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Disulfide Bond Formation Is a Determinant of Glycosylation Site Usage in the Hemagglutinin-Neuraminidase Glycoprotein of Newcastle Disease Virus

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Determinants of glycosylation site usage were explored by using the hemagglutinin-neuraminidase (HN) glycoprotein of the paramyxovirus Newcastle disease virus. The amino acid sequence of the HN protein, a type II glycoprotein, has six N-linked glycosylation addition sites, G1 to G6, two of which, G5 and G6, are not used for the addition of carbohydrate (L. McGinnes and T. Morrison, Virology 212:398–410, 1995). The sequence of this protein also has 13 cysteine residues in the ectodomain (C2 to C14). Mutation of either cysteine 13 or cysteine 14 resulted in the addition of another oligosaccharide chain to the protein. These cysteine residues flank the normally unused G6 glycosylation addition site, and mutation of the G6 site eliminated the extra glycosylation found in the cysteine mutants. These results suggested that failure to form an intramolecular disulfide bond resulted in the usage of a normally unused glycosylation site. This conclusion was confirmed by preventing cotranslational disulfide bond formation in cells by using dithiothreitol. Under these conditions, the wild-type protein acquired extra glycosylation, which was eliminated by mutation of the G6 site. These results suggest that localized folding events on the nascent chain, such as disulfide bond formation, which block access to the oligosaccharyl transferase are a determinant of glycosylation site usage.

N-linked glycosylation, a common modification of viral glycoproteins as well as cellular plasma membrane and secreted proteins, plays various roles in the folding, stability, and biological activity of proteins and is an important component of their structure and function (13, 30). The core oligosaccharide chains are preassembled on the carrier lipid, dolichol phosphate, in the rough endoplasmic reticulum (RER) and are then covalently attached through the action of oligosaccharyl transferase to polypeptides at asparagines in the sequence NXT or NXS, where X is any amino acid except proline (13). The core oligosaccharide is covalently attached to the nascent polypeptide very soon after the asparagine emerges from the translocation channel into the lumen of the RER (6, 13). Indeed, oligosaccharyl transferase is thought to be closely associated with the translocation machinery (6).

Not all NXT or NXS motifs in a protein sequence, however, are used for carbohydrate addition. There are numerous examples in the literature of proteins which remain unglycosylated at specific sites or are inefficiently glycosylated at specific sites while other sites are always utilized (30). The reasons for usage of some carbohydrate addition signals but not others are poorly understood; however, the failure to use specific addition signals suggests that there are elements, in addition to the 3-amino-acid sequence motif, involved in the recognition of a site for glycosylation. That there are no other recognized primary sequence motifs associated with N-linked glycosylation suggests the hypothesis that secondary structures on the nascent chain influence usage of glycosylation addition sites.

To test this possibility, we have explored determinants of glycosylation site usage in a viral glycoprotein, the Newcastle disease virus (NDV) hemagglutinin-neuraminidase (HN) protein, which has two unused glycosylation addition signals (17). The HN protein, a member of a family of proteins which are the attachment proteins of paramyxoviruses (24), is a type II glycoprotein with a 26-amino-acid amino-terminal cytoplasmic domain, a 22-amino-acid signal-anchor domain, and a 523-amino-acid ectodomain (24). The primary sequence has six glycosylation addition signals in the ectodomain (G1 to G6, numbered from the amino terminus of the protein) (19); however, only four sites (G1 to G4) are used for glycosylation (17). The protein also has 13 cysteine residues (C2 to C14) in the ectodomain (19). One of these, the cysteine residue closest to the membrane anchor (C2, at amino acid 123), is involved in intermolecular disulfide bond formation (22, 23), while the other 12 cysteine residues are involved in intramolecular disulfide bond formation (22). In characterizing the role of individual cysteine residues in protein maturation, we found evidence suggesting that intramolecular disulfide bond formation might play a role in glycosylation site usage (22). We report here that mutation of either of two likely linked cysteine residues allows the usage of a normally unutilized glycosylation addition signal. Furthermore, inhibition of cotranslational disulfide bond formation on the nascent chain also allows the usage of that site in the wild-type protein. Results suggest that cotranslational disulfide bond formation blocks access of oligosaccharyl transferase to the addition site.

MATERIALS AND METHODS

Cells, vectors, and viruses. Cos-7 cells, obtained from the American Type Culture Collection, were maintained in Dulbecco's modified Eagle medium supplemented with nonessential amino acids, vitamins, glutamine, penicillin, streptomycin, and 10% fetal calf serum.

The NDV HN gene (25, 26) was expressed in Cos cells by using pSVL obtained from Pharmacia (32).

NDV strain AV was purified after growth in embryonated chicken eggs by standard protocols.

Antibodies. The antibody used to detect antigenically mature HN protein was an anti-NDV antibody raised in rabbits against UV-inactivated NDV virions (strain AV) by standard protocols (32). This antibody reacts with mature HN

