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Sequence requirements for cleavage activation of influenza virus hemagglutinin expressed in mammalian cells

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ABSTRACT **Cleavage of the hemagglutinin (HA) in tissue culture systems has been correlated with virulence of avian influenza viruses. To examine the structural requirements for cleavage of the HA, the HA gene from a virulent H5 influenza virus was expressed in mammalian cells (CV-1), and the cleavage site of the HA was explored by using site-specific mutagenesis. The expressed HA protein exhibited normal cleavage, transport to the cell membrane, and ability to adsorb and to fuse erythrocytes at pH 5. Site-specific mutagenesis of the HA directly established that (*i*) most of the basic amino acids at this site are critical for cleavage activation; (*ii*) besides the connecting peptide sequence, at least one other structural feature of the HA is required for enzyme recognition; and (*iii*) the length of the connecting peptide can abrogate the structural feature(s).**

In 1918 and 1919, a worldwide influenza epidemic occurred in humans, and at least 20 million people died from influenza infection. Since then, we have not experienced such a highly virulent influenza virus in the human population. However, since then, influenza epidemics in chickens (1, 2), turkeys (3), and seals (4) have caused high mortality, which serves as a reminder that a highly virulent influenza virus may reappear in humans. It is, therefore, important to establish the molecular basis of virulence of influenza viruses, and the avian system provides a suitable model.

Although virulence of influenza viruses is a polygenic characteristic (5–7), the hemagglutinin (HA) plays an important role (7, 8). Cleavage of the HA molecule into HA1 and HA2 of influenza viruses is a prerequisite for virus infectivity (9, 10), and, in tissue culture systems, cleavage in the absence of trypsin is correlated with virulence (8). Bosch *et al.* (11), by using two-dimensional electrophoresis, have shown an association between cleavability of the HA and the presence of multiple basic amino acids at the cleavage site. We have shown that although the length of the connecting peptide varies among lethal H5 (12) and H7 (13) viruses, these viruses contain at least two pairs of basic amino acids at the cleavage site, whereas nonlethal viruses contain a single arginine at this site (Table 1). However, it has not been directly demonstrated that the presence of multiple basic amino acids at the cleavage site is important for cleavage activation. Furthermore, it is not known which amino acid residues at the cleavage site are critical.

We have shown (14, 15) that an oligosaccharide side chain in the vicinity of the cleavage site interferes with cleavage of the HA. This indicates that the structural features of the HA other than the connecting peptide sequence are also involved in cleavage activation. However, some virulent influenza viruses retain this potential glycosylation site (12), suggesting that other unknown structural feature(s) can influence cleavage in the presence of the oligosaccharide chain.

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The HA gene of influenza viruses has been expressed in various eukaryotic systems (16–19). The expressed human H2 and H3 HAs, like native viral HA, are transported to the cell surface (16, 17), adsorb erythrocytes (RBCs) (16), and induce RBC-cell fusion and polykaryon formation when exposed to low pH (20). These HAs are not cleaved in the absence of trypsin (16), whereas the single H7 avian HA expressed in insect cells was partially cleaved without additional trypsin (19). In the present study, the H5 gene from a lethal H5N8 influenza virus and several site-specific mutants of this gene were expressed in mammalian cells to determine (*i*) whether this HA gene has the necessary information for cleavage activation in mammalian cells, (*ii*) the amino acid sequence required for cleavage, (*iii*) whether the multiple basic amino acids alone provide all the necessary information for cleavage activation, and (*iv*) the importance of the length of the connecting peptide.

MATERIALS AND METHODS

Virus and Cells. A/Turkey/Ireland/ 1378/83 (H5N8) virus was obtained from D. J. Alexander (Central Veterinary Laboratory, Weybridge, U.K.). CV-1 cells were obtained from M. J. Gething (University of Texas Health Science Center).

Construction of Simian Virus 40-HA Recombinant Virus. The cloning of a full-length cDNA copy of the HA gene of influenza virus A/Turkey/Ireland/1378/83 has been reported, and the clone was designated pTH29 (12). Simian virus 40 (SV40) viral DNA (Bethesda Research Laboratories) was linealized with Hpa II and ligated to pTH29 that was linealized with Cla I and dephosphorylated (Fig. 1). The clones were screened for orientation of the HA insert toward the SV40 late promoter. One such clone (pSVHA) was then digested with *Bam*HI. The smaller fragment was isolated on the agarose gel, electroeluted, and recircularized.

