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Hemagglutinin Activation of Pathogenic Avian Influenza Viruses of Serotype H7 Requires the Protease Recognition Motif R-X-K/R-R

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The hemagglutinin of influenza virus A/FPV/Rostock/34 (H7) was altered at its multibasic cleavage site by site-directed mutagenesis and assayed for proteolytic activation after expression in CV-1 cells. The results indicated that the cellular protease responsible for activation recognizes the tetrapeptide motif R-X-K/R-R that must be presented in the correct sequence position. Studies on plaque variants of influenza virus A/fowl/Victoria/75 (H7N7) showed that alteration of the conserved sequence resulted in a loss of pathogenicity for chickens.

It has been well established that the susceptibility of the hemagglutinin to proteolytic activation by arginine-specific host proteases is essential for the pathogenicity of influenza viruses (1). The hemagglutinins of mammalian and apathogenic avian strains, which cause local infection, are cleaved only in a restricted number of cell types. On the other hand, with the pathogenic avian strains causing systemic infection, hemagglutinin is activated by proteases present in a broad range of different host cells. The key factor that determines the differential cleavability is the structure of the cleavage site of the hemagglutinin. Mammalian and apathogenic avian strains have a single arginine at this site, and plasmin (2), a factor X-like protease from allantoic fluid of chicken eggs (3), and bacterial proteases (4) have been identified as enzymes activating this type of hemagglutinin. The ubiquitous proteases responsible for activation of the hemagglutinin of the pathogenic strains have been less well characterized. They are calcium-dependent and have a neutral pH optimum (5), and they can be inhibited by specific peptidyl chloroalkyl ketones (6). It has long been known that the hemagglutinins activated by these enzymes have multiple lysine and arginine residues at their cleavage sites, and it has been shown that most of these basic amino acids are critical for cleavage activation (7). The exact sequence required for enzyme recognition, however, was not fully understood. In the present study we show that the hemagglutinin cleavage sites of the pathogenic strains have the consensus sequence R-X-K/R-R, and we also present evidence that conserved proline residues in their neighborhood are not important for cleavability.

Comparison of the published hemagglutinin sequences of the pathogenic avian influenza-A-viruses of serotype H7 reveals a number of conserved amino acids upstream of the cleavage site, notably a series of arginine and lysine residues in positions −1 to −6 and two proline residues in positions −7 or −8 and in position −10 (Table 1). To determine whether these conserved regions are important for the cleavability of the H7 hemagglutinin, we subjected a cDNA clone of the hemagglutinin of influenza virus A/FPV/Rostock/34 to site-directed mutagenesis at the cleavage site and from the panel of mutants obtained, we have selected two groups. In the first one, the arginine and lysine residues have been exchanged by each other or have been replaced by noncharged amino acids. This group comprised mutants 21, 13, 22, 7, 23, 1307, KS, 4, and 10. In the second group, comprising mutants Pro-17-Ala, Pro-18-Ala, and Pro-19, the proline residues in positions −8 and −10 have been exchanged (Table 1). To avoid distortions that might be imposed on the protein by insertions or deletions (8), only mutants with substitutions have been used.

To see whether the FPV hemagglutinin mutants were processed by host cell proteases, they were expressed in CV-1 cells using an SV40 vector and analyzed by PAGE after radiolabeling and immunoprecipitation (Fig. 1). Only mutants 21 and KS were cleaved by the endogenous enzyme, as was the case with wild type. However, all hemagglutinin mutants were cleaved by trypsin added to the medium indicating that they were exposed at the cell surface and that lack of cleavage was not the result of a transport defect. Due to release into the medium which was observed with

1 Part of this work was presented at the VIIIth International Congress of Virology in Berlin 1990.
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tion. The amino acid in position -4 of the FPV hemagglutinin has glutamic acid in this position. The amino acid in position -4 of the FPV hemagglutinin has to be arginine; if lysine is present in this position, cleavage is very inefficient as demonstrated by mutant 1307. The lysine residues in positions -5 and -6 are not essential, since they have been replaced in FPV mutant KS and in mutants SC32 and SC35 of strain A/seal/Mass/1/80 (9) by threonine or are missing in strain A/turkey/England/63 without altering cleavability.

Table 1 shows also that mutant 10 which has retained the tetrapeptide motif in a shifted position is no longer cleaved in CV-1 cells. This observation demonstrates that accessibility of the cleavage site is also essential for activation. The recognition motif is present in this mutant in a slightly shifted position and has therefore probably lost its correct steric presentation for the cellular protease. Other factors modulating accessibility of the cleavage site by steric hindrance have also been reported. Thus, interference of a carbohydrate side chain with cleavability has been observed with subtypes H5 (10, 11) and H3 (12). Furthermore, the finding that a hemagglutinin with a single arginine at the cleavage site became highly cleavable after in-
Fusion was observed with wild type and most mutants, trypsin if not cleaved by the endogenous protease, and fowl/Victoria/75 (H7N7) a number of variants with re-

