PROTEOLYTIC ACTIVATION OF INFLUENZA VIRUSES:

SUBSTRATES AND PROTEASES

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INTRODUCTION

Like many other viral glycoproteins, the hemagglutinin (HA) 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 exerted 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 has been derived mainly from studies on avian influenza viruses. The pathogenic strains of these viruses are activated by ubiquitous proteases and cause therefore systemic infection mostly leading to rapid death of the animal, whereas activation of the apathogenic strains occurs only in epithelial cells of the respiratory or the enteric tract resulting in local infection of these organs. The mammalian influenza viruses, including the human ones, resemble the apathogenic avian strains in possessing also hemagglutinins of restricted cleavability and in causing usually local infection of the respiratory tract.¹

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 HA₁ and HA₂ in the uncleaved precursor HA. With hemagglutinins of restricted cleavability, the linker consists usually 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^{1,2,3} and has later been further corroborated, when acquisition of high cleavability paralleled by an increase in the number of basic residues at the cleavage site has been observed in studies on hemagglutinin mutants generated by site-directed mutagenesis⁴ or on virus mutants adapted *in vitro* to new host cells.⁵

We will review here recent studies and work in progress from our group on proteolytic

activation of the influenza hemagglutinin. Information will be presented on the structural features of the hemagglutinin responsible for its differential cleavability and on the cellular activating proteases.

THE PROTEASE RECOGNITION MOTIF OF HEMAGGLUTININ WITH HIGH CLEAVABILITY IS ARG-X-LYS/ARG-ARG

Although it has been known for some time that high cleavability of the hemagglutinin depends on cleavage sites with multiple basic residues, it was not clear whether these amino acids have to be present in a specific consensus sequence. To answer this question, we have modulated the cleavage site of the hemagglutinin of fowl plague virus (FPV) by subjecting an M13mp11 cDNA insert to site-directed mutagenesis using the phosphorothioate method. The mutants were analyzed for cleavability in CV1 cells using the SV40 expression vector pA11SVL₃. The results are shown in Table 1. The data obtained with H7 hemagglutinin mutants 7, 13, 21, 22 and 23 indicate that a defined motif of four amino acids, of which three are basic ones, is required for proteases to recognize FPV hemagglutinin as an appropriate substrate. In general, the tetrapeptide contains arginine at position 324-HA₁, arginine or lysine at position 323-HA₁, and arginine at positions 321-HA₁. This is evidenced by the results obtained with mutants 22 and 7, which demonstrate that arginine is obligatory at position 324-HA₁. Position 323-HA₁ has to be occupied either by arginine or lysine, as an uncharged amino acid at this position presented by mutant 13 led to a hemagglutinin, which behaves like that with a single arginine at the cleavage site. The amino acid at position 322-HA₁ may be basic, acidic or neutral, as indicated by mutant 21 and the hemagglutinin of another FPV isolate, which has a glutamic acid in this position. The amino acid at position 321-HA₁ has to be basic again as indicated by mutant 23. Although it has not been tested yet for the FPV hemagglutinin whether arginine is absolutely necessary or whether it can be replaced by lysine, it is clear from the published data that the hemagglutinins of all H7 and most H5 strains with a multiple basic cleavage site contain arginine at position 321-HA₁. The only exception of these rules is the hemagglutinin of the strain A/chick/Pennsylvania/83, which contains the unusual tetrapeptide Lys-Lys-Lys-Arg at the cleavage site.⁶ However it is not yet clear, whether such a motif is recognized by the same endoproteases activating all the other highly cleavable H5 and H7 hemagglutinins or whether it is the particular structure of this H5 hemagglutinin that allows cleavage by another wide-spread protease.

Mutant 10 contains a correct consensus sequence, yet shifted by one amino acid in N-terminal direction. This mutant is not cleaved in CV1 cells. Thus, the multibasic motif has to be in the correct position to be recognized by the cellular enzyme. However, mutant 10 and the related mutant 4 are cleaved by trypsin, as are mutants 7, 13, 21, 22, and 23. In contrast to the latter ones, mutants 4 and 10 do not induce cell fusion after trypsin activation (data not shown). This finding confirms previous observations^{3,7} indicating that activation of fusion requires cleavage at the correct position, i.e. between amino acids 324-HA₁ and 1-HA₂.