Transfection and Virus Stocks. Seventy nanograms of recircularized SV40-HA recombinant DNA and the equal amount of DNA from the SV40 early deletion mutant d11055 (21) were introduced into CV-1 cells by using DEAE-dextran (22). After 5 days at 37°C, the cells and medium were frozen and thawed three times, and fresh monolayers of CV-1 cells were infected with 0.3 ml of the resulting lysate. This was repeated twice to obtain a virus stock.

Site-Specific Mutagenesis. Plasmid pTH29 was digested with *Hin*dIII and *Bam*HI. The 1.7 kilobase fragment corresponding to the entire HA gene was isolated on an agarose gel and inserted into the double-stranded replicative form of M13mp18 phage DNA. The procedures used to carry out oligonucleotide-directed mutagenesis have been described in detail by Zohler and Smith (23). The entire HA genes of the various SV40-HA recombinant genomes were sequenced by the chain-termination procedure (12) to ensure that only the desired changes were present in the molecules.

Abbreviations: HA, hemagglutinin; RBC, erythrocyte; SV40, simian virus 40.

Table 1. Comparison of the connecting peptide and flanking amino acid sequences of virulent and avirulent H5 and H7 influenza virus hemagglutinins

Virus	Subtype	Virulence	Amino acid sequence								HA1	HA2
FPV/34	(H7)	+	Pro	Glu	Pro	Ser	Lys	Lys	Arg	Glu	Lys	Arg/Gly
Tern/SA/61	(H5)	+	Pro	Gln	Arg	Glu	Thr	Arg	Arg	Gln	Lys	Arg/Gly
Ty/Ire/83	(H5)	+	Pro	Gln	—	—	Arg	Lys	Arg	Lys	Lys	Arg/Gly
Ty/Ont/66	(H5)	+	Pro	Gln	—	—	—	Arg	Arg	Lys	Lys	Arg/Gly
Ck/Scot/59	(H5)	+	Pro	Gln	—	—	—	—	Arg	Lys	Lys	Arg/Gly
Ck/Penn/83 virulent	(H5)	+	Pro	Gln	—	—	—	—	Lys	Lys	Lys	Arg/Gly
Ck/Penn/83 avirulent	(H5)	−	Pro	Gln	—	—	—	—	Lys	Lys	Lys	Arg/Gly
Avirulent H5 viruses*	(H5)	−	Pro	Gln	—	—	—	—	Arg	Glu	Thr	Arg/Gly
Avirulent H7 viruses†	(H7)	−	Pro	Glu	Asn	Pro	—	—	—	Lys	Thr	Arg/Gly

+, Kills chickens after oral inoculation. −, No disease signs after oral inoculation. Dashes are included in the sequence to allow alignment of sequences, and basic amino acids are in bold letters. Slash indicates cleavage site.
*n = 3.
†n = 6.

RESULTS

To examine the structural requirements for cleavage of the HA by site-specific mutagenesis, we first established the system to express the HA gene of a lethal influenza virus, A/Turkey/Ireland/1378/83 (H5N8) (Ty/Ire) by using the SV40 recombinant virus system. Construction of SV40-Ty/Ire recombinant virus is shown in Fig. 1. CV-1 cells infected with SV40-Ty/Ire recombinant virus were examined for cell-surface expression of the HA molecule by an indirect immunofluorescent assay (24). The HA was detected on the cell surface (Fig. 2). To determine whether the expressed HA was cleaved, infected cells were labeled with [^{3}H]mannose and [^{3}H]glucosamine, and cell lysates were immunoprecipitated with monoclonal antibodies specific to the HA molecule (777/1) (14). The expressed HA was found to be cleaved into HA1 and HA2 in the absence of trypsin (Fig. 3). The mobilities of the expressed HA1 and HA2 on gels were indistinguishable from those immunoprecipitated from the lysate of CV-1 cells infected with parental Ty/Ire influenza virus.

The biological activity of the expressed HA was examined by hemadsorption, RBC-cell fusion, and polykaryon formation. SV40-Ty/Ire-infected cells expressing HA adsorbed human and guinea pig RBC (Fig. 4) and showed RBC fusion (Fig. 5) and polykaryon formation (Fig. 6) without trypsin when exposed to pH 5.0. A/Aichi/2/68 (Aichi) (H3N2) influenza virus was used as control for the fusion assay. This HA is not cleaved in tissue culture in the absence of trypsin (Fig. 5).

