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Biological Significance of the Second Receptor Binding Site of Newcastle Disease Virus Hemagglutinin-Neuraminidase Protein

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The paramyxovirus hemagglutinin-neuraminidase (HN) is a multifunctional protein responsible for attachment to receptors containing sialic acid, neuraminidase (NA) activity, and the promotion of membrane fusion, which is induced by the fusion protein. Analysis of the three-dimensional structure of Newcastle disease virus (NDV) HN protein revealed the presence of a large pocket, which mediates both receptor binding and NA activities. Recently, a second sialic acid binding site on HN was revealed by cocrystallization of the HN with a thiosialoside Neu5Ac-2-*S*-α(2,6)Gal1OMe, suggesting that NDV HN contains an additional sialic acid binding site. To evaluate the role of the second binding site on the life cycle of NDV, we rescued mutant viruses whose HNs were mutated at Arg516, a key residue that is involved in the second binding site. Loss of the second binding site on mutant HNs was confirmed by the hemagglutination inhibition test, which uses an inhibitor designed to block the NA active site. Characterization of the biological activities of HN showed that the mutation at Arg516 had no effect on NA activity. However, the fusion promotion activity of HN was substantially reduced by the mutation. Furthermore, the mutations at Arg516 slowed the growth rate of virus in tissue culture cells. These results suggest that the second binding site facilitates virus infection and growth by enhancing the fusion promotion activity of the HN.

The *Paramyxoviridae* family includes many important human and animal pathogens, such as parainfluenza viruses, measles virus, mumps virus, and Newcastle disease virus (NDV). Two surface glycoproteins, hemagglutinin-neuraminidase (HN) and fusion (F) proteins project from the surface of the virion and play major roles in the initiation of viral infection. HN is a multifunctional molecule. HN is responsible for the attachment of virus to receptors containing sialic acid and has neuraminidase (NA) activity that hydrolyzes sialic acid residues to prevent virus self-aggregation and enhances virus spread by analogy to the role of influenza virus NA (13). Further, HN is required for membrane fusion induced by the F protein (12, 13, 15).

Previously, we isolated and crystallized the NDV HN to understand the structure-function relationship of HN (16). From this structural analysis, the three-dimensional (3D) structure of the protein was revealed (9). HN contains a large binding pocket, which mediates both receptor binding and NA activities. A comparison of the HN structure, either crystallized alone or in complex with 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid, revealed substantial conformational changes in several loops within or near the large hydrophobic surface that is involved in the fusion promotion activity of HN (15). These results suggest that receptor binding at the NA active site triggers the conformational change on HN, which in turn activates the F protein to initiate membrane fusion. In addition to the large binding pocket, we recently found a second sialic acid binding site on HN by cocrystallizing the HN with a thiosialoside [Neu5Ac-2-S-α(2,6)Gal1OMe], suggesting that NDV HN has an additional sialic acid binding site (18). The new binding site is made up of hydrophobic residues from both monomers and involves interactions with sialic acid and not galactose. The side chain of Arg516 was involved in interaction with thiosialoside, suggesting that Arg516 is one of the residues forming the second receptor binding site on HN (18) (Fig. 1). The Arg516 residue is strongly conserved among various NDV isolates (14).

In this study, we first determined whether the NDV HN on the virion contains a second sialic acid binding site around Arg516. Taking advantage of the reverse genetic system of NDV (11), we rescued two mutant NDVs whose Arg516 had been mutated. The full-length NDV cDNA used to rescue the virus was synthesized from strain Beaudette C (11). Therefore, we replaced the HN gene of the full-length cDNA with that of the Kansas strain, which we used for the crystallization of HN. Before replacing the HN gene, we substituted Cys for Tyr123 of Kansas HN cDNA in pTF1 vector (16) so that it expressed a disulfide-linked dimer molecule. We performed this substitution, because most NDV strains have disulfide-linked dimer HNs that show enhanced fusion promotion activity. The HN gene was further mutated at residue Arg516 to Ala or Ser. The F gene of the full-length NDV cDNA was also mutated at the cleavage site from dibasic to monobasic form (RRQKR to GRQGR) so that the rescued viruses possess the nonvirulent

