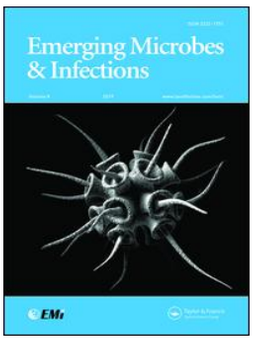

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ORIGINAL ARTICLE

Protease activation mutants elicit protective immunity against highly pathogenic avian influenza viruses of subtype H7 in chickens and mice

Ralf Wagner[1], Gülsah Gabriel[2,*], Matthias Schlesner[2,#], Nina Alex[1], Astrid Herwig[2], Ortrud Werner[3] and Hans-Dieter Klenk[2]

Protease activation mutants of the highly pathogenic avian influenza virus A/FPV/Rostock/34 (H7N1) have been generated that are fully dependent on the presence of trypsin for growth in cell culture. Unlike wild-type virus, the mutants do not induce systemic infection in chicken embryos and show low pathogenicity in both chicken embryos and adult chickens. Inactivated vaccines prepared from the mutants protected chickens and mice very efficiently against infection with highly pathogenic wild-type virus in a cross-reactive manner. The potential of these mutants to be used as veterinary and prepandemic vaccines will be discussed.
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Keywords: influenza virus; pathogenicity; protection; proteolytic activation; vaccine

INTRODUCTION

Highly pathogenic avian influenza viruses (HPAIV) cause systemic infections with lethal outcome in chickens, quail, geese and other domestic birds. The disease is a major threat to the poultry industry, since the economical damage resulting from the loss of millions of birds may be enormous.[1] The impact of these infections is dramatically illustrated by the H5N1 outbreak, with this virus now being endemic in Southeast Asia and rapidly spreading to other parts of the world.[2,3]

Although man has been thought for a long time not to be susceptible to HPAIV infection, there is now increasing evidence that these viruses can also cause disease in humans. This became quite obvious during the H5N1 outbreak,[4] but it was already observed before when avian influenza A viruses of subtype H7, including HPAIV, were found to be transmitted to man.[5–7]

The impact of these viruses for human health became most evident during a large H7N7 outbreak in poultry in 2003, when over 300 primary and secondary human infections were observed in the Netherlands. Most cases showed mild disease including conjunctivitis, but there was one fatal outcome.[8] These observations show that H7 like H5 viruses are able to cross the species barrier to man and that they have therefore the potential to cause a pandemic.

Thus, there is clearly a need for vaccines against HPAIV both to control outbreaks in birds and to be prepared for an emerging pandemic in humans. Several strategies have been employed for the generation of such vaccines. These include inactivated vaccines produced by reverse genetic techniques,[9–11] live attenuated cold-adapted vaccines,[12,13] vaccines based on recombinant adenovirus[14] and DNA vaccines.[15] Because of the ongoing H5N1 outbreak, most of the studies have focused on this subtype, whereas H7 viruses have attracted less attention. Numerous inactivated human vaccines against H5N1 viruses have been developed and licensed worldwide. However, these vaccines have so far been used only in limited numbers of subjects mostly in clinical trials since there has been no actual H5N1 pandemic. Further, protective H5N1-specific inactivated vaccines for the use in poultry have been produced in China from seed strains generated by reverse genetics.[16,17] The experiences from China's pioneering activities related to avian influenza vaccination have recently been described.[18]

The prime determinant of the high virulence of HPAIV is the polybasic cleavage site of the haemagglutinin (HA) that allows activation by furin. Since furin is a ubiquitous protease, this type of cleavage is responsible for rapid virus spread resulting in systemic infection with fatal outcome as opposed to local infection with low pathogenicity due to restricted cleavability at monobasic cleavage sites.[19] Replacement of polybasic as well as monobasic cleavage sites by valine susceptible to cleavage by elastase resulted in attenuated protease activation mutants that have the potential to be used as live vaccines.[20,21] Mutation at the cleavage site of HA proved also to be useful for the production of inactivated H5 HPAIV vaccines, since the large quantities of live virus required for this purpose precludes the use of wild-type (WT) HPAIV that would be extremely hazardous for the environment.[10]

We report here on the generation of highly attenuated H7 protease activation mutants by reverse genetics. When used as inactivated

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vaccines, these mutants protected chickens and mice with high efficacy from a lethal infection with H7 subtype viruses.

MATERIALS AND METHODS

Cells and viruses

Madin Darby canine kidney (MDCK) cells were cultured in minimal essential medium (MEM) (Gibco, Gibco Life Technologies, Darmstadt, Germany) supplemented with 10% fetal bovine serum (Invitrogen, Invitrogen Life Technologies, Darmstadt, Germany). Human embryonic kidney cells (293 cells) were maintained in Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal bovine serum. Both cell lines were cultivated at 37 ℃ and 5% CO_2. The HPAIV strains A/FPV/Rostock/34 (H7N1) (FPV) and A/chicken/Germany/R28/03 (H7N7) as well as the mouse-adapted variant of the A/seal/Mass/80 (H7N7) strain (SC35M)[22] were used.

Generation of FPV mutants

The establishment of a reversed genetics system for the rescue of FPV has been described before.[23] In brief, all eight viral gene segments were isolated and cloned into the vector pHH21 for the RNA polymerase I-driven transcription of viral RNA.[24] Next, these eight plasmids were cotransfected into 293 cells along with four additional plasmids for the expression of the subunits of the influenza virus polymerase complex. Two days post-transfection, 293 cell supernatants were passaged onto MDCK cell monolayers for the efficient amplification of contained recombinant viruses. Purification of rescued viruses was achieved by three rounds of plaque passages on MDCK cells.

