

Biologic Importance of Neuraminidase Stalk Length in Influenza A Virus

MARIA R. CASTRUCCI† AND YOSHIHIRO KAWAOKA*

*Department of Virology and Molecular Biology, St. Jude Children's Research Hospital,
332 North Lauderdale, P.O. Box 318, Memphis, Tennessee 38101-0318*

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To investigate the biologic importance of the neuraminidase (NA) stalk of influenza A virus, we generated mutant viruses of A/WSN/33 (H1N1) with stalks of various lengths (0 to 52 amino acids), by using the recently developed reverse genetics system. These mutant viruses, including one that lacked the entire stalk, replicated in tissue culture to the level of the parent virus, whose NA stalk contains 24 amino acid residues. In eggs, however, the length of the stalk was correlated with the efficiency of virus replication: the longer the stalk, the better the replication. This finding indicates that the length of the NA stalk affects the host range of influenza A viruses. The NA stalkless mutant was highly attenuated in mice; none of the animals died even after intranasal inoculation of 10^6 PFU of the virus (the dose of the parent virus required to kill 50% of mice was $10^{2.5}$ PFU). Moreover, the stalkless mutant replicated only in the respiratory organs, whereas the parent virus caused systemic infection in mice. Thus, attenuation of the virus with the deletion of the entire NA stalk raises the possibility of its use as live vaccines.

The neuraminidase (NA) of influenza A viruses, a class II glycoprotein with the amino terminus inside the cell and the carboxyl terminus outside (see reviews by Air and Laver [2] and Colman [10]), is one of two major glycoproteins on the virus surface. The NA activity helps the virus gain access to cells by catalyzing the cleavage of the α -ketosidic linkage between the terminal sialic acid and the adjacent sugar residue in mucus; it also facilitates virus release from the host cell, preventing progeny viruses from self-aggregating (25). The NA protein is a tetramer with a box-shaped head (100 by 100 by 60 Å [1 Å = 0.1 nm]) comprising four coplanar and roughly spherical subunits, as well as centrally attached stalk with a hydrophobic region by which the stalk is embedded in the viral membrane. The three-dimensional structure of the NA head, cleaved from the stalk with proteases, has been elucidated (11, 35).

The antigenic structure and the structure-function relationship of the NA head have been extensively studied (see reviews by Air et al. [3] and Webster et al. [37]), but information on the stalk region is limited. The number and sequence of the amino acid residues in the stalk region vary considerably among different viruses, even within the same subtypes (10). Since NA heads, solubilized from the virus with proteases, are antigenically indistinguishable from the intact NA (21), variation in the stalk region does not appear to be a consequence of host immune pressure. Although highly variable, the stalk regions of the different subtypes share some structural features, including at least one Cys residue and a potential glycosylation site. A spontaneous NA mutant of influenza A virus, characterized by an 18-amino-acid deletion in the stalk, lacks enzyme activity with a large substrate (fetuin) but not with a small substrate (sialyllactose) and cannot release viruses bound to erythrocytes (14). However, the effect of this stalk deletion *in vivo* remains unclear.

Recently, several groups established a reverse genetics system to rescue cloned genes in negative-strand RNA viruses (9, 23, 27, 29). Palese and colleagues have reported rescue of the chloramphenicol acetyltransferase gene (23) or influenza virus genes (15-17) derived from cDNA into influenza A viruses. Here we report the application of reverse genetics in assessing the biologic importance of the NA stalk of influenza A viruses.

MATERIALS AND METHODS

Viruses and cells. Influenza virus A/WSN/33 (H1N1) (WSN) was obtained from Thomas Chambers (University of Kentucky, Lexington, Ky.). Masahiro Ueda (The Institute of Public Health, Tokyo, Japan) provided a helper virus [WSN-HK (H1N2)] that contained the NA gene from A/Hong Kong/1/68 (H3N2) and all other genes from A/WSN/33 (H1N1) (34), which was used to rescue the WSN NA gene. The Madin-Darby bovine kidney (MDBK) cell line was cultured in Eagle's minimal essential medium containing 10% fetal calf serum. Madin-Darby canine kidney (MDCK) cells were cultured under the same conditions as MDBK cells, except that 5% calf serum was used.