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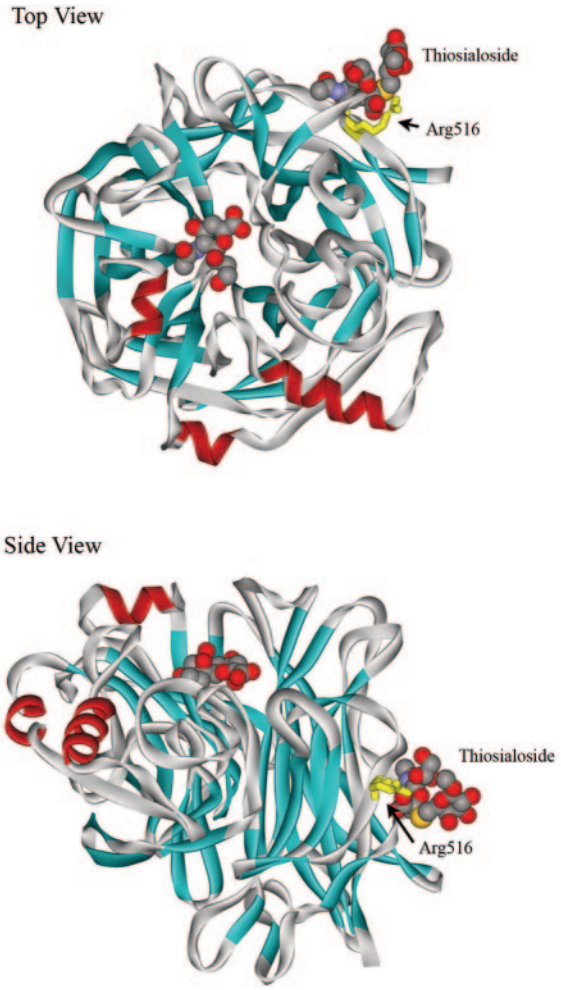

FIG. 1. 3D structure of HN showing the locations of Arg516 and thiosialoside bound at the second binding site. The side chain of Arg516 is indicated (yellow). The figure was generated with DS ViewerPro 5.0 (Accelrys, Inc.).

pathotype (17). These mutations were created using the QuikChange site-directed mutagenesis kit (Stratagene).

Mutant viruses were rescued by the reverse genetic system as described previously (4, 11). Briefly, 293T cells in 100-mm-diameter dishes were infected with UV-inactivated vTF7-3, which expresses T7 RNA polymerase. Using 64 μl of Lipofectamine (Invitrogen Life Technologies), we transfected the cells with 16 μg of mutated full-length genome NDV cDNA together with 8 μg of supporting plasmids pNP, 8 μg of pP, and 0.8 μg of pL (11). After 40 h of incubation at 37°C, cells were lysed by three cycles of freeze-thawing and then injected into embryonated eggs. In each case, mutant viruses were successfully recovered after a 48-h incubation. Mutant NDVs were cloned by plaque purification on LLC-MK_2 cells, and the stock viruses were prepared by injecting cloned virus into the embryonated eggs. To confirm the inserted mutations, viral RNAs were extracted from purified viruses and amplified by reverse transcription-PCR using the Titan One-Tube RT-PCR system (Roche). The sequencing results confirmed that the rescued mutant viruses contain mutations only at residue 516 as designed in the HN gene. Mutant viruses with Ala516 and Ser516 in their HN were designated as NDVHN516A and NDVHN516S, respectively.

First, we characterized the mutant HNs of the rescued viruses. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the purified viruses grown in embryonated eggs showed no significant difference in the virion HN contents (Fig. 2A). We next determined the NA activity of the mutant HNs. Purified wild-type NDV (NDVH-Nwt) or mutant NDVHN516A or NDVHN516S was incubated with *N*-acetylneuraminyl-lactose (Sigma) for 30 min at 37°C. NA activities were assayed by the colorimetric method of Aminoff (2), which detects *N*-acetylneuraminic acid released from the substrate. As shown in Fig. 2B, the NA activities of these mutant NDVs were almost equivalent to that of NDVH-Nwt, suggesting that mutations at residue 516 do not affect the NA activity of HN. These results correlate with the 3D structural data, which showed the additional sialic acid binding site located away from the NA catalytic site (Fig. 1).

