Original Article

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Roles of the highly conserved amino acids in the globular head and stalk region of the Newcastle disease virus HN protein in the membrane fusion process

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Summary **Newcastle disease virus (NDV), an avain paramyxovirus, has been assigned to the genus *Avulavirus* within the family *Paramyxoviridae*. It causes Newcastle disease (ND) that is a highly contagious and fatal viral disease affecting poultry and most species of birds. The hemagglutinin-neuraminidase (HN) protein of NDV has multiple functions including mediating hemadsorption (HAD), neuraminidase (NA), and fusion promotion activities affecting the process of viral attachment, entry, replication and dissemination. Fusion ability of the NDV was highly correlated to its virulence. Mutations in the HN globular head and headless HN of NDV were constructed to determinate the impact of highly conserved amino acids in the globular head of paramyxovirus HN proteins and the roles of the stalk region of HN in the fusion process. It was found that the interaction between F and HN mutants E401A, G402A, G468A, V469A, Y526A, and T527A was equal to that in F and wt HN. The mutations of G402A, G468A, V469A, and T527A had various effects on cell fusion promotion, receptor binding ability, and NA activity, but the membrane merging rate was comparable to wt HN. The elimination of hemadsorption ability and NA activity of E401A and Y526A resulted in the loss of the fusion promotion function of HN. The conclusion was that receptor binding and NA had a common active site and E401 and Y526 amino acids were essential for virus attachment, entry, and dissemination. In addition, G468A mutation made different contributions to HAD and NA, which indicated that G468 was one of the potential key amino acids in switching the two functions between receptor binding and sialic acid destruction of HN. It was also proven that the headless HN of NDV could promote the fusion event mediated by F. Thus, it revealed a novel mechanism in F activation of NDV.**

Keywords: NDV, HN protein, Fusion promotion, HN-F interaction

1. Introduction

The Paramyxovirus family has many pathogenic members in humans including measles virus, mumps virus, respiratory syncytial virus (RSV), human meta pneumovirus (hMPV), and human parainfluenza virus 1-5 (hPIV3), while others are zoonotic members such as Newcastle disease virus (NDV), Nipah virus, Hendra virus, Sendai virus and avian pneumovirus (APV).

HN proteins and F proteins co-exist on the surface of most paramyxoviruses. HN proteins are responsible for virus attachment, host-cell membrane fusion promotion, and virus dissemination. HN proteins are composed of four sections, the globular head, stalk, transmembrane region and cytotail, and N-terminus inserts into the membrane (*1,3,7*). It has multiple functions including receptor binding, neuraminidase activity, and fusion promotion. Hemagglutinin-

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neuraminidase is involved in attachment to sialic acid receptors and removal of sialic acids from infected cells. But the details of modulation between these two processes are obscure. The single F protein can not activate the fusion process in NDV. Homologous F and HN proteins of NDV are needed for the fusion process with few exceptions in most paramyxoviruses. In Nipah and Hendra viruses, attachment proteins can be used interchangeably, which suggests slightly different F and HN interaction mechanisms among these paramyxoviruses (*3*). However, no final conclusion has yet been reached on the pattern of interaction between HN and F proteins (*4,11,12,14*).

When NDV infects the host cells, the attachment protein first binds to the sialic acid on the cell surface, and then triggers the fusion (*4,5*). It has been reported that behavior of the receptor-binding promotes dramatic conformational changes of the F protein, which facilitate the membrane fusion process. There are two models of the fusion of paramyxoviruses (*2*). In the first, the HN tetramer is not tightly associated with the F protein trimer on the virus surface. Following the receptor-binding, F protein conformational changes are promoted and convert it to a pre-hairpin and then to trimer-of-hairpins formation. In the dissociation mode, some substantial evidence indicates that there are physical associations between the HN protein and F protein. In the Nipah virus and measles virus, if the interaction between the receptor-binding and the fusion protein is too tight, fusion is blocked (*12,19-22*). However, the mutations in the NDV HN weaken its association with F protein and inhibited the fusion event (*29*). The interaction of the HN proteins and F proteins varies in different stages of the paramyxoviruses membrane fusion and the exact manner remains to be determined. In addition, the conserved amino acids in the HN stalk region impact its NA activity (*23*).

In this study, highly conserved amino acids in the NDV HN globular domain were selected to determinate the variations of biological function in HN. The 401-403, 468-470, and 526-528 amino acids are extensively homologous in many paramyoxviruses (Figure 1A), which suggests they may play important roles in the virus life cycle. Site-directed mutations were constructed and the receptor-binding ability, neuraminidase activity, and cell fusion promotion capacity were detected. A headless mutation of NDV HN was also constructed to determinate its F promotion ability.

