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A Single N-Linked Oligosaccharide at Either of the Two Normal Sites Is Sufficient for Transport of Vesicular Stomatitis Virus G Protein to the Cell Surface

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We investigated the role of glycosylation in intracellular transport and cell surface expression of the vesicular stomatitis virus glycoprotein (G) in cells expressing G protein from cloned cDNA. The individual contributions of the two asparagine-linked glycans of G protein to cell surface expression were assessed by site-directed mutagenesis of the coding sequence to eliminate one or the other or both of the glycosylation sites. One oligosaccharide at either position was sufficient for cell surface expression of G protein in transfected cells, and the rates of oligosaccharide processing were similar to the rate observed for wild-type protein. However, the nonglycosylated G protein synthesized when both glycosylation sites were eliminated did not reach the cell surface. This protein did appear to reach a Golgi-like region, as determined by indirect immunofluorescence microscopy, however, and was modified with palmitic acid. It was also apparently not subject to increased proteolytic breakdown.

The carbohydrate moieties of glycoproteins have long been postulated to provide some function for the polypeptides to which they are covalently linked. Functions which have been implicated include maintenance of correct polypeptide conformation, protection from proteolytic degradation, and signals for intracellular targeting (reviewed in reference 29). Many experiments analyzing the role of asparagine-linked (N-linked) oligosaccharides on glycoproteins have used the antibiotic tunicamycin, which interferes with the synthesis of the lipid-linked precursor oligosaccharide and thus prevents addition of the core glycan to the nascent polypeptide (44, 46). The results of such experiments indicate that there is a wide range in the requirement for carbohydrates on secreted and integral membrane glycoproteins. Some proteins which normally contain N-linked oligosaccharides are not affected when they are synthesized in the presence of tunicamycin, whereas others fail to reach their correct cellular destination or are degraded (29).

The glycoprotein (G protein) of vesicular stomatitis virus (VSV) has been extensively studied as a model for glycoprotein biosynthesis. This polypeptide is an integral membrane protein which contains 511 amino acids and the following three domains: a large amino-terminal external domain, a transmembrane domain of 20 amino acids, and a highly charged cytoplasmic domain of 29 amino acids (34). In infected cells, G protein is synthesized on membrane-bound ribosomes, and two high-mannose oligosaccharides are added cotranslationally as the polypeptide is extruded into the lumen of the rough endoplasmic reticulum (rER) (35). Translocation of the protein into the lumen stops at the hydrophobic transmembrane domain, and the membrane-anchored polypeptide moves through the Golgi complex to the plasma membrane, where virus binding occurs (2, 4, 18). Several posttranslational modifications of G protein can be followed as the protein moves through the cell. These include the addition of palmitic acid to a cysteine residue in the cytoplasmic domain (25, 31, 36) and the processing of the high-mannose oligosaccharides to the complex form, which is completed in the Golgi membranes (16, 19).

Previous experiments in which tunicamycin was used to block all N-linked glycosylation indicated that the N-linked glycans of G protein are critical for intracellular transport of the protein to the plasma membrane. Virion production in VSV-infected cells treated with tunicamycin is severely inhibited as a result of the failure of nonglycosylated G protein to reach the plasma membrane (11, 23, 28). Furthermore, nonglycosylated G protein was shown to be less soluble to nonionic detergent and appeared to be in an aggregated form in intracellular membranes (12, 24). The San Juan strain of the Indiana serotype of VSV was quite stringent in its requirement for carbohydrate, whereas the Orsay strain of VSV had a less stringent requirement when it was grown at 30°C rather than at 37 to 38°C (12). The G proteins of these two strains are closely related, but they contain 10 amino acid differences (9). These amino acid differences must be responsible for the variation in carbohydrate requirement for intracellular transport of the G proteins.

The signals involved in intracellular transport of G protein are being investigated in this laboratory by using cDNA encoding the G protein cloned into a simian virus 40-based expression vector. The protein is expressed transiently at high levels under control of the late simian virus 40 promoter in COS-1 cells, which provide T antigen and thus allow extensive replication of the vector in transfected cells (13, 40). The biosynthesis, posttranslational modifications, and rate of transport of G protein to the plasma membrane in transfected cells are essentially identical to those in infected cells (32). Here we describe the use of oligonucleotide-directed mutagenesis to eliminate the consensus glycosylation sequences in the coding region in order to analyze the role of carbohydrate in the intracellular transport of G protein. In this way, the contribution of the individual glycans to this process could be assessed in the absence of

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other viral proteins and without the potential side effects of tunicamycin.

MATERIALS AND METHODS

Oligonucleotide-directed mutagenesis. The oligonucleotides used (a 22-mer and a 24-mer) were synthesized with a Systec Microsyn model 1450A automated DNA synthesizer by using the phosphoramidite chemistry. Deblocking and gel purification of the oligonucleotides were performed as previously described (31).

Site-directed mutagenesis (48) of the coding sequence for VSV G protein was carried out exactly as described previously (31), with one modification. Instead of gel purifying fully extended material, the primer extension reaction mixture was treated with S1 nuclease to remove single-stranded (nonextended) regions and used to transfect *Escherichia coli* K-12 strain JM103 directly. Approximately one-third of the primer extension reaction mixture (containing 1 μg of template DNA) was treated with 10 U of S1 nuclease (Boehringer Mannheim Biochemicals) in 20 μl of a solution containing 0.3 M NaCl, 30 mM sodium acetate (pH 4.5), and 3 mM $ZnCl_2$ for 30 min at 37°C. Plaques containing the mutant phage were identified after hybridization with the ^{32}P-labeled oligonucleotide. The DNA incorporating the mutations paired perfectly and formed a hybrid having a higher melting temperature (approximately 10°C in both cases) than the DNA containing the wild-type sequence with two mismatched bases each. The frequencies of mutants were 3% for the first glycosylation site and 4% for the second. Inserts were excised with *Xho*I from the replicative-form DNA of mutant phage and ligated into simian virus 40-based expression vector JC119 (32, 40). We prepared plasmid DNAs (pTA1 and pTA2) which contained each of the mutant G-protein genes in the correct orientation for expression. The nucleotide sequence at the site of mutation was confirmed by a DNA sequence analysis (27).

Transfection, radiolabeling, and immunoprecipitation. Transfection of COS-1 cells with plasmid DNA by using DEAE-dextran was performed as described previously (14), using 15 μg of DNA per 5-cm dish or 10 μg of DNA per 3.5-cm dish; all but the first wash with Tris-saline was eliminated. Labeling with L-[^{35}S]methionine and [9,10-^{3}H]palmitic acid and immunoprecipitation of G proteins from transfected cells were performed 40 h after transfection by previously described procedures (31, 33). When cells were labeled in the presence of tunicamycin, they were first preincubated in medium containing 2 μg of the drug (Calbiochem-Behring) per ml and then also labeled in the presence of 2 μg of drug per ml. For labeling with D-[2-^{3}H]mannose, transfected cells in 5-cm dishes were incubated, without prior washing, in 1 ml of glucose-free medium containing 5% dialyzed fetal calf serum, 0.5 mg of sodium pyruvate per ml, 2% nonessential amino acids, and 250 μCi of D-[2-^{3}H]mannose (27 Ci/mmol; New England Nuclear Corp.) per ml for 4 h at 37°C. For cell surface iodination, transfected cells in 5-cm dishes were washed twice in Dulbecco phosphate-buffered saline and incubated in 1 ml of phosphate-buffered saline containing 5 mM D-glucose, 20 μg of lactoperoxidase (Sigma), and 125 μCi of Na^{125}I (carrier free; New England Nuclear Corp.). The reaction was initiated by adding 0.1 U of galactose oxidase (Sigma Chemical Co.), and labeling was allowed to continue for 20 min at room temperature with occasional rocking. The cells were washed twice in 150 mM NaI buffered with 20 mM sodium phosphate (pH 7.4) and five more times in phosphate-buffered saline. Detergent lysis of cells and immunoprecipitation of G proteins were then performed as described above.