It is apparent from these results that the H5 molecules in CV-1 cells are synthesized, glycosylated, and transported to

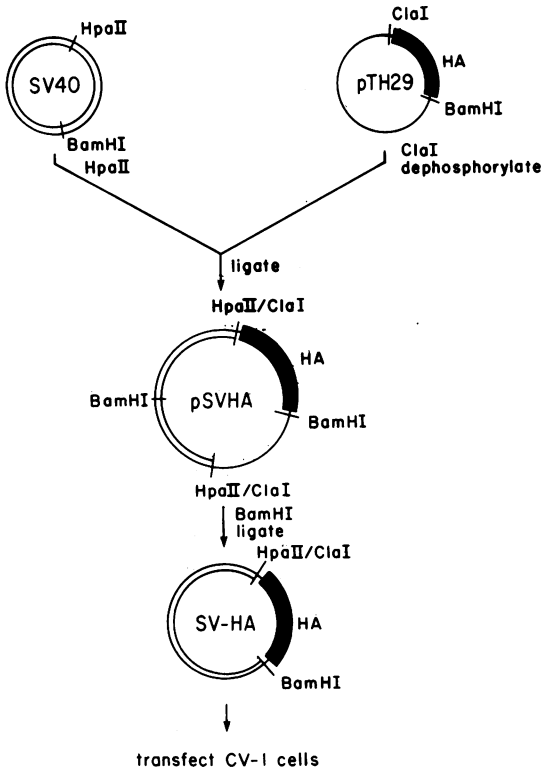

FIG. 1. Construction of SV40-Ty/Ire HA recombinant virus.

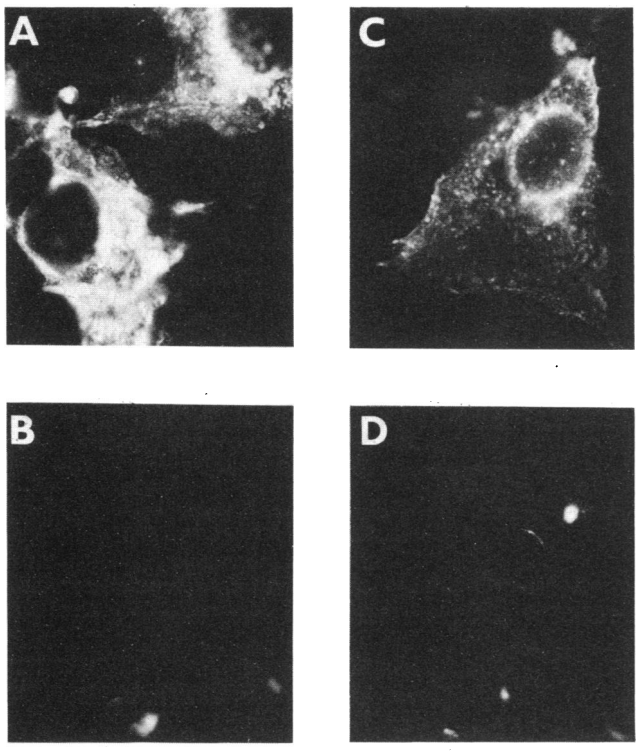

FIG. 2. Immunofluorescent antibody staining of HA in infected cells. Monolayers of CV-1 cells were infected with SV40-HA recombinant virus or were mock-infected for 56 hr. For analysis of intracellular HA (*A* and *B*), cells grown on glass slides were fixed using ice-cold methanol/actone, 1:1 (vol/vol). For analysis of surface HA (*C* and *D*), the cells were fixed with formalin [10% (vol/vol) solution in H_2O]. Indirect immunofluorescent staining was carried out as described (24). (*A* and *C*) Cells infected with SV40-HA recombinant virus. (*B* and *D*) Mock-infected CV-1 cells.

