

Loss of Glycosylation at Asn144 Alters the Substrate Preference of the N8 Influenza A Virus Neuraminidase

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ABSTRACT. Role of asparagine-linked (N-linked) oligosaccharide side chains in the maturation and the function of influenza virus neuraminidase (NA) subtype N8 was examined by site-directed mutagenesis and vaccinia virus expression system. Mutations in the consensus sequence for N-linked glycosylation at Asn 84 or 398 prevent the proper maturation of mutant NAs. On the contrary, mutation at Asn 144, that is conserved in all except two strains of influenza virus NA ever sequenced, did not affect the proper maturation and the transport of the mutant NA to the cell surface. Furthermore, this mutation led the alternation of substrate preference of this enzyme. These observations indicate that N-glycosylation at Asn 144 of N8 NA may be conserved from the functional requirement, but not from the structural necessity. — **KEY WORDS:** influenza virus, N-glycosylation, neuraminidase, site-directed mutagenesis.

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Asparagine-linked (N-linked) oligosaccharide side chains of glycoproteins not only provide solubility to nascent peptide chain within the endoplasmic reticulum (ER), but also serve as a receptor for ER chaperone(s) [4, 6]. Role of N-linked oligosaccharide side chains on glycoproteins has been studied by treatment of cells with drugs, which abolish the addition of N-linked oligosaccharide side chains [5, 15]. Recently, assessment of the role of individual sugar side chains can be done by utilizing site-directed mutagenesis of the consensus sequence (Asn-X-Thr/Ser) for oligosaccharide attachment. These studies reveal that role of the N-linked oligosaccharides on glycoprotein maturation varies from one glycoprotein to the other [2, 7, 10, 12].

Influenza virus neuraminidase (NA) is a type II glycoprotein, serving as a receptor-destroying enzyme of this virus [1]. Sequence analysis of several subtypes of NAs reveals existence of multiple putative glycosylation sites on their head region of the molecule. Although lack of glycosylation at Asn 130 in WSN and NWS strains is shown to account for the neurovirulence of these strains [8], the role of individual oligosaccharide side chains on the molecule for NA maturation has not been studied. Glycosylation site at Asn 130 in the N1 NA of WSN strain, that is homologous to Asn 144 in N8 NA, is conserved for all NAs ever sequenced, except WSN and NWS strains [1, 16]. These observations raise a possibility that Asn 144 in N8 NA is not important for NA maturation, but possesses functional importance.

N8 NA of A/duck/Ukraine/63 origin possesses 3 putative glycosylation sites at Asn 84, 144 and 398 on its head region [13]. In this study, we examined the structural and functional role of each oligosaccharide side chain on the head region of this molecule by utilizing site-directed mutagenesis. We, thus, demonstrate that abolishment of

glycosylation site at Asn 144 alters substrate preference of this enzyme.

MATERIALS AND METHODS

Virus, monoclonal antibodies (MAbs) and cells: Vaccinia virus WR strain, provided by Dr. B. Moss, was used for expression of mutant or wild type N8 NAs. MAbs raised against NA of A/duck/Ukraine/1/63 were reported elsewhere [14]. Madin-Darby canine kidney (MDCK) and human TK-143 cells were maintained in Eagle's minimal essential medium (MEM), supplemented with 5% newborn calf serum (NBCS).

Site-directed mutagenesis: NA gene of A/duck/Ukraine/1/63 cloned into pSC11 (kindly provided by Dr. B. Moss) was designated as pSCN8. Site-directed mutagenesis on pSCN8 was carried out with Transformer™ Site-Directed Mutagenesis kit (Clontech). Three mutagenic primers, designated as HG-1, HG-2, and HG-3, respectively, were used along with selection primer HindIII-mut to abolish unique HindIII site within pSC11 (HG-1; ACTTACATGAATAATAACGAAGCAATATGTGAT, HG-2; CTCAATGACAAACACTCATATGGAACAGTG AAGGAT-AGG, HG-3; GACAATTTGAATTGGAACGGA TACAGTGGATCT, HindIII-mut; CATGATTACGCCATG GTTTTGCGATCAATA). Sequence of N8 NA after mutagenesis was confirmed by autosequencer DSQ-1000 (Shimazu).

