Altered receptor specificity and fusion activity of the haemagglutinin contribute to high virulence of a mouse-adapted influenza A virus

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The viral haemagglutinin (HA) and the viral polymerase complex determine the replication fitness of a highly virulent variant of influenza A virus strain A/PR/8/34 (designated hvPR8) and its high pathogenicity in mice. We report here that the HA of the hvPR8 differs from the HA of a low virulent strain (lvPR8) by the efficiency of receptor binding and membrane fusion. hvPR8 bound to 2,6-linked as well as 2,3-linked sialic acid-containing receptors, whereas lvPR8 bound exclusively to 2,3-linked sialic acids with high avidity. Remarkably, hvPR8 infected its target cells faster than lvPR8 and tolerated an elevated pH for efficient membrane fusion. In spite of these differences, both viruses targeted type II but not type I pneumocytes in the lung of infected mice. The HA of hvPR8 differs from that of lvPR8 by 16 aa substitutions and one insertion. Mutational analyses revealed that amino acid at HA position 190 (H3 numbering) primarily determined the specificity of receptor binding, while the insertion at position 133 influenced the avidity of receptor binding. Both amino acid positions also strongly influenced viral virulence. Furthermore, leucine at position 78 and glutamate at position 354 were critical determinants of increased fusion activity and virulence of hvPR8. Our data suggest that the HA of hvPR8 enhances virulence by mediating optimal receptor binding and membrane fusion thereby promoting rapid and efficient viral entry into host cells.

INTRODUCTION

The viral haemagglutinin (HA) is a major determinant of virulence of influenza A viruses (Neumann et al., 2009). It is responsible for attachment to cellular receptors and low pH-induced fusion (Gamblin & Skehel, 2010). HA is inserted into the viral envelope as a trimeric complex and has a characteristic structure that consists of a stem and a membrane-distal, globular head domain (Skehel & Wiley, 2000; Stevens et al., 2004). The full-length HA0 precursor protein is cleaved into HA1, aa 1–328, and HA2, position 329–565. The N-terminal HA1 forms the globular head with the receptor-binding site (RBS). Both HA1 and HA2 form the stem region that is involved in fusion of the viral envelope with the cellular membrane in the endosomes. This process requires cleavage of HA0 by proteases resident in the respiratory tract, thereby restricting replication of human viruses to this organ (Bertram et al., 2010; Böttcher et al., 2006).

The RBS binds to glycoconjugates on the cell surface containing the terminal sialic acid (SA) N-acetyl neuraminic acid connected to galactose via an 2,3- or 2,6-linkage (2,3-SA and 2,6-SA, respectively) (Skehel & Wiley, 2000). Human viruses tend to bind to 2,6-SA, whereas avian viruses usually prefer 2,3-SA. The receptor-binding activity of HA is assumed to be in balance with the receptor-destroying activity of the neuraminidase (NA), which cleaves the terminal SA from sugar conjugates (Wagner et al., 2002).

Successful receptor binding leads to endocytosis of the virus. Subsequent acidification of the endosomes triggers conformational changes in HA, resulting in insertion of the N-terminal end of HA2, the so-called fusion peptide, into the target membrane. Further conformational changes in HA initiate fusion of the endosomal and viral membranes. Thus, HA determines virulence of influenza A viruses by the accessibility of the HA0 cleavage site for proteases, the specificity and affinity of HA for host cell receptors, and the efficiency to mediate membrane fusion.

Accordingly, changes in these properties were found in the HA of mouse-adapted influenza A viruses with virulence-enhancing mutations (Ward, 1997). Serial mouse passages of a clinical isolate of pandemic A/HK/1/68 (H3N2) resulted in a mouse-pathogenic virus with...
multiple alterations in HA (Brown et al., 2001) affecting receptor specificity and fusion activity (Keleta et al., 2008). Furthermore, enhanced virulence of a mouse-adapted A/Phil/82 (H3N2) virus was accompanied by optimized fusion activity at elevated pH values (Hartley et al., 1997). Recent mouse-adapted pandemic 2009 (H1N1) virus revealed a decrease of receptor-binding affinity due to changes in HA (Ilyushina et al., 2010).

