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Human Influenza Virus Hemagglutinin with High Sensitivity to Proteolytic Activation

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To examine the prerequisites for cleavage activation of the hemagglutinin of human influenza viruses, a cDNA clone obtained from strain A/Port Chalmers/1/73 (serotype H3) was subjected to site-directed mutagenesis and expressed in CV-1 cells by using a simian virus 40 vector. The number of basic residues at the cleavage site, which consists of a single arginine with wild-type hemagglutinin, was increased by inserting two, three, or four additional arginines. Like wild-type hemagglutinin, mutants with up to three additional arginines were not cleaved in CV-1 cells, but insertion of four arginines resulted in activation. When the oligosaccharide at asparagine 22 of the HA_1 subunit of the hemagglutinin was removed by site-directed mutagenesis of the respective glycosylation site, only three inserted arginines were required to obtain cleavage. Mutants containing a series of four basic residues were also generated by substituting arginine for uncharged amino acids immediately preceding the cleavage site. The observation that these mutants were not cleaved, even when the carbohydrate at asparagine 22 of HA_1 was absent, underscores the fact that the basic peptide had to be generated by insertion to obtain cleavage. The data show that the hemagglutinin of a human influenza virus can acquire high cleavability, a property known to be an important determinant for the pathogenicity of avian influenza viruses. Factors important for cleavability are the number of basic residues at the cleavage site, the oligosaccharide at asparagine 22, and the length of the carboxy terminus of HA_1.

Like many other viral glycoproteins, the hemagglutinin of influenza viruses is activated by proteolytic cleavage. Cleavage which is necessary for the fusion activity of the hemagglutinin and thus for the infectivity of the virus is initiated by host cell proteases, and the presence of an appropriate enzyme determines whether infectious virus is made in a given cell. Proteolytic activation is therefore indispensable for effective virus spread in the infected host and has been found to be a prime determinant for virus pathogenicity. This concept was derived mainly from studies on avian influenza viruses. The pathogenic strains of these viruses are activated by ubiquitous proteases and therefore cause systemic infection, mostly leading to the rapid death of the animal, whereas the apathogenic strains are activated only in epithelial cells of the respiratory or the enteric tract and results only in local infection of these organs. The mammalian influenza viruses, including the human ones, resemble the apathogenic avian strains in possessing hemagglutinins of restricted cleavability and in usually causing local infection of the respiratory tract (23).

Because of its high biological relevance, proteolytic activation has attracted substantial interest, and from a whole series of investigations the following picture emerges concerning the factors that determine the differential cleavability of the hemagglutinin. The prime determinant appears to be the structure linking the cleavage products HA_1 and HA_2 in the uncleaved precursor hemagglutinin. With hemagglutinins of restricted cleavability, the linker usually consists of a single arginine, whereas highly cleavable hemagglutinins have mostly multiple basic residues in this position. This concept was first derived from comparisons of naturally occurring strains (3, 15, 23) and was corroborated when acquisition of high cleavability paralleled by an increase in the number of basic residues at the cleavage site was observed in studies on hemagglutinin mutants generated by site-directed mutagenesis (20) or on virus mutants adapted in vitro to new host cells (26). The second important determinant appears to be a carbohydrate side chain that is present in the vicinity of the cleavage site and interferes with protease accessibility. Loss of this carbohydrate resulted in enhanced hemagglutinin cleavability and viral pathogenicity (8, 19), and the effect of steric hindrance was abolished when the number of basic residues at the cleavage site increased (21, 29). It has also been observed that insertion of a relatively long foreign peptide composed of nonbasic amino acids renders a single arginine at the cleavage site susceptible to the action of ubiquitous cellular proteases (18). Finally, point mutations at some distance from the cleavage site have also been found to increase the spectrum of permissive host cells to a certain degree (30, 35).

As already pointed out, most of these preceding studies were performed with hemagglutinins originating from avian viruses. Even more remarkably, all of the highly cleavable hemagglutinins with multiple basic residues at the cleavage site belong to just 2 of the 14 subtypes known to date, H5 and H7. The question thus arises of whether high cleavability is a specific trait compatible only with the structural features of these two serotypes or whether it may also be acquired by other hemagglutinins. It was of particular interest to find whether this is the case with hemagglutinins of human influenza viruses because of the high pathogenic potential of such a virus. To answer these questions, we cloned the hemagglutinin gene of the human strain A/Port Chalmers/1/73 (H3N2) and prepared mutants with additional arginine residues at the cleavage site. Furthermore, we obtained mutants that lacked the oligosaccharide at asparagine 22 of the HA_1 subunit. Expression of these mutants in monkey kidney cells by using a simian virus 40 (SV40) vector revealed that the hemagglutinin of the human virus can acquire high cleavability by the same mechanisms as the avian hemagglutinins.

