Role of stem glycans attached to haemagglutinin in the biological characteristics of H5N1 avian influenza virus

Xiaojian Zhang,1,2,3† Sujuan Chen,1,2,3† Da Yang,1,2,3 Xiao Wang,1,2,3 Jingjing Zhu,1,2,3 Daxin Peng1,2,3 and Xiufan Liu1,2,3

1College of Veterinary Medicine, Yangzhou University, Yangzhou, Jiangsu, 225009, PR China
2Jiangsu Co-Innovation Centre for the Prevention and Control of Important Animal Infectious Disease and Zoonoses, Yangzhou, Jiangsu, 225009, PR China
3Jiangsu Research Centre of Engineering and Technology for the Prevention and Control of Poultry Disease, Yangzhou, Jiangsu, 225009, PR China

There are three conserved N-linked glycosites, namely, Asn10, Asn23 and Asn286, in the stem region of haemagglutinin (HA) in H5N1 avian influenza viruses (AIVs). To understand the effect of glycosylation in the stem domain of HA on the biological characteristics of H5N1 AIVs, we used site-directed mutagenesis to generate different patterns of stem glycans on the HA of A/Mallard/Huadong/S/2005. The results indicated that these three N-glycans were dispensable for the replication-competent influenza viruses. However, when N-glycans at Asn10 plus either Asn23 or Asn286 were removed, the cleavability of HA was almost completely blocked, leading to a significant decrease of the growth rates of the mutant viruses in MDCK and CEF cells in comparison with that of the WT virus. Moreover, the mutant viruses lacking these oligosaccharides, particularly the N-glycan at Asn10, revealed a significant decrease in thermostability and pH stability compared with the WT virus. Interestingly, the mutant viruses induced a lower level of neutralizing antibodies against the WT virus, and most of the mutant viruses were more sensitive to neutralizing antibodies than the WT virus. Taken together, these data strongly suggest that the HA stem glycans play a critical role in HA cleavage, replication, thermostability, pH stability, and antigenicity of H5N1 AIVs.

INTRODUCTION

The H5N1 subtype avian influenza virus (AIV) infects wild birds and poultry in several places worldwide, and H5N1 AIV infection causes a serious economic loss in many countries (Chen et al., 2004; Gu et al., 2013; Li et al., 2010; Zhao et al., 2013). The virus also causes a serious and frequently fatal illness in humans who are in contact with infected birds, but the transmission between humans is limited (Claas et al., 1998; Subbarao et al., 1998; Yen et al., 2007; Yuen et al., 1998). Several lines of evidence indicate that the threat of an H5N1 pandemic is real, and that careful planning is necessary to reduce the public health impact of H5N1 AIV infection (Gao et al., 2009; Li & Chen, 2014; Song et al., 2011). Recent human isolates resistant to adamantanes and neuraminidase inhibitors and the difficulty associated with vaccine production against H5N1 AIV mutants illustrate the need for additional research on the epidemiology and biology of H5N1 influenza virus (Proença-Módena et al., 2007).

Haemagglutinin (HA) is a major surface glycoprotein responsible for viral attachment and penetration during infection and often undergoes N-linked glycosylation at several sites (Kim & Park, 2012; Rossman & Lamb, 2011). Amino acid sequence analysis has revealed that there is considerable variation in both the number and location of potential glycosylation sites among different HA subtypes and even among variants from a single subtype (Chen et al., 2012; Wang et al., 2009). In contrast, the oligosaccharides attached to the upper globular domain at variable sites of the HA molecule have been shown to modulate antigenic properties (Skehel et al., 1984; Wang et al., 2009, 2010b; Zhang et al., 2015), receptor binding (Gao et al., 2009; Liao et al., 2010; Matrosovich et al., 1999; Ohuchi et al., 1997a; Wang et al., 2010b), and resistance to collectins (Hartshorn et al., 2008; Reading et al., 2009; Tate et al., 2011). The conserved glycosylation sites are located within the stem region of the HA molecule, which has been assumed to provide the main forces that stabilize the HA trimer (Liao et al., 2010; Wiley & Skehel, 1987). In a previous study, a vaccinia virus expression system was used to study the role of stem glycosylation sites.
(12, 28 and 478) in the HA of an H7N1 influenza virus in the transport and stability of the HA protein. The loss of all three oligosaccharides in the stem region resulted in temperature sensitivity and complete transport blockage at the non-permissive temperature, whereas the loss of two oligosaccharides reduced the transport rate of HA (Roberts et al., 1993). It has been further demonstrated, by expressing mutants of the HA protein lacking stem glycans in CV1 cells, that stem glycans are important for the maintenance of the metastable conformation of the HA protein required for fusion activity (Ohuchi et al., 1997b). Moreover, it has also been demonstrated, using an RNA polymerase I-based reverse genetics system, that the N-glycan at Asn12 is crucial for virus replication (Wagner et al., 2002).

