JOURNAL OF VIROLOGY, Aug. 1989, p. 3296–3300
0022-538X/89/083296-05$02.00/0

Vol. 63, No. 8

Interplay between Carbohydrate in the Stalk and the Length of the Connecting Peptide Determines the Cleavability of Influenza Virus Hemagglutinin

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Received 13 March 1989/Accepted 28 April 1989

The ability of many viruses to replicate in host cells depends on cleavage of certain viral glycoproteins, including hemagglutinin (HA). By generating site-specific mutant HAs of two highly virulent influenza viruses, we established that the relationship between carbohydrate in the stalk and the length of the connecting peptide is a critical determinant of cleavability. HAs that lacked an oligosaccharide side chain in the stalk were cleaved regardless of the number of basic amino acids at the cleavage site, whereas those with the oligosaccharide side chain resisted cleavage unless additional basic amino acids were inserted. This finding suggests that the oligosaccharide side chain interferes with HA cleavage if the number of basic amino acids at the cleavage site is not adequate to nullify this effect. Similar interplay could influence cleavage of other viral glycoproteins, such as those of human and simian immunodeficiency viruses and paramyxoviruses.

Influenza A viruses representing 13 hemagglutinin (HA) and 9 neuraminidase subtypes have been isolated from wild waterfowl. Most of these viruses replicate in the intestinal tract of birds without causing any apparent disease symptoms. However, some influenza A viruses of the H5 and H7 subtypes are highly pathogenic; they cause systemic infection in domestic poultry, resulting in the death of large numbers of birds. In tissue culture, the HA of virulent viruses is cleaved into HA1 and HA2 in the absence of exogenous trypsin (4), and the viruses produce plaques. By contrast, the HA of avirulent viruses is not cleaved, and these viruses do not produce plaques unless trypsin is added. Comparative sequence analysis of the HA confirms the suggestion of Bosch et al. (3) that virulent influenza viruses contain a series of basic amino acids at the cleavage site, whereas all avirulent viruses contain only a single basic amino acid (16, 19). Experiments with mutants containing specific mutations of the HA gene have confirmed the need for multiple basic amino acids at the cleavage site (17).

Avirulent A/chicken/Pennsylvania/1/83 (H5N2) (Ck/Penn) influenza virus has provided insight into the function of carbohydrate on the HA. An oligosaccharide side chain in the vicinity of the cleavage site affects cleavage activation of the HA (8, 15). A single point mutation in the HA of Ck/Penn virus resulted in the loss of asparagine-linked carbohydrate in the stalk region at residue 11, leading to virulence. In the three-dimensional structure, the carbohydrate side chain is located in the vicinity of the cleavage site between HA1 and HA2.

Multiple basic amino acids have been present at the cleavage site of the HA in all virulent H5 and H7 influenza viruses studied to date, but the number of amino acids has varied (16). For example, A/turkey/Ireland/1378/83 (Ty/Ire) (H5N8) contains six basic amino acids at the cleavage site (16), whereas A/chicken/Scotland/59 (Ck/Scot) (H5N1) contains only four (Table 1). We have created a site-specific Ty/Ire mutant (MT-2) that has a deletion of two basic amino acids, leaving four basic amino acids at the HA cleavage site (18). Although the MT-2 and Ck/Scot HAs have the same amino acid sequence at the cleavage site, the former is not cleaved without exogenous trypsin (Table 1). This indicated that factors other than the number of basic amino acids at the cleavage site play a role in HA cleavage. In the present study, we sought to explain why different virulent influenza viruses have different numbers of basic amino acids at the cleavage site and why the carbohydrate in the stalk interferes with cleavage of the HA of some virulent influenza viruses but not others.

The results demonstrate that the influence of oligosaccharide side chains on cleavage activation can be circumvented by addition of basic amino acids to the cleavage site. The principles established in this and a previous study (17) may also apply to cleavage of other viral glycoproteins, such as those of human and simian immunodeficiency viruses and paramyxoviruses.