The two HA cleavage site mutants were produced by subjecting the pHH21 construct carrying the WT HA gene to the Quickchange mutagenesis procedure (Stratagene, Stratagene Agilent Technologies, Waldbronn, Germany). The following primer pairs were used in this approach: for mutant 1, GGG ATG AAG AAC GTT CCC GAA CCT TCC AAA GGA AGA GGC CTG TTT GGC GCT ATA GCA GGG (fo) and CCC TCT ATA GCG CCA AAC AGG CCT CTT CCT TTG GAA GGT TCG GGA ACG TTC TTC ATC CC (re); for mutant 2: GGG ATG AAG AAC GTT CCC GAA CCT TCC GCA GCA GCG AAA GGA AGA GGC CTG TTT GGC GCT ATA GCA GGG (fo) and CCC TGC TAT AGC GCC AAA CAG GCC TCT TCC TTT CGC TGC TGC GGA GGT TCG GGA ACG TTC TTC ATC CC (re). For the generation of the respective cleavage-mutant viruses, the resulting pHH21 constructs were included in the above mentioned reverse genetics approach and *L*-(tosylamido-2-phenyl)ethyl chloromethyl ketone-trypsin (Sigma, Taufkirchen, Germany) was added to culture media at a final concentration of 0.5 μg/mL. The genetic identity of rescued viruses was confirmed by sequencing of the complete HA gene.

Virus replication in cell culture

For growth curves, MDCK cell monolayers were inoculated at a multiplicity of infection (MOI) of 0.001 in phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (BSA) (Invitrogen) for 1 h. Unbound viruses were washed off and serum-free medium containing 0.2% BSA was added. If desired, *L*-(tosylamido-2-phenyl)ethyl chloromethyl ketone-trypsin was added to a final concentration of 0.5 μg/mL. From then on, HA titres in the supernatants were periodically monitored with chicken red blood cells (1% in saline).

For plaques assays, confluent MDCK cell monolayers in 2.5 cm dishes (in six-well plates) were inoculated with 10-fold dilutions (in PBS/0.2% BSA) of viruses for 1 h. Cells were washed and covered with an overlay of MEM containing 0.5% purified agar (Oxoid Ltd, Wesel, Germany), 0.2% BSA, and 0.001% DEAE-dextran. If applicable, cultures were supplemented with *L*-(tosylamido-2-phenyl)ethyl chloromethyl ketone-trypsin at a final concentration of 0.5 μg/mL. Cells were incubated at 37 ℃ and 5% CO_2, and plaques were visualized 3 days post-infection with 0.1% crystal violet in a 10% formaldehyde solution.

Analysis of HA cleavage activation

MDCK cells were inoculated with viruses at a MOI of 5 for 1 h. Unbound viruses were washed off with PBS and MEM was added. Four hours post-infection, the cells were washed with PBS, and 15 μCi of Redivue Pro-Mix $L^{35}S$ *in vitro* cell labelling mix (Amersham Pharmacia, Nuembrecht, Germany) were added in 2 mL of methionine and cysteine free MEM. If desired, trypsin was added to a final concentration of 1 μg/mL. After 12 h, cell supernatants were harvested and viruses collected by ultracentrifugation at 100 000 *g* for 2 h. Aliquots of these virus preparations were subjected to a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and viral protein bands were visualized by fluorography.

Replication in embryonated chicken eggs

Eleven-day-old embryonated chicken eggs were inoculated into the allantoic cavity with 2000 plaque forming units (pfu, as determined by plaque assay on MDCK cells) of viruses. At given time points post-inoculation, eggs were opened and checked for embryo viability. Next, the allantoic fluid was collected and examined for virus titres by plaque assay on MDCK cells.

Spread of viral infection to embryo tissues was addressed by *in situ* hybridisation using digoxygenin (DIG)-labelled riboprobes specific for the nucleoprotein gene of fowl plague virus (FPV-NP). The riboprobes were produced by run-off transcription of a fragment of FPV-NP (nt. 1077–1442) that had been inserted into the vector Bluescript KS+ (Stratagene). Transcription was carried out utilizing T7 RNA polymerase and the DIG-RNA labelling Kit (Roche, Roche Diagnostics GmbH, Mannheim, Germany). Riboprobes were purified from the reaction mixture by means of the RNeasy-Kit (Qiagen, Hilden, Germany) following the supplier's protocol.

Sagittal embryo cryosections (20 μm) were fixated in 4% phosphate-buffered formaldehyde solution for 1 h at room temperature (RT) and then washed three times with PBS for 10 min each. All solutions were prepared from diethylpyrocarbonate-treated water. Deproteination was achieved by proteinase K treatment (0.2 μg/mL) for 10 min at RT. Sections were washed with water and post-fixated with formaldehyde for 10 min (see above). Slides were then washed with 100 mM glycin (in PBS) and PBS for 5 min each, transferred to a solution of acetic anhydride (0.25% in 0.1 M triethanolamine, pH 8.0) and incubated for 10 min at RT. Sections were washed in PBS, dehydrated in ethanol (50% and 70%) and air dried for 15 min. Next, prehybridisation buffer (50% deionised formamide in 4×SSC (0.6 M NaCl in 0.06 M sodium citrate, pH 7.0)) was added for 1 h at 55 ℃. Prehybridisation buffer was removed and 150 μL of the DIG-labelled riboprobe previously diluted 1∶500 in hybridisation buffer (4×SSC containing 50% deionised formamide, 10% dextran solution, 1×Denhardt's solution, 0.1 mg/mL of yeast RNA, 0.1 mg/mL sheared salmon sperm DNA, 10 mM ethylenediaminetetraacetic acid) were applied for 16 h at 55 ℃ in a humid chamber. During incubation sections were sealed with coverslips. Next, washes were done at 37 ℃ with 2×SSC, and 1×SSC for 10 min each and samples subsequently digested with RNase A (20 μg/mL) (Fermentas, St. Leon-Rot, Germany) and RNase T1 (1 U/mL) (Fermentas) for 1 h at 37 ℃ (in 0.5 M NaCl, 1 mM ethylenediaminetetraacetic acid, 10 mM Tris/HCl, pH 7.6). Additional washes at 37 ℃ with 0.5×SSC, 0.2×SSC for 10 min

each followed. Finally sections were washed with 0.2×SSC for 1 h at 60 ℃.

