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Role of N-Linked Oligosaccharides in Processing and Intracellular Transport of E2 Glycoprotein of Rubella Virus

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The role of N-linked glycosylation in processing and intracellular transport of rubella virus glycoprotein E2 has been studied by expressing glycosylation mutants of E2 in COS cells. A panel of E2 glycosylation mutants were generated by oligonucleotide-directed mutagenesis. Each of the three potential N-linked glycosylation sites was eliminated separately as well as in combination with the other two sites. Expression of the E2 mutant proteins in COS cells indicated that in rubella virus M33 strain, all three sites are used for the addition of N-linked oligosaccharides. Removal of any of the glycosylation sites resulted in slower glycan processing, lower stability, and aberrant disulfide bonding of the mutant proteins, with the severity of defect depending on the number of deleted carbohydrate sites. The mutant proteins were transported to the endoplasmic reticulum and Golgi complex but were not detected on the cell surface. However, the secretion of the anchor-free form of E2 into the medium was not completely blocked by the removal of any one of its glycosylation sites. This effect was dependent on the position of the deleted glycosylation site.

Rubella virus (RV) is the sole member of the genus *Rubivirus* in the family *Togaviridae* (23) and contains three major structural proteins, the capsid protein (C; 33 kDa) and two envelope glycoproteins (E1 [58 kDa] and E2 [42 to 47 kDa]) (30). The C protein is arginine rich and is thought to interact with the genomic RNA to form the nucleocapsid, which is surrounded by a lipid bilayer containing the spike complex of the E1 and E2 glycoproteins (30). The strategy for expression of structural proteins during RV replication is similar to that of alphavirus, in which a polyprotein precursor (p110) is translated from a subgenomic RNA in the order NH_2-C-E2-E1-COOH (29, 31). The precursor is then proteolytically processed to generate each structural protein (30). E1 and E2 are transported out of the endoplasmic reticulum (ER) to the Golgi cisternae and the cell surface (13, 14, 16), where virus maturation can occur on either membrane depending on the cell type (3, 43).

RV E2 is a type I membrane glycoprotein, and its biological function remains unclear, although the protein appears to contain at least one viral neutralization epitope (11) and a strain-specific epitope (6). Expression of an RV E2 cDNA construct in COS cells showed that translocation of E2 into the lumen of the rough ER is mediated by a signal peptide residing in the C terminus of the capsid protein (13). Following translocation, asparagine-linked (N-linked) glycosylation of E2 takes place. Processing of RV E2 glycans involves at least two stable intermediates, a 39-kDa high-mannose-containing precursor and a 42-kDa form bearing some complex sugars (13, 14). The transport of E2 to the plasma membrane is inefficient, as only a small fraction of E2 is destined for the cell surface, while the majority of E2 accumulates in the ER and the Golgi cisternae (13). E2 from RV virions migrates as a diffuse band on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) at 42 to 47 kDa because of heterogeneous glycosylation (30). Furthermore, E2 is known to contain O-linked carbohydrates (20). In the presence of tunicamycin, Vero cells infected with RV and COS cells transfected with RV cDNAs produce E2 with a molecular weight of 31 kDa (5, 30). The amino acid sequence predicted from E2 cDNAs reveals a protein of 281 residues including three potential N-linked glycosylation sites in M33 (4) and HPV77 (44) strains as opposed to four in Therien (9, 41) and RA27/3 (26) strains. So far, the importance of N-linked oligosaccharides on E2 in virion assembly and infectivity is unknown.

N-linked glycosylation is one of the most common post-translational modifications of proteins in the exocytic pathway of eukaryotic cells. Animal viruses utilize host cell glycosylation machinery to synthesize and process oligosaccharides attached to viral glycoproteins. Thus, the expression of viral antigens in cells has proved to be a useful system for studying the stepwise events in glycan processing and intracellular transport along the exocytic pathway. Several approaches have been used to define the functional roles of N-linked carbohydrate addition in cells. These include the use of agents that interfere with glycosylation, elimination of each N-linked glycan addition site on the cDNA by oligonucleotide-directed mutagenesis, and use of glycan-processing-deficient cell lines. A variety of cellular and viral glycoproteins have been analyzed by these approaches. It appears that oligosaccharides on glycoproteins play a role in initiation and maintenance of folding into biologically active conformation, protecting polypeptides from proteolytic attack and influencing the antigenicity and immunogenicity of glycoproteins (reviewed in references 8, 17, and 32). Although the functional contribution of the carbohydrate varies with the glycoprotein in question, it has been generally accepted that glycosylation is necessary to aid proteins in attaining and maintaining correct tertiary structure and undergoing intracellular transport.

In this report, we analyze the role of N-linked glycosylation in the processing and intracellular transport of RV E2

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glycoprotein from M33 strain. Oligonucleotide-directed mutagenesis was used to construct a panel of E2 glycosylation mutants that were subsequently analyzed by transient expression in COS cells. Our data show that all three potential sites are used for N-linked carbohydrate addition in the E2 glycoprotein. E2 glycosylation mutants were processed to obtain some endoglycosidase H (endo H)-resistant sugar moieties, indicating that they reached the Golgi apparatus. Although none of the mutants were detected on the cell surface by indirect immunofluorescence, the single glycosylation mutants were secreted when expressed in a soluble form.

