Short Communication

Role of C596 in the C-terminal extension of the haemagglutinin–neuraminidase protein in replication and pathogenicity of a highly virulent Indonesian strain of Newcastle disease virus

Shin-Hee Kim,1 † Sa Xiao,1 † Anandan Paldurai,1 Peter L. Collins2 and Siba K. Samal1

1Virginia-Maryland Regional College of Veterinary Medicine, University of Maryland, College Park, MD, USA
2Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA

We modified the haemagglutinin–neuraminidase (HN) glycoprotein of the virulent Newcastle disease virus (NDV) strain Banjarmasin/010/10 (Ban/010) by adding C-terminal extensions similar to those found in certain avirulent NDV strains. Extension of the 571 aa wt Ban/010 HN protein to 577 and 616 aa by removal of one or two translational stop codons moderately reduced HN function and viral pathogenicity in 1-day-old and 3-week-old chickens. Substantially greater reductions were achieved by altering the 616 aa form by introducing a R596C mutation or by replacing the C-terminal extension with that of avirulent strain Ulster, which naturally contains the amino acid 596C. These results showed that extension of the C terminus of HN reduces NDV pathogenicity, and that this effect is substantially increased by the presence of 596C. These results indicate that this attenuating mechanism in avirulent strains such as Ulster can be applied directly to a highly virulent strain recently in circulation.

Newcastle disease virus (NDV) causes a highly contagious respiratory and neurological disease in chickens, leading to severe economic losses in the poultry industry worldwide (Alexander, 1989). NDV isolates vary widely in virulence. Based on virulence in chickens, NDV strains are categorized into three pathotypes: lentogenic (avirulent), mesogenic (moderately virulent) and velogenic (highly virulent) (Alexander, 1989). NDV is a member of the family Paramyxoviridae and has a non-segmented, negative-sense RNA genome consisting of six genes (3'-N-P-M-F-HN-L-5') (Lamb & Parks, 2007). The fusion (F) and haemagglutinin–neuraminidase (HN) proteins play major roles in virus infectivity and virulence (Lamb et al., 2006).

The HN protein is a multifunctional protein. It is responsible for attachment to sialic acid-containing receptors on cell surfaces, release of progeny virions from cell surface and promotion of the fusion activity of the F protein (Huang et al., 2004). The HN protein of NDV strains exists at least seven different lengths according to differences in position of the stop codon of the HN ORF: 571, 572, 577, 578, 580, 585 or 616 aa (Samal, 2011). Interestingly, the shortest length (571 aa) and the longest length (616 aa) of the HN proteins have been found in velogenic and lentogenic strains, respectively (Gorman et al., 1988, 1992). The HN proteins of intermediate length are found in all three pathotypes of NDV. The HN protein of 616 aa is detected as a precursor protein (HN0), and post-translational cleavage is required for generation of a biologically active HN protein (Nagai et al., 1976; Nagai & Klenk, 1977). Several studies have indicated that the length and sequence of the extended C terminus of the HN protein play a role in the function of the HN protein, but their role in the virulence of NDV has not been clearly evaluated. Although velogenic NDV strains with an elongated HN have never been identified in nature, we hypothesized that extension of the C terminus of HN protein would reduce the pathogenicity of velogenic strains. Therefore, in the present study, we evaluated the role of the extended length of the HN protein in NDV pathogenicity by extending the C terminus of the HN protein of a highly virulent NDV strain, Banjarmasin/010/10 (Ban/010). This strain was isolated from a diseased chicken during an outbreak in commercial chickens in Indonesia (Xiao et al., 2012a) and contains an HN protein of 571 aa. All experiments with these viruses were performed in an enhanced Biosafety Level 3 (BSL-3+) containment facility certified by the US Department of Agriculture, and all in vivo experiments were performed following the guidelines of the

†These authors contributed equally to this work.
Institutional Animal Care and Use Committee, University of Maryland, MD, USA.

Using reverse genetics, we first generated two mutant viruses with different lengths of HN protein by replacing the naturally occurring stop codon at position 572 with an arginine codon, yielding a 577 aa form (rBan/HN577), or by replacing this stop codon together with one at position 578 with arginine codon, yielding a 616 aa form (rBan/HN616) (Fig. 1a). A previous study demonstrated that the C-terminal extension of avirulent NDV strain Ulster HN protein (616 aa) blocks the neuraminidase active site and the second sialic acid-binding site, thus resulting in reduced haemadsorption and catalytic activities of the HN precursor (Yuan et al., 2012). In addition, it was found that this auto-inhibition is dependent on a disulfide bond formed by C596. Therefore, we evaluated further the role of C596 and the C-terminal sequences of strain Ulster HN protein on NDV pathogenicity by introducing an R596C mutation into rBan/HN616 (rBan/HN-R596C), and by adding the 45 aa residues of the strain Ulster C terminus into rBan/HN616 in place of the corresponding rBan/010 sequence (rBan/HN-Uls616) (Fig. 1a). Recovery of the four mutant viruses was performed as described previously for parental rBan/010 virus (Xiao et al., 2012b).

