Hemagglutinin Activation of Pathogenic Avian Influenza Viruses of Serotype H7 Requires the Protease Recognition Motif R-X-K/R-R[1]

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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 consensus sequence resulted in a loss of pathogenicity for chickens. © 1992 Academic Press, Inc.

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_{317}-Ala, Pro_{315}-Ala, and Pro^{-2}, 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

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TABLE 1

MULTIBASIC CLEAVAGE SITES OF H7 HEMAGGLUTININS

Virus/mutant	HA1 -10	-9	-8	-7	-6	-5	-4	-3	-2	-1	HA2 +1	High cleavability	Ref.
A/FPV/Rostock/34	Pro	Glu	Pro	Ser	Lys	Lys	Arg	Glu	Lys	Arg	Gly	+	(36)
A/FPV/Rostock/34	Pro	Glu	Pro	Ser	Lys	Lys	Arg	Lys	Lys	Arg	Gly	+	
Mutant 21	Pro	Glu	Pro	Ser	Lys	Lys	Arg	Asn	Lys	Arg	Gly	+	
Mutant 13	Pro	Glu	Pro	Ser	Lys	Lys	Arg	Lys	Asn	Arg	Gly	−	
Mutant 22	Pro	Glu	Pro	Ser	Lys	Lys	Arg	Lys	Lys	Lys	Gly	−	
Mutant 7	Pro	Glu	Pro	Ser	Lys	Lys	Arg	Asn	Arg	Lys	Gly	−	
Mutant 23	Pro	Glu	Pro	Ser	Lys	Lys	Gly	Lys	Lys	Arg	Gly	−	
Mutant 1307	Pro	Glu	Pro	Ser	Lys	Lys	Lys	Lys	Lys	Arg	Gly	−	
Mutant KS	Pro	Glu	Pro	Ser	Lys	Asn	Arg	Lys	Lys	Arg	Gly	+	
Mutant 4	Pro	Glu	Pro	Ser	Lys	Lys	Arg	Lys	Arg	Gly	Gly	−	
Mutant 10	Pro	Glu	Pro	Ser	Lys	Arg	Arg	Lys	Arg	Gly	Gly	−	
Mutant Pro$_{317}$-Ala	Pro	Glu	Ala	Ser	Lys	Lys	Arg	Lys	Lys	Arg	Gly	+	
Mutant Pro$_{315}$-Ala	Ala	Glu	Pro	Ser	Lys	Lys	Arg	Lys	Lys	Arg	Gly	+	
Mutant Pro^{2-}	Ala	Glu	Ala	Ser	Lys	Lys	Arg	Lys	Lys	Arg	Gly	+	
A/Fowl/Victoria/75	Pro	Glu	Ile	Pro	Lys	Lys	Arg	Glu	Lys	Arg	Gly	+	
Mutants V1, V2, V3	Pro	Glu	Ile	Pro	Lys	Lys	Lys	Glu	Lys	Arg	Gly	−	
Mutants V4, V5	Pro	Glu	Ile	Pro	Lys	Lys	Arg	Glu	Glu	Arg	Gly	−	
A/FPV/Dutch/27	Pro	Glu	Leu	Pro	Lys	Lys	Arg	Arg	Lys	Arg	Gly	+	
A/Carduelis/Germany/72	Pro	Glu	Pro	Ser	Lys	Lys	Arg	Lys	Lys	Arg	Gly	+	
A/Chick/Victoria/85	Pro	Glu	Ile	Pro	Lys	Lys	Arg	Glu	Lys	Arg	Gly	+	(37)
A/Starling/Victoria/85	Pro	Glu	Ile	Pro	Lys	Lys	Arg	Glu	Lys	Arg	Gly	+	(37)
A/Turkey/England/63	Pro	Glu	Thr	Pro	—	Lys	Arg	Arg	Arg	Arg	Gly	+	
A/Seal/Mass/1/80	Pro	Glu	Asn	Pro	Lys	Thr	—	—	—	Arg	Gly	−	(9)
Mutant SC 32	Pro	Glu	Asn	Pro	Lys	Thr	Arg	Gly	Arg	Arg	Gly	+	
Mutant SC 35	Pro	Glu	Asn	Pro	Lys	Thr	Arg	Arg	Arg	Arg	Gly	+	

Note. Amino acid exchanges observed with the mutants analyzed in this study are underlined.

wild type as well as all mutants (unpublished results), HA$_1$ was sometimes underrepresented when compared to HA$_2$.

