Trypsin-resistant protease activation mutants of an influenza virus

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New classes of mutants of influenza virus A/seal/Mass/1/80 are described in which the haemagglutinins (HA) have lost their protease cleavability by trypsin, but can be activated by elastase, chymotrypsin or thermolysin in different cell types. The same proteases that were required for activation of infectivity of the mutants also activated haemolysis and cell-fusing properties. The protease activation (pa)-mutants were non-pathogenic for chickens, but induced a protective immune response against a highly pathogenic challenge virus. The failure of the mutants to be activated by trypsin, but instead to be activated by the other proteases employed, was related to amino acid exchanges around the HA cleavage site. The cleavability of the chymotrypsin and elastase pa-mutants is most likely determined by replacement of Arg-1 by neutral amino acids such as Ile, Thr, Met or Leu, depending on the substrate specificity of the activating proteases. Cleavage activation of the thermolysin pa-mutants, on the other hand, became possible by insertion of a single Leu residue at position 4 of the HA 2 polypeptide, which compensates for the loss of the Gly residue at the N terminus of the fusion peptide due to thermolysin cleavage. The correction of the mutations in revertants confirmed the conclusions drawn from sequence analyses of the pa-mutants.

Introduction

The spike glycoprotein haemagglutinin (HA) of influenza viruses is of great importance for the uptake of virus particles by the host cell. It is responsible for the attachment of the virus to sialic acid containing cellular receptors and it is involved in virus penetration by fusion of the viral envelope with cellular membranes. To acquire fusion activity and consequent infectivity by the virus, HA, which is synthesized as the precursor molecule HA 0, has to be proteolytically cleaved during the course of intracellular transport or after virus release to yield the disulphide linked polypeptide chains HA 1 and HA 2. pH-dependent conformational changes result in the exposure and relocation of a highly conserved hydrophobic peptide, the fusion peptide, at the N terminus of the transmembrane polypeptide HA 2 which mediates membrane fusion. The structure at the cleavage site and the availability of an appropriate protease determine the potential cleavability. Although there are differences in cleavability of HA among different influenza viruses and mutants obtained therefrom, in all cases specific endoproteases attack the precursor HA 0 at a given cleavage site between the C-terminal Arg of HA 1 and the N-terminal Gly of HA 2. Correct cleavage is therefore of prime importance for virus infectivity, tropism and pathogenicity (Klenk & Rott, 1988; Nagai, 1993).

In this report we describe the isolation and characterization of new types of mutants of the influenza virus A/seal/Mass/1/80 (H7N7) (seal) virus, the HAs of which were found to be resistant to cleavage activation by trypsin. These mutants, however, are produced in the infectious form and form plaques in different cell types in the presence of chymotrypsin, elastase or thermolysin, enzymes that do not activate wild-type (wt) virus. These protease activation mutants – this expression is taken from the designation of similar mutants obtained from the parainfluenza virus Sendai virus (Scheid & Choppin, 1976) – are host range mutants. They were apathogenic for chickens, but induced a protective immunity against challenge with a highly pathogenic seal virus variant (Li et al., 1990). The relatively high frequency with which these mutants or revertants occurred provides further evidence for the variability of cleavage activation of influenza virus HA.

Methods

Virus and cells. The wt influenza virus A/seal/Mass/1/80 (H7N7) (seal) virus, obtained from R. G. Webster (Naeve & Webster, 1983) and grown in 11-day-old chicken embryos was used as the starting virus for all the studies. Infected secondary cultures of chicken embryo cells (CEC) or established lines of MDCK, BHK or CV1 cells were cultured in reinforced Eagle's medium without serum. Virus growth experiments in cell cultures and plaque assays were done in the absence of...
or presence of appropriate proteases (5 µg/ml) in the medium, similarly to experiments described previously (Klenk et al., 1975).

**Biological tests.** Haemagglutination, haemagglutination inhibition tests, haemolysis and cell fusion activity were performed as previously described (Rott et al., 1984). To examine the pathogenicity of the protease activation (pa-)mutants 8-week-old White Leghorn chickens were infected intratracheally with 105 p.f.u. After infection the individual birds were kept under isolation conditions for at least a 14 day observation period.