For H5 subtype influenza viruses, there are three highly conserved potential glycosylation sites in the stem of the HA protein: Asn10/11, Asn23 and Asn286 (H5 numbering) (Chen et al., 2012). It has been previously reported that a single point mutation in the HA of the Ck/Penn (H5N2) virus resulted in the loss of N-linked glycans in the stalk region at residue 11, leading to virulence (Deshpande et al., 1987). Moreover, it has been demonstrated that glycans in the stalk and the length of the connecting peptide determine the cleavability of the H5 influenza virus HA (Kawaoka & Webster, 1989). However, all of the above-described results are based on HA sequence analysis or only involve a single potential glycosylation site, and the functional importance of stem glycans for H5 viruses has yet to be fully defined. In this study, we generated recombinant influenza viruses lacking the N-glycans in the HA stem through site-specific mutagenesis and examined the biological characteristics and antigenicity of the resulting mutants.

**RESULTS**

**Generation of recombinant viruses differing in HA stem glycosylation**

There are three potential glycosylation sites, at Asn10/11, Asn23 and Asn286 (Fig. 1), within the HA stem, of which Asn10/11 (10NNST) is an overlapping glycosylation sequence. To remove the glycosylation site of Asn10/11, three mutants were constructed by substitution of 10NPST for 10N (10NNST) is an overlapping glycosylation sequence. To remove the glycosylation site of Asn10/11, three mutants were constructed by substitution of 10NPST for 10N, 10NPST, 10NNS for 10NNST. To remove the glycosylation sites, Asn23 and Asn286, 23NVT and 286NSS were replaced by 23NVA and 286NSG, respectively. Accordingly, the recombinant AIVs with the single, double, or triple deletion of potential glycosylation sites were generated by combination of 10NPST, 23NVA and 286NSG, and named rSΔ10/11NPST, rSΔ10/11NNSA and rSΔ10/11NNSAA, respectively. To remove the glycosylation sites Asn23 and Asn286, 23NVT and 286NSS were replaced by 23NVA and 286NSG, respectively. Accordingly, the recombinant AIVs with the single, double, or triple deletion of potential glycosylation sites were generated by combination of 10NPST, 23NVA and 286NSG, and named rSΔ10, rSΔA23, rSΔ286, rSΔA10/23, rSΔA23/286, rSΔ23/286, rSΔA10/23/286. The rescued WT virus (rS) served as control. All recombinant viruses were confirmed by reverse transcriptase PCR (RT-PCR) amplification of the viral RNA and sequence analysis. No spontaneous mutation occurred during the rescue and amplification procedure.

**Western blot analysis and indirect immunofluorescence assay**

To verify whether N-glycans were missing from the HA1 proteins of the mutant viruses, the protein profile of the recombinant viruses was determined by Western blot. As shown in Fig. 2a, HA1 of the rSΔ10/11NPST mutant virus showed a reduced molecular mass compared with that of the WT virus, HA1 of the rSΔ10/11NNSA mutant virus exhibited a similar molecular mass to that of the WT virus, while both HA0 and HA1 of the rSΔ10/11NNSAA were not detectable. However, M1 proteins were all detectable in cells infected with the three mutant viruses as well as the WT virus. The data suggested that Asn10 served as a potential glycosylation site and substitution of 10NNST for 10NPST could eliminate this overlapping glycosylation without affecting the synthesis of the HA protein.