Detection of bound DIG-riboprobes was achieved by means of alkaline phosphatase-conjugated to anti-DIG F*ab* fragments and 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium as a chromogenic substrate (all reagents from Roche). To this end, cryosections were washed with 0.3% Tween 20 for 5 min and 1% blocking reagent for 30 min (both in 150 mM NaCl, 100 mM maleic acid, pH 7.5). Next, 250 µL of anti-DIG-alkaline phosphatase F*ab* fragments (diluted 1∶5000 in 1% blocking solution) were added for 30 min at RT. Sections were washed twice for 15 min each with 0.3% Tween 20 in maleic acid buffer and equilibrated in buffer 3 (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris/HCl, pH 9.5). Chromogen solution of 250 µL (45 mg/mL nitro blue tetrazolium, 0.175 mg/mL 5-bromo-4-chloro-3-indolyl-phosphate in buffer 3) were applied and incubated in a humid chamber at RT overnight in the dark. The reaction was stopped with 10 mM Tris/HCl, pH 8.1 containing 1 mM ethylenediaminetetraacetic acid. Embryonic tissues were finally counterstained with haematoxylin–eosin.

Pathogenicity in chicken

The animal experiments were performed at the Friedrich-Loeffler-Institute, the German federal research institute for animal health, according to the guidelines of the German animal protection law. All animal protocols were approved by the state authorities of Mecklenburg-Vorpommern. Work with FPV and strain A/chicken/Germany/R28/03 (H7N7) was conducted under biosafety level 3 conditions. The virulence of influenza A viruses for chickens was estimated using the intravenous pathogenicity index (IVPI) test as follows: fresh infective allantoic fluid with a HA titre $>2^4$ was diluted 1∶10 in sterile isotonic saline and 0.1 mL of the diluted virus was injected intravenously into each of ten 6-week-old specific pathogen-free (SPF) chickens. Birds were examined at 24-h intervals for the following 10 days. At each observation, each bird was scored 0 if normal, 1 if sick, 2 if severely sick and 3 if dead. The judgment of sick and severely sick birds is a subjective clinical assessment. Normally, 'sick' birds would show one and 'severely sick' birds more than one of the following signs: respiratory involvement, depression, diarrhea, cyanosis of the exposed skin, oedema of the face and/or head and nervous signs. The IVPI is the mean score per bird per observation over the 10-day period. An index of 3.00 means that all birds died within 24 h, and an index of 0.00 means that no bird showed any clinical sign during the 10-day observation period. Any influenza A virus, regardless of subtype, giving a value of greater than 1.2 in an IVPI test, is considered to be a HPAI virus.[25]

Preparation of formalin-inactivated vaccine

Eleven-day-old embryonated chicken eggs were inoculated with recombinant viruses via the allantoic route (40 eggs per virus species). Allantoic fluids were harvested 36 h post inoculation and centrifuged for 15 min at 5000 *g* to remove debris. Next, virus was pelleted by centrifugation for 12 h at 65 000 *g*. Virus sediments were thoroughly resuspended in 2 mL of PBS containing a protease inhibitor cocktail (Calbiochem, Schwalbach, Germany) and further purified by sucrose step gradient ultracentrifugation (bottom 60%; top 20%) in a Beckman SW 41 rotor at 120 000 *g* for 8 h. The interface was removed from the gradient, and virus was pelleted for 8 h at 100 000 *g* in the same rotor. Virus pellets were resuspended to homogeneity in 2 mL of PBS containing protease inhibitor. The bulk of the material was inactivated at a protein concentration of 1 mg/mL with 0.025% formalin at 4 ℃ for 4 days. After this treatment, inactivated virus was concentrated by ultracentrifugation and pellets recovered in 1 mL of PBS with protease inhibitor. Aliquots were taken for purity analysis by Coomassie-stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis and for calculating the HA/total viral protein ratio. At different stages of purification, the protein concentrations of the virus preparations were determined by means of the bicinchoninic acid (BCA)-protein assay (Pierce, Rockford, United States) following the manufacturer's instructions.

Immunisation and protective efficacy in adult chickens

A vaccine formulation was produced by diluting 600 µL of purified virus suspension (corresponding to about 150 µg of HA) in 1.8 mL of PBS and by subsequently adding 2.4 mL of complete Freund's adjuvant. The resulting emulsion was mixed thoroughly and then injected into the pectoral muscles of SPF chickens 5 months of age at a dose of 0.5 mL per animal. Twenty-two days post-vaccination, animals were challenged by intranasal inoculation with $10^{7.9}$ 50% egg infectious dose (EID_{50}) of the HPAIV strain A/chicken/Germany/R28/03 (H7N7). As a control, unvaccinated chickens were included in the trial which received the same dose of challenge virus. Blood samples were taken for the determination of serum haemagglutination inhibition antibody titres at days 0, 9, 14 and 21 post-vaccination and days 7, 14 and 19 post-challenge. Animals were monitored for the development of clinical symptoms, mortality and shedding of the challenge virus.

Immunisation and protective efficacy in mice

The animal experiments were performed according to the guidelines of the German animal protection law. All animal protocols were approved by the relevant German authority, the Regierungspräsidium Giessen. Handling of the SC35M virus was conducted in compliance with biosafety level 3 requirements. Groups of female 5-week-old Balb/C mice (Charles River, Sulzfeld, Germany) were intramuscularly immunized with preparations of formalin inactivated FPV mutant 1 containing 7 or 15 µg HA with or without Freund′s adjuvant in a 1∶1 dilution. Four weeks after immunisation, mice were challenged intranasally with 100 median lethal doses (LD_{50}) of SC35M (H7N7) under anesthesia set by an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Mice were observed for weight loss and signs of disease for 2 weeks after immunisation and challenge, respectively.

RESULTS

Generation of FPV cleavage site mutants

The sequence at the cleavage site of FPV-HA was mutated in order to generate a motif that is no longer accessible to processing by the ubiquitous cellular protease furin.[26] Two different HA mutants were generated (Figure 1A). The first one (Mut1) represents a cleavage site typically found in mammalian and low-pathogenic avian influenza viruses.[19] In the second mutant (Mut2) the cleavage site motif of Mut1 was elongated by the addition of three alanine residues to restore the full length of the WT cleavage site. Using a reverse genetics system, these mutant HA sequences were stably incorporated into recombinant viruses.[24] We have recently developed such a system allowing us to generate recombinant FPV.[23] Mutant viruses were rescued in the presence of trypsin in cultured cells and amplified in the allantoic cavity of embryonated chicken eggs. The correct genetic identity of the rescued viruses was finally confirmed by sequencing of the HA genes.