Reverse genetics. A plasmid (pWNA15) containing the WSN NA gene was constructed as described by Huddleston and Brownlee (19). A second plasmid [pT3WSN(NA15)], containing the WSN NA gene flanked by the *Ksp632I* site and T3 RNA polymerase promoter sequence, was made by cloning the product of the polymerase chain reaction (30) made with pWNA15 as a template and with 5'-ATCGATG AATTCTCTTCGAGCGAAAGCAGGAGTTT-3' and 5'-GAGGACAAGCTTATTAACCCTCACTAAAAGTAGAAAC AAGGAGTTTTTTG-3' as primers (15). pT3WSN(NA15) contains a T3 RNA polymerase promoter upstream and a *Ksp632I* site downstream of the NA gene, so that viral sense RNA transcripts are generated when digested with *Ksp632I*, filled in with Klenow fragment, and transcribed with T3 RNA polymerase (7, 15).

Other plasmids used to rescue NA stalk mutants (Fig. 1) were constructed from pT3WSN(NA15) by either deleting or

* Corresponding author.

† Present address: Dipartimento di Virologia, Istituto Superiore di Sanità, 00161 Rome, Italy.

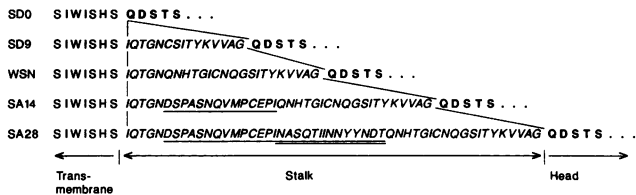


FIG. 1. Amino acid sequences of the NA stalk mutants from residues 31 to 66 of WSN NA. The stalk region is italicized, the transmembrane region is presented in roman type, and the head region is shown in boldface. A portion of the A/Tokyo/67 (H2N2) stalk is underlined and that of the A/Tern/Australia/G70C/75 (H1N9) stalk is double underlined.

inserting nucleotides in the region encoding the NA stalk, by using oligonucleotide-directed mutagenesis (20).

Nucleoprotein (NP) and polymerase (P) proteins were purified from X31 influenza A virus (H3N2) by glycerol and glycerol-caesium chloride (CsCl) gradients, as previously described (28).

An artificial NA ribonucleoprotein complex was prepared by transcription of pT3WSN(NA15) or its derivatives with T3 RNA polymerase in the presence of the NP and P, after these plasmids were digested with *Ksp632I* and filled in with Klenow fragment, as previously described (15, 16). The NA RNP complex was then transfected into 70 to 90% confluent MDBK cells infected 1 h before transfection with WSN-HK at a multiplicity of infection of 1 (23). Eighteen hours after transfection, transfectants in the supernatant were plaque on MDBK cells (34) and then plaque purified five times in MDBK cells.

Immunologic methods. Hemagglutinin (HA) titration and hemagglutination inhibition tests were performed with receptor-destroying enzyme-treated antisera in microtiter plates (26). NA and NA inhibition tests, radioimmunoprecipitations, and enzyme-linked immunosorbent assays were all performed as described in earlier publications (5, 36). Goat anti-N1 NA serum and anti-WSN NA monoclonal antibody were obtained from Robert G. Webster (St. Jude Children's Research Hospital, Memphis, Tenn.).

Virus elution assay. The ability of the NA to elute virus bound on erythrocytes was assessed as follows. Fifty microliters of twofold dilutions of virus containing the HA titers of 1:128 was incubated with 50 μ l of 0.5% chicken erythrocytes in microtiter plates at 4°C for 1 h. The microtiter plates were then stored at 37°C, and the reduction of HA titers was recorded periodically for 18 h. Calcium saline (6.8 mM CaCl_2 -154 mM NaCl in 20 mM borate buffer, pH 7.2) was used as a diluent.

Studies in mice. Six-week-old female BALB/c mice, anesthetized with methoxyflurane, were infected intranasally with 50 μ l of virus at different dilutions (three mice per dilution) and observed for 21 days to determine the minimal dose lethal to 50% of mice (MLD_{50}). To determine virus titers in the organs of infected mice, we infected three mice intranasally with virus. Virus titers in organs were determined 3 days after infection with MDCK cells (38).