Immunoprecipitated proteins were analyzed by electrophoresis in 10% polyacrylamide gels (20) in the presence of sodium dodecyl sulfate (SDS), followed by fluorography (3). For quantitation of radioactivity in the bands, films (preflashed before exposure) were scanned with a Hoeffer model GS300 densitometer, and the peaks were integrated by using a Hewlett-Packard integrator/digitizer. Several exposures of the fluorograms were scanned to ensure that the films were in the linear range for scanning.

Immunofluorescence microscopy. Indirect immunofluorescence was carried out with COS-1 cells grown on cover slips 40 h after transfection as described previously (33) by using a guinea pig anti-VSV antiserum (1:200), followed by rhodamine-conjugated goat anti-guinea pig immunoglobulin G (IgG) (1:200; Cappel Laboratories) for detection of surface G protein. After permeabilization of the fixed cells with 1% Nonidet P-40, internal G protein was stained with a rabbit anti-VSV antiserum (1:200) and fluorescein-conjugated goat anti-rabbit IgG (1:200; Cappel). For some experiments, an affinity purified anti-G protein antibody was used. This was prepared from our rabbit anti-VSV antiserum on an affinity column containing Sepharose 4B conjugated with approximately 250 μg of purified G protein recovered from an SDS gel by electroelution per ml. After application of the unfractionated antiserum to the column and extensive washing, the anti-G protein antibodies were eluted wtih 4 M $MgCl_2$ and dialyzed. The anti-G protein antibody (protein concentration, approximately 0.2 mg/ml) was used at a dilution of 1:25 for the immunofluorescence experiments; this was followed by affinity-purified fluorescein-conjugated goat anti-rabbit IgG (Cappel) at a dilution of 1:20. For localization of the Golgi complex in transfected cells, cover slips were incubated with rhodamine-conjugated wheat germ agglutinin (1:75) after permeabilized cells were stained for G protein with affinity-purified anti-G protein antibody and fluorescein-conjugated goat anti-rabbit IgG.

RESULTS

Elimination of the glycosylation sites in G protein by site-directed mutagenesis. There are two potential sites for N-linked oligosaccharide addition (Asn-X-Ser/Thr) in the amino acid sequence of G protein (the San Juan strain of the Indiana serotype of VSV), both of which must be glycosylated since G protein contains two glycans (6, 30). The first site is at asparagine 179, in the sequence Asn-Ser-Thr, and the second is at asparagine 336, in the sequence Asn-Gly-Thr (34). To eliminate these glycosylation sites, we changed the codon for the third amino acid in the sequence from threonine to alanine in both cases. This change is fairly conservative, and these two amino acids are occasionally interchanged in G proteins from the New Jersey and Indiana serotypes (8). In addition, this provided direct proof that the third amino acid in this sequence is indeed required for addition of the core oligosaccharide to asparagine residues.

The oligonucleotides used to change the codons for threonine 181 and threonine 338 to alanine are shown in Fig. 1. These oligonucleotides were used to prime second-strand DNA synthesis on single-stranded M13 DNA carrying the gene for G protein (mp8-G4) (31), and mutants were identified by differential hybridization with the oligonucleotide, as described previously (31). Both oligonucleotides contained two mismatches with the template, resulting in a 10°C difference in the melting temperature when hybrids were

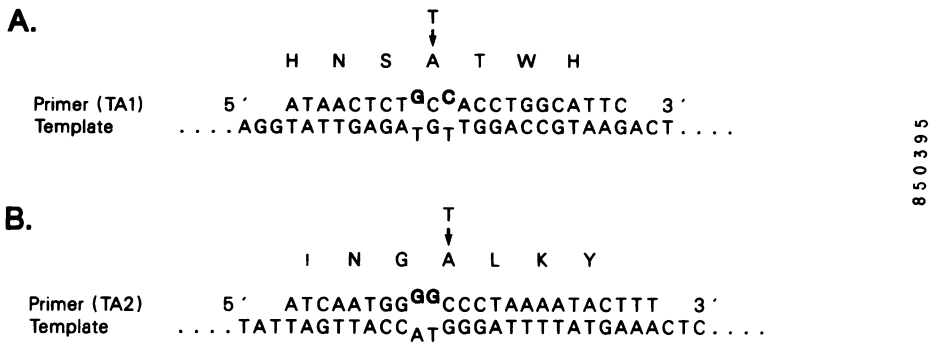

FIG. 1. Oligonucleotides used for site-directed mutagenesis. (A) The synthetic 22-mer oligonucleotide used to change the codon for threonine 181 (ACA) to the codon for alanine (GCC) in the first glycosylation site of G protein is shown hybridized to the template mp8-G4 DNA, with the corresponding amino acid sequence (single-letter code) shown for the wild-type and mutant (TA1) proteins. (B) The 24-mer oligonucleotide used to change the codon for threonine 338 (ACC) to the codon for alanine (GCC) in the second glycosylation site is shown in the same manner, with wild-type and mutant (TA2) protein sequences.

formed between mutant or wild-type phage DNA and the oligonucleotide. Fragments containing the mutant G-protein genes were prepared from phage replicative-form DNA and cloned into the *Xho*I site of the simian virus 40-based expression vector, JC119 (32, 40). Mutations were confirmed by DNA sequence analysis (27). The plasmid DNA containing the mutant insert encoding an alanine in the first glycosylation site was called pTA1, and the plasmid DNA containing the mutant insert in the second site was designated pTA2. To obtain the plasmid encoding the double mutant with both threonine-to-alanine changes (pTA1,2), the 5′ fragment of the G protein insert from pTA1 was recombined with the 3′ fragment of pTA2 at the central *Pst*I site (34).

Lack of glycosylation at the mutated sites in G protein. DNAs encoding the mutant G proteins were transfected onto COS-1 cells, and expression was analyzed and compared with expression in cells transfected with DNA encoding wild-type G protein. Cells were labeled 40 h after transfection with L-[^{35}S]methionine, and G protein was immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis. As Fig. 2A shows, the proteins encoded by pTA1 and pTA2 DNAs migrated slightly faster than wild-type G protein, and the protein encoded by pTA1,2 DNA migrated even faster. These differences are consistent with the absence of one or both N-linked oligosaccharides, each of which contributes approximately 3,000 daltons to the relative molecular mass of G protein (6). Furthermore, when transfected cells were treated with tunicamycin, the nonglycosylated G proteins all comigrated with the pTA1,2-encoded mutant G protein from untreated cells (Fig. 2B). In a parallel experiment, transfected cells were labeled with D-[2-^{3}H]mannose. As shown in Fig. 2C, the protein encoded by pTA1,2 was not detectably labeled, further demonstrating the lack of carbohydrate on this polypeptide. Identical results were obtained when transfected cells were labeled with D-[1,6-^{3}H]glucosamine (data not shown). The amount of D-[2-^{3}H]mannose incorporated into proteins encoded by pTA1 and pTA2 was about one-half that incorporated into wild-type G protein, as expected.

