A single amino acid substitution in the haemagglutinin–neuraminidase protein of Newcastle disease virus results in increased fusion promotion and decreased neuraminidase activities without changes in virus pathotype

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Attachment of Newcastle disease virus (NDV) to the host cell is mediated by the haemagglutinin–neuraminidase (HN), a multifunctional protein that has receptor recognition, neuraminidase (NA) and fusion promotion activities. The process that connects receptor binding and fusion triggering is poorly understood and amino acid residues important for the functions of the protein remain to be fully determined. During the process of generating an infectious clone of the Anhinga strain of NDV, we were able to rescue a NDV with highly increased fusogenic activity in vitro and decreased haemagglutinating activity, as compared with the wild-type parental strain. Sequencing of this recombinant virus showed a single mutation at amino acid position 192 of the HN protein (Ile—Met). In the present study, we characterized that single amino acid substitution (I192M) in three strains of NDV by assessing the NA activity and fusogenic potential of the mutated versus wild-type proteins in cell cultures. The original recombinant NDV harbouring the mutation in the HN gene was also used to characterize the phenotype of the virus in cell cultures, embryonated chicken eggs and day-old chickens. Mutation I192M results in low NA activity and highly increased cell fusion in vitro, without changes in the viral pathotype of recombinant viruses harbouring the mutation in vivo. The results obtained suggest that multiple regions of the HN-protein globular head are important for fusion promotion, and that wild-type levels of NA activity are not absolutely required for viral infection.

INTRODUCTION

Paramyxoviruses are important respiratory pathogens in humans and animals. Human parainfluenza, mumps, Newcastle disease, canine distemper and Sendai viruses are some of the pathogens included in this family (Lamb & Parks, 2007). Paramyxovirus infections are initiated by receptor recognition and binding of the virion to sialyl glycoconjugates on the host cell surface, followed by fusion of the virus lipid envelope with the membrane of the host cell (Connaris et al., 2002; Lamb & Parks, 2007). In Newcastle disease virus (NDV) infections, this process is mediated by the interaction of two viral surface glycoproteins, the haemagglutinin–neuraminidase (HN) and fusion (F) proteins.

The HN of NDV is a class II integral membrane protein, found as a tetramer in the virion, which mediates receptor recognition of sialic acid at the end of host-cell-surface proteins and possesses neuraminidase (NA) activity, necessary to prevent virus progeny self-aggregation during budding (Lamb & Parks, 2007). A third function of the protein is fusion promotion, which is thought to be mediated by interactions of the HN with the F protein (Palermo et al., 2007; Porotto et al., 2005). The F protein is a class I membrane protein present as a trimer in the virion, which is closely associated with HN in the viral membrane. Interactions of the HN tetramer with its receptor are believed to generate conformational changes in this protein, which, in turn, promote changes in the tertiary structure of the F protein, allowing the latter to go from its native structural form (a metastable state), to a pre-hairpin state (in which the fusion peptide is released and interacts with the host cell membrane), to a post-fusion form that allows the viral envelope to approximate to the host cell membrane through the formation of a hairpin structure (referred to as a six-helix bundle) of the protein trimer that results in fusion of these lipid bilayers.
The crystal structure of the HN and F proteins of NDV have been elucidated (Chen et al., 2001; Crennell et al., 2000), and a lot of information has been obtained regarding their threedimensional structure. The HN displays the six-bladed β-propeller folding typical of other known NAs (Connaris et al., 2002). The receptor recognition/binding and catalytic site for the NA activities have been located to a single large cavity at the top of the globular head of the protein (Lamb et al., 2006). A second receptor binding site (lacking NA activity) has been described for NDV and other paramyxoviruses. It is located at the interface of the monomers of the dimeric units of the HN protein that form the protein tetramers, and is believed to participate in fusion promotion (Palermo et al., 2007; Zaitsev et al., 2004). Crystallographic studies, in which the HN crystals are soaked in solutions of the inhibitor 2-deoxy-2,3-dehydro-N-acetyl neuraminic acid (Neu5Ac2en), revealed that the receptor site presents features that are common to other NAs (Connaris et al., 2002). These include a highly conserved arginine triad at positions 174, 416 and 498, which is very sensitive to mutations and is essential for receptor recognition. Mutations at these residues decrease NA, fusion promotion and haemagglutination (HA) activities to different extents (Connaris et al., 2002). Unique to HN of paramyxoviruses is a large cavity found around the O⁴ position of the receptor sialic acid molecule (when HN is bound to its receptor), which is thought to be required to effect the conformational changes associated with fusion promotion. This conserved cavity is lined with several hydrophobic amino acids, including Arg174, Ile175, Ile192 and Asp198 (Ryan et al., 2006). Previous research work has shown that mutations at positions Asp198 and Arg174 decrease receptor recognition and fusion promotion (Connaris et al., 2002; Li et al., 2004), while mutations at Ile175 can induce an increased or decreased fusogenic phenotype, depending on the strain of NDV from which the HN gene has been obtained for the in vitro work (Li et al., 2004). Receptor recognition requires the concerted interaction of some of these conserved residues with different parts of the sialic acid molecule that serves as a receptor for the virus. The major anchor points for the receptor molecule are the triad of arginine residues R174, R416 and R498, which interacts with the sialic acid carboxyl group; residues E258 and Y317, which interact with the glycerol moiety of the molecule; and residue Y299 which interacts with the methyl group of the acetamido moiety of the receptor (Connaris et al., 2002). Upon binding to the sialic acid receptor, a series of amino acid movements have been observed in crystallographic studies of the HN protein. For instance, residue R174 moves from its normal position to the large acidic cavity that is found around the O⁴ atom of the receptor molecule (Ryan et al., 2006). Movement of residue Arg174 leads to further shifts in other amino acids positions, such as Tyr526 and Lys236, which are thought to be important for the catalytic activity of the protein (Connaris et al., 2002; Crennell et al., 2000; Ryan et al., 2006). Also important for fusion promotion are structural changes that propagate through the surface of the protein upon receptor binding and that result in activation of the second binding site located in the HN-dimer interface (Zaitsev et al., 2004). After cleavage of the sialic acid receptor, a loop containing residue Asp198 moves from its original position; this leads to movement of the loops that form the second binding site including one involving residue 169, another involving residues 516, 517 and 519, and a third loop involving residues 552 and 553 (Zaitsev et al., 2004). Binding of sialic acid to this second binding site drives the F protein to its fusogenic state, thus ensuring fusion.