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MATERIALS AND METHODS

Cells and viruses. CV-1 cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. The hemagglutinin gene of the A/Port Chalmers/1/73 (H3N2) strain was derived from the MRC-11 reassortant.

Extraction of viral RNA. Influenza virus was grown in 11-day-old embryonated chicken eggs and purified by sedimentation through a 25 to 60% sucrose gradient. RNA was extracted from the purified virus as described previously (29).

Cloning of the hemagglutinin gene. cDNA was synthesized by reverse transcription of MRC-11 RNA by using a cDNA synthesis system (Amersham, London, England). For synthesis of the first cDNA strand, the oligosaccharide primer (12-mer) complementary to the 3′ terminus of the hemagglutinin segment of viral RNA was used. The second DNA strand was synthesized by using *Escherichia coli* DNA polymerase after nicking of the RNA-DNA hybride with *E. coli* RNase H. The termini of the double-stranded DNA were filled in by means of the Klenow fragment of DNA polymerase and ligated with phosphorylated *Kpn*I linker. After cleavage with *Kpn*I, the digestion mixture was subjected to agarose gel electrophoresis and visualized under UV light with ethidium bromide. The band corresponding in size to the hemagglutinin gene was cut out and ligated to pUC18 digested with *Kpn*I (pUC18-HA). White colonies were picked and screened by colony hybridization with a ^{32}P-labeled oligonucleotide probe by the method of Maniatis et al. (27).

Expression of the hemagglutinin gene in CV-1 cells. The SV40 expression vector pA11SVL$_3$ (6) was kindly supplied by D. P. Nayak, University of California, Los Angeles. Hemagglutinin cDNA was excised from plasmid pUC18-HA by *Kpn*I and inserted into the unique *Kpn*I site of pA11SVL$_3$ that is in the region controlled by the late SV40 promoter. pA11SVL$_3$-HA was digested with *Sac*I to separate SV40 DNA from plasmid DNA and was circularized by using T4 DNA ligase at a low DNA concentration to prevent religation of SV40 and plasmid DNA. The circularized DNA mixture (200 ng) was transfected into CV-1 cells together with 200 ng of helper SV40 (*dl*1055) that lacks the early region (32). Transfection was done by using DEAE-dextran, followed by chloroquine treatment at 37°C for 3 h (28). After a 4-day incubation, the transfected cells were dispersed and cultivated for several days until a cytopathic effect typical for SV40 appeared. Cells were then frozen as a source of stock virus.

Site-directed mutagenesis. Plasmid pUC18-HA was digested with *Kpn*I. The 1.7-kb fragment corresponding to the entire hemagglutinin gene was isolated on an agarose gel and then inserted into the double-stranded replicative form of M13mp18 DNA. For oligonucleotide-directed mutagenesis, we used the Amersham system, which is based on the phosphorothioate method (36). The hemagglutinin gene in M13mp18 was sequenced in the region of the mutation by the dideoxy sequencing method with hemagglutinin-specific oligonucleotide primers.

Indirect immunofluorescence. Indirect immunofluorescence of infected cells was performed by using rabbit antiserum against whole MRC-11 influenza virus as the primary antibody and fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG as the secondary antibody.

Assay of cell-cell fusion activity. Monolayers of CV-1 cells were infected with SV40-hemagglutinin recombinants. At 45 h after infection, medium was aspirated from the cells, which were then washed twice with phosphate-buffered saline (PBS). The cells were treated with buffer at pH 5.1 for 2 min and then washed twice with medium lacking serum. They were then incubated for a further 5 h in medium containing serum. They were fixed with ethanol and stained with Giemsa solution.

Radioimmunoprecipitation. At 50 h after infection, cells were incubated for 30 min in Dulbecco modified Eagle medium without methionine. [^{35}S]methionine label was added at 50 μCi/5-cm dish, left on the cultures for 2 h at 37°C, and then chased for 1 h with standard medium. When cells were treated with trypsin, it was added to the culture medium at a concentration of 10 μg/ml during the final 10 min of the labeling period. Hemagglutinin was immunoprecipitated from cell lysates with antiviral antibody and protein A-Sepharose and analyzed on 10% polyacrylamide gels under reducing conditions.