Cell surface expression of the glycosylation mutants of G protein. The transport of the mutant proteins to the plasma membrane of transfected cells was analyzed in two ways. First, indirect immunofluorescence microscopy in which a double label was used was performed (Fig. 3). Cells transfected with DNA encoding the single-site mutants pTA1 and pTA2 expressed high levels of the mutant G proteins on the cell surface, in a pattern that was indistinguishable from that of wild-type G protein. The internal patterns of the same cells (visualized after permeabilization) are also shown. However, surface labeling of cells transfected with DNA encoding the double glycosylation mutant pTA1,2 was not detected. Internal labeling of these cells demonstrated that the nonglycosylated protein specified by pTA1,2 DNA appeared in some cells in a perinuclear region characteristic of the Golgi complex (Fig. 3) (14,32).

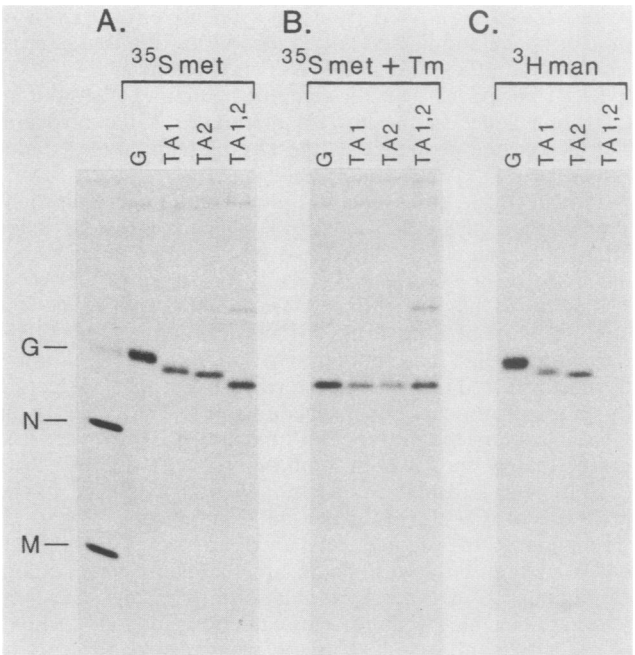

FIG. 2. Mutant G proteins are not glycosylated at the mutated glycosylation sites. COS-1 cells transfected with plasmid DNA specifying wild-type or mutant G proteins were radiolabeled 40 h after transfection, and detergent lysates of the cells were subjected to immunoprecipitation with anti-VSV antiserum, followed by SDS-polyacrylamide gel electrophoresis. Cells were labeled with 100 μCi of L-[^{35}S]methionine per ml for 1 h (A), with 100 μCi of L-[^{35}S]methionine per ml in the presence of 2 μg of tunicamycin (Tm) per ml after a 2-h of preincubation in the same concentration of the drug (B), or with 250 μCi of D-[2-^{3}H]mannose per ml for 4 h (C). VSV protein markers (G, N, M) are shown in the outside lane of this and subsequent figures.

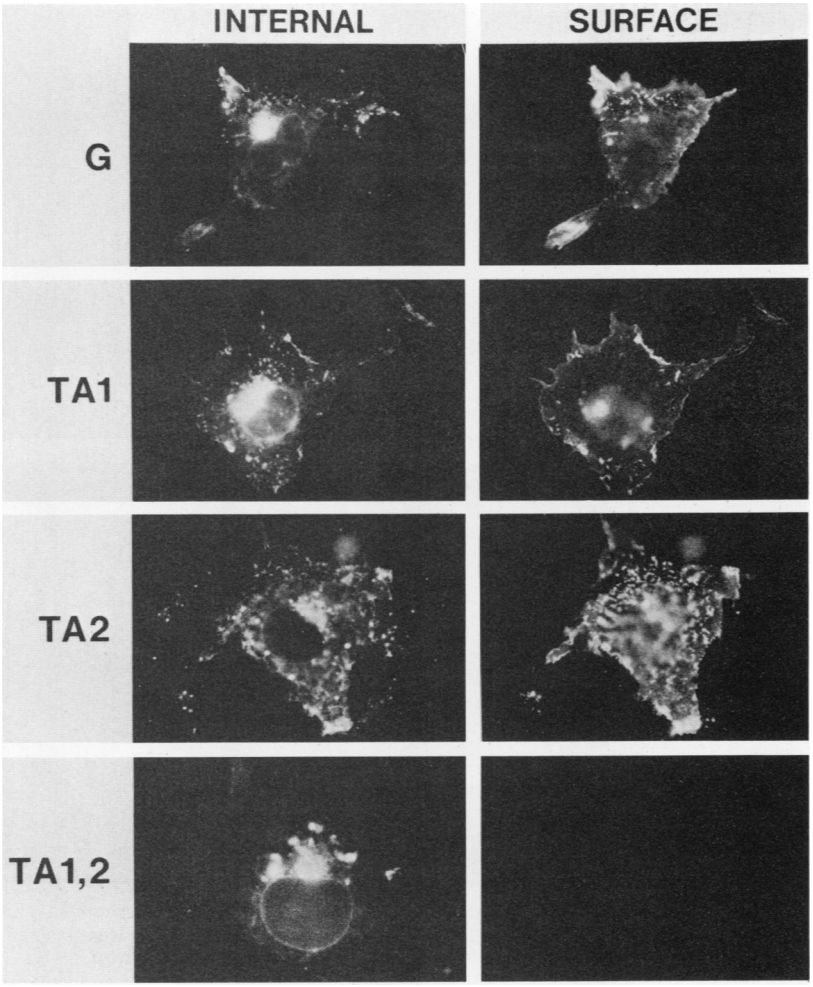

FIG. 3. Detection of mutant G proteins by indirect immunofluorescence microscopy. Transfected COS-1 cells grown on cover slips were fixed 40 h after transfection and stained as described in Materials and Methods. Each set of micrographs shown the same cell stained for surface G protein (before permeabilization) with guinea pig anti-VSV followed by rhodamine-conjugated goat anti-guinea pig IgG and after detergent permeabilization for internal G protein by using rabbit anti-VSV followed by fluorescein-conjugated goat anti-rabbit IgG.