MATERIALS AND METHODS

Viruses. Ty/Ire (H5N8) and Ck/Scot (H5N1) viruses were grown and tested in a containment laboratory in St. Jude Children's Research Hospital. These viruses are highly pathogenic in chickens (birds infected orally or intranasally with 10^2 to 10^4 50% egg infective doses of virus die within 7 days).

Purification of HA subunits. Purified egg-grown virus preparations were dialyzed against 25 mM Tris-glycine buffer (pH 8.3) containing 1% sodium dodecyl sulfate, concentrated, and then reduced and alkylated (30) before electrophoresis in sodium dodecyl sulfate–10% polyacrylamide gels (18). 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 sodium dodecyl sulfate-polyacrylamide gel electrophoresis using Coomassie brilliant blue for detection.

Amino-terminal sequence analysis. Protein samples were sequenced by automated Edman degradation on a 470A gas-phase sequencer (Applied Biosystems, Foster City,

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TABLE 1. Glycosylation site in the stalk and number of basic amino acids at the cleavage site of the HA[a]

Virus	Potential glycosylation site in the stalk				Cleavage site	HA cleavage[b]
	10	11	12	13	HA1 HA2	
Ty/Ire	Asn	Asn	Ser	Thr	Gln **Arg Lys Arg Lys Lys Arg**/Gly	+
MT-2[c]	Asn	Asn	Ser	Thr	Gln[□□] **Arg Lys Lys Arg**/Gly	−
Ck/Scot	Asn	Lys	Ser	Thr	Gln —— —— **Arg Lys Lys Arg**/Gly	+

[a] Dashes (——) indicate the deletion of sequences; boldface letters designate basic amino acids; slashes indicate the HA cleavage site. Deleted amino acids are shown by boxes. Potential glycosylation sites that contain carbohydrate and those lacking carbohydrate are emphasized with the use of solid and broken underlines, respectively. The presence of carbohydrate was demonstrated by direct amino acid sequencing in the Ty/Ire and Ck/Scot HAs and by mobility on acrylamide gel in the MT-2 HA.

[b] +, Cleaved; −, uncleaved.

[c] Site-specific mutant of the Ty/Ire HA.

Calif.) with use of the 02NVAC program and methanolic HCl conversion chemistry.

Construction of SV40-HA recombinant virus. The cloning of a full-length cDNA copy of the Ty/Ire HA gene and the construction of simian virus 40 (SV40)-Ty/Ire HA recombinant virus have been reported (17). The HA expressed in this system undergoes normal cleavage in the absence of exogenous trypsin, is transported to the cell membrane, and adsorbs to and fuses erythrocytes at pH 5.

The HA gene of Ck/Scot virus (7) was kindly provided by A. P. Kendal (Centers for Disease Control, Atlanta, Ga.). SV40-Ck/Scot HA recombinant virus was constructed in the same way as SV40-Ty/Ire virus (17).

Site-specific mutagenesis. The entire HA genes of Ty/Ire and Ck/Scot viruses were subcloned in the double-stranded replicative form of M13mp18 phage DNA. The procedures for oligonucleotide-directed mutagenesis have been described in detail by Zoller and Smith (33). The entire HA gene of each SV40-HA recombinant was sequenced by the chain-termination procedure (16) to ensure that only the desired changes were present in the molecules.

RESULTS

Ty/Ire HA, but not Ck/Scot HA, has an oligosaccharide side chain in the glycoprotein stalk. Despite wide variability in the location of potential glycosylation sites, Asn-X-Ser or Thr (29), among the HAs of different influenza viruses (10, 13, 15, 23, 28), the location of the potential glycosylation site in the stalk region is highly conserved (1). Glycosylation of this site correlates with cleavage of the HA in the Ck/Penn virus (8, 15). This observation led us to compare amino acid sequences of Ty/Ire and Ck/Scot HAs in this potential glycosylation site. The Ty/Ire HA contains the two overlapping potential glycosylation sites, whereas the Ck/Scot HA contains only one (Table 1). However, the exact position of the glycosylated Asn has not been identified because not all of the potential glycosylation sites are utilized (32).