**Radioactive labelling and SDS-PAGE.** Virions released into the liquid medium of cell cultures labelled with [35S]methionine (100 µCi/culture) and concentrated by subsequent centrifugation onto a 30% sucrose cushion were treated or not treated with the appropriate enzymes and immunoprecipitated with a monoclonal antibody raised against the seal virus HA. Slab gels were analysed by fluorography (Bosch et al., 1979).

**Nucleotide sequence analysis.** Viral RNA was extracted by the hot phenol method (Maniatis et al., 1982) from virions purified by adsorption to and elution from chicken erythrocytes. After centrifugation, nucleotide sequence determination was performed using the deoxyoligonucleotide chain termination method (Sanger et al., 1977) with viral RNA serving as template and reverse transcriptase (Daniels et al., 1985). The oligonucleotide primers used were complementary in sequence to oligonucleotides spanning the entire HA gene of the seal virus as described previously (Li et al., 1990).

**Direct amino acid sequencing.** Viral proteins were separated on a 12.5% polyacrylamide gel in which the glycine of the running buffer was replaced by tricine (Schägger & Von Jagow, 1987). The proteins were stained by KCl, and the HA2 band was cut out and electro-eluted in the presence of 0.1% SDS and 5 mM-2-mercaptoethanol. The resulting eluate was concentrated by centrifugation through an Amicon ultrafiltration membrane (cut-off Mø, 10000) and transferred to a PVDF (polyvinylidenedifluoride) membrane. Automatic N-terminal amino acid sequence determination was performed using an Applied Biosystems 477A sequencer.

**Proteases.** The following enzymes were used: TPCK (N-tosyl-L-phenylalanine chloromethyl ketone)-treated trypsin (23 U/mg, Serva), elastase from porcine pancreas (123 U/mg, Serva), TLCK (Nε-p-tosyl-L-lysine chloromethyl ketone)-treated α-chymotrypsin (80 U/mg, Fluka) and thermolysin from Bacillus thermoproteolyticus, (40 U/ml, Boehringer Mannheim). The enzymes were dissolved in sterile PBS and were used only once. They were added to the media of infected cultures after virus adsorption. For the in ovo experiments enzyme solutions were injected into the amnionic cavity of chicken embryos simultaneously with the virus.

**Results**

**Selection and growth characteristics of pa-mutants**

The pa-mutants were obtained by serial passaging the wt seal virus in non-permissive CEC in the absence of trypsin but in the presence of 5 µg/ml chymotrypsin, elastase or thermolysin. CEC were initially inoculated at an m.o.i. of 10⁻² p.f.u./cell of egg-grown wt virus. For further passages, virus containing medium obtained after incubation of infected cells for 72 h at 37 °C was used and diluted 1:10 to 1:1000, depending on the virus yield, to guarantee multiple virus growth conditions. After five to seven passages virus mutants were obtained which grew efficiently and developed clearly visible plaques in CEC when the appropriate protease was present in the culture medium. The progeny viruses were purified by three to five plaque-to-plaque passages. In this way, mutants were selected whose growth was dependent upon the presence of either chymotrypsin (Ch1–3), elastase (E1–3) or thermolysin (Th1–3).

When CEC were infected at a high m.o.i. (approx. 10 p.f.u./cell) with the elastase, thermolysin or chymotrypsin pa-mutants in the absence of exogenous proteases, virus yield, as determined by haemagglutination, was similar to that of wt-virus. Progeny viruses, however, were not infectious. They could be activated by in vitro treatment with the appropriate protease. As expected, in the cultures with no protease added no virus could be detected in the medium by haemagglutination under multiple-cyclic conditions (m.o.i. approx. 10⁻²–10⁻³ p.f.u./cell) after an incubation period of 72 h. With the appropriate protease in the medium, relatively high amounts of virus were released from the cells infected at an m.o.i. as low as 10⁻³ p.f.u./cell. The minimum concentration of the respective protease in the liquid medium which could activate the mutants in the above experiments was 0.5 µg/ml (Fig. 1). At concentrations up to 5 µg/ml the proteases did not change the activation potency. By analogy to the results obtained in the above experiments, the pa-mutants formed plaques only when the appropriate proteases were added to the agar overlay. The morphology of the plaques differed slightly among different mutants.