Of interest was the finding that the internal staining of the pTA1,2-encoded protein appeared much weaker than that of wild-type protein or pTA1- or pTA2-encoded protein (the cell shown in Fig. 3 was exceptionally bright). This suggested that a subtle conformational change could be influencing the binding of antibody to the double mutant encoded by pTA1,2. No major differences in the efficiency of immunoprecipitation were observed when this same anti-VSV antiserum was used (Fig. 2), but the precipitations were done in the presence of a low concentration of SDS, which may have eliminated small conformational differences. Therefore, we prepared an affinity-purified anti-G protein antibody from the rabbit anti-VSV serum on a column of Sepharose 4B conjugated with SDS-denatured, gel-purified G protein. This procedure probably enriched for anti-G protein antibodies which reacted wih denatured G protein. The internal staining of pTA1,2-transfected cells with this antibody was much brighter than the staining with the unfractionated rabbit antiserum, and in addition to the perinuclear, Golgi-like staining noted above, many cells were stained in a reticular pattern characteristic of the rER (Fig. 4A) (33). However, surface staining of cells with this antibody was still negative (Fig. 4B). However, we observed no differences in the efficiency of immunoprecipitation of the wild-type or glycosylation mutants when the affinity-purified anti-G protein antibody was used (data not shown). The internal staining pattern of the mutant G protein encoded by pTA1,2 obtained with the anti-G protein antibody was also compared with the pattern observed after the cells were stained with wheat germ agglutinin, which preferentially localizes in the Golgi complex due to its binding of terminal *N*-acetylglucosamine residues (47). The perinuclear regions stained by the anti-G protein antibody in cells transfected with pTA1,2 (Fig. 4C) were also stained by rhodamine-conjugated wheat germ agglutinin (Fig. 4D), suggesting that some nonglycosylated G protein was reaching the Golgi region.

In addition to immunofluorescence, cell surface expression of the glycosylation mutants was examined by lactoperoxidase-catalyzed iodination of transfected cells. The controls used included cells transfected with DNA encoding wild-type G protein, as well as mutant Δ1473, which contains a deletion in the cytoplasmic domain of G protein and is not transported past the rER of transfected cells (33). COS-1 cells were transfected with the DNAs in duplicate, and 40 h later one set of plates was used for iodination of the cell

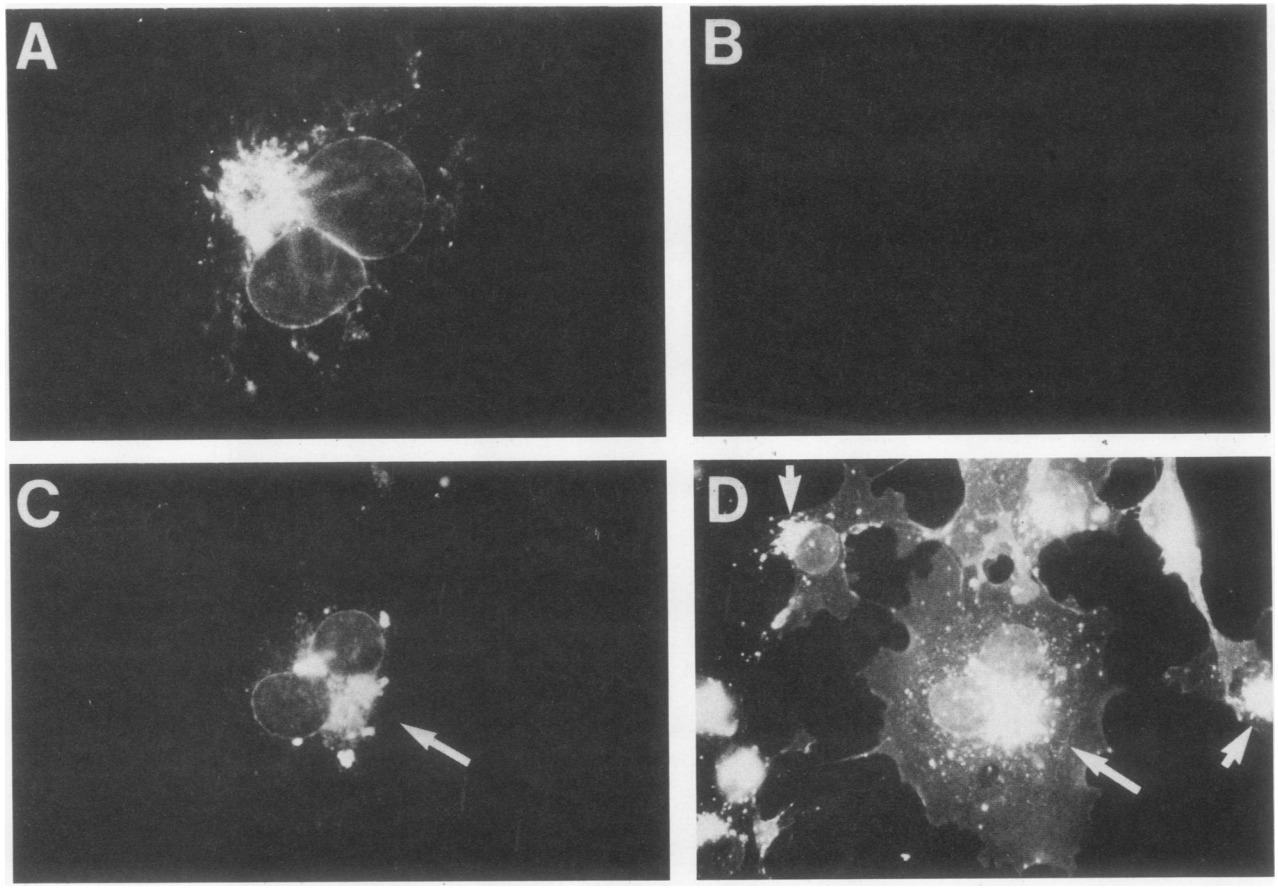

FIG. 4. Detection of the nonglycosylated G-protein mutant by using indirect immunofluorescence and an affinity-purified anti-G antibody. COS-1 cells grown on cover slips were transfected with pTA1,2 DNA and fixed 40 h later. The cells were stained for surface G protein before permeabilization with an affinity-purified anti-G protein antibody and rhodamine-conjugated goat anti-rabbit IgG (B) and for internal G protein after permeabilization by using the same anti-G protein antibody and affinity-purified fluorescein-conjugated goat anti-rabbit IgG (A). A parallel cover slip was permeabilized immediately and stained for internal G protein as described for panel A, followed by localization of the Golgi complex by staining with rhodamine-conjugated wheat germ agglutinin. (C) Staining for internal G protein. (D) Same field stained with wheat germ agglutinin. The arrows indicate the Golgi complex.

surface and the other set was labeled with L-[^{35}S]methionine to ensure that expression levels were similar. An analysis of immunoprecipitates from iodinated cells demonstrated the lack of Δ1473- and pTA1,2-encoded G proteins on the cell surface, whereas the proteins containing one glycan (pTA1- and pTA2-encoded proteins) or both glycans (wild-type protein) were readily detected (Fig. 5A). All proteins were detected at similar levels in the parallel L-[^{35}S]methionine labeling experiment (Fig. 5B). Thus, by two criteria, G protein containing only one N-linked oligosaccharide at either of the normal sites was transported to the cell surface, but G protein lacking carbohydrate was not.

