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Glycosylation affects cleavage of an H5N2 influenza virus hemagglutinin and regulates virulence

(mutation/asparagine-linked carbohydrate/A/chicken/Pennsylvania/1/83 influenza virus)

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ABSTRACT **Based on nucleotide sequence analysis of the hemagglutinin (HA) gene from the virulent and avirulent A/chicken/Pennsylvania/83 influeza viruses, it was previously postulated that acquisition of virulence was associated with a point mutation that resulted in loss of a glycosylation site. Since there are two potential glycosylation sites in this region of the HA molecule and since all Asn-Xaa-Thr/Ser sequences in the HAs of different strains are not necessarily glycosylated, the question remained open as to whether either one of these sites was glycosylated. We now provide direct evidence that a site-specific glycosylation affects cleavage of the influenza virus HA and thus virulence. We have identified the glycosylation sites on the HA1 subunit from the virulent and avirulent strains by direct structural analysis of the isolated proteins. Our results show that the only difference in glycosylation between the HA1s of the virulent and avirulent strains is the lack of an asparagine-linked carbohydrate on the virulent HA1 polypeptide at residue 11. Further, we show that the HA1s of both the avirulent and virulent viruses are not glycosylated at one potential site, while all other sites contain carbohydrate. Amino acid sequence analysis of the HA1 of an avirulent revertant of the virulent strain confirmed these findings.**

In April 1983, an avirulent (avir) influenza virus, A/chicken/Pennsylvania/1/83 (Chick/Penn) (H5N2), appeared in chickens of eastern Pennsylvania (1). By October 1983, this virus had become virulent (vir), causing up to 80% mortality in domestic poultry (1, 2). Gene reassortment studies established that the difference between the viruses was a mutation of the hemagglutinin (HA) (3), a major surface glycoprotein responsible for viral attachment to, and penetration of, host cells. Nucleotide sequencing analysis suggested that the critical mutation eliminated a possible site for the attachment of an N-linked oligosaccharide at Asn-11 of HA1 of the avirulent virus by altering a sequence for glycosylation. Preliminary evidence based on molecular weight differences in HA1 and growth in the presence of tunicamycin suggested loss of a carbohydrate (4). However, since not all Asn-Xaa-Ser/Thr sites are glycosylated in different influenza virus HAs, we do not know how many of the potential glycosylation sites contain carbohydrate, and since all of the influenza virus HAs studied to date contain a carbohydrate at this location in the HA molecule (5, 6), the question of glycosylation at this site remained unresolved.

The HA protein of influenza viruses is post-translationally modified by cleavage at a connecting peptide region into subunits HA1 and HA2 (7). This cleavage is a prerequisite for viral infectivity (8), and a correlation has been drawn between HA cleavage and virus production in tissue culture and the virulence of the strain (9–11). Although exogenously added trypsin can cleave the HA and permit infectivity *in vitro*, the mechanism by which HA is processed and cleaved *in vivo* is unknown.

To determine if altered glycosylation of the Chick/Penn HA was sufficient to explain the differences between the avirulent and virulent strains, we have identified the sites of attachment of the N-linked oligosaccharides in HA1 (vir) and HA1 (avir). Analysis of tryptic fragments by HPLC and amino-terminal sequence determination have established that the only difference in glycosylation between HA1 (vir) and HA1 (avir) is the absence of an N-linked oligosaccharide at Asn-11 in HA1 (vir). This result is a direct demonstration that a site-specific glycosylation can regulate the processing of HA and thus the virulence of a virus.

MATERIALS AND METHODS

Viruses and Viral Proteins. The avirulent A/chicken/Pennsylvania/1/83 (H5N2), highly virulent A/chicken/Pennsylvania/1370/83 (H5N2) influenza virus, and reassortants have been described (1, 3). The viruses used were handled in a P3 containment laboratory that was approved by the U.S. Department of Agriculture. Viruses were grown in 11-day-old embryonated chicken eggs and were purified by differential sedimentation through a 25–70% sucrose gradient in a Beckman SW 28 rotor. The viruses were disrupted in 1.5% *n*-octyl β-D-glucopyranoside, and viral membrane proteins were isolated from the supernatant by centrifugation in a Beckman SW 55 rotor for 2 hr at 50,000 rpm.