RESULTS

Effect of basic residues at cleavage site on cleavability of H3 hemagglutinin. To establish the identity of the H3 hemagglutinin gene used for site-directed mutagenesis, we performed nucleotide sequence analyses on the respective viral RNA and on the DNA clone derived from it. The viral RNA, which coded for a hemagglutinin polypeptide 566 amino acids long including the N-terminal signal peptide of 16 amino acids, corresponded in its sequence to that reported earlier for the Port Chalmers hemagglutinin (4), except for the four silent nucleotide exchanges T-28 to C, C-38 to T, G-224 to T, and A-621 to G. Comparison of the cloned gene with viral RNA also revealed identity, except for one point mutation resulting from the cloning procedure that involved the exchange of A-1534 to G and the exchange of amino acid 158 in the HA$_2$ subunit from tyrosine to cysteine. This exchange was subsequently repaired in all constructs as described below.

We first made three mutants, which contained inserts of two, three, and four supernumerary arginine residues at the cleavage site. Insertions of the oligonucleotides AGA AGA, AGA AGA AGA, and AGA AGA AGA CGG, located upstream of AGA 1062 to 1064 coding for arginine 329, were made by site-directed mutagenesis with oligonucleotides GAG AAA CAA ACT AGA AGA AGA GGC ATA TTC, GAG AAA CAA ACT AGA AGA AGA AGA GGC ATA TTC, and AAA CAA ACT AGA AGA AGA CGG AGG GGC ATC TTC as primers, respectively. These mutants, which all contained the glycosylation site at asparagine 22 of HA$_1$, were designated I2+, I3+, and I4+. The number of basic residues was also increased by exchanging uncharged amino acids at the cleavage site for arginine. The nucleotide exchanges ACT 1059 to 1061 to AGA generated mutant S1+, in which threonine 328 in HA$_1$ was substituted by arginine, and the nucleotide exchanges CAA ACT 1056 to 1061 to CGA AGA generated mutant S2+, in which the preceding glutamine 327 was also substituted by arginine. These exchanges were performed by using oligonucleotide GAG AAA CAA AGA AGA GGC ATA TTC for mutant S1+ and oligonucleotide CCA GAG AAA CGA AGA AGA GGC ATA TTC for mutant S2+. Examination of mutant and wild-type cDNAs by nucleotide sequence analysis revealed that the desired changes at the cleavage site had occurred. The cDNAs were then inserted into the expression vector pA11SVL$_3$.

Mutant I4+ was inserted into pA11SVL$_3$ in the correct orientation with significantly higher efficiency than the other

