Vesicular Stomatitis Virus G Proteins with Altered Glycosylation Sites Display Temperature-sensitive Intracellular Transport and Are Subject to Aberrant Intermolecular Disulfide Bonding*

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Carolyn E. Machamer‡ and John K. Rose§
From the Molecular Biology and Virology Laboratory, The Salk Institute, San Diego, California 92138

In this report we have extended our studies on a panel of vesicular stomatitis virus G proteins with altered glycosylation sites. These mutant proteins were generated by oligonucleotide-directed mutagenesis of the coding sequence to create new consensus sites for asparagine-linked oligosaccharide addition. We report that the intracellular transport of most of the mutant proteins is temperature-sensitive, implying a polypeptide folding step is affected. In addition, we find that the nonglycosylated G protein and those mutant proteins which lack oligosaccharides at the normal positions are subject to aberrant intermolecular disulfide bonding, leading to the accumulation of large complexes in the endoplasmic reticulum. These results imply that carbohydrate plays an indirect role in the intracellular transport of G protein.

Glycosylation of asparagine residues is one of the most common modifications of proteins in the exocytic pathway and occurs in all eukaryotic cells (1). We have extended our studies of the role of asparagine-linked (N-linked) glycosylation in the intracellular transport of a model plasma membrane protein, the vesicular stomatitis virus glycoprotein (VSV G protein). We have previously used gene expression and oligonucleotide-directed mutagenesis to demonstrate that the nonglycosylated G protein from tunicamycin-treated cells infected with the Orsay strain of VSV could be detected on the cell surface when cells were grown at 30°C but not at the normal temperature, 37°C (3). COS-1 cells transfected with DNAs encoding each of the mutant G proteins with altered glycosylation sites were labeled at either 37 or 30°C with [35S]methionine for 3 h at the same temperature. G proteins were immunoprecipitated, mock-treated, or treated with endo H and then electrophoresed in polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS). The proportion of each mutant protein with endo H-resistant oligosaccharides was quantitated by densitometry of the fluorograms (Table I).

EXPERIMENTAL PROCEDURES

All procedures for transfection, radiolabeling with L-[35S]methionine, immunoprecipitation, and indirect immunofluorescence microscopy for detection of the mutant G proteins with altered glycosylation sites are described in the preceding report (18). For immunofluorescence and iodination experiments involving reduced temperature, transfected cells were grown at 37°C for 16 hours and then shifted to 30°C for another 26–30 h before assay. For metabolic labeling at 30°C, transfected cells were incubated at 37°C for 42–44 h and then shifted to 30°C for 1 h before the labeling period.

For the experiment shown in Fig. 4, one set of radiolabeled cells was pretreated with 10 mM iodoacetamide in 10 mM Tris, pH 8.0, 0.15 M NaCl for 10 min on ice before lysis in detergent solution containing 10 mM iodoacetamide.

RESULTS

Effect of Reduced Temperature on Oligosaccharide Processing—In the previous report (18), we noted that for the two mutant G proteins with one oligosaccharide at a new site which were transported to the plasma membrane, only a fraction of the molecules was processed to an endoglycosidase H-resistant form. The proportion of each that was processed correlated with the level of protein that was subsequently detected at the cell surface. We tested the effect of reduced temperature on intracellular transport of the mutant proteins with altered glycosylation sites, because it was previously demonstrated that the nonglycosylated G protein from tunicamycin-treated cells infected with the Orsay strain of VSV could be detected on the cell surface when cells were grown at 30°C but not at the normal temperature, 37°C (3).