RESULTS

Preparation of NA stalk mutant viruses. The NA stalk has been assigned to different amino acid regions, depending upon the investigators (11, 13, 18). We chose the assignment of Harley et al. (18), in which the stalk region corresponds to

residues 38 to 61 (WSN numbering) (Fig. 1), because it is based on the alignment of the NA amino acid sequences of six different subtypes. In creating NA stalk mutants from the A/WSN/33 virus, we were aware that the stalks of different virus subtypes may share structural features despite the variability of their amino acid sequences; thus, insertion of unrelated amino acid sequences into the stalk region could adversely perturb its structure. We, therefore, created insertion mutants by adding amino acid residues of the NA stalk of the N2 subtype [A/Tokyo/67 (H2N2)] (SA14; 14-amino-acid insertion) or the N2 (A/Tokyo/67) and N9 [A/Tern/Australia/G70C/75 (H1N9)] subtypes (SA28; insertion of 14 amino acids from N2 and 14 from N9). For a deletion mutant, SD9, we removed nine amino acids from the region not containing the conserved features (Cys and glycosylation site). We generated another deletion mutant, SD0, that lacked the entire stalk region (Fig. 1).

After plasmids containing the mutant NA genes were prepared by oligonucleotide-directed mutagenesis (20), we attempted to rescue them in influenza A viruses. The rescue system relies on the fact that the NA gene of WSN (H1N1) virus is responsible for plaque formation of the virus in MDBK cells (34). Hence, the WSN-HK reassortant virus, which contains only the NA gene from A/Hong Kong/1/68 (H3N2) virus and the rest of the genes from WSN virus, can be used as a helper virus to rescue the WSN NA gene. Because the WSN-HK virus does not plaque on MDBK cells, the rescue of the WSN NA gene allows the virus to plaque in these cells. Eighteen hours after transfection of artificial ribonucleoprotein complex made with the parental WSN NA [WSN(NA15)] or its mutant RNA transcribed *in vitro*, we tested for the presence of virus containing the rescued NA gene in the cell supernatants. We found more than 10 PFU of virus in the supernatant for WSN(NA15) and its mutants. Surprisingly, even the mutant NA gene with the deletion of the entire stalk was rescued. By contrast, no plaques were observed when the DNA for transcription of the NA RNA was omitted from the reaction. Each virus was plaque purified five times in MDBK cells, and working stocks were made. Direct RNA sequencing of WSN(NA15) and its mutant NA genes verified their identity (Fig. 2).

These results indicate that one can manipulate (insert and delete) the NA stalk region of influenza A viruses and that the NA stalk is not essential for replication of the virus in tissue culture.

Characterization of NA stalk mutants. To assess the biologic importance of the NA stalk, we first examined replication of the NA stalk mutants in tissue culture. The titers of the mutant viruses were similar to that of WSN(NA15) virus, ranging between 10^7 and $10^{7.3}$ PFU/ml (Table 1). One exception was the SA28 virus, whose titer ($10^{8.1}$ PFU/ml) was approximately fivefold higher than that of WSN(NA15). The plaque sizes of the mutants were also similar, except that the SD0 virus produced smaller plaques in MDBK cells (approximately 1.0 mm in diameter compared with 2.5 mm for the others).

To determine if the modified NAs could be incorporated into virions, we grew the viruses in the presence of [^3H]mannose, partially purified and disrupted them with lysis buffer (36), and then immunoprecipitated them with goat anti-N1 serum. All of the NAs were detected on polyacrylamide gels (data not shown), indicating their incorporation into virions. The mobility of the NAs on the gels correspond to their stalk lengths, except in the case of the SA28 mutant, which repeatedly migrated faster than other NAs for unknown reasons (data not shown).