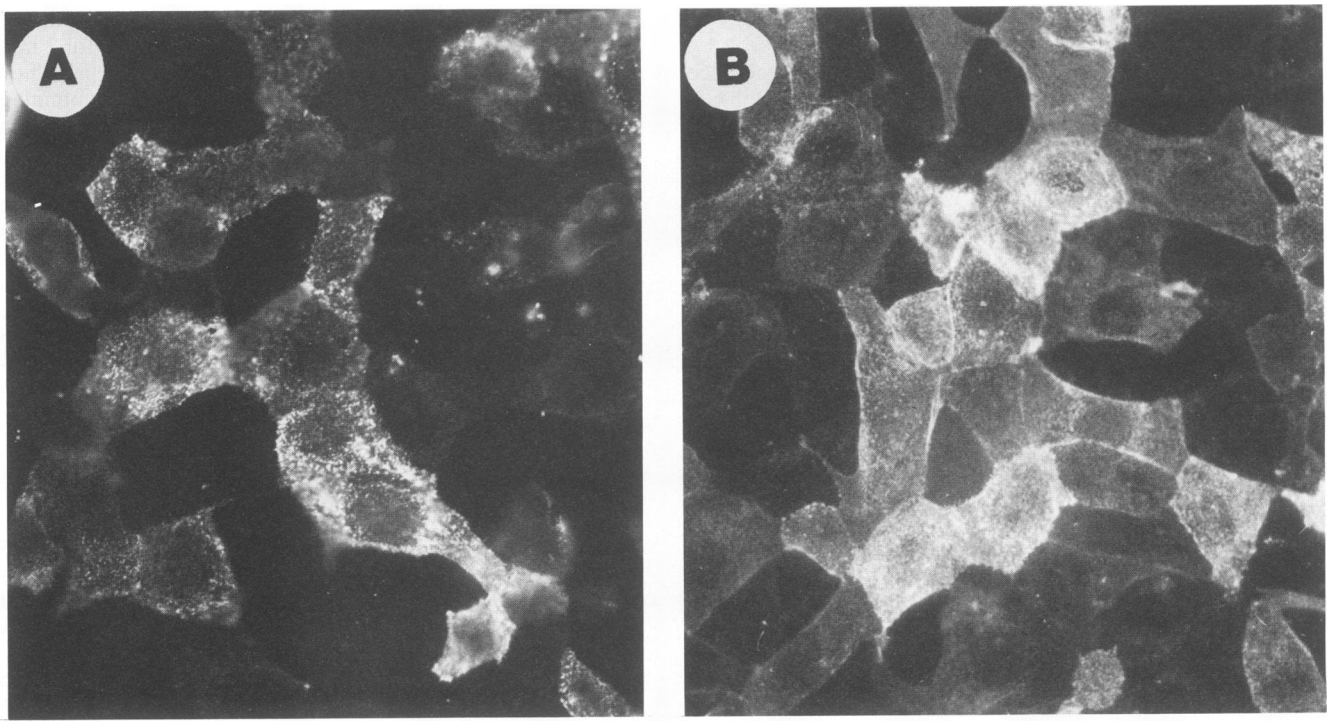

FIG. 1. Immunofluorescent staining of hemagglutinin expressed at the surface of CV-1 cells. CV-1 cells infected with wild-type (A), mutant S2+ (B), and mutant I3− (C) recombinant viruses were fixed with 1% paraformaldehyde under nonpermeabilizing conditions. Indirect immunofluorescence was carried out by using rabbit antiserum against MRC-11 virions and then using fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG.

mutants were. Therefore, I4+ cDNA was used to repair the mutation at amino acid 158 in HA_2 which was created during the cloning procedure. For this purpose, a single nucleotide exchange, TGT 1533 to 1535 to TAT, was introduced that caused cysteine to revert to tyrosine. After confirmation of the desired mutation by sequence analysis, the cDNA was inserted into pA11SVL3. A fragment containing the reversion site was excised by cleavage with *Xho*I and *Aat*II and used for replacement of the corresponding region in the wild type and in mutants I2+, I3+, S1+, and S2+, as well as in mutant I3−, to be described below. The amino acid exchange at position 158 of HA_2 was thus repaired with the wild type and all mutants described so far. CV-1 cells were then coinfected with the constructs and with helper virus, and virus stocks for infection were prepared as described in Materials and Methods.

To analyze processing and biological functions, we expressed wild-type and mutant hemagglutinins in CV-1 cells. Immunofluorescence of nonpermeabilized cells revealed that all hemagglutinins were transported to the plasma membrane, as shown in Fig. 1 for the wild type (Fig. 1A) and mutant S2+ (Fig. 1B). They were able to hemadsorb, indicating that they had receptor-binding activity (not shown). Polyacrylamide gel electrophoresis of radioactively labeled hemagglutinin shows that the wild type and mutants I2+, I3+, S1+, and S2+ were not cleaved in the absence of exogenous protease (Fig. 2A). However, the fraction of hemagglutinin exposed at the cell surface became susceptible to trypsin added to the medium (Fig. 2B). This observation indicates that CV-1 cells lack a suitable protease to cleave these hemagglutinins. Mutant I4+, in contrast, was already cleaved in part in the absence of trypsin (Fig. 2A), and exogenous enzyme was unable to cleave the residual uncleaved fraction (Fig. 2B). This observation indicates that all of the I4+ hemagglutinin that reaches the cell surface has been cleaved by endogenous cellular enzymes. This hemagglutinin was able to induce cell-cell fusion at low pH (Fig. 3C), unlike the wild type and all other mutants described so