The effect of C-terminal mutations on expression and function of the HN protein was characterized in DF1 cells (Fig. 1b–e). Expression of various lengths of HN protein was quantified by Western blotting using a serum specific to the NDV HN. This analysis confirmed the increase in molecular mass of the HN protein of C-terminally extended mutant viruses (HN\(_{578}\)) in DF1 cells (trypsin+, Fig. 1b). We further confirmed that these mutant HN proteins could be proteolytically processed in the presence of trypsin (5 \(\mu\)g ml\(^{-1}\)) in DF1 cells (trypsin+, Fig. 1b). The role of C596 on the function of the HN has also been determined using a haemadsorption assay by overlaying virus-infected cells with a 2% (v/v) suspension of chicken red blood cells (Huang et al., 2004). The red blood cells bound to the virus-infected cells were lysed, and the absorbance of the released haemoglobin was measured at 549 nm. The haemadsorption activity of the parental NDV strains, rBan/010 and Ulster, was 3.11 ± 0.47 and 0.29 ± 0.08, respectively, whereas that for the mutant viruses was 2.84 ± 0.62 for rBan/HN577, 2.91 ± 0.45 for rBan/HN616, 0.85 ± 0.27 for rBan/HN-R596C, and 0.627 ± 0.13 for rBan/HN-Uls616. This showed that all of the mutant viruses exhibited a reduced level of haemadsorption, and that this was particularly reduced for the two forms that contained the C596 residue. There was no significant difference in plaque size between the parental and mutant viruses (Fig. 1c). We next determined the in vitro growth kinetics of the viruses in the absence or presence of trypsin in DF1 cells (Fig. 1d, e). The parental rBan/010 virus replicated efficiently during the first 32 h of infection, but the titres decreased thereafter with extensive syncytium formation. Among the mutant viruses, rBan/HN577 and rBan/HN616 grew to high titres that were slightly lower than those of the parental virus. However, the two viruses did not show a significant difference (\(P<0.05\) in virus replication compared with the parental virus up to 32 h of infection in the presence or absence of trypsin (Fig. 1d, e). rBan/HN-R596C and rBan/HN-Uls616 grew slowly, and their titres were significantly lower (\(P<0.05\)) than that of rBan/010 at 16, 24 and 32 h of incubation in the presence or absence of trypsin. However, the viruses reached similar titres to the other two mutant viruses thereafter and did not show a significant difference, indicating that these two mutations noticeably affected the rate but not the final magnitude of replication of rBan/010. In general, our results showed that supplementation of trypsin did not change the in vitro replication of the HN mutant viruses, in that the pattern of replication of each virus was similar in the absence or presence of trypsin in DF1 cells. This indicated that the C-terminal extension of HN protein probably does not play a role in the in vitro replication of NDV.

The pathogenicity of the parental and mutant viruses was evaluated by mean death time (MDT) in embryonated chicken eggs and by the intracerebral pathogenicity index (ICPI) in 1-day-old chicks (Alexander, 1989). The MDT values for categorizing NDV isolates are: <60 h for velogenic strains, 60–90 h for mesogenic strains and >90 h for lentogenic strains. The ICPI is the mean of the score per bird per observation over an 8-day period. Highly virulent velogenic viruses give values approaching 2, and avirulent or lentogenic strains give values at or close to 0. The highly virulent parental virus (rBan/010) had an MDT value of 46 h and an ICPI value of 1.87. Pathogenicity tests of the mutant viruses based on MDT and ICPI showed that the C-terminal extension of the HN length in rBan/010 conferred attenuation. The order of increasing attenuation was rBan/HN577 (48 h and 1.71), rBan/HN616 (52 h and 1.79), rBan/HN-R596C (54 h and 1.62) and rBan/HN-Uls616 (59 h and 1.53). The effect was greatest when the C-terminal sequence of the HN protein of NDV strain Ulster was introduced into the rBan HN protein, which had the effect of changing the viral pathotype from velogenic to borderline mesogenic.