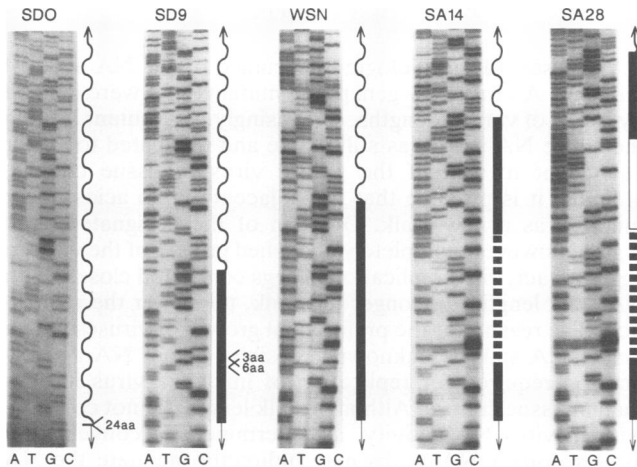


FIG. 2. Autoradiographs of direct RNA sequencing of the WSN(NA15) and its NA stalk mutants. Sequences were determined by reverse transcription with a ³²P-labeled primer (corresponding to nucleotide residues 71 to 92) and purified viral RNA (1, 6). The symbols indicate transmembrane (→), WSN stalk (■), A/Tokyo/67 (H2N2) stalk (■ ■ ■), A/Tern/Australia/G70C/75 (H11N9) stalk (□), and head (↔) regions. The SD0 NA contains 24-amino-acid deletions and SD9 contains 6- and 3-amino-acid deletions at the indicated positions.

Previous studies showed that the NA stalk length affects the elution of viruses bound to erythrocytes (14). We compared the ability of the mutant NAs to release erythrocyte-bound virions, first by performing hemagglutination at 4°C followed by incubation of the HA microtiter plates at 37°C. If the NA is active, the bound virions will be released due to the viral NA. As a control, we used the X7 strain and its spontaneous NA stalk deletion mutant, Stubby, which is defective in eluting virus from erythrocytes (14). The parent X7 strain showed a 16-fold reduction in HA titer after 4 h of incubation at 37°C, whereas the Stubby strain showed only a twofold reduction, verifying the assay system (Fig. 3). Comparison of the elution properties of the NA stalk mutants revealed good correlation between stalk length and the ability of the NA to release virions from erythrocytes. SA28 and SA14 viruses were released completely from erythrocytes by 1.5 h of incubation at 37°C, whereas longer times were required for release of SD9 and WSN(NA15). SD0 was released only slightly, even after 18 h of incubation. Thus, the longer the NA stalk length, the more active the NA is in eluting virions from erythrocytes.

To determine whether the elution activity of the NA stalk mutants correlates with their enzyme activity, we compared

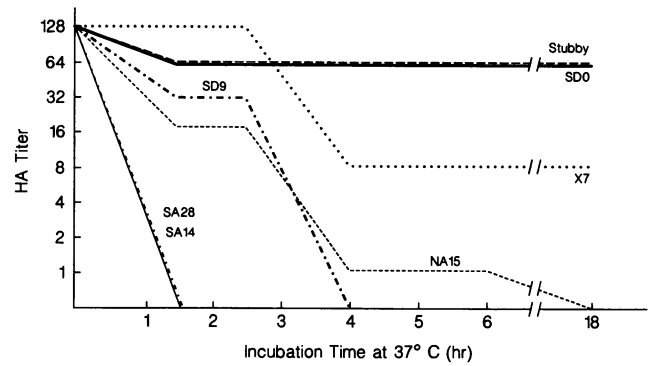


FIG. 3. Virus elution from erythrocytes. Twofold dilutions of virus containing the HA titers of 1:128 were incubated with equal volumes of 0.5% chicken erythrocytes in microtiter plates at 4°C for 1 h. The microtiter plates were then stored at 37°C, and the reduction of HA titers was recorded periodically for 18 h.

the NA activity of the viruses by using a large-molecular-weight substrate (fetuin) and a small-molecular-weight substrate (sialyllactose). All NAs preferentially cleaved *N*-acetylneuraminic acid- α 2,3 galactose linkages over *N*-acetylneuraminic acid- α 2,6 galactose linkages; however, there was no apparent relationship between NA stalk length and enzyme activity, regardless of the substrate used (data not shown).

To relate NA stalk length to the host range of the virus, we determined the 50% egg infectious dose of each stalk mutant. The results (Table 1) showed that viruses with longer stalks replicated better in eggs; the stalkless mutant, SD0, did not grow at all. These results suggest that NA stalk length affects the host range of the virus.

