m and a shortened cytoplasmic domain from G protein are functional in transport of G protein to the cell surface.

Cells that express the 1473H1 glycoprotein in which all the normal cytoplasmic domain of G protein has been removed (47) were reexamined as controls for immunofluorescence microscopy experiments. This protein contains a putative cytoplasmic domain of two to three uncharged amino acids (Pro-Ser-Ser) encoded by a synthetic DNA linker. The majority of cells that express 1473H1 were stained essentially as previously described in which the mutant protein appeared to be concentrated in a reticular ER-like pattern (Fig. 2G) but not on the cell surface (Fig. 2H). Although surface expression was not detected previously (47), we found that when the combination of antibodies was optimized for immunolabeling of the cell surface, ~20% of the cells showed labeling of the

protein on the plasma membrane (Fig. 2, I–J). It is not surprising that in previous studies immunofluorescent labeling of 1473H1 on the cell surface was undetected (47). In a preliminary experiment performed in this study utilizing the same rhodamine-conjugated second-step anti-rabbit serum used in the earlier report, results similar to those previously described were obtained. However, in this study when the sample size was significantly increased and the experiment repeated three to five times utilizing an affinity-purified second-step antibody, our ability to more accurately quantitate low levels of cell-surface fluorescence significantly increased. It is interesting to note that using the new antibody combination, immunolabeling of the plasma membrane was never observed for mutant 1473 as reported (47).

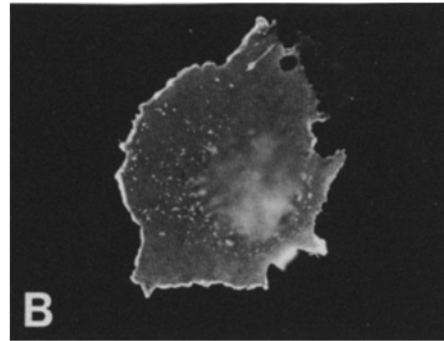
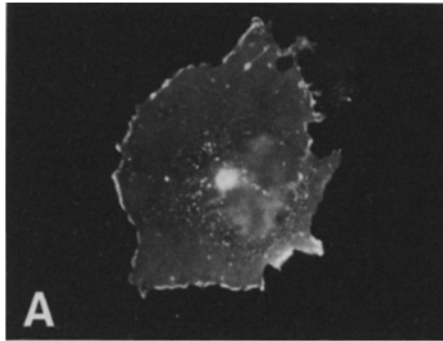
During synthesis of G protein on rough ER and nuclear membranes, there is cotranslational addition of two high mannose-type oligosaccharides (41, 49). After synthesis, G protein is rapidly transferred to a site in the Golgi complex, where its oligosaccharides are modified and become resistant to removal by the enzyme endo H (23). Thus the time course of transport of G protein to this site can be measured from its rate of acquisition of endo H-resistant oligosaccharides. To determine the time course of transport of G proteins with short cytoplasmic domains, cells transfected with pG μ , pG*, and p^Δ1473H1 were pulse-labeled with [³⁵S]methionine, then chased by incubation in medium containing unlabeled methionine for various lengths of time. Immunoprecipitates from cells at each time point were treated with endo H, and labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography (Fig. 3). The time course of transport of G protein containing a μ_m cytoplasmic domain was slower ($t_{1/2}$ = 105 min) than that observed for the wild type

Figure 2. Double-label indirect immunofluorescence microscopy showing surface and internal staining of wild type and mutant G proteins. COS-1 cells grown on coverslips were transfected with plasmids encoding the indicated proteins. Approximately 40–44 h after transfection, cells were fixed and cell-surface G proteins were labeled with rabbit anti-VSV and rhodamine- or Texas Red-conjugated anti-rabbit IgG. After permeabilization with detergent, the internal G protein was stained with mouse anti-VSV and fluorescein-conjugated anti-mouse IgG. The same field is shown in each pair of fluorescence micrographs. Typical internal (left panels) and surface (right panels) labeling patterns for each of the indicated proteins is shown. Bar, ~10 μ m.

