Role of overlapping glycosylation sequons in antigenic properties, intracellular transport and biological activities of influenza A/H2N2 virus haemagglutinin

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The haemagglutinin (HA) protein of influenza A/H2N2 virus possesses five oligosaccharide attachment sites, two of which have overlapping glycosylation sequons at positions 20–23 (NNST) and 169–172 (NNTS). Here, the role of these two oligosaccharide attachment sites is investigated with regard to antigenic property, intracellular transport and biological activity of the HA protein. Glycosylation-site HA mutants with mutation(s) in their overlapping glycosylated sequons, each of which had one or two oligosaccharide attachment sites removed, were constructed. Comparison of electrophoretic mobility between the wt and mutant HA proteins showed that both Asn residues 20 and 21 and Asn residues 169 and 170 could be used for glycosylation. Analysis of reactivity of the mutants with anti-HA monoclonal antibodies suggested that amino acid changes at these two positions result in a conformational change of the HA molecule. Even if oligosaccharide chains linked to Asn 20 or 21 and Asn 169 or 170 are eliminated, the antigenic properties, intracellular transport and biological activities are not influenced strongly. Thus it is reasonable to conclude that the two overlapping glycosylation sequons at positions 20–23 and 169–172 are conserved among all of the HAs of influenza A/H2N2 viruses because conservation of the amino acid sequence itself rather than that of N-glycosylation is essential for the formation of the proper conformation, intracellular transport and biological activities of the H2 subtype HA.

Introduction

The haemagglutinin (HA) protein of influenza A virus is a type I integral membrane protein with an ectodomain composed of a globular head and a stem region (Wilson et al., 1981). Both of these regions carry N-linked oligosaccharide chains. Important information about the biological significance of glycosylation has been obtained: (i) addition of carbohydrates to antigenic sites interferes with the access of antibodies (Skehel et al., 1984); (ii) sialylation of oligosaccharide chains near the receptor-binding site prevents receptor binding (Ohuchi et al., 1995); (iii) oligosaccharides near the cleavage site modulate proteolytic activation, influencing the pathogenicity of the virus (Deshpande et al., 1987; Ohuchi et al., 1989); (iv) stem oligosaccharides stabilize the HA in a form susceptible to the conformational change needed for fusion (Ohuchi et al., 1997). Together, these observations support the notion that the individual oligosaccharide chains on the HA play distinct roles in the biosynthesis and biological function of the glycoprotein.

We previously investigated the antigenic structure of the influenza A/Kayano/57 (H2N2) virus HA by using anti-HA monoclonal antibodies (mAbs) and escape mutants selected by these antibodies and identified six distinct antigenic sites, designated I-A to I-D, II-A and II-B (Tsuchiya et al., 2001). We demonstrated that most of the escape mutants selected by mAbs to site I-A, I-B or I-C acquired a novel glycosylation site at position 160, 187 or 131, respectively, showing that influenza A/H2N2 viruses have the potential to acquire at least one additional oligosaccharide on the tip of the HA (Tsuchiya et al., 2001). Interestingly, however, examination of the available HA amino acid sequences of influenza A/H2N2 viruses showed that none of the HA molecules had obtained a new glycosylation site on the tip and had only one carbohydrate chain at position 169 or 170 [two glycosylation sequons overlap each other at residues 169–172 (NNNTS)]. We recently showed that HA glycosylation-site mutants, which
acquired one to three oligosaccharide chains at position 160, 187 or 131 by site-directed mutagenesis, are transported to the cell surface efficiently but exhibit a moderate or drastic decrease in both receptor-binding and cell-fusing activities (Tsuchiya et al., 2002).