Virulence in mice. In mice, the minimum dose lethal to 50% of the mice of each of the NA stalk mutants ($10^{3.2}$ to $10^{3.7}$ PFU) was moderately higher than that of WSN(NA15) ($10^{2.5}$ PFU), with the exception of SD0. There were no deaths among mice inoculated with doses of SD0 as high as 10^6 PFU. We next examined the dose of SD0, compared with WSN(NA15), required to infect 50% of mice. As shown in Table 2, both viruses replicated better in the lungs than in the nasal turbinates. Virus titers in these organs depended on the size of the virus inoculum. The 50% infectious dose for SD0 virus ($10^{1.84}$ PFU; calculated from the data presented in Table 2) was at least 70-fold higher than that for WSN(NA15) virus (<1 PFU).

The tissue distribution of the SD0 and WSN(NA15) viruses is reported in Table 3. In mice infected with WSN(NA15), we recovered the virus from a variety of

TABLE 1. Correlation between NA stalk length and virus replication in tissue culture and eggs

Virus	No. of amino acid residues in the NA stalk ^a	Titer in MDCK cells (log ₁₀ [PFU/ml])	Replication in eggs (log ₁₀ [EID ₅₀ ^b /ml])	PFU/EID ₅₀
Wild type [WSN(NA15)]	24	7.3	5.5	63
SD0	0	7.3	— ^c	—
SD9	15	7.0	4.5	350
SA14	38	7.0	7.0	1
SA28	52	8.1	8.5	0.4

^a Based on assignment of Harley et al. (18).
^b EID₅₀, 50% egg infective dose.
^c —, SD0 virus did not grow in eggs, even after inoculation of 10⁷ PFU of virus.

TABLE 2. Recovery of virus from the organs of mice infected with different doses of SD0 and WSN(NA15)^a

Virus and amt of virus inoculated into each mouse [PFU (log ₁₀)]	Infectivity titer (mean log ₁₀ [PFU/g] ± SD)	
	Lung	Nasal turbinate
WSN(NA15)		
0	5.22 ± 0.69	< ^b
1	6.84 ± 0.39	<
2	8.06 ± 0.16	<
3	8.70 ± 0.09	4.32 ± 0.49
4	8.95 ± 0.13	5.47 ± 0.14
SD0		
0	<	<
1	<	<
2	3.81 ± 0.63 ^c	<
3	4.44 ± 0.80	<
4	5.80 ± 0.36	<
5	5.90 ± 0.50	2.89 ± 1.06
6	6.61 ± 0.60	4.42 ± 1.26

^a Six-week-old female BALB/c mice were infected intranasally with 50 μl of virus at different dilutions (three mice per dilution). Three days after infection, tissue samples were collected, and a 10% tissue suspension was prepared and assayed for virus, as described by Webster et al. (38). Samples of virus isolated from each organ were identified in hemagglutination inhibition tests with specific antisera (26).

^b <, virus was not recovered from any of the mice (less than 10² PFU/g).

^c Virus was recovered from only two of the three mice infected.

organs, including the brain. In one animal, virus was recovered from all the organs examined, suggesting the presence of viremia. These data show that WSN strain causes systemic infection in mice. By contrast, in SD0-infected mice, virus was recovered only from the respiratory organs; moreover, virus titers in the organs were at least 100-fold lower than those of WSN(NA15)-infected mice. These findings indicate that the stalkless mutant SD0 is highly attenuated and has lost its neurovirulence.

TABLE 3. Tissue distribution of SD0 and WSN(NA15) viruses in mice^a

Organ	Infectivity titer (mean log ₁₀ [PFU/g] ± SD)	
	SD0	WSN(NA15)
Brain	< ^b	4.16 ± 0.67
Nasal turbinate	3.75 ± 0.69	8.53 ± 0.48
Trachea	2.83 ± 1.30	7.15 ± 0.09
Lung	6.74 ± 0.24	8.88 ± 0.05
Liver	<	2.30 ^c
Spleen	<	3.84 ± 0.28
Kidney	<	2.30 ^d
Jejunum	<	4.18 ± 0.85
Ileum	<	2.48 ^c
Colon	<	3.84 ± 0.28

^a Three mice were inoculated intranasally with 10⁶ PFU of virus, and tissue samples were collected 3 days after infection. To avoid contamination with virus from respiratory tissues, we collected the samples in the following order: brain, jejunum, ileum, colon, spleen, liver, kidney, nasal turbinate, trachea, and lung. A 10% tissue suspension was prepared, and virus was assayed as described by Webster et al. (38). Samples of virus isolated from each organ were identified in hemagglutination inhibition tests with specific antisera (26).