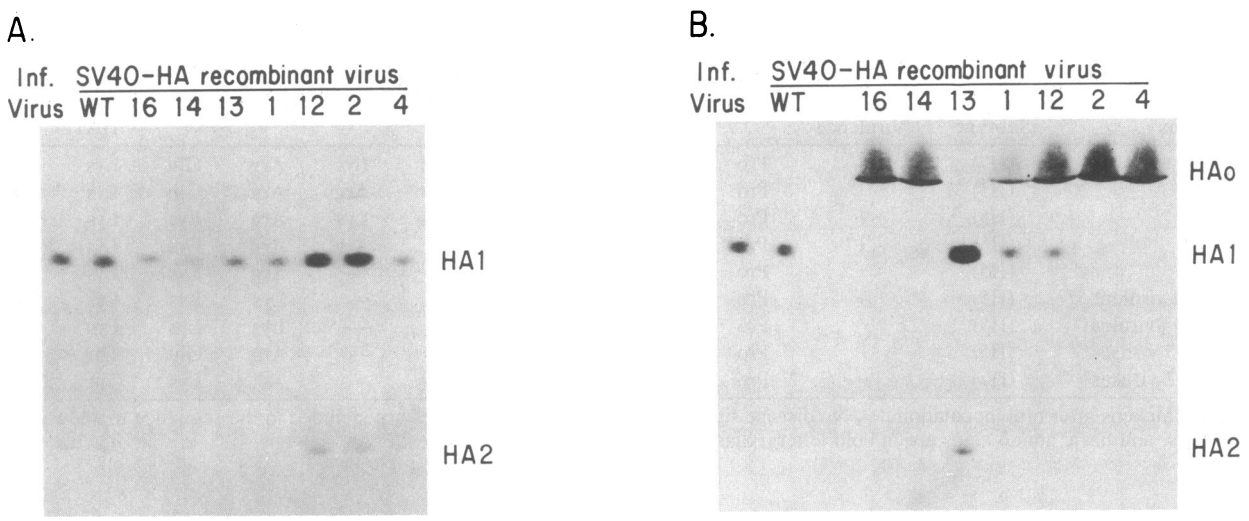

FIG. 3. Comparison of the HAs produced in SV40-HA or influenza virus-infected cells (Inf. virus). To examine cleavage of the HA molecule expressed in CV-1 cells, infected cell monolayers 15 hr after influenza virus (Ty/Ire) infection and 36 hr after SV40-HA recombinant virus infection were labeled with [^{3}H]mannose and [^{3}H]glucosamine for 15 hr at 37°C. Cell extracts were immunoprecipitated with monoclonal antibodies to the HA and analyzed on a 12.5% $NaDodSO_4$/polyacrylamide gel as described (25). (*A*) Cells were treated with trypsin (1 μg/ml) for 10 min at the end of the labeling period. (*B*) No trypsin treatment. WT, wild type; 16, MT-16; 14, MT-14; 13, MT-13; 1, MT-1; 12, MT-12; 2, MT-2; 4, MT-4.

the cell membrane. These results also show that an H5 HA molecule from a lethal avian influenza virus contains sufficient information to be cleaved in mammalian cells in the absence of trypsin and that no other influenza viral gene products are involved in cleavage.

To examine the amino acid sequence requirements at the activation site of the HA molecule for cleavage, site-specific mutagenesis was done on the Ty/Ire HA gene subcloned in M13mp18. The mutant HAs were tested for cleavage and fusion activity with and without trypsinization. No significant differences in cell-surface expression and hemadsorption were observed between the site-specific mutant and parent HAs. Furthermore, when these mutant HAs were trypsinized prior to the subsequent assays, they were indistinguishable from the parent HA in electrophoretic mobility (Fig. 3*A*) and in fusion activity (data not shown).

We first made a mutant HA (MT-4) containing the same sequence at the cleavage site as avirulent H5 HAs (Tables 1 and 2); two basic amino acids were deleted, and two amino acid substitutions were made, Lys → Glu and Lys → Thr. The mutant MT-4 HA was not cleaved (Fig.3) and did not show fusion activity (Fig. 5), indicating that the amino acid sequence at the cleavage site of the HA does affect susceptibility of the molecule to cleavage enzyme(s).