In our laboratory, the mesogenic NDV strain Anhinga/US(FL)/44083/93 (Anh) was previously used to generate an infectious clone and for reverse genetics studies using this viral strain (Estevez et al., 2007). During rescue, one of the recombinant viruses obtained showed a marked increase in fusogenic activity in vitro and low haemagglutinating activity as compared with the parental strain. Sequence analysis of the genome of this virus showed a single amino acid substitution at position 192 (Ile→Met) of the HN gene. This Ile amino acid at position 192 turned out to be highly conserved in the HN protein of most analysed NDV strains.

To date, no work has been performed characterizing HN proteins with mutations at position Ile192, despite its being a conserved residue in all NDV strains and despite it being part of a highly conserved structural element of the NDV HN protein. In the present study, we characterized an amino acid mutation of Ile to Met at position 192 in HN proteins from three different strains of NDV in vitro, and in vivo for a recombinant virus harbouring this mutation.

RESULTS

Mutation I192M in expressed NDV HN proteins decreases NA activity and haemadsorption, and increases fusion promotion

To determine if the mutation I192M in the HN of NDV affects its functionality, the transiently expressed mutant or wild-type HN proteins from the plasmids were assessed for NA activity, haemadsorption (HAd) and fusion promotion activity. As summarized in Table 1, Vero cell-expressed mutant HN proteins harbouring the I192M amino acid substitution showed a significantly decreased (P<0.05) NA activity in comparison with the wild-type protein in the viral strains assessed (ranging from 8.7 to 16.22 % of that of the Anh-wt HN protein), as shown by the results of the fluorimetric NA test. This diminished activity was not due
to differences in expression levels of the proteins from the plasmid constructs since all the proteins were expressed to similar levels, as demonstrated by the ELISA test performed on transfected monolayers. Similarly, haemadsorption was diminished in the mutated forms of the protein when compared with wild-type (8.4–23.5 % of the levels found in the Anh-wt HN protein) regardless of the NDV strain used to obtain the HN genes, as observed in the HAd test performed (Table 1). Despite diminished HAd and NA activity, the mutant proteins showed a significant increase (P<0.05) in fusion promotion in the strains tested in this study, as evidenced by a two- to fourfold increase in the average number of nuclei in 20 fusion foci induced by the expressed mutant HN proteins, compared with that induced by the wild-type forms of these HN proteins (Table 2).

