Journal of General Virology (2002), **83**, 601–609. Printed in Great Britain

N-Glycans attached to the stem domain of haemagglutinin efficiently regulate influenza A virus replication

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The haemagglutinin (HA) protein of fowl plague virus A/FPV/Rostock/34 (H7N1) contains three *N*-linked oligosaccharide side chains in its stem domain. These stem glycans, which are attached to the Asn residues at positions 12, 28 and 478, are highly conserved throughout all HA protein sequences analysed to date. In a previous study, in which mutant HA proteins lacking individual stem glycosylation sites had been expressed from an SV-40 vector, it was shown that these glycans maintain the HA protein in the metastable form required for fusion activity. In the present study, the functional role of the stem *N*-glycans for virus replication was investigated using recombinant influenza viruses generated by an RNA polymerase I-based system. Studies in Madin–Darby canine kidney cells and embryonated chickens' eggs revealed that the *N*-glycan at Asn^{12} is crucial for virus replication. In both culture systems, growth of virus lacking this glycan (mutant cg1) was completely blocked at 37 °C and inhibited at 33 °C. Loss of the glycan from Asn^{478} (mutant cg3) caused less striking, but still measurable, effects. Interestingly, it was not possible to generate mutant viruses containing the HA protein lacking the *N*-glycan at Asn^{28}. It is concluded from this that the *N*-glycan at Asn^{28} is indispensable for the formation of replication-competent influenza viruses. When compared to viruses containing wild-type HA protein, mutants cg1 and cg3 showed a significantly decreased pH stability. Taken together, these data show that the HA stem glycans are potent regulators of influenza virus replication.

Introduction

Influenza A and B viruses contain two spike glycoproteins: the haemagglutinin (HA) and the neuraminidase (NA). The HA protein is the most abundant protein on the virus surface. It is the prototype of a class I transmembrane glycoprotein and is embedded in the virus membrane as a homotrimer of noncovalently linked monomers. Each monomer consists of a globular head region connected to a fibrous stalk domain (Wilson *et al.*, 1981). Both of these regions carry *N*-linked oligosaccharide side chains (Keil *et al.*, 1985). The HA protein plays an essential role during virus entry (Skehel & Wiley, 2000; Steinhauer & Wharton, 1998). Infection is initiated by binding of the HA protein to sialic acid-containing receptors on the surface of target cells. Following internalization of bound viruses by receptor-mediated endocytosis, the HA protein induces fusion of the virus envelope with the endosomal membrane. This fusion reaction is an absolute requirement for the delivery of viral nucleocapsids into the cytoplasm of the infected cell, thus triggering the generation of progeny viruses. To show fusion activity, the HA protein has to undergo a biphasic activation process. The first step involves cleavage by host proteases into two disulfide-linked subunits, HA1 and HA2 (Klenk & Garten, 1994). Proteolytic cleavage renders the protein in a metastable form, which, upon acidification, allows the molecule to undergo an irreversible conformational change to gain fusion activity (Bullough *et al.*, 1994; Carr & Kim, 1993). Adoption of this fusion-competent state involves a series of highly ordered refolding steps, leading ultimately to the extrusion of the hydrophobic fusion peptide towards the endosomal target membrane (Shangguan *et al.*, 1998; Hughson, 1995). Changes in the HA protein sequence that destabilize the metastable conformation, decrease monomer interactions within the trimers or inhibit the extensive acid-triggered molecular rearrangements have been

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shown to interfere with fusion activity or to alter the pH optimum of fusion (Daniels *et al.*, 1985; Doms *et al.*, 1986; Wharton *et al.*, 1986; Godley *et al.*, 1992; Kemble *et al.*, 1992; Steinhauer *et al.*, 1996). In particular, *N*-glycans attached to the stem region of the HA protein of fowl plague virus (FPV) were shown to influence the functional properties of the HA protein. Interestingly, oligosaccharides decorating this HA protein domain are extremely conserved (Nobusawa *et al.*, 1991). *N*-Glycosylation sites at Asn^{12} and Asn^{478} (H7 subtype numbering) are present in all known HA protein sequences and the site at Asn^{28} appears in most strains analysed to date. In contrast, *N*-glycans attached to other regions of the HA protein show considerable variation in structure and number among different influenza A viruses (Matrosovich *et al.*, 1999; Mir-Shekari *et al.*, 1997; Inkster *et al.*, 1993). This high conservation of stem glycans suggested that they play an important structural or functional role. Support of this concept came from a study revealing that FPV HA mutants lacking the stem glycans show a temperature-sensitive phenotype and suffer from a complete transport block at the nonpermissive temperature (Roberts *et al.*, 1993). Investigations on FPV HA were extended subsequently by expressing mutants lacking individual stem glycans in CV1 cells from an SV-40 vector. By this approach, it was demonstrated that stem glycans are important for the maintenance of the metastable conformation of the HA protein required for fusion activity (Ohuchi *et al.*, 1997b).