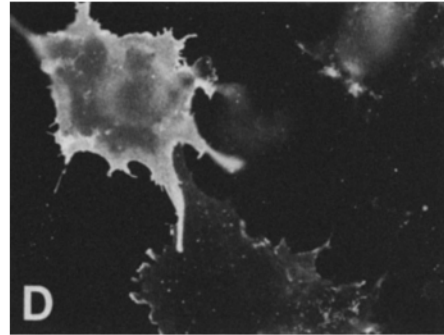
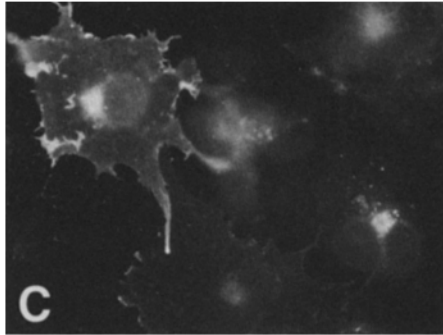
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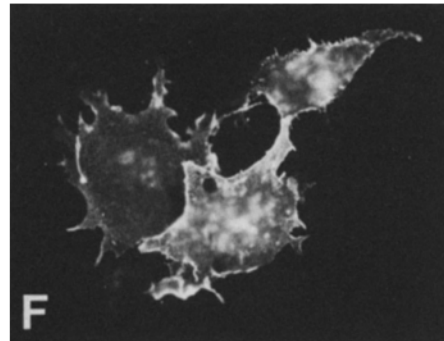
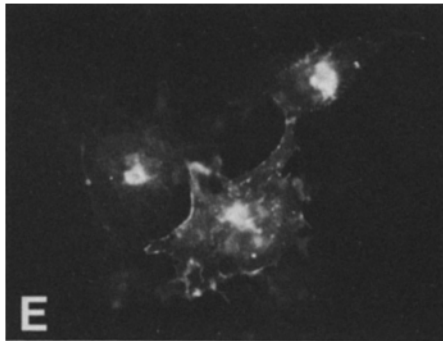
G



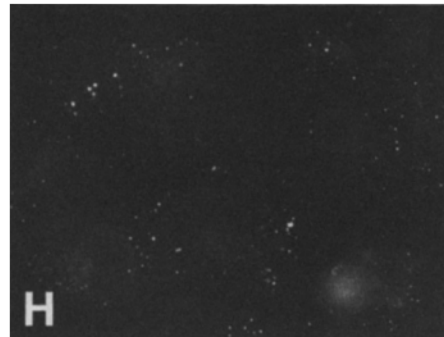
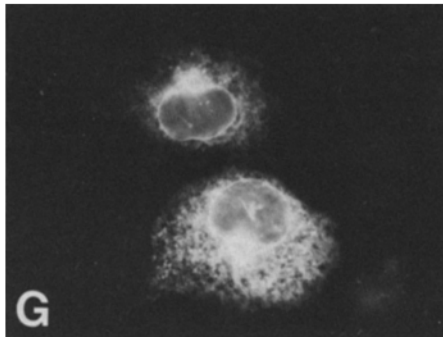
G μ



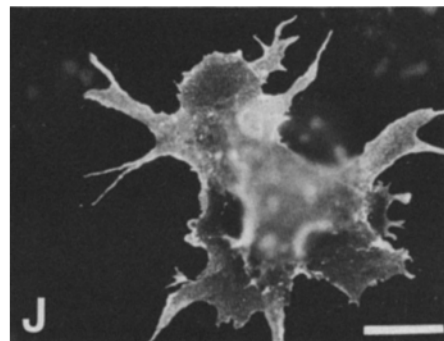
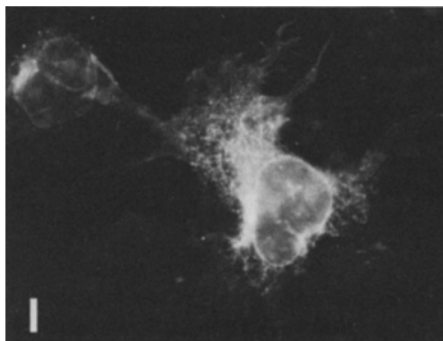
G*