We recently described a highly virulent A/PR/8/34 virus (hvPR8) that was generated by serial lung passages in Mx1-positive mice (Grimm et al., 2007; Haller, 1981). These mice exhibit a strong interferon-regulated antiviral defence against influenza A viruses due to the expression of a functional, interferon-induced Mx1 protein (Haller et al., 1979). hvPR8 showed enhanced replication and elevated virulence in Mx1-mice when compared with a regular PR8 virus, called lvPR8. These characteristics of hvPR8 were mostly determined by segment 1 encoding the PB2 subunit of the viral polymerase (Rolling et al., 2009) and segment 4 coding for HA (Grimm et al., 2007). The aim of the present study was to identify critical properties and accompanying amino acid changes in HA that determine the enhanced virulence of hvPR8 in the Mx1-positive mouse model. We found that the influence of HA on enhanced replication of hvPR8 involved a number of amino acid changes in the globular head as well as the stem region of HA. A detailed analysis of single and multiple amino acid exchanges identified positions in hvPR8 critically influencing receptor binding and fusion activity.

RESULTS

Efficient cell entry of hvPR8 depends on viral glycoproteins

Previous studies revealed that abortive infection of murine L929 cells with hvPR8 resulted in a much faster intracellular accumulation of the viral nucleoprotein (NP) as compared with infection with lvPR8 (Grimm et al., 2007). To determine the contribution of the two viral glycoproteins HA and NA to this phenotype, we infected L929 cells with viruses with swapped segments 4 and 6. In cells infected with hvPR8 or lvPR/hvHN carrying HA and NA of the highly virulent virus, NP accumulated with fast kinetics (Fig. 1). In contrast, in cells infected with lvPR8 or hvPR/hvHN, which carry HA and NA of hvPR8, accumulation of NP was delayed. This result suggested that the glycoprotein-dependent difference in early virus replication was due to more efficient binding of hvPR8 particles to cell surface receptors, more efficient fusion of virus and host cell membranes, or both.

Enhanced binding of hvHA to 2,6-linked SA

To determine the receptor-binding specificity of hvPR8 compared to lvPR8, we tested the viruses for binding to fetuin that was enzymically modified to carry either terminal 2,3- or 2,6-linked SA. We found that hvPR8 showed moderate affinity for both 2,3- and 2,6-linked SA, whereas lvPR8 exclusively bound to 2,3-linked SA albeit with enhanced affinity (Fig. 2).
Next, we tested whether the observed difference in receptor-binding specificity might translate into a different cell tropism in vivo. To compensate for differences in replication speed between the two viruses (Grimm et al., 2007; Rolling et al., 2009), mice were infected intranasally with a high infectious dose corresponding to a 100-fold LD$_{50}$ each. At 48 h post-infection, the lungs were analysed by staining simultaneously for viral NP and markers for type I and type II pneumocytes. A clear co-localization of NP with the marker for type II but not type I pneumocytes was observed with both viruses (Fig. 3), suggesting a similar cell tropism of hvPR8 and lvPR8 in the lung.

Residues in HA conferring virulence and influencing receptor binding

The amino acid sequences of the HAs of hvPR8 and lvPR8 differ from each other in 17 positions (Grimm et al., 2007). To determine which of these differences might confer virulence, we individually mutated the specific amino acids in the HA of hvPR8 and tested the resulting virus mutants for virulence in Mx1-positive mice. This study was performed with a virus that contains all segments from hvPR8 except for the NA-coding segment 6, which was derived from lvPR8. We previously reported that the combination of lvNA with lvHA resulted in a complete loss of virulence of hvPR8 (Grimm et al., 2007). We therefore expected that introduction of attenuating mutations into hvHA might show the strongest phenotype when combined with lvNA. Viruses carrying mutations in segment 4 were rescued in two independent approaches and were used to confirm the phenotypes of the mutation in HA. The recombinant viruses grew to comparably high titres in embryonated chicken eggs, within a range between $1 \times 10^8$ and $4 \times 10^8$ p.f.u. ml$^{-1}$, excluding general attenuating effects on virus replication or packaging. If amino acids in hvHA were mutated individually to the respective amino acids in lvPR8, we observed a minor decrease in virulence for positions 78, 133, 193 and 354 (H3 numbering) (Table 1). Interestingly, there was a prominent decrease in virulence, measured by determination of viral lung titres and LD$_{50}$, if arginine at position 133$^+$ in hvHA (133$^+$ designates an amino acid insertion in hvPR8 compared with lvPR8) was deleted (Fig. 4a and Table 1). Interestingly, the latter virus, designated hvHA(del R133$^+$), showed higher affinity for 2,3-linked as well as 2,6-linked SA compared with wild-type hvHA when tested in the fetuin-binding assay (Fig. 5). With respect to its binding affinity for 2,3-linked SA, hvHA(del R133$^+$) thus closely resembled lvPR8.