MODULATION OF THE ACCESSIBILITY OF THE CLEAVAGE SITE BY CARBOHYDRATE

Besides the number and sequence of basic residues at the cleavage site, other factors determining cleavability have also been identified. Thus, it has been observed that insertion of a relatively long foreign peptide composed of non-basic amino acids renders a single arginine at the cleavage site susceptible to the action of ubiquitous cellular proteases.⁸ Also,

Table 1. Hemagglutinin variants of influenza virus A/FPV/Rostock/34 (H7) obtained by site-directed mutagenesis.

Hemagglutinin	Oligosaccharides at Asn 12 of HA ₁	HA ₁ 315 316 317 318 319 320 321 322 323 324	Cleavage site HA ₂ 1	Activation in CV1 cells
Wild type	+	Pro-Glu-Pro-Ser-Lys-Lys-Arg-Lys-Lys-Arg	Gly	+
Mut 21	+	Pro-Glu-Pro-Ser-Lys-Lys-Arg- <u>Asn</u> -Lys-Arg	Gly	+
Mut 13	+	Pro-Glu-Pro-Ser-Lys-Lys-Arg-Lys- <u>Asn</u> -Arg	Gly	-
Mut 22	+	Pro-Glu-Pro-Ser-Lys-Lys-Arg-Lys-Lys- <u>Lys</u>	Gly	-
Mut 7	+	Pro-Glu-Pro-Ser-Lys-Lys-Arg- <u>Asn</u> - <u>Arg</u> - <u>Lys</u>	Gly	-
Mut 23	+	Pro-Glu-Pro-Ser-Lys-Lys- <u>Gly</u> -Lys-Lys-Arg	Gly	-
Mut 4	+	Pro-Glu-Pro-Ser-Lys-Lys-Arg-Lys- <u>Arg</u> - <u>Gly</u>	Gly	-
Mut 10	+	Pro-Glu-Pro-Ser-Lys- <u>Arg</u> -Lys- <u>Arg</u> - <u>Gly</u>	Gly	-

Substitutions of amino acids are indicated by underlining.

point mutations at some distance from the cleavage site have been found to increase the spectrum of permissive host cells to a certain degree, too.^{9,10} Another important determinant is 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 pathogenicity of the avian influenza virus A/chick/Pennsylvania/83 (H5N2).^{11,12}

The interaction of this oligosaccharide with the cleavage site has been further corroborated in a study employing plaque variants of the apathogenic strain A/chick/Pennsylvania/1/83 (Table 2). Unlike the wild type, the plaque variants contain a hemagglutinin that is cleaved in chick embryo cells and MDCK cells. The variants differ also from the wild type by their pathogenicity for chickens. Nucleotide sequence and oligosaccharide analysis of the hemagglutinin has revealed that, unlike natural isolates with increased pathogenicity,¹² the variants obtained *in vitro* have retained an oligosaccharide at asparagine 11 that is believed to interfere with the cleavage site of the wild type. However, all variants showed mutations in the hemagglutinin resulting in an increased number of basic groups at the cleavage site. These observations demonstrate that masking of the cleavage site by an oligosaccharide is overcome by an enhancement of the basic charge at the cleavage site.

HEMAGGLUTININ OF HUMAN INFLUENZA VIRUS ACQUIRES HIGH SENSITIVITY TO PROTEOLYTIC ACTIVATION AFTER MUTATION AT THE CLEAVAGE SITE

The studies described so far were performed on 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 two of the fourteen subtypes known to date, H5 and H7. The question thus arises, 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 out if this is the case with hemagglutinins of human influenza viruses, because of the high pathogenic potential such a virus might have. In a study on the hemagglutinin of the human strain A/Port Chalmers/1/73 (H3N2) we could show that this hemagglutinin indeed can undergo a change in substrate specificity and become sensitive to the ubiquitous proteases. This has been accomplished 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 HA₁. All of the hemagglutinin mutants appeared at the plasma membrane as demonstrated by surface immunofluorescence and hemadsorption, and even those 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 3 indicate that, in the presence of the carbohydrate at asparagine 22, an insertion of 4 additional arginines, i.e. a total sequence of 5 arginines, is required at the cleavage site to obtain cleavage in CV-1 cells. This observation is in good agreement with a report by Gething and coworkers⁷ who, using the same approach, observed cleavage of another human H3 hemagglutinin after insertion of 4 arginines, whereas 1 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, but not by more residues, when the oligosaccharide at asparagine 22 is missing. Two conclusions can be drawn from these observations: (1) in agreement with the studies on H7 hemagglutinin described above the consensus sequence Arg-X-Lys/Arg-Arg is the minimal requirement for recognition by the ubiquitous proteases, and (2) by the insertion of an additional arginine at the cleavage site the masking effect of the oligosaccharide at asparagine 22 of HA₁ can be abolished, exactly as has been observed with the H5 hemagglutinin.^{6,13} Thus, interplay