1473HI



1473HI



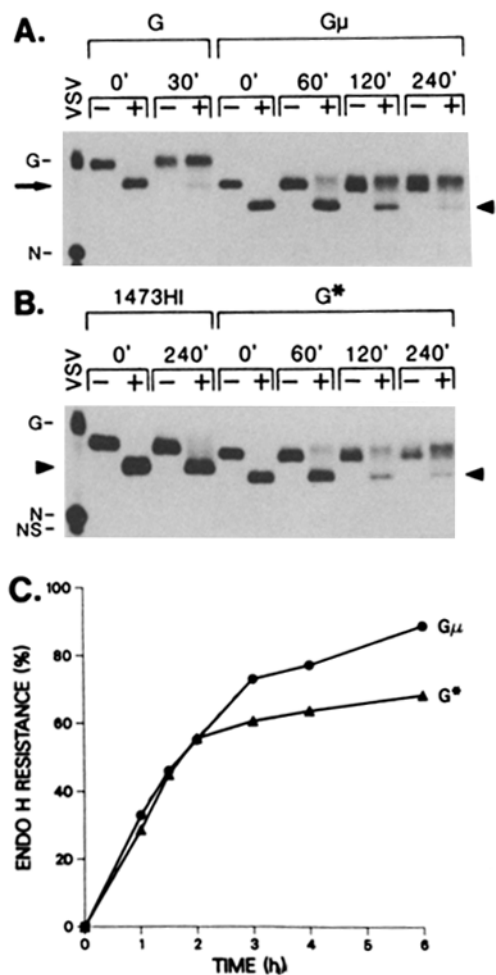


Figure 3. Time course of transport of mutant and wild type G proteins. 5-cm dishes of COS-1 cells were transfected with 10 μ g of pSVGL (encoding the wild type G protein), pG μ , pG*, or p^Δ1473H1. At 40 h posttransfection, cells were pulse-labeled for 15 min with [³⁵S]methionine, then chased by incubation in medium containing unlabeled methionine for the indicated times (in minutes). Immunoprecipitates of cells from each time point were incubated in the presence (+) or absence (-) of endo H and analyzed by SDS-polyacrylamide gel electrophoresis. Fluorograms of the dried gels are shown (A and B). The mobility of G proteins after removal of oligosaccharides by endo H is indicated for the wild type (arrow) and mutant (arrowheads) G proteins. As expected, the apparent molecular weights of G proteins with shortened cytoplasmic domains determined on SDS-polyacrylamide gels were less than that observed for the wild type G protein. Preexposed x-ray films obtained from this and similar experiments were quantified by densitometry and the rate of acquisition of endo H-resistant oligosaccharides for each of the glycoproteins was calculated. Results presented are the means of at least two experiments (C).

G protein ($t_{1/2} = 20$ min). G protein containing only the first three normal amino acids in its cytoplasmic domain had a similarly reduced rate of acquisition of endo H-resistant oligosaccharides. We also found that in some cases, 4 or 6 h after synthesis, as much as 15–25% of 1473H1 glycoprotein molecules had acquired resistance to cleavage by endo H.

Rates of μ_m and G Protein Transport in a B Cell Line

To determine if the rate of transport of G protein with a μ_m cytoplasmic domain was similar to the normal rate of trans-

port of IgM to the cell surface, we analyzed the μ_m glycoprotein produced in the B lymphoma cell line WEHI 231. IgM produced by these cells has been well characterized by others (42) and the nucleotide sequences of the genes encoding its membrane (μ_m) and secreted (μ_s) forms have been determined (44). WEHI 231 cells have characteristics of an undifferentiated B lymphocyte that produces μ_m and μ_s mRNAs in similar amounts, both of which are translated into protein (34). They synthesize large quantities of monomeric IgM as a membrane receptor, but they do not secrete IgM due to lack of synthesis of the J polypeptide chain and possibly other components required to assemble the pentameric form for secretion (28). It has been determined that biosynthesis and maturation of μ_m occurs via a pathway similar to the VSV G protein from the rough ER through the Golgi complex to the plasma membrane (36, 53, 60), however an accurate rate of transport for this protein is difficult to extrapolate from existing data (17, 60). Thus, we performed an experiment similar to that performed on COS-transfected cells to measure the rate of acquisition of endo H-resistant oligosaccharides by immunoglobulin μ_m molecules.

WEHI 231 cells were pulse-labeled with [³⁵S]methionine, then chased by incubation in medium containing unlabeled methionine for various lengths of time. Detergent extracts of cells harvested at each time point were subjected to immunoprecipitation with anti- μ or anti- κ sera. To identify the mature form of IgM on the cell surface, WEHI 231 cells were subjected to radioiodination followed by immunoprecipitation with antisera directed against μ heavy chains. Immunoprecipitated proteins were reduced, alkylated, and precipitated with acetone before incubation with endo H and analysis by SDS-polyacrylamide gel electrophoresis and fluorography (Fig. 4). The half-time for acquisition of endo H-resistant oligosaccharides by G μ in COS-1 cells was ~ 2 h and similarly the half-time for acquisition of such oligosaccharides by ³⁵S-labeled μ_m was 2 h in WEHI 231 cells. The electrophoretic mobility of the [¹²⁵I]-labeled cell surface form of μ_m increases after incubation with endo H (Fig. 4, lanes 16 and 17) as 1–2 oligosaccharides on the mature protein are unprocessed and of the high mannose-type, thus susceptible to removal by endo H (25). Virtually all the ³⁵S-labeled μ_m was mature 8 h after synthesis (Fig. 4, lanes 14 and 15) and comigrated with the ¹²⁵I-labeled μ_m , consistent with the fact that (given a half-time of 2 h) $\sim 94\%$ of the μ_m molecules have passed through the Golgi complex. Although turnover of both μ_s and μ_m occurred during this experiment, analysis by densitometry of radioactivity in each form of μ_m at the time points shown in Fig. 4 as well as earlier time points (e.g., 30 min) indicated that no selective degradation of the endo H-sensitive form of μ_m occurred in these cells (data not shown; 17). Thus, the ratio of μ_m molecules in the endo H-resistant form to total μ_m molecules can be used to determine a time course of transport of IgM from the rough ER through the Golgi complex in WEHI 231 cells.