MATERIALS AND METHODS

Oligonucleotide-directed mutagenesis. The cDNA insert encoding RV E2 protein (14) in the expression vector pCMV5 (2) was subcloned into the *Eco*RI and *Hin*dIII sites of M13mp18 (25). Oligonucleotide-directed mutagenesis was performed on single-stranded uracil-containing templates by the method of Kunkel (19). The oligonucleotides used to eliminate each consensus sequence for N-linked glycan addition were pGTCGCTGGCTTGTCGGTG, which introduced two nucleotide changes (underlined), resulting in a single amino acid change of asparagine 53 to glutamine; pCCAGTCGCCCAGGTTGTA, which destroyed a glycosylation site by changing serine 73 to glycine; and pGCTG CTAATGAGGCC, which changed asparagine 115 to isoleucine. Double and triple mutants were generated by ligating the restriction fragment containing the appropriate wild-type or mutant site(s). All the mutants were confirmed by DNA sequence analysis (36) and subcloned into the expression vector pCMV5 (2).

Transfection, metabolic labeling, and immunoprecipitation. COS cells were transfected with recombinant plasmids according to the method of Adams and Rose (1) with modifications (16). Metabolic labeling and immunoprecipitation were carried out as described previously (13, 16).

***N*-Glycanase and endo H digestions.** *N*-Glycanase (Genzyme) treatment was performed as recommended by the manufacturers. Immunoprecipitates to be digested with *N*-glycanase were adjusted to 100 mM sodium phosphate (pH 8.6)–1% Nonidet P-40–100 mM EDTA–0.5% β-mercaptoethanol–0.1% SDS and incubated with different amounts of enzyme for at least 8 h (for complete digestion) or for 10 min (for partial digestion) at 37°C. Endo H glycosidase digestion was performed as previously described (37).

Indirect immunofluorescence. Transfected COS cells grown on polylysine-coated 9-mm glass coverslips were washed three times with phosphate-buffered saline (PBS) with 0.7 mM $CaCl_2$ and 0.3 mM $MgCl_2$, fixed for 20 min at room temperature in 2% formaldehyde-PBS, and washed with PBS. Some cells were permeabilized with 0.1% Nonidet P-40–PBS for 30 min and incubated with rhodamine-conjugated wheat germ agglutinin (WGA) or concanavalin A (ConA) prior to being blocked by 1% bovine serum albumin (BSA)–PBS. Coverslips were overlaid with diluted human serum (1:200), incubated for 60 min at room temperature, and then washed with BSA-PBS. Incubation with secondary antibody, i.e., fluorescein-conjugated goat anti-human immunoglobulin G (1:100; Kirkegaard and Perry Laboratories), was for 60 min. Coverslips were washed, mounted, examined by epifluorescence, and photographed.

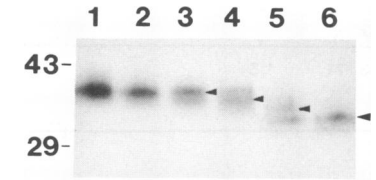

FIG. 1. Determination of the number of N-linked glycans on RV E2. [^{35}S]methionine-labeled E2 was incubated with no (lane 1), 10 mU (lane 2), 20 mU (lane 3), 50 mU (lane 4), 100 mU (lane 5), and 300 mU (lane 6) of *N*-glycanase (Boehringer Mannheim) for 10 min at 37°C, separated by SDS-PAGE, and fluorographed. Positions of molecular size markers are shown on the left (in kilodaltons).

RESULTS

Determination of functional N-linked glycosylation sites in E2 glycoprotein. The predicted amino acid sequence indicates that E2 from RV M33 strain contains three putative N-linked glycosylation sites (4). However, it remains unknown how many sites are actually used. To characterize the number of oligosaccharide side chains on wild-type E2, recombinant plasmid pCMV5-E2 (14), which contains E2 cDNA in a mammalian expression vector pCMV5 (2) downstream from the human cytomegalovirus immediate early gene promoter, was used to transfect COS cells. Transfected COS cells (40 to 50 h posttransfection) were metabolically labeled with 100 μCi of [^{35}S]methionine for 30 min at 37°C and lysed. E2 protein was immunoprecipitated with human anti-RV serum, digested with a serially diluted *N*-glycanase, and subjected to SDS-PAGE and fluorography. *N*-Glycanase hydrolyzes the glucosylamine linkage of all types of N-linked oligosaccharides on glycoproteins to give free oligosaccharides and polypeptides (33). This partial digestion generated four species with molecular sizes of 39, 36, 33.5, and 31 kDa (Fig. 1). It is likely that these species corresponded to E2 with three, two, one, and no carbohydrate side chain(s), suggesting that wild-type E2 glycoprotein normally has three N-linked oligosaccharide chains. It has been reported previously that digestion of E2 from RV virion by *N*-glycanase changed the apparent molecular weight dramatically and resulted in a smeared appearance on SDS-PAGE (20).