The substrate specificity of the Ch and E pa-mutants could be changed after passaging in CEC in the presence of the alternative enzyme. Thus, ECh mutants could be obtained from elastase pa-mutants, which were produced in infectious form and formed plaques only when chymotrypsin was added to the medium. Conversely, infectious Ch1E mutants were isolated after passaging the Ch1 pa-mutant, which grew efficiently and formed plaques only when the selecting elastase was present. These ECh and Ch1E mutants were found to be genetically stable upon continuous passages under these conditions.

In addition, mutants derived from the E3 pa-mutant were selected, the HA of which was activated by elastase in combination with chymotrypsin. To this end CEC were infected with mutant E3 at an m.o.i. of 10⁻² p.f.u./cell, incubated for 72 h and passed three times in the presence of 5 µg/ml elastase plus chymotrypsin (5 µg/ml) for 72 h. The progeny mutants, designated E3CE, were purified by three plaque passages. As expected, CEC infected with the plaque purified E3CE mutants formed infectious virus and produced plaques when elastase and chymotrypsin were present in the overlay. The progeny of different plaques from these mutants showed the same
Influenza virus protease activation mutants

Fig. 1. Replication of seal influenza virus chymotrypsin (Ch1), elastase (El) and thermolysin (Th1) activation mutants in relation to the concentrations of activating proteases in the culture medium. CEC were infected at an m.o.i. of $10^{-3}$ p.f.u./cell and kept in the presence of the respective protease for 72 h at 37 °C. HA titres of the culture medium were then determined.

<table>
<thead>
<tr>
<th>Chymotrypsin</th>
<th>Elastase</th>
<th>Thermolysin</th>
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<tbody>
<tr>
<td>0.2 μg/ml</td>
<td>2.5 μg/ml</td>
<td>0.5 μg/ml</td>
</tr>
<tr>
<td>2.0 μg/ml</td>
<td>5.0 μg/ml</td>
<td>10 μg/ml</td>
</tr>
</tbody>
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Fig. 2. Plaque formation by the E3CE1 pa-mutant, diluted $10^{-4}$ and incubated for 72 h at 37 °C in the presence of 5 μg/ml elastase or chymotrypsin in the agar overlay.

Host specificity of the pa-mutants

The requirement for a specific protease for the activation of the pa-mutants has been established by the finding that cell types other than CEC, such as MDCK, BHK and CV1 cells, also produced infectious virus under multiple-cycle conditions only when the appropriate protease was present in the culture medium. Similarly, 11-day-old chicken embryos infected into the allantoic cavity with $10^{9}$ p.f.u. (small inoculum) of pa-mutants produced high amounts of infectious virus only when the inoculum contained 50 μg/ml of the appropriate protease. In the absence of exogenous protease the virus titre was not higher than $10^{2}$-10$^{3}$ p.f.u./ml. This is in contrast to the wt-virus, where a high virus yield was obtained in chicken eggs without application of additional protease.
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**Fig. 3.** Proteolytic cleavage of the haemagglutinin of wt-seal virus (a) and its pa-mutants Th1 (b), Ch2 (c), E3 (d) and E3CE1 (e). CEC were labelled with 100 μCi [35S]methionine 5–20 h after infection with 10 p.f.u./cell. Virus obtained from the medium was treated with 5 μg/ml chymotrypsin (Ch), elastase (E), trypsin (T) or thermolysin (Th) or not treated (–). HA was immunoprecipitated with an HA
(H7)-specific monoclonal antibody by standard procedures. SDS-PAGE was carried out as described previously (Bosch et al., 1979).

(Li et al., 1990). Thus, the pa-mutants were found to have a host range different from the wt-virus.