When the temperature was lowered to 30°C, a large increase in the proportion of protein resistant to endo H was observed for both of the mutant G proteins with a single oligosaccharide at a new site which can be detected at the cell surface at 37°C (QN1/TA1,2 and EN2/TA1,2). After 3 h of chase at 37°C, the fraction of QN1/TA1,2 and EN2/TA1,2 with endo H-resistant oligosaccharides was 42 and 15%, respectively, whereas this fraction increased to 78 and 75% when cells were labeled and chased at the lower temperature. In addition, two mutant proteins which could not be detected on the cell surface at 37°C showed some processing of oligosaccharides at 30°C. A proportion of both QN2/TA1,2 and KT/TA1,2 possess endo H-resistant oligosaccharides after 3 h at 30°C (23 and 46%, respectively), whereas no endo H resistance was detected at 37°C for either protein. Thus, the intracellular transport of these proteins appears to be tem...
proteins with processed oligosaccharides when cells were grown at 30°C. The mutant proteins VT/TA1,2 and EN1/TA1,2 were not detected on the plasma membrane of cells grown at either temperature (Fig. 1) nor was the mutant protein VT with three oligosaccharides (not shown). These results correlate with the processing of the oligosaccharides shown in Table I.

In addition to the glycosylated mutant proteins which could be detected on the cell surface at reduced temperature, the nonglycosylated protein TA1,2 was also observed on the plasma membrane of some transfected cells by indirect immunofluorescence microscopy (Fig. 1). Thus, glycosylation of the G protein of the San Juan strain of VSV is not absolutely required for transport of the protein to the cell surface.

To confirm the results obtained with indirect immunofluorescence microscopy, lactoperoxidase-catalyzed iodination was employed to label cell surface proteins of transfected cells grown at 30°C. Immunoprecipitates from these cells were compared to those from a parallel set of transfected cells biosynthetically labeled with L-[35S]methionine (Fig. 2). The differing levels of expression of mutant G proteins in transfected cells have been previously noted (2) and do not appear to result from decreased stability of the proteins. The mutant proteins that can be detected on the plasma membrane by indirect immunofluorescence microscopy are also labeled when cell surface proteins are iodinated. These results are consistent with the results obtained for oligosaccharide processing and with immunofluorescence microscopy. The fact that a low level of the nonglycosylated protein TA1,2 can be detected on the cell surface at the reduced temperature whereas the mutant proteins VT, VT/TA1,2, and EN1/TA1,2 cannot suggests that the addition of an oligosaccharide to the new sites in these proteins is incompatible with intracellular transport of G protein.

Temperature Sensitivity of the Mutant G Proteins Is Reversible—The increase in transport of the mutant G proteins at 30°C compared to 37°C implied that the carbohydrate might be influencing a polypeptide folding step required for intracellular transport. Since N-linked oligosaccharides are temperature-sensitive, at least through the Golgi region.

Transport of the Mutant Proteins to the Plasma Membrane Is Temperature-sensitive—The increased fraction of mutant proteins with processed oligosaccharides when cells were grown at 30°C instead of 37°C suggested that a greater proportion of these molecules might reach the cell surface under these conditions. To address this question, we performed double-label indirect immunofluorescence microscopy on transfected cells grown at 30°C. COS-1 cells grown on coverslips were shifted from 37°C to 30°C 16 h after transfection (before G protein can be detected) and incubated another 28 h before fixation. Surface G protein was detected by staining nonpermeabilized cells with an antibody specific for G protein followed by a rhodamine-conjugated second antibody. Internal G protein was then detected after detergent permeabilization with G protein-specific antibody followed by a fluorescein-conjugated second antibody. Fig. 1 presents the results obtained for the mutant proteins with only one oligosaccharide at a new site. In addition to QN1/TA1,2 and EN2/TA1,2 which can be detected on the plasma membrane in cells grown at 37°C (18), the mutant proteins QN2/TA1,2 and KT/TA1,2 can also be detected on the plasma membrane of cells grown at 30°C.

### Table I: Glycosylation and Aberrant Disulfide Bonding

| Protein          | Percent of proteins resistant to endo H | Temperature |
|------------------|-----------------------------------------|-------------|
| G (wild-type)    | >98%                                    | 37°C        |
| QN1              | >98%                                    | 30°C        |
| QN1/TA1,2        | 42%                                     | 37°C        |
| QN2              | 40%                                     | 30°C        |
| QN2/TA1,2        | <2%                                     | 37°C        |
| KT               | <2%                                     | 30°C        |
| KT/TA1,2         | <2%                                     | 37°C        |
| VT               | <2%                                     | 30°C        |
| VT/TA1,2         | <2%                                     | 37°C        |
| EN1/TA1,2        | <2%                                     | 30°C        |
| EN2/TA1,2        | 15%                                     | 37°C        |
|                 |                                         |             |