Rate of transport of mutant G proteins containing one N-linked oligosaccharide. As G protein passes through the Golgi complex, the outer mannose residues of the core oligosaccharides are trimmed and other sugars (galactose, *N*-acetylglucosamine, *N*-acetylneuraminic acid, and fucose) are added. The initial high-mannose glycans are susceptible to digestion with endo-*N*-acetylglucosaminidase H (endoH), whereas the processed or complex oligosaccharides are resistant (45). Thus, the half-time for acquisition of resistance to endoH is a measure of the rate of transport of proteins from the rER through the region of the Golgi complex, where this processing step occurs (probably the medial Golgi region); this rate has been measured to be approximately 15 to 20 min for wild-type G protein in transfected COS-1 cells (32). The acquisition of resistance to endoH by the single oligosaccharide present on pTA1- and pTA2-encoded proteins was measured in a pulse-chase labeling experiment. When cells were chased for 60 min with unlabeled methionine after a 15-min pulse with L-[^{35}S]methionine, virtually all of the G protein specified by pTA1 and pTA2 DNAs was resistant to endoH, as was wild-type G protein (Fig. 6). As expected, the nonglycosylated G protein specified by pTA1,2 DNA was not susceptible to digestion with the enzyme (data not shown). With shorter chase periods, the kinetics of acquisition of endoH resistance by pTA1- and pTA2- encoded proteins appeared to be slightly slower than the kinetics for wild-type G protein, with half-times of approximately 20 min (compared with 15 min for wild-type G protein) (Fig. 6). This difference may not be significant since it was not observed in one of three experiments and because the actual determination of the half-time after a relatively long pulse label (15 min) was probably not precise. Thus, the absence of either N-linked oligosaccharide had only a small effect on the rate of transport of G

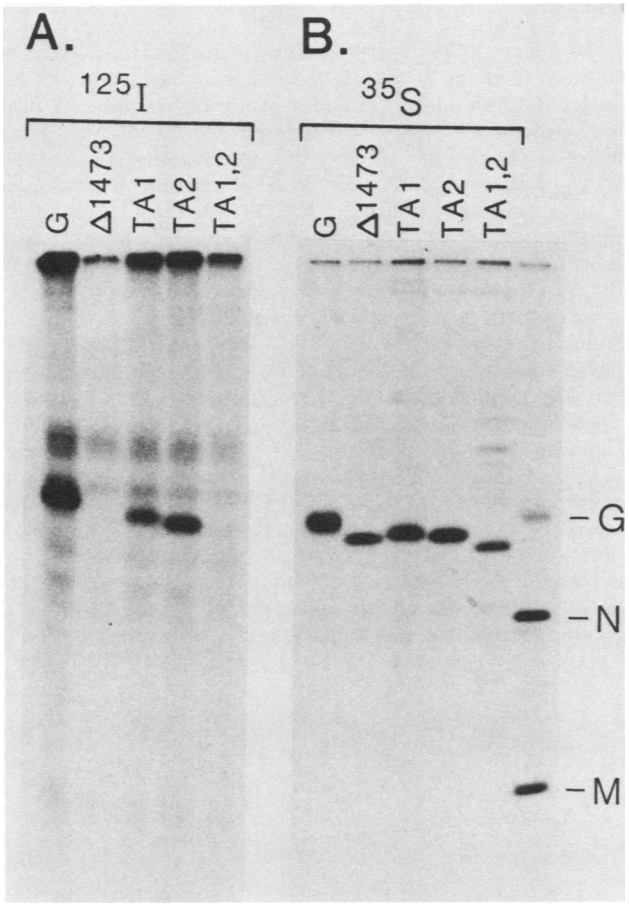

FIG. 5. Lactoperoxidase-catalyzed iodination of cell surface G proteins. Parallel dishes of transfected COS-1 cells were either surface labeled with ^{125}I as described in the text (A) or labeled with 100 μCi of L-[^{35}S]methionine (B), and detergent lysates of the cells were immunoprecipitated with anti-VSV antiserum and electrophoresed on an SDS-polyacrylamide gel.

protein from the rER through the medial region of the Golgi complex. Together with the data from immunofluorescence and iodination experiments, this suggests that mutant G proteins containing one glycan at either of the two normal sites are efficiently transported to the cell surface.

Stability of G proteins lacking carbohydrate in transfected cells. The level of expression of the G-protein mutants possessing one or neither oligosaccharide appeared to be somewhat lower than the level of expression of wild-type G protein when immunoprecipitates from radiolabeled cells were examined. An analysis of transfected cells by immunofluorescence suggested that similar numbers of cells (approximately 10%) were expressing wild-type and mutant G proteins. The lower level of mutant G proteins in immunoprecipitates did not appear to be the result of a decreased affinity of the antiserum used in immunoprecipitation for the mutant G proteins, since the same results were obtained with an affinity-purified anti-G protein antibody which probably recognized denatured G protein. To examine the intracellular stability of these mutant proteins and to address the possibility that the nonglycosylated G protein was expressed at the cell surface but immediately degraded or shed, a long chase after labeling with L-[^{35}S]methionine was used. Figure 7 shows the level of G protein in transfected cells after a 24-h chase compared with the level after a 1-h chase of cells labeled for 1 h. The proportion of the pTA1,2-encoded G protein remaining after the long chase was similar to the proportion of wild-type protein or protein in the single-site mutants (about 15% for all). The same results were obtained in two other similar experiments, as well as when shorter periods of pulse-labeling and chase were used (data not shown). This eliminates the possibility that a major proportion of nonglycosylated G protein reaches the cell surface but is rapidly degraded and thus not detected. It also indicates that the carbohydrate probably does not protect G protein from intracellular proteolytic degradation, as postulated for some glycoproteins (29). The lower level of expression of G-protein mutants remains unexplained, but has been

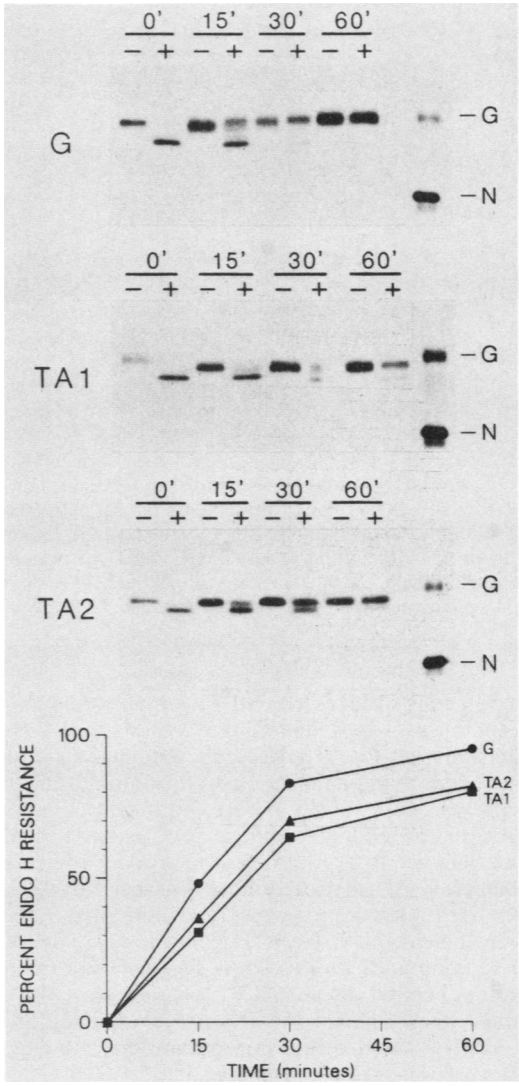

FIG. 6. Kinetics of acquisition of resistance to endoH. Transfected COS-1 cells were pulse-labeled for 15 min with 100 μCi of L-[^{35}S[methionine per ml. Parallel dishes were harvested immediately or after a chase with unlabeled methionine of 15, 30, or 60 min. Immunoprecipitates from each lysate were divided in half and digested with endoH (+) or mock digested (−) for 16 h at 37°C; this was followed by SDS-polyacrylamide gel electrophoresis. The graph shows the quantitation of this data obtained by scanning the fluorograms.