**Revertants of pa-mutants**

In contrast to the above results with small inocula, all pa-mutants grew to high titres in the absence of added proteases in the chicken embryo when the eggs were infected with high inocula, e.g. ≥ 10⁷ p.f.u. This means that added protease is not required for a single-cycle replication when a sufficiently large portion of endodermal cells of the allantoic membrane, which are permissive for wt-virus (Rott et al., 1980), are infected with the initial inoculum, resulting in large amounts of virus. The viruses obtained under these conditions were activated in cell cultures by trypsin after a single egg passage, i.e. the progeny viruses behaved similar to the wt-virus. Selection of these revertants (which were designated Ch1Egg, E1–3Egg and Th2Egg) may be brought about by the blood clotting factor Xa, a trypsin-like protease which is present in the allantoic fluid of chicken embryos (Gotoh et al., 1990; Ogasawara et al., 1992).

Similarly, when CEC were infected with a high m.o.i. (10 p.f.u./cell) of either E or Th mutants, revertants, designated ET or ThT, could be obtained after two to three passages in the presence of 5 μg/ml trypsin and the absence of other exogenous proteases in the medium. The revertants grew efficiently during continued passages in CEC and produced plaques when trypsin was present in the medium. The progeny of such plaques preserved the characteristics of wt-virus, including the failure to be activated by proteases other than trypsin.

**Fusogenic activity of the pa-mutants**

As was demonstrated above, each pa-mutant is characterized by its susceptibility to activation by the protease which was used for selection. To ascertain that activation is correlated with the fusogenic properties of the mutants, virus-induced haemolytic and cell-to-cell fusion activities were determined. As expected, all pa-mutants are capable of lysing chicken erythrocytes only when the respective protease, which activated the mutants, was added to the virus producing system or when isolated virus particles grown in the absence of activated enzymes were treated in vitro with the appropriate protease. The pH optimum of haemolysis for most pa-mutants was in the same range as that of wt-virus, i.e. 5.4, with the exception of the Th mutants where the threshold of pH decreased to 5.0, suggesting changes in the conformation of the fusion peptide of those mutants (Daniels et al., 1983, 1985; Gething et al., 1986). Similarly, the same proteases that activated infectivity and haemolytic properties also activated virus-induced fusion-from-within of CV1 cells, when the culture medium was adjusted to pH 5.0 (not shown). This indicates that cell membrane-associated HA is activated by the respective protease added to the medium. It should be noted that monolayers infected with Th mutants and treated with thermolysin showed a more rapid rounding of cells than those infected with the other pa-mutants and treated with the respective enzymes.

**Pathogenic properties of the pa-mutants**

Pathogenicity of representative pa-mutants of all types was studied in chickens to assess the effect of altered HA
substrate specificity. Eight-week-old White Leghorn chickens inoculated intratracheally with \( \approx 10^6 \) p.f.u. of pre-activated viruses did not develop any signs of disease during a 14-day observation period. Low titres of virus was recovered 2 days after infection only from the lung, not from the brain, heart, liver, kidney, spleen or pancreas, with the exception of mutant Th1 which could not be detected in any organ. The virus recovered from the lung had the same characteristics as the inoculated mutants, i.e. they were activated only by their specific activation proteases.

Nevertheless, all of the pa-mutants, including Th1, must have undergone at least a single replication cycle, probably in the respiratory tract, since all infected birds developed specific HA inhibiting antibodies with titres of 2\(^{-4}\) to 2\(^{-5}\). Furthermore, all infected chickens were protected against challenge with \( 10^6 \) p.f.u. of the highly pathogenic wt-virus variant SC35 (Li et al., 1990) 14 days after inoculation. They showed no signs of disease during a 21 day observation period, whereas the challenge virus, as a control, caused fowl plague-like pathology with death of the birds 2–3 days after infection.

**HA cleavability of pa-mutants**

To determine the cleavability of the HA from the pa-mutants PAGE analyses were performed. \([^{35}S]\)Methionine-labelled virus released into the culture medium and partially purified by centrifugation was used in all experiments. The viruses were treated or not treated with the different proteases, immunoprecipitated using an HA(H7)-specific monoclonal antibody and analysed on a SDS–12.5% polyacrylamide gel. As shown in Fig. 3 for representative mutants, the HA of most pa-mutants was only cleaved when the virus was treated with the appropriate protease. One exception was found for the Th mutants as shown for Th1, which were cleaved by thermolysin and by trypsin, although the virus was activated only by thermolysin. Another exception was found with the E3CE mutants where cleavability by elastase as well as by chymotrypsin coincided with activation by both enzymes. As expected, the HAs of the revertant viruses were proteolytically processed only by trypsin, analogous to the wt-virus (not shown). Taken together, the PAGE analyses confirmed previous findings that protease-dependent activation of influenza viruses correlates with cleavage of HA\(_0\) into the HA\(_1\) and HA\(_2\) polypeptides.