To determine whether the difference in glycosylation in this region would explain why the mutant of the Ty/Ire HA (MT-2), which has the same connecting peptide as the Ck/Scot HA, is not cleaved, we directly sequenced the amino acids of the isolated HA1 protein. Glycosylated residues are not detected directly in sequence analyses. Amino-terminal sequence analysis demonstrated that Asn-11 is glycosylated on the Ty/Ire HA1, since a single Asn was recovered at position 10 but not at position 11. In the Ck/Scot HA1, Asn was recovered at position 10, demonstrating that this position is not glycosylated (data not shown).

These analyses establish that the Ty/Ire HA but not the Ck/Scot HA has carbohydrate in the stalk of the HA.

Carbohydrate in the stalk interferes with cleavage of the HA. Because the amount of HA expressed in the SV40 system is insufficient for direct sequencing, we could not examine glycosylation at Asn-10 and Asn-11 of MT-2. It is likely, however, that the glycosylation pattern of MT-2 is similar to that of Ty/Ire: the HA1 and HA2 of the two HA proteins showed the same mobilities on polyacrylamide gels when exogenous trypsin was added to cleave the MT-2 HA (17). These data indicate that Asn-11 of MT-2 is also glycosylated.

The carbohydrate at Asn-11 of the HA has been shown to be a key determinant in modulating cleavage of the Ck/Penn HA (8, 15), making it reasonable to ask whether carbohydrate at the same position would interfere with cleavage of the MT-2 HA. A mutant (MT-9) characterized by deletion of the potential glycosylation site at position 11 was created by site-specific mutagenesis (residue 13 was changed from Thr to Lys). It was cleaved and showed fusion activity without exogenous trypsin (see Fig. 1 and 3 and Table 2), indicating that the oligosaccharide at Asn-11 interferes with cleavage. The HA1 of the MT-9 HA migrated faster on polyacrylamide gels than did the HA1 of Ty/Ire (Fig. 1), confirming a loss of carbohydrate at Asn-11.

That carbohydrate interferes with HA cleavage was further confirmed in the SV40 system using a different virus.

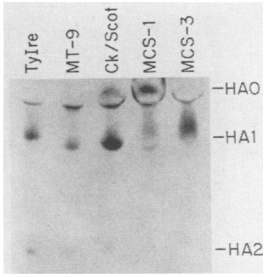

FIG. 1. Comparison of the HAs produced in SV40-HA. Cleavage of the HA molecule expressed in CV-1 cells was examined by infecting cell monolayers with SV40-HA recombinant virus; 36 h later the HAs were labeled with [^{3}H]mannose and [^{3}H]glucosamine for 15 h at 37°C. Cell extracts were immunoprecipitated with monoclonal antibodies to the HA and analyzed on a 12.5% polyacrylamide gel as described (18).

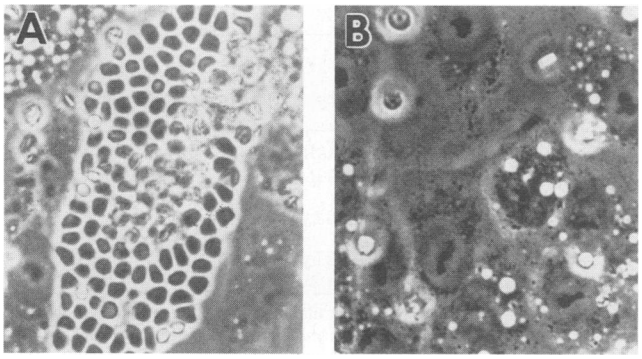

FIG. 2. Hemadsorption by the HA expressed in CV-1 cells infected with SV40-Ck/Scot HA recombinant virus. At 56 to 60 h after infection with SV40-HA recombinant viruses, cells were overlaid at room temperature with a 1% human erythrocyte suspension in isotonic phosphate-buffered saline (PBS). After 20 min, unbound erythrocytes were removed by washing with PBS. (A) Cells infected with SV40-Ck/Scot HA recombinant virus; (B) mock-infected cells.

