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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Amino acid changes in the individual mutants.

(B) Primary sequence in the region of cysteines 13 and 14.

(C) Positions of the cysteine residues and glycosylation addition sites marked. TM, transmembrane. (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.

**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 [35S]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.

**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

<table>
<thead>
<tr>
<th>DNAa</th>
<th>HNa</th>
<th>HNb</th>
<th>% of total HN glycosylated at site 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt 1</td>
<td>C13S</td>
<td>37</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>C14S</td>
<td>64</td>
<td>66</td>
</tr>
<tr>
<td>Expt 2</td>
<td>C13S</td>
<td>58</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>C14S</td>
<td>99</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>C14Sa</td>
<td>81</td>
<td>68</td>
</tr>
<tr>
<td>Expt 3</td>
<td>C13S</td>
<td>49</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>C14S</td>
<td>91</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>C14a</td>
<td>71</td>
<td>58</td>
</tr>
</tbody>
</table>

* Calculated as HNb/(HNb + HNa) × 100.

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 the candidate cysteines were expressed and their use of glycosylation site 6 analyzed.
the six glycosylation addition sites were synthesized in the presence of DTT and then precipitated with antibodies 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-
ment proteins have not yet been defined. However, based on comparisons of sequences, Colman et al. (5) have 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 diagrammed 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 diagrammed 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 to 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

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

<table>
<thead>
<tr>
<th>Antibody</th>
<th>HNa</th>
<th>HNb</th>
<th>% of total HN glycosylated at site 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt 1, anti-AC/AS</td>
<td>108</td>
<td>40</td>
<td>27</td>
</tr>
<tr>
<td>Expt 2, anti-AC/AS</td>
<td>75</td>
<td>27</td>
<td>26</td>
</tr>
<tr>
<td>Expt 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-4</td>
<td>61</td>
<td>18</td>
<td>22</td>
</tr>
<tr>
<td>Anti-14</td>
<td>84</td>
<td>26</td>
<td>24</td>
</tr>
<tr>
<td>Anti-3</td>
<td>43</td>
<td>13</td>
<td>23</td>
</tr>
<tr>
<td>Anti-2</td>
<td>61</td>
<td>18</td>
<td>23</td>
</tr>
</tbody>
</table>

*Measured as densitometer units. HNa, HN glycosylated at sites G1 to G4; HNb, HN glycosylated at sites G1 to G4 and G6.

*Calculated as HNb/(HNa + HNb) × 100.
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. Liviet 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.

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.

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site is properly folded, stable, and secreted by NIH 3T3 cells. J. Biol. Chem. 268:11456–11462.