To study the role of stem glycans so far, only vector-expressed HA proteins have been analysed. We have now generated recombinant influenza viruses lacking the *N*-glycans in the HA stem to analyse the effects of these mutations in infection. For the production of the mutant viruses, we used an RNA polymerase I-based reverse genetics system described previously (Wagner *et al.*, 2000; Pleschka *et al.*, 1996; Zobel *et al.*, 1993). Employing this strategy, we were able to demonstrate that stem glycans are important determinants of efficient influenza virus replication. While viruses lacking the glycan at Asn^{478} were only marginally affected, growth of viruses lacking the glycan at Asn^{12} was totally blocked at 37 °C and severely impeded at 33 °C. Most interestingly, it was not possible to obtain recombinant viruses with the HA protein that lacked the glycan at Asn^{28}. Thus, it appears that this glycan is indispensable for the generation of replication-competent influenza viruses. Moreover, we found that loss of stem glycans lowered significantly the pH stability of the respective viruses, indicating that stem glycans are effective stabilizers of the native conformation of the HA protein in the virus particle.

Methods

■ **Cells and viruses.** Human embryonic kidney cells (293) and Madin–Darby bovine kidney (MDBK) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4·5 g/l glucose (ICN) supplemented with 10% foetal calf serum (FCS) (Life Technologies). Madin–Darby canine kidney (MDCK) cells were grown in MEM containing 10% FCS. All cells were maintained at 37 °C and 5% CO_2.

The influenza virus reassortant WSN-HK (Schulman & Palese, 1977) was used. This virus contains the N2 subtype NA gene of the A/Hong Kong/8/68 virus strain and the residual genes of the A/WSN/33 virus strain. The reassortant was amplified in 11-day-old embryonated chickens' eggs.

■ **Construction of plasmids.** The plasmid PolI-SapI/HA containing the wild-type HA gene of FPV (A/FPV/Rostock/34) (H7N1) in genomic orientation under the control of the human RNA polymerase I promoter has been described before (Wagner *et al.*, 2000). Expression plasmids pHMG-PB1, pHMG-PB2, pHMG-PA and pHMG-NP, encoding the proteins of the influenza virus polymerase complex under the control of a hydroxymethyl–glutaryl–coenzyme A reductase promoter, were kindly provided by J. Pavlovic (University of Zürich, Zürich, Switzerland). The *N*-glycosylation sites in the HA protein stem at positions 12, 28, and 478 were eliminated using the Quickchange Mutagenesis kit (Stratagene), according to the manufacturer's protocol. Thr^{14} was exchanged for Leu using the oligonucleotides 5′ GGACATCATGCTGTATCAAATGG-TTTAAAAGTAAACACACTACTG 3′ and 5′ CAGTGAGTGTGT-TTACTTTTAAACCATTTGATACAGCATGATGTCC 3′ as primers to obtain the cg1 mutant of the FPV HA sequence. Primers contained a *Dra*I restriction site to confer a genetic tag to the mutated sequence. Thr^{30} was exchanged for Gly using the primers 5′ GGAGTAGAAGTTGTCA-ATGCCGGCGAAACAGTGGAGCGGACAAACATCCC 3′ and 5′ GGGATGTTTGTCCGCTCCACTGTTTCGCCGGCATTGACAACT-TCTACTCC 3′ to generate the cg2 mutant HA sequence. These primers contained a *Nae*I restriction site. To obtain the cg3 mutant HA sequence, Thr^{480} was exchanged for Gly with the primer pair 5′ GGCTAGTATA-AGGAACAATGC-ATATGATCACAGCAAATACAG 3′ and 5′ CT-GTATTTGCTGTGATCATATGCATTGTTCCTTATACTAGCC 3′. These primers contained an *Nsi*I restriction site serving as the genetic tag. To distinguish between the HA protein from authentic FPV and plasmid-based wild-type FPV HA, the latter sequence was modified by the introduction of a *Pvu*II site at position 1149, as reported previously (Wagner *et al.*, 2000).

■ **Expression of the HA protein in 293 cells.** Confluent 293 cell monolayers were trypsinized from a 75 cm^2 flask and pelleted by centrifugation at 1000 ***g*** for 5 min. After resuspending in culture medium, one-third of the cell suspension was transferred to a 6 cm culture dish and then transfected with plasmids pHMG-PB1 (1 μg), pHMG-PB2 (1 μg), pHMG-PA (1 μg) and pHMG-NP (2 μg) to express the influenza virus polymerase complex and with the PolI-SapI plasmid encoding the respective versions of the FPV HA sequence (4 μg). Transfection was carried out using the LipofectAMINE 2000 reagent (Life Technologies), according to the supplier's instructions. Cells were incubated at 37 °C. At 2 days after transfection, 293 cells were fixed with 4% paraformaldehyde. The HA protein expressed on the cell surface was detected by indirect immunfluorescence using an H7 subtype HA-specific monoclonal antibody from mouse as the primary antibody and a fluorescein-conjugated swine anti-mouse antiserum as the secondary antibody.

■ **Rescue of recombinant viruses.** Confluent 293 cell monolayers were transfected as outlined above. At 36 h post-transfection, cells were infected with the WSN-HK (H1N2) helper virus at an m.o.i. of 2. Progeny viruses were harvested 18 h post-infection and passaged onto MDBK cell monolayers in the absence of trypsin to select for recombinant viruses expressing the FPV HA protein. Infected MDBK cells were cultivated at either 33 or 37 °C and monitored for the appearance of liquid plaques over the next few days. Recombinant viruses were purified by three

plaque passages on MDBK cells under selection conditions and virus stock solutions were produced in MDCK cells.