As summarized in Table 1, the following conclusions can be drawn from the data described so far. The results obtained with mutants 7, 13, 21, 22, and 23 indicate that the motif Arg$_{-4}$-X$_{-3}$-Lys/Arg$_{-2}$-Arg$_{-1}$ is required for proteases present in CV-1 or equivalent cells to recognize the FPV hemagglutinin as an appropriate substrate. Arginine is obligatory in position −1, since mutants 7 and 22 which have lysine in this position, are not cleaved. Lysine present mostly in position −2 can be replaced by arginine as is the case with A/turkey/England/63, but not by nonbasic amino acids, such as asparagine in mutant 13 or glutamic acid in mutants V4 and V5 of A/fowl/Victoria/75 (see below). The amino acid in position −3 may be basic, acidic, or neutral as indicated by mutant 21 and the hemagglutinin of another FPV isolate that have 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-

sertion of a bulging peptide suggests also a mechanism of steric alteration (8).

Finally, the results obtained on mutants Pro_{317}-Ala, Pro_{315}-Ala, and Pro^{2-} indicate that the proline residues in positions −8 and −10 are not essential for cleavability (Table 1). Since proline is known to have distinct effects on peptide folding and since the proline residues in positions −8 and particularly in position −10 are conserved, this observation was unexpected. It is also remarkable in the light of the finding that the F protein of Sendai virus acquires high cleavability when a proline residue is introduced at position −2 next to lysine or arginine at position −1 of the cleavage site (13). It will be interesting to see whether or not the enzymes recognizing the motifs R-X-K/R-R and P-K/R which both appear to be ubiquitous belong to the same type of proteases.

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_2 (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,
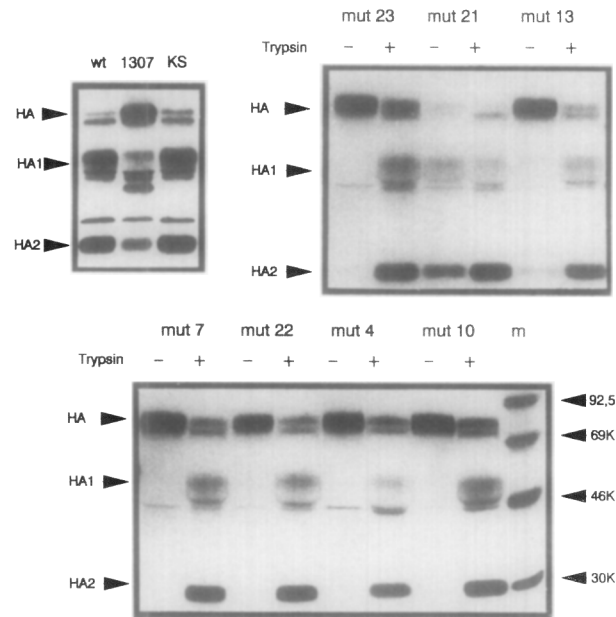

FIG. 1. Proteolytic processing of FPV hemagglutinin obtained by site-directed mutagenesis. For mutagenesis, the cDNA of the HA gene of fowl plague virus (A/FPV/Rostock/34 H7N1) was available in the cloned form inserted into M13mp11 (33). Mutagenesis was done with a commercial kit (Amersham, Braunschweig, Germany) based on the phosphorothioate method (34) using ssDNA of M13mp11-HA as template and the following oligonucleotides: 5'ACT GGG ATG AAG AAC GTT C/GCC GAA C/GCT TCC3' (1000–1030), 5'CCT TCC AAA/C AAA/C A/GGG AAA AAA AGA GGC GTG3'(1024–1054), 5'CCT TCC AAA AGA AGG AAA AGA GG3'(1024–1038), and 5'AGG AAA/C AA/G/A/C A/GA/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 dideoxynucleotide 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 pA11SVl3. 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. [^{35}S]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 l-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 endoglucosaminidase H-sensitive (data not shown) and, thus, represents hemagglutinin with immature carbohydrate side chains.