Purification of HA Subunits. Preparations of the viral membrane proteins were dialyzed against 25 mM Tris/glycine buffer, pH 8.3, containing 1% $NaDodSO_4$, concentrated, then reduced and alkylated (12) before electrophoresis in $NaDodSO_4$/10% polyacrylamide gels (13). The HA1 and HA2 proteins were visualized by Coomassie brilliant blue staining, excised from the gel, and electroeluted (14). The proteins were judged to be greater than 99% pure when analyzed by $NaDodSO_4$/PAGE by using Coomassie brilliant blue for detection (Fig. 1).

Enzymatic Digests and HPLC Analysis of Tryptic Fragments. The concentration of protein in the electroeluted fractions was determined either by comparison to known amounts of protein standards subjected to $NaDodSO_4$/PAGE and stained with Coomassie blue (15) or by fluorescent protein assay with bovine serum albumin as standard (16). Enzymatic digestions were performed on aliquots of the electroeluted samples containing approximately 100 μg of protein. These samples were precipitated by the method of Wessel and Flugge (17) before additional manipulation. For deglycosylation, the precipitated protein was resuspended in 50 μl of 0.5% $LiDodSO_4$/10 mM dithiothreitol and heated at 70°C for 30 min. Ninety microliters of 0.25 M sodium

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Abbreviations: avir, avirulent; vir, virulent; rev, revertant; HA, hemagglutinin; Chick/Penn, A/chicken/Pennsylvania/1/83; FPV, A/FPV/Rostock/34.

phosphate, pH 8.6/10 mM EDTA was added at ambient temperature followed by 10 μl of 10% Nonidet P-40 (NP-40). The sample was incubated with 5 units (as defined by the supplier) of *N*-glycanase (peptide *N*-glycosidase F; Genzyme, Norwalk, CT) at 30°C for 18 hr. Control samples were incubations without *N*-glycanase. The incubation conditions appear adequate to completely deglycosylate the protein, as only one sharp band was observed on $NaDodSO_4$/PAGE and further incubation with additional *N*-glycanase did not increase the protein mobility (data not shown). Analysis of the isolated glycopeptides, presented in *Results*, established that deglycosylation was complete. Samples were reprecipitated as above and aliquots were either sequenced directly or digested with trypsin. Trypsin digestions were performed in a 0.05 M NH_4HCO_3, pH 8.2, buffer system with a sample-to-trypsin mass ratio of 25:1. The digests were fractionated on a reverse-phase C_{18} column (120T-TSK, obtained from LKB) developed with a gradient of acetonitrile in 0.1% trifluroacetic acid. High-performance chromatography was performed on an LKB pump/controller system using a Kratos 783 variable wavelength detector. Peptides were automatically collected with the peak detector feature of the LKB SuperRac fraction collector.

Amino-Terminal Sequence Analysis. Protein or peptide samples were sequenced by automated Edman degradation on a 470A gas-phase sequencer (Applied Biosystems, Foster City, CA) using the 02NVAC program and methanolic HCl conversion chemistry. The phenylthiohydantoin derivatives of the residues were identified and quantitated by a Cyano column (IBM) HPLC procedure similar to the one described by Hunkapiller and Hood (18). The phenylthiohydantoin derivatives of aspartic and glutamic residues were identified directly as their methyl esters. Total net yields were calculated for each cycle, after correction for background and lag values, which were usually less than 1%. Repetitive yields were calculated by linear regression analysis of all cycles except those yielding serine, threonine, histidine, and cysteine.

RESULTS

The HA1 Protein of the Avirulent Strain Is More Extensively Glycosylated than Is HA1 of the Virulent Strain. Chick/Penn HA1 (avir) has a slower mobility than does the HA1 (vir) when compared by $NaDodSO_4$/PAGE (4). This difference, equivalent to approximately 3 kDa, is illustrated in Fig. 1, which shows the purified HA1 proteins of the avirulent and virulent strains in lanes 2 and 4, respectively. Digestion with *N*-glycanase eliminated this difference in mobility (Fig. 1, lanes 1 and 3), strongly suggesting that these HA1 forms differ by the number of N-linked oligosaccharide chains. The minor difference in mobility between the polypeptides deglycosylated with *N*-glycanase is due to a net charge difference of 2 units between these molecules. These charge differences lead to anomalous mobility on $NaDodSO_4$ gels (V.A.F., unpublished results).