■ **Characterization of recombinant viruses.** Plaque-purified recombinant viruses were used for the infection of MDCK cells. At 3–4 days post-infection, supernatants were collected and cleared of cellular debris by centrifugation at 2000 ***g***. Viruses were pelleted from the supernatants by ultracentrifugation at 100000 ***g*** for 1 h. RNA was extracted from the virus pellet in a final volume of 50 μl of highly purified water with the High Pure RNA Isolation kit (Roche), according to the manufacturer's instructions. Of the isolated RNA, 10 μl was subjected to RT–PCR using the OneStep RT–PCR kit (Qiagen) with primer pairs 5′ GGCCAGTCCGGACGGATTGATTTTC 3′ and 5′ CATGATGCCCCGAAGCTAAACC 3′ (for the wild-type HA protein), 5′ ATGAACACTCAAATCCTGG 3′ and 5′ ATTGTCTGTATTTGACAGGAGCC 3′ (for the cg1 HA protein) and 5′ GGCAACTGGGATGAAGAACG 3′ and 5′ CATGATGCCCCGAAGCTAAACC 3′ (for the cg3 HA protein). RT–PCR products were digested with *Pvu*II (wild-type HA), *Dra*I (cg1 HA) or *Nsi*I (cg3 HA), respectively. Cleavage products were examined by electrophoresis on a 1·4% agarose gel.

To analyse the virus HA protein, MDCK cells were infected with recombinant viruses at an m.o.i. of 2. At 8 h post-infection, 20 μCi Redivue Pro-mix L^{35}S *in vitro* cell-labelling mix (Amersham Pharmacia) was added in 2 ml of MEM lacking methionine and cysteine. After 12 h, radioactively labelled viruses were pelleted from the supernatants. Viruses were lysed in 500 μl radioimmunoprecipitation buffer (150 mM NaCl, 1% Triton X-100, 0·1% SDS, 1% deoxycholate, 10 mM EDTA, 1 mM PMSF, 10 mM iodoacetamide, 5000 U aprotinin and 20 mM Tris–HCl pH 8·8). The FPV HA protein was immunoprecipitated from the lysate by adding an FPV HA-specific monoclonal antibody (1:250) and 30 μl protein A–Sepharose (Sigma) (1:10 in water). One-half of the precipitated material was digested for 6 h with 500 U of peptide:*N*-glycosidase F (PNGase F) (New England Biolabs), while the other half remained untreated. Samples were resolved by 10% SDS–PAGE and visualized by fluorography.

■ **Flow cytometric analysis of the HA protein in infected cells.** MDCK cell monolayers were inoculated with recombinant viruses at an m.o.i. of 2 in PBS containing 0·2% BSA (ICN) for 1 h. Cells were washed and serum-free MEM containing 0·2% BSA was added. After 12 h of incubation at 33 °C, cells were detached from the dish by trypsin treatment, washed with PBS and fixed with 2% paraformaldehyde at 4 °C for 1 h. Fixed cells were stained with an H7 subtype HA-specific monoclonal antibody followed by a fluorescein-conjugated swine anti-mouse immunoglobulin. After suspension in 1 ml PBS, cells were subjected to FACs analysis (Becton Dickinson).

■ **Analysis of virus growth.** For growth curves, MDCK cell monolayers were infected for 1 h with recombinant viruses at an m.o.i. of 0·001 in PBS containing 0·2% BSA. Unbound virus was washed away and serum-free MEM containing 0·2% BSA was added. Cells were incubated at either 33 or 37 °C and HA titres in the supernatants were monitored periodically with chicken red blood cells (1% in saline).

Embryonated 11-day-old chickens' eggs were inoculated into the allantoic cavity with 10^4 p.f.u. of recombinant viruses. Eggs were incubated at either 33 or 37 °C for 48 h. The allantoic fluid was then harvested and monitored for virus content by plaque assay on MDCK cells.

■ **pH stability of recombinant viruses.** Aliquots containing 10^7 recombinant viruses were incubated in the absence of target membranes in 130 mM NaCl and 20 mM sodium acetate with the pH value ranging from 6·0 to 5·3 (Korte *et al.*, 1999). After 30 min at 37 °C, samples were neutralized (pH 7·4) immediately and kept on ice. Remaining infectivity in the samples was determined subsequently by plaque assay on MDCK cells.

Results

Generation and molecular characterization of recombinant viruses

To study the impact of oligosaccharide side chains decorating the stem of the FPV HA protein on the growth of intact influenza viruses, mutant HA cDNAs lacking the glycan attachment sites at Asn^{12}, Asn^{28} or Asn^{478} were inserted in genomic orientation under the transcriptional control of the human RNA polymerase I promoter, as described before (Wagner *et al.*, 2000). For analytical reasons (see below), endonuclease restriction motifs were introduced as tag sites into the HA cDNAs. A *Pvu*II site was added at position 1150 to the wild-type HA cDNA. The HA sequences encoding the mutants cg1, cg2 and cg3 were modified by the introduction of novel *Dra*I, *Nae*I and *Nsi*I sites, respectively (for the phenotypes and nomenclature of mutants see Fig. 1). Generated plasmids were then tested for their ability to express mutant HA proteins using an influenza virus polymerase-dependent system. After transfection into 293 cells, RNA polymerase I-based transcription produces a virus-like HA RNA gene that is translated by proteins of the influenza virus polymerase complex present in the cells after cotransfection of the respective expression plasmids (Pleschka *et al.*, 1996). All mutant HA proteins could readily be expressed by this approach in 293 cells (Fig. 2).