Recombinant viruses harbouring mutation I192M in the HN protein are viable and can be rescued by reverse genetics

Our laboratory has previously developed a reverse genetics system for pathogenesis studies using an infectious clone of the Anhinga strain of NDV. Taking advantage of this system, we rescued recombinant NDV of the Anhinga strain harbouring mutation HN I192M, named rAnh-I192M. The growth kinetics of rAnh-I192M were compared with those of the rescued wild-type Anhinga strain, rAnh-wt, in cell culture and in embryonated chicken eggs. Mutated rAnh-I192M virus grew to titres similar to those of the wild-type recombinant virus in embryonated chicken eggs (log10 9.4 and 9.1 TCID50 ml–1, respectively), while exhibiting an accelerated growth pattern in Vero-cell monolayers in comparison with wild-type virus, as demonstrated by the virus growth curve assay performed (Fig. 1).

Table 1. NA, cell expression of HN and HAd activities of wild-type and mutant HN proteins

The biological activities of the wild-type and mutated HN proteins are shown. All values are indicated as a percentage of the activity of the wild-type Anh HN protein in each test. NA and haemadsorption (HAd) activities are corrected for the level of protein expression. Strains: Anh, Anhinga/US(FL)/44083/93; TkND, Turkey/US(ND)/43084/92; CA02, Game fowl/US(CA)/211472/02.

<table>
<thead>
<tr>
<th>Protein</th>
<th>NA</th>
<th>HAd</th>
<th>Cell expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anh HN wt</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Anh HN I192M</td>
<td>8.7*</td>
<td>23.5*</td>
<td>106.9</td>
</tr>
<tr>
<td>TkND HN wt</td>
<td>106.1</td>
<td>101.9</td>
<td>106.6</td>
</tr>
<tr>
<td>TkND HN I192M</td>
<td>8.7*</td>
<td>8.4*</td>
<td>91.8</td>
</tr>
<tr>
<td>CA02 HN wt</td>
<td>106.2</td>
<td>62.3</td>
<td>99.3</td>
</tr>
<tr>
<td>CA02 HN I192M</td>
<td>16.2*</td>
<td>8.9*</td>
<td>109.3</td>
</tr>
</tbody>
</table>

*Statistically significant differences, P<0.05.

Table 2. Fusion promotion

The nuclei counts of fused cells are presented (mean of 20 fusion events).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Nuclei count (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anh HN wt</td>
<td>5.35</td>
</tr>
<tr>
<td>Anh HN I192M</td>
<td>12.50*</td>
</tr>
<tr>
<td>TkND HN wt</td>
<td>5.85</td>
</tr>
<tr>
<td>TkND HN I192M</td>
<td>27.60*</td>
</tr>
<tr>
<td>CA02 HN wt</td>
<td>4.80</td>
</tr>
<tr>
<td>CA02 HN I192M</td>
<td>10.40*</td>
</tr>
</tbody>
</table>

*The number of nuclei in fused cells was significantly different (P<0.05) when mutated and wild-type HN proteins of the same virus strain were compared.

The mutant virus rAnh-I192M shows an increased fusogenic phenotype and diminished HA and NA activities

When comparing the phenotypes of the rescued viruses in cell culture, it was found that Vero-cell monolayers infected with the same m.o.i. as wild-type or mutated virus showed marked differences in their fusion activities by 24 h post-inoculation. The rAnh-I192M virus induces larger fusion foci than wild-type virus (Fig. 2), which increased markedly by the second day post-inoculation (not shown). The microplate HA test performed with these viruses showed that mutant virus has a lower HA titre when compared with the wild-type strain (32 and 512, respectively). The NA activity of virus rAnh-I192M was
also diminished in comparison with virus rAnh-wt, mutated virus having roughly 24% of the NA activity of the rescued wild-type strain. These results are detailed in Table 3.