Potential sites for glycosylation occur when the Asn residue is in the consensus sequence Asn–X–Ser/Thr, where X can be any amino acid except Pro (Kornfeld & Kornfeld, 1985). The HA of influenza A/H2N2 virus contains five N-linked glycosylation sites and two glycosylation sequons overlap at positions 20–23 (NNST) and 169–172 (NNTS) (Waterfield et al., 1980; Brown et al., 1981) (Fig. 1). The overlapping glycosylation sequons at positions 20–23 are also present in H1, H6, H8, H11 and H12 subtype HA s and those at positions 169–172 are present in H13 subtype HA (Nobusawa et al., 1991). In the present study, we explored the role of these two oligosaccharide chains in the antigenic properties, intracellular transport and biological activities of the HA protein by eliminating each of the overlapping glycosylation sequons by site-specific mutagenesis. The data obtained suggest that the oligosaccharide chains linked to Asn 20 or 21 and Asn 169 or 170 are not essential for the intracellular transport and biological activity. It is reasonable to conclude that the two overlapping glycosylation sequons present at positions 20–23 and 169–172 are conserved among all of the HAs of influenza A/H2N2 viruses because conservation of the amino acid sequence itself in these glycosylation sequons is critical for the formation of the proper conformation, intracellular transport and biological activities of the H2 subtype HA.

Methods

- **Virus and cells.** The A/Kayano/57 strain of influenza A/H2N2 virus was propagated in the allantoic cavities of 10-day-old embryonated hens’ eggs. COS-1 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% foetal calf serum (FCS).

- **Antibodies.** Antibodies to egg-grown influenza A/Kayano/57 virus were raised in rabbits as described previously (Yokota et al., 1983). Neutralizing mAbs to the influenza A/Kayano/57 virus HA were prepared previously (Tsuchiya et al., 2001) and were demonstrated by operational mapping to be directed against six nonoverlapping or partially overlapping antigenic sites, designated I-A to I-D, II-A and II-B (Tsuchiya et al., 2001).

- **Plasmid construction and oligonucleotide-directed mutagenesis.** The wt HA gene cDNA of influenza A/Kayano/57 virus was generated from viral RNA using AMV Reverse Transcriptase XL (Life Sciences) and oligonucleotide primers complementary to positions 1–25 of RNA segment 4 and amplified by PCR using a plus-sense primer, corresponding to positions 41–61 and containing a NotI site, and a minus-sense primer, corresponding to positions 1759–1731 and containing a SpeI site. The PCR product was cut with Nol|SpeI and subcloned into the Nol–SpeI sites of the transient expression vector, pME18S (Takebe et al., 1988). The mutated HA gene cDNAs with mutation(s) in two overlapping glycosylated sequons located at positions 20–23 (NNST) or 169–172 (NNTS) were constructed with mutant primers. The mutant primers were designed so that the Asn-, Thr- or Ser-encoding codon would be replaced by an Ala-encoding codon. In a particular mutant, 20-NNTA (see Fig. 7), the Ser-encoding codon was changed into a Thr-encoding codon. PCR products were excised by digestion with Nol|SpeI and self-ligated and then used for transfection. Nucleotide sequences of all mutant cDNAs in pME18S were confirmed by dideoxynucleotide chain-terminating sequencing.

- **Transfection, radioisotopic labelling and immunoprecipitation.** Subconfluent monolayers of COS-1 cells in 3-5 cm Petri dishes were transfected with 1 µg of the recombinant pME18S plasmid containing a wt or mutant HA gene using LipofectAMINE Reagent (Invitrogen). Unless otherwise noted, cells were labelled with 10 µCi/ml (3.7 × 10⁸ Bq/ml) [³⁵S]methionine (ARC) for 20 min in methionine-free DMEM at 48 h post-transfection. Then, cells were disrupted in 0.01 M Tris–HCl (pH 7.4), containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl and a cocktail of protease inhibitors (Hongo et al., 1997) and immunoprecipitated as described (Sugawara et al., 1987) using rabbit antiserum or anti-HA mAbs. The resulting immunoprecipitates were analysed by SDS–PAGE on 13% gels containing 4 M urea under reducing conditions.

- **Endoglycosidase H (endo H) digestion.** Immunoprecipitated HA proteins were digested with endo H (30 µl) for 16 h at 37 °C, as described (Hongo et al., 1997), precipitated with acetone and analysed by SDS–PAGE.