To obtain recombinant viruses, transfected 293 cells were then infected with the influenza virus reassortant WSN-HK (H1N2) as helper virus. Selection for recombinant viruses was achieved by passaging rescue supernatants from 293 cells onto MDBK cell monolayers and cultivating in the absence of trypsin at either 33 or 37 °C. Since only the FPV HA protein but not the H1 subtype HA protein of the helper virus is activated by the cellular protease furin (Stieneke-Gröber *et al.*, 1992), recombinant viruses will propagate, whereas helper virus replication is inhibited. Following this approach, we were able to generate recombinant viruses containing the FPV HA protein lacking either the glycans from Asn^{12} (mutant cg1) or Asn^{478} (mutant cg3). Rescue of mutant cg1 viruses was achieved only at 33 °C, while wild-type and cg3 viruses could be obtained at 33 and 37 °C. However, it was interesting to see that it was not possible to obtain recombinant viruses expressing cg2 mutant HA protein in which the glycan attachment site at Asn^{28} had been deleted. This points strongly to the fact that this glycan is indispensable for the generation of replication-competent influenza viruses. It remains to be seen whether the cg2 HA protein is incorporated into viruses or not.

The recombinant identity of the rescued viruses was confirmed by RT–PCR analysis of viral RNA. To this end, isolated viral RNA was employed as a template for RT–PCR

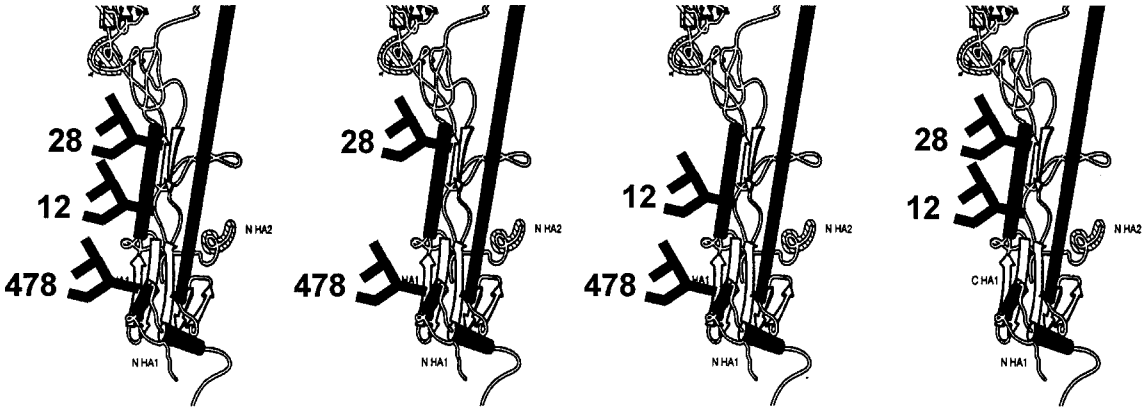

Fig. 1. Schematic representation showing the stem region of the FPV HA protein. Glycosylation pattern and nomenclature of the mutant HA proteins lacking individual *N*-glycans from the stem domain are depicted.

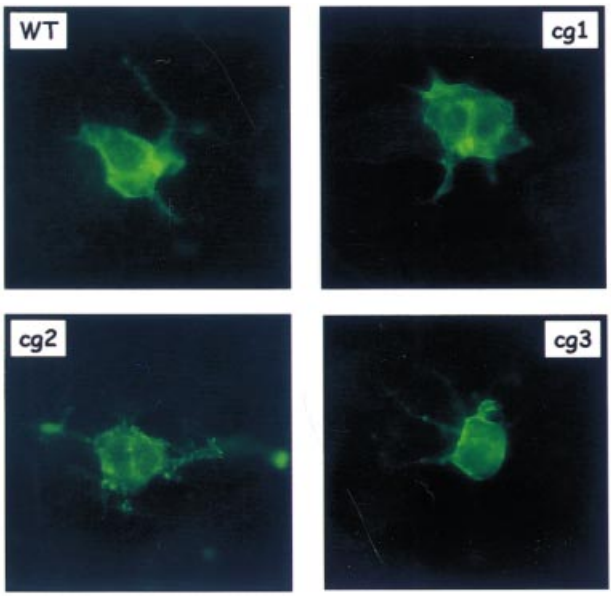

Fig. 2. Expression of wild-type (WT) and stem glycosylation mutants of the HA protein (cg1, cg2 and cg3) in 293 cells. Cells were transfected with plasmids expressing the subunits of the influenza virus polymerase along with a plasmid expressing the respective version of the HA gene. Cells were fixed and stained with an HA-specific monoclonal antibody from mouse and fluorescein-conjugated anti-mouse immunoglobulins.