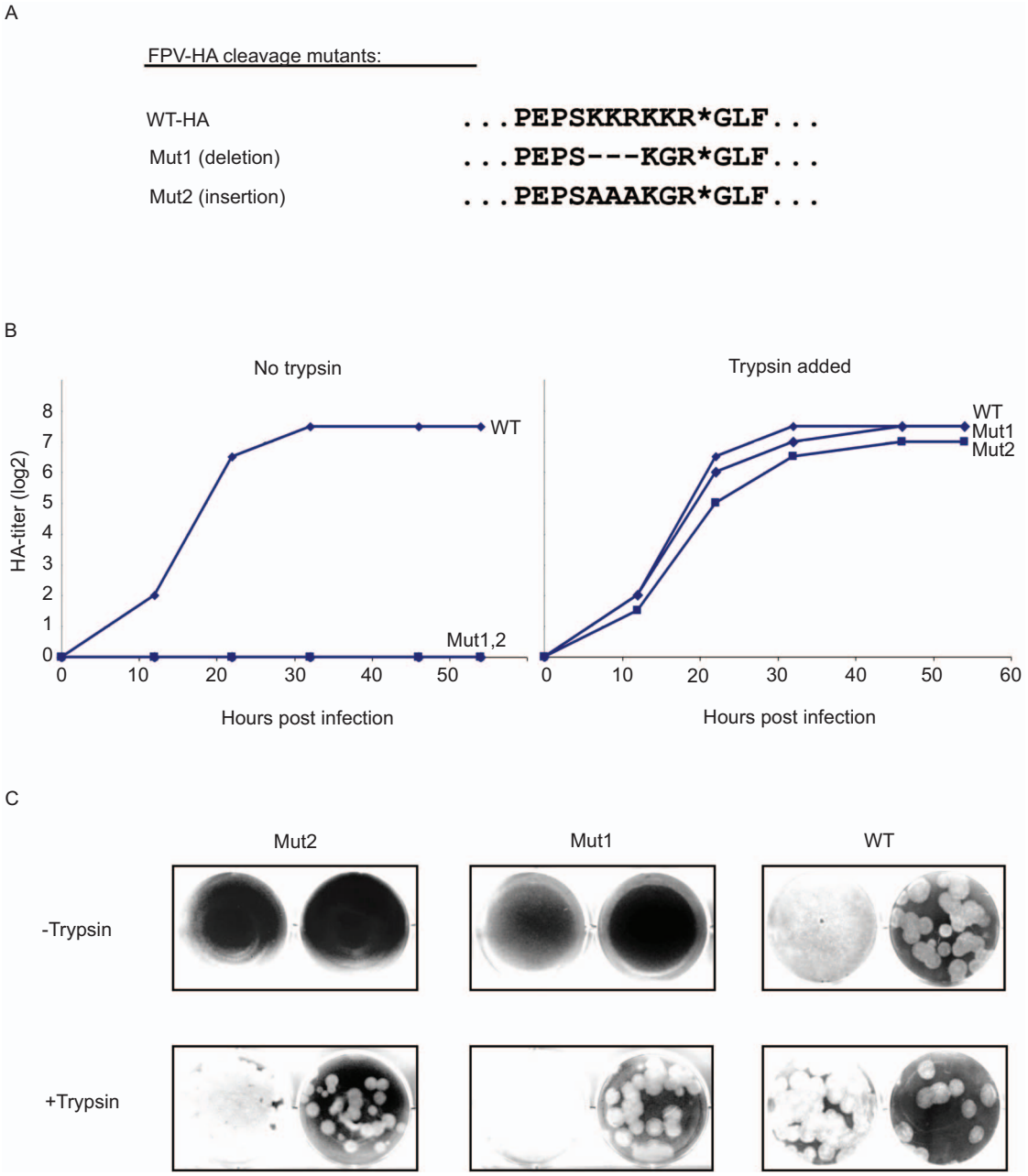

Figure 1 Characterisation of FPV mutants. (**A**) Cleavage site motifs of WT and mutant viruses. The WT cleavage site sequence of FPV and the two mutants produced for the generation of recombinant viruses are depicted. Asterisks indicate the site of cleavage. (**B** and **C**) Replication of viruses in MDCK cell culture in the absence and presence of trypsin. (**B**) Cells were inoculated at a MOI of 0.001. At the indicated time points, culture supernatants were monitored for HA titres using chicken red blood cells. (**C**) Spread of virus infection traced by plaque formation. Monolayers seeded in six-well plates (diameter per well: 35 mm) were inoculated with the indicated viruses and covered with an agarose-containing medium overlay. At 3 days post-infection, plaques were visualized by staining cell monolayers with a crystal violet solution.

Replication in MDCK cells

First, we looked at the replication of the virus mutants in MDCK cells. Growth curves showed that, in accordance with the existing furin recognition motif in HA, WT virus was able to replicate in these cells in the absence of exogenously added trypsin. In contrast, replication of both cleavage site mutants was found to be strictly dependent on the presence of trypsin in the culture medium (Figure 1B). The situation was the same when the ability of these viruses to produce plaques in the MDCK cell monolayer was assayed. Virus mutants were unable to spread from cell to cell in the absence of trypsin, while large plaques were obtained when trypsin was added to the medium (Figure 1C). Again, production of plaques with WT viruses was identical irrespective of the presence of trypsin. The growth restrictions of the virus mutants were due to the lack of proteolytic activation of HA in the absence of trypsin. This became clear when we examined the protein pattern of virus released from MDCK cells that had been infected with mutants at a high multiplicity of infection. Only in cells treated with trypsin, the HA precursor is cleaved into its subunits HA_1 and HA_2 (Figure 2). No cleavage products are detectable if trypsin was omitted. Due to its accessibility to cleavage by the cellular protease furin, for

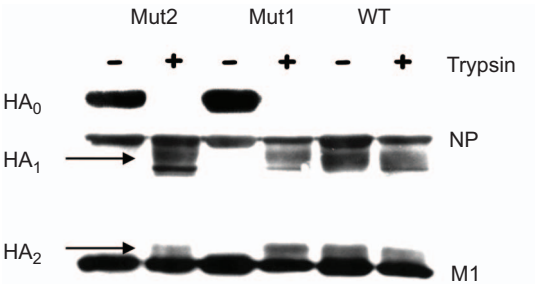

Figure 2 Analysis of cleavage activation of HA. MDCK cells were infected at a MOI of 5 and incubated in ^{35}S-methionine/cysteine containing medium in the absence or presence of trypsin. After 12 h, metabolically labelled progeny viruses were collected from the cell supernatants by ultracentrifugation and aliquots of the virus preparations were applied to a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Protein bands were visualized by fluorography. The uncleaved HA precursor (HA_0) and the cleavage products HA_1 and HA_2 are indicated.