The HA1 Protein of the Avirulent Strain Is Glycosylated at Residue Asn-11 Whereas HA1 of the Virulent Strain Is Not. Nucleotide sequence analysis of the HA genes from the virulent and avirulent viruses suggested that the predicted amino acid change, at residue 13, eliminated a potential glycosylation site at Asn-11 on HA1 (vir) by replacing a Thr with a Lys (4). However, the nucleotide sequence of this region also indicates a possible glycosylation site at Asn-10 for the HA1s of both viruses. To determine the actual glycosylation at either site, the *N*-glycanase-digested HA1 proteins were sequenced through this region, starting from the amino terminus of the intact proteins. The analysis and observed sequence is shown in Table 1. The consequence of deglycosylation by *N*-glycanase is the conversion of the

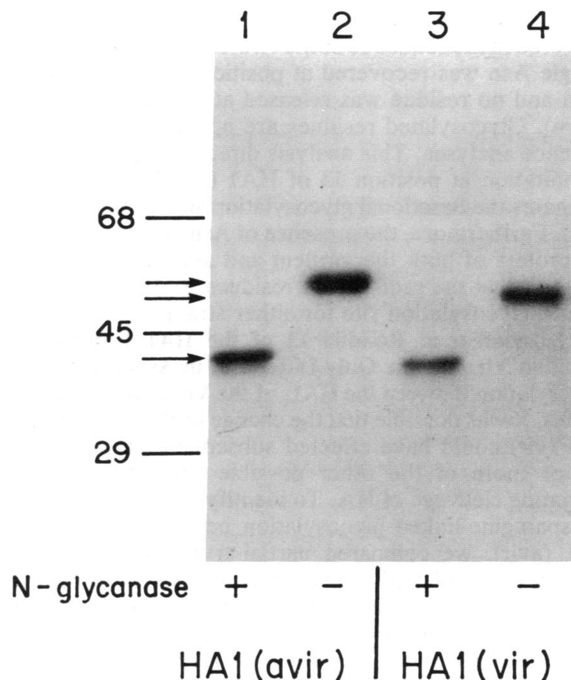

FIG. 1. The apparent mass difference between HA1 (vir) and HA1 (avir) is due to the content of N-linked oligosaccharide. HA1 (vir) and HA1 (avir) were purified by electroelution from $NaDodSO_4$/polyacrylamide gels and samples were digested by *N*-glycanase. Digested and control samples were analyzed on $NaDodSO_4$/10–20% polyacrylamide gradient gels stained with Coomassie blue. Lanes: 1, HA1 (avir) digested with *N*-glycanase; 2, untreated HA1 (avir); 3, HA1 (vir) digested with *N*-glycanase; 4, untreated HA1 (vir). Standards were run in adjacent lanes but are not shown; their masses in kDa are given on the left.

glycosylated Asn to an Asp residue at that position in the sequence (19). The amino-terminal sequences demonstrated that neither Asn-10 nor Asn-11 is glycosylated on the HA1 (vir) protein, since Asn was recovered at both positions. In contrast, Asp was recovered only at position 11 in HA1 (avir), demonstrating that this position is glycosylated. Amino-terminal sequence analysis of the glycosylated HA1

Table 1. Partial amino-terminal sequence of deglycosylated HA1 of virulent and avirulent strains

	HA1 (avir)		HA1 (vir)		HA1 (rev)	
Cycle	Amino acid	Yield, pmol	Amino acid	Yield, pmol	Amino acid	Yield, pmol
1	Asp	35	Asp	59	Asp	270
2	Gln	17	Gln	32	Gln	174
3	Ile	23	Ile	47	Ile	249
4	Cys	ND	Cys	ND	Cys	ND
5	Ile	15	Ile	39	Ile	227
6	Gly	11	Gly	31	Gly	162
7	Tyr	14	Tyr	36	Tyr	208
8	His	ND	His	22	His	125
9	Ala	16	Ala	27	Ala	222
10	Asn	11	Asn	17	Asn	159
11	Asp*	12	Asn	21	Asp*	164
	Asn	<0.5	Asp	<0.5	Asn	<1
12					Ser	29
13					Thr	67

