Sialidase, receptor-binding and fusion-promotion activities of Newcastle disease virus haemagglutinin–neuraminidase glycoprotein: a mutational and kinetic study

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Mutations were generated in residues at the putative catalytic site of the haemagglutinin–neuraminidase (HN) protein of Newcastle disease virus Clone 30 strain (Arg498, Glu258, Tyr262, Tyr317 and Ser418) and their effects on its three associated activities were studied. Expression of the mutant proteins at the surface of HeLa cells was similar to that of the wild-type. Sialidase, receptor-binding and fusion-promotion activities were affected to different degrees for all mutants studied. Mutant Arg498Lys lost most of its sialidase activity, although it retained most of the receptor-binding activity, suggesting that, for the former activity, besides the presence of a basic residue, the proximity to the substrate molecule is also important, as Lys is shorter than Arg. Proximity also seems to be important in substrate recognition, since Tyr262Phe retained most of its sialidase activity while Tyr262Ser lost most of it. Also, Ser418Ala displayed most of the wild-type sialidase activity. However, a kinetic and thermodynamic study of the sialidase activity of the Tyr262Ser and Ser418Ala mutants was performed and revealed that the hydroxyl group of these residues also plays an important role in catalysis, since such activity was much less effective than that of the wild-type and these mutations modified their activation energy for sialidase catalysis. The discrepancy of the modifications in sialidase and receptor-binding activities in the mutants analysed does not account for the topological coincidence of the two sites. These results also suggest that the globular head of HN protein may play a role in fusion-promotion activity.

INTRODUCTION

Members of the family Paramyxoviridae such as Newcastle disease virus (NDV) are enveloped, negative-strand RNA viruses that encode two transmembrane glycoproteins: an attachment protein and a fusion protein. The attachment protein, the haemagglutinin–neuraminidase (HN) or sialidase, interacts with sialic acid located at the termini of host glycoconjugates through its haemadsorption (HAd) activity. Moreover, HN possesses sialidase activity to hydrolyse sialic acid residues from progeny virion particles to prevent virus self-aggregation (Lamb & Kolakofsky, 2001). In addition to these activities, the HN protein also displays a fusion-promotion activity.

HN is a type-II protein with a single transmembrane segment having a terminal globular head in which HAd and sialidase activities reside, together with residues probably involved in fusion promotion. The structure of the HN protein displays the six-bladed β-propeller fold typical of other known sialidases (Varghese et al., 1983; Crennell et al., 1993, 1994, 2000; Gaskell et al., 1995; Sagrera et al., 2001). Recently, the crystal structure of the NDV HN globular head has been determined, both alone and complexed with the inhibitor 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (Crennell et al., 2000), revealing an active site that shares many features with other sialidases: (i) a triarginyl cluster (Arg174, Arg416 and Arg498) that encircles the carboxyl moiety of the inhibitor; (ii) a glutamic acid residue (Glu547) that stabilizes the Arg174 boundary; (iii) a tyrosine residue (Tyr526) whose hydroxyl group is positioned near the C1–C2 bond of the inhibitor interacting with Glu401; and (iv) a hydrophobic interaction between the methyl group of the inhibitor and the face of Tyr299. Unlike other sialidases, the NDV HN crystal structure shows extensive interactions with all three hydroxyls of the inhibitor glycerol group, directly involving Glu258, Tyr262, Tyr317 and Ser418.

The above structural studies provide no evidence for a second sialic acid-binding site on HN and this suggests that this protein has a single site that could switch between being
a catalytic site and a binding site (Crennell et al., 2000). This notion is in agreement with the results of earlier papers that proposed a single site that would be responsible for both activities (Scheid & Choppin, 1974; Merz et al., 1981) or two sites in close proximity (Thompson & Portner, 1987). However, it differs from the findings of a large number of studies based on monoclonal antibody (mAb)-binding data and mutant studies, which favour the existence of two separate sites (Ray & Compan, 1986; Gorman et al., 1990; Mirza et al., 1994; Muñoz-Barroso et al., 1997; Iorio et al., 2001).

