Role of the Cytoplasmic Tail Amino Acid Sequences of Newcastle Disease Virus Hemagglutinin-Neuraminidase Protein in Virion Incorporation, Cell Fusion, and Pathogenicity

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To determine the role of amino acid sequences of the hemagglutinin-neuraminidase (HN) cytoplasmic tail in Newcastle disease virus (NDV) replication and pathogenicity, we generated recombinant NDVs with a deletion or point mutation in the N-terminal cytoplasmic tail. The first 2-amino-acid deletion in the cytoplasmic tail did not affect the biological characteristics of NDV. However, a 4-amino-acid deletion and the substitution of alanine for serine at position 6 affected cell fusion, pathogenicity, and colocalization of the HN and M proteins of NDV, indicating that these residues of the HN cytoplasmic tail are critical for its specific incorporation into virions.

Newcastle disease virus (NDV) causes a highly contagious respiratory and neurologic disease in chickens, leading to severe economic losses in the poultry industry worldwide (1). NDV is a member of the family Paramyxoviridae and has a nonsegmented, negative-sense RNA genome consisting of six genes (3′-NP-P-M-F-HN-L-5′) (7). Infection of host cells by NDV is accomplished through the interaction of two surface glycoproteins, the fusion (F) and hemagglutinin-neuraminidase (HN) proteins. The F protein directs the membrane fusion between the viral and cellular membranes, while the HN protein mediates attachment to sialic acid, has neuraminidase activity, and plays a role in fusion promotion (4).

The HN protein of NDV is a type II transmembrane glycoprotein and possesses three spatially distinct domains: the ectodomain, transmembrane domain, and cytoplasmic tail. The globular ectodomain contains the sites for receptor binding and neuraminidase activity, and the transmembrane domain anchors to viral envelopes (8). The cytoplasmic tail domain contains 26 highly conserved amino acids whose functions are not well-known. In a plasmid-based expression system, truncation (23 amino acids) of the cytoplasmic tail caused improper orientation of the HN protein in the membrane insertion (13). In other paramyxoviruses, cytoplasmic tails of the HN proteins are known to play crucial roles in virus budding and assembly (10, 12). Our unsuccessful attempt to recover a recombinant NDV (rNDV) with complete deletion of the HN cytoplasmic tail did not affect their in vitro replication (Fig. 1B). Although the rBC/HNΔ2 and rBC/HNΔ4, respectively, indicated that only these 4 amino acids are dispensable in generating infectious virions. Since rNDV containing 6-amino-acid deletion of the HN cytoplasmic tail could not be recovered, we wanted to know the role of amino acids at positions 5 and 6 in NDV replicates. The serine residue at position 6 is a potential phosphorylation site. Therefore, to determine whether phosphorylation at this site is crucial for recovery of NDV, we additionally generated rNDVs with substitution of alanine and glutamic acid for serine (rBC/HNS6A and rBC/HNS6E, respectively) to confirm its crucial role in the recovery of rNDV.

In vitro replication of recovered viruses was determined by plaque assay in virus-infected DF-1 cells at a multiplicity of infection (MOI) of 0.01 (5). All mutant viruses and the parental virus, rBC, grew to similar titers, indicating that alteration of the HN cytoplasmic tails did not affect their in vitro replication (Fig. 1B). Although the rBC/HNΔ4 mutant had grown well up to 24 h postinfection, a reduction of the viral titer was detected thereafter with rapid and extensive induction of syncytia. Therefore, we determined fusion promotion activity of the mutant viruses by quantitating syncytia in virus-infected Vero cells at an MOI of 0.1 at 30 h postinfection (8) and confirmed increased fusion promotion activity of rBC/HNΔ4 followed by rBC/HNS6A compared to that of rBC (Fig. 1C). Similarly, enhanced fusion activity was observed in other cytoplasmic tail-truncated paramyxoviruses, such as simian virus 5 and measles virus (2, 9). It has been postulated that interaction of matrix (M) protein with the cytoplasmic tails of the glycoproteins involves in a fusion-refractory conformation at the early stage of viral maturation (2). Therefore, these altered HN cytoplasmic...
FIG. 1. Constructs of recombinant NDVs containing a deletion or point mutation in the N-terminal cytoplasmic tail of the HN protein and replication and fusion index of recovered viruses in infected cells. (A) Consecutively, 6 nt, 12 nt, or 18 nt of mRNA of the HN cytoplasmic tail in a full-length antigenomic cDNA of NDV was deleted. Deletions in the HN cytoplasmic tails are indicated by the large boldface dashes. In addition, serine at position 6 was substituted with alanine and glutamic acid was substituted by changing guanine to cytidine and adenosine, respectively. (B) In vitro replication of the mutant viruses was determined in virus-infected DF-1 cells at an MOI of 0.01. The viral titers were determined by plaque assay. (C) The fusion index was determined in virus-infected Vero cells at an MOI of 0.1. Cells were stained with hematoxylin-eosin, and the fusion index was calculated as a mean number of nuclei per cell. The assay was performed three times.
FIG. 2. Effect of alteration of the HN cytoplasmic tail on incorporation of the HN proteins into viral particles and their surface expression in DF-1 cells. (A) Surface expression of the NDV HN protein in DF-1 cells was analyzed by a fluorescence-activated cell sorter. At 24 h postinfection, DF-1 cells infected with each virus were stained with monoclonal antibody against the HN protein followed by anti-Alexa Fluor 488 conjugate. (B) Ultracentrifuge-purified viruses from infected allantoic fluid samples were separated by electrophoresis, and the gel was then stained with Coomassie brilliant blue. (C) Ratios of HN protein to M-protein levels from the parental virus and the HN cytoplasmic tail mutant viruses were quantified.
In general, the levels of the HN protein contents on the surfaces of virus-infected cells and in the virus particles were more affected by point mutation of serine than by truncation of the cytoplasmic tail. We analyzed surface expression of the HN protein on virus-infected DF-1 cells at an MOI of 0.1. At 24 h postinfection, the cells were labeled with a monoclonal antibody against the NDV HN protein followed by anti-Alexa Fluor 488 conjugate, fixed with 4% paraformaldehyde, and analyzed by a fluorescence-activated cell sorter (AriaII; BD Bioscience) with Flowjo program (Tree Star, Inc.) (Fig. 2A). The percentages of cells expressing the HN proteins were 89 (rBC/HNΔ2) and 84 (rBC/HNS6E), 50 (rBC/HNΔ4) and 51 (rBC/HNS6A), and 62 (rBC/HNΔ6E) and 58 (rBC/HNS6E) with Flowjo program (Tree Star, Inc.) (Fig. 2A). In contrast, the MDTs of rBC/HNΔ4 and rBC/HNS6A were 50 h and 51 h, respectively. Increased pathogenicity of these two mutants was also confirmed by an intracerebral pathogenicity index (ICPI) test in 1-day-old SPF chicks (1). The scale of the ICPI value in evaluating the virulence of NDV strains is from 0.00 (avirulent strains) to 2.00 (highly virulent NDV strains). The rBC/HNΔ4 virus had the highest ICPI value (1.61), followed by rBC/HNS6A (ICPI value of 1.58), among the parental and mutant viruses, probably due to their enhanced fusion promotion activity. In contrast, rBC/HNS6E had the lowest ICPI value (1.41), which would be associated with decreased HN protein content detected in the viral particles and virus-infected cells.