- **Trypsin treatment of transfected cells.** Transfected COS-1 cells

![Fig. 1. Location of N-glycosylation sites of the HA of influenza A/H2N2 virus. The positions of the N-glycosylation sites are shown on the three-dimensional structure of H3 subtype HA (Wilson et al., 1981). Numbers indicate Asn residues in the first position of the glycosylation sequon. Sites A–E indicate the positions of the five antigenic sites identified on the H3 molecule.](image_url)
Results and Discussion

Expression, antigenic properties and intracellular transport of HA mutants with amino acid change(s) in overlapping glycosylation sequons present at positions 20–23 and 169–172

Wt HA possesses overlapping glycosylation sequons at two positions: 20 NNST and 169 NNTS. First, to identify the Asn residue used for N-glycosylation, the following six HA mutants were constructed: 20-NNAT, 20-NNSA, 20-NNAA, 169-NNAS, 169-NNTA and 169-NNAA (the mutated amino acid residue is underlined). COS-1 cells transfected with either wt cDNA or each of the six mutant cDNAs labelled with [35S]methionine were immunoprecipitated with rabbit antiserum and the resulting precipitates were analysed by SDS–PAGE. As shown in Fig. 2(A), mutant 20-NNAA (in which both glycosylation sequons were eliminated) exhibited a faster electrophoretic mobility compared with that of wt HA. In contrast, the mobility of 20-NNAT was indistinguishable from that of wt HA, while 20-NNSA generated two distinct bands: one comigrated with wt HA and one comigrated with 20-NNAA. These results suggest that both Asn residues 20 and 21 can be used for N-glycosylation, although the Thr → Ala change at position 23 decreased the efficiency of N-glycosylation considerably.

Mutant 169-NNAA showed a faster electrophoretic mobility than wt HA, as expected. Most of the 169-NNAS and 169-NNTA molecules comigrated with wt HA, although some of them displayed the same mobility as that of 169-NNAA, a finding that suggests that both Asn residues 169 and 170 can be used for glycosylation. The amounts of wt and mutant HAs expressed in transfected cells were determined by measuring the radioactivity of the HA bands after SDS–PAGE of cell lysates labelled with [35S]methionine and the expression levels of all the mutant HAs were comparable to that of wt HA (data not shown). It should be noted, however, that the amount of 169-NNAS immunoprecipitated with rabbit antiserum was much lower than those of wt HA and 169-NNTA.

To investigate the antigenic properties of the individual HA mutants, COS-1 cells expressing each of the six mutant HAs were labelled with [35S]methionine, immunoprecipitated with each of the six anti-HA mAbs and the resulting precipitates were analysed by SDS–PAGE.

Fig. 2. (A) Expression of HA glycosylation-site mutants. COS-1 cells transfected with wt or mutated HA gene cDNAs were labelled with [35S]methionine for 20 min at 48 h post-transfection. Cells were then immunoprecipitated with rabbit antiserum and the resulting immunoprecipitates were analysed by SDS–PAGE. (B) Reactivity of glycosylation-site mutants with anti-HA mAbs. COS-1 cells expressing wt or mutant HA were labelled with [35S]methionine for 20 min at 48 h post-transfection and immunoprecipitated with each of the six anti-HA mAbs. The resulting precipitates were analysed by SDS–PAGE.
resulting precipitates were analysed by SDS–PAGE. As demonstrated in Fig. 2(B), all mutants 20-NNAT, 20-NNSA and 20-NNAA, like wt HA, reacted with all of the mAbs used. Interestingly, however, it became evident that 169-NNAS, unlike wt HA and 169-NNTA, was unreactive with any of the anti-HA mAbs used and that the reactivity of 169-NNAA was weak compared with wt HA and 169-NNTA, suggesting that the amino acid change at position 171 (Thr→Ala) results in an extensive, conformational change of the HA molecule and that this drastic, conformational change is restored partially by an amino acid substitution at position 172 (Ser→Ala).

To see whether the six HA mutants described above acquire resistance to endo H, indicative of the conversion of carbohydrate chains from high mannose-type to complex one, which takes place in the Golgi cisternae (Tarentino & Maley, 1974), COS-1 cells expressing wt HA or each of the mutant HAs were labelled with [35S]methionine for 20 min at 48 h post-transfection and then chased for 4 h. Immediately after a pulse, or after a subsequent chase, cells were immunoprecipitated with rabbit antiserum and the resulting precipitates were treated with endo H followed by analysis with SDS–PAGE. As can be seen in Fig. 3(A), mutants 20-NNAT, 20-NNSA and 20-NNAA became endo H-resistant during a chase, showing that all these mutant HAs are transported to the medial Golgi compartment. In contrast, 169-NNAS remained endo H-sensitive even after a 4 h chase, although 169-NNTA acquired resistance to endo H. Interestingly, however, most of the 169-NNAA molecules, like 169-NNTA, became endo H-resistant, suggesting that the amino acid change at position 171 (Thr→Ala) affects the transport of the HA molecule from the endoplasmic reticulum to the Golgi apparatus.