The hemagglutination (HA) activity of the purified mutant viruses was measured by a standard HA test using 0.5% chicken red blood cells (cRBC), and the results were compared

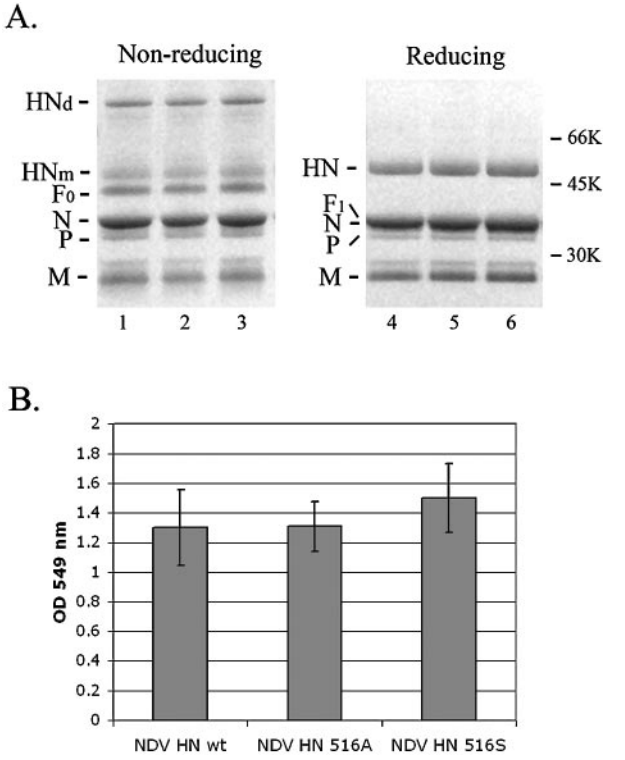

FIG. 2. Characterization of NDVHNwt, NDVHN516A, and NDVHN516S. (A) SDS-PAGE analysis of purified viruses. Seven micrograms of purified NDVHNwt (lanes 1 and 4), NDVHN516A (lanes 2 and 5), or NDVHN516S (lanes 3 and 6) was fractionated in SDS–10% polyacrylamide gels in reducing or nonreducing conditions. The positions of molecular size standards (in thousands [K]) are shown to the right of the gel. HNd, HN dimer; HNm, HN monomer. (B) NA activities of NDVHNwt, NDVHN516A, and NDVHN516S. One microgram of each purified virus was used to analyze viral NA activity by the colorimetric method. All values are the averages ± standard deviations (error bars) from four independent experiments. OD 549 nm, optical density at 549 nm.

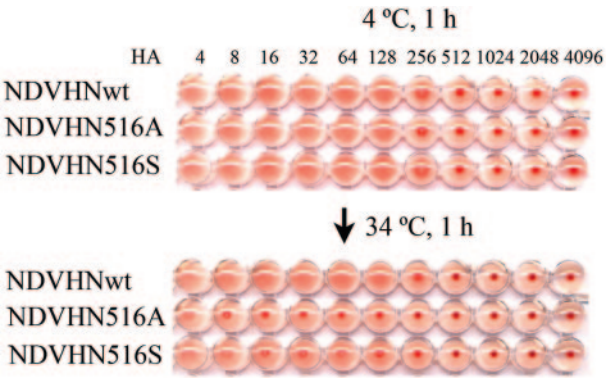

FIG. 3. Virus adsorption and elution from RBC. Attachment activity of wild-type and mutant NDV was analyzed by the HA assay. Each virus (10 μg/ml) was serially diluted (1:2 ratio) in phosphate-buffered saline (pH 7.2) and incubated with an equal volume of 0.5% cRBC in a 96-well plate for 1 h at 4°C. The plate was then shifted to 34°C and incubated for 1 h.