**Table 3. Biological activities and pathotyping tests of recombinant viruses**

<table>
<thead>
<tr>
<th>Virus</th>
<th>HA</th>
<th>NA</th>
<th>MDT (h)</th>
<th>ICPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>rAnh-wt</td>
<td>512</td>
<td>100%</td>
<td>89</td>
<td>0.91</td>
</tr>
<tr>
<td>rAnh-I192M</td>
<td>32</td>
<td>23.9%</td>
<td>85</td>
<td>1.10</td>
</tr>
</tbody>
</table>

**Presence of mutation I192M in the HN of the rAnh-I192M virus did not affect the virus pathotype**

The NDV Anhinga strain has been previously characterized as a mesogenic virus by standard pathotyping tests (King & Seal, 1998). The pathotype of rAnh-wt and rAnh-I192M viruses was assessed by the mean death time (MDT) and intracerebral pathogenicity index (ICPI) tests. As shown in Table 3, the MDT in embryonated chicken eggs was 89 h for rAnh-wt, and 85 h for rAnh-I192M virus, while the ICPI values were 0.91 and 1.10, respectively. These results indicate that these viruses can be classified as mesogens, according to the classification used by Alexander (1998). Despite diminished HA and NA activities, the I192M mutation in the recombinant virus did not result in a pathotype change.

**DISCUSSION**

In this study we have characterized the effects of a mutation at position 192 (Ile→Met) of HN proteins derived from selected NDV strains, as well as characterizing a recombinant virus harbouring this mutation, using *in vitro* and *in vivo* tests. This I192M mutation resulted in decreased HAd and NA activities and increased fusion promotion activity, but no pathotype change.

The HN protein of paramyxoviruses is highly important for the biology of this family of viruses. It determines tropism, mediates virus penetration by its interaction with the F protein and prevents virus aggregation during budding. Receptor recognition and the NA activity of the protein are topographically located to a cavity in the globular head of HN, which is lined with highly conserved amino acid residues, not only in paramyxoviruses but also in other known NAs (Connaris *et al.*, 2002; Ryan *et al.*, 2006). In our experiment, we have found a substitution for one of these conserved amino acids (I192M) that results in changes in the activity of the HN protein. This substitution of isoleucine with methionine, which is a non-polar amino acid, preserves the hydrophobic character of the conserved cavity. Our computer modelling shows that Ile192 is at the base of the loop that contains residue Asp198, a residue that is thought to be important for the catalytic activity of site I and whose movement is necessary for activation of the second binding site (Fig. 3a) (Zaitsev *et al.*, 2004). The side chain of the isoleucine is also part of the large conserved cavity unique to paramyxoviruses. The effects observed with the mutation I192M may result from several different mechanisms. The presence of the larger side chain of the methionine residue in the lining of the cavity in site I of the protein might prevent normal coupling of the sialic acid molecule in the binding site by steric hindrance or disruption of the native conformation of the binding site. This may account for the diminished HAd activity shown by the cell culture-expressed mutant HN protein, as well as for the low HA titre of the rescued virus harbouring the mutation.
The effect of the mutation on the activity of NA is interesting, as it may be related to the highly fusogenic phenotype of the mutant protein. As described previously, Met192 is located in the loop that contains residue Asp198 which is important for NA activity. Upon receptor binding this loop is located above the sugar ring of the substrate, where Asp198 is thought to participate in the hydrolysis of the molecule (Zaitsev et al., 2004). After hydrolysis, the loop moves away from the substrate, and this movement seems to propagate through the protein, resulting in activation of the second binding site and triggering of fusion by the F protein. It is conceivable that the mutation shifts this loop away from the active site, which may result in diminished contact of Asp198 with the substrate (thus diminishing NA activity), and causing movements in the protein that can trigger fusion promotion more effectively.

Previous studies have shown that the HN protein forms a tetramer in the viral surface, and that the stalk region is also implicated in the activation of the fusion activity of the F protein (Yuan et al., 2005). Met192 is located away from the stalk region, so it seems unlikely that the fusogenic phenotype of the mutant protein is the result of influences of the mutation on the stalk region.

As shown by the rescue of virus harbouring the mutated HN protein, mutation I192M is not disruptive for the biology of the virus. The rescued virus was infective in \textit{in vitro} systems as well as \textit{in vivo}. Interestingly, the rescued virus showed a growth advantage in Vero cell cultures. It is possible that, although the virus has a lower receptor affinity and diminished NA activity, the increased fusion promotion compensates for these phenotypes, resulting in increased virus titres in infected monolayers. This mutation did not result in attenuation of the virus, as shown by the pathotyping tests performed. This is interesting because it seems to indicate that the HA and NA activity levels are not correlated with pathogenicity in NDV. Moreover, these results indicate that there is some flexibility in these HN-protein functions, and also that wild-type levels of these activities are not absolutely required for virus infectivity.