The HA of Ck/Scot, expressed in CV-1 cells, showed normal cleavage in the absence of trypsin (Fig. 1), transport to the cell membrane, and ability to adsorb to (Fig. 2) and fuse (Fig. 3) erythrocytes at pH 5 and to produce polykaryon (Fig. 4). To determine whether carbohydrate in the stalk could inhibit cleavage of the Ck/Scot HA, we created a new glycosylation site by changing Lys to Asn at residue 11. The amino acid sequence at residues 10 to 13 of this mutant (MCS-1) was the same as that of the Ty/Ire HA (Table 2). The majority of the MCS-1 HA molecules were not cleaved (Fig. 1) and did not show fusion activity (Fig. 3). In the presence of exogenous trypsin, the MCS-1 HA was cleaved into HA1 and HA2. The mobility of the MCS-1 HA1 was slower than that of the Ck/Scot HA1 (data not shown), confirming addition of an oligosaccharide side chain to the newly created glycosylation site at Asn-11. We conclude that the carbohydrate at Asn-11 does inhibit cleavage of the HA.

Insertion of basic amino acids at the cleavage site of the HA circumvents the interference with cleavage by carbohydrate in the stalk. Why does the oligosaccharide at Asn-11 interfere with cleavage of the Ck/Scot HA, but not with that of the Ty/Ire HA? The explanation could lie in Ty/Ire having six basic amino acids at the HA cleavage site, whereas Ck/Scot has only four. This suggests that additional basic amino acids are required to abrogate structural interference with cleavage by the carbohydrate side chain. To test this possibility, we inserted two basic amino acids (Arg-Lys) into the cleavage site of the MCS-1 HA. The resulting mutant (MCS-3) has six basic amino acids at the cleavage site, as well as carbohydrate in the stalk (Table 2), and fully restored cleavability and fusion activity (Fig. 1, Table 2).

These results indicate that the carbohydrate in the stalk and the number of basic amino acids are interrelated determinants of cleavage of the HA: an increased number of basic amino acids will circumvent interference by the carbohydrate.

DISCUSSION

The number of basic amino acids at the cleavage site (17) and the presence or absence of carbohydrate in the stalk influence the cleavage activation of HAs (8, 15). Why carbohydrate interferes with the cleavage of certain HAs but not others was uncertain, nor was it clear why the same number of basic amino acids at the cleavage site was sufficient for cleavage of some but not all HAs. We demonstrate an interrelationship between the stalk carbohydrate and the number of basic amino acids that appears to determine the cleavability of the HA. When the HA stalk lacks carbohydrate, the number of basic amino acids at the cleavage site can be as small as four consecutive basic residues; however, when the stalk contains carbohydrate, extra basic amino acids are needed to initiate cleavage by a protease. Because multiple basic amino acids are found at the cleavage sites of many viral glycoproteins (2, 9, 25, 27), including those of human immunodeficiency virus types 1 (24) and 2 (12), simian immunodeficiency virus (5), and paramyxoviruses (6, 11, 21, 31), the principles established in this study may apply to other viral glycoproteins as well. The importance of the number of basic amino acids, but not carbohydrate, for cleavage activation of the viral glycoprotein has also been shown in the SV5 fusion protein (22).