whereas wild-type hemagglutinin was readily cleaved in these cells (Fig. 3B). RNA sequence analysis of the Victoria hemagglutinin and its variants demonstrated
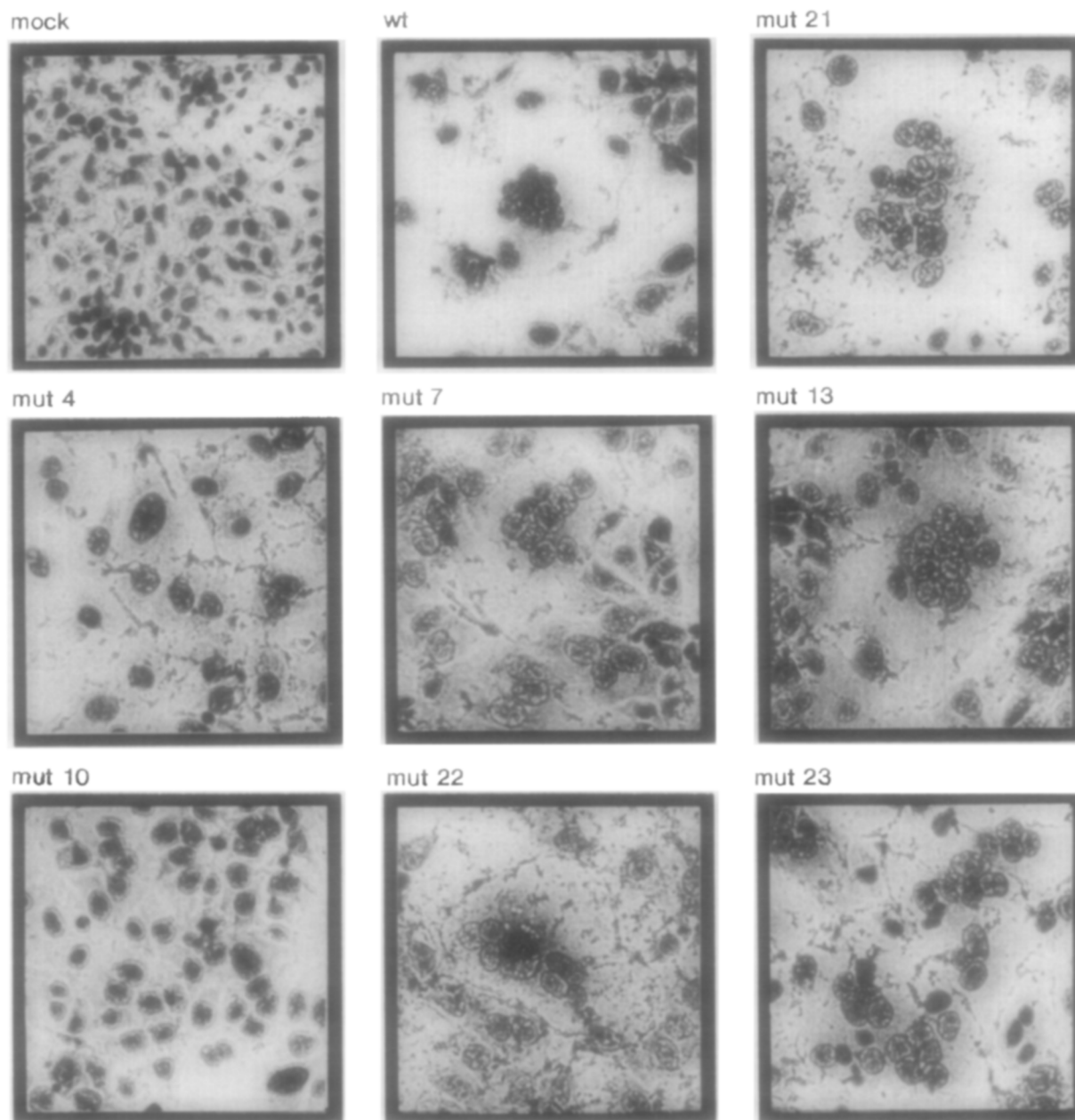
FIG. 2. Cell–cell fusion activity of FPV hemagglutinin mutants obtained by site-directed mutagenesis. Monolayers of CV-1 cells were infected with SV40 HA-recombinant viruses. 45 hr after infection, the cells were freed from the medium, washed twice with PBS, and treated with prewarmed pH5 medium (10 mM MES, 10 mM HEPES in PBS) for 3 min. Then the cells were washed twice with DMEM lacking serum and incubated further in DMEM containing serum for 5 hr. The cells were fixed and stained with Giemsa solution. The results obtained with wild-type hemagglutinin (wt) and the mutants indicated are shown.