Construction and expression of RV E2 glycosylation mutants. To confirm the above-mentioned observation, a panel of E2 glycosylation mutants was constructed and expressed transiently in COS cells. Oligonucleotide-directed mutagenesis was employed to introduce one or two nucleotide changes in the codons encoding asparagine or serine, resulting in a single amino acid substitution at each potential glycosylation site. The addition of N-linked oligosaccharides was prevented by changing the Asn-X-Ser consensus sequence at asparagine residues 53, 71, and 115 to Gln-X-Ser, Asn-X-Gly, and Ile-X-Ser, respectively. The mutants in which consensus sequences were altered singly are referred to as G1, G2, and G3; the double mutant is referred to as G12; and the triple mutant is referred to as G123 (Fig. 2). The positions are numbered sequentially from the N terminus of E2.

The cDNA inserts of RV E2 mutants were subcloned into the pCMV5 vector (2) and used to transfect COS cells. The expression of mutant proteins was analyzed by metabolic labeling, radioimmunoprecipitation, and SDS-PAGE as described previously (13, 16). Wild-type E2 expressed a prominent 39-kDa glycoprotein and a 42-kDa glycoprotein (Fig. 3A), which correspond to isoforms of E2 containing high

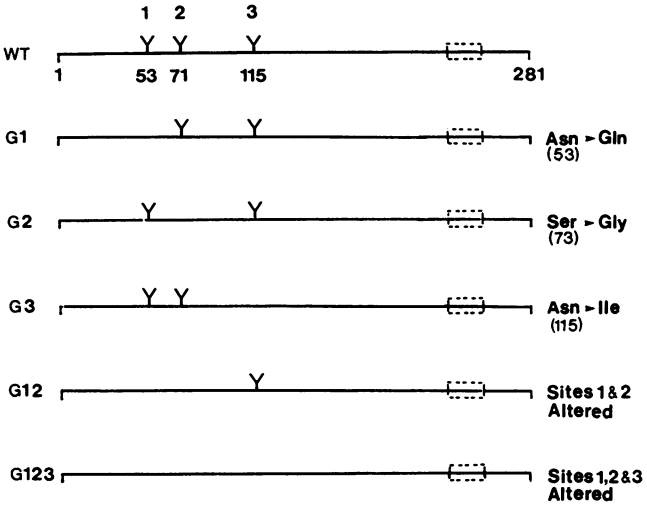

FIG. 2. Schematic representation of wild-type (WT) and glycosylation mutants of RV E2. The E2 protein contains three N-linked glycosylation sites at residues 53, 71, and 115, as indicated by branched structures (Y). The putative transmembrane region is located near the C terminus of E2 (⋯). The first residue of mature E2 is glycine 1, and the C-terminal residue of E2 before E1 is glycine 281.

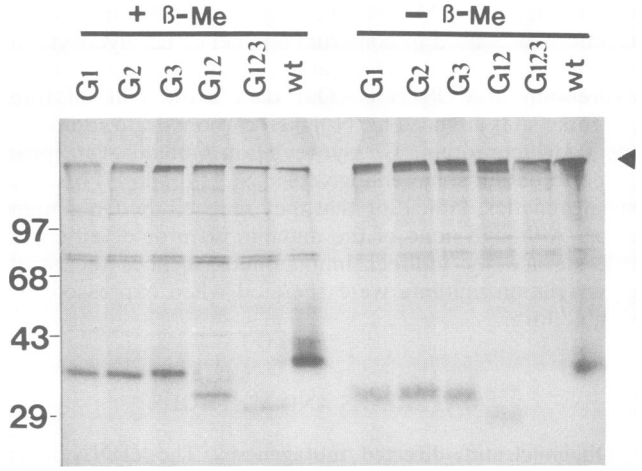

FIG. 4. Formation of aberrant disulfide bonding in E2 glycosylation mutants. Transfected cells were pulse-labeled with 100 μCi of [^{35}S]methionine for 30 min and chased with excess methionine for 2 h. RV-specific proteins were analyzed by immunoprecipitation with human anti-RV serum, separated on 11% SDS-PAGE with or without β-mercaptoethanol (β-Me), and fluorographed. Positions of molecular size markers are shown on the left (in kilodaltons), and the arrow indicates the start of the separating gel.

mannose and complex sugars, respectively (13). The electrophoretic mobilities of the mutant proteins increased proportionally with the number of inactivated glycosylation sites (Fig. 3A). Removal of any single glycosylation site at position 1, 2, or 3 resulted in synthesis of a major 36-kDa glycoprotein, while the double mutant G12 and the triple mutant G123 directed the synthesis of proteins which migrated at 33.5 and 31 kDa, respectively (Fig. 3A).

To verify that the differences in electrophoretic mobility between wild-type and mutant E2 were due to the numbers of N-linked oligosaccharide side chains attached, some transfected cells were treated with tunicamycin. Tunicamycin at a low concentration efficiently inhibits N-linked glycosylation without interfering with protein synthesis in cells (8). In the presence of 3 μg of tunicamycin per ml, all the E2 polypeptides synthesized in cells transfected with wild-type or different glycosylation mutant cDNA constructs had the same molecular weight as the triple mutant, G123 (Fig. 3A, +Tm). Tunicamycin did not affect the apparent molecular weight of G123 from transfected cells (Fig. 3) nor did digestion with *N*-glycanase (Fig. 3B). These results suggest that all three potential N-linked glycosylation sites are normally used and that the difference in molecular weight between mutant and wild-type E2 is due to the number of carbohydrate chains attached.