When critical changes were combined that lie in close proximity to the RBS, namely at position 131, 133, 193 and 193, both the LD$_{50}$ in Mx1-positive mice and the binding affinity for 2,3-linked and 2,6-linked SA did not increase much further compared to hvHA(del R133$^+$) (Table 1 and Fig. 5), suggesting that the deletion of arginine at position 133$^+$ is the most decisive change.

### Table 1. Virulence of viruses with various mutations in HA

<table>
<thead>
<tr>
<th>Nature of HA mutations</th>
<th>LD$_{50}$ (f.f.u.)</th>
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<tbody>
<tr>
<td>hvHA</td>
<td>$6 \times 10^2$</td>
</tr>
<tr>
<td>hvHA(L78P)</td>
<td>$2 \times 10^3$</td>
</tr>
<tr>
<td>hvHA(T131N)</td>
<td>$&lt;10^4$</td>
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<tr>
<td>hvHA(T133N)</td>
<td>$5 \times 10^3$</td>
</tr>
<tr>
<td>hvHA(del R133$^+$)$^+$</td>
<td>$2 \times 10^4$</td>
</tr>
<tr>
<td>hvHA(D190E)</td>
<td>$&lt;10^4$</td>
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<tr>
<td>hvHA(K193N)</td>
<td>$2 \times 10^3$</td>
</tr>
<tr>
<td>hvHA(T131N/T133N/del R133$^+$)</td>
<td>$4 \times 10^4$</td>
</tr>
<tr>
<td>hvHA(T131N/T133N/del R133$^+$/K193N)</td>
<td>$5 \times 10^4$</td>
</tr>
<tr>
<td>hvHA(T131N/T133N/del R133$^+$)/D190E/K193N</td>
<td>$5 \times 10^5$</td>
</tr>
<tr>
<td>hvHA(S328Y)</td>
<td>$&lt;10^3$</td>
</tr>
<tr>
<td>hvHA(Q354H)</td>
<td>$3 \times 10^3$</td>
</tr>
<tr>
<td>hvHA(L78P/Q354H)</td>
<td>$5 \times 10^3$</td>
</tr>
<tr>
<td>lvHA</td>
<td>$&gt;10^6$</td>
</tr>
<tr>
<td>lvHA(P78L/H354Q)</td>
<td>$10^7$</td>
</tr>
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</table>

*LD$_{50}$ values were determined by infecting groups of Mx1$^{+/+}$ mice with various doses of the indicated viruses. Each virus dose was applied to 6–8 mice. Animals were killed if severely ill or if weight loss exceeded 25%.

$^+$ In hvHA(del R133$^+$) the supernumerary arginine at position 133$^+$ in HA of hvPR8 was deleted.

\[D190E/K193N\]

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**Fig. 3.** The glycoproteins of hvPR8 do not affect cell tropism in vivo. Mx1-positive mice were infected with 100 LD$_{50}$ of lvPR8 and hvPR8, respectively. At 48 h p.i. lungs were taken, inflation fixed and embedded in paraffin. Lung sections were stained with various doses of the indicated viruses. Each virus dose was applied to 6–8 mice. Animals were killed if severely ill or if weight loss exceeded 25%.

