Importance of conserved amino acids at the cleavage site of the haemagglutinin of a virulent avian influenza A virus

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The virulence of avian influenza A viruses depends on the cleavability of the haemagglutinin (HA) by an intracellular protease at multiple basic amino acids. Although previous studies have demonstrated the importance of these amino acids for processing by the cellular protease, with emphasis on conserved residues near the cleavage site, the minimal requirements for cleavage remain unknown. By expressing site-specific mutants of the HA of a virulent avian influenza virus, A/turkey/Ireland/1378/85 (H5N8), in the simian virus 40 system and testing for their cleavability by an endogenous protease in CV-1 cells, and their fusion activity in a polyclaryon formation assay, we were able to show that glycine at the amino terminus of HA2 is not essential for cleavage and that maximal cleavage requires at least five basic residues at the cleavage site, when carbohydrate is nearby. Moreover, we confirmed, that a conserved proline upstream of the cleavage site is not essential for HA cleavage or fusion activity, and that lysine replacement of the carboxyl-terminal arginine of HA1 abolishes cleavability. These findings should help identify the proteases responsible for intracellular cleavage of the HA of virulent avian influenza viruses.

The haemagglutinin (HA) molecule of influenza viruses is responsible for attachment of the virus to cell surface receptors and subsequent fusion of viral and cell membranes leading to internalization of the nucleocapsid (Lamb, 1989; Wiley & Skehel, 1987). Before this process can take place, the HA molecule must undergo a low pH-induced conformational change preceded by specific cleavage of the molecule into two disulphide-linked moieties, HA1 and HA2 (Lamb, 1989; Waterfield et al., 1980). HA cleavage in tissue culture by an endogenous cellular protease correlates with the presence of multiple basic amino acids at the site of cleavage (Bosch et al., 1981; Kawaoka & Webster, 1988). Moreover, this property appears limited to virulent strains of influenza virus (Bosch et al., 1981).

Intracellular HA cleavage requires basic residues at the cleavage site (Kawaoka & Webster, 1988). Additional basic amino acids are required upstream, in the so-called connecting peptide, to prevent the interference by glycosylation at the amino-terminal region of HA1 (Kawaoka & Webster, 1989). Although previous studies established the positions of the basic residues needed for complete HA cleavage (Kawaoka & Webster 1988, 1989), they did not address the minimal number or nature of the additional residues required for maximal HA cleavage.

In addition to the basic residues at the site of HA cleavage, several other amino acid residues surrounding the cleavage site are conserved in all 14 subtypes of influenza A virus (Nobusawa et al., 1991; Kawaoka et al., 1990), even in those HAs cleaved at a single arginine. Although basic amino acids at this site are essential for intracellular HA cleavage (Kawaoka & Webster, 1988), the conserved residues may also be important for recognition by an intracellular protease. In this study, we investigated the importance of two structural features at the cleavage site: conserved residues and the number of basic amino acids.

To determine the structural requirements for HA cleavage by an intracellular protease, we expressed mutant HAs in the simian virus 40 (SV40) system in CV-1 cells and examined their cleavability and fusion activity. There were no appreciable differences in cell surface expression as determined by haemadsorption between the site-specific mutant and parent HAs. Furthermore, when these mutant HAs had been trypsinized prior to the subsequent assays, they were all cleaved into HA1 and HA2 moieties and were indistinguishable by fusion activity (data not shown).

Comparison of the amino acid sequences at the cleavage site of the HA revealed conservation, among all the HAs examined to date, of two amino acids: the proline proximal to the carboxyl terminus of the HA1 and the amino-terminal glycine of the HA2. An arginine residue, the carboxyl-terminal residue of the HA1, was conserved in all but the H14 strains (Nobusawa et al.,...
The strictly conserved proline upstream of the cleavage site may be important in presentation of the cleavage site to an intracellular protease owing to its strong turn-breaking potential. We therefore constructed two mutant HAs, MT23 and MT25, in which the conserved proline was changed to tyrosine and glycine, respectively. In neither case was there a significant change in either cleavability by intracellular proteases or in fusion activity (Fig. 1), indicating that the proline residue is not essential for recognition by the protease.

