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Amino acids adjacent to the haemagglutinin cleavage site are relevant for virulence of avian influenza viruses of subtype H5

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The prime virulence determinant of highly pathogenic avian influenza viruses (HPAIVs) is the polybasic haemagglutinin (HA) cleavage site. However, engineering of a polybasic cleavage site into an avian influenza virus of low pathogenicity does not result in transformation into an HPAIV, indicating the importance of other adaptations. Here, the influence of amino acids adjacent to the HA cleavage site on virulence was studied. Most HPAIVs of subtype H5 carry serine or threonine at position 346 (corresponding to position 323 according to H3 numbering), whereas almost all low-pathogenic H5 viruses have valine. Moreover, all H5 low-pathogenic strains carry threonine at position 351 (corresponding to position 328 according to H3 numbering), suggesting that acquisition of a polybasic cleavage site involves several steps. This study generated a virus mutant derived from HPAIV A/Swan/Germany/R65/06 H5N1 (R65) with a monobasic cleavage site, $R65_{mono}$-S-ER, and the following additional mutants: $R65_{mono}$-V-ER with serine changed to valine at position 346, and $R65_{mono}$-S-ETR and $R65_{mono}$-V-ETR with threonine inserted at position 351. Moreover, in the R65 HA, serine was replaced with valine at position 346 (R65-V). Infection of chickens with $R65_{mono}$-S-ETR or $R65_{mono}$-S-ER led to slight transient respiratory symptoms, whereas R65-infected animals died within 2 days. However, chickens infected with R65-V survived longer than R65-infected animals, indicating that serine 346 in R65 HA contributes to virulence. These data suggest that evolution of H5 HPAIVs from low-pathogenic precursors, besides acquisition of a polybasic cleavage site, involves adaptation of neighbouring regions.

INTRODUCTION

Highly pathogenic avian influenza viruses (HPAIVs), which cause devastating losses in gallinaceous birds, invariably specify haemagglutinin (HA) subtypes H5 or H7 with a polybasic cleavage site (Garten & Klenk, 2008; Neumann & Kawaoka, 2006). At this site, the precursor HA_0 has to be cleaved into the HA_1 and HA_2 subunits to mediate fusion between virion and endosomal membranes (Huang *et al.*, 1980; Maeda & Ohnishi, 1980; White *et al.*, 1981, 1982). The polybasic cleavage site of HPAIVs is susceptible to the ubiquitous protease furin (Stieneke-Grober *et al.*, 1992), facilitating systemic spread and lethal disease in galliformes. In contrast, low-pathogenic avian influenza viruses (LPAIVs) have an HA cleavage site with only one basic arginine or lysine residue (Bosch *et al.*, 1979; Garten *et al.*, 1981; Gunther *et al.*, 1993; Kawaoka *et al.*, 1990) that can be cleaved only by proteases with monobasic specificity. Accordingly, LPAIV infection in birds is confined to the digestive or respiratory tracts and leads to milder illness or no disease. Therefore, the polybasic HA cleavage site is considered the prime virulence determinant of influenza viruses (Bosch *et al.*, 1979; Garten *et al.*, 1981, 1982; Horimoto & Kawaoka, 1994; Senne *et al.*, 1996).

HPAIVs are known to evolve from LPAIVs (Garcia *et al.*, 1996; Garten & Klenk, 2008; Horimoto *et al.*, 1995b; Kawaoka & Webster, 1985; Perdue *et al.*, 1997; Rohm *et al.*, 1995) by insertion mutations at the cleavage site region. Several highly pathogenic strains have acquired fragments from the 28S rRNA sequence (Khatchikian *et al.*, 1989) or a gene segment derived from the same or a different virus strain (Morsy *et al.*, 1994; Pasick *et al.*, 2005; Suarez *et al.*, 2004) leading to elongation of the cleavage site region. Polybasic HA cleavage sites may also be generated by polymerase slippage (Garcia *et al.*, 1996; Perdue *et al.*, 1997). However, whereas transformation of the polybasic cleavage site to a monobasic motif results in a drastic reduction in virulence (Horimoto & Kawaoka, 1994), conversion of the monobasic HA cleavage site of LPAIVs to a polybasic motif may or may not lead to HPAIVs (Bogs *et al.*, 2010; Munster *et al.*, 2010; Stech *et al.*, 2009). Thus,

evolution from LPAIVs to HPAIVs appears to require, in addition to the acquisition of a polybasic motif, additional adaptations within and/or outside the HA gene. In this study, we analysed the immediate vicinity of the HA cleavage site for its contribution to viral virulence. By reverse genetics, we generated different mutants from HPAIV A/Swan/Germany/R65/06 (H5N1) (R65) (Weber *et al.*, 2007) with a monobasic cleavage site but altered neighbouring regions, and investigated their *in vitro* properties and pathogenicity in chickens.