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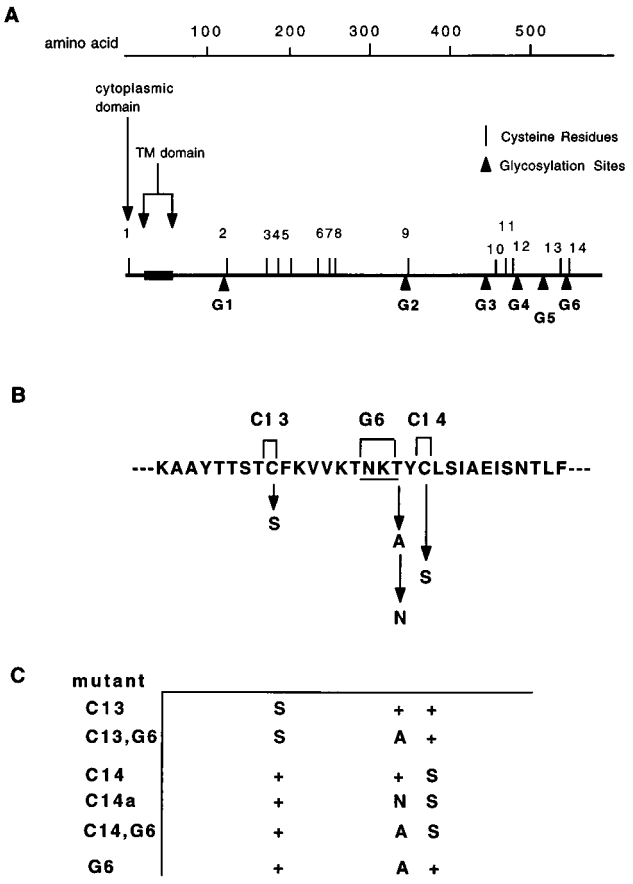

FIG. 1. Primary structure of the HN protein and mutants of the HN protein. (A) Linear diagram of the primary sequence of the HN glycoprotein, with the positions of the cysteine residues and glycosylation addition sites marked. TM, transmembrane. (B) Primary sequence in the region of cysteines 13 and 14. (C) Amino acid changes in the individual mutants. +, no change.

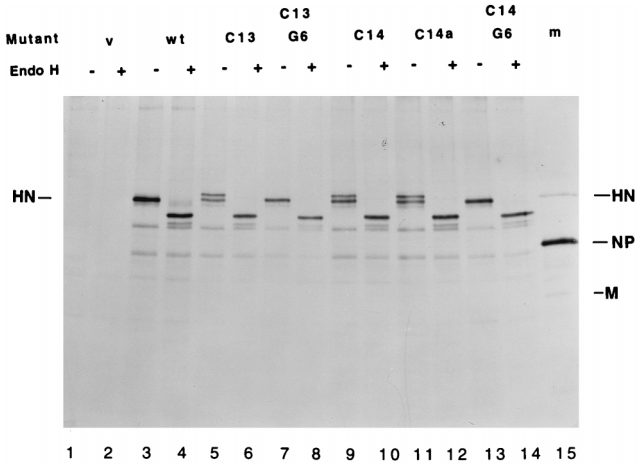

FIG. 2. Expression of wild-type and mutant proteins. Cos cells transfected with wild-type and mutant proteins were radioactively labeled with [^{35}S]methionine for 2 h at 48 h posttransfection. Proteins present in cell extracts were precipitated with a mix of anti-NDV, anti-AC, and anti-AS antibodies, and the resulting precipitated proteins were digested (+) or mock digested (−) with endo H and then electrophoresed on 10% polyacrylamide gels in the presence of reducing agent. Mutant DNAs are indicated at the top of the figure in lanes 5 through 14. v, vector-transfected cells; wt, wild-type DNA; m, marker proteins from NDV-infected cells; NP, nucleocapsid protein; M, membrane protein.

protein but not with nascent protein (22, 32). Monoclonal antibodies specific for the NDV HN protein were the generous gift of Ron Iorio. Antibodies used were anti-2b, anti-4a, and anti-14c (7–10). These monoclonal antibodies also react only to mature protein (20). The antibody used to detect the nascent or unfolded HN protein was a mix of rabbit antibody raised against the *Alu*I-to-*Sma*I fragment (amino acids 117 to 515) (anti-AS) of the HN gene expressed in bacteria as a TrpE fusion protein and anti-AC, a rabbit antibody raised against the *Alu*I-to-*Cla*I fragment (amino acids 117 to 268) of the HN protein expressed as a TrpE fusion protein (32).

Site-specific mutagenesis. Mutant genes were derived by using the Scultor mutagenesis kit (Amersham Co.). The appropriate oligomers, 15 to 18 nucleotides in length, were used in the mutagenesis reactions to make the changes shown in Fig. 1B and C. All mutant DNAs were sequenced in their entirety to verify that the rest of the gene was unchanged in the mutagenesis reactions.

Infections. Cos cells were infected with NDV at a multiplicity of infection of 15, as previously described (20). Labeling with [^{35}S]methionine was accomplished at 5 to 6 h posttransfection as previously described, and cell extracts were prepared as described below.

Transfections. Transfections using DEAE-dextran and chloroquine were done essentially as previously described (14, 32). Cos cells were incubated with a mix consisting of 0.25 ml of Tris-buffered saline (25 mM Tris, 136 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 [pH 7.4]), 1.25 μl of DEAE-dextran (100 mg/ml) in 0.15 M Tris-HCl (pH 7.5), and 1 μg of DNA for 30 min at 37°C. The DNA-dextran mix was replaced with chloroquine (100 μM final concentration) in 2 ml of OptiMem, and the cells were incubated for 4 to 5 h at 37°C. The chloroquine was replaced with 2 ml of Cos medium.

Radiolabeling and immunoprecipitation of protein. Transfected or infected cells were radiolabeled at 37°C in Dulbecco modified Eagle medium lacking methionine but containing 100 μCi of [^{35}S]methionine (Amersham) per ml. At the end of the labeling period, cells were washed in phosphate-buffered saline and lysed in reticulocyte standard buffer (0.01 M Tris-HCl [pH 7.4]–0.01 M NaCl) containing 1% Triton X-100, 0.5% sodium deoxycholate, 10 mM iodoacetamide, 10 mM *N*-ethylmaleimide, 40 mU of hexokinase, 20 mM glucose, and 2 mM $CaCl_2$ as previously described (18, 22, 28). Nuclei were removed by centrifugation. Immunoprecipitation of NDV proteins was accomplished as previously described (22, 27).