Formation of aberrant disulfide bonds in the glycosylation mutants. The possible formation of aberrant disulfide bonds in E2 glycosylation mutants was examined by pulse-chase analysis. COS cells were transfected with recombinant plasmids containing E2 glycosylation mutant cDNAs, labeled with [^{35}S]methionine for 30 min, and incubated with a chase medium (with excess unlabeled methionine) for 2 h. Cells were lysed, and E2 polypeptides were immunoprecipitated with human anti-RV serum and separated by SDS-PAGE under reducing and nonreducing conditions. Wild-type and mutant E2 proteins migrated slightly faster in the absence of β-mercaptoethanol than in its presence (Fig. 4), implying the existence of intramolecular disulfide bonds in E2 that have

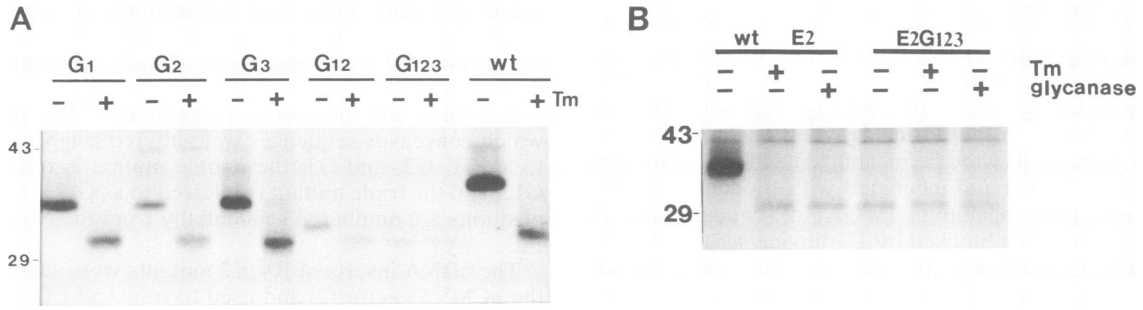

FIG. 3. Expression of wild-type (wt) and glycosylation mutants of E2 in COS cells. (A) Transfected cells were labeled with [^{35}S]methionine for 30 min in the presence (+) or the absence (−) of 3 μg of tunicamycin (Tm) per ml. RV-specific proteins were immunoprecipitated with human anti-RV serum and separated by 11% SDS-PAGE. (B) Some immunoprecipitated E2 proteins were treated with 100 mU of *N*-glycanase at 37°C overnight (+glycanase) and subjected to SDS-PAGE and autoradiography. Positions of molecular size markers are shown on the left (in kilodaltons).

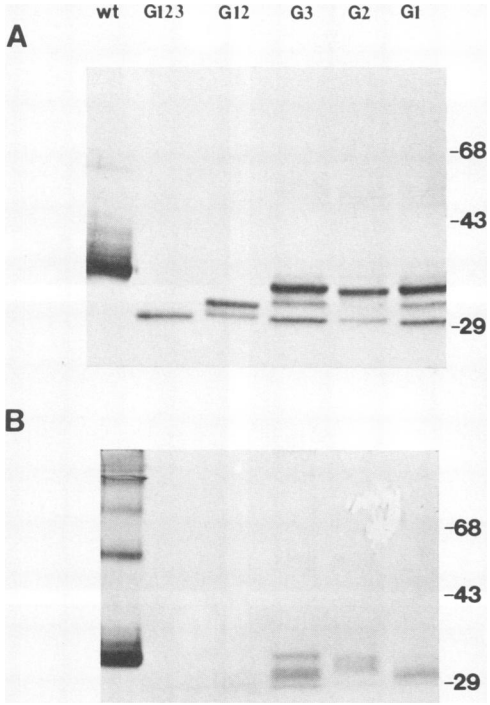

FIG. 5. Western blot (immunoblot) analysis of steady-state wild-type (wt) and mutant E2 proteins in transfected cells under reducing and nonreducing conditions. Transfected COS cells were lysed (40 h posttransfection) with RIPA buffer (50 mM Tris-HCl [pH 7.5], 1% Triton X-100, 10 mM EDTA, 0.15 M NaCl, 0.1% SDS, 1% sodium deoxycholate) containing 10 mM iodoacetamide. Cytoplasmic extracts were electrophoresed on 11% reducing (A) and nonreducing (B) gels. The proteins were transferred to cellulose nitrate membranes. Membranes were blocked in 4% milk powder in TBS (0.15 M NaCl, 0.02 M Tris-HCl [pH 7.5]) and incubated with human anti-RV serum (1:200 dilution). The proteins were visualized with alkaline phosphatase-conjugated anti-human immunoglobulin G. Positions of molecular size markers are shown on the right (in kilodaltons).