Parallel immunoprecipitations of detergent extracts with antibodies directed against either μ or κ chains demonstrated that excess free heavy or light chains were detectable immediately after synthesis (Fig. 4, lanes 4 and 6) and not at later times (Fig. 4, lanes 2, 3, 8, and 12) indicating that the time course of transport of μ_m cannot be attributed to a deficiency in synthesis of light chains by cells at this stage of B cell differentiation. In fact, the association between the two chains

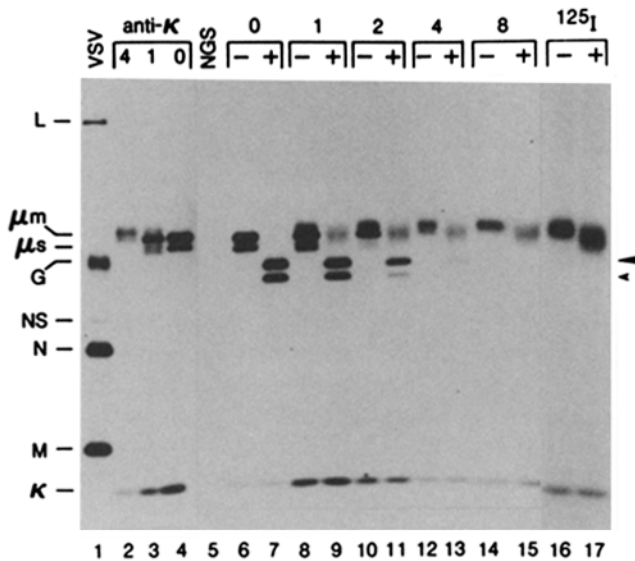


Figure 4. Time course of transport of μ_m in a B cell line. WEHI 231 cells were pulse-labeled for 10 min with [35 S]methionine, then chased by incubation in medium containing unlabeled methionine for the indicated times (in hours). IgM molecules on the cell surface were labeled with [125 I] using lactoperoxidase. Detergent extracts from 1.2×10^6 labeled cells were subjected to immunoprecipitation with antisera directed against μ heavy chains (lanes 6–17), or normal goat serum (lane 5, control). Also, extracts from 6×10^5 cells were subjected to immunoprecipitation with antiserum directed against κ light chains (lanes 2–4). Immunoprecipitates were reduced and alkylated before incubation in the presence (+) or absence (–) of endo H. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis and a fluorograph of the dried gel is shown. The apparent molecular weight of μ_m and μ_s synthesized by these cells is indicated. The electrophoretic mobility of μ_m and μ_s after removal of oligosaccharides by endo H is shown by large and small arrowheads, respectively. No IgM is secreted from these cells and μ_s is degraded intracellularly (39; our unpublished results). Molecular weight markers are the VSV proteins: L (240,000), G (65,000), N (50,000), and M (27,000).

appeared complete as early as 30 min after synthesis, since the ratio of $\mu_m:\kappa$ at this and all later times of chase (Fig. 4, lanes 8–15) closely resembled the steady-state ratio of the two polypeptides (data not shown). These results indicated that in our pulse-chase experiments, we were examining assembled IgM molecules rather than a pool of free μ_m s blocked in transport in rough ER due to a deficiency of association with κ light chains. Similar rates of intracellular transport of μ_m s have been suggested for IgM molecules synthesized in murine spleen cell populations (60).

To determine whether WEHI 231 cells have a generalized defect in ability to transport glycoproteins, we also measured the time course of transport of G protein in VSV-infected WEHI 231 cells. WEHI 231 cells were infected with VSV for 5 h and pulse-chase experiments were performed. Detergent extracts of cells from each time point were subjected to immunoprecipitation with antiserum directed against VSV and immunoprecipitated proteins were treated with endo H as described for transfected COS-1 cells. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography (Fig. 5). The half-time of acquisition of endo H-resistant oligosaccharides in the Golgi complex by G protein was 20 min, similar to G protein in other infected cell types and in cells that express the cloned G gene (Fig. 6A; 26, 48).