FIG. 1. Double-label indirect immunofluorescence microscopy of transfected cells grown at 30°C. COS-1 cells grown on coverslips were transfected with DNA encoding wild-type or mutant G proteins with altered glycosylation sites, incubated for 16 h at 37°C, and then at 30°C for a further 28 h before fixation. Surface G protein was labeled with rabbit anti-VSV and rhodamine-conjugated goat anti-rabbit IgG. Following permeabilization with detergent, internal G protein was labeled with a mixture of rabbit anti-VSV and a rabbit antiserum specific for the carboxyl terminus of G protein followed by fluorescein-conjugated goat anti-rabbit IgG. Each set of micrographs shows the same cell stained for surface (right panels) and internal (left panels) G proteins. Bar, ~10 μm.
added cotranslationally to G protein (4), they could be expected to influence the folding of a polypeptide as it is synthesized. To determine if the temperature-sensitive step in transport of the mutant G proteins was confined to folding during biosynthesis, we asked whether the temperature at which the proteins were synthesized determined the proportion of molecules which were subsequently transported. Transfected cells were pulse-labeled for 15 min at 37 or 30 °C and chased at 37 or 30 °C. G proteins were immunoprecipitated and treated with endo H, electrophoresed in SDS-polyacrylamide gels, and the proportion of endo H-resistant oligosaccharides was quantitated by densitometry of the fluorograms. The data obtained for two of the mutant G proteins, QN1/TA1,2 and EN2/TA1,2 are shown in Fig. 3. The data show that the temperature at which the transfected cells are chased, not that at which they are labeled, determines the proportion of molecules which will be transported through the Golgi complex. Similar results were obtained for the other mutant proteins which are transported only at the lower temperature (QN2/TA1,2 and KT/TA1,2, not shown). Thus, the temperature-sensitive step is not confined exclusively to cotranslational folding, and it is completely reversible.

**Nonglycosylated G Protein Is Subject to Aberrant Interchain Disulfide Bonding**—To investigate the possibility of improper folding of the nonglycosylated G protein, we examined the mobility of TA1,2 in SDS-polyacrylamide gels under nonreducing conditions. Transfected cells expressing either wild-type glycosylated G protein or the mutant G protein TA1,2 lacking both glycosylation sites were labeled with L-[35S]methionine, and immunoprecipitated proteins were electrophoresed in the presence or absence of reducing agent (Fig. 4). When nonreduced, glycosylated G protein migrates with a slightly faster mobility than when reduced. This implies the existence of at least one intramolecular disulfide bond which maintains a more compact structure of G protein when not reduced (5). In contrast to the glycosylated protein, nonglycosylated TA1,2 does not even enter the separating gel when nonreduced. This suggests that interchain disulfide bonds are formed in the absence of the two N-linked oligosaccharides normally added to G protein. This does not appear to be an artifact of detergent solubilization (6), since TA1,2 isolated from cells pretreated and lysed in the presence of iodoacetamide behaves in the same fashion (Fig. 4). Nonglycosylated G protein from tunicamycin-treated transfected COS-1 cells or VSV-infected COS-1 cells is also found in these large disulfide-linked aggregates (data not shown).

The time course for formation of the aberrant interchain disulfide bonds was analyzed in a pulse-chase labeling experiment. After a 15-min label, only a small fraction of the nonglycosylated G protein runs as monomer under nonreducing conditions, most being found in an aggregate at the top of the separating gel (Fig. 5). With time, the amount of aggregated G protein at the top of the separating gel decreases, and the amount at the top of the stacking gel increases. Thus, even early after synthesis, G protein lacking oligosaccharides exists in large disulfide-linked aggregates. These aggregates appear to increase in size as the protein accumulates in the endoplasmic reticulum. A variable level of two labeled polypeptides with mobilities greater than G protein is often observed in immunoprecipitates of the nonglycosylated G protein TA1,2 (Figs. 4 and 5) and some of the other mutant G proteins lacking oligosaccharides at the normal sites (18). These probably represent SDS-resistant dimers and trimers (18), and are included in the disulfide-linked aggregates of
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**FIG. 4.** The nonglycosylated G protein is subject to aberrant intermolecular disulfide bonding. Transfected COS-1 were labeled for 1 h with 50 μCi of L-[35S]methionine at 37 °C and lysed in the absence (−) or presence (+) of 10 mM iodoacetamide (IAA). Immunoprecipitated G proteins were electrophoresed in an SDS-polyacrylamide gel following elution at 100 °C in sample buffer with 5% 2-mercaptoethanol or without reducing agent.