To further examine whether the six mutant HAs can be transported to the cell surface, COS-1 cells transfected with wt cDNA or each of the mutant cDNAs were pulse-labelled for 20 min at 48 h post-transfection and chased for 4 h. Cells were treated with TPCK–trypsin during the last 15 min of the chase to cleave the HA protein expressed on the cell surface into the disulfide-linked HA1 and HA2 subunits. Fig. 3(B) shows that 20-NNAT, 20-NNSA and 20-NNAA were all cleaved by TPCK–trypsin into HA1 and HA2, indicating that these mutants were all transported to the cell surface and that glycosylation at Asn residues 20 or 21 is not a prerequisite for the surface expression of the HA molecule. This is consistent with previous findings that no individual stem oligosaccharide is necessary or sufficient for the intracellular transport of the HA molecule (Gallagher et al., 1992; Roberts et al., 1993; Ohuchi et al., 1997). As expected from the results of Fig. 3(A), 169-NNAS remained resistant to digestion with TPCK–trypsin, whereas 169-NNTA and 169-NNAA, like wt HA,
Glycosylation of influenza A/H2N2 virus HA

Fig. 4. (A) Expression of HA glycosylation-site mutants. COS-1 cells transfected with wt or mutated HA gene cDNAs were labelled with \([35S]\)methionine for 20 min at 48 h post-transfection. Cells were then immunoprecipitated with rabbit antiserum and the resulting immunoprecipitates were analysed by SDS–PAGE. (B) Reactivity of glycosylation-site mutants with anti-HA mAbs. COS-1 cells transfected with wt cDNA or each of glycosylation-site mutant cDNAs were labelled with \([35S]\)methionine and then immunoprecipitated with each of the six anti-HA mAbs. The immunoprecipitates obtained were subjected to SDS–PAGE.

were cleaved into HA1 and HA2 (Fig. 3B). These observations showed that N-glycosylation at Asn residues 169 or 170 is not required for the surface expression of HA and that the Thr → Ala change at position 171 prevents the intracellular transport of HA.

The results described above raised the possibility that, with respect to the overlapping glycosylation sequons present at positions 169–172, conservation of the amino acid sequence itself rather than that of N-glycosylation is critical for the formation of the proper conformation and intracellular transport of the HA molecule. To further confirm this notion, an additional six mutant HAs (20-ANST, 20-NAST, 20-AAST, 169-ANTS, 169-NATS and 169-AATS), in which Asn residues were replaced by Ala residues, were constructed. COS-1 cells expressing wt HA or each of these mutant HAs were labelled with \([35S]\)methionine, immunoprecipitated with rabbit antiserum and then analysed by SDS–PAGE (Fig. 4A). The expression levels of mutant HAs were comparable to that of wt HA, as described above. Clearly, the amounts of 169-NATS and 169-AATS recovered in the immunoprecipitates were much lower than those of wt and the other mutant HAs. To validate this observation, immunoprecipitation was carried out with six anti-HA mAbs to six different antigenic sites (Fig. 4B). 169-NATS and 169-AATS, in contrast to wt HA and the other mutant HAs, were completely unreactive with any of the anti-HA mAbs used, showing that the amino acid change at position 170 (Asn → Ala) prevents the formation of all of the neutralizing epitopes tested. Thus it is reasonable to conclude that the overlapping glycosylation sequons at positions 169–172 is conserved among all of the HAs of influenza A/H2N2 viruses because conservation of the amino acid sequence itself in these glycosylation sequons is essential for the formation of the proper conformation of H2 subtype HA. This is not incompatible with the previous report showing that the presence of oligosaccharides on the globular head of HA is not necessarily required for influenza virus replication, since most of the HAs of influenza viruses isolated from aquatic birds do not contain oligosaccharides on their tips (Inkster et al., 1993; Matrosovich et al., 1999).