RESULTS

Generation of recombinant viruses

Comprehensive comparison of HA cleavage sites and their adjacent regions have revealed that H5 HPAIVs carry, at position 346 (corresponding position 323 according to H3 numbering; Ha *et al.*, 2001; Nobusawa *et al.*, 1991), either serine or threonine, with the exception of few strains. In contrast, almost all LPAIVs strains possess valine at this position. Furthermore, H5 LPAIVs, except for a few strains, carry the cleavage site motif ETR↓G. This observation suggests that acquisition of a polybasic cleavage site might also involve the loss of the threonine residue at the P2 position of the cleavage site. Therefore, we analysed whether the serine residue at position 346 is involved in virulence and whether the ETR↓G motif is essential for LPAIVs.

First, we changed the polybasic stretch in the HA cleavage site of R65 (aa 351–355) to a single basic residue resulting in the cleavage site ER↓G. However, among the LPAIVs with subtype H5, we found such a motif in none of the surveyed H5 sequences available in GenBank (Bao *et al.*, 2008); almost all other HAs carried the site ETR↓G, with only a very few specifying XTR↓G. Accordingly, we generated a second mutant with threonine inserted at position 351 (Table 1). As we observed that the majority of H5 HPAIVs carried a serine or threonine at position 346, whereas almost all LPAIVs have valine at the corresponding position, we replaced serine 346 with valine in the R65 HA and in the two monobasic HA variants ER and ETR (Table 1). By co-transfection of the appropriate HA plasmids with plasmids encoding the other seven R65 gene segments (PB2, PB1, PA, NP, NA, M and NS genes) (Stech *et al.*, 2008), we rescued the parent virus R65 and its mutants R65-V, $R65_{mono}$-S-ETR, $R65_{mono}$-V-ETR, $R65_{mono}$-S-ER and $R65_{mono}$-V-ER.

In vitro properties

To investigate replication of the viruses *in vitro*, we performed plaque assays in the presence or absence of trypsin. R65 and R65-V, which both carry polybasic HA cleavage sites, were able to form plaques in the absence of any exogenous protease (Fig. 1a). However, the monobasic mutants $R65_{mono}$-S-ETR, $R65_{mono}$-V-ETR, $R65_{mono}$-S-ER and $R65_{mono}$-V-ER showed plaque formation only after addition of trypsin (Fig. 1a and data not shown), thus displaying the phenotype of a LPAIV.

To study cleavage of the HA precursor (HA_0) directly, infected MDCK-H cells were subjected to Western blot analysis. After infection with the monobasic cleavage site mutants $R65_{mono}$-S-ETR, $R65_{mono}$-V-ETR, $R65_{mono}$-S-ER and $R65_{mono}$-V-ER, the HA subunits HA_1 and HA_2 were detected only after addition of trypsin to the medium (Fig. 1b), whereas HA_0 of R65 and R65-V was cleaved independently of trypsin, as expected.

In multicycle growth kinetics, all viruses reached similar titres in the presence of trypsin. In the absence of any exogenous protease, replication of R65 and R65-V was unaffected, whereas the monobasic cleavage site mutants $R65_{mono}$-S-ETR, $R65_{mono}$-V-ETR, $R65_{mono}$-S-ER and $R65_{mono}$-V-ER reached comparable titres at 8 h but stagnated from then on at levels 4–5 orders of magnitude lower (Fig. 1c). These different kinetics suggested that they were able to complete only one replication cycle in the absence of exogenous protease. However, final titres differed to some extent, with $R65_{mono}$-S-ETR as the most and $R65_{mono}$-V-ER as the least efficiently growing virus, with $R65_{mono}$-V-ETR and $R65_{mono}$-S-ER having intermediate titres. Taken together, these data demonstrated that removal of the polybasic HA cleavage site in R65 resulted in the *in vitro* phenotype of a LPAIV and suggest that threonine 351 and serine 346 may facilitate replication of the monobasic cleavage site mutants.