Since stem glycosylations are thought to play a role in appropriate folding and HA conformation, the HA expression of the three mutant viruses was further evaluated by indirect immunofluorescence assay. As shown in Fig. 3, no marked difference in the intracellular distribution of the HA protein between the WT virus and the three mutant viruses was observed. Thus, it appeared that the loss of the glycosylation site at Asn10/11 by substitution of 10NNST for 10NPST did not result in accumulation of the HA protein in the endoplasmic reticulum. Therefore, combined with Western blot data, the rSΔ10/11NPST (abbreviated as rSΔ10) mutant virus was chosen for subsequent study.

**Fig. 1.** Location of N-glycosylation sites on the HA of the H5N1 AIV A/Mallard/Huadong/S/2005 strain. Ribbon diagram of monomeric H5 HA structures were generated by the SWISS-MODEL automated protein structure homology modelling server, and the image were obtained using the RasTop (version 2.2) program. The carbohydrates observed in the electron-density map are coloured green, and all of the Asn sites in the HA1 protein are labelled. The location of the polybasic cleavage site is highlighted in red and surrounded by a dashed line.
Compared with the WT HA1 protein, the HA1 proteins from the single, double or triple deletion mutant viruses showed reduced molecular masses corresponding to the absence of one, two or three oligosaccharide chains at the conserved glycosites (Fig. 2b). However, owing to the absence of two oligosaccharide chains, HA1 protein from the rS\textsubscript{D10/23} or rS\textsubscript{D10/286} mutant virus was not detectable (Fig. 2b), indicating that the removal of these oligosaccharide chains significantly affected the cleavage of the H5N1 AVI HA protein. These data confirmed that the HA proteins of the mutant viruses were glycosylated as expected and that the cleavability was affected by deletion of stem glycosylation.

To evaluate the total amount of HA protein in the infected cells, equal loading of protein samples was calibrated by M1 protein probed with an anti-M1 antibody (Fig. 2b). The HA expression level of the rS\textsubscript{D10} and rS\textsubscript{D23} mutant viruses was higher than that of the WT virus, while a slight decrease was observed in the HA expression level of the rS\textsubscript{D10/286} and rS\textsubscript{D10/23/286} mutant viruses.

**Virus growth**

To assess the effects of the loss of N-linked glycosylation on the growth rates of the recombinant viruses, chicken embryo fibroblast (CEF) and Madin–Darby canine kidney (MDCK) cells were infected with the recombinant viruses at an m.o.i. of 0.001. In CEF cells, the loss of glycans had marginal effects on the virus yields compared with the WT virus, with the exception of the rS\textsubscript{D10/23} and rS\textsubscript{D10/286} viruses, which showed retarded growth until 72 h post-infection (p.i.) (Fig. 4a). In MDCK cells, the rS\textsubscript{D10/23} and rS\textsubscript{D10/286} mutant viruses exhibited markedly diminished growth at 72 h p.i. and rS\textsubscript{D10/23/286} showed a 10-fold difference in the TCID\textsubscript{50} value compared with the WT virus (Fig. 4b), while the other viruses replicated in a similar manner to the WT virus. All rescued viruses replicated well in 10-day-old specific-pathogen-free (SPF) eggs or in CEF cells (\textgreater 10\textsuperscript{8} TCID\textsubscript{50} ml\textsuperscript{-1}), except for the rS\textsubscript{D10/23} and rS\textsubscript{D10/286} mutant viruses, which showed a decreased virus titre compared with the WT virus. Moreover, the rS\textsubscript{D10/23}, rS\textsubscript{D10/286} and rS\textsubscript{D10/23/286} mutant viruses exhibited significant attenuation in embryonic eggs according to mean times to death of the recombinant viruses. All of the recombinant viruses produced similar plaque sizes in MDCK cells (Fig. 5). However, the mutant viruses with loss of 10N glycosylation...
(especially for rSΔ10 and rSΔ10/23) exhibited a decrease in the clarity of the plaques compared with the WT virus.

**Thermal stability of the recombinant viruses**

To analyse their thermostabilities, the WT and seven mutant viruses with different stem glycosylation patterns were exposed to 37 °C for 5 days. All of the mutant viruses exhibited a significant decrease in titre compared with the WT virus, and the rSΔ10, rSΔ10/23 and rSΔ10/286 mutant viruses were completely inactivated within 3 days when incubated at 37 °C (P<0.05; Fig. 6a). When incubated at 42 °C, all of the mutant viruses showed a similar pattern, and the rSΔ10, rSΔ10/23 and rSΔ10/286 mutant viruses were completely inactivated within 1 day (Fig. 6b), indicating that deletion of 10N glycosylation in the stem of HA exhibits a significant effect on the thermostability of the H5N1 virus in vitro.