The amino acid residue immediately downstream of the cleavage site affects the cleavability of prorenin, which is processed at multiple basic amino acids (Oda et al., 1991). We therefore examined the importance of the conserved first residue of HA2, a glycine, for HA cleavage. The semiconservative substitution from glycine to alanine (MT22) did not affect the cleavability of the HA (Fig. 1), although fusion activity was reduced to about 40% of the level of the wild-type HA (Table 1). This indicates that the glycine at the amino terminus of the HA2 is not conserved for cleavability but because it promotes the fusion activity.

Although the H14 HA contains lysine at the carboxyl end of the HA1 (Kawaoka et al., 1990), all known viral glycoproteins and prohormones containing multiple basic amino acids at the cleavage site have arginine immediately upstream of the cleavage site (Barr, 1991). To determine the importance of this residue for cleavage

![Fig. 1. Comparison of the cleavage of mutant HAs by an intracellular protease. The plasmid pTH29, which contains the full-length HA gene from A/turkey/Ireland/1378/85 (Kawaoka & Webster, 1988), was used to generate site-directed mutants by the method of Kunkel (1985). SV40-HA recombinant viruses were prepared as described (Kawaoka & Webster, 1988). Cleavage of the HA was examined in infected monolayers of CV-1 cells labelled with [3H]mannose and [3H]glucoseamine, as previously described (Kawaoka & Webster, 1988). Cell lysates were immunoprecipitated with anti-H5 antiserum and analysed on a polyacrylamide gel. Lane 1, wild-type. Lanes 2 to 7, MT22, -23, -25, -34, -41 and -42, respectively.](image)

### Table 1. Amino acid sequences at the cleavage site of HA mutants and their biological properties*

<table>
<thead>
<tr>
<th>Mutant HA</th>
<th>Amino acid sequence at the cleavage site</th>
<th>Cleavage</th>
<th>Polykaryon formation %†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td>PQRKKRKKR / G</td>
<td>+</td>
<td>100</td>
</tr>
<tr>
<td>MT22</td>
<td>PQRKKRKKR / G</td>
<td>-</td>
<td>11</td>
</tr>
<tr>
<td>MT25</td>
<td>PQRKKRKKR / G</td>
<td>±</td>
<td>29</td>
</tr>
<tr>
<td>MT23</td>
<td>PQRKKRKKR / G</td>
<td>+</td>
<td>40</td>
</tr>
<tr>
<td>MT24</td>
<td>PQRKKRKKR / G</td>
<td>+</td>
<td>100</td>
</tr>
<tr>
<td>MT25</td>
<td>PQRKKRKKR / G</td>
<td>+</td>
<td>92</td>
</tr>
<tr>
<td>MT34</td>
<td>PQRKKRKKR / G</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>MT41</td>
<td>PQRKKRKKR / G</td>
<td>+</td>
<td>90</td>
</tr>
<tr>
<td>MT42</td>
<td>PQRKKRKKR / G</td>
<td>+</td>
<td>60</td>
</tr>
</tbody>
</table>

* Mutated or deleted amino acids are shown in boxes; cleavage sites are indicated by slashes (/), basic amino acids by boldface type. +, cleaved; ±, partially cleaved; -, uncleaved.
† The extent of cell fusion was determined by calculating the average percentage of fused cells in three microscopic fields.
‡ These previously reported mutants (Kawaoka & Webster, 1988) are included for comparison.

In this study, the strictly conserved proline proximal to the cleavage site was not essential for HA cleavage by intracellular proteases; however, the conservative change of the arginine at the carboxyl end of the HA1 to another basic residue, lysine, prevented HA cleavage by proteases. All viral glycoproteins and prohormones cleaved at multiple basic residues contain an arginine immediately upstream of the cleavage site (Hosaka et al., 1991,
Dong et al., 1992, Kawaoka & Webster, 1988). In a spontaneous mutation of this residue to a lysine in the envelope protein of a murine leukaemia provirus, infectivity was severely impaired (Freed & Risser, 1987), supporting the absolute requirement for an arginine at this position. Recently, Vey et al. (1992) obtained results identical to ours by replacing the proline and arginine residues in a virulent avian influenza virus of the H7 HA subtype. The important role, in the cellular protease-induced cleavage, of the first (Vey et al., 1992), second (Kawaoka & Webster, 1988) and fourth (Kawaoka & Webster, 1988) basic residues from the carboxyl end of the HA1, and the irrelevance of the third (Kawaoka & Webster, 1988) basic residue, led Vey et al. (1992) to recognize a conserved sequence motif, Arg-xxx-Arg/Lys-Arg, found at the cleavage site of virulent avian virus HAs (Kawaoka et al., 1987). Results of the present study support that observation.