Table 1. Recombinant viruses with their HA cleavage site regions

Name	Description	HA cleavage site
R65	A/Swan/Germany/R65/06 (H5N1), polybasic cleavage site, serine at position 346	N<u>S</u>PQGE*RRRKK*R↓G
R65-V	R65, serine replaced with valine at position 346	N<u>V</u>PQGE*RRRKK*R↓G
$R65_{mono}$-S-ETR	R65, monobasic cleavage site, threonine inserted at position 351	N<u>S</u>PQGE**T**——R↓G
$R65_{mono}$-V-ETR	R65, monobasic cleavage site, threonine inserted at position 351 and valine at position 346	N<u>V</u>PQGE**T**——R↓G
$R65_{mono}$-S-ER	R65, monobasic cleavage site	N<u>S</u>PQGE———R↓G
$R65_{mono}$-V-ER	R65, monobasic cleavage site, valine at position 346	N<u>V</u>PQGE———R↓G

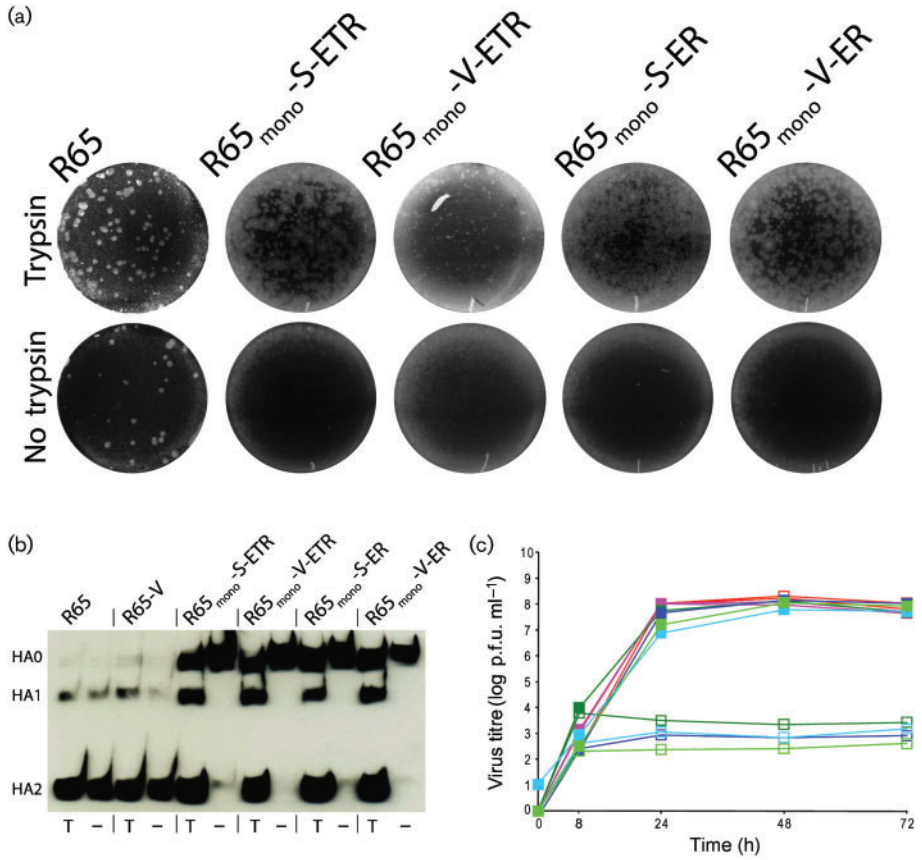

Fig. 1. *In vitro* replication properties of the engineered mutant viruses. (a) Plaque assays of the monobasic cleavage site mutants R65$_{mono}$-S-ETR, R65$_{mono}$-V-ETR, R65$_{mono}$-S-ER and R65$_{mono}$-V-ER in MDCK cells in the presence and absence of trypsin compared with wild-type HPAIV R65. (b) Western blots of MDCK-H cells infected with R65, R65-V, R65$_{mono}$-S-ETR, R65$_{mono}$-V-ETR, R65$_{mono}$-S-ER or R65$_{mono}$-V-ER at an m.o.i. of 0.1 in the presence (T) or absence (–) of trypsin. (c) Growth curves from MDCK-H cells inoculated with R65 (red), R65-V (magenta), R65$_{mono}$-S-ETR (green), R65$_{mono}$-V-ETR (blue), R65$_{mono}$-S-ER (light blue) or R65$_{mono}$-V-ER (light green) at an m.o.i. of 10^{-3} in the presence (filled squares) or absence (open squares) of trypsin. Viral titres in the supernatant were determined at the indicated times by plaque assay on MDCK-H cells in the presence of trypsin.

Pathogenicity in chicken

To study virulence in the natural host, we inoculated chickens via the oculo-nasal route with 10^6 $TCID_{50}$ R65 or R65-V. All R65-infected chickens died on day 2 post-inoculation (p.i.); eight of the ten animals displayed significant symptoms by day 1 p.i. In contrast, in the R65-V-infected group, only five animals exhibited symptoms at day 1 p.i., whilst five animals died on day 2 p.i. and the other five died on day 3 p.i. (Fig. 2a).