WT HA, there is no need for trypsin to achieve proteolytical processing. Hence, the mutations introduced into the HA sequence totally abrogated furin-mediated proteolytic activation. As a consequence, replication of the mutant viruses in MDCK cell culture is strictly dependent on exogenously added proteases targeting monobasic cleavage sites, such as trypsin.

Replication and pathogenicity in embryonated chicken eggs

Growth to high titres in embryonated chicken eggs is a key requirement for a virus designated to serve as a candidate seed strain for vaccine production. Therefore, we examined the replication of FPV mutants in this system. Thirty hours post-inoculation into the allantoic cavity, allantoic fluids were harvested and monitored for viral titres by plaque assay on MDCK cells in the presence of trypsin. It turned out that all three virus types reached almost the same titres in a range of 5.2×10^8–6.4×10^8 pfu/mL (Figure 3A). Thus, while changes in the HA cleavage site motif drastically inhibit growth of mutant viruses in cell culture, replication in embryonated chicken eggs is not affected.

We next examined the pathogenicity of our set of viruses in embryonated chicken eggs by comparing the survival rates of embryos at different time points after inoculation. Embryos from WT FPV-infected eggs started to die at 18 h post-infection. Twenty-four hours post-infection, all embryos within this group were dead (Figure 3B). In contrast, embryos in eggs infected with the mutant viruses did not die until 48 h following inoculation. Accordingly, by solely converting the HA cleavage site motif, FPV can be efficiently attenuated and transformed into a non-pathogenic avian virus. This finding was confirmed when we analysed the spread of the infection within the embryo tissues by *in situ* hybridisation. In this assay, a DIG-labelled RNA probe specific for the FPV-NP segment was used to detect viral replication in embryo cryosections. All organs of WT-infected embryos showed clear signs of infection (Figure 4). The infection was confined to the endothelia of blood vessels as already reported previously.[27] However, no evidence of infection was detected in embryo tissues from eggs infected with the mutants indicating that these viruses are unable to cross the chorio-allantoic membrane, a typical feature of low pathogenic avian influenza viruses (LPAIV).[28] Taken together, these results

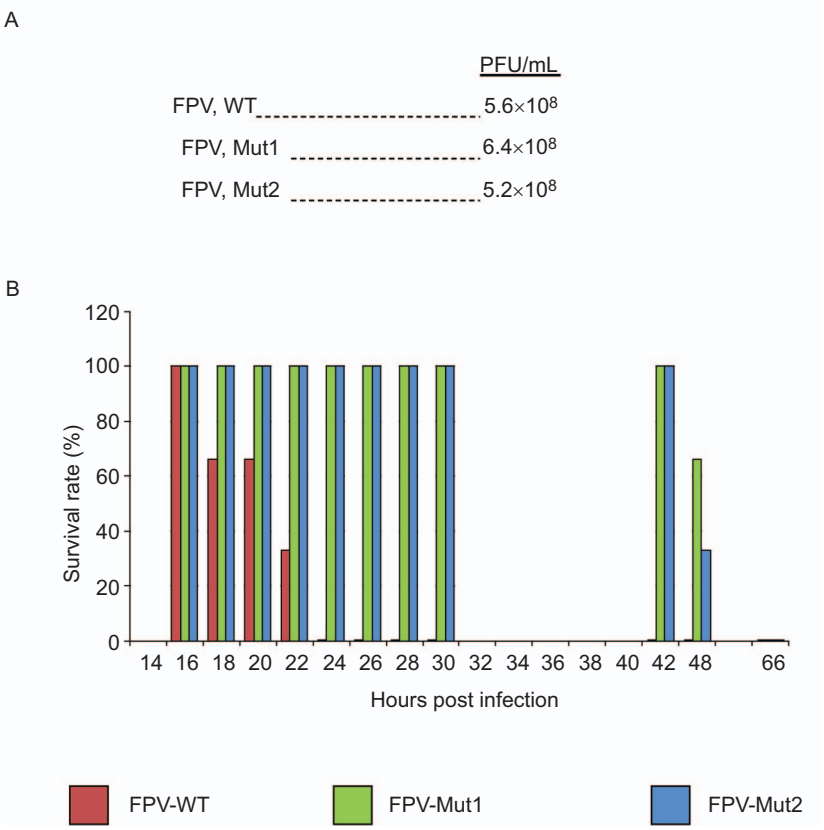

Figure 3 Pathogenicity and virus yields in embryonated chicken eggs. (**A**) Virus yields in allantoic fluids 30 h post-inoculation. (**B**) Mortality of embryos over time. Eleven-day-old embryonated eggs were inoculated by the allantoic route with 2000 pfu/egg. At the indicated time points post-inoculation, eggs were opened and checked visually for viability of embryos. The survival rates are given as per cent values based on the total number of eggs infected per virus species.