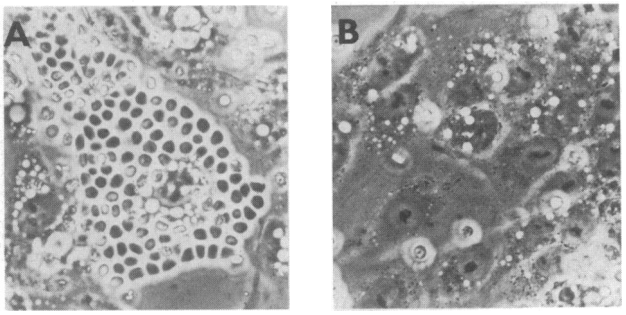

FIG. 4. Hemadsorpsin by the HA expressed in CV-1 cells infected with SV40-HA recombinant virus. At 56–60 hr after infection with SV40-HA recombinant viruses, cells were overlaid at room temperature with a 1% human RBC suspension in isotonic phosphate-buffered saline (PBS). After 20 min, unbound RBCs were removed by washing with PBS. (*A*) Cells infected with SV40-HA recombinant virus: (*B*) Mock-infected cells.

Comparison of the amino acid sequence of virulent and avirulent influenza viruses (12, 13) at the cleavage site (Table 1) show that the minimal requirement for cleavage activation is that the HA molecule must contain at least two pairs of basic amino acids at this site. We, therefore, made a mutant HA (MT-16) containing two mutations in the middle of the parental series of basic amino acids (Arg → Thr and Lys → Gln) leaving two pairs of basic amino acids separated by two

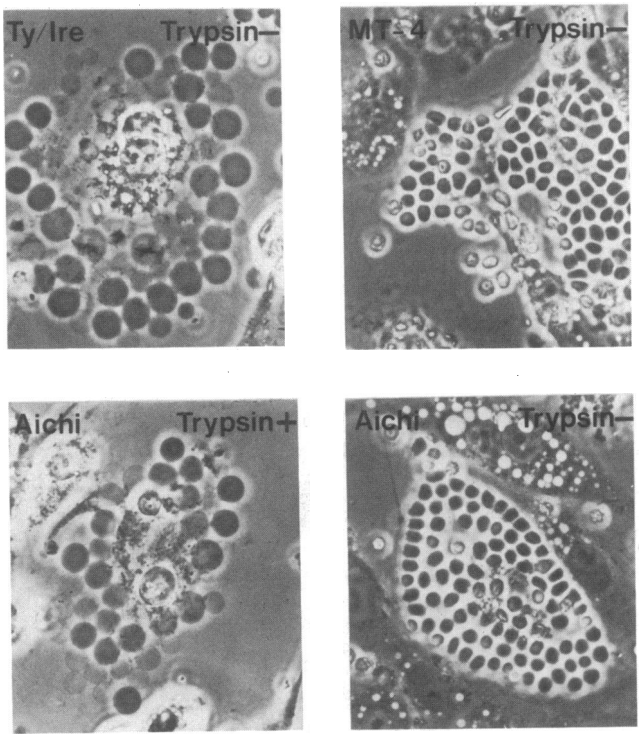

FIG. 5. RBC-cell fusion activity of the expressed HA in the SV40 recombinant system. Fifty-six hours after SV40-HA recombinant virus infection and 12 hr after influenza virus (Aichi/2/68) infection, the cells were washed with PBS. Human RBCs were bound to the infected cells for 15 min at 37°C. Prewarmed PBS (pH 5.0) was added for 3 min, after which the cells were placed in growth medium for 30 min to 2 hr.

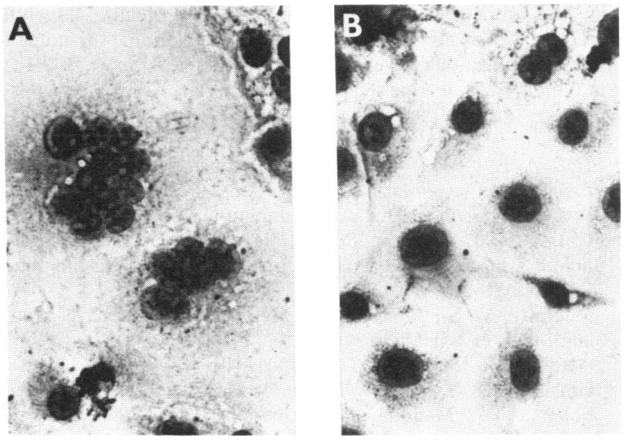

FIG. 6. Polykaryon formation by CV-1 cells infected with SV40-HA recombinant virus. At 56 hr after infection with the SV40-HA recombinant virus, the monolayers were incubated for 3 min at 37°C with PBS (pH 5). PBS was then removed, and the cells were incubated 7 hr in tissue culture medium. (*A*) Cells infected with SV40-HA recombinant virus: (*B*) Mock-infected cells.