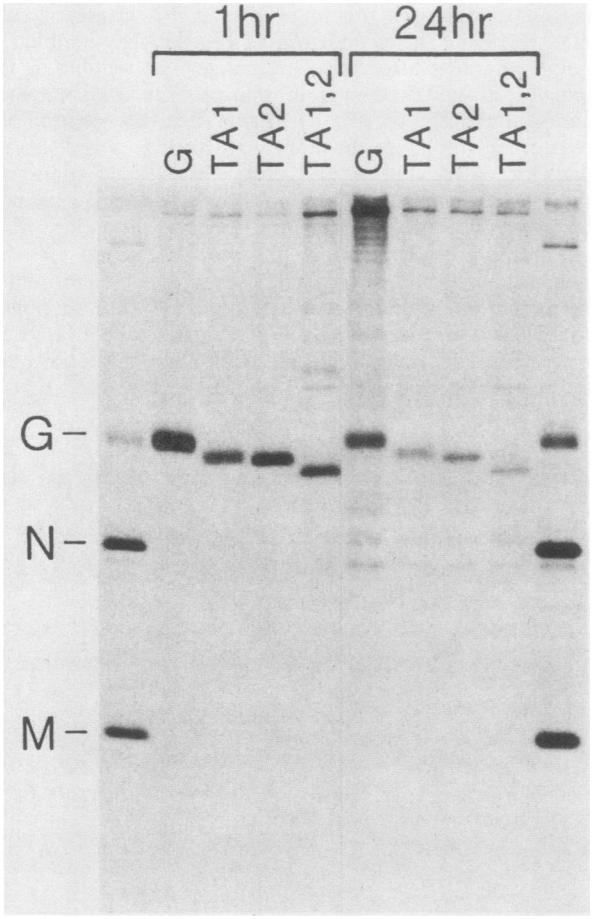

FIG. 7. Stability of the glycosylation mutants in transfected cells. Transfected COS-1 cells were labeled for 1 h with 100 μCi of L-[^{35}S]methionine, and parallel dishes were incubated in medium containing unlabeled methionine for either 1 or 24 h. G proteins were immunoprecipitated from detergent lysates of the cells and analyzed on an SDS-polyacrylamide gel.

observed in cells transfected with plasmids containing other mutations in the G-protein-coding region as well (32).

Modification of the glycosylation mutants with palmitic acid. The addition of palmitic acid to the cytoplasmic domain of G protein occurs shortly before the oligosaccharides are processed to the endoglycosidase H-resistant form and is therefore believed to occur just as the polypeptide enters the Golgi complex (37). To determine whether fatty acid was esterified to the G-protein glycosylation mutants, transfected cells were labeled with [9,10-^{3}H]palmitic acid, and G proteins were immunoprecipitated. As Fig. 8 shows, the mutant G protein encoded by pTA1,2 was labeled with [9,10-^{3}H]palmitic acid, as were the G proteins encoded by pTA1 and pTA2. By scanning the fluorogram and comparing the intensities of the [9,10-^{3}H]palmitic acid-labeled G proteins with the intensities of the parallel L-[^{35}S]methionine-labeled G proteins (to correct for variability in levels of expression), we found that the mutant G protein lacking both glycosylation sites was labeled as efficiently as wild-type G protein was with [9,10-^{3}H]palmitic acid. This indicates that the double glycosylation mutant is transported to the compartment where fatty acid is esterified to the protein, and thus the block in its transport must be after this point.

DISCUSSION

The experiments described above were designed to examine the role of the two N-linked oligosaccharides of VSV G protein in intracellular transport of the polypeptide. A transient expression system in which we used cloned cDNA encoding G protein (32) and the recently developed techniques of oligonucleotide-directed mutagenesis (48) afforded us the opportunity to assess the contribution of each of the oligosaccharides of G protein and to examine the transport of G protein lacking carbohydrate without the potential side effects of tunicamycin (5, 26). We eliminated one or the other or both of the glycosylation sites in the DNA encoding G protein by replacing the codon for threonine (the third amino acid in the consensus sequence at both glycosylation sites) with the codon for alanine. Examination of the G proteins expressed from these DNAs in transfected cells demonstrated that the mutated sites were not glycosylated, thus confirming the predicted locations of the two sites (34) and the importance of the third amino acid in the glycosylation consensus sequence (42). Somewhat surprisingly, G protein containing one N-linked oligosaccharide at either of the normal sites appeared to be transported to the cell surface nearly as efficiently as the wild-type protein. In contrast, G protein lacking both glycosylation sites was not detected at

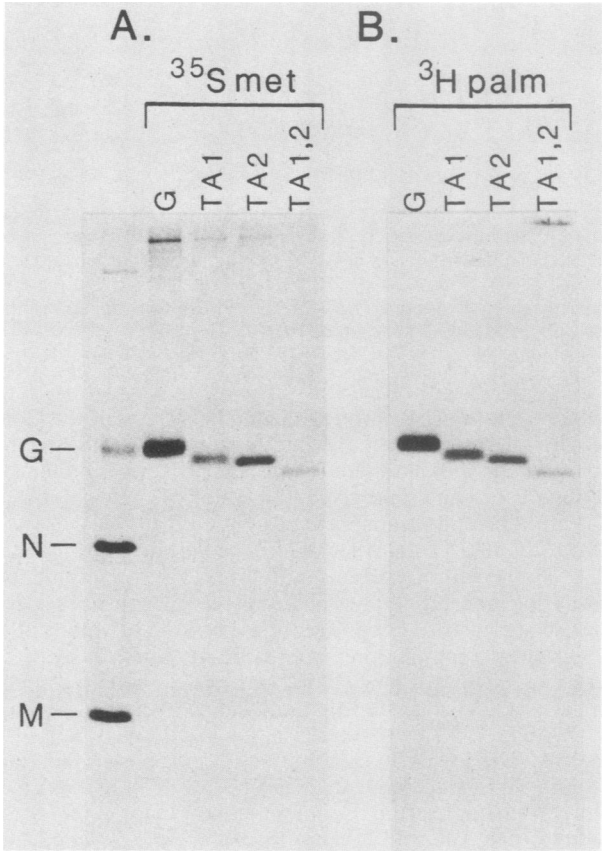

FIG. 8. Addition of palmitic acid to the glycosylation mutants of G protein. Parallel dishes of transfected COS-1 cells were labeled for 6 h with 50 μCi of L-[^{35}S]methionine (A) or 2.5 mCi of [9,10-^{3}H]palmitic acid (B) as described in the text. G proteins were electrophoresed on an SDS-polyacrylamide gel after immunoprecipitation from cell lysates. (A) Exposure for 20 h. (B) Exposure for 6 days.

the cell surface. However, this protein could be detected in a Golgi-like region by indirect immunofluorescence microscopy and was modified with palmitic acid as efficiently as wild-type protein.