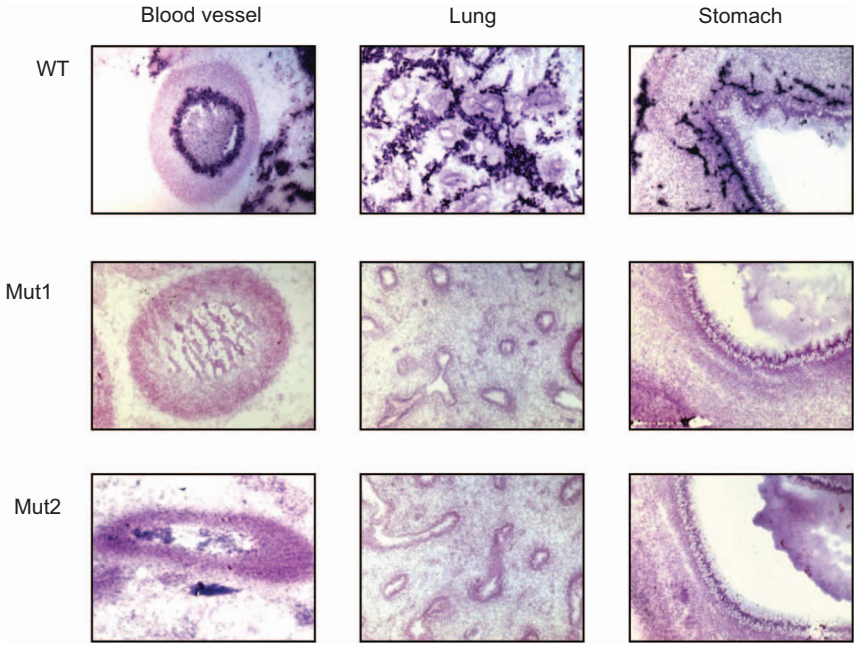

Figure 4 Spread of virus infection to embryonic tissues. Eleven-day-old embryonated chicken eggs were inoculated as outlined in the legend to Figure 3. At 48 h post-inoculation, embryos were removed for the preparation of sagittal cryosections. Virus infection of the depicted tissues was detected by means of *in situ* hybridisation employing a digoxygenin-labelled riboprobe specific for the FPV-NP gene. Embryonic tissues were visualized by common haematoxylin–eosin staining (magnification: ×63).

demonstrate that, although the FPV mutants can grow to high titres in the allantoic cavity of chicken eggs, they otherwise behave as typical LPAIV which fail to infect and to rapidly kill chicken embryos.

Pathogenicity in adult chickens

To employ these FPV mutants as seed strains for the production of influenza vaccines, it is of crucial importance to make sure that their non-pathogenic phenotype in cell culture and embryonated chicken eggs is retained upon infection of adult animals. To prove this, three groups of 10 chickens each were infected intravenously. All 10 animals infected with WT viruses developed typical severe signs of fowl plague and seven out of ten died. In contrast, all of the chickens infected with the virus mutants survived. Only one animal suffered transiently from mild diarrhea and slight depression. The other animals showed no disease symptoms, thus providing striking evidence for the low pathogenicity of the mutant viruses (Figure 5). The intravenous pathogenicity indices of the viruses were 2.34, 0.0 and 0.07 for WT, Mut1 and Mut2, respectively.

Hence, owing to their capability for high yield growth in embryonated chicken eggs on the one hand and their complete attenuation in chicken embryos and adult birds on the other hand, the engineered FPV mutants are perfectly suited for the safe production in bulk amounts that is a key prerequisite for vaccine manufacturing.

Immunogenicity and protective efficacy of formalin-inactivated vaccine in chickens

Next, the potential of FPV mutant 1 to serve as antigenic component of an inactivated vaccine for the protection of adult chickens from an HPAIV challenge was investigated. To this end, the virus was grown in large scale in embryonated chicken eggs and purified from allantoic fluids by centrifugation through a sucrose gradient. Inactivated whole-virus vaccine was prepared by formalin treatment. The absence of any residual infectivity in the inactivated virus preparation was confirmed by plaque assay on MDCK cells (data not shown). Aliquots of the purified vaccine preparation containing approximately 15 μg of HA protein were mixed with Freund's adjuvant and employed for the intramuscular immunisation of a group of nine SPF chickens. On day 22 post-vaccination, the birds were challenged oculo-nasally with $10^{7.9}$ EID_{50} of the HPAIV A/chicken/Germany/R28/03 (H7N7), a virus that has an intravenous pathogenicity index in chickens of 2.93. Additionally, three unvaccinated birds were included in the challenge experiment as a control. Over a period of 40 days post-vaccination, the animals were monitored for clinical symptoms of infection and the development of a H7N7-specific serum antibody response as determined by hemagglutination inhibition (HI) test. No serum antibodies directed against the H7N7 virus were found in blood samples taken before vaccination. At day 9 post-vaccination, however, specific HI titres were detectable in the serum of all vaccinated chickens which reached peaks of 2^{11} in vaccinated animals at the end of the experiment (Table 1). No such antibody titres were found in non-vaccinated control animals. Apart from a mild form of diarrhea at day 3 post-infection, none of the vaccinated animals showed signs of avian influenza and all chickens stayed perfectly healthy until the end of the trial. In one animal, transient shedding of H7N7 virus was detected in oropharyngeal and cloacal swabs. In contrast, unvaccinated control animals rapidly developed typical symptoms of severe avian influenza (head edema, ruffled feathers, diarrhea, depression) and died at day 3 post-infection.

Taken together, these results clearly demonstrate that the inactivated FPV mutant 1 has the potential to elicit a high-titre antibody response that completely protects birds from a lethal challenge even with another HPAIV strain of the H7 subtype.