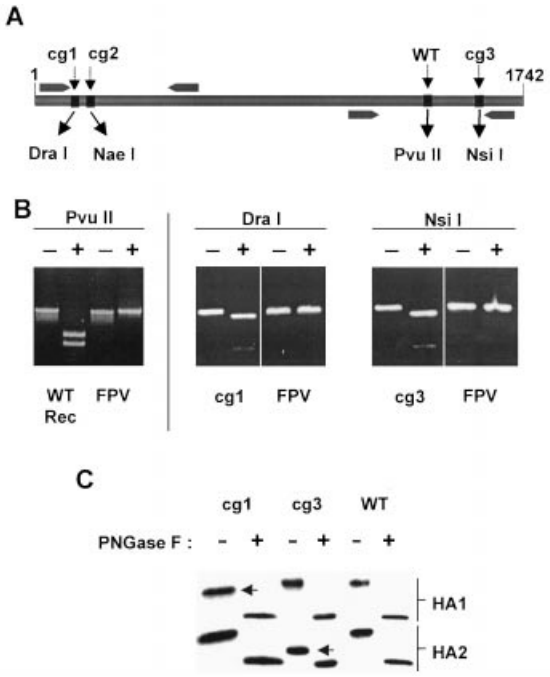

Fig. 3. Characterization of recombinant viruses. (A) Scheme of the cDNA used for the generation of recombinants. Positions of endonuclease restriction motifs introduced as genetic tag sites for individual mutants are indicated. The binding sites of specific oligonucleotide primers used in RT–PCR are shown by arrows. (B) RT–PCR analysis of RNA isolated from wild-type (WT), cg1 and cg3 recombinant viruses. RT–PCR products were incubated with endonucleases, as indicated, and separated on an agarose gel. RNA isolated from FPV was used as a control. (C) Analysis of the glycosylation pattern of the HA protein from recombinant viruses. The HA protein was immunoprecipitated from ^{35}S-labelled viruses. One-half of the material was treated with PNGase F (+), while the other half remained untreated (−). The protein profile was analysed by SDS–PAGE and bands were visualized by fluorography. Arrows point to bands showing reduced molecular masses, a result of missing *N*-glycans.

with two sets of HA-specific primers encompassing the introduced genetic tag sites mentioned above (see Fig. 3A). RT–PCR fragments were digested with the respective restriction endonucleases and analysed by agarose gel electrophoresis. The rescued viruses all proved positive when subjected to this assay (Fig. 3B). RNA obtained from the wild-type HA protein-carrying virus was cleaved with *Pvu*II, while that from the cg1 virus was cleaved with *Dra*I. RT–PCR fragments obtained with the cg3 virus were susceptible to *Nsi*I digestion. No sensitivity to these enzymes was seen with RT–PCR products transcribed from FPV RNA. Thus, restriction analysis revealed clearly that the plasmid-based mutated FPV HA genes had been incorporated stably into rescued viruses. Additionally, sequencing of the whole HA gene isolated from mutant viruses was performed to ascertain

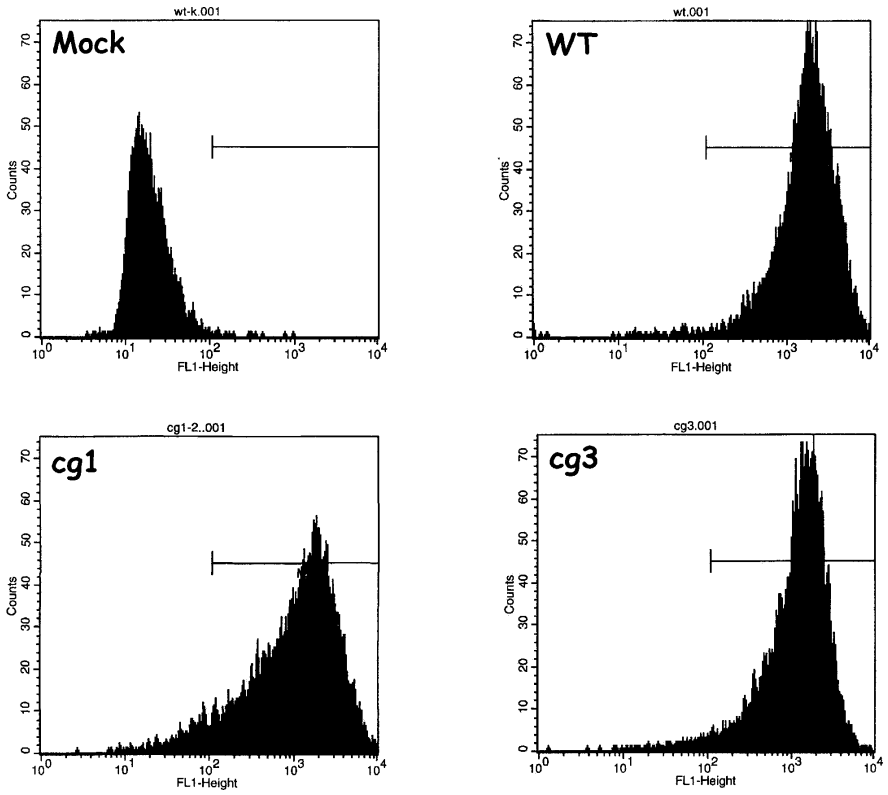

Fig. 4. Comparison of the surface expression of wild-type (WT) and mutant (cg1 and cg3) HA proteins in virus-infected MDCK cells. At 12 h after infection, cells were fixed with paraformaldehyde and immunostained using an HA-specific monoclonal antibody from mouse and a fluorescein-conjugated anti-mouse immunoglobulin. Uninfected cells were used as a control (Mock).