In our previous study, decreased HN protein contents in virus particles due to complete deletion of S’ untranslated regions of the HN gene also resulted in attenuation of the virus in chickens (14). Consistently, rBC/HNΔ2 showed biological characteristics and pathogenicity similar to those of the parental virus, suggesting that aspartic acid and arginine are indispensable for the HN cytoplasmic tail of NDV.

The M protein plays a major role in virus assembly through its interaction with envelope glycoproteins and with the membranes of infected cells (11). To gain insight into the function of the amino acid sequences of the HN cytoplasmic tail in virus assembly, colocalization of the HN and M proteins was determined by confocal microscopy (LSM 510; Zeiss). Detection of the M and HN proteins was facilitated by coexpressing M protein and each altered HN protein using the pCAGGS expression system in 293T cells. In particular, the open reading frame of the M gene had been fused with an influenza virus hemagglutinin epitope tag (7 amino acid residues) followed by a stop codon and cloned into pCAGGS. After 24 h of transfection, the cells were fixed, permeabilized, stained with a monoclonal antibody against the NDV HN protein followed by anti-Alexa Fluor 488 and anti-HA Alexa Fluor 594 conjugates, and analyzed by confocal microscopy. The M and wild-type HN proteins were distributed in the nucleus and cytoplasm and in the cytoplasm, respectively, leading to their colocalization in the cytoplasm of infected cells (Fig. 3A). In contrast, cytoplasmic tail-altered HN proteins (4-amino-acid deletion and substitution of alanine for serine) were dominantly found on the cell surface with their colocalization with the M protein, indicating reduction of specificity in membrane insertion of these HN proteins (Fig. 3B and C). Furthermore, no colocalization of the 6-amino-acid deletion of cytoplasmic tail-altered HN protein with the M protein was detected (Fig. 3D), suggesting that this alteration had affected incorporation of the HN protein into virus particles and consequently virus recovery. Other paramyxoviruses (e.g., simian virus 5 and human respiratory syncyial virus) also showed a loss of intracellular interaction between the M protein and glycoproteins containing cytoplasmic tail-truncated domains (3, 12).

In summary, we demonstrate that the cytoplasmic tail of HN plays a crucial role in the NDV life cycle. Our data suggest that the first 2 amino acids of the cytoplasmic tail are not absolutely required for NDV replication, but amino acids at positions 4

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**TABLE 1. Pathogenicity of the HN cytoplasmic tail mutant viruses in embryonated eggs and chicks**

<table>
<thead>
<tr>
<th>Virus</th>
<th>MDT (h)*</th>
<th>ICPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>rBC</td>
<td>58</td>
<td>1.49</td>
</tr>
<tr>
<td>rBC/HNA2</td>
<td>59</td>
<td>1.51</td>
</tr>
<tr>
<td>rBC/HNΔ4</td>
<td>50</td>
<td>1.61</td>
</tr>
<tr>
<td>rBC/HNS6A</td>
<td>51</td>
<td>1.58</td>
</tr>
<tr>
<td>rBC/HNΔ6E</td>
<td>62</td>
<td>1.41</td>
</tr>
</tbody>
</table>

* The mean time (in hours) for the minimum lethal dose of virus to kill all the inoculated embryos. NDV strains were classified by the following criteria: virulent strains take <60 h to kill embryos, intermediate virulent strains take 60 to 90 h to kill embryos, and avirulent strains take >90 h to kill embryos.

b Pathogenicity of NDV in 1-day-old SPF chicks was evaluated by the ICPI value: virulent strains had ICPI values of 1.5 to 2.0, intermediate virulent strains had ICPI values of 1.0 to 1.5, and avirulent strains had ICPI values of 0.0 to 0.5.
through 6 are critical for specific insertion of the HN protein into virion particles. Furthermore, our results indicate that the cytoplasmic tail of HN protein modulates the fusion activity of NDV. It will also be necessary to determine whether alteration of the HN cytoplasmic tail can affect interaction of the HN protein with the F protein.

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