2. Materials and Methods

2.1. *Cells and viruses*

BHK-21 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Thermo Scientific, San Jose, CA, USA) supplemented with 1% glutamine, 1% penicilin-streptomycin (Invitrogen, California, USA), and 10% fetal calf setum (Gibco, California, USA). Wild-type (wt) vaccinia virus was produced in BHK-21 cells and used to quantify cell fusion. Recombinant vaccinia virus vTF7-3 was used to provide T7 RNA polymerase in the vaccinia-T7 RNA polymerase expression system (*25*). Recombinant vaccinia virus vTF7-3 was also maintained in BHK-21 cells.

2.2. *Recombinant Plasmid Vectors and the Transient Expression System*

NDV HN and F genes were inserted into pBluescript(+) (pBSK+) at the BamHI site, as previously described (*26*). The stalk region of the NDV HN gene (HNs) was amplified by PCR and inserted into the pEGFP-N3 vector. Wt or mutated HN proteins were co-expressed in BHK-21 cells with the F protein by the vaccinia-T7 RNA polymerase expression system. All experiments were performed in 35-mm plates seeded a day earlier with the same amount of cells.

2.3. *Site-directed mutagenesis*

Oligonucleotide primers (Sangon Biotech Co. Ltd., Shanghai, China) complementary with appropriate sequence of the NDV HN gene were designed to mutate the amino acids in the conserved domains of the NDV HN globular head. Overlapping PCR was used to product each pair of the recombinant plasmids. Two products with a short homologous sequence were co-transformed into *Escherichia coli* TG1 cells, and they recombined to form a complete plasmid. All mutants were sequenced to verify the proper mutation.

2.4. *Expression eEfficiency of HN glycoprotein on the cell surface*

The expression levels of mutant HN proteins on the cell surface were assayed with Fluorescence-activated Cell Sorter (FACS) (Roche, Indianapolis, IN, USA) analysis with a mixture of two mouse MAbs (Chemicon International, Inc., Temecula, California, USA) specific for the NDV HN protein, as previously described (*28*). The secondary antibody was rabbit anti-mouse IgG (ZSGB-BIO, Beijing, China). Cell surface expression efficiency was quantified as the mean fluorescence intensity per cell. Medium fluorescence intensities were normalized to the medium fluorescence intensities of NDV wt HN. Cells transfected with only the vector and wt HN genes co-expressed with the F gene were also incubated as negative and positive controls, respectively.

2.5. *Indirect immunofluorescence assay (IFA)*

IFA was used to detect whether the R403A, Y470A, and T528A mutants were intracellularly expressed

(Figure 2A). BHK-21 cells were plated on 35-mm plates containing a cover glass and transfected as previously described (*33*). The primary antibody and the secondary antibody were the same as in the FACS assay. The monolayers were fixed with acetone 24 h post co-expression F and mutated HN. Photographs of the cells were immediately taken by fluorescence microscope (OLYMPUS, Japan).

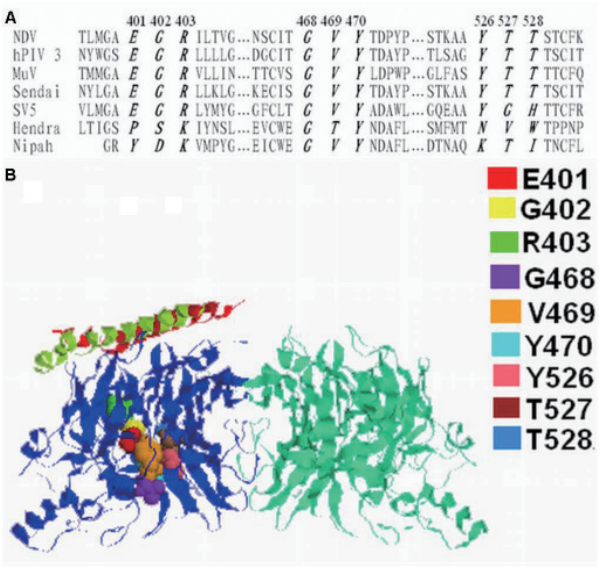

Figure 1. The position of conserved amino acids mutated in the NDV HN global head. (A) The selected amino acid positions of HN in NDV, hPIV 3, MuV, Sendai virus, SV5, Hendra virus, and Nipah virus. The conserved amino acids E401, G402, R403, G468, V469, Y470, Y526, T527, and T528 to be mutated are marked in italic and bold. **(B)** The 3-dimensional structure of NDV HN and locations of mutated residues. The 401-403, 468-470, and 526-528 amino acids are in β4-S1, β5-S1, and β6-S1 sheets, respectively. The mutants are marked in different colors. The figure was generated by RasMol, using the structure of Yuan et al (PDB IDs, 3T1E).