tions +1 and +2 (14) and amino acid exchanges in the 

tions -2 and -1. Compatible with previous studies by 

It was then of interest to find out if the mutant hemagglutinins were able to fuse after cleavage. Hemagglutinin was therefore expressed in CV-1 cells, treated with trypsin if not cleaved by the endogenous protease, and examined for its capacity to induce cell fusion after exposure to low pH. Except for mutants 4 and 10, cell fusion was observed with wild type and most mutants, regardless as to whether they were activated by trypsin or the CV-1 cell enzyme (Fig. 2). The inability of mutants 4 and 10 to induce fusion demonstrates that hemagglutinin is not activated if it is cleaved between positions -2 and -1. Compatible with previous studies by us and others showing that cleavage between positions +1 and +2 (14) and amino acid exchanges in the amino terminal sequence of HA, (15) do not result in activation, this observation underlines the concept that the fusion peptide has a highly specific structure.

The studies employing site-directed mutagenesis on hemagglutinin cDNA have been extended by an analysis of influenza virus variants with altered hemagglutinin cleavability. In previous work, pathogenic variants with increased hemagglutinin cleavability could be obtained, when apathogenic avian influenza virus strains were adapted to non-permissive host cells (11, 9, 16). We have now derived from the pathogenic strain A/ fowl/Victoria/75 (H7N7) a number of variants with restricted hemagglutinin cleavability. This was accomplished by selecting plaque variants that were able to undergo multiple replication cycles in MDCK cells and in CEC only in the presence of trypsin (Fig. 3A). Whereas chickens died 4–6 days after infection with wild-type virus, birds infected with the plaque variants survived the observation period of 14 days without showing disease symptoms. Thus, the plaque variants had lost pathogenicity for chickens. As had to be expected from these observations, the mutants were resistant to the endogenous protease present in CEC, whereas wild-type hemagglutinin was readily cleaved in these cells (Fig. 3B). RNA sequence analysis of the Victoria hemagglutinin and its variants demonstrated

**Fig. 1.** Proteolytic processing of FPV hemagglutinin obtained by site-directed mutagenesis. For mutagenesis, the cDNA of the HA gene of fowl plaque virus (A/FPV/Rostock/34 H7N1) was available in the cloned form inserted into M13mpl 1 (33). Mutagenesis was done with a commercial kit (Amerham, Braunschweig, Germany) based on the phosphorothionate method (34) using ssDNA of M13mpl 1-HA as template and the following oligonucleotides: 5'ACT GGG ATG AAG AAC GTT C/GGC GAA C/GCT TCC3' (1000–1030), 5'CCT TCC AAA C/AA A/GGG AAA AGA GCC GTG3(1024–1054), 5'CCT TCC AAA AGA AGG AAA AGA G3(1024–1038), and 5'AGG AAA/C AA/G/A C/GAG/GA GGC CTG3(1036–1054). Mutations were screened by G- or A-track analysis, respectively. HA genes containing the desired mutations were then analyzed using the di-deoxynucleotide chain-termination sequencing technique (35). Of the 26 different mutants obtained, 10 were selected for vectorial expression in CV-1 cells. Except for the desired mutations, they contained no other amino acid exchanges. The mutated HA genes were excised from the phage replicative form DNA with BglII and ligated into the compatible BamHI site of the simian virus 40 expression vector pA1 lSVl3. Subconfluent CV-1 cells, which were grown in Dulbecco’s modified Eagle’s medium (DMEM) in the presence of 10% fetal calf serum, were cotransfected with the SV40-HA recombinant genomes and the SV40 helper genomes using either DEAE-Dextran or Lipofectin (BRL, Berlin, Germany). 56 hr after infection with FPV HA recombinant SV40, CV 1 cells were incubated in DMEM without methionine for 60 min. [35S]Methionine (100 μCi/ml) was added to the cell cultures for 15 min at 37°. The radioactive labeling was chased by replacement of the radioactive medium by DMEM containing 10 mM I-methionine at 37° for 60 min before the cells were solubilized by adding RIPA buffer at 0°. The hemagglutinin was immunoprecipitated from the cell lysates by standard procedures using an anti-FPV serum raised in rabbits, separated by SDS-

**Fig. 2.** Mutations in the cleavage site of hemagglutinin. Mutations were screened by G- or A-track analysis, respectively. HA genes containing the desired mutations were then analyzed using the di-deoxynucleotide chain-termination sequencing technique (35). Of the 26 different mutants obtained, 10 were selected for vectorial expression in CV-1 cells. Except for the desired mutations, they contained no other amino acid exchanges. The mutated HA genes were excised from the phage replicative form DNA with BglII and ligated into the compatible BamHI site of the simian virus 40 expression vector pA1 lSVl3. Subconfluent CV-1 cells, which were grown in Dulbecco’s modified Eagle’s medium (DMEM) in the presence of 10% fetal calf serum, were cotransfected with the SV40-HA recombinant genomes and the SV40 helper genomes using either DEAE-Dextran or Lipofectin (BRL, Berlin, Germany). 56 hr after infection with FPV HA recombinant SV40, CV 1 cells were incubated in DMEM without methionine for 60 min. [35S]Methionine (100 μCi/ml) was added to the cell cultures for 15 min at 37°. The radioactive labeling was chased by replacement of the radioactive medium by DMEM containing 10 mM I-methionine at 37° for 60 min before the cells were solubilized by adding RIPA buffer at 0°. The hemagglutinin was immunoprecipitated from the cell lysates by standard procedures using an anti-FPV serum raised in rabbits, separated by SDS-