To evaluate further the role of the length of HN in neurovirulence, the growth kinetics and lethality of the parental and chimaeric viruses were monitored following inoculation of 1-day-old chicks (10\(^3\) p.f.u. per bird) via the intracerebral route. Infected chickens were sacrificed daily and brain tissue was collected for virus titration (Fig. 2). All of the chicks infected with rBan/010 virus were dead at 2 days post-infection (p.i.), and the virus titres were consistently higher than those of the other groups (\(>7.0\) log\(_{10}\) TCID\(_{50}\) g\(^{-1}\); \(P<0.05\)). In contrast, reduced virus replication and delays in mortality were observed in chicks infected with the four mutant viruses, consistent with their reduced ICPI values noted above. The rBan/HN577 and rBan/HN616 viruses reached their highest titres (7.0 log\(_{10}\) TCID\(_{50}\) g\(^{-1}\)) at 3 days p.i. followed by 100% mortality by the next day, whereas rBan/HN-R596C and
rBan/HN-Uls616 reached their highest titres at 4 days p.i. followed by 100% mortality by the next day. The titres of rBan/HN577 and rBan/HN616 showed significant differences from those of rBan/HN-R596C and rBan/HN-Uls616 ($P<0.05$). Our results suggest that extension of the HN C terminus to two different lengths (577 and 616 aa)

Fig. 1. Mutants of NDV strain Ban/010 containing extensions to the C terminus of the HN protein, and characterization of HN expression and virus replication in vitro. (a) The length of the rBan/010 HN protein was extended by replacing naturally occurring stop codons at positions 572 and 578 with arginine (R) residues (rBan/HN577 and HN616). In addition, the R596C mutation was introduced in rBan/HN616 (rBan/HN-R596C). Also, the 45 aa of the strain Ulster HN C terminus were added into wt HN rBan/010 in place of the existing sequence (indicated by a box) (rBan/HN-Uls616). In the first three mutants (rBan/HN577, rBan/HN616 and rBan/HN-R596C), the introduced mutations are shown in bold. In the last virus (rBan/Uls616), the bold letters identify amino acids that differ from the Ban/010 sequence. The C-terminal 45 aa extensions in rBan/HN616 and rBan/HN-Uls616 had 55% sequence identity. (b) Expression of the HN proteins of parental and mutant viruses evaluated in infected DF1 cells at an m.o.i. of 0.1 in the absence or presence of trypsin (5 μg ml$^{-1}$). Cell lysates were subjected to gel electrophoresis under denaturing and reducing conditions, and analysed by Western blotting using an anti-NDV HN mAb. (c) Plaque morphology of parental and mutant viruses in DF-1 cells visualized by staining with crystal violet 4 days p.i. (d, e) Growth kinetics of parental and mutant viruses in DF1 cells (m.o.i. of 0.01). Viral titres were determined in the absence (d) or presence (e) of trypsin (5 μg ml$^{-1}$). Results are shown as mean ± SD of duplicate samples. * indicates position of stop codon.
Chickens were inoculated intranasally with 200 infected by a route mimicking natural topical infection. The parental and mutant viruses in 3-week-old chickens

We next evaluated the replication and tissue tropism of comparable levels of attenuation, suggesting the importance of residue C596 present in both viruses.

The cleavage site sequence of the F protein is a major determinant of NDV virulence (Peeters et al., 1999; Panda et al., 2004). Virulent NDV strains contain a multibasic cleavage site (with the general consensus sequence RRKR↓F) containing the furin protease motif [RXR/KR↓], which is readily cleaved in cell culture by intracellular furin or furin-like protease. Avirulent NDV strains lack this polybasic site and depend on extracellular protease for cleavage. However, mesogenic and velogenic NDV strains can have the same cleavage site sequence but differ in virulence. This indicates that additional viral factors contribute to NDV virulence. The HN protein has also been shown to determine the tropism of NDV and to contribute to virulence (Huang et al., 2004). Interestingly, some lentogenic NDV strains have evolved to express a determinant of NDV virulence (Peeters et al., 1999; Panda et al., 2004).

Fig. 2. Growth kinetics of NDV strain Ban/010 HN mutant viruses in the brains of infected 1-day-old chicks. Ten 1-day-old specific-pathogen-free chicks per group were inoculated with 10^3 p.f.u. each virus via the intracerebral route. Two birds in each surviving group were sacrificed daily up to 4 days p.i. Brain samples were collected and virus titres were determined by limiting dilution on DF1 cells. Results are represented as mean ± SD for two chicks.

conferred similar degrees of virulence and levels of replication in the brains of chicks. Furthermore, each of the other two modifications (introduction of the R596C mutation in rBan/HN616 or replacement of its C-terminal extension by that of strain Ulster) led to greater and comparable levels of attenuation, suggesting the importance of residue C596 present in both viruses.