2.6. *Quantification of cell fusion*

Monolayers of BHK-21 seeded 1 day earlier with 4 × 10^5 cells/35 mm plate were co-transfected with the desired HN and F genes, washed with PBS, fixed with methanol for 5 min, and stained with Giemsa stain. The Reporter Gene Method was used to quantify the cell fusion (*34,35*). The plasmid pG1NT7β-gal encoding β-galactosidase was transfected into BHK-21 cells. At 16 h post transfection, these cells were mixed with those transfected with F and HN genes. After 5 h incubation at 37°C, monolayers were lysed with lysis buffer and the extract was assayed for β-galactosidase activity using high sensitivity β-galactosidase assay kit (Stratagene, La Jolla, CA, USA). The level of fusion was quantified by determination of the absorbance at 590 nm with an automated ELISA reader. The cells transfected with only the F gene were used as a negative control to eliminate the background fusion.

2.7. *Neuraminidase (NA) assay*

Transfection was preformed as previously described. To each well 0.5 mL of 625 mg/mL of neuramin-lactose (Sigma, St. Louis, USA) in 0.1 M sodium acetate (pH 6.0) was added and the plates were incubated at 37°C for 1 h. NA activity was quantified by measuring the intensities of the colors in the butanol layer at 549 nm minus the background of cells expressing only the vector (*29-32*).

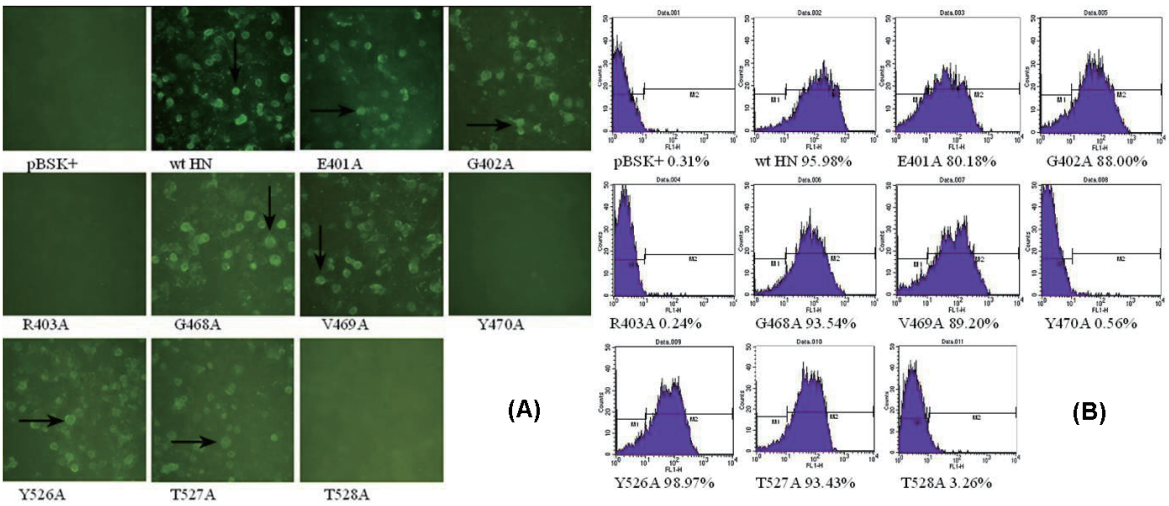

Figure 2. Expression efficiency of mutations in NDV HN head. (A) Indirect immunofluorescent assay (IIFA) of NDV HN mutations. BHK-21 cells were plated on 35-mm plates containing a cover glass and transfected as previously described. The primary antibody and the FITC labelled secondary antibody were the same as in the FACS assay. The monolayers were fixed with acetone 24h post co-expression F and mutated HN. Photographs of the cells were immediately taken by fluorescence microscope (OLYMPUS, Japan). The arrows point to the HN expression cells. **(B)** FACS data shows the expression efficiency of HN mutations by fluorescence intensity. The mutations of E401A, G402A, G468A, V469A, Y526A, and T527A were successfully expressed on the cell surface. The mutations R403A, Y470A, and T528A were not detected by FACS or IIFA. A mixture of monoclonal antibodies for NDV HN was used as primary antibodies in IIFA and FACS assays. The second antibody was fluorescein isothiocyanate-conjugated (FITC) goat anti-mouse IgG with a 1:200 dilution.