**Sequence changes to the HA of pa-mutants**

To determine the amino acid sequence required for enzyme recognition the HA genes of all the pa-mutants obtained were analysed by nucleotide sequencing and the amino acid sequences were predicted from the sequences thus obtained. In addition, direct amino acid sequencing of the N-terminal region of HA\(_2\) was performed. The results obtained from the region at the cleavage site are summarized in Table 1. Notably, only with the thermolysin mutants, as described below, and the elastase mutant E1 contained mutations in the fusion peptide. Mutant E1 contained a Phe in place of Leu at the second position of HA\(_2\), which did not alter the fusogenic properties of the mutant. With the exception of the Th-mutants, amino acid exchanges were found in all pa-mutants at the C terminus of HA\(_1\), which prevented cleavage activation by trypsin. The chymotrypsin activation mutants have only one amino acid exchange, Arg-1 for Met, resulting in the loss of the basic cleavage site for trypsin. Therefore, the conclusion appears to be justified that this amino acid exchange is responsible for the altered cleavability of the Ch-mutants. Accordingly, the same amino acid exchange found with mutants E1Ch and E3Ch led to the altered substrate specificity, although one (E3Ch) or two (E1Ch) additional mutations were present. The elastase mutants E1 and E2 are characterized by the amino acid exchange Arg-1 for Thr in HA\(_1\), so that in these cases Thr can be regarded as being responsible for cleavage. On the other hand, with mutant E3 Arg-1 was replaced by Ile and Lys-3 by Met. Ile was also found to be the C-terminal amino acid of HA\(_2\) in the Ch1E1–3-mutants. In connection with the occurrence of Ile at the cleavage site of the F-protein of elastase activation mutants of Sendai virus (Hsu et al., 1987; Itoh et al., 1987; Tashiro et al., 1992a), it can be assumed that the cleavage site of E3 and that of the Ch1E1–3-mutants should be Ile, and that the other amino acid exchanges can be neglected. With the pa-mutants E3CE1 and E3CE2, which were activated by both elastase and chymotrypsin, Arg-1 was replaced by Leu. Since Leu is a substrate for both enzymes, this recognition site is supposedly critical for HA cleavage. In any case, Gly-1 was present as the N-terminal residue of HA\(_2\) as shown by direct amino acid sequencing. [The structural changes of the thermolysin mutants have been described elsewhere (Orlich & Rott, 1994). It is noticeable that with these mutants no amino acid exchange was found in the C-terminal region of HA\(_1\), but an additional Leu was inserted at position 4 of HA\(_2\). By direct amino acid sequencing, the thermolysin cleavage site was revealed to be between Gly-1 and Leu-2 of HA\(_2\), i.e. the cleavage site of these mutants was shifted by one amino acid towards the C terminus of HA\(_2\).]

With most revertants obtained, either in cell culture or in the chicken embryo, when trypsin or trypsin-like proteases were present in the environment, Arg-1 was reintroduced at the cleavage site with the exception of E1Egg, where instead of Arg-1, Lys was found. It should be noted that Lys, although unusual, serves in trypsin
Table 1. Amino acid sequences around the cleavage site of the HAs of protease activation mutants of the seal influenza virus and their revertants