In hvHA(del R133$^+$) the supernumerary arginine at position 133$^+$ in HA of hvPR8 was deleted.
We also analysed the impact of alterations at position 190 that was previously reported to modulate affinity of HA for 2,6-linked SA (Glaser et al., 2005). Although the D190E exchange alone in hvHA did not influence virulence of the corresponding virus in mice, the combination of D190E with changes at positions 131, 133, 133+ and 193 resulted in a large increase in LD50 (Table 1). However, these five exchanges in hvHA showed only a moderate effect on viral replication in the lungs of infected Mx1-positive animals at 30 h post-infection (Fig. 4a). Interestingly, the D190E exchange in the context of the other mutations was accompanied by a loss of binding to 2,6-linked SA (Fig. 5).

We also tested whether the exchange of critical amino acids in HA of lvPR8 would enhance virulence. The virus carrying lvHA with substitutions P78L and H354Q was substantially more virulent than parental lvHA virus (Fig. 4b and Table 1). Unfortunately, we were not able to study the effects of amino acid exchanges at positions 131, 133, 133+, 190 and 193. Mutations at these positions in lvHA either did not yield viable viruses, or the recombinant viruses were unstable and acquired additional mutations during the rescue.

**Fusion activity of hvPR8**

Besides receptor binding, the fusogenic activity of HA determines the virulence of influenza viruses. This function is dependent on post-translational cleavage of HA0 into HA1 and HA2 by trypsin-like proteases. Because hvHA differs from lvHA at position 328, S328Y, directly adjacent to the arginine at position 329 of the protease cleavage site of PR8-HA, we compared the cleavage of hvHA and lvHA by trypsin. The HAs of hvPR8 and lvPR8 did not show significant differences in the accumulation of cleaved HA1 when cleavage was analysed in transfected 293T cells incubated with increasing amounts of trypsin in the culture medium (data not shown). Additionally, we studied the effect of an exchange of serine at position 328 into tyrosine on virulence of hvHA(S328Y). This mutated virus showed no attenuation in Mx1-mice when compared with the virus

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**Fig. 4.** Growth behaviour of recombinant hvPR8 with specific mutations in HA. B6.A2G-Mx1 mice (n=6) were infected with 1000 p.f.u. of PR8 viruses encoding all segments of hvPR8 except segment 6 and carrying the indicated amino acid exchanges in hvHA (a) or lvHA (b), respectively. At 30 h p.i., the lungs were removed and virus titres were determined by plaque assay on MDCK cells.

**Fig. 5.** Receptor-binding specificity of viruses with mutations in HA. Viruses encoding all segments of hvPR8 except segment 6 of lvPR8 and carrying the indicated amino acid exchanges in hvHA were tested for binding specificity in a solid-phase assay with modified fetuin as described in Fig. 2. PBS (Ctrl), A/mallard/Alberta/47/98 (H4N1) (Avian) and A/Memphis/14/96 (H1N1) (Human) served as controls. Because of the variation of the absolute values of affinity constants between different experiments, the graph shows data for one experiment representative of three independent experiments with similar results.
carrying the wild-type hvHA (Table 1), indicating that the amino acid difference at position 328 does not contribute to the higher virulence of hvPR8.

To analyse fusion activity of the two HA molecules, Madin–Darby canine kidney (MDCK) cells were infected in the presence of increasing amounts of NH4Cl in the medium. NH4Cl counteracts acidification of the endosomal compartment and thus prevents conformational changes in HA that are necessary for fusion (Matlin et al., 1981). Successful viral fusion, entry and uncoating were monitored by staining the cells for newly synthesized NP at 6 h post-infection. Infection rates of untreated cells were set to 100 %. Viruses carrying hvHA entered the cells even in the presence of 3 mM NH4Cl in the culture medium, whereas viruses carrying lvHA did not initiate entry and expression of viral NP at NH4Cl concentrations above 1.5 mM (Fig. 6a). This result suggested that viruses expressing HA of lvPR8 need stronger acidification for activation of fusion, whereas HA of hvPR8 is able to mediate membrane fusion at relatively high pH.

To directly measure the influence of the pH on membrane fusion activity of the HA proteins, hvPR8 or lvPR8 viruses were mixed with human erythrocytes and incubated for 20 min at 37 °C in buffers with different pH values. Structural changes in the HA then initiated membrane fusion and haemolysis. By measuring absorption of the released haemoglobin, we determined the fusion activity of HA. Whereas decreasing the pH to 5.8 was sufficient for hvPR8 to mediate fusion, lvPR8 only induced fusion at pH 5.3 and lower (Fig. 6b). This result suggested that viruses expressing HA of lvPR8 need stronger acidification for activation of fusion, whereas HA of hvPR8 is able to mediate membrane fusion at relatively high pH.