2.8. *Hemadsorption (HAD) assay*

The receptor-binding activity of mutant genes was detected by the ability to absorb guinea pig erythrocytes (*26*). The monolayers of BHK-21 that expressed NDV HN proteins were incubated for 30 min at 4°C with 2% guinea pig erythrocytes diluted with PBS supplemented with 1% of each $CaCl_2$ and $MgCl_2$. Then the cells were treated with 50 mM NH4Cl at 4°C until all erythtocytes were lysed, and the lysate was clarified by centrifugation. The absorbance density was determined at 540 nm on the UV spectrophotometer (Shimadzu, Kyoto, Japan) minus the background which was obtained with cells expressing only the vector. All work with animals followed National Institutes of Health guidelines and was approved by the Animal Care Committee of Shandong University (Approved protocol no. 20130305).

2.9. *Hemifusion assay*

The lipid probe octadecyl rhodamine B (R18) (Invitrogen, California, USA) was used to determine the ability of HN mutations to promote hemifusion from RBCs to BHK-21 cells co-transfected with HN and F genes as the protocol previously described (*29*). RBCs were washed and re-suspended with PBS, then labeled with R18 (1 mg/mL in ethanol) at room temperature for 30 min in the dark. RBCs were washed 5 times with ice-cold PBS to remove the uncombined R18 and re-suspended in PBS (0.1% hematocrit). Labeled RBCs were added to co-transfected monolayers and incubated at 4°C for 30 min. Cells were washed with PBS to remove unbonded RBCs, then incubated and the fluorescence was visualized using a fluorescence microscope (OLYMPUS, Japan) at 37°C.

2.10. *Co-immunoprecipitation (Co-Ip) assay*

Twenty-four hours after transfection, the interaction between mutated HN and F at the surface of BHK-21 cells was detected by the co-IP assay as previously described (*29,34*). The wt F proteins and a cleavage site mutant form of F proteins were used. The F (Fcsm) proteins that efficiently interacted with HN proteins without fusion activity were used to avoid the impact of HN and F interaction in or after the fusion process. The monolayers were co-transfected with 1 μg HN and 1 μg Fcsm for each well. Cells were washed 3 times with cold PBS. The AminoLink Plus Coupling Resin (Thermo Scientific, San Jose, CA, USA) were incubated with MAb specific for NDV F (AbBioSci Inc., Changzhou, China) for 2 h at room temperature. Cells were lysed by ice-cold IP lysis buffer on ice for 5 min with periodic mixing. The lysate was added into the resins and incubated with gentle mixing and at 4°C overnight. The proteins were eluted by elution buffer and heated at 100°C for 5 min for Western blot.

2.11. *Fusion promotion of the HN stalk region*

The stalk region (HNs) of NDV HN was constructed by PCR amplification from the full-length of NDV HN in pBSK(+)-HN. The PCR products were cloned into the pEGFP-N3 vector. The nucleotide sequences were verified by BIOSUNE (Beijing, China). The NDV wt F and HNs were expressed by transient transfection of the pBSK-F and HNs vectors in BHK-21 cells. Transfected BHK-21 cells were placed at 37 or 42°C to allow fusion. At 24 h post-transfection, the monolayers were washed with PBS, fixed by methanol, stained using Giemsa stain, and then photographed.

3. Results

3.1. *Site-directed mutation*

The selected amino acids were highly conserved in paramyxovirus (Figure 1A) and mutations were successfully constructed. Alanine substitution was individually introduced into each amino acid position. Each mutation was inserted into pBluescript(+)(pBSK+) and sequence analysis confirmed no additional mutations in the recombinant plasmids. Hydrophilic amino acids E401, G402, R403, G468, Y470, Y526, T527, and T528 were mutated to hydrophobic amino acid alanine. V469 is a hydrophobic amino acid, but it was changed to alanine.

3.2. *Cell surface expression efficiency of mutant NDV HN proteins*

IFA was used to determine HN mutant proteins expression qualitatively intracellular or extracellular. There was no detectable fluorescence in substitutions of R403A, Y470A, or T528A mutants. Green fluoresence was detected in E401, G402, G468, V469, Y526, and T527 mutants (Figure 2A). The FACS was used to determine expression efficiency of mutant NDV HN proteins. The results of FACS indicated that mutants were successfully expressed on the cell surface except for R403A, Y470A, and T528A mutants. To some extent, expression efficiency of the rest of the mutants was similar to wt HN from 69.3% to 102.9% (Figure 2B and Table 1) on average of three independent experiments.