Construction of vaccinia recombinants: Recombination of mutant or wild type NA gene with vaccinia WR strain was done as described elsewhere [9]. Resulting vaccinia recombinants, with N8 genes mutated by HG-1, HG-2 and HG-3 were designated as HG-1-Vac, HG-2-Vac and HG-3-Vac, respectively. Recombinant with wild type N8 NA was designated as N8-Vac.

Fluorescence-activated cell sorting (FACS) analysis: A confluent monolayer of MDCK cells was infected with vaccinia recombinant (m.o.i.=4) for 1 hr. After 2 hr

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additional incubation, cells were suspended by trypsinization at 37°C [11]. Cells were centrifuged (15,000 × g) for 1 min and washed twice with PBS, followed by fixation with 3% paraformaldehyde in PBS at 4°C for 30 min. After washed twice with PBS, cells were incubated with MAb N8-4 (1:200 dilution in PBS) at 4°C overnight. After washed twice with PBS, they were incubated with FITC-labeled anti-mouse IgG conjugate (Cappel) for 1 hr at room temperature. Cells were washed and resuspended in 1% NBSCS-PBS for FACS analysis. Analysis was done with EPICS Profile II (Coulter).

Neuraminidase (NA) assay: For NA assay of cell-associated NA, virus-infected cells were solubilized by cell lysis buffer (50 mM Tris-HCl; pH7.5, 100 mM NaCl, 1% Triton X-100, 20 mM Iodoacetamide). Samples were subjected to NA assay by method recommended by the World Health Organization [3] with 4 different substrates. Fetuin (1.25 mg/100 μ l), 3'-N-acetylneuraminlactose (3'-NAL; 100 μ g/100 μ l), 6'-N-acetylneuraminlactose (6'-NAL; 100 μ g/100 μ l), or 3'-sialyllactosamine (3'-SAL; 103 μ g/100 μ l) in 0.2 M phosphate buffer (pH 5.9) was used as a substrate.

Radioimmunoprecipitation (RIP): MDCK cells in 24-well culture plate were infected with vaccinia recombinant (m.o.i.=4) for 1 hr, followed by additional 2 hr incubation period with 5% NBSCS-MEM. Then, medium was replaced with methionin-free MEM for methionin starvation. After 15 min, cells were labeled with 100 μ Ci of Tran-³⁵S label (ICN Biomedicals, Inc.) for 1 hr. Cells were lysed by cell lysis buffer. Lysates were precleared with untreated Protein A Sepharose 4 Fast Flow (Pharmacia Biotech), followed by incubation with Protein A coated with MAb N8-10 for 1 hr. After washed with RIP buffer (50 mM Tris-HCl; pH 7.5, 100 mM NaCl, 1% TrironX-100, 20 mM Iodoacetamide), samples were suspended in sample buffer and incubated for 3 min at 100°C. Supernatants were applied for SDS-PAGE analysis with 10% acrylamide gel.

RESULTS

Expression of the mutant NAs by recombinant vaccinia viruses: Three NA-expressing vaccinia recombinant viruses with mutant NA, as well as wild type NA (designated as N8-Vac), were constructed (Fig. 1). Mutations of HG-1-Vac and HG-3-Vac were intended for conservative changes for amino acid, whereas that of HG-2-Vac was to make adjacent region completely homologous to that found in NWS strain. That is, amino acids 137 to 146 of HG-2-Vac NA were completely homologous to amino acids 123 to 132 of NWS strain [13, 16].

Expression of NAs with proper conformation was examined by RIP with MAb N8-10 (Fig. 2). This MAb recognizes the conformation-dependent epitope on the N8 NA monomer. NAs could be precipitated by MAb N8-10 from the cells infected with either N8-Vac or HG-2-Vac. The NA precipitated from HG-2-Vac infected cells showed faster migration in SDS-PAGE, indicating that the