In nature, does an avirulent influenza virus become highly virulent by insertion of basic amino acids or mutations at the cleavage site? Comparative sequence analysis of the connecting peptides of virulent and avirulent influenza viruses (16, 19) indicates that two or more mutations are required to convert the connecting peptide of avirulent viruses to the virulent form. It is therefore more likely that avirulent viruses that already contain multiple basic amino acids at the cleavage site could become virulent by loss of carbohydrate in the stalk, since a single mutation is sufficient for this

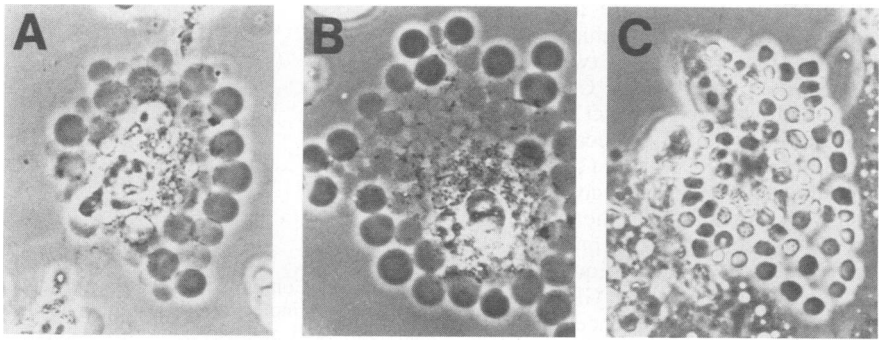

FIG. 3. Erythrocyte fusion activity of the HA expressed in the SV40 recombinant system. At 56 h after SV40-HA recombinant virus infection, the cells were washed with phosphate-buffered saline (PBS). Human erythrocytes were bound to the infected cells for 15 min at 37°C. Prewarmed PBS (pH 5.0) was added for 3 min, after which the cells were placed in growth medium for 30 min to 2 h. (A) MT-9; (B) SV40-Ck/Scot HA; (C) SV40-MCS-1 HA.

TABLE 2. Influence of stalk carbohydrate and the number of basic amino acids at the cleavage site on the biologic activities of the HA[a]

Site-specific mutant	Amino acid sequence at: Potential glycosylation site in the stalk (10 11 12 13)	Cleavage site	HA cleavage[b]	Erythrocyte fusion[c]	Polykaryon formation[d]
Ty/Ire series		HA1 HA2			
Parent	Asn Asn Ser Thr	Gln **Arg Lys Arg Lys Lys Arg**/Gly	+	+	+
MT-2	Asn Asn Ser Thr	Gln [ ] [ ] **Arg Lys Lys Arg**/Gly	−	−	−
MT-9	Asn Asn Ser [Lys]	Gln [ ] [ ] **Arg Lys Lys Arg**/Gly	+	+	+
Ck/Scot series					
Parent	Asn Lys Ser Thr	Gln —— —— **Arg Lys Lys Arg**/Gly	+	+	+
MCS-1	Asn [Asn] Ser Thr	Gln —— —— **Arg Lys Lys Arg**/Gly	−	−	−
MCS-3	Asn [Asn] Ser Thr	Gln [Arg] [Lys] **Arg Lys Lys Arg**/Gly	+	+	+

[a] Dashes (——) indicate the deletion of sequences; boldface letters indicate basic amino acids; slashes indicate the HA cleavage site. Mutated or deleted amino acids are shown in boxes. Potential glycosylation sites that contain carbohydrate and those lacking carbohydrate are emphasized with the use of solid and broken underlines, respectively. The presence of carbohydrate was demonstrated by direct amino acid sequencing in the Ty/Ire and Ck/Scot HAs and by mobility on acrylamide gel in the MT-2, MT-9, MCS-1, and MCS-3 HAs.

[b] +, Cleaved; −, uncleaved.

[c] +, 80% of the adsorbed human erythrocytes were fused; –, fusion of erythrocytes not observed.