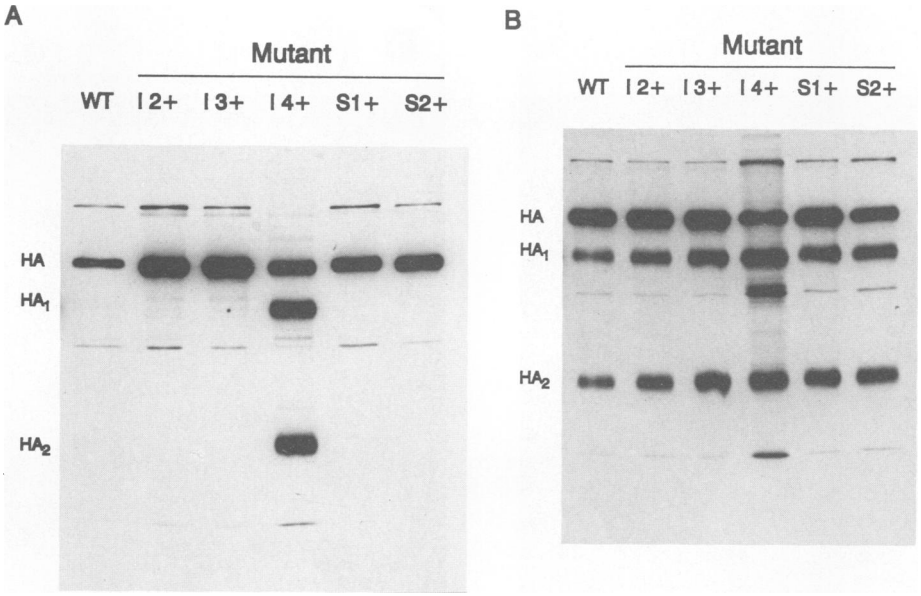

FIG. 2. Proteolytic cleavage of wild-type and mutant hemagglutinins I2+, I3+, I4+, S1+, and S2+ in CV-1 cells. At 50 h after infection with recombinant viruses, CV-1 cells were labeled for 2 h with [^{35}S]methionine. Untreated cells (A) and cells treated with trypsin (10 μg/ml) for 10 min (B) were analyzed by immunoprecipitation and polyacrylamide gel electrophoresis. HA, Hemagglutinin.

far (Fig. 3A and B), which could do so only after trypsin activation (data not shown). These findings indicate that the H3 hemagglutinin acquires cleavability by endogenous CV-1 proteases when four additional arginine residues are inserted at the cleavage site.

Effect of oligosaccharide at asparagine 22 on cleavability of H3 hemagglutinin. To examine the effect of the oligosaccharide attached to asparagine 22 of HA_1, we eliminated the respective glycosylation site by substituting threonine 24 for isoleucine by site-directed mutagenesis. To this end, triplet ACA 147 to 149 was changed to ATA by using I3+ cDNA as a template. The mutated hemagglutinin gene was subcloned into $pA11SVL_3$, and the amino acid exchange at position 158 of HA_2 was repaired as described above. This mutant, which had an insert of three arginines at the cleavage site and lacked the glycosylation site at asparagine 22, was designated I3−. To vary the sequence at the cleavage site, *Cvn*I-*Aat*II fragments were excised from mutants I2+ and S2+ and used for replacement of the corresponding region in $pA11SVL_3$-HA/I3−. The mutants obtained in this way were called I2− and S2−, respectively. Sequence analysis confirmed that the desired mutations had occurred (data not shown).

When the mutants lacking the oligosaccharide at asparagine 22 of HA_1 were expressed in CV-1 cells, they were transported to the cell surface as demonstrated by immunofluorescent staining (Fig. 1C) and hemadsorption (data not shown). Cleavage was examined by polyacrylamide gel electrophoresis following immunoprecipitation of radioactively labeled hemagglutinin (Fig. 4). After trypsin treatment, three polypeptide bands were observed with all mutants, corresponding to HA_1, HA_2, and residual uncleaved hemagglutinin. When compared with mutants I2+, I3+, and S2+, mutants I2−, I3−, and S2− showed higher electrophoretic mobilities in their hemagglutinin and HA_1 bands, reflecting the lack of an oligosaccharide (Fig. 4B). In the absence of trypsin, the hemagglutinin of mutant I3− was cleaved into HA_1 and HA_2 but that of I2− and S2− was not (Fig. 4A). As a consequence, only mutant I3− showed cell fusion activity in the absence of trypsin (Fig. 3D). Thus, in the absence of the carbohydrate at asparagine 22, only three inserted arginine residues were required to make H3 hemagglutinin highly cleavable.