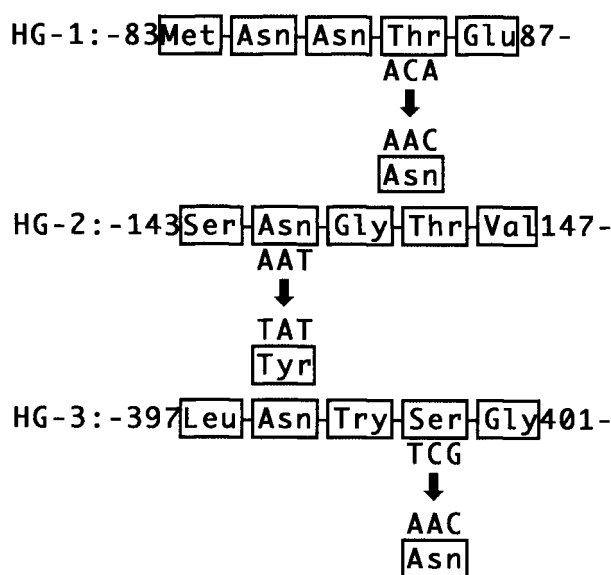


Fig. 1. Diagram of amino acid changes in each NA mutant. Amino acid sequence of wild type NA is drawn at the top and that of mutant at the bottom for each mutant. Nucleotide triplets are written below the mutated amino acids.

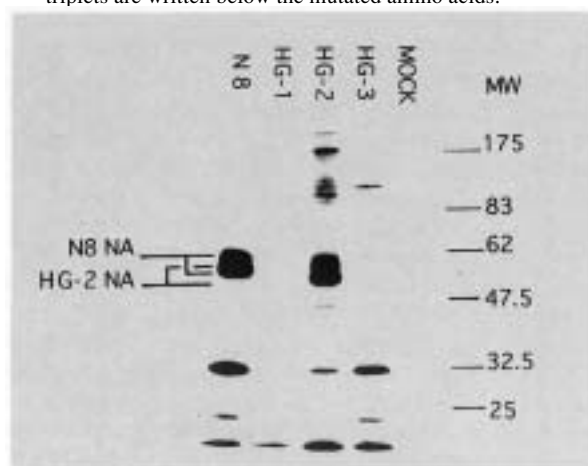


Fig. 2. Radioimmunoprecipitation of mutant NAs and wild type NA expressed from vaccinia recombinants.

elimination of sugar chain at the intended site was successful. No precipitable NAs were observed for HG-1-Vac or HG-3-Vac. This indicates that elimination of glycosylation site at Asn 144 does not prevent NA folding, on the other hand, HG-1-Vac and HG-3-Vac cannot express NA with proper folding. Since conformation-independent antibody was not available for N8 NA at hand, we were unable to confirm further if unfolded NA peptide exists within the cells infected by HG-1-Vac or HG-3-Vac.

Cell surface transport of HG-2 NA: To examine if mutant NA expressed by HG-2-Vac (HG-2 NA) is transport competent within the host cell, expression of NA at the cell surface was quantitated by FACS analysis (Fig. 3). MAb

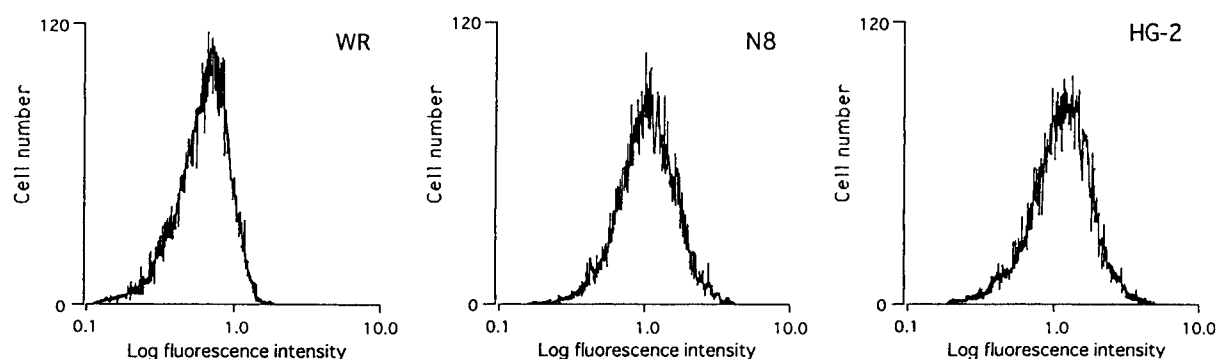


Fig. 3. FACS analysis of vaccinia recombinant-infected cells. The infected cells were stained with MAb N8-4.

N8-4, used for FACS analysis, is the tetramer specific MAb, making it able to measure mature NAs on the cell surface.