In summary, we have shown that residue Ile192 can affect NA activity, fusion promotion and receptor recognition in NDV, regardless of viral strain. It is likely that the observed fusogenic phenotype is the result of changes in the dynamics of fusion triggering by sialic acid binding. It was also shown that mutation I192M is compatible with the biology of this virus and that it does not induce changes in the viral pathotype.

**METHODS**

**Cell cultures.** Vero-cell monolayers (CCL-81; ATCC) were used for plasmid expression of wild-type and mutated forms of HN proteins derived from the viruses used in this study. Rescue of recombinant viruses was performed using HEP-2 cell monolayers (CCL-23; ATCC). Propagation of rescued viruses was performed on 9-day-old embryonated chicken eggs (obtained from the specific pathogen free white leghorn flock maintained by Southeast Poultry Research Laboratory). All cell monolayers were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U ml$^{-1}$ penicillin, 100 µg ml$^{-1}$ streptomycin, 0.25 µg ml$^{-1}$ amphotericin B (Thermo Scientific) and 10 % FBS (Invitrogen) and kept at 37°C under a 5 % CO$_2$ atmosphere unless otherwise indicated.

**Viruses and viral RNA extraction.** The mesogenic NDV strain Anhinga/US(FL)/44083/93 (Anh) and the velogenic strains Turkey/US(ND)/43084/92 (TkND) and Game fowl/US(CA)/211472/02 (CA02), obtained from a repository bank at the Southeast Poultry Research Laboratory (USDA–ARS), were propagated in 9-day-old embryonated specific-pathogen free white leghorn flock maintained by Southeast Poultry Research Laboratory. All cell monolayers were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U ml$^{-1}$ penicillin, 100 µg ml$^{-1}$ streptomycin, 0.25 µg ml$^{-1}$ amphotericin B (Thermo Scientific) and 10 % FBS (Invitrogen) and kept at 37°C under a 5 % CO$_2$ atmosphere unless otherwise indicated.
Characterization of NDV HN mutation I192M

of Dr Bernard Moss, Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health) was used during viral rescue and plasmid-derived HN expression in vitro to provide the T7 bacteriophage polymerase (Wyatt et al., 1995).

Construction of HN- and F-protein expression plasmids. The entire coding regions of the HN and F genes of NDV strains Anh, TkND and CA02 were amplified from viral RNA by RT-PCR and ligated into protein-expression plasmid pTM1 (kindly provided by Dr B. Moss) (Moss et al., 1990). The plasmids obtained were named pAnh–HNwt, pTkND–HNwt, pCA02–HNwt, pAnh–F, pTkND–F and pCA02–F and encoded within them are the HN and F protein genes derived from the parental strains described. Transcription from these plasmids is driven by the bacteriophage T7 RNA polymerase. Primers used to amplify the HN and F coding regions are available upon request.

Construction of an infectious clone and supporting protein expression plasmids of the Anhinga strain of NDV. A reverse-genetics system for the generation of recombinant viruses of the NDV Anhinga strain was developed in our laboratory previously (Estevez et al., 2007). cDNAs encoding the complete complement of the genome of the NDV Anhinga strain, and the nucleoprotein (N), phosphoprotein (P) and polymerase (L) protein genes were generated by RT-PCR and ligated into a modified pBlueScript-based vector (complement of the virus genome) (Stratagene) or the pTM protein expression plasmid (N, P, L protein genes), as described elsewhere (Estevez et al., 2007). Transcription from the modified pBlueScript-based vector is also driven by the T7 RNA polymerase. The final construct containing the whole virus genome was named pFLC–Anhwt. Protein expression plasmids were named pTM–N, pTM–P and pTM–L.

Site-directed mutagenesis of HN proteins. Plasmids pAnh–HNwt, pTkND–HNwt, pCA02–HNwt and pFLC–Anhwt were modified to contain a single nucleotide mutation (T→A) at position 576 of the coding region of the HN protein. This mutation changes the highly conserved isoleucine residue at position 192 to methionine. The exchange was performed by site-directed mutagenesis using a QuikChange Multi Site-Directed Mutagenesis kit (Stratagene) following the manufacturer’s recommendations. Primers used for the mutagenesis reactions are available upon request. Plasmids harbouring the mutation were named pAnh–HNI192M, pTkND–HNI192M, pCA02–HNI192M and pFLC–AnhI192M.