neutral amino acids (Table 2). This mutant HA was not cleaved and did not show fusion activity (Fig. 3 and Table 2). Therefore, two pairs of basic amino acids alone were not sufficient for cleavage activation. From examination of the amino acid sequences (Table 1) of the virulent viruses, it is apparent that a virulent influenza strain with only two pairs of basic amino acids (e.g., Tern/SA/61) have a single neutral amino acid between the basic pairs instead of two as in MT-16. To examine the importance of the number and the position of the nonbasic amino acids between the two pairs of basic amino acids at the cleavage site, the mutant HAs MT-14 and MT-13 were tested. The HA containing a mutation at the fourth residue from the carboxylterminus of the HA1 from Arg → Thr (MT-14) was not cleaved and, therefore, did not show fusion activity (Fig. 3 and Table 2). In contrast, the mutant HA containing glutamine instead of lysine in the middle of the multiple basic amino acids (MT-13) was cleaved and showed fusion activity as efficiently as the parent Ty/Ire HA (Fig. 3 and Table 2). These results suggest that there is a critical basic amino acid in this region of the molecule; a nonbasic amino acid at the third residue from the carboxylterminus of HA1 does not affect cleavage activation of the HA molecule, whereas a nonbasic amino acid at fourth residue from the carboxylterminus abolishes cleavage.

One of the differences between the HAs of lethal and nonlethal influenza viruses besides the presence of the multiple basic amino acids is that the majority of the HAs of nonlethal viruses contain threonine as the second amino acid from the carboxylterminus of the HA1. The exception is the nonlethal Chicken/Pennsylvania/83 virus (14) (Table 1), and this will be discussed later. We, therefore, introduced a threonine at this position in the Ty/Ire HA. This mutant HA (MT-1) was partially cleaved (Fig. 3). However, fusion activity of this mutant was not detected (Table 2). It is not known whether lack of fusion activity of MT-1 HA was due to an insufficient amount of the cleaved HA or the cleavage by another cellular enzyme at an inappropriate position. There is precedent for this; chymotrypsin can cleave an avirulent HA but does not activate the fusion activity (26).

The total number of basic amino acids at the cleavage site varies among lethal influenza viruses (Table 1); the minimal number was four as detected in the Chicken/Scotland HA. To examine the importance of the number of basic amino acids, we changed the first two basic amino acids in the series of six basic amino acids of the Ty/Ire HA to neutral (threonine) and acidic (glutaminic acid) amino acids (MT-12) (Table 2). Although a small amount of the cleaved product was detected, the majority of this mutant HA was uncleaved (Fig. 3). Fusion activity of this HA was not detected (Table 2), which suggested that the presence of the first two basic amino acids is necessary in Ty/Ire HA for cleavage activation or that these basic amino acids are unnecessary, but nonbasic amino acids in the same positions prevent cleavage. We, therefore, generated a mutant with these two amino acids deleted, producing a connecting peptide sequence identical to that of the cleavable Ck/Scot/59 HA (Table 1). This mutant HA (MT-2) was not cleaved (Fig. 3). Similarly, neither of the mutant HAs (MT-18 and MT-3) having a deletion of the two amino acids from the partially (MT-1) and completely (MT-13) cleavable mutant HAs were cleaved and, therefore, did not show fusion activity (Table 2).

Lack of susceptibility to cleavage enzyme(s) of the MT-2 HA that contains exactly the same sequence at the cleavage site as the cleavable Ck/Scot HA suggests that there are additional structural feature(s) besides basic amino acids involved in determining cleavage activation and that the structural feature(s) can be modulated by increasing the length of the connecting peptide. These results suggest that the various lengths of the connecting peptide among virulent influenza viruses might be required to accommodate the structural feature(s) such as carbohydrate side chains or conformational changes in the vicinity of the cleavage site.