Immunogenicity and protective efficacy of formalin-inactivated vaccine in mice

Further, we investigated the potential of the inactivated mutant 1 vaccine for its ability to cross-protect against a lethal challenge with

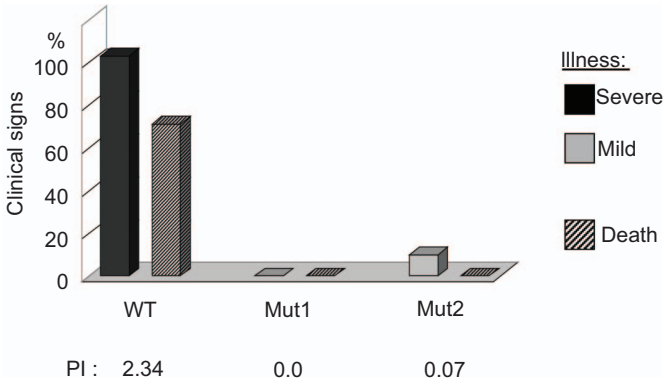

Figure 5 Pathogenicity of FPV mutants in adult chicken. Three groups of ten 6-week old SPF chickens each were inoculated intravenously with fresh infective allantoic fluid (HA titre $>2^4$) containing WT, Mut1 or Mut2 viruses. From then on, birds were examined at 24-h intervals for the following 10 days. At each observation, the birds are examined for clinical signs of disease and scored 0 if normal, 1 if mildly sick, 2 if severely sick and 3 if dead (for details, see the section on 'Materials and methods'). The intravenous PI is the mean score of birds per group over the entire observation period. PI, pathogenicity index.

another related but antigenically distinct H7 virus, the mouse-adapted strain SC35M.[29] Therefore, groups of mice (n=5) were immunized intramuscularly according to the following protocol: (i) one dose of non-adjuvanted vaccine containing 7 μg of HA; (ii) one dose of vaccine containing 7 μg HA in Freund's adjuvant; (iii) two doses of adjuvanted vaccine containing 7 μg HA and administered 2 weeks apart; and (iv) one dose of adjuvanted vaccine containing 15 μg HA. As a control, one group remained unvaccinated. The vaccine was tolerated very well by all animals irrespective of the immunisation scheme as judged from body weight measurements (Figure 6A). Four weeks after the single immunisation or two weeks after the second immunisation, mice were challenged intranasally with $100LD_{50}$ of SC35M. During the subsequent observation period, all vaccinated animals survived indicating that they were robustly protected against a lethal challenge with SC35M (Figure 6B). Groups receiving one dose of 7 μg HA either with or without adjuvant exhibited a temporary weight loss of approximately 20%. Weight loss was lower (app. 10%) in those groups having received either one adjuvanted dose of 15 μg HA or two adjuvanted doses 7 μg HA. All control mice succumbed to infection within 5 days post-challenge.

Accordingly, the results for vaccine efficacy in mice very closely correlated with those observed in chickens. Taken together, these data clearly demonstrate that the FPV-based vaccine is highly effective to induce cross-protection in animals against a lethal infection by an antigenically different influenza strain of the same subtype.

DISCUSSION

Proteolytic activation of influenza virus HA by cleavage into its subunits HA_1 and HA_2 has long been known to be a prime determinant of virulence.[19,30] Additional factors either encoded by the virus itself or provided by the host cell significantly contribute to host restriction, transmission and pathogenicity, and therefore, influenza virulence clearly is to be regarded a multifactorial trait.[31,32] In terms of proteolytic activation, two major classes of viruses can be distinguished. LPAIV contain HA with monobasic cleavage motifs susceptible to trypsin-like proteases present only in the respiratory or gastro-intestinal tract. In contrast, the HA of HPAIV has a multibasic cleavage site that is activated by the ubiquitous cellular protease furin, thus allowing for a systemic spread of the infection.[19,26,33] Attempts to confer high pathogenicity by solely introducing a multibasic cleavage site into the HA of LPAIV of H3- and H5-subtypes have so far proved unsuccessful,[34,35] clearly indicating that a multibasic HA cleavage site alone is not *per se* sufficient for the development of a HP phenotype for these subtypes.

In this study, we have generated and characterized protease activation mutants of the HPAIV A/FPV/Rostock/34 (H7N1). Using a reverse genetics approach, the long loop between HA_1 and HA_2 containing the multibasic cleavage site was converted into either a short loop (mutant 1) or into a long loop (mutant 2), each containing a single arginine as cleavage motif. The rationale for creating these different mutants was to trace potential impacts of HA cleavage loop conformation and accessibility on proteolytic activation and resulting viral pathogenicity.[36] Replication and pathogenicity of the mutants were analysed in cell culture, chicken embryos and chickens. In contrast to WT FPV, both virus mutants exhibited a classical low pathogenic phenotype as judged from the following findings: (i) cleavage activation and multistep replication in cell culture were fully dependent on the presence of exogenously added trypsin; (ii) growth in embryonated chicken eggs was identical to that observed for typical LPAIV; and (iii) the IVPIs of the mutants in chickens were 0.0 (mutant 1) and 0.07 (mutant 2) as compared to 2.34 of WT virus. This clearly reveals that the multibasic cleavage site is the key virulence factor of FPV and that its removal is sufficient to convert FPV into a virus with classical LPAIV phenotype. No significant differences regarding replicative properties or pathogenicity were detected between mutants 1 and 2 indicating that length and conformation of the cleavage loop have no effect on the accessibility of the monobasic cleavage site to the activating protease.

Table 1 Immunogenicity and protective efficacy in chickens of the inactivated vaccine prepared from FPV mutant 1

	HI-GMT[a] (log 2)							**Clinical manifestations**[b]	
	Days post vaccination				Days post challenge			Severe symptoms or death	Survival
	0	9	14	21	7	14	19		
Vaccinated animals	**0**	**5.23** (1.6)	**7.07** (1.9)	**8.51** (2.0)	**10.54** (0.5)	**10.19** (0.8)	**11.0** (0.0)	**0**	**100**
Control animals	**0**	—	—	—	**0**	—	—	**100**	**0**

A group of nine SPF-chickens was immunized intramuscularly with an adjuvanted vaccine preparation containing approximately 15 μg HA per dose. Three control animals remained unvaccinated in a separate cage. At day 22 post-vaccination, all animals were challenged with $10^{7.9}$ EID_{50} units of influenza A/chicken/Germany/R28/03 (H7N7). For the following 19 days, the animals were monitored for signs of infection (such as fever, oedema, ruffled feathers, diarrhoea, depression), mortality and shedding of the challenge virus. Serum antibodies were determined by HI assay.

[a] Geometric mean values of HI-titres and standard deviations (in brackets). Titres are determined with virus strain A/chicken/Germany/R28/03 (H7N7).

[b] Percentage of animals.