**FIG. 5.** Time course of formation of aberrant disulfide-bonded complexes. Transfected COS-1 cells expressing either wild-type G protein or the nonglycosylated protein TA1,2 were pulse-labeled for 15 min with 50 μCi of L-[35S]methionine at 37 °C followed by incubation in the presence of excess unlabeled methionine for the times indicated. Immunoprecipitates were split in half and reduced with 5% 2-mercaptoethanol or run under nonreducing conditions. The upper arrow marks the top of the stacking gel, and the lower arrow the top of the separating gel.

TA1,2, since they do not enter the gel under nonreducing conditions (Figs. 4 and 5).

**Mutant G Proteins Which Lack Carbohydrate at the Normal Sites Are Subject to Aberrant Interchain Disulfide Bonding—** The formation of large disulfide-linked complexes in the endoplasmic reticulum could prevent transport of the nonglycosylated G protein to the cell surface. To determine if the formation of these complexes correlated with lack of transport, the panel of mutant proteins was electrophoresed under nonreducing conditions. As seen in Fig. 6, the mutant proteins which lack carbohydrate at the normal sites have an increased level of aberrant interchain disulfide bonding as compared to wild-type G protein or the mutant proteins with three oligosaccharides. Only a fraction of QN1/TA1,2 and EN2/TA1,2, the proteins with one oligosaccharide at a new site which can be transported to the plasma membrane, runs as a monomer. When immunoprecipitates from iodinated transfected cells were run under nonreducing conditions, all of the labeled material ran as a monomer, suggesting that the aberrantly disulfide-bonded polypeptides were not transported to the plasma membrane (data not shown). However, the mutant protein VT, which has three oligosaccharides but is not transported out of the endoplasmic reticulum, runs as a monomer under nonreducing conditions. Thus, not all molecules that fail to be transported accumulate in disulfide-linked aggregates. The presence of carbohydrate at the normal sites seems to be important in preventing cross-linking of polypeptide chains, especially as the nontransported G protein accumulates in the endoplasmic reticulum.

**DISCUSSION**

In this report we have analyzed a panel of mutant G proteins with altered glycosylation sites generated by oligonucleotide-directed mutagenesis of the coding sequence. We show that the N-linked oligosaccharides added to the VSV G protein can influence a post-translational folding step and protect the protein from forming aberrant intermolecular disulfide bonds.

In contrast to the results reported for tunicamycin-treated cells infected with the San Juan strain of VSV (3), we were able to detect a low level of nonglycosylated San Juan G protein on the plasma membrane of transfected cells grown at 30 °C. This difference could be the result of increased sensitivity when transfected cells are analyzed since G protein is allowed to accumulate over 2 days rather than several hours as in the case of infected cells. Alternatively, the two amino acid substitutions in the nonglycosylated G protein introduced to eliminate the glycosylation sites (both threonine to alanine...
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The formation of large disulfide-linked aggregates in the endoplasmic reticulum could explain the inability of mutant G proteins to be transported. These aggregates could be prevented from entering transport vesicles for steric reasons. For most of the mutant proteins, the formation of these aggregates was found to correlate with lack of transport. However, formation of disulfide-linked aggregates is not the only reason for lack of transport of mutant G proteins.