In a second experiment, chickens were infected oculo-nasally with 10^6 $TCID_{50}$ of the monobasic cleavage site mutants R65$_{mono}$-S-ETR, R65$_{mono}$-V-ETR, R65$_{mono}$-S-ER, and R65$_{mono}$-V-ER. Over a period of 10 days, R65$_{mono}$-S-ETR-infected and, to a lesser extent, R65$_{mono}$-S-ER-infected chickens developed very mild sporadic respiratory symptoms, whereas R65$_{mono}$-V-ETR- and R65$_{mono}$-V-ER-infected animals did not develop any clinical signs (Fig. 2b). Five chickens infected with R65$_{mono}$-V-ETR were challenged on day 16 p.i. with a lethal dose of 10^6 EID_{50} native R65 and observed for 7 days. All animals survived without any sign of illness.

Taken together, these minor differences in virulence suggested that serine 346 might facilitate replication of the low-pathogenic monobasic cleavage site mutants and the high-pathogenic parental virus R65 in chicken.

Virus shedding

To investigate the extent of virus shedding, oral and cloacal swabs were subjected to virus titration and quantitative real-time RT-PCR. One R65-infected, moribund animal sampled on day 2 p.i. exhibited $10^{4.9}$ $TCID_{50}$ ml^{-1} in the oral swab and $10^{4.3}$ $TCID_{50}$ ml^{-1} in the cloacal swab. From the R65-V-infected animals, we analysed five animals on day 2 p.i., yielding oral swab titres between $10^{3.4}$ and $10^{5.3}$

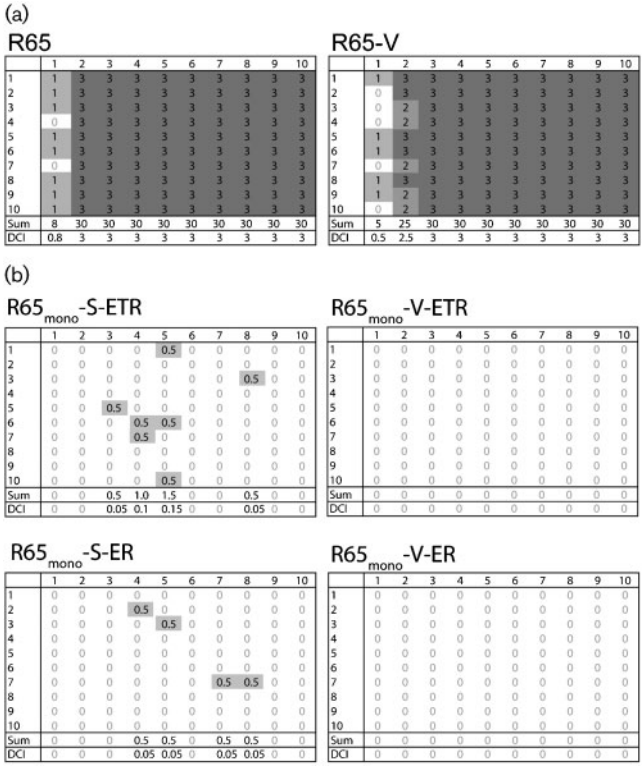

Fig. 2. Virulence in chickens. Survival and disease after oculonasal inoculation with 10^6 $TCID_{50}$ R65 or R65-V (a) or 10^6 $TCID_{50}$ $R65_{mono}$-S-ETR, $R65_{mono}$-V-ETR, $R65_{mono}$-S-ER or $R65_{mono}$-V-ER (b). The birds were observed for 10 days for clinical signs and classified as healthy (0), slightly ill (0.5), ill (1), severely ill (2) or dead (3); the daily clinical (DCI) score was calculated from the sum of individual clinical scores from all birds divided by the number of animals per group (ten chickens).

$TCID_{50}$ ml^{-1} and cloacal swab titres between $10^{3.1}$ and $10^{4.5}$ $TCID_{50}$ ml^{-1}. No virus was detected from either oral or cloacal swabs taken on day 4 p.i. from animals infected with the monobasic cleavage site mutants $R65_{mono}$-S-ETR, $R65_{mono}$-V-ETR, $R65_{mono}$-S-ER or $R65_{mono}$-V-ER. Furthermore, no virus could be detected on days 2, 5 or 7 in the oral and cloacal swabs from any of the five $R65_{mono}$-V-ETR-infected animals after challenge with R65.