Fig. 2. Growth kinetics of NDV strain Ban/010 HN mutant viruses in the brains of infected 1-day-old chicks. Ten 1-day-old specific-pathogen-free chicks per group were inoculated with 10^3 p.f.u. each virus via the intracerebral route. Two birds in each surviving group were sacrificed daily up to 4 days p.i. Brain samples were collected and virus titres were determined by limiting dilution on DF1 cells. Results are represented as mean ± SD for two chicks.

We next evaluated the replication and tissue tropism of the parental and mutant viruses in 3-week-old chickens infected by a route mimicking natural topical infection. Chickens were inoculated intranasally with 200 μl each virus (10^5 p.f.u. per bird). Three birds from each group were sacrificed at 4 days p.i. and tissue samples (trachea, lungs, spleen and brain) were collected. The virus titres in homogenized tissue samples were determined by a limiting end-point dilution assay (Kim et al., 2012). Three additional birds from each group were observed for clinical signs during the 5-day experiment (clinical score, 0 ± 0.0; Fig. 3a). These chickens started to show significantly different clinical score from those of chickens infected with parental virus (rBan/010) at 2 days p.i. and from the other two mutant viruses (rBan/HN577 and rBan/HN616) at 3 days p.i. Similarly, only the parental virus was able to replicate in all of the collected tissues (P<0.05) (Fig. 3b). This velogenic virus replicated to high titres in the trachea, lung and spleen (>7.0 log10 TCID50 g^-1), with a lower level of replication in the brain. In contrast, replication of the rBan/HN577 and rBan/HN616 viruses was mostly restricted to the upper respiratory tract, and the two viruses did not show significantly different levels of replication in the trachea (P<0.05) (Fig. 3b). Among the mutant viruses, only rBan/HN577 was able to replicate in the lungs, with a titre of 2.1 log10 TCID50 g^-1, but this was a significantly low level compared with that of the parental virus (P<0.05). In contrast, replication of rBan/HN-R596C and rBan/HN-Uls616 was not detected in any of the collected tissues. These results indicated that each of the C-terminal HN mutations affected tissue tropism and replication of velogenic Ban/010 virus in 3-week-old chickens.

The cleavage site sequence of the F protein is a major determinant of NDV virulence (Peeters et al., 1999; Panda et al., 2004). Virulent NDV strains contain a multibasic cleavage site (with the general consensus sequence RRKR↓F) containing the furin protease motif [RXR/KR↓], which is readily cleaved in cell culture by intracellular furin or furin-like protease. Avirulent NDV strains lack this polybasic site and depend on extracellular protease for cleavage. However, mesogenic and velogenic NDV strains can have the same cleavage site sequence but differ in virulence. This indicates that additional viral factors contribute to NDV virulence. The HN protein has also been shown to determine the tropism of NDV and to contribute to virulence (Huang et al., 2004). Interestingly, some lentogenic NDV strains have evolved to express a precursor form of HN, called HN0, which contains an additional ~45 aa C-terminal extension that is cleaved by host-cell protease. As the presence of this C-terminal extension on HN0 is known to inhibit major functions of the HN protein, such as receptor binding and neuraminidase activities, this can be an important factor in moderating NDV pathogenicity, and cleavage may be essential to the virus life cycle (Yuan et al., 2012). However, a previous study using an avirulent NDV backbone suggested that the addition of a C-terminal extension to the HN protein did not confer attenuation as measured by an ICPI assay (Römer-Oberdörfer et al., 2003). In contrast, in the present study, the introduction of several different C-terminal extensions to the HN protein of the virulent