3.3. *Fusion promotion ability of individual mutants*

Giemsa staining (Figure 3A) and the reporter gene method were used to test the qualitative and quantitative effect of the individual mutation, respectively. The cell fusion promotion activities of all mutants decreased, to some extent, compared with the wt HN and the fusion activity of mutated protein E401A and Y526A almost disappeared (Figure 4). The mutations of G402A, G468A, V469A, and T527A exhibited various effects

Table 1. Functional profile of the mutants in the globular head of NDV HN

Amino acid substitutions	Position of β-Sheet	Avg cell surface expression (% of wt)	Avg cell fusion (% of wt)	Avg NA (% of wt)	Avg HAd (% of wt)
E401A	B4-S1	69.3 ± 13.4	2.43 ± 1.9	10.1 ± 0.6	7.3 ± 1.0
G402A	B4-S1	101.4 ± 8.5	53.4 ± 1.8	72.8 ± 1.9	71.5 ± 1.8
R403A	B4-S1	—	—	—	—
G468A	B5-S1	94.6 ± 2.6	70.0 ± 3.0	35.3 ± 0.9	93.9 ± 9.1
V469A	B5-S1	92.0 ± 4.7	12.4 ± 0.8	68.1 ± 2.0	46.5 ± 1.6
Y470A	B5-S1	—	—	—	—
Y526A	B6-S1	84.1 ± 25.7	3.2 ± 0.4	0.4 ± 0.5	7.9 ± 1.7
T527A	B6-S1	102.9 ± 23.0	54.2 ± 4.3	35.5 ± 2.9	49.2 ± 7.7
T528A	B6-S1	—	—	—	—

The average of cell surface expression, cell fusion, NA activity, and HAD ability were determined by FACS, Report Gene Method, NA assays, and HAD assays, respectively. The data are averages of three independent experiments.

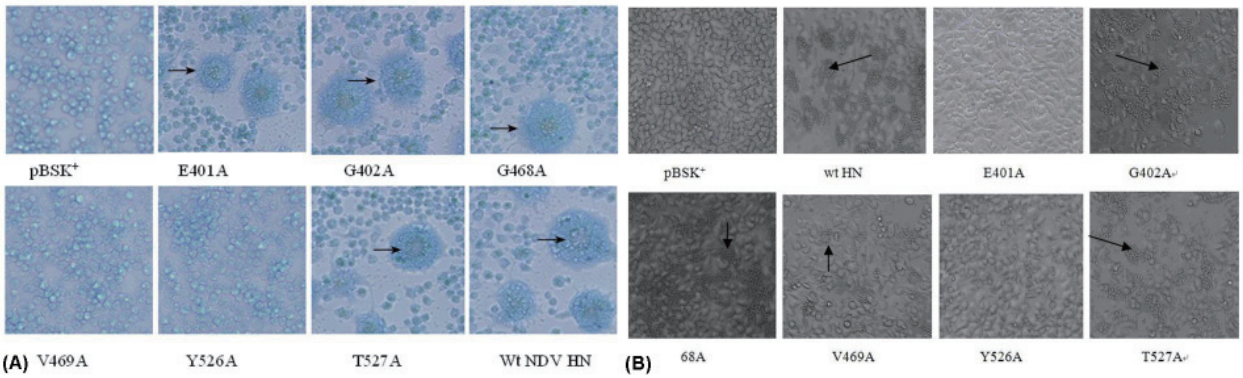

Figure 3. Detection of cell fusion ability and hemadsorption ability of mutation proteins in the NDV HN global head. (A) Giemsa staining results of NDV HN mutations in the presence with F co-transfection (24h post-transfection) in BHK-21 monolayers. The arrows point to the syncytia. **(B)** The monolayers that expressed HN were incubated with 2% guinea pig erythrocytes suspension at 4°C for 30 min. The redundant erythrocytes were removed by washing with PBS 3 times and photomicrographs were taken. The attached guinea pig erythrocytes are indicated by arrows.

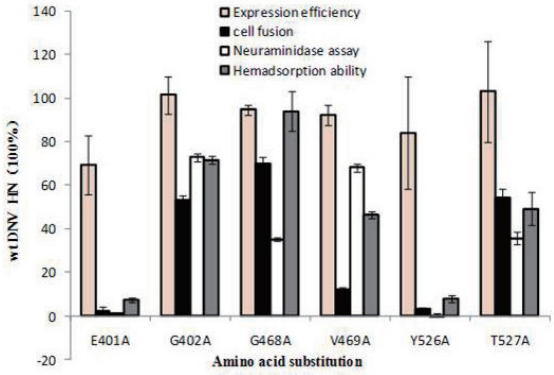

Figure 4. Expression efficiency and function of mutational NDV HN proteins. Comparison of mutant NDV HN expression efficiency, cell fusion activity, NA assay, and HAD ability (% of wt HN). The data represent the means of three independent determinations plus standard deviations.

on cell fusion promotion, receptor binding ability, and NA activity.