**Low-pH stability of the recombinant viruses**

To evaluate the low-pH stability of the recombinant viruses, their HA titres were measured after pre-incubation for 10 min under the following acidic conditions (pH 4.0, 5.0 and 6.0). The results showed that all of the viruses were inactivated at pH 4.0. The rSΔ286 mutant virus maintained the HA titre at pH 5.0 and 6.0, as did the WT virus. The rSΔ10/23/286 mutant virus exhibited enhanced susceptibility at pH 5.0, and the other mutant viruses showed a significant decrease in HA titre at pH 5.0 and 6.0 compared with the WT virus (P<0.05; Fig. 7), indicating that the loss of 10N and 23N glycosylation in the stem of HA plays a part in the low-pH stability of the H5N1 virus in vitro.

**Antigenicity**

To evaluate the effect of stem glycosylation on the antigenic properties of the H5 viruses, we tested the neutralizing activities of sera obtained from chickens immunized with each of the glycosylation mutant viruses against each other. As shown in Table 1, the mutant viruses induced lower neutralizing antibody titres against the WT virus than did the WT virus. However, the mutant viruses were more susceptible to antisera induced by the WT or mutant viruses than the WT virus, suggesting that the stem glycosylation of the HA proteins influences the antigenicity and stability of the H5N1 AIV.

**DISCUSSION**

It has been shown that HA stem glycans are potent regulators of influenza virus (H7N1) replication (Wagner et al., 2002).

---

**Fig. 4.** Growth curves of mutants in CEF (a) and MDCK (b) cells. The cell monolayers were infected with recombinant viruses bearing WT or glycan mutant HAs from S virus at an m.o.i. of 0.001, and TCID50 virus titres were measured in the supernatants at the time points indicated. The error bars represent SD of the means from three independent experiments. The statistical significance of the differences in the growth properties between the WT virus and mutant viruses was assessed through a Mann–Whitney test (*P<0.05).
For example, the N-glycan at Asn28 (H7 numbering) is indispensable for the formation of replication-competent influenza viruses in the RNA polymerase I-based reverse genetics system. In this study, we were able to rescue all of the mutant viruses lacking any of the stem glycans using an eight-plasmid DNA transfection system (Hoffmann et al., 2000). Thus, it appears that stem glycans are dispensable for the generation of replication-competent influenza viruses. Previous studies in MDCK cells and embryonated chicken eggs have revealed that the N-glycan at Asn12 is crucial for virus replication (Wagner et al., 2002). It has been proven that the HA sequence at positions 10–12, namely Asn-Asn-Ser, in either avirulent or virulent strains of H5N2 AIV is not a glycosylation site and that the HA protein in an avirulent H5N2 AIV is glycosylated at residue Asn11 (Deshpande et al., 1987). In the present study, the H5N1 AIV A/Mallard/Huadong/S/2005 strain possesses the same sequence, Asn-Asn-Ser-Thr, at positions 10–13. We used the 10NPST, 10NNSA or 10NNAA changes to remove the potential glycosylation site and confirmed that Asn10 served as a potential glycosylation site, and that the 10NPST change could eliminate this overlapping glycosylation without affecting the synthesis of the HA protein. The mutant viruses with a single N-glycan deletion at Asn10, Asn23 or Asn286 showed a similar growth rate to the WT virus in both CEF and MDCK cells; however, the rSΔ10/23 and rSΔ10/286 mutant viruses exhibited a strong decrease in replication. We conclude that glycans at Asn10 are involved in H5N1 virus replication in vitro, and there is a synergistic effect between stem glycans on virus replication. This finding corresponds closely with our other results, which show that the absence of the N-glycan at Asn10 has the most severe effect on thermal stability and low-pH stability.