It was interesting to note that similar to human pre-B cell lines (12), infection of WEHI 231 cells or another murine B lymphoma line A20.2J (64) did not result in efficient shut-off of host cell protein synthesis. Yet, infection with VSV of X16c8.5 cells, another murine B lymphoma line which expresses IgM and IgD on the cell surface (30), did result in efficient shut-off of synthesis of endogenous proteins (data not shown). Nonetheless, the time course of transport of G protein in all B cell lines tested was virtually identical and indicated that normal transport mechanisms were functional in these cells.

Transport of G protein with the Cytoplasmic Domain of an Influenza Virus Hemagglutinin Protein

Since G protein with a μ_m cytoplasmic domain appeared to be transported to the cell surface with kinetics that closely resembled transport of IgM to the surface of B cells, we constructed a G gene which encoded the cytoplasmic domain of another viral glycoprotein transported to the cell surface as rapidly as G protein. We chose the carboxyl-terminal domain

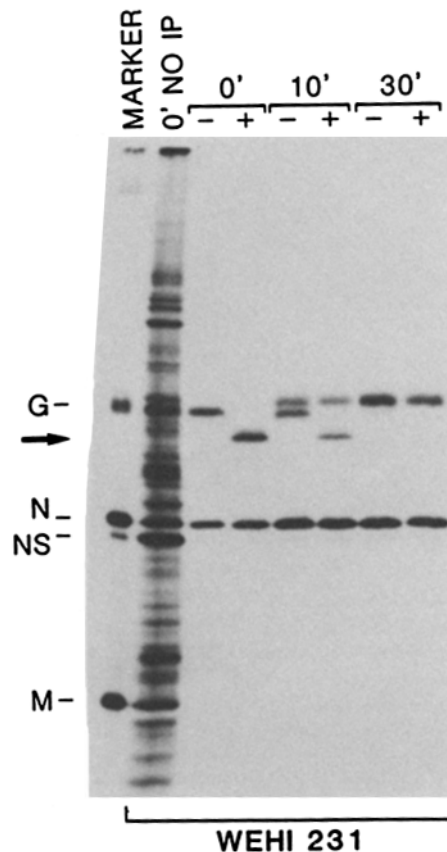


Figure 5. Time course of transport of G protein in VSV-infected B cells. WEHI 231 cells were infected with 30 plaque-forming units of VSV/cell for 5 h. Infected cells were pulse-labeled with [35 S]methionine for 5 min, then chased by incubation in medium containing unlabeled methionine for the indicated times (in minutes). Detergent extracts were analyzed directly (lane 0' NO IP) or after immunoprecipitation with anti-VSV serum. Immunoprecipitates of cells from each time point were incubated in the presence (+) or absence (–) of endo H and analyzed by SDS-polyacrylamide gel electrophoresis. A fluorograph of the dried gel is shown. The arrow indicates the apparent molecular weight of G protein after removal of oligosaccharides by endo H.