Cell surface expression of wild-type and mutated HN proteins. Vero-cell monolayers in 96-well plate format were grown to 95% confluency overnight in DMEM containing 10% PBS without antibiotics. These monolayers were transfected with 0.1 μg of protein expression plasmid pAnh–HNwt, pTkND–HNwt, pCA02–HNwt, pAnh–HN192M, pTkND–HN192M or pCA02–HN192M, using 24 wells per plasmid construct. Transfection was performed with the aid of Lipofectamine 2000 transfection reagent (Invitrogen), at an inclusion rate of 0.2 μl per well. Recombinant MVA/T7 was added at an m.o.i. of 3. Transfected cell monolayers were incubated for 5 h at 37 °C and then the supernatant was discarded and replaced with DMEM containing 100 U ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin, 0.25 μg ml⁻¹ amphotericin B and 10% PBS. After 19 h of incubation, the cell surface expression of the HN protein was detected using a cell ELISA system as described by Bousse et al. (1995), with some modifications. Briefly, the cell monolayers were washed once with PBS and fixed, using a 4% paraformaldehyde solution, for 8 min at room temperature. The cells were washed with PBS, overlaid with 100 μl of a 1:250 dilution of an anti-NDV chicken polyclonal serum stock in PBS and incubated at 4 °C for 45 min. The cell monolayers were washed again and incubated with a 1:5000 dilution of the secondary rabbit anti-chicken antibody conjugated with HRP (Fitzgerald Industries) for 30 min at room temperature. After a final set of washes, the cells were reacted with 200 μl of a 1× solution of Sigma Fast OPD reagent (Sigma-Aldrich). After 30 min of incubation, the A₄₅₀ of the reaction was read in a Biotek Synergy HT Microplate Reader (Biotek Instruments).

Neuraminidase activity. NA activity was determined by a fluorimetric procedure as described by Potier et al. (1979), with some modifications. Vero-cell monolayers in 96-well-plate formats were transfected using 0.1 μg of protein expression plasmid encoding the wild-type NDV HN proteins (pAnh–HNwt, pTkND–HNwt, pCA02–HNwt) or the mutated proteins (pAnh–HN192M, pTkND–HN192M or pCA02–HN192M), and 0.2 μl of Lipofectamine 2000 and MVA/T7 (m.o.i. of 3), as described previously. After 18 h of incubation at 37 °C the transfected cells were washed with PBS and overlaid with 30 μl of 0.1 M sodium acetate buffer containing 1 mM of the substrate 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MU-Neu5Ac; Sigma-Aldrich). Cells were incubated for 1 h at 37 °C. The reaction was stopped by the addition of 0.25 M glycine buffer (0.25 M glycine in ethanol). The fluorescence of the 4-methylumbellifereone released in the supernatant after cleavage by the HN proteins present in the monolayers was determined using a fluorometer at wavelengths of 365 nm for excitation and 450 nm for emission.

HAd test. HA activity was assayed as described previously (Li et al., 2004) with modifications. Briefly, Vero cells in 24-well-plates were transfected with the same expression plasmids and procedures utilized in the NA activity assay. The transfected monolayers were washed with PBS, before adding a 3% (v/v) chicken red blood cell (CRBC) suspension in PBS, followed by incubation at 4 °C for 30 min. After removal of unbound CRBC by repeated washings with ice-cold PBS, bound cells were lysed using a red blood cell lysis buffer (30 mM Tris, pH 7.4, 5 mM EDTA, 150 mM NaCl, and 0.5% NP-40). A₅₅₀ was measured.

Fusion promotion. To assess the differences in the fusion promotion capability of wild-type versus mutated HN proteins, Vero-cell monolayers in six-well formats were transfected with 0.2 μg of pAnh–HNwt, pTkND–HNwt, pCA02–HN192M, pTkND–HNI192M or pCA02–HN192M, and 0.2 μg of the homotypic F protein of each of the NDV strains used. Differences in fusion promotion were assessed by counting the mean number of nuclei present in twenty fusion foci per HN protein type at 24 h post-transfection.