**Fig. 3.** Proteolytic processing of FPV hemagglutinin obtained by site-directed mutagenesis. For mutagenesis, the cDNA of the HA gene of fowl plaque virus (A/FPV/Rostock/34 H7N1) was available in the cloned form inserted into M13mpl 1 (33). Mutagenesis was done with a commercial kit (Amerham, Braunschweig, Germany) based on the phosphorothionate method (34) using ssDNA of M13mpl 1-HA as template and the following oligonucleotides: 5'ACT GGG ATG AAG AAC GTT C/GGC GAA C/GCT TCC3' (1000–1030), 5'CCT TCC AAA C/AA A/GGG AAA AGA GCC GTG3(1024–1054), 5'CCT TCC AAA AGA AGG AAA AGA G3(1024–1038), and 5'AGG AAA/C AA/G/A C/GAG/GA GGC CTG3(1036–1054). Mutations were screened by G- or A-track analysis, respectively. HA genes containing the desired mutations were then analyzed using the di-deoxynucleotide chain-termination sequencing technique (35). Of the 26 different mutants obtained, 10 were selected for vectorial expression in CV-1 cells. Except for the desired mutations, they contained no other amino acid exchanges. The mutated HA genes were excised from the phage replicative form DNA with BglII and ligated into the compatible BamHI site of the simian virus 40 expression vector pA1 lSVl3. Subconfluent CV-1 cells, which were grown in Dulbecco’s modified Eagle’s medium (DMEM) in the presence of 10% fetal calf serum, were cotransfected with the SV40-HA recombinant genomes and the SV40 helper genomes using either DEAE-Dextran or Lipofectin (BRL, Berlin, Germany). 56 hr after infection with FPV HA recombinant SV40, CV 1 cells were incubated in DMEM without methionine for 60 min. [35S]Methionine (100 μCi/ml) was added to the cell cultures for 15 min at 37°. The radioactive labeling was chased by replacement of the radioactive medium by DMEM containing 10 mM I-methionine at 37° for 60 min before the cells were solubilized by adding RIPA buffer at 0°. The hemagglutinin was immunoprecipitated from the cell lysates by standard procedures using an anti-FPV serum raised in rabbits, separated by SDS-PAGE under reducing conditions, and analyzed by fluorography. Of the two HA bands seen in many samples, the faster migrating one is endoglucoaminidase H-sensitive (data not shown) and, thus, represents hemagglutinin with immature carbohydrate side chains.
that, besides a silent mutation in variant V3, only mutations had occurred that resulted in amino acid exchanges at the cleavage site. These were the exchange of arginine at position -4 for lysine with variants V1, V2, and V3 and the exchange of lysine at position -2 for glutamic acid with variants V4 and V5 (Table 1). These observations further substantiate the requirement of the consensus sequence R-X-K/R-R for high cleavability and they show that alteration of this motif results in a decrease of pathogenicity.

This sequence is not only found with all pathogenic H7 viruses, but also with most pathogenic H5 strains (7). Furthermore H3 hemagglutinin acquires high cleavability after insertion of this motif by site directed mutagenesis (15, 12). The only exception are pathogenic A/chick/Pennsylvania/83 isolates which have the unusual tetrapeptide K-K-K-R at the cleavage site (7). It thus appears that in this particular case the hemagglutinin structure allows a lysine in position -4. The motif R-X-K/R-R is also found in the F protein of paramyxovi-
A pathogenic variants of influenza virus A/fowl/Victoria/75 with altered hemagglutinin cleavability. A pathogenic variants were prepared by infecting CEC with egg-grown wild-type virus at a m.o.i. of 1–0.001 PFU/cell in the presence of trypsin (5 μg/ml). Progeny virus was subjected to a plaque passage in CEC. Three days after incubation in the absence of trypsin, visible plaques were marked, and a second overlay containing 10 μg/ml trypsin was added to the dishes. Additional plaques were picked 3 days later and subjected to another round of growth in CEC cultures followed by plaque passage with and without trypsin as before. This procedure was repeated several times, until generation of plaques depended completely on the presence of trypsin. Five variants designated V1–V5, were obtained in this way. (A) Virus replication in the absence (a) and presence (0) of trypsin of wild type and variants V3 and V5 in CEC cultures infected at a m.o.i. of 0.0001 PFU/cell. (B) Hemagglutinin cleavage of wild type and variants V1–V5 in CEC. Hemagglutinin was labeled with [35S]methionine and, after immunoprecipitation with an H7-specific monoclonal antibody, analyzed by PAGE.

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