RNA quantification by real-time RT-PCR corresponded to the $TCID_{50}$ titres from oral and cloacal swabs. High RNA copy numbers were found in the swabs of R65- and R65-V-infected animals on day 2 p.i. in the range of 10^9–10^{10} copies ml^{-1}. Animals infected with the monobasic cleavage site mutants showed considerably lower RNA copy numbers on day 2 p.i., with approximately 10^5 copies ml^{-1} in oral swabs and 10^4 copies ml^{-1} in cloacal swabs. After challenge with R65, RNA could be detected only in the cloacal swab of one animal on day 7 with 10^3 copies ml^{-1}, a level close to the published detection limit (Spackman *et al.*, 2002). From all other animals sampled on days 2, 5 and 7 p.i., no RNA was detected.

Infection with R65 and R65-V resulted in shedding of similar amounts of infectious virus and viral genome copies, indicating that virus shedding from moribund chickens does not reflect the observed differences in the course of the disease. Although successful immunization against R65 challenge demonstrated efficient inoculation of birds, shedding of the live monobasic cleavage site mutants could not be demonstrated, indicating severely restricted replication *in vivo* due to removal of the polybasic HA cleavage site.

Organ tropism and tissue lesions in chicken

The brain, trachea, lung, heart, kidney, spleen, pancreas, caecum and duodenum were sampled after euthanasia of moribund chickens infected with R65 (three animals, day 2 p.i.) or R65-V (four animals, days 2 and 3 p.i.). Infected birds from both groups displayed wide-spread organ tropism accompanied by notable intralesional influenza virus antigen distribution (Table 2, Fig. 3). However, in organs of birds infected with the $R65_{mono}$-S-ETR, $R65_{mono}$-V-ETR, $R65_{mono}$-S-ER or $R65_{mono}$-V-ER mutants, euthanized on day 4 (two animals), neither histological lesions nor influenza virus antigen could be observed. Taken together, in the animals that were already moribund on day 2 p.i., the extent of organ tropism and the severity of lesions were not affected by the valine at position 346 in the R65 HA.

DISCUSSION

HPAIVs evolve from low-pathogenic precursors by acquisition of a polybasic HA cleavage site (Garcia *et al.*, 1996; Garten & Klenk, 2008; Horimoto *et al.*, 1995b; Kawaoka & Webster, 1985; Pasick *et al.*, 2005; Perdue *et al.*, 1997; Suarez *et al.*, 2004). Whereas back-conversion of this polybasic cleavage site to the initial monobasic motif results in a drastic reduction in virulence (Horimoto & Kawaoka, 1994), the contrary mutation by insertion of a polybasic stretch into the HA cleavage site of a LPAIV may or may not lead to a HPAIV (Bogs *et al.*, 2010; Munster *et al.*, 2010; Stech *et al.*, 2009). These different findings suggest that, along with acquisition of a polybasic cleavage site, its vicinity requires adaptation. Sequence surveys (Bao *et al.*, 2008) have revealed that most HPAIV H5 strains carry either serine or threonine at position 346 in their HA (corresponding to position 323 according to H3 numbering; Ha *et al.*, 2001; Nobusawa *et al.*, 1991), whereas almost all low-pathogenic H5 isolates have a valine at this position. Moreover, H5 LPAIVs, except for a few strains, specify a threonine at P2 of their HA cleavage sites. These observations led us to analyse whether these alterations have an impact on virulence.

In this study, we generated monobasic cleavage site mutants from HPAIV R65 (Weber *et al.*, 2007) with different amino acid exchanges in the vicinity of the cleavage site. *In vitro*, these variants were trypsin dependent

Table 2. Organ tropism and tissue lesions

Organs were taken from R65-infected chickens on day 2 p.i. and R65-V-infected chickens on days 2 and 3 p.i., and from animals infected with $R65_{mono}$-S-ETR, $R65_{mono}$-V-ETR, $R65_{mono}$-S-ER or $R65_{mono}$-V-ER on day 4 p.i. The tissue samples were subjected to immunohistochemical (IHC) detection of influenza virus nucleoprotein antigen and haematoxylin–eosin staining, with the results shown as −, negative; +, focal; ++, multifocal; +++, multifocal coalescent or diffuse. BALT, Bronchial-associated lymphoid tissue; PNS, peripheral nervous system.