Viral rescue from infectious clones. Recombinant viruses encoding wild-type and mutated forms of the HN protein of the NDV Anhinga strain were generated by transfection of HEP-2-cell monolayers as described (Estevez et al., 2007). HEP-2-cell monolayers were grown to 90% confluency and co-transfected with plasmid pFLC–Anhwt or pFLC–AnhI192M, plus supporting plasmids pTM–N, pTM–P and pTM–L, with the addition of Lipofectamine 2000 (3 μl per well) and MVA/T7 (m.o.i. of 3). After 3 days of incubation, 400 μl of the cell supernatants were injected into the allantoic cavity of 9-day-old embryonated SPF chicken eggs to amplify the rescued viruses. These viral stocks were further amplified in eggs and their titres determined by infecting Vero-cell monolayers with 50 μl of serial tenfold dilutions of the original stock. Titres were calculated by the Reed & Muench (1938) method and are expressed as TCID₅₀.

Recombinant virus growth curves. Single-step growth curves were determined for the rescued Anhinga viruses encoding the wild-type and I192M mutant forms of the HN protein. Briefly, Vero-cell monolayers in six-well plate formats were infected at an m.o.i. of 10. Six hundred microlitre samples of cell supernatant were collected at 9, 20, 31 and 45 h post-infection, which were frozen at −80 °C until used. Virus titres of the collected samples were assessed by inoculation of Vero-cell monolayers (96-well formats) with 50 μl of serial tenfold dilutions (three replicates per dilution) of each of the time-point samples collected. Calculation of titres was performed using the Reed &
Muench (1938) method, taking as an end point the highest dilution in which the cytopathic effect of the virus was observed.

**Pathotyping of wild-type and mutated viruses.** Recombinant virus pathotypes were assessed using MDT in embryonated chicken eggs and ICPI tests as described by Alexander (1998). The MDT was performed with samples of viruses in allantoic fluid by inoculating 9-day-old SPF chicken eggs with 100 µl of a serial tenfold dilution in PBS, of the viruses encoding the wild-type or I192M mutant HN gene (five eggs per dilution). Embryo mortality was recorded daily by candling the inoculated eggs twice a day. The ICPI was assessed by inoculating day-old SPF chicks intraocularly with a 1:10 dilution of infective allantoic fluid of the wild-type and mutant viruses. The birds were observed daily for 8 days and scored normal, sick or dead. Calculations of the MDT and ICPI were performed as described (Alexander, 1998).

**Fusion phenotype, HA and NA activities of rescued viruses.** The fusion phenotype of the rescued viruses was assessed by inoculating Vero-cell monolayers with the wild-type and recombinant viruses at an m.o.i. of 0.5. Fusion foci were photographed at 24 h post-inoculation. HA titres of the viruses were obtained by using the microplate HA test. Fifty microlitres of a 1×10^6 TCID_{50} ml^{-1} stock sample of each virus was used in triplicate to perform twofold serial dilutions in PBS, which were followed by the addition of 50 µl of a 0.5% CRBC solution (0.51% (v/v) in PBS). The HA titre was defined as the reciprocal of the last dilution in which HA of the chicken RBC was observed. The NA activity of the viral stocks was determined using the fluorometric assay as described previously, with modifications. Twenty microlitres of a 1×10^6 TCID_{50} ml^{-1} stock sample of each virus was diluted in 30 µl of 0.1 M sodium acetate buffer containing 1 mM of MU-Neu5Ac. The reaction was incubated at 37°C for 1 h, and stopped by adding 150 µl of the 0.25 M glycine buffer. The fluorescence of the 4-methylumbelliferone released during the reaction was determined using a fluorometer at wavelengths of 365 nm for excitation and 450 nm for emission.

**Sequencing and molecular modelling.** All sequences were confirmed by dideoxy chain-termination sequencing, using an Applied Biosystems-PRISM fluorescent big dye sequencing kit and an ABI 3730 DNA sequencer (ABI). Nucleotide sequence editing, assembling and analysis were performed using the DNASTAR program (DNASTAR). The three-dimensional structures of the wild-type and I192M mutant HN proteins were generated with the aid of the Swiss-PdbViewer program (Guex & Peitsch, 1997), which is freely available at http://www.expasy.org/spdbv/. The structure of mutant I192M was modelled using the coordinates of the Newcastle HN crystal structure (PDB code 1EBU) and the program MODELLER (Martí-Renom et al., 2000).

**Statistical analysis.** Statistical significance of the differences found was analysed using a single factor ANOVA test followed by Tukey’s test, performed with the aid of the data analysis package included in the Excel program (Microsoft).

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**REFERENCES**