HA is synthesized as a precursor (HA0) in the endoplasmic reticulum and then exported to the cell surface via the Golgi network. On the cell surface, HA0 is cleaved by specific host proteases into HA1 and HA2 (Skehel & Wiley, 2000). It has been previously reported that site-specific glycosylation affects the cleavage of the H5N2 AIV HA and thus virulence (Deshpande et al., 1987). Presumably, the presence of glycosylation at Asn10/11 may mask a recognition signal required by the cleavage enzyme or perturb a structural feature such that it no longer serves as a substrate for the processing enzyme. Further study confirmed that the relationship between carbohydrates in the stalk and the length of the connecting peptide is a critical determinant of cleavability (Kawaoka & Webster, 1989). HAs lacking an oligosaccharide side chain at residue Asn10/11 were cleaved with four basic amino acids at the cleavage site, whereas those with an oligosaccharide side chain resisted cleavage unless additional basic amino acids were inserted. In this study, the HA of the WT virus with five basic amino acids at the cleavage site was cleaved into HA1 and HA2 efficiently even if the strain possessed a potential glycosylation site at residue Asn10. However, HAs that lacked glycosylation at Asn10 resisted cleavage, and the cleavage of the HAs was almost completely blocked by the deletion of glycosylation at Asn10 in combination with either Asn23 or Asn286. It is most likely that the absence of carbohydrates may perturb a structural feature such that it no longer serves as a substrate for the processing enzyme.

The deletion of glycans in the HA stem region leads to a change in the HA structure (Liao et al., 2010), thereby possibly affecting the stability of the resulting mutant viruses. It has been shown that temperature is negatively correlated with the persistence time (Brown et al., 2007, 2009). In this study, we found that the deletion of any N-glycan in the stem of HA exhibits a significant effect on the thermostability, and this effect was particularly observed with the deletion of an N-glycan at Asn10. In fact, the mutant viruses lacking an oligosaccharide side chain at residue Asn10 are much more thermosensitive than the WT virus (Negovetich & Webster, 2010). In addition to temperature, the persistence of virus in the environment is also affected by pH (Brown et al., 2009). It has been reported that the stem glycan can be regarded as a potent stabilizer of the metastable conformation of the HA protein, preventing premature denaturation (Wagner et al., 2002). Consistently, our results showed that the deletion of glycans at Asn10 and Asn23 had a significant effect on the haemagglutination activity of the mutant viruses under acidic conditions (pH 5.0 and 6.0) compared with the WT virus. These data indicate that the glycan at Asn10 is important for the maintenance of the thermostability and pH stability of H5N1 AIVs.
Fig. 6. Activity of the mutant viruses at 37 and 42 °C. The WT virus and mutant viruses were incubated at 37 °C (a) or 42 °C (b) for 5 days. The TCID_{50} titres of the aliquots were determined on CEF cells. The error bars denote SD of the mean of four independent titres at each time point. An asterisk indicates that the titre of the mutant virus was significantly different from those of the WT virus at the indicated time points, as determined by the Mann–Whitney test (*P<0.05).

Fig. 7. Low-pH stability of the mutant viruses. The recombinant viruses were incubated in each buffer at 37 °C for 10 min, and the viral titres were then determined by haemagglutination assay. The results are presented as log_{2} HA titres at the indicated pH conditions. The error bars denote SD of the mean of three independent titres at each time point. The statistical significance of the differences in low-pH stability between the WT virus and mutant viruses was assessed through the Mann–Whitney test (*P<0.05).
The acquisition of N-linked glycosylation sites on the HA protein is thought to play a beneficial role in virus variation by shielding antigenic sites from interaction with antibodies (Kobayashi & Suzuki, 2012; Wanneck et al., 2011). The removal of glycosylation sites on the HA protein can expose conserved epitopes hidden by large glycans to elicit an immune response that recognizes HA variants at a higher titre (Wang et al., 2009). Broadly neutralizing HA2 stem-specific monoclonal antibodies with neutralizing activity for H1 clade viruses (Ekiert et al., 2009; Sui et al., 2009) and H3 subtype viruses (Bommakanti et al., 2010; Wang et al., 2010a) have been developed. These studies indicate that epitopes in the stem of HA are conserved and accessible to antibodies. In the present study, the mutant viruses lacking stem glycosylation of the HA are susceptible to antibodies induced by the WT virus; however, the mutant viruses failed to induce higher neutralizing antibodies against the WT virus. Although some mutant viruses showed higher HA protein expression as compared with the WT virus, they still induced lower levels of neutralizing antibodies against the WT virus. Presumably, the deletion of stem glycans may expose more epitopes, resulting in virus inactivation, and decrease the stability of the HA trimer, resulting in the induction of a poor antibody response.