To further analyse the fusogenic activity of HA, a fusion assay was established using transfected HA-expressing cells (Su et al., 2008). To this end, 293T cells were co-transfected with expression constructs for HA and a T7-driven
luciferase reporter gene. In a second 293T cell population, only the recombinant bacteriophage T7 polymerase was transiently transfected. Then, these two cell types were mixed and incubated with trypsin and buffers with different pH values to initiate fusion activity of HA. Fusion of the cells led to the expression of firefly luciferase that served as a direct measure of HA fusion activity. In this virus-free test system, hvHA-expressing cells showed enhanced reporter gene expression at pH 5.5. lvHA initiated luciferase expression only at pH 5.3 (Fig. 6c).

Residues in hvHA that influence fusion activity

Neither single amino acid exchanges at positions 78 and 354 nor single exchanges of amino acids in the RBS influenced the ability of hvHA to mediate fusion in the T7-based reporter system at pH 5.5 (Fig. 7a). Interestingly, when positions 78 and 354 in hvHA were both exchanged to the corresponding residues of lvHA, fusion activity was reduced (Fig. 7b). Furthermore, simultaneous exchange of P78L and H354Q in lvHA significantly increased fusion activity of lvHA at pH 5.5 (Fig. 7b). Positions 78 and 354 also influenced virulence in mice: the virus carrying hvHA(L78P/Q354H) was less virulent than the virus containing wild-type hvHA (Fig. 4a and Table 1). On the other hand, the virus lvHA(P78L/H354Q) showed enhanced virulence compared with the virus encoding wild-type hvHA (Fig. 4b and Table 1). Although changes in these positions had only a moderate effect on LD50 when compared to the changes in the RBS of hvPR8, the data indicate that positions 78 and 354 in the HA protein of hvPR8 contribute to fusion at elevated pH and to increased virulence of the virus in mice.

DISCUSSION

Here, we describe the molecular analysis of HA in a mouse-adapted A/PR/8/34 (hvPR8) with enhanced virulence in Mx1-positive mice. Analyses of reassortant viruses demonstrated a rapid and efficient entry of the viruses mediated by the glycoproteins of hvPR8. To identify the molecular basis of this phenotype, receptor-binding specificity and fusion activity of hvHA were determined and critical amino acids in hvHA were identified.

We found a dual specificity of hvHA for avian-like 2,3- and human-like 2,6-SA receptors. This is consistent with previous characterizations of PR8-HA receptor-binding properties and co-crystallization of PR8-HA with both SA receptors (Gamblin et al., 2004; Rogers & D’Souza, 1989; Suzuki et al., 1987). By contrast, viruses carrying lvHA did not bind to 2,6-SA and bound only to 2,3-SA-containing receptors albeit with higher affinity. We showed that the altered receptor-binding profile of hvPR8 compared to lvPR8 did not translate into a different cell tropism in the alveolar epithelium in mice, as both viruses infected type II pneumocytes. To date, only a limited number of studies have been performed to analyse SA distribution in the respiratory tract of mice, detecting either both 2,3- and 2,6-SA structures (Glaser et al., 2007; Ning et al., 2009) or exclusively 2,3-SA in the murine alveolar epithelium (Gagneux et al., 2003; Ibricevic et al., 2006; Pekosz et al., 2009). Thus, it remains open to how exactly the distinctive receptor-binding profile of hvPR8 facilitates virus replication in the mouse respiratory tract.