Table 2. Correlation of glycosylation at Asn 11, cleavage site, and cleavability of the hemagglutinin with pathogenicity of influenza/A/chick/Pennsylvania (H5N2) variants.¹³

Virus strain	Oligo-saccharide at Asn 11 of HA ₁	Cleavage site					HA ₂	Cleav-ability	Chickens sick	Chickens killed	Pathogenicity
		HA ₁	320	321	322	323	324	325	1		
Penn/1/83	+	Pro-Gln-Lys-Lys-Lys-Arg	Gly							0/3	apathogenic
Penn/1370/83	-	Pro-Gln-Lys-Lys-Lys-Arg	Gly							3/3	pathogenic
8 t	+	<u>His</u> -Gln- <u>Arg</u> -Lys-Lys-Arg	Gly							3/3	pathogenic
9	+	Pro- <u>Lys</u> -Lys-Lys-Lys-Arg	Gly							2/2	pathogenic
13	+	Pro- <u>Arg</u> -Lys-Lys-Lys-Arg	Gly						2/2	2/2	pathogenic
33	+	Pro- <u>Lys</u> -Lys-Lys-Lys-Arg	Gly						2/2	2/2	pathogenic
46	+	Pro- <u>Lys</u> -Lys-Lys-Lys-Arg	Gly						2/3	1/3	pathogenic
47	+	<u>His</u> -Gln- <u>Arg</u> -Lys-Lys-Arg	Gly							2/2	pathogenic

Substitutions of amino acids are indicated by underlining.

between this carbohydrate and the cleavage site is a feature common to H5 and H3 hemagglutinins.

It is interesting to see that mutant S2- retained restricted cleavability, although it displayed, like mutant I3-, a sequence of four basic amino acids at the cleavage site and the loss of the oligosaccharide at asparagine 22 of HA₁. 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,⁸ remains to be seen. Our data indicate also that the basic sequence must not be generated on the expense of the carboxy-terminal end of HA1. 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 HA₁ has a spacer function which, in addition to the basic residues and the oligosaccharide at asparagine 22 of HA₁, is another determinant of cleavability.

The change in cleavability observed with the mutants analyzed here is clearly of different quality than that reported previously for variants of influenza virus X-31 adapted to MDCK cells.¹⁰ 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 non-permissive. 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 HA1, nor the carbohydrate at asparagine 22, but was caused by a point mutation at position 17 of HA₁. It is interesting to see, however, that this mutation resulted in the exchange of histidine for arginine and that the mutation site was located within < 10 Å of the amino-terminus of HA₂. Thus, there is some similarity to the mutants analyzed here in that a new arginine residue was introduced in the vicinity of the cleavage site, too.

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 virus¹⁴ 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 could be possible.

THE NATURE OF THE ACTIVATION PROTEASES

The H3 hemagglutinin of naturally occurring virus and other hemagglutinins of restricted cleavability are activated by 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 paramyxoviruses at a single arginine residue has recently been isolated from chicken embryo and identified as a protease highly homologous to the blood-clotting factor X, a member of the prothrombin family.¹⁵

Evidence is now emerging that a different family of proteases is responsible for endo-proteolytic cleavage at the carboxyl terminus of multiple basic residues which is a common post-translational modification of membrane and secretory proteins. Such proteins include precursors of peptide hormones, neuropeptides, growth factors, the vitamin K-dependent coagulation factors, serum albumins and the insulin receptor, as well as the hemagglutinin of the pathogenic avian influenza viruses, the F protein of several paramyxoviruses, the precursor to the E2 protein of alphaviruses, the E2 protein of coronaviruses, the envelope glycoprotein of retroviruses, the Lassa virus glycoprotein, and the gB protein of human cyto-

Table 3. Hemagglutinin variants of influenza virus A/Port Chalmers/1/73 (H3) obtained by site-directed mutagenesis.¹⁶