The significance of the glycine residue at the HA2 amino terminus for recognition of the HA cleavage enzyme was not clear at the outset of this study. Alteration of the amino acid residue immediately downstream of the prorenin processing site affected the cleavability of the proprotein (Oda et al., 1991), suggesting a possible role for this residue in cleavage by a cellular protease at a site of multiple basic amino acids. This prediction was not substantiated by our results; that is, substitution of alanine for the highly conserved glycine at the amino terminus of the HA2 (MT22) did not alter cleavability. Thus the glycine is conserved not for HA cleavage but for fusion activity (Garten et al., 1981; Gething et al., 1986), although alanine residues are found at the analogous position of human immunodeficiency virus type 1 gp41, the fusion peptide of the molecule (McCune et al., 1988).

Ohuchi et al. (1989) reported that the HAs of variant H5 viruses containing five basic residues at the cleavage site are cleaved by intracellular proteases, but the extent was limited and the importance of the P6 arginine residue was not examined. In the present study, we systematically examined the relation of the number of basic amino acid residues at the HA cleavage site to the cleavability of this molecule. We showed that the deletion, but not the alteration, of the P6 arginine reduces cleavability when the HA contains the carbohydrate near the cleavage site. Hence the P6 residue may be important for the abolition of possible steric hindrance of HA cleavage by the carbohydrate, but it does not have to be basic in nature, at least for cleavage by an endogenous protease in CV-1 cells. The basic nature of the P5 position, by contrast, is critical for HA cleavage, because alteration of the P6 and P5 residues to non-basic residues appreciably reduces cleavability (Kawaoka & Webster, 1988). Thus, for the H5 HA-containing carbohydrate, at least five basic amino acids are required for maximal HA cleavage.

Because of the structural similarities noted for cleavage of viral glycoproteins and prohormones, understanding the processing of prohormones by eukaryotic cellular proteases would probably contribute to our understanding of viral glycoprotein activation. The search for mammalian enzymes that cleave prohormones recently led to identification of calcium-dependent subtilisin-like proteases (Barr, 1991). After identification and structural determination of a yeast protease, Kex2, that cleaves the killer toxin at dibasic sites (Fuller et al., 1989), three eukaryotic homologues, furin, PC2 and PC1 or PC3, were identified (for a review, see Barr, 1991). These enzymes differ in cleavage specificities and tissue tropism. Whereas both PC2 and PC3 cleave at the dibasic residues R-R, K-R or K-K (Korner et al., 1991; Benjannet et al., 1991; Thomas et al., 1991), furin cleaves at tetrabasic (R-X-K/R-R) (Nagahama et al., 1991) and/or dibasic (K-R or R-R) sequences (Bresnahan et al., 1990). Moreover, furin is ubiquitously and constitutively expressed (Hatsuzawa et al., 1990) and located in the Golgi complex (Misumi et al., 1991), whereas both PC2 and PC3 are inducible and tissue-specific (Birch et al., 1991), and both appear to act beyond the Golgi complex (Nagahama et al., 1991). Thus, the structural requirements (Kawaoka & Webster, 1988; Dong et al., 1992; Hosaka et al., 1991) and intracellular location (Walker et al., 1992; Nagahama et al., 1991) of viral glycoprotein cleavage suggest that furin, or a closely related enzyme, is the cleavage protease. Indeed, Stieneke-Gröber et al. (1992) recently reported that human furin and a protease antigenically cross-reacting with furin in Madin-Darby bovine kidney cells cleave the HA of another virulent virus, A/FPV/Rostock/34 (H7N1). The availability of genes encoding prohormone processing enzymes, together with mutant influenza virus HA genes, should allow a detailed study of HA-protease interactions.

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