DISCUSSION

Endoproteolytic cleavage at the carboxyl terminus of multiple basic residues is a common posttranslational modification of membrane and secretory proteins. Such proteins include precursors of peptide hormones, neuropeptides, growth factors, the vitamin K-dependent coagulation factors, serum albumins (13), and the insulin receptor (40), as well as the hemagglutinin of the pathogenic avian influenza viruses (23), the F protein of several paramyxoviruses (5, 9, 31, 39), the precursor to the E2 protein of alphaviruses (14, 34), the E2 protein of coronaviruses (2), the envelope glycoprotein of retroviruses (33, 37), the Lassa virus glycoprotein (1), and the gB protein of human cytomegalovirus (38). The enzymes cleaving at multibasic residues have not been identified yet, but it is known that, for example, the protease activating the hemagglutinin of fowl plague virus is Ca^{2+} dependent and membrane bound, and has a neutral pH optimum (22). It has long been known that cleavage of this hemagglutinin occurs within the cell (24), and according to recent evidence, the responsible protease may be located in the *trans*-Golgi network (7). Such enzymes appear to be highly conserved, since the fowl plague virus hemagglutinin is activated not only in virtually all mammalian and avian cells analyzed but also in invertebrate tissues (25). It was therefore interesting to find that a protease resembling these enzymes in its catalytic and other biochemical properties exists in the yeast *Saccharomyces cerevisiae* (12). This Kex2 protease, which has been well characterized on the genetic level and partially purified, can process mammalian hormone and neuropeptide precursors, and genes encoding polypeptides homologous to Kex2 have been discovered in human