In summary, stem glycans are dispensable for the generation of replication-competent H5N1 influenza viruses, but stem glycans contribute to HA cleavability and replication in tissue culture. Moreover, the deletion of stem glycans decreases the thermostability and low-pH stability, which could result in antibody susceptibility of the mutant viruses and induction of a lower antibody response. This study underscores the importance of N-glycans attached to the stem domain of the HA protein for the replication, thermostability, pH stability and antigenicity of influenza viruses and may be used as a method for the production of temperature-sensitive, live attenuated influenza virus vaccines.

**METHODS**

**Viruses and cells.** A/Mallard/Huadong/S/2005 (S, H5N1) (Tang et al., 2009) was isolated in eastern China by our laboratory and identified as a highly pathogenic H5N1 AIV. All WT AIV and mutant strains were propagated in 10-day-old SPF embryonated chicken eggs. MDCK cells, human embryonic kidney (293T) cells (obtained from the American Type Culture Collection) and CEF cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% FBS (PAA) at 37 °C under 5% CO2.

**Mutagenesis and virus rescue.** Site-directed mutagenesis of the HA gene of the H5N1 AIV S strain was performed by overlap-PCR with following primers. To remove N-glycosylation sites at 10/11N, Asn11 was exchanged for Pro using the primers 5'-CATGCAAAACCCCCTCGACAGA-GA-3' and 5'-TCTGTCGAGGGTTTGCATG-3' (substitution nucleotides are underlined); Thr13 was exchanged for Ala using the primers 5'-CAAAACACTCGAGAGACGGTTCG-3' and 5'-CAAACCTCGTCTGCGAGCGTTG-3' (substitution nucleotides are underlined).

**Site-directed mutagenesis of the HA gene of the recombinant viruses at an m.o.i. of 2 for 1 h at 35 °C under 5% CO2.** The RNAs of the propagated viruses at 35 °C were cotransfected into a mixture of 293T and MDCK cells using the FuGENE HD Transfection Reagent (Promega). After 48 h, the culture mixtures were inoculated into 10-day-old SPF eggs to amplify the rescued viruses at 35 °C. The allantoic fluids were tested individually for the presence of infectious virus through a standard haemagglutination assay using chicken red blood cells (Killian, 2008). The RNAs of the propagated rescue viruses were extracted and amplified, and each viral gene segment was sequenced to ensure the absence of unwanted mutations.

**Western blot analysis.** To analyse the virus HA protein, CEF cells were infected with the recombinant viruses at an m.o.i. of 2 for 1 h at 37 °C under 5% CO2. The infected cells were washed three times with

---

**Table 1. Neutralization titres of chicken sera induced by the WT virus and the mutant viruses**

<table>
<thead>
<tr>
<th>Virus</th>
<th>rS (WT)</th>
<th>rSΔA10</th>
<th>rSΔA23</th>
<th>rSΔA286</th>
<th>rSΔA10/23</th>
<th>rSΔA10/286</th>
<th>rSΔA23/286</th>
<th>rSΔA10/23/286</th>
</tr>
</thead>
<tbody>
<tr>
<td>rS (WT)</td>
<td>1280†</td>
<td>80</td>
<td>160</td>
<td>40</td>
<td>20</td>
<td>160</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>rSΔA10</td>
<td>2560</td>
<td>160</td>
<td>320</td>
<td>160</td>
<td>40</td>
<td>320</td>
<td>160</td>
<td>160</td>
</tr>
<tr>
<td>rSΔA23</td>
<td>5120</td>
<td>640</td>
<td>160</td>
<td>320</td>
<td>160</td>
<td>320</td>
<td>320</td>
<td>320</td>
</tr>
<tr>
<td>rSΔA286</td>
<td>10 240</td>
<td>320</td>
<td>160</td>
<td>160</td>
<td>640</td>
<td>160</td>
<td>640</td>
<td>640</td>
</tr>
<tr>
<td>rSΔA10/23</td>
<td>10 240</td>
<td>1280</td>
<td>1280</td>
<td>640</td>
<td>320</td>
<td>10 240</td>
<td>2560</td>
<td>640</td>
</tr>
<tr>
<td>rSΔA10/286</td>
<td>10 240</td>
<td>10 240</td>
<td>2560</td>
<td>10 240</td>
<td>320</td>
<td>2560</td>
<td>1280</td>
<td>2560</td>
</tr>
<tr>
<td>rSΔA23/286</td>
<td>320</td>
<td>5120</td>
<td>640</td>
<td>160</td>
<td>640</td>
<td>160</td>
<td>1280</td>
<td>2560</td>
</tr>
<tr>
<td>rSΔA10/23/286</td>
<td>2560</td>
<td>320</td>
<td>640</td>
<td>160</td>
<td>40</td>
<td>2560</td>
<td>160</td>
<td>320</td>
</tr>
</tbody>
</table>