Virus	Test	Brain	Trachea	Lung	Heart	Kidney	Spleen	Pancreas	Caecum	Duodenum
R65	IHC (n=3)	++/++/++	++/+/++	++/+++/++	++/+++/+	++/++/++	+++/++/+++	++/++/++	++/++/++	++/++/++
	IHC-positive cell types	Neurons, glial cells, endothelium	Epithelium, muscle cells, endothelium, mononuclear cells	Pneumocytes I and II, mononuclear cells, endothelium, parabronchial epithelium	Cardiac myocytes, endothelium	Tubular and glomerular epithelium	Mononuclear cells, endothelium	Acinar cells, endothelium	Endothelium, epithelium, mononuclear cells, intramural ganglia	Endothelium, mononuclear cells, epithelium, spindle-shaped cells
	Histopathology (H&E)	Neuronal necrosis, glial cell proliferation, malacia	None	Pneumonia: heterophilic with fibrin, histiocytosis, congestion, oedema; single pneumocyte necrosis, intralesional rod-like bacteria	Cardiac myocyte degeneration	Tubular degeneration	Lymphocyte and macrophage degeneration and necrosis, histiocytosis	Acinar necrosis	BALT: lymphocyte necrosis and degeneration, histiocytosis	BALT: lymphocyte necrosis, degeneration of intramural ganglia
R65-V	IHC (n=4)	++/++/+++/++	++/+/+/+	++/+++/++/++	++/++/++/++	++/++/++/++	++/++/++/++	+++/+++/++/++	++/++/+/+	++/++/++/+
	IHC-positive cell types	Neurons, glial cells, ependymal cells, endothelium	Neurons (PNS), endothelium, smooth muscle cells, mononuclear cells	Pneumocytes I and II, mononuclear cells, endothelium	Cardiac myocytes, neurons (PNS), endothelium	Tubular and glomerular epithelium	Endothelium, mononuclear cells	Acinar cells, endothelium	Endothelium, mononuclear cells, spindle-shaped cells, intramural ganglia	Endothelium, mononuclear cells, epithelium, spindle-shaped cells, intramural ganglia
	Histopathology (H&E)	Neuronal necrosis, glial cell proliferation, malacia	None	Pneumonia, heterophilic with fibrin and histiocytosis, congestion, oedema, single pneumocyte necrosis	Myocardial degeneration and necrosis	Tubular degeneration and necrosis, heterophilic nephritis	Depletion, lymphocyte and macrophage necrosis, histiocytosis	Acinar necrosis, focal lympho-histiocytic pancreatitis	BALT: lymphocyte depletion, necrosis and degeneration, histiocytosis	BALT: lymphocyte and macrophage necrosis, epithelial cell necrosis, histiocytosis

Table 2. cont.

Virus	Test	Brain	Trachea	Lung	Heart	Kidney	Spleen	Pancreas	Caecum	Duodenum
$R65_{mono}$-S-ETR, $R65_{mono}$-V-ETR, $R65_{mono}$-S-ER, $R65_{mono}$-V-ER	IHC ($n=2$)	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
	Histopathology (H&E)	None	None	None	None	None	None	None	None	None

and thus displayed the phenotype of a LPAIV. Serine at position 346 in the R65 HA appeared to be beneficial at the early stages of replication, as $R65_{mono}$-S-ETR replicated more efficiently than $R65_{mono}$-V-ETR, and $R65_{mono}$-S-ER was superior to $R65_{mono}$-V-ER, which was most apparent in the absence of exogenous protease. As $R65_{mono}$-V-ER had the lowest titre, the cleavage site motif ER ↓ G may be detrimental compared with ETR ↓ G as commonly found in H5 LPAIVs. This disadvantage could be overcome by the exchange of valine to serine at position 346, as shown with $R65_{mono}$-S-ER; its titre equalled that of $R65_{mono}$-V-ETR after 8 h but was higher than that of $R65_{mono}$-V-ER. Therefore, a serine at position 346 may facilitate replication of the monobasic HA cleavage site mutants independently of cleavage efficiency *in vitro*. In chicken, all these mutants exhibited low pathogenicity. However, it is likely that the removal of the polybasic cleavage site led to masking of additional virulence determinants, as only the two serine mutants $R65_{mono}$-S-ETR and $R65_{mono}$-S-ER caused mild sporadic respiratory symptoms and no monobasic cleavage site mutants could be reisolated from swabs. Furthermore, replacement of serine 346 with valine in R65-V led to prolonged survival to a minor degree compared with R65-infected animals. Both viruses were still high pathogenic, as there were no significant differences in virus shedding or organ tropism. However, the possible explanation that the animals had not received exactly the same amount of virus stands in contrast to our previous finding that R65 had 100 % lethality over a range of decreasing inoculation dosages from 10^6 to 10^4 EID_{50} (unpublished data). Overall, these observations suggest that serine 346 in the R65 HA might contribute to virulence in chicken.