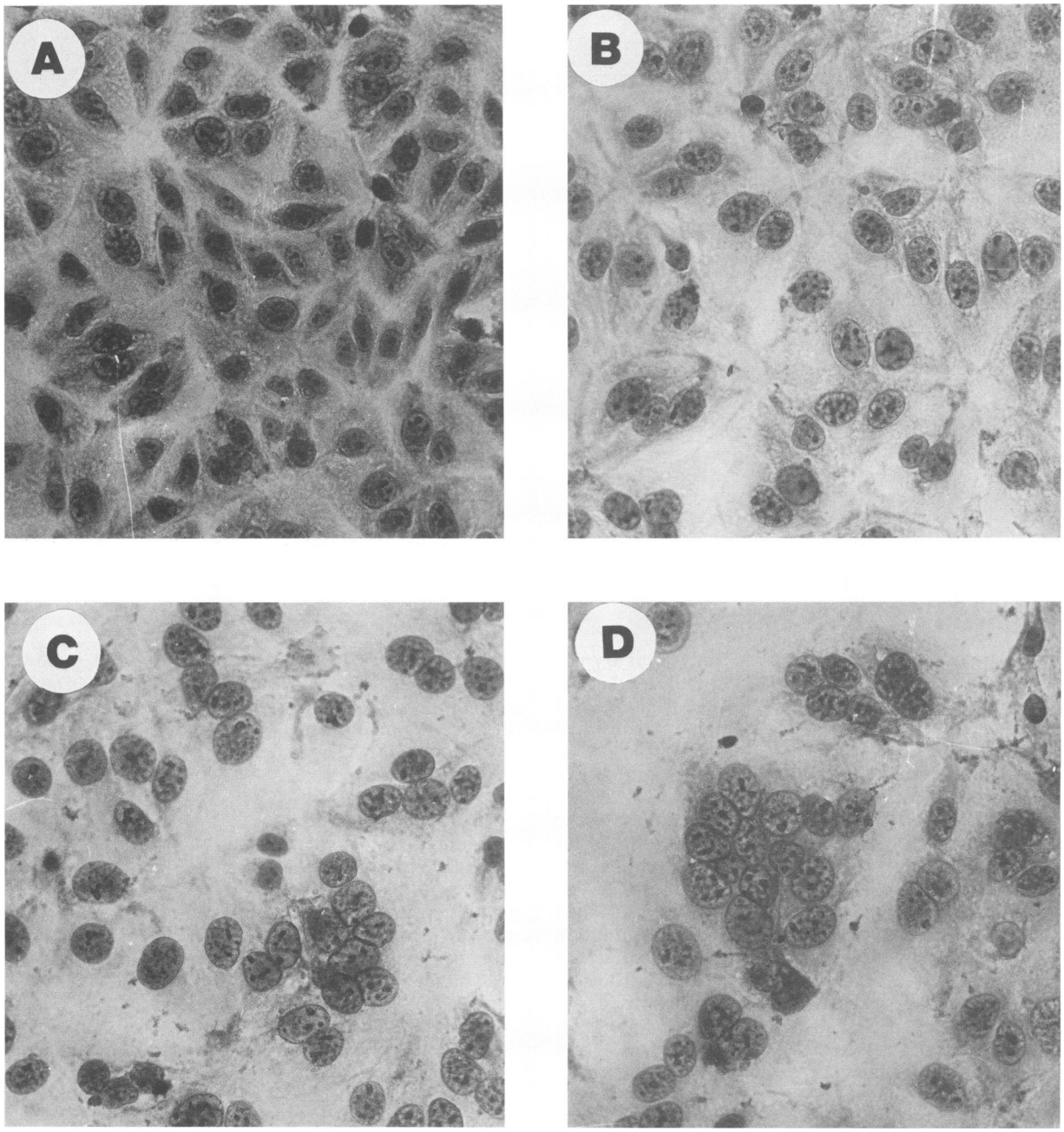

FIG. 3. Cell fusion of CV-1 cells expressing mutants S1+ (A), I3+ (B), I4+ (C), and I3− (D). At 45 h after infection, cells were treated for 2 min with pH 5.1 buffer and incubated for another 5 h in medium containing serum. The cells were then fixed with ethanol and stained with Giemsa solution.

and mouse tissues (11). Although evidence that this enzyme can also cleave the fowl plague virus hemagglutinin has not been obtained yet, it appears that hemagglutinins with multiple basic residues at their cleavage site are activated by a family of ubiquitous proteases, of which Kex2 may be a prototype. However, the H3 hemagglutinin and other hemagglutinins of restricted cleavability are activated by different enzymes, which, according to the information available so far, appear to be present only in epithelial cells. Such an enzyme which activates influenza virus hemagglutinin and F protein of paramyxoviurses at a single arginine residue has been recently isolated from chicken embryos and identified as a protease highly homologous to the blood-clotting factor X, a member of the prothrombin family (17).

In the present study, we have shown that the H3 hemagglutinin of a human influenza A virus can undergo a change in substrate specificity and become sensitive to the ubiquitous proteases. We have done this by designing hemagglutinin mutants that varied in the number of arginine residues at the cleavage site and in the presence of the oligosaccharide at asparagine 22 of HA_1. All of the hemagglutinin mutants appeared at the plasma membrane as demonstrated by

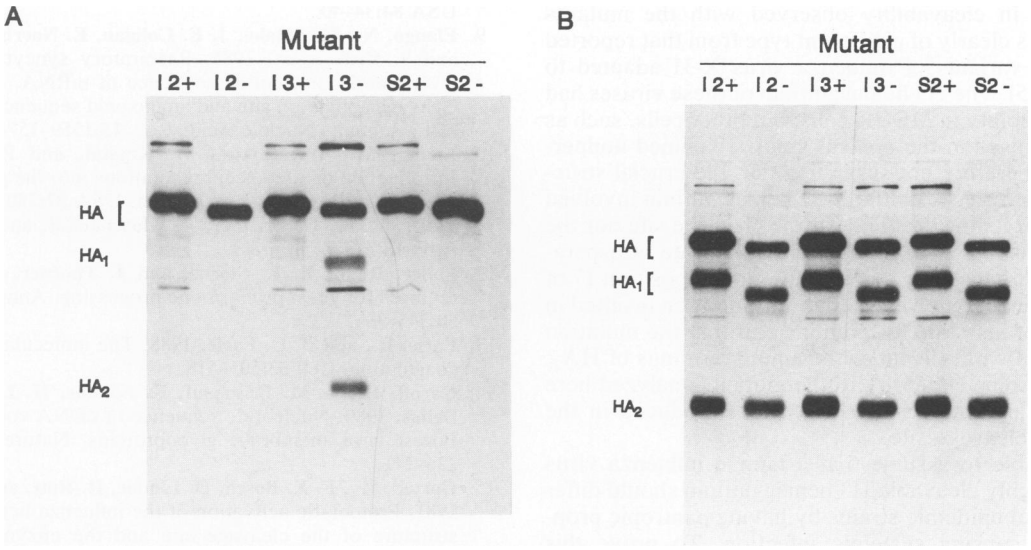

FIG. 4. Proteolytic cleavage of mutants I2−, I3−, and S2−. CV-1 cells were infected, labeled, and analyzed by polyacrylamide gel electrophoresis as described in the legend to Fig. 2. (A) No trypsin treatment. (B) Trypsin treatment. HA, Hemagglutinin.