*The chickens were immunized with the indicated viruses, with different HA glycosylations, and serum samples were collected for the neutralization assay on CEF cells. The values represent reciprocal geometric mean antibody titres from three animals.

†The neutralization titres against homologous viruses are shown in bold.
For the plaque assays, MDCK cells were seeded into 12-well plates and lysed with 200 μl whole-cell lysis buffer (protease inhibitor mixture, 150 mM NaCl, 1 % Triton X-100, 20 mM Tris/HCl, pH 7.5) (Beyotime) on ice for 30 min. The cell lysates were collected with a cell scraper, and the whole-cell extract was harvested by centrifugation at 13 000 r.p.m. (Eppendorf 5417R) and 4 °C for 10 min. The proteins were separated by electrophoresis on a 12 % Tris-glycine gel and transferred to a nitrocellulose membrane. The membrane was blocked in 5 % skimmed milk, incubated with mAb 3A9 (anti-HA1 of H5N1 AIV) and mouse serum (anti-M1 of H5N1 AIV), followed by incubation with horseradish peroxidase-conjugated goat anti-mouse antibodies (EMD Chemicals). The protein bands were visualized using enhanced chemiluminescence (Thermo Fisher Scientific) on radiographic film.

Indirect immunofluorescence assay. CEF cells were infected with the recombinant viruses at an m.o.i. of 2 for 1 h at 37 °C under 5 % CO2, and then 2 ml DMEM was added. After incubation for 12 h, the cells were fixed with 4 % paraformaldehyde in PBS for 30 min. The cultures were incubated with 0.5 % Triton X-100 for 10 min and then washed with 10 % BSA in PBS for 30 min. The cultures were then incubated with mAb 3A9 (anti-HA) at 37 °C for 1 h. The cells were then washed three times with PBS and incubated at 37 °C for 1 h with FITC-coupled goat anti-mouse secondary antibodies (Santa Cruz). After incubation with secondary antibodies, cells were washed three times with PBS and incubated with DAPI (Beyotime) for 5 min and ER-Tracker Red (Beyotime) for 15 min. Cells were observed using a Zeiss LSM 710 META/AxioVert 200 confocal microscope with a × 40 lens.

Analysis of virus growth. For the growth curves, monolayer cells (CEF and MDCK) were infected with the recombinant viruses at an m.o.i. of 0.001 in DMEM for 1 h. The unbound viruses were washed with PBS, and serum-free DMEM was then added. At 12 h p.i., the cells were lysed with 200 μl whole-cell lysis buffer (protease inhibitor mixture, 150 mM NaCl, 1 % Triton X-100, 20 mM Tris/HCl, pH 7.5) (Beyotime) on ice for 30 min. The cell lysates were collected with a cell scraper, and the whole-cell extract was harvested by centrifugation at 13 000 r.p.m. (Eppendorf 5417R) and 4 °C for 10 min. The proteins were separated by electrophoresis on a 12 % Tris-glycine gel and transferred to a nitrocellulose membrane. The membrane was blocked in 5 % skimmed milk, incubated with mAb 3A9 (anti-HA1 of H5N1 AIV) and mouse serum (anti-M1 of H5N1 AIV), followed by incubation with horseradish peroxidase-conjugated goat anti-mouse antibodies (EMD Chemicals). The protein bands were visualized using enhanced chemiluminescence (Thermo Fisher Scientific) on radiographic film.