Table 2. Amino acid sequence at the cleavage site of the SV40-HA recombinant viruses and their biological activities

SV40-HA mutant	Amino acid sequence at the cleavage site							HA cleavage*	RBC-cell fusion†	Polykaryon formation‡
						HA1	HA2			
Parent	Gln	Arg	Lys	Arg	Lys	Lys	Arg/Gly	+	+	+
MT-4	Gln	[ ]	[ ]	Arg	[Glu]	[Thr]	Arg/Gly	−	−	−
MT-16	Gln	Arg	Lys	[Thr]	[Gln]	Lys	Arg/Gly	−	−	−
MT-14	Gln	Arg	Lys	[Thr]	Lys	Lys	Arg/Gly	−	−	−
MT-13	Gln	Arg	Lys	Arg	[Gln]	Lys	Arg/Gly	+	+	+
MT-1	Gln	Arg	Lys	Arg	Lys	[Thr]	Arg/Gly	±	−	−
MT-12	Gln	[Thr]	[Glu]	Arg	Lys	Lys	Arg/Gly	±	−	−
MT-2	Gln	[ ]	[ ]	Arg	Lys	Lys	Arg/Gly	−	−	−
MT-18	Gln	[ ]	[ ]	Arg	[Gln]	Lys	Arg/Gly	−	−	−
MT-3	Gln	[ ]	[ ]	Arg	Lys	[Thr]	Arg/Gly	−	−	−

All strains synthesized HA. The mutated or deleted amino acids are shown in boxes. The slashes indicate the cleavage site.
*+, Cleaved; ±, partially cleaved; −, uncleaved.
†+, Of the adsorbed human RBC >80% were fused; −, no fusion was observed.
‡+, Of cells >80% were fused; −, no polykaryon formation was observed.

DISCUSSION

The avirulent Ck/Penn/83 influenza virus does not conform to the established pattern of other nonpathogenic H5 and H7 influenza virus (Table 1). This virus possesses the same series of basic amino acids found in the connecting peptide as the lethal virus (virulent Ck/Penn/83) that emerged in October 1983 (14). Nucleotide sequence analysis suggested that the critical difference between the HAs of these two H5 viruses was a point mutation that resulted in loss of a glycosylation site from the nonpathogenic strain (14). This was confirmed by direct amino acid sequence analysis (15); the nonpathogenic virus contains a carbohydrate at asparagine-11, whereas the pathogenic virus does not. The proximity of this glycosylation site to the cleaved peptide in the three-dimensional structure of HA suggests that glycosylation at this site might directly interfere with processing and result in a nonpathogenic strain. Similar results have been shown by Rott *et al.* (27), the HA of the H3 variants, having a mutation in the vicinity of the cleavage site in the three-dimensional structure but not in the connecting peptide, is cleaved in the absence of trypsin. With these results, the current study demonstrates that (*i*) the structure of the connecting peptide is crucial for cleavage activation (arginine at the fourth position from the carboxyl terminus is essential, whereas lysine at the third position is not; lysine as the second residue increases susceptibility to cleavage). (*ii*) other structural features of the HA molecule are also involved in cleavage activation; and (*iii*) these structural requirements can be circumvented by insertion of basic amino acids in the connecting peptide.

The nature of the enzyme(s) involved in cleavage activation of the HA is not known. The present study indicated that the specificity of the enzyme(s) is similar to those involved in hormone processing in recognizing more than a single basic amino acid (28). Defective hormones containing a mutation at a pair of basic amino acids at the cleavge site are no longer cleaved by processing enzymes (29, 30), resembling the molecular feature detected at the cleavage site of the HAs of lethal and nonlethal influenza viruses. In the case of hormone processing, the basic amino acids are removed by carboxypeptidase B-like enzymes (28) after cleavage at a pair of basic amino acids by a trypsin-like enzyme. Similar processing has been observed in influenza viruses by direct amino acid sequencing of the cleaved HA *in vivo* (26). Removal of the basic amino acids at the carboxyl-terminus of the HA1 after cleavage may not be essential for influenza viruses, because incomplete processing of these basic amino acids by carboxypeptidase B-like enzymes has been observed (26). Thus, it is likely that lethal influenza viruses utilize the hormone processing system for processing their HA.

Among the 13 HA subtypes of influenza viruses, cleavable HA in tissue culture systems without trypsin has been recognized only in the H5 (12) and H7 (8) HAs. Other HAs so far sequenced contain only a single arginine at the cleavage site (31, 32). It would be worthwhile to determine whether simple addition of multiple basic amino acids at the cleavage site of human influenza viruses is sufficient for cleavage activation or whether some other mutations are also required. This would elucidate the connection between the enzymatic cleavage of HA from human influenza viruses and the lethality of the viruses.

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