Similar level of surface expression was observed for HG-2 NA and wild type NA after 2 hr incubation period. Fluorescence intensity of the cells infected with these two recombinants was significantly higher than that of the cells infected by wild type vaccinia WR strain. This confirms that HG-2 NA is as competent for cell surface transport as wild type NA in MDCK cells. Cytopathic effect of WR strain prevented the FACS analysis after longer incubation period.

Change of substrate preference of HG-2 NA: Since it was demonstrated that the elimination of glycosylation site at Asn 144 did not prevent proper folding and cell surface transport, we next examined the effect of the elimination on NA activity (Fig. 4). When enzymatic activities of HG-2 and N8 NAs were examined using fetuin as a substrate, there was no significant difference between the two NAs. On the contrary, differences in substrate preference between HG-2 and N8 NAs were apparent when 3'-NAL and 3'-SLN were used as substrates. HG-2 NA shows significant difference in substrate preference between 3'-NAL and 3'-SLN. No significant difference was observed for N8-NA between 3'-NAL and 3'-SLN. For 3'-SLN, HG-2 NA is 80% as effective as N8 NA, even though they are similarly efficient on fetuin as a substrate.

These observations suggest that loss of side chain at Asn 144 alters substrate preference of N8 NA, making it less efficient for small substrates such as 3'-SLN and 3'-NAL. HG-2 and N8 NAs did not use 6'-NAL as a substrate and HG-1, HG-3 NAs and WR strain showed no enzymatic activity on fetuin and 3'-NAL (data not shown).

DISCUSSION

Importance of N-linked oligosaccharide side chains for the structure and/or the function of glycoproteins has been demonstrated. For influenza virus NA, loss of N-glycosylation at Asn 130 determines the neurovirulence of WSN and NWS strains [8]. In this study, we demonstrate that loss of N-glycosylation at Asn 144 (HG-2 NA), corresponding to Asn130 in the N8 NA, does not influence the maturation and the transport of the mutated NA of A/

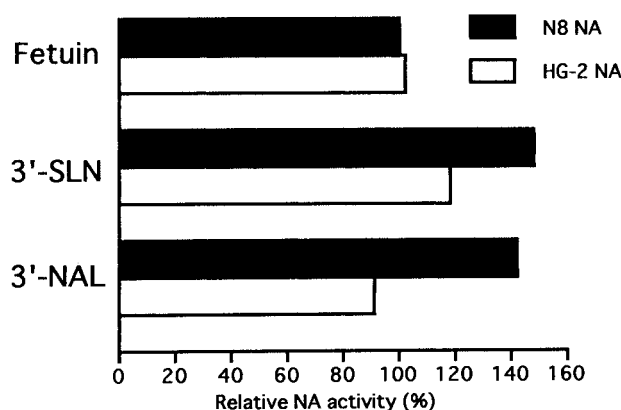


Fig. 4. NA activity of vaccinia recombinant-infected cells. Relative NA activity is expressed as percentage of that of wild type NA using fetuin as a substrate. Amount of the NAs expressed is standardized based on the surface expression of NAs determined by FACS analysis.

duck/Ukraine origin to the cell surface. On the other hand, vaccinia recombinants carrying NA gene with the mutation leading the lack of N-glycosylation at either Asn 83 or 398 (HG-1 or HG-3) could not express structurally or functionally mature NA. This suggests that N-glycosylations at Asn 83 and 398 may be important for NA maturation, although we could not rule out the possibility that amino acid change itself in HG-1 and HG-3 NAs disrupts tertiary structure of the NA.

N-glycosylation site at Asn 144 (in the N8 subtype) is conserved for influenza virus NAs of all subtypes at the homologous position, except for NWS and WSN strains [1, 16]. Abolishment of N-glycosylation at this site does not result in the disruption of NA structure in the case of HG-2 NA as well as NWS and WSN strains. Conservation of this glycosylation site in influenza virus NAs may be functionally required.

Li *et al.* [8] constructed a Gly⁺ mutant virus that has additional glycosylation site at position 130 on WSN genetic background. This mutant showed lower enzyme activities on both fetuin and 2-(4-methylumbelliferil)- α -D-N-acetylneuraminic acid, indicating that addition of N-linked oligosaccharide at Asn 130 decreases the efficacy of this

enzyme. When enzyme activities with different substrates were compared between NAs of WS strain and its neurovirulent derivatives, the enzymes from neurovirulent strains showed an increased preference for small substrates [16]. In contrast, we observed a decreased preference of HG-2 NA for small substrates, 3'-NAL and 3'-SLN, although it worked as efficiently as wild type NA on fetuin as a substrate. These observations indicate that glycosylation at Asn 144 (N8 numbering) is important for the determination of substrate preference of this enzyme, although how to affect on the preference may differ among different subtypes.