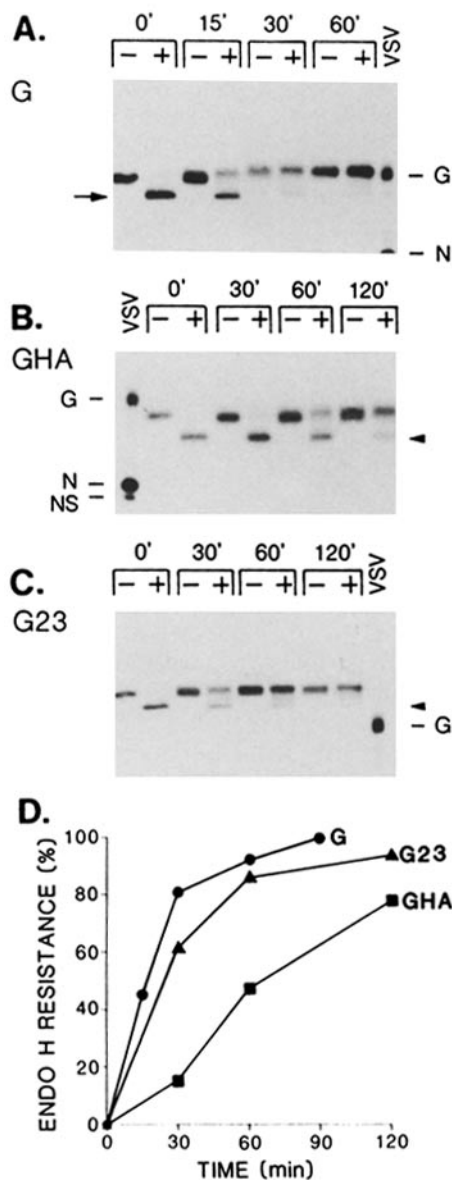


Figure 6. Time course of transport of G proteins with other viral glycoprotein cytoplasmic domains. 5-cm dishes of COS-1 cells were transfected with plasmid DNA encoding the indicated proteins. At 40 h posttransfection cells were pulse-labeled for 15 min with [³⁵S] methionine, then chased by incubation in medium containing unlabeled methionine for the indicated times (in minutes). Immunoprecipitates of cells from each time point were incubated in the presence (+) or absence (-) of endo H and analyzed by SDS-polyacrylamide gel electrophoresis. Fluorograms of the dried gels are shown (A-C). The mobility of G proteins after removal of oligosaccharides by endo H is indicated for the wild type (arrow) and mutant (arrowheads) G proteins. The apparent molecular weights of mutant G proteins determined on SDS-polyacrylamide gels was consistent with the substitution of a 10 amino acid cytoplasmic domain (GHA) or a 130 amino acid cytoplasmic domain (G23) for the normal 29 amino acid cytoplasmic domain of wild type G protein. Preexposed x-ray films obtained from this and similar experiments were quantified by densitometry and the rate of acquisition of endo H-resistant oligosaccharides by each glycoprotein was calculated. Results presented are the means of at least two experiments (D).

of the HA glycoprotein of influenza virus which contains 10 amino acid residues presumably exposed on the cytoplasmic side of the membrane. Nucleotides encoding the cytoplasmic

domain of an influenza virus HA (strain A/Aichi/2/68; 61) were substituted for the first 30 nucleotides encoding the cytoplasmic domain of G protein (pGHA) as described in Fig. 1 and in Materials and Methods. When double-label indirect immunofluorescence microscopy was performed on COS-1 cells transfected with pGHA, the patterns of cell surface and internal staining were similar to those observed for the wild type G protein in transfected cells (data not shown). We also performed pulse-chase experiments to determine the time course of transport through the Golgi complex of GHA in transfected COS-1 cells. Immunoprecipitates from detergent extracts of cells harvested at each time point were treated with endo H, and labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography (Fig. 6). The half-time of acquisition of endo H-resistant oligosaccharides by GHA ($t_{1/2} = 60$ min) was slower than for wild type G protein but was not as slow as for G_{μ} or G^* . We measured the time course of transport of HA in influenza virus-infected COS-1 cells and the half-time of acquisition of endo H-resistant oligosaccharides by an HA encoded by influenza virus strain A/Aichi/2/68 was ~20–30 min, as has been reported by others for HA encoded by another strain of influenza virus (19).

Transport of G Protein with the Cytoplasmic Domain of a Coronavirus p23 Protein

We also examined the ability of the cytoplasmic domain from a viral glycoprotein that is not normally destined for incorporation into the plasma membrane to substitute for the cytoplasmic domain of G protein. Coronaviruses such as IBV bud from intracellular membranes and their small glycoproteins, designated p23 for IBV, appear only in intracellular membranes in virus-infected cells (16, 40). The sequences of cDNAs encoding p23 predict a polypeptide of 225 amino acids which has a short extracytoplasmic domain containing two sites for addition of N-linked oligosaccharides followed by three hydrophobic domains which are believed to span the membrane three times. The carboxyl-terminal 123 amino acids are relatively hydrophilic and probably constitute the cytoplasmic domain of the protein (9; Rose, J., unpublished results).

Although our attempts to express the full-length p23 protein from cloned cDNA have been unsuccessful, we wanted to determine if its cytoplasmic domain could prevent transport of G protein to the cell surface. A cDNA-encoding G protein containing the putative cytoplasmic domain of the IBV p23 glycoprotein (pG23) was constructed as described in Materials and Methods. The cDNA constructed encoded a hybrid protein consisting of the extracellular and transmembrane domains of G protein and a cytoplasmic domain consisting of three amino acid residues derived from G protein fused to the carboxyl-terminal 128 amino acids of p23 which included its putative cytoplasmic domain. Double-label indirect immunofluorescence microscopy of COS-1 cells transfected with pG23 showed both cell surface and internal staining similar to that observed for wild type G protein (data not shown). We also measured the rate of acquisition of endo H-resistant oligosaccharides by G23 (Fig. 6) and the half-time was 25 min, almost as fast as G protein with its normal cytoplasmic domain.