Furthermore, our data suggest that threonine at position P2 may facilitate replication efficiency corresponding to the predominance of this amino acid in HA sequences of H5 LPAIVs. Remarkably, there are some H5 HPAIVs that have polybasic HA cleavage motifs with threonine at P2 (Garcia *et al.*, 1996; Garten & Klenk, 2008; Horimoto *et al.*, 1995a; Pasick *et al.*, 2005; Perdue *et al.*, 1996, 1997; Saito *et al.*, 1994; Suarez *et al.*, 2004; Wood *et al.*, 1993). These examples indicate that threonine at P2 does not impair the virulence of HPAIVs. Therefore, loss of P2 threonine, as often observed during evolution from LPAIVs into HPAIVs, is not required for the highly virulent phenotype, but could result from different molecular mechanisms of polybasic cleavage site acquisition (Garcia *et al.*, 1996; Perdue *et al.*, 1996, 1997).

Taken together, the theoretical considerations and experimental observations suggested that serine or threonine at position 346 may facilitate replication of both LPAIVs and HPAIVs, whereas threonine at P2 is beneficial for the efficient replication of H5 LPAIVs only. We therefore propose that evolution from low-pathogenic precursors to HPAIVs requires not only acquisition of a polybasic HA cleavage site but also adaptive changes in neighbouring regions.

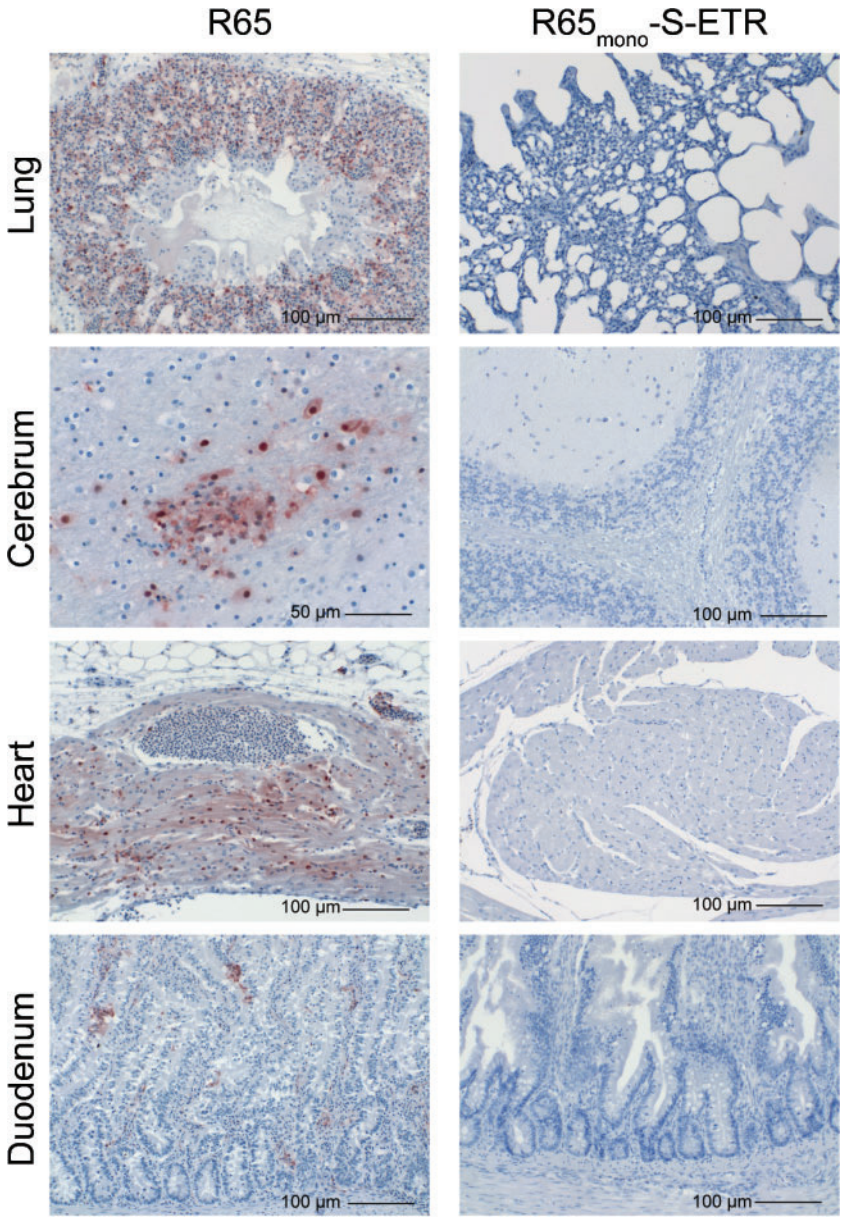

Fig. 3. Virus organ tropism. Immunohistochemical detection of influenza A virus nucleoprotein (brown) distribution in the lung, cerebrum, heart and duodenum of moribund chickens euthanized on day 2 after infection with 10^6 $TCID_{50}$ R65 and from chickens euthanized on day 4 after infection with 10^6 $TCID_{50}$ $R65_{mono}$-S-ETR.