^b <, virus was not recovered from any of the mice (less than 10² PFU/g).

^c Virus was recovered from only one of three mice infected.

^d Virus was recovered from only two of three mice infected.

DISCUSSION

We assessed the biologic importance of the NA stalk of influenza A viruses by generating mutants that were defined by stalks of various lengths. Surprisingly, the mutant lacking the entire NA stalk was still viable and replicated to a titer equivalent to that of the parent virus in tissue culture, although it is possible that the adjacent amino acids might function as a new stalk. Deletion of the designated stalk region, however, completely abolished growth of the virus in eggs. In fact, virus replication in eggs correlated closely with NA stalk length: the longer the stalk, the better the replication. The reason for the preferential growth of viruses with a longer NA stalk is unknown. Possibly, higher NA activity may be required for replication of influenza virus in eggs than in tissue culture. Although stalk length did not correlate with *in vitro* NA activity, as determined by conventional assays, longer NA stalks may indirectly facilitate the NA function in natural settings, as demonstrated by results of the virus elution assay.

Why is the SD0 virus with complete deletion of the NA stalk highly attenuated in mice? Insertion of up to 28 amino acid residues into the NA stalk region did not affect virulence of the virus significantly. SD0 virus was inefficient in eluting the virus from erythrocytes compared with those with the longer NA stalk. Thus, the attenuated phenotype of SD0 may reflect the mutant's inability to detach virions from receptor analogs in extracellular glycoconjugates such as mucin, which traps the virus and prevents its attachment to cell surface receptors.

How does deletion of the stalk affect the ability of the NA to elute the virus from the erythrocyte surface? Els et al. (14) showed that a spontaneous mutant with an NA stalk deletion (leaving 18 amino acid residues) has deficient enzyme activity with a large substrate (fetuin) but not with a small substrate (sialyllactose) and does not release viruses efficiently from erythrocytes, a property those authors attribute to steric hindrance. By contrast, we did not observe any differences in the NA activity among the mutants with different substrates, although stalk length was correlated with ability to elute virus. This discrepancy may reflect differences in the level of steric hindrance of NA activity by the HA between the viruses, which depends on the ratio of HA to NA molecules on virions.

The stalkless SD0 virus was highly attenuated in mice, raising the possibility that it could be used as a live vaccine. Cold-adapted live vaccines, now in clinical trials (8), hold promise for use in the general population (31–33). The major concern with such vaccines is that the limited number of attenuating mutations (12) might permit generation of a revertant virus in the field, as illustrated by experience with polio vaccines (4). One way to avoid this hazard would be to introduce multiple attenuating mutations into the viral genes. Deletion of the entire NA stalk in the SD0 mutant makes the likelihood of the generation of revertant viruses remote. We recently showed that influenza A viruses can also be attenuated by inserting a foreign amino acid sequence into a viral protein (7). A similar result was achieved by altering the noncoding region of the influenza virus gene (24). Thus, the generation of influenza viruses with modified viral genes by reverse genetics could lead to the production of safe live influenza vaccines.

In this study, we showed that one can insert up to 28 amino acid residues into the NA stalk of influenza A virus, providing an avenue for insertion of foreign linear epitopes into this region of the molecule. Li et al. (22) also demon-

strated the incorporation of a foreign epitope into the HA molecules. Thus, these findings open the way for use of influenza virus as a vaccine vector to immunize against other unrelated infectious agents. Most investigators do not consider the NA stalk to be a highly immunogenic region. This perception may not be entirely valid, since antibodies to the NA molecule are usually measured by NA inhibition assays, which detect only those antibodies which inhibit the catalytic activity located on the top of the molecule. It is therefore possible that linear epitopes inserted into the NA stalk are still sufficiently immunogenic to confer protective immunity.

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