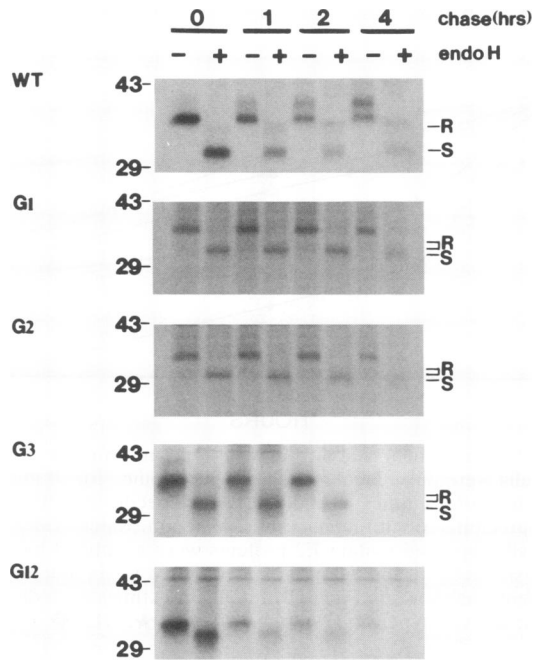

FIG. 6. Time course for glycan processing of wild-type (WT) and mutant E2 proteins. Cells were pulse-labeled with [^{35}S]methionine for 30 min and chased for various times as indicated. Some immunoprecipitated samples were digested with endo H for at least 8 h (+endo H). Endo H-resistant (R) and -sensitive (S) oligosaccharide-containing proteins are indicated. Positions of molecular size markers are shown on the left (in kilodaltons).

also been observed in many other glycoproteins (21, 22, 40). The G12 protein ran as a diffuse band, and the G123 protein was not detectable on the gel under nonreducing conditions, although in the presence of β-mercaptoethanol, there existed clear sharp bands for these mutant proteins (Fig. 4). These results suggest the formation of aberrant intermolecular disulfide bonds that cause the proteins to migrate as diffuse smears when disulfide bonds are not disrupted.

The possible formation of aberrant intermolecular disulfide bonds in E2 mutants was further analyzed by immunoblots (39). COS cells were transfected with wild-type and mutant recombinant plasmids. Cell lysates were prepared 48 h after transfection and analyzed directly by SDS-PAGE under reducing and nonreducing conditions. Transferred proteins were probed with human anti-RV sera. Under reducing conditions, single glycosylation mutants had a predominant 36-kDa and minor 34.5- and 32.5-kDa high-mannose glycoprotein species (Fig. 5A). Two high-mannose glycoprotein species at 33.5 and 32 kDa were detected in the double mutant (Fig. 5A). Only the 31-kDa unglycosylated E2 protein was observed in the triple mutant (Fig. 5A). Under nonreducing conditions, the samples migrated slightly faster because of the presence of intramolecular disulfide bonds (Fig. 5B). Deletion of any glycosylation site from E2 seemed either to abolish the binding of antibodies to E2 or to reduce the amount of monomeric forms, especially in G12 and G123 (Fig. 5B). It is possible that these mutant proteins exist as alternatively folded structures that are not recognized by anti-RV serum and that the antigenic sites in G12 and G123 forms are detectable only after unfolding of these proteins by cleavage of intramolecular disulfide bonds. These findings suggest that the pattern of disulfide bonding for E2 glycosylation mutants is heterogeneous and that glycosylation may be important in preventing aberrant disulfide bond formation.

Glycan processing and intracellular stability of E2 proteins. The kinetics of processing and the turnover rate of the E2 mutant proteins were examined by pulse-chase experiments followed by densitometric analysis of processed proteins. Transfected COS cells were pulse-labeled with [^{35}S]methionine for 30 min and chased for various times. The cell lysates were immunoprecipitated with human anti-RV serum, and one half of each sample was digested with endo H glycosidase. After a 30-min pulse-label, wild-type E2 was found predominantly in the 39-kDa form, and removal of high-mannose glycans by digestion with endo H (38) reduced the molecular size to 32 kDa (Fig. 6). Approximately 25, 40, and 50% of wild-type E2 was found to possess complex-type sugar after 1-, 2-, and 4-h chase periods, respectively (Fig. 6). In contrast, G1, G2, and G3 mutant proteins containing complex-type glycans represented only 17, 14, and 10% of the total amount of each mutant protein after a 2-h chase (Fig. 6). No endo H resistance was observed for the double mutant, G12 (Fig. 6). As the acquisition of endo H resistance is believed to be indicative of transport of the glycoproteins through the medial Golgi apparatus, it is evident that re-