Previous work in which tunicamycin and G proteins from two strains of VSV were used suggested that polypeptide conformation is the critical factor for intracellular transport. The nonglycosylated G protein of the San Juan strain of VSV is not transported to the cell surface when virus is grown in tunicamycin-treated cells either at the normal temperature (37 to 38°C) or at a lower temperature (30°C) (12). However, nonglycosylated G protein from another strain, the Orsay strain, whose amino acid sequence differs at 10 positions from the sequence of San Juan G protein (9), was partially transported to the cell surface at the lower temperature (12). The results of experiments designed to measure the solubility in nonionic detergent and the aggregation of nonglycosylated G proteins synthesized in the presence of tunicamycin were interpreted to indicate that the conformation of G protein was altered in the absence of carbohydrate (12, 24). The aggregation was most dramatic for the San Juan G protein, but was also evident for the Orsay G protein at the higher temperature. This strain difference was also noted in assays for aggregation of G proteins from VSV-infected mutant cell lines in which a truncated core oligosaccharide was transferred to the polypeptide chain or in which the oligosaccharides remained in the high-mannose form due to a lack of specific glycosyltransferases (10). The differences in the primary structure between the two G proteins must be responsible for this variation in solubility and aggregation when they are synthesized in the presence of tunicamycin, although it is not obvious which of the changes is responsible.

In light of these findings, we expected that one or the other oligosaccharide or perhaps both oligosaccharides on the San Juan G protein would be required for maintaining a polypeptide conformation essential for intracellular transport. The fact that either oligosaccharide promotes efficient transport to the cell surface may simply imply that an oligosaccharide at either position can maintain a proper polypeptide conformation. Alternatively, our results could be interpreted as evidence that the carbohydrate is a direct signal for intracellular transport, since it can function at two positions in the polypeptide chain.

One criterion of a transport signal is that it be able to function at multiple sites in the polypeptide chain (17). Thus, a function for carbohydrate in signaling transport of G protein to the cell surface could be tested by introducing glycosylation sites at several new positions in G protein lacking the two normal sites. However, N-linked glycans are large, hydrophilic modifications that could easily disrupt protein folding and cause protein denaturation. Indeed, a single amino acid substitution in G protein can completely block its transport from the rER (9), presumably because it causes protein denaturation. Therefore, new sites for introduction of carbohydrate will need to be chosen judiciously. Our initial results on introduction of new sites show that at least one new glycosylation site does promote G-protein transport to the cell surface, while a second site does not (Machamer and Rose, unpublished data). However, the second site inhibits transport when it is placed in the wild-type background and therefore may interfere with the proper folding of the protein.

In a related study we found that glycosylation at two different sites in a membrane-anchored form of rat growth hormone promotes its transport to the cell surface (J.-L. Guan, C. E. Machamer, and J. K. Rose, Cell, in press). The transport of the nonglycosylated form of this protein is normally blocked in the Golgi region (14). These results are also consistent with a role for N-linked glycans in signaling cell surface transport of this protein.

One of the best known examples of a specific signal for intracellular targeting does involve carbohydrate; the mannose-6-phosphate residues found on lysosomal enzymes direct the transport of these enzymes from the Golgi complex to lysosomes via a receptor specific for this phosphorylated sugar (reviewed in reference 39). It has been shown that the cytoplasmic domain of G protein is important for its efficient transfer from the rER to the Golgi complex (33). The cytoplasmic region of the polypeptide could interact with a receptor which somehow directs incorporation of G protein into transport vesicles. Similarly, a second receptor recognizing some portion of the external domain of G protein could be involved in the transport from the Golgi complex to the plasma membrane. The oligosaccharides of glycoproteins are well suited for providing such a signal because of their diversity and because of the widespread existence of exquisitely specific carbohydrate-binding proteins (lectins) as potential receptors for these signals (29).

There is ample data indicating that carbohydrate cannot be an essential signal for cell surface transport of all proteins. First, cell surface expression or secretion of some proteins which normally contain N-linked carbohydrate is not inhibited by tunicamycin (29). Examples include secretion of IgG from plasmacytoma cells (15), secretion of transferrin (43), and cell surface expression of some class II histocompatibility antigens (38). Even different class I histocompatibility antigens in the same cell demonstrate varied responses to tunicamycin treatment, from no effect to complete inhibition of surface expression (22). Some of the variability in the effects of tunicamycin could be explained if some glycoproteins are modified in other ways which allow transport when the addition of their carbohydrate is prevented by tunicamycin. The IgG from tunicamycin-treated hybridoma cells was recently shown to possess sulfated tyrosine residues, a modification not present on the glycoprotein synthesized in the absence of the drug (1). Also, the results obtained by using tunicamycin must be interpreted with caution, since glycosylation of all cellular proteins is inhibited in treated cells; thus, any proteins utilizing host cell glycoproteins in intracellular transport could be indirectly affected. Furthermore, tunicamycin is a mixture of components, some of which exhibit biological activities other than inhibition of N-linked glycosylation (26).

In addition to the studies in which tunicamycin was used, there is other evidence that N-linked oligosaccharides are not important for transport of all secreted and plasma membrane proteins. Not all secreted proteins are glycosylated (7), and there is a recent example of an influenza virus protein (M_2) that is transported to the cell surface but not glycosylated (21). Also, carbohydrate is not sufficient to direct proteins to the cell surface because there are examples of glycosylated proteins, such as galactosyl transferase, which reside in intracellular membranes (41).

We describe here a new approach for studying the role of glycosylation in protein transport. This approach eliminated the complications of treating cells with general inhibitors of glycosylation and allowed us to assess the importance of individual glycosylation sites. Our results suggest that carbohydrate on VSV G protein may play a more direct role in signaling transport than postulated previously (12). Indeed, N-linked carbohydrate on some glycoproteins may play a

dual role, both as a signal for transport and as an important structural component of the polypeptide.