In this study, we confirmed that abolishment of N-glycosylation site at Asn 144 of N8 NA did not affect its proper maturation and transport to the cell surface. Additionally, this mutation led the change of substrate preference of this enzyme. These observations suggest that glycosylation site at Asn 144 is conserved probably for functional importance rather than structural requirement. This expands our current knowledge on the structural and functional importance of N-linked oligosaccharide side chain on this glycoprotein.

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REFERENCES

1. Air, G. M. and Laver, W.G. 1989. The neuraminidase of influenza virus. *Proteins* 6: 341-356.
2. Alkhatib, G., Shen, S.-H., Breiedis, D., Richardson C., Massie, B., Weinberg, R., Smith, D., Taylor, J., Paoletti, E., and Roder, J. 1994. Functional analysis of N-linked glycosylation mutants of the measles virus fusion protein synthesized by recombinant vaccinia virus vector. *J. Virol.* 68: 1522-1531.
3. Aymard-Henry, M., Colman, M. T., Dowdle, W.R., Laver, W. G., Schild, G. C., and Webster, R. G. 1973. Influenza virus neuraminidase-inhibition test procedures. *Bull. WHO* 48: 199-202.
4. Doms, R. W., Lamb, R. A., Rose, J. K., and Helenius, A. 1993. Folding and assembly of viral membrane proteins. *Virology* 193: 545-562.
5. Gallagher, P. J., Henneberry, J. M., Sambrook, J. F., and Gething, M. -J. H. 1992. Glycosylation requirements for intracellular transport and function of the hemagglutinin of influenza virus. *J. Virol.* 66: 7136-7145.
6. Helenius, A. 1994. How N-linked oligosaccharides affect glycoprotein folding in the endoplasmic reticulum. *Mol. Biol. Cell* 5: 253-265.
7. Hu, A., Cattaneo, R., Schwartz, S., and Norrby, E. 1994. Role of N-linked oligosaccharide chains in the processing and antigenicity of measles virus hemagglutinin protein. *J. Gen. Virol.* 75: 1043-1052.
8. Li, S., Schulman, J., Itamura, S., and Palese, P. 1993. Glycosylation of neuraminidase determines the neurovirulence of influenza A/WSN/33 virus. *J. Virol.* 67: 6667-6673.
9. Mackett, M., Smith, G. L., and Moss, B. 1982. General method for production and selection of infectious vaccinia virus recombinants expressing foreign genes. *J. Virol.* 49: 857-864.
10. McGinnes, L. R. and Morrison, T. G. 1996. The role of individual oligosaccharide chains in the activities of the HN glycoprotein of newcastle disease virus. *Virology* 212: 398-410.
11. Mitnaul, L. J., Castrucci, M. R., Murti, K. G., and Kawaoka, Y. 1995. The cytoplasmic tail of influenza A virus neuraminidase(NA) affects NA incorporation into virions, virion morphology, and virulence in mice but is not essential for virus replication. *J. Virol.* 70: 873-879.
12. Roberts, P. C., Garten, W., and Klenk, H.-D. 1993. Role of conserved glycosylation sites in maturation and transport of influenza A virus hemagglutinin. *J. Virol.* 67: 3048-3060.
13. Saito, T., Kawaoka, Y., and Webster, R. G. 1993. Phylogenetic analysis of the N8 neuraminidase gene of influenza A viruses. *Virology* 193: 868-876.
14. Saito, T., Taylor, G., Laver, W. G., Kawaoka, Y., and Webster, R. G. 1994. Antigenicity of the N8 influenza A virus neuraminidase:existence of an epitope at the subunit interface of the neuraminidase. *J. Virol.* 68: 1790-1796.
15. Saito, T., Taylor, G., and Webster, R. G. 1995. Steps in maturation of influenza A virus neuraminidase. *J. Virol.* 69: 5011-5017.
16. Ward, A. C. 1995. Changes in the neuraminidase of neurovirulent influenza virus strains. *Virus Genes* 10: 253-260.