Hemagglutinin	Oligosacchride at Asn 22 of HA ₁	Cleavage site			Activation in CV-1 cells
		HA ₁ 326	327	HA ₂ 329	
Wild type	+	Lys-Gln-Thr	-----	Arg Gly	-
Mutant I2+	+	Lys-Gln-Thr	-----	<u>Arg-Arg</u> -Arg Gly	-
Mutant I3+	+	Lys-Gln-Thr	-----	<u>Arg-Arg-Arg</u> -Arg Gly	-
Mutant I4+	+	Lys-Gln-Thr	<u>Arg-Arg-Arg</u> -Arg	Arg Gly	+
Mutant S1+	+	Lys-Gln- <u>Arg</u>	-----	Arg Gly	-
Mutant S2+	+	Lys- <u>Arg-Arg</u>	-----	Arg Gly	-
Mutant I2-	-	Lys-Gln-Thr	-----	<u>Arg-Arg-Arg</u> -Arg Gly	-
Mutant I3-	-	Lys-Gln-Thr	-----	<u>Arg-Arg-Arg</u> -Arg Gly	+
Mutant S2-	-	Lys- <u>Arg-Arg</u>	-----	Arg Gly	-

Insertions and substitutions of amino acids are indicated by underlining.

Figure 1. Identification of a furin-related protein in partially purified preparations of the FPV-activating protease. The endoprotease activating the FPV hemagglutinin was partially purified from microsomal preparations of MDBK cells employing solubilization with octyl glucoside, sucrose density gradient centrifugation, anion exchange chromatography on DEAE-Sepharose and Mono-Q, and Superose 12 gel filtration. The left panel shows that, after gel filtration, the protease can be detected in fractions 13 and 14 using FPV with uncleaved hemagglutinin¹⁷ as substrate. The right panel shows that Western blotting with a furin-specific antiserum²³ identifies a 85 kD protein in these fractions.

megalovirus (for references see Ohuchi et al.¹⁶). The enzymes cleaving at multibasic residues have not been identified yet, but it is known that, for example, the protease activating the FPV hemagglutinin is Ca^{++} -dependent, membrane-bound, and has a neutral pH optimum.¹⁷ It has long been known that cleavage of this hemagglutinin occurs within the cell,¹⁸ and according to recent evidence the responsible protease may be located in the trans-Golgi network.¹⁹ Such enzymes appear to be highly conserved, since the FPV hemagglutinin is not only activated in virtually all mammalian and avian cells analyzed, but also in invertebrate tissues.²⁰ It was therefore interesting to see that a protease resembling these enzymes in its catalytic and other biochemical properties exists in the yeast *Saccharomyces cerevisiae*.²¹ 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 and mouse tissues.²² The protease activating the FPV hemagglutinin has been partially purified from MDBK cells. The isolation procedure involved cell fractionation, gradient centrifugation, and various chromatographic procedures. In enzyme preparations obtained in this way, we have detected a 85 kD protein by immunoblotting, with an antiserum raised against furin, one of the mammalian Kex2 analogues (Figure 1). These observations indicate that the ubiquitous proteases activating the FPV hemagglutinin and related viral glycoproteins belong to a family of subtilisin-like enzymes of which Kex2 and furin are known to be members.

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REFERENCES

1. H.-D. Klenk and R. Rott, The molecular biology of influenza virus pathogenicity, *Adv. Virus Res.* 34:247 (1988).
2. F.X. Bosch, W. Garten, H.-D. Klenk, and R. Rott, Proteolytic cleavage of influenza virus hemag-