Indirect immunofluorescence assay. CEF cells were infected with the recombinant viruses at an m.o.i. of 2 for 1 h at 37 °C under 5 % CO2, and then 2 ml DMEM was added. After incubation for 12 h, the cells were fixed with 4 % paraformaldehyde in PBS for 30 min, saturated with PBS containing 0.5 % Triton X-100 for 10 min and then washed with 10 % BSA in PBS for 30 min. The cells were incubated with mAb 3A9 (anti-HA) at 37 °C for 1 h. The cells were then washed three times with PBS and incubated at 37 °C for 1 h with FITC-coupled goat anti-mouse secondary antibodies (Santa Cruz). After incubation with secondary antibodies, cells were washed three times with PBS and incubated with DAPI (Beyotime) for 5 min and ER-Tracker Red (Beyotime) for 15 min. Cells were observed using a Zeiss LSM 710 META/AxioVert 200 confocal microscope with a × 40 lens.

Analysis of virus growth. For the growth curves, monolayer cells (CEF and MDCK) were infected with the recombinant viruses at an m.o.i. of 0.001 in DMEM for 1 h. The unbound viruses were washed with PBS, and serum-free DMEM was added. The cells were incubated at 37 °C under 5 % CO2, and then 2 ml DMEM was added. After incubation for 1 h, the cells were washed three times with PBS and then overlaid with DMEM containing 0.8 % agar (Sigma) and 2 % FBS. The infected cells were incubated for an additional 72 h, and the plaques were visualized by staining with 0.1 % crystal violet in a 10 % formaldehyde solution (Wagner et al., 2001). For each virus, 10 well-spaced plaques were measured using the GNU image manipulation program (version 2.8; www.gimp.org), as described previously (Negovetich & Webster, 2010).

Thermostability. The thermostability of the viruses was determined as described previously (Negovetich & Webster, 2010; Yamanouchi & Barrett, 1994). Prior to the incubations, the TCID50 values on CEF cells of the recombinant viruses in allantoic fluid were determined. The starting titres (10 ° TCID50 ml−1) were standardized by diluting the virus samples in DMEM containing 0.5 % BSA and 0.1 ml aliquots were transferred into vials. The viruses in the sealed vials were incubated at either 37 or 42 °C for up to 5 days. After incubation, the aliquots were quickly cooled in ice-water and stored at −80 °C until TCID50 titre determination on CEF cells.

Low-pH stability. Fifty microlitres of each virus was incubated with 50 μl 100 mM acetate buffer (pH 4.0 and 5.0), 100 mM phosphate buffer (pH 6.0) or PBS (pH 7.2) at 37 °C for 10 min, and the samples were then neutralized immediately with PBS (pH 7.4) and maintained on ice (Takehashi et al., 2010; Wagner et al., 2002). The virus titres were determined by haemagglutination assay.

Antigenicity. All of the animal studies were approved by the Jiangsu Administrative Committee for Laboratory Animals (Permission number SYXKSU-2007-0005), and complied with the guidelines for laboratory animal welfare and ethics of the Jiangsu Administrative Committee for Laboratory Animals. Four-week-old SPF white Leghorn chickens (four chickens in each group) were inoculated intramuscularly with 0.2 ml inactivated vaccine preparations containing 106 EID50 each virus mixed with Freund’s adjuvant (Sigma) and boosted after 2 weeks. Sera were collected on day 21 after the second immunization, and the serum antibody levels against the homologous and heterologous viruses were determined using a neutralization assay (Wang et al., 2010b). Briefly, twofold serial dilutions of chicken serum starting from 1:2 dilution were incubated with an equal volume of the indicated viruses at a concentration of 100 TCID50 (50 μl)−1 in a 96-well plate for 1 h at 37 °C. The virus–serum mixture was transferred to monolayers of CEF cells and incubated at 37 °C for 4 days. The neutralizing antibody titres were defined as the reciprocal of the highest serum dilution that completely neutralized the appropriate virus as defined by the absence of a cell pathology effect on day 4 p.i.

Statistical analysis. The viral titres and antibody titres are expressed as the means ± SD. The statistical analyses were performed using an independent-sample t-test. Differences with a P-value of less than 0.05 were regarded to be statistically significant.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (31372450, 31402229), the Special Fund for Agro-Scientific Research in the Public Interest (201003012), the Major State Basic Research Development Program of China (973 Program) (2011CB 505003), the National High-Tech Research and Development Program of China (2011AA10A209), and a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions. We are grateful to Professor Xiulong Xu for his language editing of our manuscript.

REFERENCES