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LITERATURE CITED

1. **Baeuerle, P. A., and W. B. Huttner.** 1984. Inhibition of N-glycosylation induces tyrosine sulphation of hybridoma immunoglobulin G. EMBO J. **3**:2209–2215.
2. **Bergmann, J. E., K. T. Tokuyasu, and S. J. Singer.** 1981. Passage of an integral membrane protein, the vesicular stomatitis virus glycoprotein, through the Golgi apparatus in route to the plasma membrane. Proc. Natl. Acad. Sci. USA **78**:1746–1750.
3. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.
4. **Chatis, P. A., and T. G. Morrison.** 1979. Vesicular stomatitis virus glycoprotein is anchored to intracellular membranes near its carboxyl end and is proteolytically cleaved at its amino terminus. J. Virol. **29**:957–963.
5. **Duksin, D., and W. C. Mchoney.** 1982. Relationship of the structure and biological activity of the natural homologues of tunicamycin. J. Biol. Chem. **257**:3105–3109.
6. **Etchison, J. R., and J. J. Holland.** 1974. Carbohydrate content of the membrane glycoprotein of vesicular stomatitis virus grown in four mammalian cell lines. Proc. Natl. Acad. Sci. USA **71**:4011–4014.
7. **Eylar, E. H.** 1965. The biological role of glycoproteins. J. Theor. Biol. **10**:89–113.
8. **Gallione, C. J., and J. K. Rose.** 1983. Nucleotide sequence of a cDNA clone encoding the entire glycoprotein from the New Jersey serotype of vesicular stomatitis virus. J. Virol. **46**:162–169.
9. **Gallione, C. J., and J. K. Rose.** 1985. A single amino acid substitution in a hydrophobic domain causes temperature-sensitive cell surface transport of a mutant viral glycoprotein. J. Virol. **54**:374–382.
10. **Gibson, R., S. Kornfeld, and S. Schlesinger.** 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.
11. **Gibson, R., R. Leavitt, S. Kornfeld, and S. Schlesinger.** 1978. Synthesis and infectivity of vesicular stomatitis virus containing nonglycosylated G protein. Cell **13**:671–679.
12. **Gibson, R., S. Schlesinger, and S. Kornfeld.** 1979. The nonglycosylated glycoprotein of vesicular stomatitis virus is temperature-sensitive and undergoes intracellular aggregation at elevated temperatures. J. Biol. Chem. **254**:3600–3607.
13. **Gluzman, Y.** 1981. SV40-transformed simian cells support the replication of early SV40 mutants. Cell **23**:177–182.
14. **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.
15. **Hickman, J., and S. Kornfeld.** 1978. Effect of tunicamycin on IgM, IgA and IgG secretion by mouse plasmacytoma cells. J. Immunol. **121**:990–996.
16. **Hubbard, S. C., and R. J. Ivatt.** 1981. Synthesis and processing of asparagine-linked oligosaccharides. Annu. Rev. Biochem. **50**:553–583.
17. **Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith.** 1985. A short amino acid sequence able to specify nuclear location. Cell **39**:499–509.
18. **Katz, F. N., J. E. Rothman, V. R. Lingappa, G. Blobel, and H. F. Lodish.** 1977. Membrane assembly in vitro: synthesis, glycosylation, and asymmetric insertion of a transmembrane protein. Proc. Natl. Acad. Sci. USA **74**:3278–3282.
19. **Knipe, D. M., D. Baltimore, and H. F. Lodish.** 1977. Separate pathways of maturation of the major structural proteins of vesicular stomatitis virus. J. Virol. **21**:1128–1139.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–684.
21. **Lamb, R. A., S. L. Zebedee, and C. D. Richardson.** 1985. Influenza virus M_2 protein is an integral membrane protein expressed on the infected cell surface. Cell **40**:627–633.
22. **Landolfi, N., R. Rich, and R. G. Cook.** 1985. Differential glycosylation requirements for the cell surface expression of class I molecules. J. Immunol. **134**:423–430.
23. **Leavitt, R., S. Schlesinger, and S. Kornfeld.** 1977. Tunicamycin inhibits glycosylation and multiplication of Sindbis and vesicular stomatitis viruses. J. Virol. **21**:375–385.
24. **Leavitt, R., S. Schlesinger, and S. Kornfeld.** 1977. Impaired intracellular migration and altered solubility of nonglycosylated glycoproteins of vesicular stomatitis virus and Sindbis virus. J. Biol. Chem. **252**:9018–9023.
25. **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.
26. **Mahoney, W. C., and D. Duksin.** 1979. Biological activities of the two major components of tunicamycin. J. Biol. Chem. **254**:6572–6576.
27. **Maxam, A. M., and W. Gilbert.** 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. USA **74**:560–564.
28. **Morrison, T. G., and C. O. McQuain, and D. Simpson.** 1978. Assembly of viral membranes: maturation of the vesicular stomatitis virus glycoprotein in the presence of tunicamycin. J. Virol. **28**:368–374.
29. **Olden, K., J. B. Parent, and S. L. White.** 1982. Carbohydrate moieties of glycoproteins. A re-evaluation of their function. Biochim. Biophys. Acta **650**:209–232.
30. **Robertson, J. S., J. R. Etchison, and D. F. Summers.** 1976. Glycosylation sites of vesicular stomatitis virus glycoprotein. J. Virol. **19**:871–878.
31. **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.
32. **Rose, J. K., and J. E. Bergmann.** 1982. Expression from cloned cDNA of cell surface and secreted forms of the glycoprotein of vesicular stomatitis virus in eukaryotic cells. Cell **30**:753–762.
33. **Rose, J. K., and J. E. Bergmann.** 1983. Altered cytoplasmic domains affect intracellular transport of the vesicular stomatitis virus glycoprotein. Cell **34**:513–524.
34. **Rose, J. K., and C. J. Gallione.** 1981. Nucleotide sequences of the mRNAs encoding the VSV G and M proteins as determined from cDNA clones containing the complete coding regions. J. Virol. **39**:519–528.
35. **Rothman, J. E., and H. F. Lodish.** 1977. Synchronized transmembrane insertion and glycosylation of a nascent membrane protein. Nature (London) **269**:775–780.
36. **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.
37. **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.
38. **Shackelford, D. A., and J. L. Strominger.** 1983. Analysis of the oligosaccharides on the HLA-DR and DC-1 B cell antigens. J. Immunol. **130**:274–282.
39. **Sly, W. S.** 1982. The uptake and transport of lysosomal enzymes, p. 3–25. *In* M. I. Horowitz (ed.), The glycoconjugates, vol. 4. Academic Press, Inc., New York.

40. **Sprague, J., J. H. Condra, H. Arnheiter, and R. A. Lazzarini.** 1983. Expression of a recombinant DNA gene coding for the vesicular stomatitis virus nucleocapsid protein. J. Virol. **45**:773–781.
41. **Strous, G. J. A. M., and E. G. Berger.** 1982. Biosynthesis, intracellular transport and release of the Golgi enzyme galactosyltrransferase (lactose synthtase A protein) in HeLa cells. J. Biol. Chem. **257**:7623–7628.
42. **Struck, D. K., and W. J. Lennarz.** 1980. The function of saccharide-lipids in synthesis of glycoproteins, p. 35–83. *In* W. C. Lennarz (ed.), The biochemistry of glycoproteins and proteoglycans. Plenum Publishing Corp., New York.
43. **Struck, D. K., P. B. Siuta, M. D. Lane, and W. J. Lennarz.** 1978. Effect of tunicamycin on the secretion of serum proteins by primary cultures of rat and chick hepatocytes. Studies on transferrin, very low density lipoprotein and serum albumin. J. Biol. Chem. **253**:5332–5337.
44. **Takasuki, A., K. Kohno, and G. Tamura.** 1975. Inhibition of biosynthesis of polyisoprenol sugars in chick embryo microsomes by tunicamycin. Agric. Biol. Chem. **39**:2089–2091.
45. **Tarentino, A. L., and F. Maley.** 1974. Purification and properties of an endo-β-acetylglucosaminidase from *Streptomyces griseus*. J. Biol. Chem. **249**:811–817.
46. **Tkacz, J. S., and J. O. Lampen.** 1975. Tunicamycin inhibition of polyisoprenyl N-acetylglucosaminyl pyrophosphate formation in calf liver microsomes. Biochem. Biophys. Res. Commun. **65**:248–257.
47. **Virtanen, I., P. Ekblom, and P. Laurila.** 1978. Subcellular compartmentalization of saccharide moieties in cultured normal and malignant cells. J. Cell Biol. **85**:429–434.
48. **Zoller, M. J., and M. Smith.** 1982. Oligonucleotide-directed mutagenesis using M13-derived vectors: an efficient and general procedure for the production of point mutations in any fragment. Nucleic Acids Res. **10**:6487–6500.