Discussion

The transport of membrane and secretory proteins to the cell surface is thought to occur along the same basic pathway from the ER, through the three to four compartments of the Golgi complex to the cell surface (51). In principle, one can imagine that sorting of proteins within this pathway occurs in two ways. In the "positive signal" model, recognition of sorting signals by specific factors would result in direct movement of proteins to specific locations within the cell. In the "negative signal" model, all proteins would move (perhaps with phospholipids or cholesterol destined for various cellular membranes) unless they were delayed at specific sites after recognition by specific factors. It is well documented that different proteins move through the exocytic pathway at different rates (22, 56), and this observation could be reconciled with either model for sorting. That is, proteins could have different affinities for the same transport factors resulting in different rates of transport, or they could have different affinities for resident components along the pathway, and thus move through the cell at different rates.

Given the complexity of the transport process which presumably requires that some proteins segregate into transport vesicles while others do not, it seems likely that both positive and negative signals operate within the exocytic pathway. In a transmembrane protein, the sorting signals could reside in the cytoplasmic domain, the transmembrane domain, the extracytoplasmic domain or in some combination of these domains. In fact, mutations in any of these domains of VSV G protein can arrest transit from the rough ER or Golgi complex, whereas other changes in these domains have little or no effect on transport of G protein to the plasma membrane (2, 33, 47). In an initial search for sorting signals in the VSV G protein, two considerations led us to analyze mutations affecting the cytoplasmic domain (47). First, this domain is separated from the remainder of the protein by an α -helical segment of 20 amino acid residues that spans the membrane, and thus it seemed unlikely that changes in the cytoplasmic domain would affect the folding of the extracytoplasmic domain of the protein on the other side of the membrane. The second consideration was that the cytoplasmic domain is localized where it might be expected to interact with proteins or other factors involved in formation of transport vesicles or in subsequent fusion of these vesicles with various membrane compartments in the cell. The results of the earlier studies indicated that the structure of the cytoplasmic domain is important in signaling a normal rate of transport of G protein to the cell surface (47). We undertook the experiments described here to extend these earlier studies and to ask specifically if cytoplasmic domains from other integral membrane proteins could substitute for the normal cytoplasmic domain of VSV G protein in transport to the plasma membrane.

In the earlier experiments it was found that substitution of two to three uncharged amino acids for the normal cytoplasmic domain of G protein (mutant 1473H1) resulted in a G protein that accumulated in the rough ER and Golgi membranes, but not on the cell surface (47). In fact, we show here (using a more sensitive reagent) that the 1473H1 protein was detected on the plasma membrane of ~20% of the cells expressing this protein. Also, we detected a small percentage of conversion of the oligosaccharides on the protein to a form resistant to cleavage by endo H, indicating some transfer of

1473H1 from the rough ER through the Golgi complex. From these experiments we conclude that the two to three uncharged amino acids in the cytoplasmic domain of 1473H1 were sufficient for expression of G protein on the plasma membrane. Thus, none of the normal cytoplasmic domain was essential for transport of G protein to the cell surface. However, the rate of transport of this protein lacking the entire normal cytoplasmic domain was at least 30-fold lower than the rate of transport of the wild type G protein. This slow rate of transport of 1473H1 could account for the fact that only a small proportion of COS-1 cells expressing 1473H1 had detectable levels of this protein on the cell surface at 40 h after transfection. That is, in the transient expression system used here, most of the cells may not have had sufficient time to transport and accumulate detectable levels of the mutant protein on the cell surface after initiation of protein synthesis.

The results obtained with a new mutant G* demonstrated that the presence of only the three membrane-proximal amino acids (Arg-Val-Gly) from the normal cytoplasmic domain resulted in significantly greater expression of G protein on the cell surface than observed with mutant 1473H1. However the rate of acquisition of endo H-resistant oligosaccharides by G* was sixfold slower than for the wild type G protein. A second mutant (G μ) with the three amino acid cytoplasmic domain from the immunoglobulin μ membrane heavy chain (Lys-Val-Lys) showed a rate of acquisition of endo H-resistant oligosaccharides and level of expression on the plasma membrane virtually identical to that of G*. The presence of the one basic amino acid residue in these short cytoplasmic domains could be responsible for promoting the fivefold increase in the rate of transport of the mutant proteins to the cell surface (as compared with 1473H1). In a recent report, rates of processing were determined for H-2L^d with mutations in the cytoplasmic domain and, similar to our results, the presence of basic amino acid residues greatly enhanced expression of mutant H-2L^d on the cell surface (66). Also, these results are consistent with observations of Lehrman et al. in which a mutation in the low density lipoprotein receptor that leaves three of the 50 normal amino acid residues in the cytoplasmic domain generates a mutant protein that is transported to the plasma membrane but at a rate slower than the wild type protein (31).