3.4. *HAD ability and NA activity of HN mutants*

The HAD assay was confirmed by the ability to adsorb guinea pig erythrocytes at 4°C to reveal the receptor binding ability (Figure 3B). Mutant G468A retained a similar HAD ability as the wt NDV HN (93.9%). G402A decreased to 71.5% of wt NDV HN. The hemabsorption abilities of V469A and T527A were 46.5% and 49.1% respectively. E401A and Y526A significantly affected the receptor binding ability (7.3% and 7.9%, respectively). Sialyllactose colorimetrically was used to quantify the NA activity of each mutated protein. Compared with wt NDV HN, mutated proteins G402A, V469A, G468A and T527A had 72.7%, 68.1%, 35.3%, and 35.5% of the NA activity, respectively. Neuraminidase activity of E401A and Y526A was almost undetectable, less than 5% (1.09%, and 0.38%) of the wt HN protein (Figure 4 and Table 1).

3.5. *Hemifusion assay*

R-18 labeled RBCs were used to detect the lipid-mixing rate and fusion efficiency (Figure 5). The BHK-21 monolayers of wt HN or E401A, G402A, G468A, V469A, and T527A co-transfection with F obtained the maximum fluorescence almost at the same time at 37°C. We attempted to quantify the lipid-mixing rate for these mutations, but the lipid-mixing process was accomplished in a few seconds. The time of fusion promotion was too short to distinguish the variation among wt HN and HN mutations. The number of syncytia of E401A, G402A, V469A, and T527A was

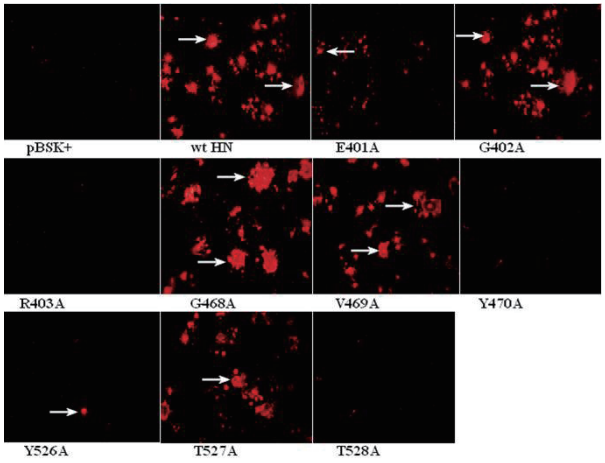

Figure 5. Cell fusion of R18-labeled RBCs with HN and F protein co-transfection BHK-21 cells. The labeled RBCs were incubated with HN and F co-expressed monolayers at 4°C for 30 min. Monolayers were washed with ice-cold PBS to remove the unbound RBCs, incubated at 37°C for 5 min, and detected using fluorescence microscope. Arrows indicate lipid mixing.

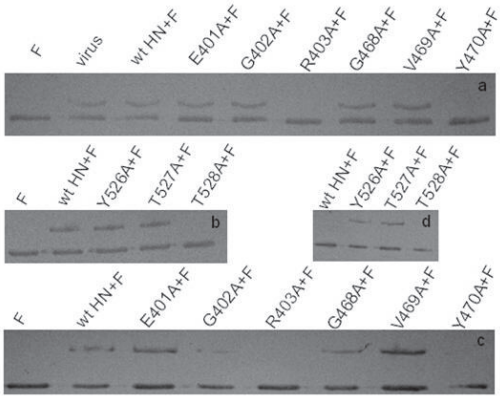

Figure 6. Co-immunoprecipitation of NDV HN and NDV F. The AminoLink Plus Coupling Resin was incubated with MAb specific for NDV F for 2 h at room temperature. The monolayers co-expressed with F and HN genes were lysed by ice-cold IP Lysis Buffer on ice for 5 min with periodic mixing. The lysate was added into the resins and incubated for overnight at 4°C. The proteins were eluted by Elution Buffer and heated at 100°C for 5 min for Western Blot. A mixture of an anti-F MAb and two anti-HN MAbs was used for Western Blot. Monolayers transfected F alone, co-transfected with wt HN and F, and virus supernatant were used as controls. **a** and **b** show the wt HN or HN mutations co-expression with Fcsm in BHK-21 monolayers. The wt HN or HN mutations and wt F were co-expressed in BHK-21 monolayers in **c** and **d**. The grayscale of the photograph was analyzed by Image J 1.48V.

less than that in wt HN and the diameter of the syncytias for HN mutations was smaller. It was proven that the decrease of receptor-binding ability in HN had no impact on the rate of lipid-mixing in the fusion promotion process but the fusion efficiency declined at the end of the lipid-mixing event.