[d] +, >80% of cells were fused; −, no polykaryon formation observed.

change. This was demonstrated in the Ck/Penn virus in 1983 (15). If, however, the pressure for selecting the virus containing the multiple basic amino acids in the connecting peptide exists, a virulent influenza virus could emerge from an avirulent virus by insertion of basic amino acids at the cleavage site of the HA. Recently, Ohuchi et al. (20) showed that this can occur; after blind passages of an avirulent Ck/Penn virus in tissue culture in the absence of trypsin, highly virulent variants that contained additional basic amino acids at the cleavage site were isolated.

Steric hindrance is the simplest mechanism by which stalk carbohydrate could interfere with the cleavage of HA. The Asn residue at position 11 is approximately 13 Å (1.3 nm) from the carboxyl terminus of HA1 and 1.9 nm from the amino terminus of HA2. Although the X-ray data for only 25% of the hexose rings in the X-31 HA structure are available, it is known that the nine-hexose oligosaccharide bound to the human immunoglobulin Fc domain extends approximately 2.5 nm from Asn-297 (Clayton Naeve, personal communication). It is, therefore, conceivable that the oligosaccharide side chain could interfere with HA cleavage by steric hindrance. How does addition of two basic amino acids circumvent interference by oligosaccharides? Perhaps such an addition resolves the steric hindrance, or the extra positive charge conferred by the amino acids may be important for interaction between cleavage enzymes and their substrates. The latter idea is supported by the observation that a mutant of the Ty/Ire HA, in which the first two basic amino acids are replaced by nonbasic amino acids (not deleted) at the cleavage site, showed decreased susceptibility to cleavage enzymes (17). The precise mechanism, however, cannot be determined until the three-dimensional structure of the uncleaved HA becomes available.

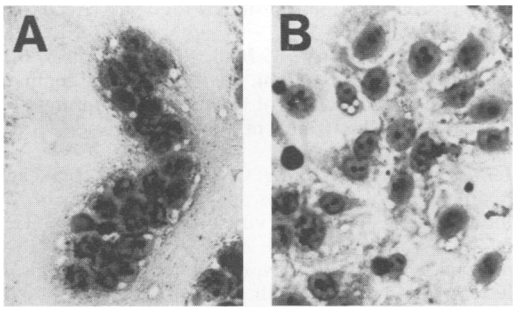

FIG. 4. Polykaryon formation by CV-1 cells infected with SV40-Ck/Scot HA recombinant virus. At 56 h after infection with the SV40-HA recombinant virus, the monolayers were incubated for 3 min at 37°C with phosphate-buffered saline (PBS) (pH 5). PBS was then removed, and the cells were incubated for 7 h in tissue culture medium. (A) Cells infected with SV40-CK/Scot HA recombinant virus; (B) mock-infected cells

Among the HAs of mammalian influenza viruses examined thus far, none has contained multiple basic amino acids at the cleavage site. Whether the causative agent of the human epidemic of lethal influenza in 1918 to 1919 contained a series of basic amino acids at this region is not known. However, the HA of human influenza virus can become cleavable in tissue culture in the absence of trypsin; a mutant of A/Aichi/2/68 virus produces cleaved HA without exogenous trypsin by a mutation in the stalk region rather than in the cleavage site (26). These studies suggest that human influenza virus could become highly virulent by acquiring cleavability of the HA.

ACKNOWLEDGMENTS

We thank Victor Fried for amino acid sequence analyses and Clayton Naeve for preparation of oligonucleotides and for calculations of the distance between amino acid residues and the length of the oligosaccharide in the three-dimensional structure of the HA molecule. Daniel Channell and Scott Krauss provided excellent technical assistance, and we thank John Gilbert for writing assistance and Dayna Anderson for typing the manuscript.

This work was supported by Public Health Service research contract AI 52586 from the National Institute of Allergy and Infectious Diseases, by Public Health Service Cancer Center Support (CORE) grant CA 21765, and by American Lebanese Syrian Associated Charities.

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