The observed residues are as predicted from the nucleotide sequence except position 11, where Asp* appears after deglycosylation of Asn in that position. Both Asn and Asp values are presented for residue 11 to demonstrate the point made in the text. Repetitive yield is 93%. ND, not quantitated.

proteins confirmed this result, since both Asn residues were recovered in the sequence of the virulent strain, whereas only a single Asn was recovered at position 10 for the avirulent strain and no residue was released at position 11 (data not shown). Glycosylated residues are not detected directly in sequence analyses. This analysis directly demonstrates that the mutation at position 13 of HA1 (vir) from Thr to Lys eliminates the functional glycosylation site at Asn-11 of HA1 (avir). Furthermore, the presence of Asn at position 10 in the HA protein of both the virulent and avirulent strains demonstrates that the sequence at residues 10–12, Asn-Asn-Ser, is not a glycosylation site for either strain.

Glycosylation at Residue 11 of the HA1 Protein of the Avirulent Virus Is the Only Difference in Asparagine-Linked Glycosylation Between the HA1s of the Virulent and Avirulent Strains. It was possible that the change in glycosylation of the HA1 (vir) could have affected subsequent glycosylation at one or more of the other possible sites, thus indirectly regulating cleavage of HA. To identify any other differences in asparagine-linked glycosylation between HA1 (vir) and HA1 (avir), we compared partial tryptic digests of these glycosylated and *N*-glycanase-treated HA1 proteins by high-resolution reverse-phase HPLC. The comparative maps of the glycosylated and deglycosylated forms of HA1 (avir) and HA1 (vir) are shown in Fig. 2 *A* and *B* respectively. The upper profile in each of these panels is a chromatogram of the deglycosylated peptides. Differences observed between the glycosylated and deglycosylated digests for each HA1 protein should reflect only those peptides whose mobility has been altered by removal of the carbohydrate and the concomitant change of the asparagine to aspartate; peptides whose mobility is not altered by deglycosylation are those not containing asparagine-linked carbohydrate. The glycopeptides appear as broad heterogeneous peaks, whereas the deglycosylated peptides are in sharp and well-resolved peaks.

After deglycosylation, 10 new peptides appear that are common to digests of HA1 (vir) and HA1 (avir), as can be seen in the direct comparison maps (Fig. 2*D*). These peptides are indicated by arabic numerals. Direct amino-terminal sequence analysis of these peptides identified which glycosylation sites were represented. Sufficient sequence information was obtained to identify these peptides (Table 2), which represent all predicted *N*-glycosylation sites in HA1 and demonstrate that all these potential glycosylation sites are used in both HA1 (vir) and HA1 (avir). The fact that there are more glycopeptides than glycosylation sites is due to incomplete tryptic cleavage.

Several differences between the glycosylated and deglycosylated peptide maps of HA1 (vir) and HA1 (avir) were noted. One glycopeptide, designated A, is present in digests of the glycosylated HA1 (avir) but not in glycosylated HA1