that, besides a silent mutation in variant V3, only mutations had occurred that resulted in amino acid exchanges at the cleavage site. These was 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-
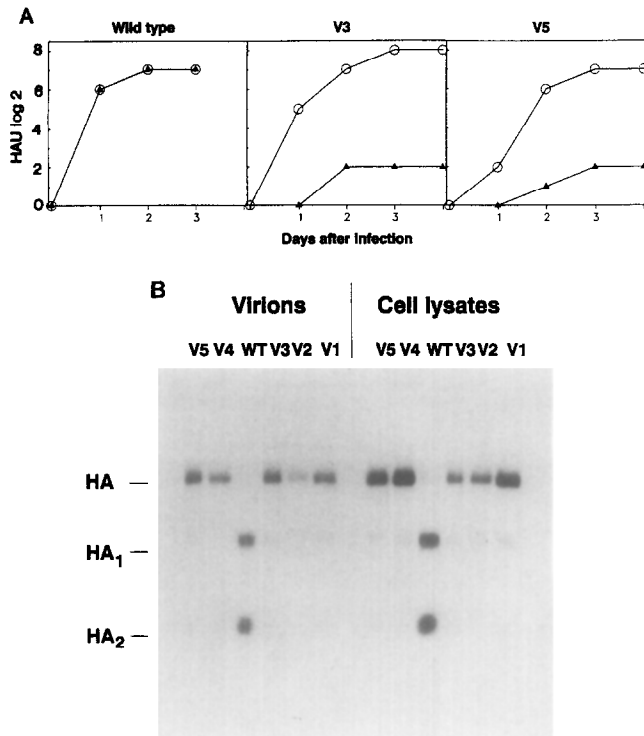
FIG. 3. Apathogenic variants of influenza virus A/fowl/Victoria/75 with altered hemagglutinin cleavability. Apathogenic variants were prepared by infecting CEC with egg-grown wild-type virus at a m.o.i. of 1–0.001 PFU/cell in the presence of trypsin (5 μg/ml). Progeny virus was subjected to a plaque 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 (▲) and presence (O) 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.

ruses (17) and with other highly cleavable glycoproteins present in togaviruses (18), coronaviruses (19), retroviruses (20), and cytomegalovirus (21). Deviations from this motif are not compatible with high cleavability, as shown with Rous sarcoma virus (22), HIV (23), SV5 (24), and NDV (25). The characteristic basic motif is not restricted to viral glycoproteins. It is also present in a variety of cellular proteins which are most likely activated by the same type of enzymes. Examples of such precursor proteins are insulin receptor (26), insulin-like growth factor I receptor (27), von Willebrand factor (28), and uvomorulin (29). All of these proteins do not show sequence homology to the influenza virus hemagglutinin downstream the cleavage site (positions +1, +2, +3, etc.) Thus, it appears that this region,

although highly conserved among the different hemagglutinin serotypes, is not important for the consensus sequence of the cleavage site.

The ubiquitous protease responsible for the activation of the hemagglutinins containing several basic amino acids has not been identified yet. In previous studies it has been shown to be a Ca^{2+}-dependent membrane-bound enzyme with a neutral pH optimum (5). In these properties it resembles the Kex2 protease from yeast that cleaves pro-α factor and pro-killer toxin at Lys-Arg and Arg-Arg sites (30). An analogue found in mammalian tissues is furin, a human precursor protein-processing endoprotease with sequence homology and shared topology to the Kex2 protease (31). Furin is localized in the Golgi compartment, is also Ca^{2+}-dependent and membrane-associated. Its substrate specificity is also characterized by recognition of the basic tetrapeptide motif described above (32). Whether furin is the protease activating viral proteins of the FPV hemagglutinin type is the subject of current studies.

ACKNOWLEDGMENTS

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