The HAs of hvPR8 and lvPR8 differ by 17 aa residues. Among them is an insertion of an additional arginine between positions 133 and 134 (H3 numbering) in hvHA, here designated R133+. This amino acid is located within...
the 130-loop that together with the 220-loop form two sides of the RBS. The loops are known to interact directly with the sialyloligosaccharide moiety of the receptor (Gamblin et al., 2004; Gamblin & Skehel, 2010). Deletion of R133+ in hvHA led to a significant increase in the binding affinity to both SA receptors and to a strong reduction of virulence. It is conceivable that a drastic change like the deletion of a positively charged arginine in the 130 loop has an impact on receptor binding. The 190-helix is the third structural component of the RBS with contacts to both terminal SA moiety and to more distant sugar residues of the receptor (Gamblin et al., 2004). Several studies described the importance of position 190 for the receptor specificity of HA. Aspartic acid instead of glutamic acid (E190D) is believed to result in a broader receptor-binding pocket size allowing 2,3-SA as well as 2,6-SA binding (Stevens et al., 2004). Accordingly, exchange D190E in the HA of the pandemic H1N1 virus of 1918 drastically reduced binding to the 2,6-SA receptor (Glaser et al., 2005) and reduced pathogenicity in mice (Qi et al., 2009) as well as transmission in ferrets (Tumpey et al., 2007). Interestingly, in our study, exchange D190E had no effect on the virulence of hvPR8. However, when exchange D190E was combined with exchanges at positions 193 and in the 130-loop, the corresponding virus showed a 10-fold increase in its LD50+ accompanied by a complete loss of binding to 2,6-SA but almost unchanged binding to 2,3-SA. Thus, amino acids R133+ and D190 were crucial for the overall reduction of receptor-binding activity and dual receptor specificity of hvHA, respectively. Furthermore, both positions significantly influenced virulence of hvPR8. A reduced receptor-binding affinity could enable hvPR8 to escape soluble SA-containing receptor analogues in the bronchial mucus, as previously envisaged for human influenza viruses (Couceiro et al., 1993; Lamblin et al., 2001; Reading et al., 2008). Additionally, a reduction of receptor-binding affinity could promote efficient infection of the host cells by allowing repetitive rounds of receptor binding and dissociation until optimal conditions for endocytosis are found, as proposed previously (Ohuchi et al., 2006).

We further observed that hvHA induced cell entry and fusion at a pH value 0.5 unit higher compared with lvHA. The ability of hvPR8 to mediate fusion at an elevated pH makes the virus competent for early and efficient uncoating and minimizes the risk of premature lysosomal degradation (Yoshimura & Ohnishi, 1984). Among the residues influencing virulence, we identified position 78 in combination with position 354 to optimize fusion activity of hvHA. The two positions were not involved in receptor binding and had a smaller but significant effect on virulence compared with positions 190 and 133+. Amino acid exchanges influencing fusion activity are normally found within or near the fusion peptide or at interfaces between domains that rearrange upon acidification (Daniels et al., 1985; Lin et al., 1997; Reed et al., 2009; Thoennes et al., 2008). Accordingly, position 354 lies in the vicinity to the fusion peptide that comprises the N-terminal 23 aa (aa 330–352) of the HA2 subunit (Cross et al., 2009). One may speculate that the H354Q exchange has a destabilizing effect on the fusion peptide, thereby lowering the energy barrier that is needed for membrane fusion, as described for other mutations within or near the fusion peptide (Daniels et al., 1985). The second virulence determining position 78 has a non-typical position for an amino acid influencing pH-regulated fusion, in that it lies underneath the globular head of HA at the outer face of the trimeric HA complex. hvHA has a leucine instead of proline at position 78. This amino acid exchange could induce a conformational change that influences fusion activity of other parts of HA.

Amino acid exchanges in HA that result in fusion activity at elevated pH values were previously described for cell culture-adapted influenza viruses (Daniels et al., 1985; Lin et al., 1997) as well as viruses serially passaged in mice (Keleta et al., 2008; Smeenk et al., 1996). A mouse-adapted virus derived from the pandemic A/HK/1/68 (H3N2) showed changes in HA that promoted fusion at elevated pH (Keleta et al., 2008). Likewise, another study using strain A/Netherlands/219/03 showed that an exchange at position 352, which is close to position 354 in HA of hvPR8, allowed fusion activity at elevated pH and correlated with increased virulence in mice (Ilyushina et al., 2007). Furthermore, it was shown that fusion at higher pH augments virulence of influenza viruses also in ducks (Reed et al., 2010). However, none of the sequence alterations in HA described for duck viruses were close to the amino acid positions critical for mouse-adapted hvPR8. The previous and our findings indicate that early uncoating at higher pH levels is advantageous for the infecting virus, enabling it to leave the hostile endosomal compartment as early as possible to escape degradation in the endo-lysosomal compartment (Yoshimura & Ohnishi, 1984).