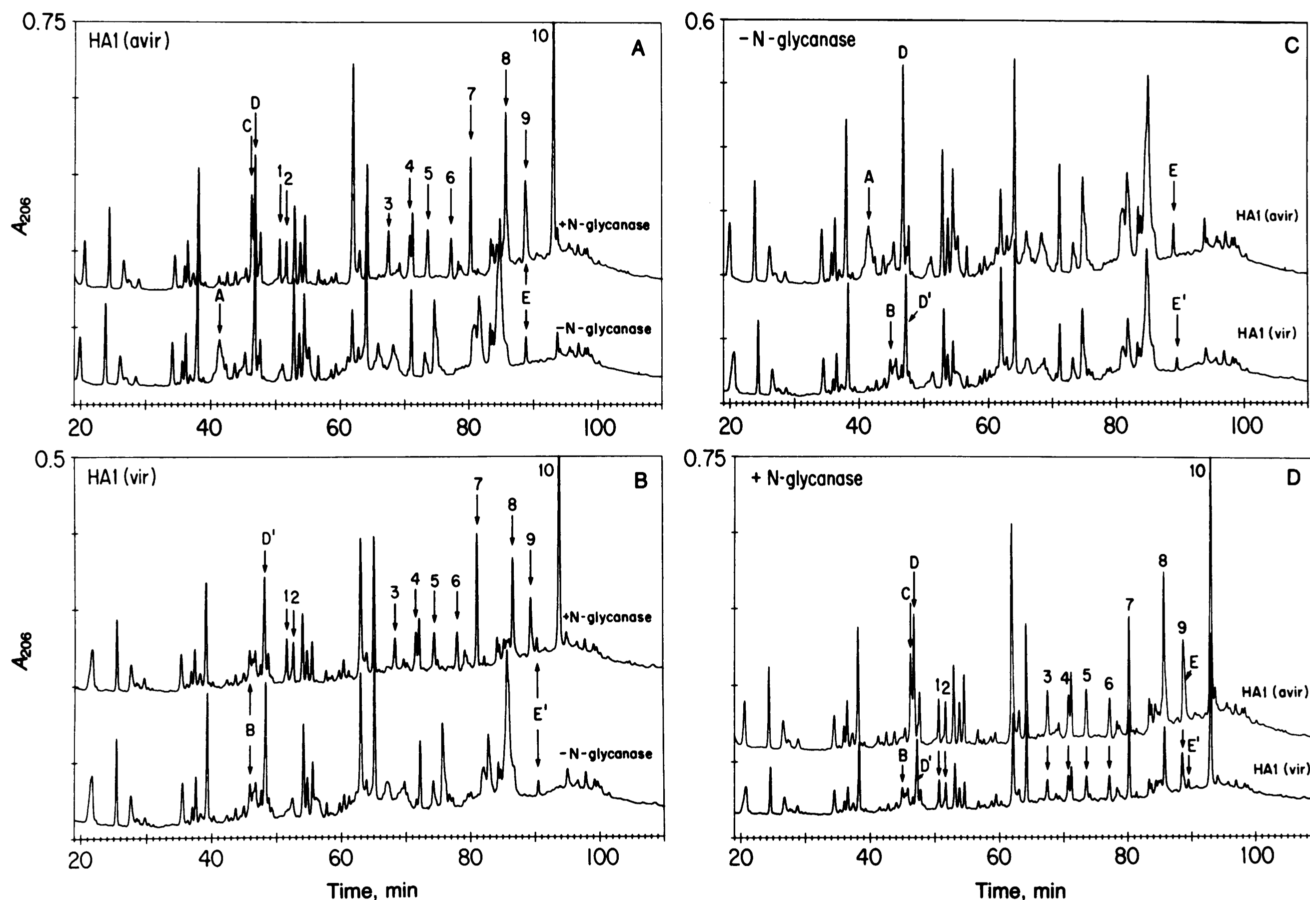

FIG. 2. Identification of glycopeptides by comparative analysis of tryptic digests by HPLC. HA1s from the virulent and avirulent strains were purified by electroelution from gels. One portion of the isolated HA1 was digested by *N*-glycanase to remove N-linked oligosaccharides and an undigested portion served as a control. The samples were digested by trypsin and analyzed by reverse-phase HPLC. The four different digests are compared in separate panels to aid identification: (*A*) Comparison of tryptic digest of HA1 (avir) deglycosylated (+*N*-glycanase), upper profile, and glycosylated (−*N*-glycanase), lower profile. (*B*) Comparison of tryptic digests of deglycosylated and glycosylated HA1 (vir), as above. (*C*) Comparison of tryptic digest of glycosylated (−*N*-glycanase) HA1 (avir), upper profile, and HA1 (vir), lower profile. (*D*) Comparison of tryptic digests of deglycosylated (+*N*-glycanase) HA1 (avir), upper profile, and HA1 (vir), lower profile. Peptides 1–10 are glycopeptides common to HA1 (avir) and HA1 (vir); peptides A and C are glycopeptides found only in HA1 (avir); peptide B is a nonglycosylated peptide found only in HA1 (vir); peptides D and E and D′ and E′ are nonglycosylated peptides found in HA1 (avir) and HA1 (vir), respectively. Peptide sequences are shown in Table 2.