rBan/HN577, which were significantly different compared to those of rBan/HN-R596C and rBan/HN-Uls616 (P<0.05). However, all chickens infected with these two viruses began to recover at 5 days p.i. None of the chickens infected with rBan/HN-R596C and rBan/HN-Uls616 had any apparent clinical signs consistently during the 7-day experiment (clinical score, 0 ± 0.00; Fig. 3a). These chickens started to show significantly different clinical score from those of chickens infected with parental virus (rBan/010) at 2 days p.i. and from the other two mutant viruses (rBan/HN577 and rBan/HN616) at 3 days p.i. Similarly, only the parental virus was able to replicate in all of the collected tissues (P<0.05) (Fig. 3b). This velogenic virus replicated to high titres in the trachea, lung and spleen (>7.0 log10 TCID50 g^-1), with a lower level of replication in the brain. In contrast, replication of the rBan/HN577 and rBan/HN616 viruses was mostly restricted to the upper respiratory tract, and the two viruses did not show significantly different levels of replication in the trachea (P<0.05) (Fig. 3b). Among the mutant viruses, only rBan/HN577 was able to replicate in the lungs, with a titre of 2.1 log10 TCID50 g^-1, but this was a significantly low level compared with that of the parental virus (P<0.05). In contrast, replication of rBan/HN-R596C and rBan/HN-Uls616 was not detected in any of the collected tissues. These results indicated that each of the C-terminal HN mutations affected tissue tropism and replication of velogenic Ban/010 virus in 3-week-old chickens.
Ban/010 strain resulted in mutants with variously decreased levels of pathogenicity and replication \textit{in vivo} as measured by several different assays: MDT, ICPI and replication efficiency and lethality in 1-day-old chicks and 3-week-old chickens. Extension of the 571 aa wt Ban/010 HN to 577 and 616 aa moderately reduced HN function and viral pathogenicity. A greater reduction in HN function and viral pathogenicity was achieved by further altering the 616 aa form by introduction of the C596 residue or by swapping the C-terminal extension of the 616 aa form with that of strain Ulster, which naturally contains the C596 residue. For each of these four mutants, the C-terminal modifications had little effect on replication \textit{in vitro}, indicating that their \textit{in vivo} attenuation was not

**Fig. 3.** Clinical signs and replication of Ban/010 HN mutant viruses in 3-week-old chickens. Chickens in groups of six were inoculated with each virus (100 p.f.u. per bird) by the intranasal route. (a) Clinical signs in the infected chickens (three birds each) were scored daily (0, healthy; 1, sick; 2, paralysis; 3, prostration; 4, dead) and the daily mean for each group is shown. Results are represented as mean ± SD of three chickens. (b) Three birds from each group were sacrificed at 4 days p.i., and virus titres in the collected brain, trachea, lung and spleen were determined by limiting dilution on DF1 cells. Results are represented as mean ± SD. The existence of significant differences (P<0.05) among the groups is indicated using different letters on each column.
simply due to direct impairment of the viral replicative machinery. This indicates an important role for these extended sequences in modulating NDV pathogenicity. The observation that each of the four mutant viruses was attenuated suggested that the various C-terminal extensions conferred an attenuating effect that was not dependent on a particular length or sequence. The further inclusion of the C596 residue provided a strongly attenuating effect. The finding that these two mutants had very similar levels of virulence supports the importance of the C596 residue and the lesser importance of the sequence of the rest of the C-terminal extension. In strain Ulster, the C596 residue was recently shown to participate in forming a disulfide bond that regulated HN activities and neuraminidase domain dimerization (Yuan et al., 2012). In the crystal structure, the dimerized neuraminidase domain containing the C-terminal extension extends along the outside of the sialidase \( \beta \)-propeller domain and inserts C-terminal residues into the neuraminidase domain active site. The C-terminal extension also engages a secondary sialic acid-binding site present in NDV HN proteins and blocks its attachment function. These results clarify how the Ulster HN C-terminal extension leads to an auto-inhibited state of HN and the requirement for proteolytic activation of HN. The interchain disulfide bond formed by C596 is therefore critical for maintaining the auto-inhibited state of the strain Ulster. This previous study did not address pathogenicity, but the present results confirm the importance of the C596 residue and, by implication, the interchain disulfide bond, in NDV replication in vivo, tissue tropism, neurovirulence and pathogenicity, and contribute to our understanding of the avirulent pathotype of NDV strains. Interestingly, this attenuating mechanism could be directly transferred to the HN protein of a velogenic strain recently in circulation.

Acknowledgements

We thank Daniel Rockemann, Girmay Gebrelul, Yonas Araya, Andrea Ferrero-Perez and our laboratory members for excellent technical assistance; and Dr Bernard Moss [National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH)] for providing the vaccinia T7 recombinant virus and the pTM1 plasmid. This research was supported by NIAID contract N01-AI-60009 (85% support) and the NIAID, NIH, Intramural Research Program (15% support). The views expressed herein do not necessarily reflect the official policies of the Department of Health and Human Services, nor does mention of trade names, commercial practices or organizations imply endorsement by the US Government.

References