Endoglycosidase H digestion. Cells transfected with wild-type or mutant DNAs were radioactively labeled for 2 h and chased with nonradioactive medium for 2 h. HN proteins were precipitated with a mix of anti-AC/AS and anti-NDV antibodies. The precipitated protein was resuspended in buffer H (1% sodium dodecyl sulfate–0.150 M Tris-HCl [pH 7.5]) and boiled for 5 min. Phenylmethylsulfonyl fluoride (final concentration, 3 mM) and citrate buffer (pH 5.5; final concentration, 0.1 M) were added. Endoglycosidase H (endo H) (1.5 mU; Boehringer Mannheim) was added to half the sample, and the sample was incubated for 24 h at 37°C.

RESULTS

Glycosylation of the HN protein with mutations in cysteine residues. That intramolecular disulfide bond formation might influence glycosylation of the HN protein was suggested from properties of mutants with alterations in cysteine residues (22). The approximate locations of the 13 cysteine residues in the linear sequence of the ectodomain of the HN protein (19) are diagramed in Fig. 1A. Characterization of the expression of proteins with alterations in each of the cysteine residues revealed that mutation of either cysteine 13 or cysteine 14 (Fig. 1C) resulted in two polypeptides, one which comigrated with the wild-type protein and one which was approximately 3 kDa larger than the wild-type protein (22) (Fig. 2, lanes 5 and 9). Similar results were obtained with the mutant C14a, in which cysteine 14 was eliminated and the glycosylation addition site was moved slightly (Fig. 2, lane 11). That the increased molecular size of the larger species was due to glycosylation is shown by digestion of the proteins with endo H. Labeling conditions were such that the wild-type protein was a mixture of endo H-sensitive and -resistant forms and proteins were precipitated with a mix of antibodies specific for both mature and nascent protein. It has been previously shown that the mature form of the HN protein is partially resistant to endo H, thus migrating slightly faster than the undigested control (31) (Fig. 2, lane 4, top band). The C13 and C14 mutations block maturation of the protein (22); thus, the proteins contained no

TABLE 1. Quantitation of amounts of mutant HN protein glycosylated at site 6

DNA[a]	HNa[b]	HNb[b]	% of total HN glycosylated at site 6[c]
Expt 1			
C13S	37	55	59
C14S	64	66	51
Expt 2			
C13S	58	56	49
C14S	99	62	39
C14Sa	81	68	46
Expt 3			
C13S	49	65	57
C14S	91	71	44
C14a	71	58	56

[a] Experiments were performed with a mix of antibodies.
[b] Measured as densitometer units. HNa, HN glycosylated at sites G1 to G4; HNb, HN glycosylated at sites G1 to G4 and G6.
[c] Calculated as HNb/(HNa + HNb) × 100.

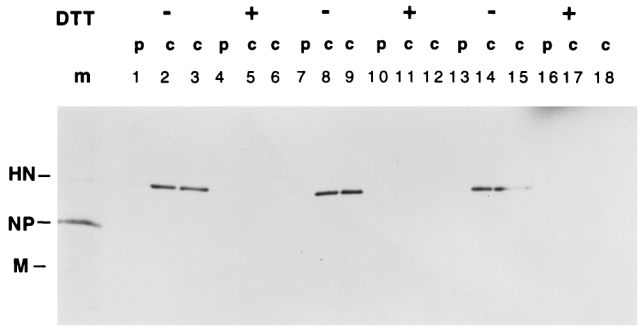

FIG. 3. Incubation of cells with DTT blocks folding of the HN protein. Cells infected with NDV were pulse-labeled (p) with [^{35}S]methionine for 5 min and then chased (c) in nonradioactive medium. Precipitates shown in lanes 1 to 3, 7 to 9, and 13 to 15 were from cells not treated with DTT (−), while precipitates shown in lanes 4 to 6, 10 to 12, and 16 to 18 were from cells treated with 5 mM DTT during both the pulse-labeling and the chase (+). Nonradioactive chases were for 1 h (lanes 2, 5, 8, 11, 14, and 17) or 2 h (lanes 3, 6, 9, 12, 15, and 18). Proteins present in the resulting cell extracts were precipitated with anti-4a (lanes 1 to 6), anti-14c (lanes 7 to 12), or anti-2b (lanes 13 to 18). The resulting precipitated proteins were electrophoresed in the presence of reducing agent on 10% polyacrylamide gels. m, marker proteins from infected cell extracts; NP, nucleocapsid protein; M, membrane protein.

partially resistant material. The endo H-sensitive forms of both mutant and wild-type proteins comigrated, as is shown in Fig. 2 (lanes 4, 6, 10, and 12). Thus, the increased molecular size of C13 and C14 mutant proteins was due to altered glycosylation.

The minor band migrating just below the undigested and digested HN protein was likely due to initiation of protein synthesis at a methionine located just within the transmembrane domain of the protein (17, 19). It is seen in reticulocyte cell-free translation product made in the absence of membranes (data not shown).

Glycosylation site usage in cysteine mutants. One interpretation of the altered glycosylation of the mutant HN protein is that elimination of either cysteine residue 13 or cysteine residue 14 allows the glycosylation of a normally unused glycosylation addition site. The HN protein of the AV strain of NDV has six glycosylation addition sites in its sequence (19), the positions of which are shown in Fig. 1A. Glycosylation addition sites 1, 2, 3, and 4 are used, while sites 5 and 6 are not used (17). One of the unused sites, G6, is located between cysteine residues 13 and 14 (Fig. 1A and B), suggesting that failure to form an intramolecular disulfide bond using cysteine 13 or 14 might make a normally inaccessible glycosylation site 6 accessible to oligosaccharyl transferase. To test this idea, double mutants were constructed which combined mutation of glycosylation site 6 with a mutation in either cysteine 13 or cysteine 14 (Fig. 1C). Both double mutants resulted in only one polypeptide, which comigrated with the wild-type protein (Fig. 2, lanes 7 and 13). Thus, the elimination of site 6 eliminated the larger-molecular-size species found in proteins with a mutation in cysteine 13 or 14.