In summary, we hypothesize that the HA of hvPR8 displayed two strategies to enhance virulence in vivo. They involve distinct mutations in the HA molecule leading to (i) optimized receptor binding and (ii) increased fusion activity in a less acidic environment. It is conceivable that, upon introduction into the human population, animal influenza A viruses like the recent pandemic H1N1 strain will be subject to similar changes during adaptation to the human host.

**METHODS**

**Viruses.** All the experiments presented in this study were carried out with recombinant viruses generated by reverse genetics. hvPR8 and lvPR8 strains were described previously (Grimm et al., 2007). Virus stocks were produced in 9-day-old embryonated chicken eggs. Virus titres were determined by infecting MDCK cells with 10-fold serial dilutions in PBS containing 0.3% BSA. Infected cells were detected with a rabbit anti-PR8 serum and donkey anti-rabbit Alexa Fluor 555 (Invitrogen). Virus titres are expressed as f.f.u. For the receptor-binding...
studies, the allantoic fluids were clarified by low-speed centrifugation; the viruses were pelleted by high-speed centrifugation, resuspended in PBS containing 60 % glycerol and stored at −20 °C.

**Generation of recombinant viruses.** Mutations were introduced into the HA encoding cDNA by PCR. The resulting cDNAs were cloned into the ambisense expression vector pDZ (Quinlivan et al., 2005). For rescue of viruses, eight pDZ plasmids were transfected into co-cultures of 293T and MDCK cells as described previously (Grimm et al., 2007). Viruses in the supernatants were plaque purified on MDCK cells. For each recombinant virus, two independent rescued virus stocks were generated and the mutations confirmed by sequencing. All rescued viruses grew well in eggs and reached comparable titres of about 10^8 p.f.u. ml⁻¹.

**Infection of L929 cells and Western blot analysis.** L929 cells were infected at an m.o.i. of 0.5 for the indicated times and lysed in buffer containing 50 mM Tris pH 7.5, 280 mM NaCl, 0.5 % Nonidet P-40, 2 mM EDTA and 0.5 % Triton X-100. Lysates were treated with SDS and 2-mercaptoethanol. Proteins were separated by SDS-PAGE and transferred onto PVDF membranes. The blots were probed with monoclonal mouse anti-β-actin (Sigma) antibodies. HRP-labelled secondary antibodies and the ECL detection system (Roche) were used to detect primary antibodies.

**Mice.** B6.A2G-Mx1 mice (Stacheli et al., 1985) carrying intact Mx1 alleles on the C57BL/6 background (Mex1^{+/−}) were bred locally. Six- to 8-week-old animals were used for the experiments that were performed in accordance with the local Animal Care Committee and the Regierungspässedienst, Freiburg, Germany. For infection, groups of animals (n=6-8) were anesthetized by intraperitoneal injection of a mixture of ketamine (100 μg per gram of body weight) and xylazine (5 μg per gram of body weight) and infected intranasally with 50 μl virus dilution. Animals were euthanized if severe symptoms developed or if weight loss exceeded 25 %. LD₅₀ values were calculated as described previously (Reed & Muench, 1938).

**Virus lung titres.** Anaesthetized mice were infected intranasally with 1000 p.f.u. of the recombinant viruses. At 30 h post-infection (p.i.), the lungs were collected and homogenized in PBS using the FastPrep24 (MP Biomedicals). Tissue debris was removed by low-speed centrifugation and virus titres were determined by plaque assay.

**Lung histology.** Mouse lungs were inflation fixed with PBS containing 4 % formaldehyde pH 7.0, embedded in paraffin and cut into 5 μm slices. For staining, sections were bathed in a series of solutions with decreasing concentrations of ethanol. After permeabilization of tissues with 0.1 % Triton X-100, non-specific antibody binding was blocked with 1 % goat serum for 30 min. As primary antibodies served hamster anti-podoplanin (Abcam) for detection of type I pneumocytes, rabbit anti-pro-surfactant protein C (Chemicon Millipore) for detection of type II pneumocytes and a human anti-influenza A serum for detection of virus. Goat anti-hamster Cy3 (Dianova), donkey anti-rabbit Alexa Fluor 555 (Invitrogen) and goat anti-human DTAF (Dianova) were used as secondary antibodies.