Fig. 5 demonstrates the results of experiments where endo H sensitivity of the six HA mutants tested under the same

![Fig. 5. Endo H digestion of HA glycosylation-site mutants. COS-1 cells expressing wt or mutant HA were pulse-labelled with \([35S]\)methionine for 20 min at 48 h post-transfection, chased for 4 h and then immunoprecipitated with rabbit antiserum. The resulting precipitates were digested (+) or mock-digested (−) with endo H and analysed by SDS–PAGE.](image-url)
Most (69–84%) of the 20-ANST and 20-NAST molecules acquired endo H resistance during a 4 h chase (Fig. 5A). It should be noted, however, that more than half the molecules (61%) of mutant 20-AAST remained sensitive to digestion with endo H, suggesting that substitution of both the Asn residues at positions 20 and 21 results in partial inhibition of the transport of HA to the Golgi apparatus. This is supported by the results of Fig. 6(A) showing that 20-AAST was transported to the cell surface only very inefficiently. Fig. 5(B) shows that mutants 169-NATS and 169-AATS, unlike wt HA and 169-ANTS, were completely sensitive to endo H even after a 4 h chase, indicating that they cannot move out from the endoplasmic reticulum. This is supported by the results of Fig. 6(B), which demonstrated that these two mutants were not expressed on the cell surface at all. The failure of 169-NATS and 169-AATS to be transported to the Golgi apparatus is likely to be due to their failure to establish the proper conformation (see Fig. 4B). Additionally, it should be noted that about 51% of the 169-ANTS molecules remained sensitive to endo H. This is consistent with the fact that its surface expression was poor compared with wt HA, as shown in Fig. 6(B). Thus, the amino acid change at residue 169 (Asn → Ala), like that at position 171 (Thr → Ala), also influences the intracellular transport of the HA molecule.

Asn–X–Thr is used for N-glycosylation more efficiently than Asn–X–Ser at overlapping glycosylation sequons at positions 20–23

Fig. 2 shows that both Asn residues 20 and 21 can be used for N-glycosylation but the latter is glycosylated more efficiently than the former, raising the possibility that between the two glycosylation consensus sequences Asn–X–Thr and Asn–X–Ser, the former may be used more efficiently than the latter. To examine this possibility, we constructed one mutant HA (20-NNTA) and compared the efficiency of its glycosylation with another mutant, 20-NNSA. Fig. 7 demonstrated that while only half of the 20-NNSA molecules were glycosylated, all the 20-NNTA molecules were glycosylated. An earlier report by Bause & Legler (1981) demonstrated using a series of hexapeptides as substrates and a calf liver microsomal fraction as an enzyme source that replacement of Thr by Ser results in a fourfold decrease in $V_{\text{max}}$ for glycosyl transfer. Our finding is consistent with their report.

Biological activities of mutant HAs

To investigate the receptor-binding activity of the 12 mutant HAs constructed here, COS-1 cells transfected with wt cDNA or each of the mutant cDNAs were examined for haemadsorption at 48 h post-transfection according to the procedures described in Methods. As shown in the left column of Table 1, the extent of haemadsorption of cells expressing each of the mutants 20-NNAT, 169-NNTA and 169-NNAA was comparable to that of cells expressing wt HA. The fact that 169-NNAA exhibited a high level of haemadsorption activity indicates that an oligosaccharide chain linked to Asn at position 169 or Asn at position 170 is not necessary for the H2 subtype HA to show receptor-binding activity. The other mutant HAs displayed lower levels of haemadsorption activity than that of wt HA (2–77% of wt). The levels of haemad-
Table 1. Biological activities of HA glycosylation-site mutants

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<th>Haemadsorption (%) of wt</th>
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<tr>
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Haemadsorption was measured as the percentage of haemoglobin released compared to wt. The average of three separate experiments is shown. Cell-fusing activity is scored as strong (++), slightly lower than wt (+), significantly decreased compared with wt (±) or weak (no activity detected, –). We thank Dr. Y. Takebe (National Institute of Infectious Diseases) for providing the expression vector, pME18S. This work was supported by a research fellowship of the Japanese Society for the Promotion of Science for Young Scientists, a grant-in-aid for scientific research from the Japanese Ministry of Education, Culture, Sports, Science and Technology and a research grant provided by the Japanese Ministry of Health, Labour and Welfare.

References


Conformational change that results in a decrease in intracellular transport and biological activity.


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