3.6. *Co-IP assay*

If the fusion could not be promoted, the interaction between HN and F was not impacted with co-expression

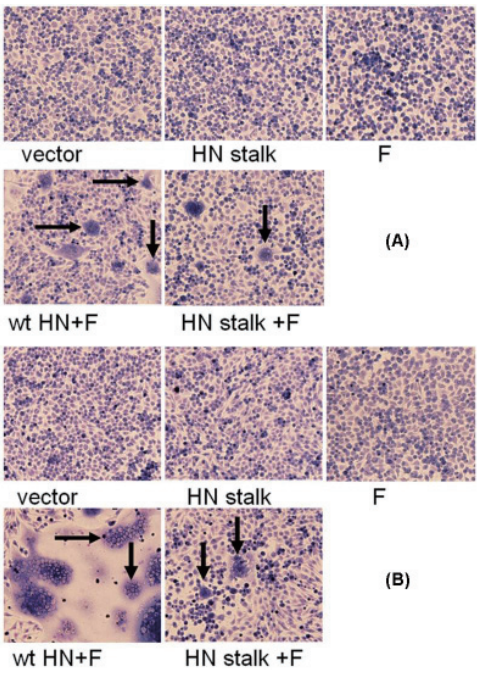

Figure 7. The F promotion capacity of the HN stalk by co-expression F and HN stalk genes. BHK-21 monolayers were infected with recombinant vaccina virus and co-transfected with wt HN or HNs and F genes. At 24 h post-transfection, the monolayers were fixed with methanol for 10 min and stained with Giemsa stain. Syncytias are indicated by arrows. BHK-21 monolayers were incubated at 37°C **(A)** and 42°C **(B)**.

Fcsm and wt HN or HN mutations (Figures 6a and 6b). The monolayers co-expressed wt F and wt HN or mutant HN detected varying degrees of fusion (Figures 6c and 6d). Nevertheless, the interaction between Fcsm and HN mutations were comparable to that in wt HN. It was concluded that the interaction of F and HN proteins was not related with the receptor binding ability of HN. The globular head of HN did not participate in the interaction and the interaction of HN and F disappeared in the stage of post-fusion.

3.7. *Fusion promotion of NDV HNs*

In the monolayers co-transfacted with F and HNs, syncytia were detected at 37°C and 42°C (Figures 7A and 7B). But the number of syncytia of HNs was less than that in wt HN and the size of syncytia in HNs was smaller. It indicated that the headless HN could promote the fusion process in NDV, but the fusion efficiency declined. It was inconsistent with the theory that the receptor binding event promotes the fusion process mediated by F. However, there is no convincing explanation for the promotion mechanism of HNs in NDV.

4. Discussion

The head region of NDV HN has three functions, receptor binding ability, fusion promotion, and NA activity. We determined the roles of the highly

conserved amino acids in the head region of NDV HN proteins. Some amino acids changes in the HN globular head removed the F promotion ability of HN protein and restrained syncytia formation. Fewer and smaller syncytias were detected in the HN mutations. It is thought that the disappearance of receptor binding ability is responsible for these changes based on previous studies. However, a headless HN recombinant protein was also constructed to test the roles of the HN stalk region in the F promotion process. It was found that the headless HN promoted the fusion process though the fusion extent was weaker than wt HN.

The expression efficiency of nine NDV HN mutants was determined by FACS. The mutant proteins E401A, G402A, G468A, V469A, Y526A, and T527A were successfully expressed, but the mutants R403A, Y470A, and T528A were not detected by FACS. Indirect immunofluorescence assay was also used to determine expression of R403A, Y470A, and T528A mutants and no intracellular or extracellular fluorescence was found. It is possible that the mutants affected the protein translation or transport process of the recombinant proteins. No reasonable reason was established in this study. It can not be excluded that the monoclonal antibody binding site was changed so that the MAbs could not bind to the NDV HN proteins.