with that of NDVHNwt. Wild-type NDV provided 128 HA units at a concentration of 10 μg/ml. NDV516A or NDV516S showed the same HA titer at the same concentration (Fig. 3, top panel), indicating that mutation at residue 516 did not affect HA activity at 4°C. This was probably because the mutation at residue 516 has no effect on the receptor binding at the NA catalytic site as described above. The presence of the second binding site on wild-type HN but not on mutant HNs was determined by shifting the same HA plate to 34°C (Fig. 3, bottom panel). Mutant viruses were eluted from the cRBC after 1 h of incubation at 34°C, while NDVHNwt did not, suggesting that mutant HNs bound to sialic acid-containing receptor only through the NA catalytic site, which was cleaved by NA activity at elevated temperatures. In contrast, wild-type virus likely remained attached to the cRBC through the second HN binding site even at higher temperatures. This result suggests that wild-type HN on the virion binds to sialic acid-containing receptors through two separated sites, one at the NA catalytic site and the other around residue 516. Similar results were reported with NDV HN, which possessed mutations at the hydrophobic surface (8). Mutations at the predicted dimer interface of NDV HN significantly impaired their ability to adsorb to RBCs at 37°C but not at 4°C. Although mutated residues (Phe220, Ser222, and Leu224) were not located close to the Arg516, structural analysis of the HN-thiosialoside complex indicated that HN dimer interaction is required for the formation of the second binding site (18).

To further confirm that wild-type HN possesses a second receptor binding site around residue 516, we determined the HA activity of HN in the presence of NA inhibitor BCX-2798 (1). BCX-2798 was designed on the basis of the 3D structure of the catalytic site of NDV HN. The compound is highly effective in inhibiting NA activities in vitro and checks the growth of human parainfluenza viruses type 1, 2, and 3 in LLC-MK_2 cells (1). If wild-type HN possesses two receptor binding sites, one at the NA catalytic site and the other around residue 516, BCX-2798 will not inhibit the HA activity of wild-type HN because it blocks only the NA catalytic site. To test this possibility, BCX-2798 was serially diluted and incubated with eight HA units of NDVHNwt, NDVHN516A, or NDVHN516S. After 30-min incubation at room temperature, cRBC (0.5% [vol/vol]) were added and incubated at 4°C for 45 min. As shown in Fig. 4, BCX-2798 did not inhibit the HA activity of NDVHNwt even at 5,000 nM. In sharp contrast, the HA activity of NDVHN516A and NDVHN516S was almost completely inhibited by 20 and 78 nM BCX-2798, respectively. These results together with our structural data indicate that NDV HN has a second sialic acid binding site on the protein and that residue 516 is involved in the formation of the site.

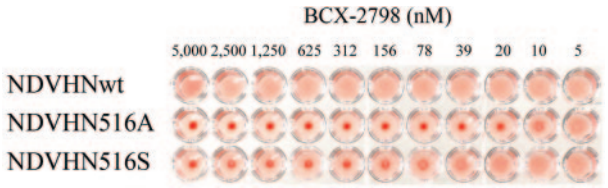

FIG. 4. HA inhibition of wild-type and mutant HNs by BCX-2798. Purified wild-type (NDVHNwt) or mutant NDVs (NDVHN516A and NDVHN516S) were incubated with serially diluted BCX-2798 before the addition of RBC.

Next we evaluated the role of the second binding site on the biological activity of HN using mutant HNs expressed in cultured cells. HN proteins were expressed in HeLa $T4^+$ cells using a vaccinia virus T7 transient-expression system (10). We characterized their NA and receptor binding activities and their cell surface expression for comparison with those of wild-type HN. Both mutant HNs were expressed at levels similar to that of wild-type HN (Table 1). NA activities of the expressed mutant HNs were almost equivalent to that of the wild type as expected from the results of characterization of rescued viruses (Fig. 2B). Receptor binding activity of the expressed mutant HNs was determined by a hemadsorption (HAD) assay with guinea pig red blood cells at 4°C. Mutations at R516 resulted in a slight reduction in their HAD activity, possibly due to the loss of the second binding site (Table 1).

The fusion promotion activity of these HNs was determined by coexpressing each HN together with NDV F in HeLa $T4^+$ cells. Fusion activity was scored by syncytium formation examined with a light microscope as described previously (5, 7).