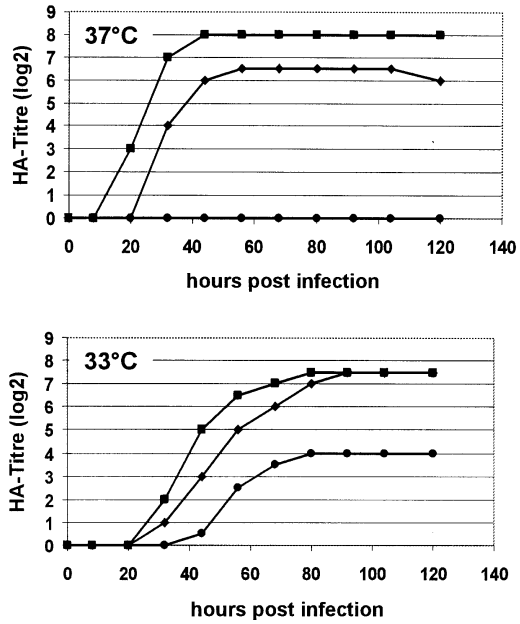

Fig. 5. Replication of recombinant viruses in MDCK cells at 37 and 33 °C. Cell monolayers were infected at a low m.o.i. and supernatants were monitored for HA titres at the time-points indicated. ■, wild-type; ●, cg1; ◆, cg3.

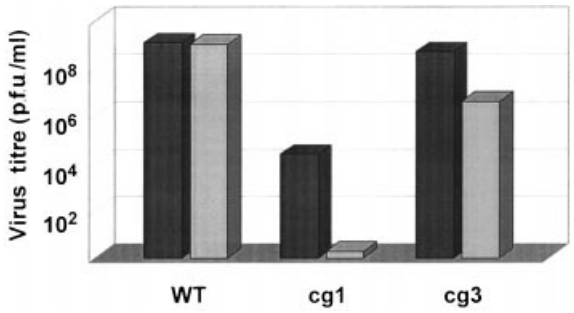

Fig. 6. Growth of recombinant viruses in 11-day-old embryonated chickens' eggs. 1000 p.f.u. of the respective virus was inoculated into the allantoic cavity. At 48 h post-infection, allantoic fluid was harvested and virus titres were determined by plaque assay on MDCK cells. Chickens' eggs were incubated at either 37 (grey) or 33 °C (black).

that no spontaneous mutations had been acquired during the rescue and amplification procedure (data not shown).

We then looked at the protein profile of the recombinant viruses to ascertain if *N*-glycans are, in fact, missing from the HA protein of the mutant viruses. The HA protein was isolated by immunoprecipitation from radioactively labelled viruses produced in MDCK cells. When examined in SDS–PAGE and

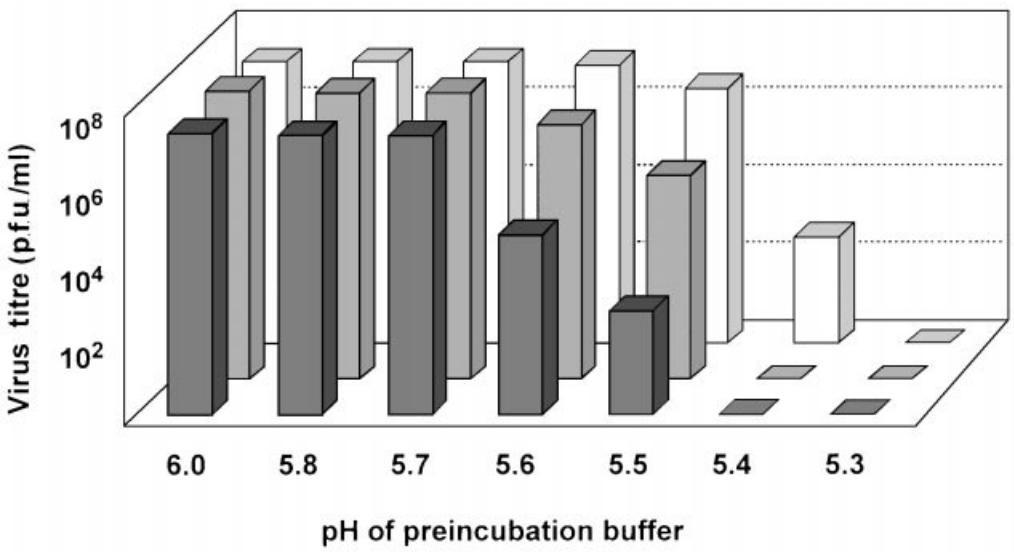

Fig. 7. Comparison of the acid stability of recombinant viruses. 10^7 p.f.u. of the respective virus was preincubated for 30 min with low pH buffers ranging from pH 6·0 to 5·3. Samples were then neutralized immediately and the infectivity remaining was determined by plaque assay on MDCK cells. Wild-type viruses are represented in white, whereas cg1 and cg3 viruses are represented in black or grey, respectively.

compared to the wild-type HA protein, the HA protein from the cg1 and cg3 viruses showed a reduced molecular mass in the HA1 and HA2 subunit, respectively (Fig. 3C). By PNGase F treatment, we confirmed that the observed differences in molecular mass actually reflected the loss of *N*-linked oligosaccharides.

To exclude any adverse effects of the cg mutations on the transport and surface expression of the HA protein, MDCK cells infected with recombinant cg viruses at high multiplicity were subjected to flow cytometry using an HA-specific monoclonal antibody. In these experiments, the HA protein accumulated to roughly the same amount at the surface of infected cells, irrespective of the mutation present in the stem of the molecule (Fig. 4).