<table>
<thead>
<tr>
<th>Virus</th>
<th>Activated by</th>
<th>Sequences*</th>
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<tr>
<td></td>
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<td>HA1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCA</td>
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<tr>
<td>Chl-3</td>
<td>Trypsin</td>
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<td>ChlE1</td>
<td>Elastase</td>
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<tr>
<td>ChlE3</td>
<td>Elastase</td>
<td></td>
</tr>
<tr>
<td>E1</td>
<td>Elastase</td>
<td></td>
</tr>
<tr>
<td>E1T</td>
<td>Trypsin</td>
<td></td>
</tr>
<tr>
<td>E1Egg</td>
<td>Trypsin</td>
<td></td>
</tr>
<tr>
<td>E1Ch</td>
<td>Chymotrypsin</td>
<td></td>
</tr>
<tr>
<td>E3</td>
<td>Elastase</td>
<td></td>
</tr>
<tr>
<td>E3Egg</td>
<td>Trypsin</td>
<td></td>
</tr>
<tr>
<td>E3Ch</td>
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<tr>
<td>E3CE1</td>
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<td></td>
</tr>
<tr>
<td>E3CE2</td>
<td>Chymotrypsin</td>
<td></td>
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* As predicted from nucleotide sequences (capital letters) or determined by direct amino acid sequencing (lower case letters). The sequence of the wt-virus was taken from Li et al. (1990).

Discussion

Limited proteolytic cleavage of the HA of influenza viruses at a specific cleavage site was found to be of crucial importance for the activation of viral infectivity. Results obtained by comparison of naturally occurring influenza viruses (for references see Klenk & Rott, 1988), by selecting mutants on the basis of their cleavage properties (for references see Rott, 1992) or by site-specific mutagenesis of HAs in *in vitro* expression systems (for references see Horimoto & Kawaoka, 1994) indicate that, in principle, two structural features determine HA cleavability: a single Arg, present at the HA cleavage site of most mammalian and apathogenic avian influenza viruses, and, on the other hand, an insertion of a series of multibasic amino acids with the main enzyme recognition motif Arg-X-Lys/Arg-Arg (where X is a non-basic amino acid) found at the cleavage site of the highly pathogenic avian influenza viruses (Vey et al., 1992). Correspondingly, two groups of proteases appeared to be responsible for HA cleavage activation. The first includes trypsin-like proteases which cleave behind the single Arg, such as trypsin itself (Klenk & Rott, 1988), plasmin (Lazarowitz & Choppin, 1975), the blood clotting factor Xa (Gotoh et al., 1990; Ogasawara et al., 1992), tryptase Clara (Kido et al., 1992; Tashiro et al., 1992), recognition and this is also the case for H1N1 influenza virus A/Tübingen/12/85 (Günther et al., 1993). Another exception was found with revertants obtained from the thermolysin pa-mutants (Orlich & Rott, 1994). In this case the insertion of Leu at position 4 of HA2 was compensated for by the removal of Leu-2 (Th2T) or Phe-3 (Th1T), so that the number of amino acids of the fusion peptide became the same as that of the other pa-mutants and of wt-virus.
In our search for further HA activating proteases three main questions were of foremost interest. (i) Is it possible to obtain mutants with alterations in HA cleavage activation under the selection pressure of enzymes possessing totally different substrate specificities, such as elastase, chymotrypsin and thermolysin which were found to activate mutants of the parainfluenza Sendai virus? (ii) What are the sequence requirements for HA cleavage activation of those mutants? (iii) Do these mutants cause disease?

Only a few passages of the seal influenza wt-virus in CEC in the presence of either of the proteases employed, i.e. chymotrypsin, elastase or thermolysin, were necessary to recover the desired pa-mutants. These replicated under multiple cycle growth conditions and produced plaques only when the appropriate protease was present in the medium. The correlation found between virus activation, fusogenic properties and cleavage of HA by all the different enzymes employed confirmed previous findings that proteolytic cleavage at a specific site and exposure of a functional fusion peptide is essential for membrane fusion and for infectivity. The relatively high frequency with which all the pa-mutants appeared suggests that they were selected from a heterogeneous virus population. They were genetically stable, at least in cell culture, as long as the respective enzymes were present in the culture medium. However, when the activating proteases were exchanged, again very frequently either revertants or mutants with another substrate specificity could be obtained. This could mean that mutations at the region of the HA cleavage site occur frequently but express their effect only when the virus is exposed to a different environment. This became particularly obvious when chicken embryos were infected with large inocula ($\geq 10^7$ p.f.u.) of pa-mutants in the absence of additional proteases. Under these conditions revertant viruses were immediately isolated which were activated by trypsin, like the wt-virus. From the estimation made by Tyrrell et al. (1954) it can be concluded that the number of endodermal cells of the allantoic membrane, permissive for growth of all influenza viruses (Rott et al., 1980), is less than $10^7$. Thus, high m.o.i. permits the production of a large amount of virus during a single cycle of reproduction which is released into the allantoic cavity, where the cleavage active trypsin-like blood clotting factor Xa could act for selection. These findings underline again that the enzyme present under particular environmental conditions represents the selective barrier for activation. Other environmental conditions obviously govern the outcome of infection of other organ systems in chickens. After intratracheal infection of chickens with pa-mutants, leading to an asymptomatic course of infection, only progeny with the characteristics of the inoculated virus could be reisolated. The finding that the pa-mutants were non-pathogenic suggests that the enzymes used in these studies are not present, at least in the respiratory tract of chickens. However, a single cycle of replication must have taken place, since this inoculation was sufficient to produce a solid protective immune response against an otherwise lethal challenge. Since the pa-mutants were found to induce a protective immunity in chickens, this type of mutant could serve as a basis for the preparation of influenza virus live vaccines, as already proposed for Sendai virus (for references see Wang et al., 1994).