TABLE 1. Biological activities of mutant HNs

HN	Expression[a]	HAD activity[b]	NA activity[b]	Fusion[c]
Wild-type	1.00	1.00	1.00	1.00
516A	1.07 ± 0.10	0.86 ± 0.04	1.03 ± 0.06	0.40 ± 0.03
516S	1.10 ± 0.25	0.79 ± 0.13	1.05 ± 0.05	0.48 ± 0.05

[a] Cell surface expression levels of mutant HN proteins relative to the level of wild-type HN. The expression of HN proteins was quantitated by a cell surface enzyme-linked immunosorbent assay using a cocktail of HN-specific monoclonal antibody (N1, N3, N6, and N7) (15). The values are averages ± standard deviations from three independent experiments.

[b] HAD and NA activities of mutant HNs are expressed as normalized values relative to the amount of HN expressed at the cell surface. Each value is relative to the activity of wild-type HN. All values are averages ± standard deviations from three independent experiments.

[c] Fusion promotion activities of mutant HNs are expressed relative to the activity of wild-type HN. Cell fusion was calculated as the ratio of the total number of nuclei in multinuclear cells to the total number of nuclei in the field. Five randomly chosen fields in which about 1,000 nuclei were counted were used in each experiment (5). The values are averages ± standard deviations from three independent experiments.

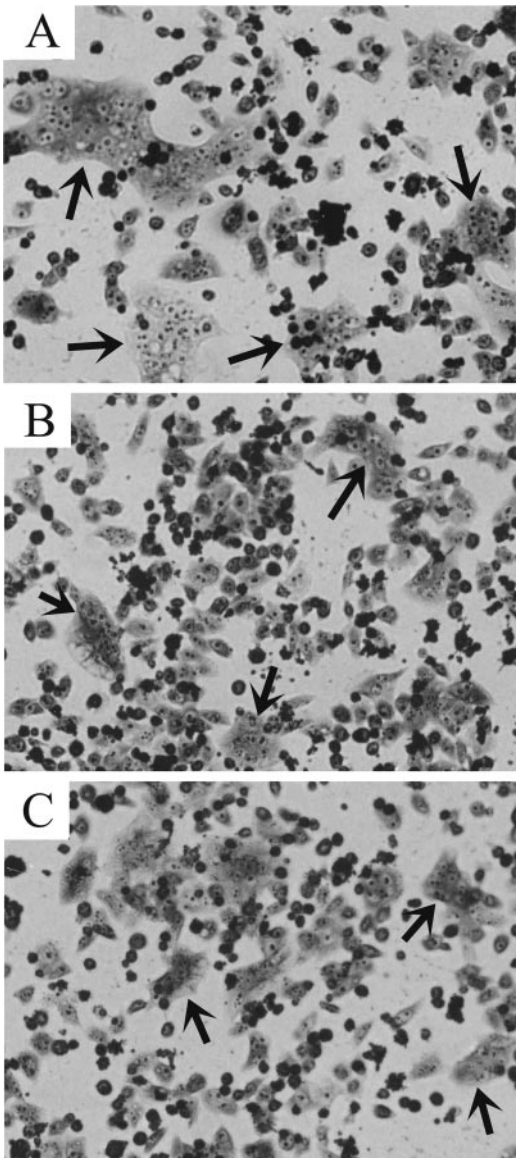

FIG. 5. Syncytium formation by HN and F proteins expressed from cDNAs. NDV F was expressed in HeLa $T4^+$ cells together with (A) NDVHNwt, (B) NDVHN516A, or (C) NDVHN516S by vaccinia virus-T7 RNA polymerase expression system (5, 10). The cells were fixed with 3% paraformaldehyde and stained with toluidine blue. Syncytia are indicated by the arrows.

Coexpression of the wild-type HN and F fused 25% of the cells. In contrast, expression of HN516A or HN516S with NDV F resulted in syncytium formation in only 10 and 12% of the cells, respectively (Fig. 5 and Table 1). This difference is not due to the level of expression of HN because almost equivalent amounts of HN were expressed at the cell surface (Table 1). These results indicate that wild-type HN that has an additional sialic acid binding site promotes membrane fusion more efficiently than mutant HNs. These results suggest that receptor binding through the second binding site plays a role in the membrane fusion induced by HN and F.