Endo H digestion of these double mutants also resulted in a polypeptide which comigrated with endo H-digested wild-type protein (Fig. 2, lanes 8 and 14). This result shows that only the glycosylation of the C13 and C14 mutant proteins has been altered by the G6 mutation. Proteolytic cleavage cannot account for the decrease in molecular size of the protein.

The amount of total mutant HN protein synthesized that was glycosylated at site 6 ranged between 59 and 39% (Table 1). The C13 mutant protein consistently contained more glycosylation at site 6 than the C14 mutant protein, while the C14a mutant protein contained only slightly lower amounts than the C13 mutant protein.

Glycosylation site usage under reducing conditions. The results above suggest that failure to form intramolecular disulfide bonds using cysteine 13 or 14 resulted in the usage of a normally unused glycosylation addition site 6. Thus, if intramolecular disulfide bond formation is inhibited during the expression of the wild-type protein, it would be predicted that glycosylation site 6 would be utilized. It has been previously shown that treatment of cells with dithiothreitol (DTT) results in the synthesis of an unfolded protein without intramolecular disulfide bonds (3, 16, 33). Formation of intramolecular disulfide bonds and, therefore, folding of the molecule can occur only after removal of DTT (3, 16, 33).

To determine if DTT treatment of HN protein-expressing cells blocks intramolecular disulfide bond formation and, therefore, folding, monoclonal antibodies which react only with the mature, folded form of HN protein (20, 22) were used to precipitate proteins from DTT-treated and untreated cells. We have previously shown that representative monoclonal antibodies to six different sites on the HN protein do not react to nascent HN protein (20), as illustrated in Fig. 3, lanes 1, 7, and 13, for three of these antibodies. Reactivity appears as the protein matures (20) (Fig. 3, lanes 2, 3, 8, 9, 14, and 15). However, the HN protein synthesized in cells incubated in 5 mM DTT and chased in the presence of DTT remained antigenically immature and unreactive to the antibodies (Fig. 3, lanes 5, 6, 11, 12, 17, and 18). Infected cells were used in order to label significant amounts of protein in a 5-min pulse-labeling. Thus, DTT treatment of cells expressing the HN protein also blocked the maturation of this glycoprotein, a result consistent with inhibition of disulfide bond formation.

The protein synthesized in the presence of DTT and precipitated with antibody specific for nascent protein resolved into two bands (Fig. 4B, lane 2), while the wild-type protein similarly labeled in the absence of DTT migrated as a single band (Fig. 4A, lane 2). In several experiments, 26 to 27% of the total HN protein was found in a species which migrated with a molecular size approximately 3 kDa larger than that of protein made in the absence of DTT. The results presented above lead to the hypothesis that it was usage of site 6 that was responsible for the higher-molecular-size polypeptide seen in DTT-treated cells. To explore this hypothesis, proteins mutated in each of

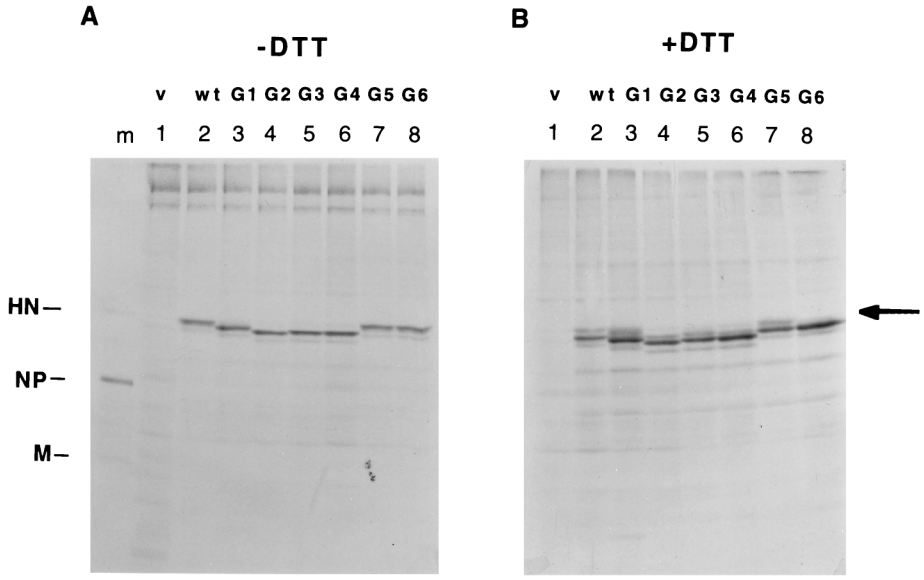

FIG. 4. Electrophoresis of nascent wild-type and mutant proteins made in the presence or absence of DTT. Cos cells transfected with wild-type (lane 2) or mutant (lanes 3 to 8) DNAs were radioactively labeled with [^{35}S]methionine for 1 h in the absence (A) or presence (B) of 5 mM DTT. Proteins present in the resulting cell extracts were precipitated with a mix of anti-AC and anti-AS antibodies, and the resulting precipitated protein was electrophoresed on polyacrylamide gels in the presence of reducing agent. Panel A was exposed to film for 1 day, and panel B was exposed for 2 days to compensate for the reduction in incorporation due to DTT (3, 16, 33). m, marker proteins present in total infected cell extracts; v, vector-transfected cells; wt, wild-type DNA; NP, nucleocapsid protein; M, membrane protein. The arrow indicates the polypeptide glycosylated at site 6. As previously described (17), elimination of carbohydrate at the G1 position results in a protein which migrates slightly slower than protein missing either the G2, the G3, or the G4 oligosaccharide but faster than the fully glycosylated protein.

the six glycosylation addition sites were synthesized in the presence of DTT and then precipitated with antibody specific for immature protein. Clearly, all expressed proteins resolved into two species, except the protein with a mutation in site 6 (Fig. 4B, lane 8), showing that it was indeed utilization of site 6 that accounted for the additional carbohydrate in the presence of DTT. Thus, prevention of intramolecular disulfide bond formation by DTT, as well as by mutation, resulted in the usage of the normally unused glycosylation site 6.