METHODS

Ethical statement. Animal experiments were evaluated by the responsible ethics committee of the State Office for Agriculture, Food Safety and Fishery in Mecklenburg-Western Pomerania (LALFF M-V) and gained governmental approval (registration number LALLF M-V/TSD/7221.3-1.1-018/07).

Cells and viruses. Human embryonic kidney (HEK293T), Madin–Darby canine kidney (MDCK) and Madin–Darby canine kidney H cells (MDCK-H; Matrosovich *et al.*, 2003) were cultured in minimal essential medium containing 10 % FBS. Native A/Swan/Germany/R65/06 (H5N1) virus (R65) (Weber *et al.*, 2007) was propagated in embryonated chicken eggs.

Generation of recombinant viruses. Recombinant viruses were rescued as described elsewhere (Gabriel *et al.*, 2005; Hoffmann *et al.*, 2000) using plasmids encoding the eight viral gene segments of R65 (H5N1) (Stech *et al.*, 2008). Modifications of the HA cleavage site region of R65 (GenBank accession no. DQ464354) were performed by site-directed QuikChange mutagenesis (primer sequences available on request). Rescued viruses were propagated in 11-day-old embryonated chicken eggs (monobasic HA cleavage site mutants) or in MDCK cells (R65 and R65V). The presence of the introduced changes and the absence of unwanted mutations in the HA gene as well as the gene composition of the generated viruses were verified by sequencing of RT-PCR amplicons obtained from viral RNA (data not shown). All viruses with polybasic cleavage sites were handled under Biosafety Level 3+ conditions.

Plaque assays and growth curves. Plaque assays were performed on MDCK and MDCK-H cells either in the presence of trypsin that had been treated with *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK, 2 µg ml^{-1}; Sigma) or in the absence of any exogenous protease. Multicycle replication was assessed after infection of MDCK-H cells in the presence (2 µg ml^{-1}) or absence of TPCK-treated trypsin at an m.o.i. of 10^{-3} (four independent experiments). Virus titres in the supernatant were determined by plaque assay on

MDCK cells in the presence of 2 μg TPCK-treated trypsin ml^{-1} at 0, 8, 24, 48, 72 and 96 h p.i.

Western blots. MDCK-H cells were infected at an m.o.i. of 0.1 in the presence of 2 μg TCPK-treated trypsin ml^{-1} or in the absence of any exogenous protease. After 24 h, cells were lysed, and proteins were separated by 10 % SDS-PAGE and electrotransferred to nitrocellulose membranes. HA was detected using a polyclonal rabbit anti-H5 serum (Pavlova *et al.*, 2009) (diluted 1 : 20 000, incubated overnight) and a secondary HRP-conjugated goat anti-rabbit antibody (BioVision) (diluted 1 : 10 000 and incubated for 1 h at room temperature), followed by chemiluminescence (Supersignal West Pico Chemiluminescent Substrate kit; Pierce).

Animal experiments. Sixteen 2-week-old specific-pathogen-free White Leghorn chickens (Lohmann) were infected oculo-nasally with 10^6 $TCID_{50}$ per animal. The birds were observed daily for clinical symptoms and classified according to Office International des Épizooties guidelines as healthy (0), sick (1), severely sick (2) or dead (3) (Alexander, 2008). Birds with mild respiratory symptoms were scored as 0.5. When birds were too sick to eat or drink, they were euthanized and scored as dead on the next observation day (Alexander, 2008).

Virus shedding. Virus shedding was analysed from oral and cloacal swabs by determining $TCID_{50}$ and by real-time RT-PCR. $TCID_{50}$ (Kalthoff *et al.*, 2008) was established on MDCK cells with the addition of 2 μg TPCK-treated trypsin ml^{-1}. The starting dilution for titration was 10^{-1}. Viral RNA from swab samples was quantified by real-time RT-PCR of the M gene with a detection limit of 10^3 copies ml^{-1}, as described previously (Spackman *et al.*, 2002; Veits *et al.*, 2006).

Histopathology and immunohistochemistry. Samples from brain, trachea, lung, heart, kidney, spleen, pancreas, caecum and duodenum were formalin fixed and processed for paraffin wax embedding according to standard procedures. Immunohistochemistry for detection of influenza A virus nucleoprotein (NP) and haematoxylin–eosin staining was performed as described previously (Kalthoff *et al.*, 2008).

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