For example, the mutant protein VT with three oligosaccharides (Fig. 6) or the ts045 G protein do not form these disulfide-linked aggregates, yet are not transported. We have recently found that the mutant protein VT is not immunoprecipitated by several monoclonal antibodies which appear to be conformation-dependent, suggesting it may be denatured. Thus, either denaturation of the polypeptide or the formation of large disulfide-bonded aggregates may be sufficient to cause a block in intracellular transport. In fact, the aberrant disulfide-linked aggregates may be secondary to accumulation of certain types of misfolded molecules in the endoplasmic reticulum.

Our studies of the mutant G proteins with altered glycosylation sites confirm and extend the conclusions reached using tunicamycin and VSV-infected cells (3, 14, 15). The primary role of carbohydrate in intracellular transport of G protein appears to be that of promoting proper polypeptide folding and protecting molecules from aberrant disulfide bonding. Although there is a surprising degree of flexibility in the location of the oligosaccharide side chain which promotes transport of G protein (18), this apparently reflects the ability of an oligosaccharide to promote folding, rather than demonstrating a direct role of carbohydrate in signaling intracellular transport.

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changes, Ref. 2) could be responsible for the low level of nonglycosylated protein detected at the cell surface at 30 °C.

Our finding that the intracellular transport of the mutant G proteins with altered glycosylation sites is temperature-sensitive is consistent with the results of Gibson et al. (3) regarding the transport of the nonglycosylated Orsay G protein in tunicamycin-treated VSV-infected cells. The temperature sensitivity of these proteins implies that the oligosaccharides are influencing a polypeptide folding step. Increased temperature is known to increase hydrophobic interactions in proteins and thus affect folding of polypeptide chains (7, 8). Single amino acid changes which result in temperature-sensitive misfolding of the P22 phage spike protein, which also forms a trimer, have been well documented (9), and a single amino acid change in the G protein from the Orsay VSV strain ts045 is responsible for its temperature-sensitive aggregation and block in transport (10, 11). The presence of the large hydrophilic N-linked oligosaccharides at new sites in G protein could certainly influence the proximity of specific amino acids in the polypeptide backbone.

Presumably, the proper folding of at least a fraction of the two mutant G proteins with a single oligosaccharide at a new site (QN1/TAI, 2 and EN2/TAI, 2) can occur at 37 °C, but this fraction increases when the temperature is lowered to 30 °C. Also, a fraction of two other mutant proteins with single oligosaccharides (QN2/TAI, 2 and KT/TAI, 2) as well as the nonglycosylated polypeptide protein TAI, 2 can also fold properly at the lower temperature. The temperature-sensitive step may be folding that occurs after synthesis of G protein is complete, since the fraction that is transported is determined by the temperature at which the transfected cells are incubated during the chase period, rather than the temperature during the labeling period. This could represent an oligomerization step, since G protein has recently been shown to form a trimer before transport from the endoplasmic reticulum (11, 12). Alternatively, it could implicate a folding step required of monomers before oligomerization can occur. We have recently analyzed trimer formation at 37 °C of several of the mutant G proteins with altered glycosylation sites. The mutant proteins which are not transported fail to form trimers but instead appear as heterogeneous aggregates in transfected cells.

We also report here that the nonglycosylated G protein and those mutant G proteins which lack oligosaccharides at the normal sites are subject to aberrant intermolecular disulfide bonding. This results in large aggregates which appear to be conformation-dependent, suggesting it may be denatured. Thus, either denaturation of the polypeptide or the formation of large disulfide-bonded aggregates may be sufficient to cause a block in intracellular transport. In fact, the aberrant disulfide-linked aggregates may be secondary to accumulation of certain types of misfolded molecules in the endoplasmic reticulum.

Our studies of the mutant G proteins with altered glycosylation sites confirm and extend the conclusions reached using tunicamycin and VSV-infected cells (3, 14, 15). The primary role of carbohydrate in intracellular transport of G protein appears to be that of promoting proper polypeptide folding and protecting molecules from aberrant disulfide bonding. Although there is a surprising degree of flexibility in the location of the oligosaccharide side chain which promotes transport of G protein (18), this apparently reflects the ability of an oligosaccharide to promote folding, rather than demonstrating a direct role of carbohydrate in signaling intracellular transport.

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