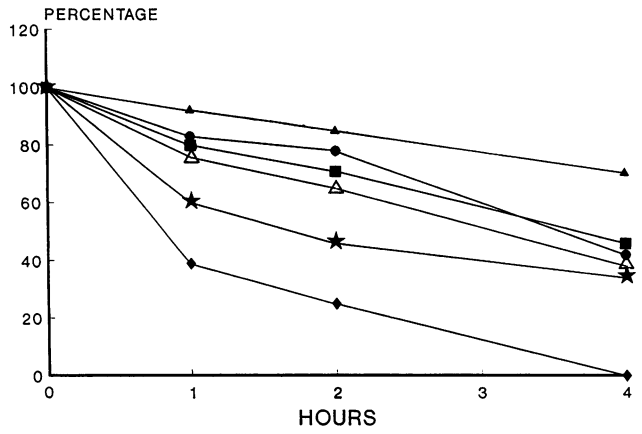

FIG. 7. Intracellular stability of wild-type and mutant E2 proteins. Cells were pulse-labeled with [^{35}S]methionine for 30 min and chased for various times as indicated. RV-specific proteins were immunoprecipitated with human anti-RV serum. Rates of degradation of wild-type and mutant E2 proteins were quantified by scanning densitometry of the X-ray films from three to six independent experiments, as shown in Fig. 6. Different chase times are indicated. Symbols: ▲, wild type; ●, G1; ■, G2; △, G3; ★, G12; ◆, G123.

moval of glycosyl moieties impairs the transport of E2 mutant proteins. This effect is dependent on both the position and the number of glycosylation sites altered.

To determine the turnover rate of wild-type and mutant E2, immunoprecipitates from transfected COS cells were fractionated by SDS-PAGE and quantitated by densitometric analysis of the autoradiographs (Fig. 7). Wild-type E2 was relatively stable in COS cells, with 70% of E2 remaining after a 4-h chase. By contrast, the mutants exhibited a higher turnover rate. The half-lives for mutant proteins in the cells were 3 h for G1, G2, and G3; 2 h for G12; and 30 to 60 min for G123. It could be that the mutant proteins were not properly folded and transported because of an altered glycosylation pattern and were rapidly degraded, as has been reported for some other glycoproteins (24).

Intracellular localization of mutant E2 proteins. The subcellular localization of E2 mutant proteins was examined by indirect immunofluorescence. Cells expressing wild-type E2 exhibited staining throughout the cytoplasmic reticulum as well as in the juxtanuclear region (Fig. 8a). The single, double, and triple glycosylation mutant proteins displayed a predominantly reticular staining pattern as well as Golgi-like staining (Fig. 8c, e, and g). To visualize the distribution of E2 protein in the ER and the Golgi, fluorescent-conjugated WGA and ConA were used as markers for the compartments. WGA has been shown to label *trans* Golgi cisternae, associated vesicles, and the cell surface (38) by binding to clustered terminal *N*-acetylneuraminic acid residues as well as *N*-acetylglucosamine-containing oligosaccharide chains on glycoproteins (42). Costaining of transfected COS cells with human anti-RV serum and fluorescein-conjugated WGA revealed that wild-type E2 was concentrated in the Golgi region (Fig. 8b) while the mutant E2 proteins were distributed throughout the reticulum network and the Golgi region (Fig. 8d and f). A strong reticular staining, which colocalized with ConA, was observed in COS cells transfected with glycosylation mutants (Fig. 8h). In addition, unlike wild-type E2, which has been shown to exhibit a limited amount of cell surface expression (14), the glycosylation mutants had no detectable cell surface signals (data not shown). Elimination