Replication of recombinant viruses

So far, effects caused by the loss of *N*-glycans from the HA stem had only been studied by the solitary expression of recombinant protein in CV1 cells (Ohuchi *et al*., 1997b; Roberts *et al*., 1993; Gallagher *et al*., 1992). With our set of mutant cg viruses, it became possible to examine the impact of individual HA stem glycans on the replication of intact influenza viruses. To this end, MDCK cells were infected with recombinant viruses at low m.o.i. and cultivated at either 33 and 37 °C. Growth of progeny viruses in culture supernatants was monitored for the following 5 days. HA titres obtained with the cg3 mutant (lacking the *N*-glycan at Asn^{478}) were quite similar to those of wild-type virus (Fig. 5). Only at 37 °C did we observe a slight reduction of cg3 virus titres when compared to the wild-type virus. However, loss of the *N*-glycan from Asn^{12} (in cg1 viruses) had a dramatic effect on virus replication in MDCK cells. Growth of the cg1 virus was blocked totally at 37 °C and decreased about 20-fold at 33 °C.

Next, we examined the propagation of mutant viruses in 11-day-old embryonated chickens' eggs inoculated via the allantoic route. Virus yields in the allantoic fluid were determined 48 h post-infection. Here, the effects of missing stem glycans turned out to be even more drastic than in MDCK cells (Fig. 6). Replication of the cg3 virus was unaffected at 33 °C but reduced about 100-fold at 37 °C. The cg1 virus showed a highly attenuated phenotype, since its replication was impeded severely by more than four log steps at 33 °C and blocked completely at 37 °C.

These results highlight the relevance of individual *N*-glycans attached to the HA stem for influenza virus growth. While the glycan at Asn^{478} is only of minor importance, the glycan attached to Asn^{12} represents a major determinant for efficient influenza virus replication in cell culture and embryonated chickens' eggs.

Functional impact of stem glycans

It was now of interest to get some insight into the mechanism by which stem oligosaccharides might promote influenza virus replication. From our previous studies on the expression of the mutant HA proteins in CV1 cells, we knew already that the loss of stem glycans interferes with the pH stability of the molecule (Ohuchi *et al*., 1997b). Accordingly, we next tested the stability of the mutant cg viruses by incubation for 30 min at different pH prior to plaque titration. The viruses analysed showed marked differences in their response to this acid preincubation (Fig. 7). Inactivation of virus containing the wild-type HA protein was observed only

at pH values lower than 5·5, whereas the mutant cg viruses displayed a higher pH instability, with inactivation starting already at pH 5·6 and total inactivation occurring at pH 5·4. This enhanced susceptibility to low pH treatment was very distinct with the cg1 viruses and less pronounced with the cg3 viruses. These results most likely reflect a premature acid-induced denaturation of the FPV HA protein lacking the *N*-glycans from the stem region. Therefore, stem glycans can be regarded as potent stabilizers of the metastable conformation of the HA protein preventing premature denaturation, with the glycan at Asn^{12} being dominant and that at Asn^{478} being of minor importance.

Discussion

Attachment of oligosaccharide side chains to asparagine residues of the nascent polypeptide chain is a common cotranslational modification affecting both structural and functional features of the respective glycoproteins (reviewed by Lis & Sharon, 1993; Varki, 1993). With the HA protein of influenza viruses, there is considerable variation in *N*-glycans located in the area of the globular head domain, while those linked to the stem of the molecule are highly conserved (Nobusawa *et al*., 1991). Here we report on the generation of recombinant viruses lacking these conserved *N*-glycans from the stem of the FPV HA protein. Since our current knowledge on the functional aspects of HA stem glycosylation resulted solely from the expression of the HA protein in cell culture (Ohuchi *et al*., 1997b; Roberts *et al*., 1993; Gallagher *et al*., 1992), the present study provides experimental data on the role of individual stem glycans in influenza virus replication. We found that loss of these glycans affected strongly the replication of viruses in different culture systems. Growth of the cg1 mutant lacking the glycan at Asn^{12} was blocked in MDCK cells as well as in embryonated chickens' eggs at 37 °C and severely inhibited at 33 °C. These effects were less striking but still evident with the cg3 mutant (lacking the *N*-glycan at Asn^{478}). From the observation that the cg2 mutant lacking the glycan at Asn^{28} could not be generated, we conclude that this glycan is an absolute requirement for the formation of replication-competent influenza viruses in our system. This finding corresponds closely with our earlier results where the absence of the *N*-glycan at Asn^{28} proved to have the most severe effect on the transport rate and the trimerization efficiency (Roberts *et al*., 1993) as well as on syncytia formation activity and the pH optimum of FPV HA-induced fusion (Ohuchi *et al*., 1997b). This is quite remarkable because the glycan at this position, in contrast to those attached to Asn^{12} and Asn^{478}, which are present in all HA subtypes analysed so far, is not strictly conserved but is found in only 10 of the 15 known HA subtypes. Therefore, it appears that this special glycan is essential to meet the structural demands of some HA subtypes, while others gain their structural integrity by different mechanisms.