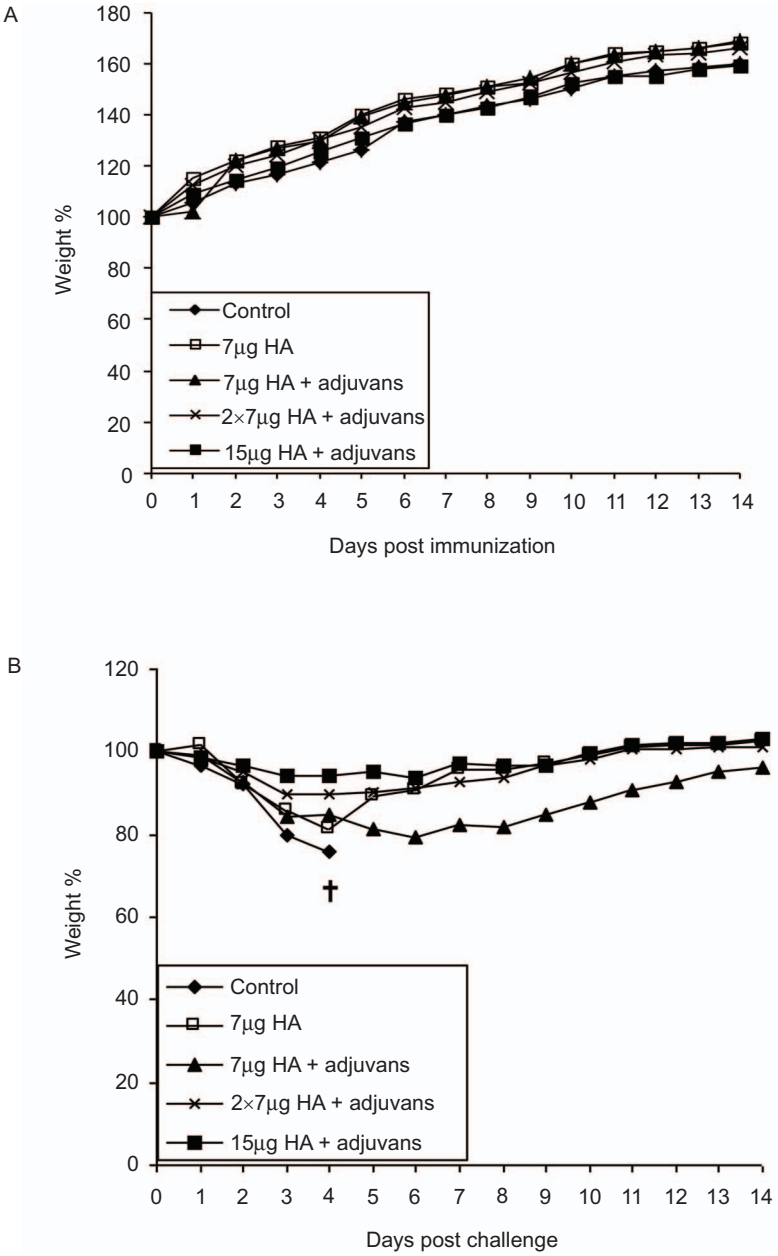

Figure 6 Immunisation of mice with formalin-inactivated vaccine prepared from FPV mutant 1 (H7N1) and subsequent challenge with 100LD_{50} of SC35M (H7N7). Groups of five mice each were immunized intramuscularly as indicated in the boxes. An unvaccinated group was used as a control. Mice were monitored for 14 days after immunisation (**A**) and after challenge (**B**) for weight loss and signs of disease.

Virus yields of WT and mutant FPV obtained in allantoic fluids were within the same range, indicating that the extensive loss of pathogenicity does not interfere with efficient replication in embryonated chicken eggs. Very importantly, during multiple passages in embryonated chicken eggs, we never observed viral escape mutants, indicating that the introduced cleavage site mutations are very stable. Because of these properties, we concluded that the FPV cleavage mutants might be promising candidates for safe and large-scale production of an avian influenza vaccine. In order to prove this concept, an inactivated vaccine produced from egg-grown FPV mutant 1 was prepared and used for the immunisation of chickens. Three weeks after vaccination, the animals were challenged with a lethal dose of the HPAIV isolate A/chicken/R28/03 (H7N7) that shares about 93% sequence identity in the HA protein with FPV. All vaccinated animals were fully protected against the challenge with this non-homologous virus and survived without showing signs of severe disease. In contrast, all non-vaccinated animals developed classical fowl plague symptoms and died within 3 days following challenge. These results clearly demonstrate that an inactivated vaccine based on a protease activation mutant of FPV as seed virus can be readily produced in chicken eggs and very efficiently protects chickens from severe clinical disease and death caused by a non-homologous but related HPAIV.

We also tested the efficacy of the vaccine in a mammalian challenge system by analysing its ability to protect mice from lethal infection with SC35M virus (H7N7).[29] All HA-antigen dosages and formulations tested protected mice from a lethal challenge while all non-vaccinated animals

died within 5 days after infection. As the HAs of FPV mutant 1 and SC35M display only 85% overall sequence homology, these results clearly demonstrate that the vaccine elicits a potent crossreactive immune protection. It can also be concluded from these observations that the vaccine is not restricted to the use in birds, but that it may also be applicable to a broader range of species. Hence, our data strongly indicate that FPV protease activation mutants constitute very useful seed strains for the rapid and large-scale production of safe and efficient H7-specific vaccines in a (pre)pandemic situation.

In a more general scenario, the FPV cleavage mutants might also represent a powerful alternative to strain A/PR/8/34 that is currently used almost exclusively as donor of the internal viral genes for the generation of reassortant seed strains on which human influenza vaccines are based.[37,38] FPV grows to very high titres in eggs as well as in cell cultures, and as shown here, the yields of the mutants are similar to those obtained with WT virus. In this context, it is important to note that while most influenza vaccines are still produced in embryonated chicken eggs, several cell culture-based vaccine products have recently been licensed for seasonal as well as for pandemic indications.[39–41] Therefore, the use of high-growth FPV reassortants with HA and NA proteins of circulating viruses generated on the basis of the cleavage mutants might be of considerable advantage especially in a pandemic situation where timely availability of large numbers of vaccine doses is of utmost importance for the immediate and efficient protection of vulnerable population groups.

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