Interestingly, these results show that inhibition of intramolecular disulfide bond formation with DTT did not appear to increase the usage of the G5 site. There was no species of HN protein made in the presence of DTT that was eliminated by mutating the G5 addition site.

Folding of the HN protein glycosylated at site 6. Previous characterization of proteins with mutations in cysteine 13 or 14 showed that these proteins failed to fold properly (22). It seemed possible that glycosylation at site 6 was at least partially responsible for this folding defect. We therefore determined if removal of glycosylation site 6 facilitated the folding of C13 or C14 mutant proteins. Folding was monitored by reactivity of the proteins to conformationally sensitive monoclonal antibodies (20) (Fig. 5). As previously reported, mutants C13 and C14 failed to acquire mature antigenic sites (Fig. 5, lanes 3 and 5). The elimination of site 6 in these mutants did not restore the ability of these mutants to fold, since the double mutants C13,G6 and C14,G6 also failed to acquire mature antigenic determinants (Fig. 5, lanes 4 and 6). Failure to fold could not be attributed to the mutation in site 6, since a protein defective only in site 6 did acquire mature antigenic sites (Fig. 5, lane 7). Thus, it is likely that the failure to form the disulfide bond, rather than the secondary effect of glycosylation, is inhibitory to maturation. This conclusion is supported by the observation (Fig. 2) that the double mutants do not acquire endo H partial resistance, as does the wild-type protein.

The effect of glycosylation at site 6 on maturation of the wild-type protein was explored by characterizing the antigenic sites formed on the protein synthesized in the presence of DTT and chased in the absence of DTT to allow posttranslational disulfide bond formation. In material precipitated by four different conformationally sensitive monoclonal antibodies, a double band was resolved, suggesting that the protein species glycosylated at site 6 formed antigenic sites, as did the faster-migrating protein not glycosylated at this position (Fig. 5, lane 9). Table 2 shows a quantitation of the amount of material present in both species after precipitation with different antibodies. The ratio of protein glycosylated at site 6 to protein not glycosylated in this position was similar for all antibodies. However, in another study (21), we have shown that while the HN protein synthesized in the presence of DTT and then allowed to fold after removal of DTT acquires conformationally sensitive antigenic sites, the protein is folded in a different sequence and shows no biological activity (21). This result suggests that the conformation of the protein folded after removal of DTT is abnormal. This abnormal folding cannot, however, be attributed to glycosylation at site 6, since HN protein not glycosylated at site 6 is abnormally folded.

DISCUSSION

The results presented above show that a normally unused glycosylation addition site in the sequence of the HN protein of NDV is likely not used because intramolecular disulfide bond formation in the vicinity of the site interferes with the addition of the oligosaccharide. Usage of the site can occur when either of two nearby cysteine residues is mutated or by inhibition of cotranslational disulfide bond formation. Mutation of either cysteine residue resulted in, on average, approximately 50% of the protein being glycosylated at site 6, while inhibition of cotranslational disulfide bond formation resulted in usage of the site in 26 to 27% of the molecules.