- glutinins, Primary structure of the connecting peptide between HA1 and HA2 determines proteolytic cleavability and pathogenicity of influenza viruses, *Virology* 113:725 (1981).
3. W. Garten, F.X. Bosch, D. Linder, R. Rott, and H.-D. Klenk, Proteolytic activation of the influenza hemagglutinin: The structure of the cleavage site and the enzymes involved in cleavage, *Virology* 115:361 (1981).
 4. Y. Kawaoka and R.G. Webster, Sequence requirements for cleavage activation of influenza virus hemagglutinin expressed in mammalian cells, *Proc. Natl. Acad. Sci. USA* 85:321 (1988).
 5. S. Li, M. Orlich, and R. Rott, Generation of seal influenza virus variants pathogenic for chickens because of hemagglutinin cleavage site changes. *J. Virol.* 64:3297 (1990).
 6. Y. Kawaoka and R.G. Webster, Interplay between carbohydrate in the stalk and the length of the connecting peptide determines the cleavability of influenza virus hemagglutinin, *J. Virol.* 63:3296 (1989).
 7. M.-J. Gething, J. Henneberry, and J. Sambrook, Fusion activity of the hemagglutinin of influenza virus, *Curr. Top. Membr. Trans.* 13:339 (1989).
 8. D. Katchikian, M. Orlich, and R. Rott, Increased viral pathogenicity after insertion of a 28S ribosomal RNA sequence into the haemagglutinin gene of an influenza virus, *Nature* 340:156 (1989).
 9. M. Orlich, D. Khatchikian, A. Teigler, and R. Rott, Structural variation occurring in the hemagglutinin of influenza virus A/Turkey/Oregon/71 during adaptation to different cell types, *Virology* 176:531 (1990).
 10. R. Rott, M. Orlich, H.-D. Klenk, M.L. Wang, J.J. Skehel, and D.C. Wiley, Studies on the adaptation of influenza viruses to MDCK cells, *EMBO J.* 3:3328 (1984).
 11. K.L. Deshpande, V.A. Fried, M. Ando, and R.G. Webster, Glycosylation affects cleavage of an H5N2 influenza virus hemagglutinin and regulates virulence, *Proc. Natl. Acad. Sci. USA* 84:34 (1987).
 12. Y. Kawaoka, C.W. Naeve, and R.G. Webster, Is virulence of H5N2 influenza viruses in chickens associated with loss of carbohydrate from the hemagglutinin? *Virology* 139:303 (1984).
 13. M. Ohuchi, M. Orlich, R. Ohuchi, B.E. Simpson, W. Garten, H.-D. Klenk, and R. Rott, Mutations at the cleavage site of the hemagglutinin alter the pathogenicity of influenza virus A/chick/Penn/83 (H5N2), *Virology* 168:274 (1989).
 14. M. Enami, W. Luytjes, M. Krystal, and P. Palese, Introduction of site-specific mutations into the genome of influenza virus, *Proc. Natl. Acad. Sci. USA* 87:3802 (1990).
 15. D. Gotoh, T. Ogasawara, T. Toyoda, N.M. Inocencio, M. Hamaguchi, and Y. Nagai, An endoprotease homologous to the blood clotting factor X as a determinant of viral tropism in chick embryo, *EMBO J.* 9:4189 (1990).
 16. R. Ohuchi, M. Ohuchi, W. Garten, and H.-D. Klenk, Human influenza virus hemagglutinin with high sensitivity to proteolytic activation, *J. Virol.* 65:3530 (1991).
 17. H.-D. Klenk, W. Garten, and R. Rott, Inhibition of proteolytic cleavage of the hemagglutinin of influenza virus by calcium-specific ionophore A23187, *EMBO J.* 3:2911 (1984).
 18. H.-D. Klenk, W. Wöllert, R. Rott, and C. Scholtissek, Association of influenza virus proteins with cytoplasmatic fractions, *Virology* 57:28 (1974).
 19. I. de Curtis and K. Simons, Isolation of exocytotic carrier vesicles from BHK cells, *Cell* 58:719 (1989).
 20. K. Kuroda, A. Gröner, K. Frese, D. Drenckhahn, C. Hauser, R. Rott, W. Doerfler, and H.-D. Klenk, Synthesis of biologically active influenza virus hemagglutinin in insect larvae, *J. Virol.* 63:1677 (1989).
 21. R.S. Fuller, R.E. Sterne, and J. Thorner, Enzymes required for yeast prohormone processing, *Ann. Rev. Physiol.* 50:345 (1988).
 22. R.S. Fuller, C. Brenner, P. Gluschauf, and C.A. Wilcox, The yeast prohormone-processing Kex2 protease, an enzyme with specificity for paired basic residues, in: "Advances in Life Sciences," (1991), in press.
 23. P.A. Bresnahan, R. Ladne, L. Thomas, I. Thorner, H.L. Gibson, A.J. Brake, P.J. Barr, and G. Thomas, Human fur gene encodes a yeast Kex2-like endoprotein that cleaves pro- β -NGF *in vivo*, *Cell. Biol.* 111:2851 (1990).