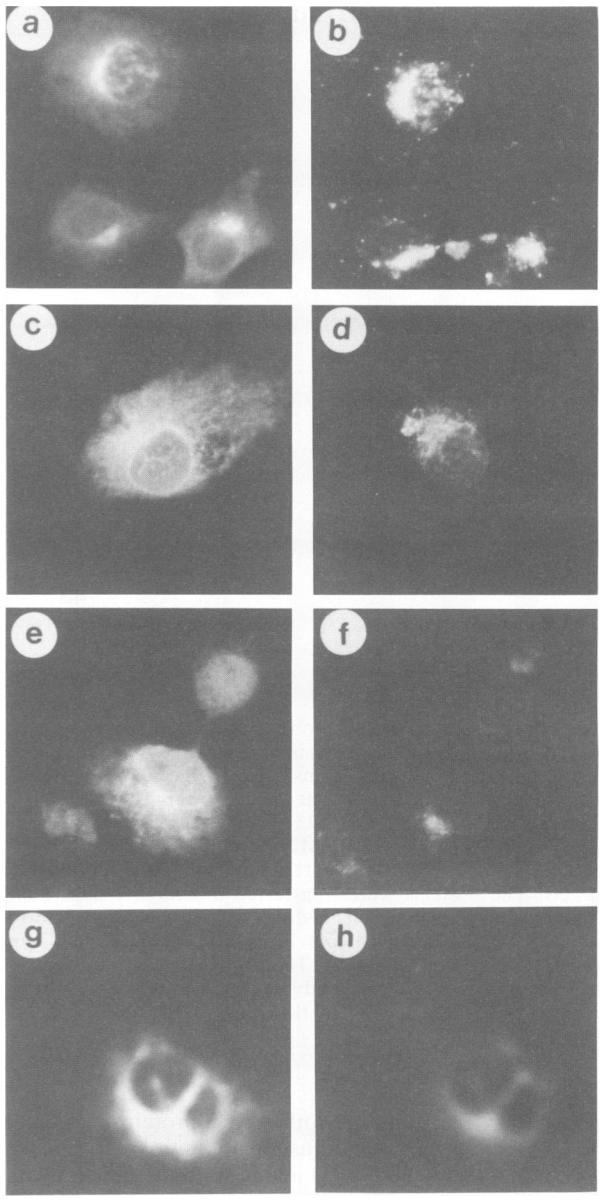

FIG. 8. Indirect immunofluorescence of wild-type and mutant E2 proteins in COS cells. Cells were permeabilized prior to addition of rhodamine-conjugated WGA or ConA and anti-RV serum. After the cells were washed, a secondary antibody (fluorescein-conjugated goat anti-human immunoglobulin G) was added. (a) Wild type, anti-RV; (b) wild type, TRITC-WGA; (c) G2, anti-RV; (d) G2, TRITC-WGA; (e) G12, anti-RV; (f) G12, TRITC-WGA; (g) G123, anti-RV; (h) G123, TRITC-ConA.

of any of the glycosylation sites in E2 seemed to impair the intracellular transport and block the cell surface expression of E2.

Secretion of an anchor-free form of wild-type and mutant E2 proteins. To analyze the transport behavior of E2 mutants in the secretory pathway, we constructed a panel of truncated E2 glycosylation mutants, each of which had 68 amino acids deleted from the hydrophobic C terminus (4). The truncated form of wild-type E2 was previously observed to be secreted into the culture medium as a 37-kDa endo H-resistant glycoprotein (15a). The truncated forms of E2

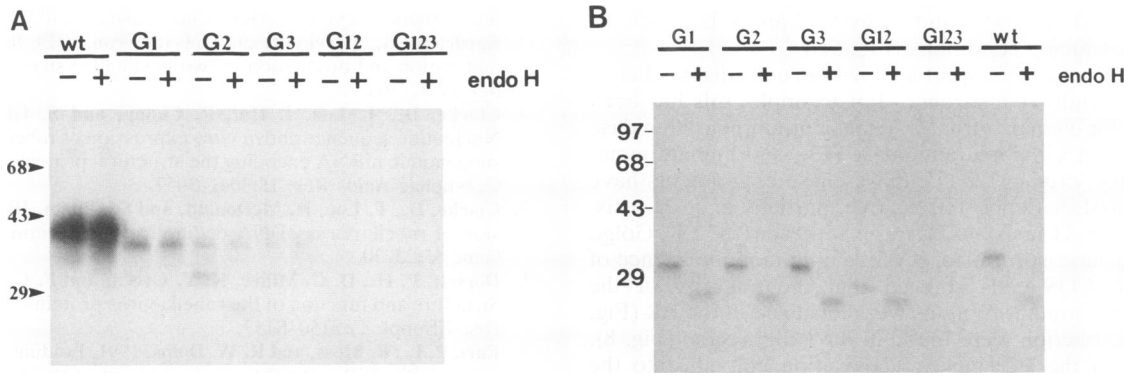

FIG. 9. Intracellular processing and secretion of a soluble form of wild-type (wt) and mutant E2 proteins. Cells were labeled with [^{35}S]methionine for 30 min and chased for 4 h. (A) Immunoprecipitated samples from culture media of cells transfected with anchorless wild-type and mutant E2 cDNA constructs. (B) Intracellular forms of each anchorless E2 protein of wild-type and glycosylation mutants. Equal volumes of each sample were incubated at 37°C for at least 8 h with (+) or without (−) endo H and separated by 11% SDS-PAGE. Positions of molecular size markers are shown on the left (in kilodaltons).

single glycosylation mutants (G1, G2, and G3) but not double (G12) and triple (G123) mutants were also secreted into the culture medium, although not as efficiently as the anchorless wild-type E2 (Fig. 9A). In addition, the efficiency of secretion appeared to depend on the position of the deleted glycosylation site. The deletion of the glycosylation site proximal to the C terminus (G3) had a more profound inhibitory effect on secretion than did deletion of the central site (G2) and that proximal to the N terminus (G1) (Fig. 9A). The intercellular forms of anchorless wild-type E2 and E2 glycosylation mutants were found to be sensitive to endo H digestion (Fig. 9B), whereas the secreted E2 was endo H resistant (Fig. 9A). However, expression of the truncated triple mutant was not detected intracellularly (Fig. 9B). A 31-kDa endo H-sensitive protein species was found in the culture medium of the G2-transfected cells (Fig. 9A). This probably was due to some cell lysis during the chase period that released the intracellular G2 mutant protein into the medium. Taken together, it was evident that the single glycosylation mutants G1, G2, and G3 were transported out of the ER through the Golgi to the cell surface and that they then exited the cell into the culture medium, although not as efficiently as the otherwise unaltered anchorless E2.