**Receptor-binding assay.** Receptor specificity was assayed using monospecific fetuin-HRP conjugates containing either 2,6-linked- or 2,3-linked SA moieties as described previously (Matrosovich et al., 2003). In brief, dilutions of purified viruses were adsorbed to fetuin-coated 96-well plates (Greiner Bio-One). Then the wells were incubated with serial dilutions of HRP-labelled resialylated fetuins (either 2,3-fetuin or 2,6-fetuin) for 1 h at 4°C in PBS containing 0.02 % BSA, 0.02 % Tween 80 and 2 μM neuraminidase inhibitor osetamivir carboxylate. After washing with ice-cold 0.02 % Tween 80 in PBS, bound HRP-fetuin was determined by the peroxidase activity present in the wells. The data were converted to A490/C versus A490 Scatchard plots, arbitrarily taking the concentration of fetuin-HRP stocks for 1000 U ml⁻¹. The affinity constants of the virus–fetuin complexes were determined from the slopes of these plots.

**HA cleavage by trypsin.** 293T cells were transfected with the expression vector pDZ encoding hvHA or lvHA. After an incubation period of 48 h, the cells were treated with TPCK-trypsin (Sigma) at different concentrations for 20 min at 37°C. Subsequently, the cells were lysed and subjected to Western blot analysis. For detection of HA, rabbit anti-PR8 serum and donkey anti-rabbit HRP (GE Healthcare) antibodies were used.

**Infection of MDCK cells in the presence of NH₄Cl.** MDCK cells were infected with an m.o.i. of 1 of viruses in the presence of the indicated concentrations of NH₄Cl diluted in PBS–0.3 % BSA. After an incubation period of 1 h at room temperature, the inoculum was replaced by culture medium containing NH₄Cl at the same concentrations used before. At 6 h p.i., cells were fixed and virus-infected cells were detected using a rabbit anti-PR8 serum as primary and donkey anti-rabbit Alexa Fluor 555 antibody (Invitrogen) as secondary antibodies.

**Haemolysis assay.** Allantoic fluid containing 1 x 10⁸ f.e.u. of virus was incubated with human erythrocytes (1 % diluted in PBS) for 10 min on ice. The mixture was divided into aliquots of 0.5 ml that were subsequently centrifuged for 1 min at 100 g. The pellets were incubated for 20 min at 37°C with 0.5 ml of 0.15 M citrate-buffered saline with the indicated pH values. After centrifugation of the samples for 1 min at 10 000 g, the levels of the virus-induced haemolysis were determined by measuring the haemoglobin in the supernatants at 575 nm.

**Reporter fusion assay.** The assay was performed as described previously (Su et al., 2008). 293T cells were transfected using Nanofectin (PAA Laboratories) with pDZ expression plasmids encoding HA (2 μg), pTM1-FFLuc encoding firefly luciferase (FF-Luc) under the control of the T7 promoter (1 μg) and pRL-SV40 encoding renilla-luciferase (Ren-Luc) under the control of the simian virus 40 (SV40) promoter (50 ng). Other cells were transfected with a chicken β-actin promoter expression plasmid, pCAGGS, for the T7 polymerase (3 μg). At 40 h after transfection, cells from the two transfections were mixed in a six-well dish and incubated for 5 h at 37°C. Then the cells were treated with TPCK-trypsin (1 μg ml⁻¹) for 2 min at 37°C and fusion was induced by changing the cell culture medium to PBS adjusted to pH 5.1, 5.3 or 5.5 for 2 min at 37°C. After an additional incubation period of 7 h at 37°C in cell culture medium, luciferase activity was determined in the cell lysates by using the dual-luciferase assay system (Promega). Firefly luciferase activity was normalized to renilla luciferase activity.

**Nucleotide sequence accession numbers.** GenBank accession numbers for the genomic segments of hvPR8 are EF190971–EF190978, and those of lvPR8 are EF190979–EF190986.

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