Table 2. Location of glycosylated asparagine residues on the virulent and avirulent HA1 polypeptides

Peptide	Sequence	Yield, pmol	Repetitive yield, %
1	^{23}_D*V T_ V T H A Q D I L E K K	235	93
2	^{23}_D*V T_ V T H A Q D I L E K	200	94
3	^{277}C Q T P L G A I _D*S S_ M P F H	170	95
4	Not determined	200	ND
5	^{277}C Q T P L(G A I _D*S S_ M . . . .)	210	ND
6	^{277}C Q T P L G A I _D*S S_ M P F H	10	93
7	^{163}T Y _D*N T_ N V E D L L I L	315	95
8	^{163}T Y _D*N T_ N V E D L . . .	150	96
9	^{225}M E F F W T I L R P _D*D T_ I S	50	95
10	^{225}M E F F W T I L R P _D*D T_ I S	200	96
A	Not determined		
B	^{1}D Q I C I G Y H A _N N S_ K K	90	97
C	^{1}D Q I C I G Y H A N _D*S T_ K	400	96
D	^{120}N S W _A_ N H D A S S G V S S A C P H L G R	200	95
D′	^{120}N S W _T_ N H D A S S G V S S A	400	95
E	^{54}D C S V A G L L L G N P M C D _E_ F L N A P E . .	38	93
E′	^{54}D C S . . . . _D_	ND	ND

The standard one-letter residue symbols are used. Italics indicate consensus sequence for potential glycosylation sites. D* is D (Asp) generated from N (Asn) by deglycosylation with *N*-glycanase. ND, not done. Dots indicate peptides were not analyzed at these positions. A single residue italicized indicates the predicted difference between the avirulent and virulent strains.

(vir) (Fig. 2 *A* and *C*). In addition, a deglycosylated peptide, designated C, is present in the digests of HA1 (avir) but not in HA1 (vir) (Fig. 2 *A* and *D*). Direct sequence analysis of peptide C demonstrated that the peptide is glycosylated at Asn-11 (Table 2) and, thus, is the only glycopeptide difference between HA1 (vir) and HA1 (avir). Conversely, a nonglycosylated peptide, peptide B, is present in HA1 (vir) but not in HA1 (avir) (Fig. 2 *B*, *D*, and *C*). Amino-terminal sequence analysis of peptide B (Table 2) demonstrated that it is the predicted amino-terminal peptide of HA1 (vir) that was not glycosylated at either Asn-10 or Asn-11, which confirmed the direct amino-terminal sequence analysis.

Finally, there were two differences in digests of HA1 (vir) and HA1 (avir) that are not a function of glycosylation. One of the differences is between peptides D and D′. Peptide D of HA1 (avir) contains the second mutation at position 123, from alanine to threonine, that was predicted by nucleotide sequencing (Table 2). The other difference illustrated in Fig. 2 *C* and *D* is between peptides E and E′ for the HA1 (avir) and HA1 (vir), respectively. Peptide E of HA1 (avir) elutes earlier than E′ of HA1 (vir) and forms a shoulder on the glycopeptide 9 peak. Direct amino-terminal sequence analysis of peptides E and E′ demonstrated that they contain the third mutation that distinguishes HA1 (avir) from HA1 (vir), respectively, from glutamate to aspartate at position 69 in the sequence (Table 2). Thus, our analyses have identified all the mutations that distinguish HA1 (vir) from HA1 (avir) and show that the only direct effect of these changes on glycosylation is the lack of N-linked oligosaccharide to the asparagine at position 11 in HA1 (vir). The peptide analysis also established that corrections to the nucleotide-derived sequence are necessary; Val-61 is replaced by Leu and Val-282 is replaced by Gly.

The Asparagine Residue at Position 11 Is Glycosylated in an Avirulent Revertant of a Virulent Virus. An avirulent revertant of the virulent virus, which no longer has a cleaved HA without trypsin, has an HA1 gene sequence that differs from that of its parent by a single point mutation, resulting in a predicted amino acid change at residue 13 (3). This change, from lysine to threonine, caused reversion to a sequence containing a potential glycosylation site previously predicted for the avirulent virus (4). To determine whether or not the HA of the revertant virus [HA1 (rev)] was glycosylated at Asn-11, as in the avirulent virus, the amino terminus of HA1 (rev) was sequenced before and after *N*-glycanase treatment (Table 1). The results show that HA1 (rev), like HA1 (avir), was glycosylated at Asn-11. This suggests strongly that glycosylation at Asn-11 is sufficient to prevent cleavage of the Chick/Penn HA.