By contrast, the localization of the regions of the HN protein required for fusion promotion has not been fully elucidated. Although it is clear that the stalk region is crucial for fusion promotion (Sergel et al., 1993; Tanabayashi & Compan, 1996; Stone-Hulslander & Morrison, 1999), there is disagreement as regards the involvement of the transmembrane region and the globular head (Tsru dome et al., 1996; Stone-Hulslander & Morrison, 1999), there is disagreement as regards the involvement of the transmembrane region and the globular head (Tsru dome et al., 1996; Stone-Hulslander & Morrison, 1999). By contrast, the localization of the regions of the HN protein required for fusion promotion has not been fully elucidated. Although it is clear that the stalk region is crucial for fusion promotion (Sergel et al., 1993; Tanabayashi & Compan, 1996; Stone-Hulslander & Morrison, 1999), there is disagreement as regards the involvement of the transmembrane region and the globular head (Tsru dome et al., 1996; Stone-Hulslander & Morrison, 1999). In an attempt to shed some light on the controversy concerning the topological relationship of sialidase and HAd activities, we performed a mutational analysis of residues Glu258, Tyr262, Tyr317, Ser418 and Arg498, identified as part of the active site of the HN protein (Crennell et al., 2000). To investigate further the involvement of the above residues, a complete kinetic study of the sialidase activity was performed, along with a thermal inactivation assay, and we analysed the ability of the mutants to promote fusion.

METHODS

Viruses and cell lines. The lentogenic ‘Clone 30’ strain of NDV was obtained from Intervet Laboratories (Salamanca, Spain). HeLa cells obtained from the ATCC were grown in Dulbecco’s modified Eagle’s medium (DMEM; BioWhittaker) supplemented with L-glutamine (580 mg l⁻¹), penicillin (100 U ml⁻¹), streptomycin (100 μg ml⁻¹) and 10% heat-inactivated fetal bovine serum (FBS).

Recombinant plasmid vectors and site-directed mutagenesis. The hn and f genes were cloned in the pGEM-T Easy vector system (Promega) as described in Sagre rara et al. (2001). Mutagenesis was performed using the Stratagene QuickChange site-directed mutagenesis kit (Stratagene Cloning Systems), following the manufacturer’s instructions. The wild-type (wt) hn, mutated hn and f sequences from pGEM-hn and pGEM-f were subcloned between the NcoI and Sall sites of pTM1 (generously donated by Adolfo García-Sastre) so that HN and fusion (F) protein expression could be driven by the T7 promoter.

Transient-expression system. Wt and mutant proteins were expressed in HeLa cells using VTF7-3 vaccinia virus (Fuerst et al., 1986), a generous gift from Bernard Moss. Cell monolayers were infected with the recombinant vaccinia virus at an m.o.i. of 1. After 1 h at 37°C, the monolayers were washed with DMEM without serum or antibiotics and transfected with 6 μg DNA diluted in 50 μl OptiMEM I (Gibco-BRL) and 3 μl Lipofectamine 2000 reagent (Invitrogen) diluted in 100 μl OptiMEM I according to the protocols recommended by the supplier. After 6 h at 37°C, the medium containing the DNA–Lipofectamine 2000 complexes was removed and replaced with 2 ml fresh medium, after which the monolayers were incubated for an additional 24 h at 37°C.

Quantification of cell expression of HN by fluorescence-activated cell sorting (FACS) analysis. Transfected cells were removed from plates using a scraper, washed with PBS, pelleted by centrifugation and incubated for 15 min at room temperature in the presence of mAb against NDV HN protein (anti-HN mAb), a generous gift from Adolfo García-Sastre. After centrifugation, the cells were resuspended in PBS and incubated with anti-mouse immunoglobulins/FITC (Dako) for 15 min in the dark at room temperature, followed by the addition of 2 ml FACS lysing solution (Becton-Dickinson) and incubation for 10 min. The cells were then pelleted by centrifugation, washed with PBS and resuspended in 200 μl PBS for analysis in a FACSscan flow cytometer (Becton-Dickinson). Cells transfected with the vector alone were used as background.