The intramolecular disulfide bonds in paramyxovirus attach-

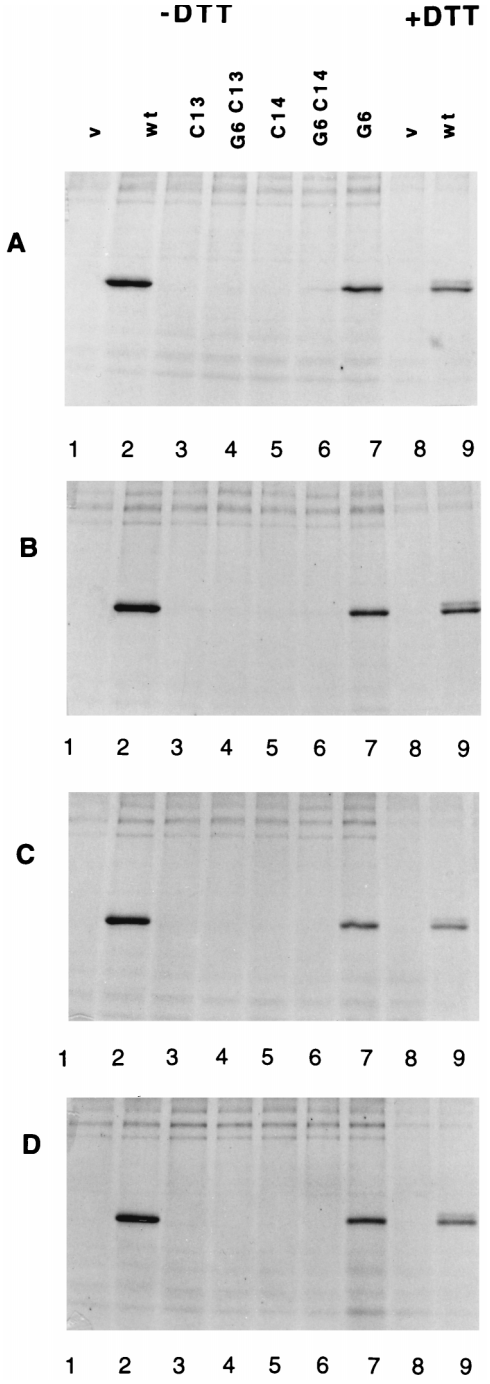

FIG. 5. Maturation of mutant and wild-type polypeptides. Cos cells transfected with wild-type or mutant DNAs were radioactively labeled with [^{35}S]methionine for 15 min in the absence (lanes 1 to 6) or presence (lanes 8 and 9) of DTT and then chased for 2 h in nonradioactive medium without DTT. Proteins present in the resulting cell extracts were precipitated with anti-4a (A), anti-14c (B), anti-3a (C), and anti-2b (D). v, vector-transfected cells; wt, wild-type DNA.

ment proteins have not yet been defined. However, based on comparisons of sequences, Colman et al. (5) have proposed that this family of proteins is folded in much the same way as the influenza neuraminidase glycoprotein, a protein whose crystal structure and intramolecular disulfide bonds have been defined (4). Based on the comparison, Colman et al. have

TABLE 2. Quantitation of amounts of wild-type HN protein glycosylated at site 6 in the presence of DTT

Antibody	HNa[a]	HNb[a]	% of total HN glycosylated at site 6[b]
Expt 1, anti-AC/AS	108	40	27
Expt 2, anti-AC/AS	75	27	26
Expt 3			
Anti-4	61	18	22
Anti-14	84	26	24
Anti-3	43	13	23
Anti-2	61	18	23

[a] Measured as densitometer units. HNa, HN glycosylated at sites G1 to G4; HNb, HN glycosylated at sites G1 to G4 and G6.
[b] Calculated as HNb/(HNa + HNb) × 100.

proposed that cysteine residues 13 and 14 are linked (5). Indeed, we have shown that cysteine 13 and cysteine 14 mutants have similar phenotypes, which are different from those of proteins with mutations in other cysteine residues (22), a finding which strongly supports the idea that these two residues are linked.

Linkage of cysteines 13 and 14 would provide an explanation for their role in the usage of glycosylation site 6. It has been shown by Nilsson and von Heijne (29) that oligosaccharyl transferase can transfer oligosaccharides to nascent polypeptide chains only after the addition site is 30 to 40 Å from the RER membrane or after 12 to 14 amino acids carboxy-terminal to the site have emerged into the lumen of the RER, as diagramed in Fig. 6A. Given these results, folding events in the region of a glycosylation site which occur on the nascent chain before the site is 12 to 14 amino acids from the RER membrane might have the potential to block oligosaccharide addition. Intramolecular disulfide bond formation might be such a folding event. Indeed, cysteine 14 is only 4 amino acids from site 6. If cysteines 13 and 14 link immediately after cysteine 14 has emerged from the translocation channel, the linkage would occur prior to accessibility of the glycosylation site to the transferase, as diagramed in Fig. 6C. Formation of the intramolecular disulfide bond would serve to sequester the site, preventing its usage (Fig. 6D). Indeed, we have previously reported that mutations in cysteine 13 or 14 block maturation of the HN protein very early in a folding sequence (22), results consistent with cotranslational formation of this disulfide bond.

There are reports in the literature suggesting that intramolecular disulfide bond formation may influence glycosylation site usage in other proteins. Kane suggested that an unused site in procathepsin L is not used because of a nearby intramolecular disulfide bond (11). Allen et al. (1) showed that an inefficiently used glycosylation addition site in plasminogen activator is more efficiently used if intramolecular disulfide bond formation is inhibited with DTT. A survey of the literature, however, makes it clear that the restriction of glycosylation site usage cannot always be attributed to disulfide bond formation. There are examples of unused sites very distant from cysteine residues. Indeed, glycosylation site 5 in the HN sequence is not located near cysteine residues, nor was this site used if intramolecular disulfide bond formation was inhibited. Thus, a more general hypothesis for the failure to use sites is that localized folding events on the nascent chain which block access of the oligosaccharyl transferase to the site are a determinant of glycosylation site usage. Disulfide bond formation may be only one such folding event. For example, Altmann et