DISCUSSION

Protein movement from the ER to the medial Golgi apparatus has been identified as the rate-limiting step in the exocytic pathway (35), as measured by the acquisition of a variety of organelle-specific posttranslational modifications. Regarding the intracellular transport rate, several viral glycoproteins that have been extensively investigated fall into two categories. The first group includes the vesicular stomatitis virus G protein (34) and influenza virus hemagglutinin (10), which move quickly along the exocytic pathway. After a 15-min chase, 50% of the oligosaccharides on vesicular stomatitis virus G protein and 25% on influenza virus hemagglutinin acquire endo H resistance (10, 34). The second group contains human immunodeficiency virus type 1 envelope protein (Env) (7) and simian virus 5 hemagglutinin-neuraminidase (28), for which acquisition of endo H resistance was observed within 80 and 60 min postlabeling, respectively. We found that the carbohydrates on wild-type RV E2 were converted to complex-type sugar moieties by 1 h postlabeling. However, the conversion was not complete even after an 8-h chase (data not shown), reflecting a slow movement of RV E2 from the ER to the Golgi apparatus.

Our data showed that E2 contains three potential oligosaccharide addition sites and that all three potential N-linked glycosylation sites were utilized (Fig. 1 and 3). Inactivation of these functional sites impaired the processing as well as the intracellular stability of E2 proteins (Fig. 6 and 7), the severity of the defect depending on both the number and the position of the glycosylation site deleted. Deletion of one N-linked glycosylation site on RV E2 considerably reduced the rate of transport, as determined by the fraction of proteins that acquired endo H-resistant carbohydrates (Fig. 6). The glycosylation site proximal to the N terminus (G1) seems to be less important than the site proximal to the C terminus (G3), as judged by the fraction of molecules containing endo H-resistant carbohydrates for the membrane-bound form and by the secretion ratios of their anchorless counterparts (Fig. 6 and 9). Oligosaccharide at each glycosylation site plays a different functional role, as has been noted previously in other glycoproteins (24, 27). In addition, studies on other glycoproteins by the same approach have shown that glycosylation on all the predicted sites is not a prerequisite for folding, assembly, and transport of the protein (12). It has been suggested that the contribution of each carbohydrate chain varies depending on its location in a different conformational circumstance of a particular protein. RV E2 is rich in cysteine and undergoes intramolecular disulfide bonding (Fig. 5). Inspection of the amino acid sequence of RV E2 reveals that the G2 and G3 glycosylation sites are flanked by two cysteine residues (4). It is possible that the oligosaccharides attached to the G2 and G3 sites are important in preventing improper intramolecular disulfide bond formation, whereas glycosylation at the G1 site has less effect on proper folding and transport. The diffuse or smeared appearances of nonreduced mutant G12 and G123 proteins on an immunoblot probably reflect the formation of aberrant intermolecular disulfide bonds. Thus, it appears that oligosaccharide addition is required for proper intramolecular disulfide bonding to promote correct folding, which in turn is essential for efficient transport (40). Removal of a glycosylation site leads to formation of improper intramolecular disulfide bonds and protein misfolding. Dramatic alteration in protein conformation could be the consequence when glycosylation sites are inactivated. This may account

for diminished antibody binding by G12 and G123 proteins under nonreducing conditions (Fig. 5B).

Proteins that are transported slowly in cells display heterogeneity in endo H resistance. For example, this has been observed for simian virus 5 hemagglutinin-neuraminidase (28), influenza virus neuraminidase (18), and human immunodeficiency virus gp120 (7). Pulse-chase experiments have demonstrated that endo H-sensitive, partially endo H-resistant, and endo H-resistant E2 forms represent the ER, Golgi, and cell surface isoforms of RV E2. Immunofluorescence of transfected COS cells showed that the majority of the glycosylation mutant proteins were localized in the ER (Fig. 8). A small fraction were found in the Golgi region (Fig. 8). Transport of the E2 single glycosylation mutants into the Golgi compartment was evidenced by the presence of partially endo H-resistant bands after the chase period (Fig. 6) as well as by the secretion of the C-terminal-truncated form of E2 single glycosylation mutants (Fig. 9A). Thus, the transport of E2 to the Golgi apparatus appeared to be significantly affected but not completely blocked by the absence of any one of the N-linked oligosaccharides.

The anchorless E2 single glycosylation mutants were secreted into the culture medium, although less efficiently. The oligosaccharides on the secreted forms of wild-type and mutant E2 were completely endo H resistant, as was E2 from the RV virion (20), suggesting that carbohydrates attached to these proteins are modified by Golgi enzymes. This finding indicates that the soluble forms of E2 single glycosylation mutants are transported through the normal exocytic route. Inability to detect cell surface expression of E2 single glycosylation mutants could be due either to the low sensitivity of indirect immunofluorescence in our experiments or to the fact that mutant E2 proteins were quickly and extensively internalized from the plasma membrane, as has been observed for other glycoproteins (28).

The role of glycosylation generally varies for any given protein, and no concrete rules exist for predicting the phenotype of a particular glycosylation mutant. Analysis of this type is further complicated by the fact that when glycosylation sites are changed by site-directed mutagenesis, targeting and/or processing kinetics may differ as a consequence of the amino acid substitution itself rather than of lack of glycosylation per se. Glycosylation may also be important for expression of immunogenic epitopes. However, in this case, human anti-RV sera seemed to recognizes the glycosylation mutants just as well as the wild-type RV antigens did. Studies are in progress to determine whether RV E2 is internalized and degraded rapidly and to examine the capacities of the E2 glycosylation mutant proteins to function in RV virions.

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