surface immunofluorescence and hemadsorption, and even the mutants that were not cleaved by the host cell protease could be activated by trypsin added to the culture media. These observations indicate that regular folding and surface transport of the hemagglutinin was not affected by the mutations. The results, as summarized in Table 1, indicate that in the presence of the carbohydrate at asparagine 22, an insertion of four additional arginines, i.e., a total sequence of five arginines, is required at the cleavage site to obtain cleavage in CV-1 cells. This observation is in good agreement with results of Gething et al. (16), who, using the same approach, observed cleavage of another human H3 hemagglutinin after insertion of four arginines, whereas one additional arginine had no effect. It is also clear from our data that the number of inserted basic amino acids can be reduced by one residue, but not by more, when the oligosaccharide at asparagine 22 is missing. Two conclusions can be drawn from these observations. (i) Studies on mutants of the hemagglutinin of serotype H5 (20) and on the F protein of SV5 (31) had suggested that a series of four basic amino acids is the minimal requirement for recognition by the ubiquitous proteases. Our findings support this notion. (ii) By the insertion of an additional arginine at the cleavage site, the masking effect of the oligosaccharide at asparagine 22 of HA_1 can be abolished, exactly as has been observed with the H5 hemagglutinin (21, 29). Thus, interplay between this carbohydrate and the cleavage site is a feature common to H5 and H3 hemagglutinins.

It is interesting that mutant S2− retained restricted cleavability, although, like mutant I3−, it displayed a sequence of four basic amino acids at the cleavage site and the loss of the oligosaccharide at asparagine 22 of HA_1. In contrast to mutant I3−, however, the basic quadruplet was not the result of an insertion, but of substitutions at glutamic acid 327 and threonine 328. Thus, it is clear that the H3 hemagglutinin can acquire high cleavability only if the basic sequence is introduced by an insertion mechanism. Whether the insert improves the accessibility of the cleavage site by bulging, as has been suggested for an H7 hemagglutinin (18), remains to be seen. Our data also indicate that the basic sequence must not be generated at the expense of the carboxy-terminal end of HA_1. Since these amino acids are not conserved, one can conclude that their number is more important than their individual structures. Thus, it appears that the carboxy terminus of HA_1 has a spacer function which, in addition to the basic residues and the oligosaccharide at asparagine 22 of HA_1, is another determinant of cleavability.

TABLE 1. Hemagglutinin variants of influenza virus A/Port Chalmers/1/73 (H3) obtained by site-directed mutagenesis

Hemagglutinin	Oligosaccharide at Asn-22 of HA_1	Cleavage site[a]									Activation in CV-1 cells
		326	327	328					HA_1 329	HA_2 1	
Wild type	+	Lys	Gln	Thr					Arg	Gly	−
Mutant I2+	+	Lys	Gln	Thr			**Arg**	**Arg**	Arg	Gly	−
Mutant I3+	+	Lys	Gln	Thr		**Arg**	**Arg**	**Arg**	Arg	Gly	−
Mutant I4+	+	Lys	Gln	Thr	**Arg**	**Arg**	**Arg**	**Arg**	Arg	Gly	+
Mutant S1+	+	Lys	Gln	**Arg**					Arg	Gly	−
Mutant S2+	+	Lys	**Arg**	**Arg**					Arg	Gly	−
Mutant I2−	−	Lys	Gln	Thr			**Arg**	**Arg**	Arg	Gly	−
Mutant I3−	−	Lys	Gln	Thr		**Arg**	**Arg**	**Arg**	Arg	Gly	+
Mutant S2−	−	Lys	**Arg**	**Arg**					Arg	Gly	−

[a] Inserted and substituted amino acids are indicated by boldface letters.

The change in cleavability observed with the mutants analyzed here is clearly of a different type from that reported previously for variants of influenza virus X-31 adapted to MDCK cells (35). The H3 hemagglutinin of these viruses had acquired cleavability in MDCK cells, but other cells, such as the CV-1 cells used in the present study, remained nonpermissive. It is therefore not surprising that the crucial structural change observed with the X-31 hemagglutinin involved neither the intervening sequence at the cleavage site nor the carboxy terminus of HA_1, nor the carbohydrate at asparagine 22, but was caused by a point mutation at position 17 of HA_1. It is interesting, however, that this mutation resulted in the exchange of histidine for arginine and that the mutation site was located within 1 nm of the amino terminus of HA_2. Thus, there is some similarity to the mutants analyzed here in that a new arginine residue was also introduced in the vicinity of the cleavage site.

It is reasonable to assume that a human influenza virus containing a highly cleavable H3 hemagglutinin should differ from the present epidemic strains by having pantropic properties and by causing systemic infection. To prove this concept would require construction of recombinant virus (10) containing this hemagglutinin, but its unusual pathogenic potential precludes such an approach. Whether human influenza viruses of this kind can occur in nature is another open question. Although this has not been observed so far, our data show that, in principle, it is possible.

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