When tested for their response to low pH treatment, stem glycosylation mutant viruses displayed a higher susceptibility than virus carrying the wild-type HA protein, as demonstrated by a premature loss of infectivity upon acidification. Again, this effect was distinct with the cg1 mutants and less pronounced with the cg3 mutants. Previous studies on several virus strains occurring naturally have revealed that the threshold pH that causes loss of virus infectivity is dependent on the HA subtype (Scholtissek, 1985). By assaying hydrophobicity, sensitivity to protease digestion, exposure of antibody epitopes, appearance in electron microscopy and fusion activity, it was shown subsequently that HA subtypes differ in the structural rearrangements, which develop upon acidification of virus particles (Korte *et al*., 1999; Puri *et al*., 1990). For example, pretreatment of X31 virus (H3 subtype) at pH 5 in the absence of target membranes led to virus inactivation due to an irreversible denaturation of the HA protein, while the A/Japan/305/57 virus strain (H2 subtype) retained infectivity after this treatment. Taken together, these results demonstrate that premature acid-induced denaturation of influenza viruses is indicative of the structural instability of the HA protein. Accordingly, it becomes clear that the observed low pH response of the mutant cg viruses reflects the lability of the HA molecule lacking its stem glycans. In an earlier study, we found that the pH required for optimal fusion activity of wild-type, cg1 and cg3 mutant HA proteins expressed in CV1 cells in the presence of ammonium chloride was 5·0 when assayed by syncytia formation activity (Ohuchi *et al*., 1997b). Here we show that infectivity of cg mutant virus particles is already totally lost at pH 5·4. These observations suggest that pH dependence of infectivity is a more sensitive assay for the stability of the FPV HA protein than pH dependence of syncytia formation induced by the vector-expressed HA protein. It is also possible that, in addition to the HA protein, there are other factors determining the pH dependence of FPV infectivity. In any case, FPV appears to differ in this respect from other influenza A viruses, such as X31, where the pH for optimal fusion is identical to the pH that gives complete inactivation of virus infectivity (Korte *et al*., 1999). Differences in the experimental setting might also serve as an explanation for varying results obtained with solitary expressed HA protein and mutant viruses. In particular, cg3 mutant HA protein expressed in CV1 cells was affected more strongly in syncytia formation activity than the cg1 mutant HA protein, whereas growth of recombinant cg1 viruses was restricted much more than that of the cg3 viruses. Furthermore, it cannot be ruled out that ammonium chloride used to stabilize vector-expressed HA proteins during their transport to the cell surface might influence the pH response of the molecule.

Likewise, it has been established that heat is capable to destabilize the HA protein (Carr *et al*., 1997). Hence, the temperature-sensitive replication observed with the cg1 viruses also reflects the instability of the mutant HA protein, which is prone to denaturation at 37 °C.

For the HA proteins of influenza viruses, there are at least two steps where stabilization of the correct protein conformation is critical. Firstly, the metastable state emerging from proteolytic activation needs to be preserved in order to avoid a premature switch to the low pH structure (Steinhauer *et al.*, 1996; Bullough *et al.*, 1994; Carr & Kim, 1994). Secondly, adoption of the acid induced fusion active conformation is a complex process of extensive refolding, probably involving structural intermediates that require transient stabilization (Korte *et al.*, 1999; Shangguan *et al.*, 1998; Stegmann *et al.*, 1990). *N*-Glycans have been known for a long time to play a crucial role in maintaining the structure and stability of glycoproteins by mediating contact with the aqueous environment (Varki, 1993). Our results on the pH stability of mutant viruses demonstrate clearly that the stem glycan at Asn^{12} is crucial to prevent a premature denaturation of the HA protein in mature virions, thereby maintaining infectivity. It has been shown before that amino acid exchanges in the stem domain affect significantly the stability of the HA protein, thereby altering the pH required for HA-mediated fusion to occur (Steinhauer *et al.*, 1991; Doms *et al.*, 1986; Daniels *et al.*, 1985). The data obtained in the present study reveal that not only amino acids but also highly conserved *N*-glycans in the stem contribute strongly to the stability and function of the HA protein.

In an earlier study, we demonstrated that glycans flanking the receptor-binding site regulate receptor-binding activity of the FPV HA protein very efficiently (Ohuchi *et al.*, 1997a). When FPV HA mutants lacking these glycans were introduced stably into recombinant viruses, growth of these viruses in cell culture turned out to be significantly restricted. These growth restrictions could be attributed to incomplete release of progeny viruses from host cells owing to the enhanced receptor affinity of the mutant HA proteins (Wagner *et al.*, 2000). Considering the results of the present study, it becomes evident that *N*-glycans neighbouring the receptor-binding site and those decorating the HA stem exert their function during virus replication by totally different mechanisms. Hence, *N*-glycans are very efficient and versatile regulators of structural and functional properties of virus glycoproteins.

Given the high conservation of HA stem glycans, it is reasonable to assume that the growth restrictions seen with the FPV HA mutant viruses also apply for viruses containing other HA subtypes. Deletion of glycans from the HA stem might, therefore, constitute a general experimental approach for the production of temperature-sensitive, attenuated influenza viruses. Such virus mutants are likely to represent an excellent source for the production of live, attenuated influenza virus vaccines. In this respect, it will now be interesting to examine the pathogenesis and the host tropism of our panel of mutant cg viruses.

We are grateful to Peter Palese and Adolfo Garcia-Sastre for kindly providing the reassortant helper viruses and the PolI-SapI vector. This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 286) and from the Fonds der Chemischen Industrie. T.W. was a recipient of a fellowship of the Deutsches Krebsforschungszentrum (Infektionsforschung, AIDS-Stipendienprogramm).

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Received 13 September 2001; Accepted 15 November 2001
Published ahead of print (27 November 2001) in JGV Direct as DOI 10.1099/vir.0.18104-0