It was interesting that the time course of transport of G μ in COS-1 cells was virtually identical to the time course measured for μ_m in WEHI 231 cells. The time course we observed also resembled the rate of acquisition of endo H-resistant oligosaccharides by μ_m in the human B lymphoblastoid cell line Daudi in which the pulse-labeled μ_m and μ_s forms are more difficult to resolve on SDS-polyacrylamide gels (17). A similar transit time has been suggested for δ_m molecules in B1-8 δ .1 cells (3). The μ_m molecules synthesized in these two cell lines and δ_m molecules differ in their extracellular domains with respect to amino acid composition and extent of glycosylation yet their transmembrane and cytoplasmic domains are identical (6). Thus, very different glycoproteins, μ_m , δ_m , and G μ , which contain an identical cytoplasmic domain consisting of the three amino acids Lys-Val-Lys, are transported to the cell surface at very similar rates. These results would be consistent with a model of intracellular glycoprotein transport in which important interactions occur on the cytoplasmic side of intracellular membranes. In the case of these three proteins we would suggest that the amino acid sequence

in the cytoplasmic domain could be providing a significant, although perhaps weak, transport signal. In the case of wild type G protein, the complete cytoplasmic domain might provide for stronger interactions and result in its more rapid rate of transport.

In this study we demonstrated that G protein with an influenza virus HA cytoplasmic domain was transported three times more slowly than either the wild type G or HA protein in COS-1 cells. A reasonable interpretation of this result is that the structure of the cytoplasmic domain of HA is less important than that of G protein in signaling efficient transfer to the cell surface. That mutations in the cytoplasmic domain of HA have a much less profound effect on the rate of acquisition of endo H-resistant oligosaccharides by mutant HA molecules (15) than do mutations in the cytoplasmic domain of the VSV G protein (47) is consistent with this hypothesis. Thus, the HA protein may rely more heavily than G protein on extracytoplasmic signals to promote its rapid transport to the cell surface. Because the HA protein is known to be a trimer (63) while G protein may be a monomer (14), an alternative explanation is that the extracellular and transmembrane domains of G protein are not structurally compatible with the HA cytoplasmic domain. For example, perhaps the cytoplasmic domain of HA is efficiently recognized in a trimeric configuration, and since such a conformation is unexpected in the hybrid protein, the rate of transport observed for GHA was reduced as compared with either parent molecule.

Addition of 127 amino acids which constitute the cytoplasmic domain of IBV p23 to G* greatly increased the hybrid protein's rate of intracellular transport. This observation was interesting in view of the fact that coronaviruses encode two transmembrane glycoproteins, one that is transported to the surface of infected cells, and the other that remains localized in rough ER and Golgi membranes (16, 40). The location of p23 in these intracellular membranes is believed to direct budding of coronaviruses from infected cells. We conclude from our experiment that the cytoplasmic domain of p23 cannot halt G protein in intracellular membranes. Instead, it increases the rate of transport of G protein with three of its normal amino acids in the cytoplasmic domain, at least from the rough ER through the Golgi complex. Other domains of p23 may provide signals that halt transfer of p23 beyond the Golgi complex. Further experiments on expression of p23 itself and other G-p23 hybrid proteins are in progress and may allow identification of features of the p23 protein that are critical for targeting to intracellular membranes.

The results described here demonstrate that precise replacement of cytoplasmic domains, as can be achieved by site-directed mutagenesis, can result in production of hybrid proteins whose functional domains can be directly assayed in the absence of endogenous or extraneous amino acids. This is in contrast to expression of chimeric cDNAs generated by recombination at restriction sites which can result in novel properties which resemble neither parent glycoprotein (37). The experiments also illustrate the complexity involved in analysis of the sorting problem using recombinant DNA and gene expression technology. Although the simple methods we describe allow the precise replacement of small protein domains with the corresponding domains from other proteins, the results obtained with these proteins are not always con-

sistent with simple models. However, considering that proteins potentially contain signals in more than one domain that function actively to promote transport, as well as signals that may retard transport at certain stages, it is possible to arrive at consistent models to explain the results without invoking effects of the cytoplasmic domain on the three-dimensional structure of the extracytoplasmic domain of the protein as suggested by Doyle et al. (15). Having precise constructs encoding hybrid glycoproteins correctly targeted to the plasma membrane of transfected cells, we can now begin to assess directly the biological roles of the cytoplasmic domain of G protein in targeting to specific plasma membrane domains in epithelial cells, in assembly of VSV virions, in lateral diffusion of G protein, and in immune recognition.

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