By characterizing the determinants that affect the differences in HA cleavage activation of the pa-mutants it became evident that in all cases mutations occurred around the cleavage site. With the elastase pa-mutants the amino acid exchange Arg-I for Ile is most probably responsible for the altered cleavability. This assumption is supported by the observation that a similar mutation renders the F-protein of Sendai virus mutants sensitive for activation by elastase (Hsu et al., 1987; Itoh et al., 1987; Tashiro et al., 1992a), although, in contrast to the pa-mutants described here, some of the respective Sendai virus mutants (KDe), were activated in the chicken embryo and in MDCK cells in the absence of the exogeneous protease (Tashiro et al., 1992a). These results suggest that in addition to Ile-1 other structural features, such as the three-dimensional structure of the cleavage site, could determine cleavability by elastase-like proteases, and that the host proteases in MDCK cells and in chicken embryos that activate the F-protein of the Sendai virus KDe-mutants are similar, although not identical, to elastase. All the chymotrypsin pa-mutants were found to have Met instead of Arg-1. The differences between these findings and those obtained previously with corresponding mutants of Sendai virus (Hsu et al., 1987; Itoh et al., 1987; Tashiro et al., 1992a), where the F-protein is cleaved by chymotrypsin before Gln-3, might be explained by different conformations at the cleavage sites or by the absence of amino acids with similar sensitivity to chymotrypsin around the cleavage sites of the Ch-mutants described here. Exchange of Arg-I for Leu was obviously decisive for the activation of mutants E3CE1 and E3CE2 by both elastase and chymotrypsin, since both enzymes recognize Leu as a substrate and Gly was found to be present at the N terminus of HA2 of these mutants. Of special interest are the thermolysin activation mutants, as discussed in more detail elsewhere (Orlich & Rott, 1994). No mutations...
were detected at the C-terminal region of HA$_2$. As one might expect from the substrate specificity of thermolysin, HA cleavage occurred before Gly-1, the N-terminal amino acid of HA$_2$ of all influenza A viruses. Maintenance of the fusogenic properties of the Th-mutants in spite of the absence of Gly-1, which was found previously to be essential for the induction of HA-mediated membrane fusion (Garten et al., 1981), was obviously due to the insertion of the apolar residue Leu at position 4 of HA$_2$, which is thought to compensate for the missing Gly. Thus it can be concluded that it is not a conserved amino acid sequence but the number of hydrophobic residues that determines the fusogenic property of the influenza virus fusion peptide. The finding that HA$_2$ of the Th mutants, which has an additional Leu residue, is cleaved but not activated by trypsin, is in agreement with this conclusion.

In a more general context the data obtained in this study indicate that the structure of the HA cleavage site is more variable than was previously thought. Study of mutants activated by thermolysin could give further information on the structural features of the fusion peptide that are necessary for membrane fusion and on alterations that prevent cleavage activation by trypsin or trypsin-like proteases despite the presence of Arg-1 at the cleavage site. Biologically, it might be possible that one or other of the mutants could cause an unusual disease or a persistent infection, if the mutant could reach an organ in the infected host which possesses enzymes with appropriate substrate specificities.

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