NDV, SV5, and hPIV3 are members of paramyxoviridae virus with six β-sheets in the globular head of the HN protein. Highly conserved animo acids found in the S1 region of each β-sheet in these viruses suggested that HAD ability and NA activity of HN protein are related to these conserved amino acids in paramyxoviruses. For the mutants used in this study, 401-403 amino acids are in β4-S1 sheet, 468-470 amino acids are in β5-S1 sheet, and 526-528 amino acids are in β6-S1 sheet. E401, G468, and Y526A are in the external part of the pocket-like structure in globular head of the HN protein that are necessary for the receptor-binding ability and neuraminidase activity. The G402, V469A, and T527A on the inner side of E401A, G468A, and Y526A are less important for the receptor-binding ability and neuraminidase activity. The R403, Y470, and T528 in the internal part of the receptor-binding motif of the NDV HN protein are not expressed on the cell surface. It is possible that the highly conserved amino acids in β-sheet of the external part of the globular head of the HN are vitally important for the receptor-binding ability and neuraminidase activity. It has been confirmed that both the receptor binding and nueraminidase activity are mediated by the same activity site in the NDV HN protein globular head. The conformation change of the NDV HN protein is able to switch between the two different states (*6,18*). The virus attachment process is implemented by HN protein binding with the sialic acids on the host cell surface and the virus dissemination depends on the removal of sialic acids on the cell surface by HN nueraminidase activity. There may be something balancing mechanism between the virus switching these two functions of the HN protein and the conserved amino acids may provide some novel information.

Murrell *et al*. reported that in 4-GU-DANA-resistant HPIV3 virus variant (ZM1) the receptor-binding ability and NA activity were higher than that in the parent virus. It suggested that the receptor-binding ability was closely related with neuraminidase activity. The virus increased neuraminidase activity to promote virus release when its hemagglutinin ability was improved. In the same mutant, the impact on receptor-binding ability, neuraminidase activity, and cell fusion is different (*36*). The mutations in NDV HN demonstrated that R174L, R416L, R498L, E547Q, Y526L, and I175E impacted the receptor binding ability and NA activity. We have proved that decrease of hemadsorption ability for the mutants G402A (71.5%), V469A (46.5%), and T527A (49.1%) correlated with the loss of neuraminidase activity (72.7%, 68.1%, and 35.5%, respectively). However, G468A had a different result from other mutants. The hemadsorption ability of G468A was 93.9% of the wt HN but neuraminidase activity was only 35.3% of the wt HN proteins. A conclusion can be drawn that E401, Y526, G402, V469, and T527 play the same role in the receptor-binding and receptor remove process, but G468 plays a different role in these two processes. It may be one of the key amino acids in switching the functions between the receptor binding and destruction process. Previous studies have shown that G403A in NDV HN did not impact the biological function of HN, but we found that the G403A mutation did not express intracellularly or on the cell surface.

The 3D structure of HN global head demonstrated that side chains of E401 and Y526 are hydrogen bonding to sialic acid ligands. Changing the side chains of E401 and Y526 to E401A and Y526A almost eliminated the receptor binding ability and NA activity of the HN protein in this study. The hemadsorption ability and neuraminidase activity of mutants E401A (2.43%, 3.17%) and Y526A (1.09%, 0.38%) almost disappeared compared with the wt HN. It could indicate that E401 and Y526 are essential for both virus attachment and dissemination.

The structure of the globular head in NDV HN proteins revealed that the β-sheet propeller motif forms a NA activity site. It has been reported that K147, Y526, and E547 constituted the NA activity domain in accordance with the conclusion in this research (*6*). The NA activity of Y526A declined to 0.38% of the wt NDV HN. Reduction of HAD ability and cell fusion were comparable in mutants G402A, G468A, and T527A. It can be assumed that the decline of cell fusion promotion was caused by the decrease of receptor recognition ability. However, the decline of cell fusion promotion ability (12.4 ± 0.8%) of V469A was lower than that of receptor-binding ability (46.5 ± 1.6%) and NA activity (68.1 ± 2.0%). Thus, V469A impacts the hemagglutinin,

sialidase, and fusion promotion activity in the virus life cycle.

However, there was a contradiction that the headless HN of NDV promoted the fusion process. Related theories agree that the head of HN is necessary for the F protein promotion in the membrane fusion process. Some conserved amino acids (E401A and Y526A) change in the HN protein of NDV which lead to incapacity of receptor binding, and also loss of the fusion promotion function and syncytia formation ability. In these mutations, the whole stalk region of HN exists and did nothing for the F promotion. It has been reported that the head of HN was responsible for the virus attachment and useless for the F promotion process. However, we think that the globular head of HN suppresses the F promotion and the stalk region of HN has the ability to promote the F conformation change. It possibly revealed a new model of the HN and F interaction. In the family of paramyxovirus, there is a similar flexible linker structure between the globular head and stalk region that was the potential for the movement of the globular head in HN proteins. The globular head of HN binding to sialic acids is separated from F by the flexible linker structure between the globular head and stalk region of HN. Then, the stalk region of HN promoted the conformation changes of F protein to activate the fusion process.

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