How does the second receptor binding site contribute to membrane fusion? The results of our previous structural (9) and biochemical (15) analyses suggest that the binding of a sialic acid receptor to the NA catalytic site induces a conformational change on the hydrophobic surface of HN, which triggers the membrane fusion induced by F protein. This hypothetical model proposes that the structural change triggered by receptor binding induces dissociation between HN and F, which results in the hydrophobic fusion peptide of the F protein being inserted into the target cellular membrane (12, 15). Further conformational changes of the F protein merge the cellular and viral membranes by forming a coiled-coil structure between the two heptad repeat regions near the fusion peptide and transmembrane domain of the F protein (3, 6). Apparently, viral membrane must be in close proximity to the target membrane during the whole process for efficient membrane fusion. Therefore, a possible role of the second binding site may be to maintain the close proximity of the target membrane and virus during the fusion process. Receptor binding through the NA active site is essential for the fusion promotion activity of HN because it induces a series of conformational changes on HN, which triggers fusion by F protein. However, hydrolysis of the sialosides by NA will result in the separation of the two membranes. The attachment of HN protein to the cellular receptor through the second binding site will hold the target membrane in close proximity to the virus throughout the process, which may contribute to the efficient membrane fusion mediated by HN and F proteins.

Finally, we evaluated the role of the second binding site on viral growth in tissue culture cells. Duplicate wells of LLC-MK_2 cells in six-well plates were infected with wild-type NDV, NDVHN516A, or NDVHN516S at a multiplicity of infection of 0.01 for 1 h at room temperature. After the cells were washed three times with phosphate-buffered saline (pH 7.2), the cells were cultured at 37°C in minimal essential medium supplemented with 0.15% bovine serum albumin and 2 μg of trypsin per ml. At the indicated time points, 200-μl aliquots of medium were taken and replaced with equal volumes of fresh medium. Titration of each virus sample indicated that growth of both mutants, especially NDVHN516S, was slower than that of wild-type NDV (Fig. 6). Both mutant viruses, however, reached the same titer as wild-type NDV 72 h after infection.

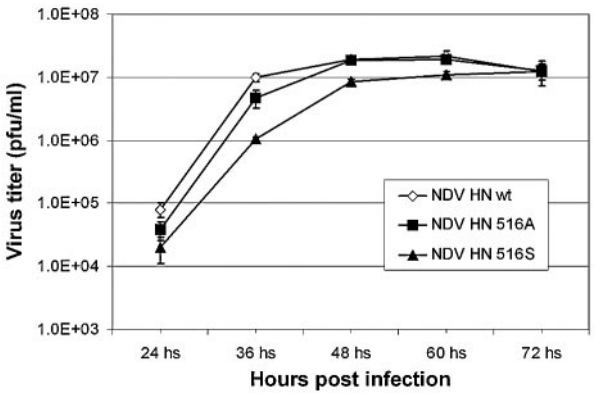

FIG. 6. Growth kinetics of wild-type and mutant NDVs in LLC-MK_2 cells. Virus titers of the wild type (NDVHNwt) and two mutants (NDVHN516A and NDVHN516S) were measured at the times indicated on the *x* axis by plaque assay with LLC-MK_2 cells. 1.0E+03, 1×10^3.

These results indicate that the loss of the second binding site slows the multistep growth of the virus, possibly due to the less efficient fusion promotion activity of HN.

In this study, we confirmed the presence of an additional receptor binding site on NDV HN using mutant viruses rescued by the reverse genetic method. Our data for mutant viruses indicates that Arg516 is involved in the formation of the second binding site as suggested by our structural data (18). Mutations at Arg516 did not significantly affect the NA activity or the HA activity at low temperatures. However, the mutation did reduce the fusion promotion activity of the HN protein. Mutant viruses that lack the second site grow to a titer similar to that of wild-type virus in cultured cells. This indicates that receptor binding through the second site is not essential for viral infection. However, the kinetic study of virus growth suggests that the second binding site does enhance the efficiency of multistep growth in cells (Fig. 6). Receptor binding through the second site probably contributes to efficient viral infection by enhancing the fusion promotion activity of the HN protein.

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