DISCUSSION

We have determined the sites at which N-linked oligosaccharides are attached to the HA1 subunit of the avirulent and virulent Chick/Penn (H5N2) virus and provide evidence that site-specific glycosylation at Asn-11 can influence cleavage of the Chick/Penn HA in tissue culture and, thus, the acquisition of virulence *in vivo*. Virulence is considered to be a polygenic property (20–23). However, reassortant studies have shown that the avirulent Chick/Penn virus contains seven of the eight genes necessary for virulence, requiring only mutation of the HA gene for the acquisition of virulence (3). Our study confirms the predictions of Kawaoka *et al.* (4) and Webster *et al.* (3) indicating a relationship between glycosylation of the Chick/Penn HA and the acquisition of virulence.

Through direct amino acid sequence analysis, we have shown that the HA1 subunits of the avirulent and avirulent revertant viruses differ from the subunit of the virulent virus by containing a carbohydrate at Asn-11. An Asn residue can be glycosylated if it is part of the sequence Asn-Xaa-Thr/Ser (24); however, glycosylation did not occur on any of the three Chick/Penn HA1 subunits at the first potential site, Asn-10, while it did take place at all four remaining sites.

There are precedents for lack of glycosylation among influenza virus HAs. The HA1 subunits of A/Japan/57 (H2) and A/FPV/Rostock/34 (FPV) (H7) also are not glycosylated at all potential sites (6, 11). Absence of glycosylation at the predicted sites in these two viruses has been attributed either to the placement of an Asp or a Pro in the variable

position of the glycosylation sequence (25, 26) or to steric hindrance when the potential sites were adjacent (6). Neither explanation applies to the HA1 of the virulent Chick/Penn virus, which contains the sequence $\overset{10}{\text{Asn}}$-Asn-Ser-Lys.

The proximity of the amino-terminal glycosylation site to the cleavage peptide in HA, as seen in three-dimensional modeling (4), suggests that glycosylation at that site could directly interfere with proper processing and, thus, lead to avirulence. It is likely, however, that other structural features of the HA molecule are required for proper proteolytic processing of HA. For example, the well-characterized FPV (H7) strain has an HA molecule that is glycosylated at a site near its amino terminus but is still processed to HA1 and HA2 in culture and is highly virulent (9, 10). Presumably, FPV HA processing occurs through an alternative mechanism.

It has been proposed that cleavage of the FPV HA depends on the presence of several consecutive basic amino acids at the cleavage site in the connecting peptide region between HA1 and HA2 (27, 28). The deduced amino acid sequence of the putative connecting peptide is the same for the virulent, avirulent, and revertant Chick/Penn HA and is similar to that of the FPV HA in that it contains at least four basic amino acids at the predicted carboxyl terminus of HA1 (4). Only influenza viruses of the H5 and H7 subtypes have been found to be virulent for avian species (9), and it is possible that the type of connecting peptide sequence represented by FPV is important in determining HA cleavage in infected birds. Thus, it is likely that both the sequence of the putative connecting peptide and glycosylation at Asn-11 are important in determining cleavage of the Chick/Penn HA.

Glycosylation of the influenza virus HA is important for protection against proteolytic degradation (29, 30) and for antigenic recognition (31), and its importance for stabilization of HA conformation and subunit interaction (32) has been suggested. Studies of another viral membrane glycoprotein, the G protein of vesicular stomatitis virus, have shown that glycosylation is important for transportation from the Golgi apparatus to the cell surface (33, 34). Our studies suggest a further role for site-specific glycosylation in directly regulating HA protein processing.

It is still unresolved how glycosylation affects cleavage of the Chick/Penn HA. The presence of carbohydrate may mask a recognition signal required by the cleavage enzyme or perturb a structural feature so that it no longer serves as a substrate for the processing enzyme. Alternatively, the carbohydrate may be involved in generating the correct signal for transporting the HA through the Golgi apparatus to encounter the cleavage enzyme and/or for proper assembly of cleaved HA onto virions. It is also possible that the presence of a basic residue, the lysine at position 13 in HA1 (vir), plays an additional role by serving as part of the recognition signal for the processing enzyme. The molecular events involved in processing of the HA to HA1 and HA2 require further study.

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