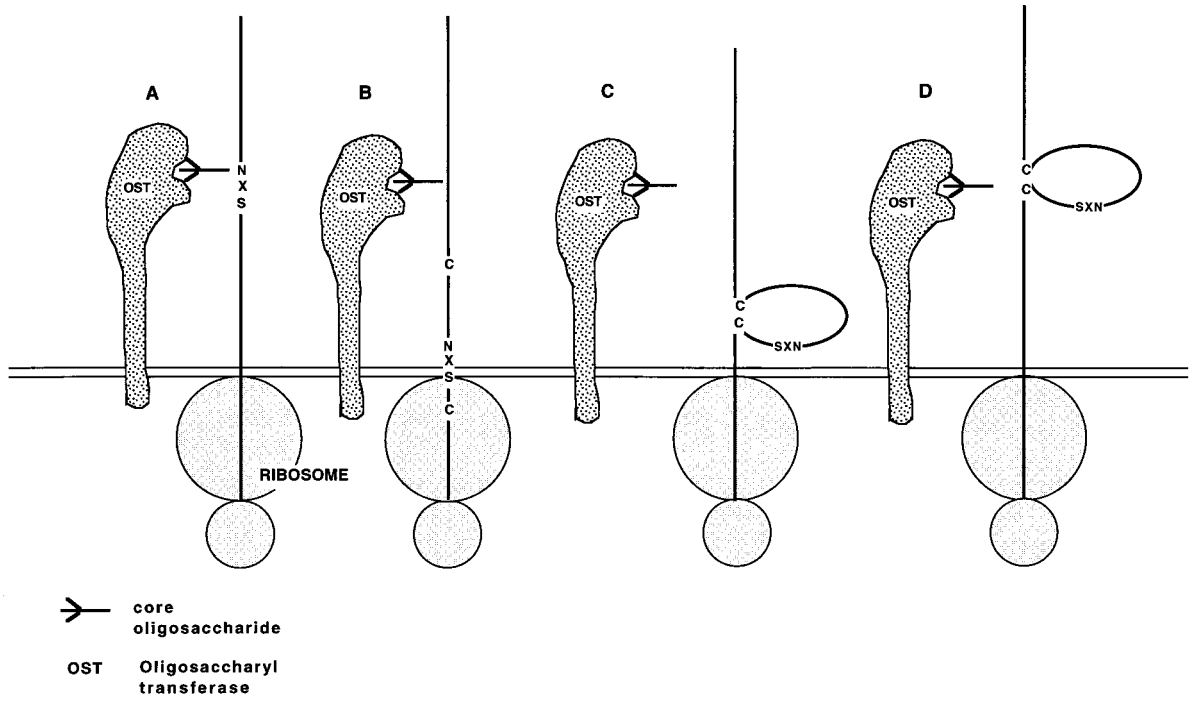

FIG. 6. Proposed mechanism for failure to use glycosylation site 6. (A) The active site of oligosaccharyl transferase (OST) is positioned such that the glycosylation addition site is recognized only when 12 to 13 amino acids have emerged from the translocation pore, as proposed by Nilsson and von Heijne (29). (B) Cysteine 13 and then glycosylation site 6 emerge from the translocation pore, followed by cysteine 14. (C) Upon the appearance of cysteine 14, a disulfide bond forms. (D) Translocation proceeds, but folding blocks access of the oligosaccharyl transferase to the glycosylation addition site.

al. (2) showed that introduction of prolines into predicted α helices resulted in hyperglycosylation of murine granulocyte-macrophage colony-stimulating factor and speculated that a more relaxed conformation of a nascent chain allows greater exposure of Asn residues to oligosaccharyl transferase.

Unused glycosylation sites may also be located in specific regions of the nascent chain which interact with other ER proteins, blocking access to oligosaccharyl transferase. Livi et al. (15) have reported that a glycosylation addition site close to the signal sequence cleavage site is not used and suggest that signal peptidase can block access of oligosaccharyl transferase to the amino terminus of the nascent chain. Kim and Cunningham (12) have shown that the murine retrovirus Env protein can bind to its receptor molecule in the RER, preventing glycosylation at a specific site.

Inhibition of the disulfide bond by synthesis of protein in the presence of DTT or by mutation resulted in the usage of site 6 in approximately 26 to 59%, respectively, of the molecules made in the cell. Clearly, the site was not used at 100% efficiency under either of these conditions. Thus, it is likely that there is some folding of this region of the nascent chain, independent of disulfide bond formation, which decreases the efficiency of glycosylation at site 6. Indeed, this region must be folded in such a way as to place the cysteine residues in proximity. This folding may decrease the accessibility of the site to oligosaccharyl transferase, resulting in less than 100% efficiency of usage. Consistently, the site was used more efficiently in mutant proteins than in wild-type protein made in the presence of DTT. Perhaps single amino acid changes present in the mutants affect the folding in this region of the molecule, increasing the accessibility of the site to oligosaccharyl transferase.

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REFERENCES

1. **Allen, S., H. Y. Naim, and N. J. Bulleid.** 1994. Intracellular folding of tissue-type plasminogen activator. J. Biol. Chem. **270:**4797–4804.
2. **Altmann, S. W., G. D. Johnson, and M. B. Prystowky.** 1991. Single proline substitutions in predicted α helices of murine granulocyte-macrophage colony stimulating factor result in a loss of bioactivity and altered glycosylation. J. Biol. Chem. **266:**5333–5341.
3. **Braakman, I., J. Helenius, and A. Helenius.** 1992. Manipulating disulfide bond formation and protein folding in the endoplasmic reticulum. EMBO J. **11:**1717–1722.
4. **Colman, P. M.** 1989. Influenza virus neuraminidase: enzyme and antigen, p. 175–218. *In* R. M. Krug (ed.), The influenza viruses. Plenum Publishing Corp., New York, N.Y.
5. **Colman, P. M., P. A. Hoyne, and M. C. Lawrence.** 1993. Sequence and structure alignment of paramyxovirus hemagglutinin-neuraminidase with influenza virus neuraminidase. J. Virol. **67:**2972–2980.
6. **Gilmore, R.** 1993. Protein translocation across the endoplasmic reticulum: a tunnel with toll booths at entry and exit. Cell **75:**589–592.
7. **Iorio, R. M., R. L. Glickman, A. M. Riel, J. P. Sheehan, and M. A. Bratt.** 1989. Functional and neutralization profile of seven overlapping antigenic sites on the HN glycoprotein of Newcastle disease virus: monoclonal antibodies to some sites prevent viral attachment. Virus Res. **13:**245–262.
8. **Iorio, R. M., R. L. Glickman, A. M. Riel, J. P. Sheehan, and M. A. Bratt.** 1989. Identification of amino acid residues important to the neuraminidase activity of the glycoprotein of Newcastle disease virus. Virology **173:**196–204.
9. **Iorio, R. M., R. L. Glickman, and J. P. Sheehan.** 1992. Inhibition of fusion by neutralizing monoclonal antibodies of the hemagglutinin-neuraminidase glycoprotein of Newcastle disease virus. J. Gen. Virol. **73:**1167–1176.
10. **Iorio, R. M., R. J. Syddall, R. L. Glickman, A. M. Riel, J. P. Sheehan, and M. A. Bratt.** 1991. Neutralization map of the hemagglutinin-neuraminidase glycoprotein of Newcastle disease virus: domains recognized by monoclonal antibodies that prevent receptor recognition. J. Virol. **65:**4999–5006.
11. **Kane, S. E.** 1993. Mouse procathepsin L lacking a functional glycosylation

site is properly folded, stable, and secreted by NIH 3T3 cells. J. Biol. Chem. **268:**11456–11462.
12. **Kim, J. W., and J. M. Cunningham.** 1993. N linked glycosylation of receptor for murine ecotropic retrovirus is altered in virus infected cells. J. Biol. Chem. **268:**16316–16320.
13. **Kornfeld, R., and S. Kornfeld.** 1985. Assembly of asparagine-linked oligosaccharides. Annu. Rev. Biochem. **54:**631–664.
14. **Levesque, J.-P., P. Sanilvestri, A. Hatzfeld, and J. Hatzfeld.** 1991. DNA transfection in Cos cells: a low cost serum free method compared to Lipofectin. BioTechniques **11:**313–318.
15. **Livi, G. P., J. S. Lillquist, L. M. Miles, A. Ferrara, G. M. Sathe, P. L. Simon, C. A. Meyers, J. A. Gorman, and P. R. Young.** 1991. Secretion of N-glycosylated interleukin-1β in *Saccharomyces cerevisiae* using a leader peptide from *Candida albicans*. J. Biol. Chem. **266:**15348–15355.
16. **Lodish, H. F., and N. Kong.** 1993. The secretory pathway is normal in dithiothreitol-treated cells, but disulfide-bonded proteins are reduced and reversibly retained in the endoplasmic reticulum. J. Biol. Chem. **268:**20598–20605.
17. **McGinnes, L., and T. Morrison.** 1995. Role of individual oligosaccharide chains in the maturation and activities of the HN protein of NDV. Virology **212:**398–410.
18. **McGinnes, L., T. Sergel, and T. G. Morrison.** 1993. Mutations in the transmembrane domain of the HN protein of Newcastle disease virus affect the structure and activity of the protein. Virology **196:**101–110.
19. **McGinnes, L., A. Wilde, and T. Morrison.** 1987. Nucleotide sequence of the gene encoding the Newcastle disease virus hemagglutinin-neuraminidase protein and comparisons of paramyxovirus hemagglutinin-neuraminidase sequences. Virus Res. **7:**187–202.
20. **McGinnes, L. W., and T. G. Morrison.** 1994. Conformationally sensitive antigenic determinants on the HN glycoprotein of Newcastle disease virus form with different kinetics. Virology **199:**255–264.
21. **McGinnes, L. W., and T. G. Morrison.** 1996. Role of cotranslational disulfide bond formation in the folding of the hemagglutinin-neuraminidase protein of Newcastle disease virus. Virology **224:**465–476.
22. **McGinnes, L. W., and T. G. Morrison.** 1994. The role of the individual cysteine residues in the formation of the mature, antigenic HN protein of Newcastle disease virus. Virology **200:**470–483.
23. **Mirza, A. M., J. P. Sheehan, L. W. Hardy, R. L. Glickman, and R. M. Iorio.** 1993. Structure and function of a membrane anchor-less form of the hemagglutinin-neuraminidase glycoprotein of Newcastle disease virus. J. Biol. Chem. **268:**21425–21431.
24. **Morrison, T., and A. Portner.** 1991. Structure, function, and intracellular processing of the glycoproteins of *Paramyxoviridae*, p. 347–382. *In* D. Kingsbury (ed.), The paramyxoviruses. Plenum Press, New York, N.Y.
25. **Morrison, T. G., and L. W. McGinnes.** 1989. Avian cells expressing the Newcastle disease virus HN protein are resistant to NDV infection. Virology **171:**10–17.
26. **Morrison, T. G., C. McQuain, and L. McGinnes.** 1991. Complementation between avirulent Newcastle disease virus and a fusion protein expressed from a retrovirus vector: requirements for membrane fusion. J. Virol. **65:**813–822.
27. **Morrison, T. G., C. McQuain, K. F. O'Connell, and L. W. McGinnes.** 1990. Mature, cell-associated HN protein of Newcastle disease virus exists in two forms differentiated by posttranslational modifications. Virus Res. **15:**113–134.
28. **Morrison, T. G., L. Ward, and A. Semerjian.** 1985. Intracellular processing of the Newcastle disease virus fusion glycoprotein. J. Virol. **53:**851–857.
29. **Nilsson, I., and G. von Heijne.** 1993. Determination of the distance between the oligosaccharyltransferase active site and the endoplasmic reticulum membrane. J. Biol. Chem. **268:**5798–5801.
30. **Rademacher, T. W., R. B. Parekh, and R. A. Dwek.** 1988. Glycobiology. Annu. Rev. Biochem. **57:**786–830.
31. **Schwalbe, J. C., and L. E. Hightower.** 1982. Maturation of the envelope glycoproteins of Newcastle disease virus on cellular membranes. J. Virol. **41:**947–957.
32. **Sergel, T., L. W. McGinnes, M. E. Peeples, and T. G. Morrison.** 1993. The attachment function of the Newcastle disease virus hemagglutinin-neuraminidase protein can be separated from fusion promotion by mutation. Virology **193:**717–726.
33. **Tatu, U., I. Braakman, and A. Helenius.** 1993. Membrane glycoprotein folding, oligomerization, and intracellular transport: effects of dithiothreitol in living cells. EMBO J. **12:**2151–2157.