Sialidase assays. HeLa cells transfected with wt or mutated HN protein expression plasmids were washed once in PBS, scraped from the dish, pelleted at 4°C, resuspended in lysis buffer (10 mM Tris/ HCl, 1 M NaCl, 10 mM CaCl₂, 2% Triton X-100, 1 mM PMSF, pH 7.5) and incubated on ice for 30 min. Lysates were then centrifuged at 120 000 r.p.m. for 30 min at 4°C. The pellets were removed and the supernatants were assayed for sialidase (EC 3.2.1.18) activity, determined by spectrophotometry using 2’-4-(methylumbelliferyl) -α-D-N-acetylenuraminic acid (4MU-N-acetylenuraminic acid) as substrate (García-Sastre et al., 1991).

Enzyme kinetics and thermal inactivation. The sialidase activity of the HN protein was assayed using substrate concentrations ranging from 0.05 to 10 mM. The kinetic data were fitted by non-linear least-squares regression with the Enzyme Kinetics version 1.0 module of Sigma Plot version 7.0 (Jandel Scientific) to two different models, Michaelis–Menten and substrate inhibition. Choice between fits was based on the goodness of each individual fit according to their R² values.

Thermal inactivation experiments of sialidase activity were performed using cellular extracts obtained from cells transfected with wt and mutant HN protein-expression plasmids as described above. Samples were incubated at the desired temperature and collected at different times and sialidase activity was assayed. Data representing loss of activity according to the time of incubation were fitted to an exponential decay equation and the velocity constant of this decay was represented against temperature in the usual way for the Arrhenius equation (Sagrera et al., 1995). The activation energy (Ea) for the thermal denaturation of HN for wt and mutants was estimated.

HAd assays. HeLa cells (3·5×10⁵) grown in monolayers were infected with VTF7-3 vaccinia virus at an m.o.i. of 1 and transfected as described above. At 24 h post-transfection, HAd activity was evaluated as described by Iorio et al. (2001) with the modifications described by Ferreira et al., 2004.

Fusion assays. Cell fusion was quantified with a vaccinia-based reporter gene assay described previously (Nussbaum et al., 1994). Briefly, a monolayer of HeLa cells was infected with VTF7-3 vaccinia virus at an m.o.i. of 5. At 1·5 h post-infection, the cells were co-transfected with wt or mutated pTM1-hn and wt pTM1-f as described above and maintained at 37°C for 24 h. A second monolayer of the designated cell type was infected with the vaccinia virus VCB21R (kindly provided by Bernard Moss) at an m.o.i. of 5. At 1·5 h post-infection, cells were detached by trypsinization, suspended at a density of 2·5×10⁶ cells ml⁻¹ in DMEM supplemented with 2·5% FBS (DMEM-2%FBS) and placed in a CO₂ tissue-culture incubator at 31°C for 16 h. Following overnight incubation, the first monolayer was detached by trypsinization and both cell populations were washed once with DMEM-2%, centrifuged and resuspended in...
DMEM-2. Cell fusion was performed by incubating the two cell populations together for 6 h at 37 °C, as described previously (Nussbaum et al., 1995). β-Galactosidase activity was then quantified as described by Nussbaum et al. (1995), except that NP-40 was added to each well to a final concentration of 1% (v/v) and rates of substrate hydrolysis were determined at 37 °C for 30 min. Background absorbance was calculated from cells expressing the vector alone and subtracted.

RESULTS AND DISCUSSION

The recombinant plasmid pTM1-hn was used to express the HN protein in HeLa cells. Protein expression was analysed by FACS, using an anti-HN mAb (Fig. 1). To characterize the expression of HN protein at the cell surface, we decided to analyse certain other parameters of the sialidase activity of the NDV HN protein. In particular, we examined the optimum pH, the enzyme kinetics and the thermodynamics.

Analysis of the pH-dependence of wt HN sialidase activity revealed an optimum pH of 6-5 (data not shown), a value similar to the optimum pH of the HN sialidase activity reported by our group for intact viruses (García-Sastre et al., 1991). When sialidase activity versus substrate concentration was studied (not shown), we found that the best fit for sialidase kinetics corresponded to the substrate inhibition model ($R^2 = 0.98193$) at a significant level in comparison with the Michaelis–Menten model ($R^2 = 0.95799$). We found similar results for two of the mutants studied.

![Fig. 1](http://vir.sgmjournals.org)

**Fig. 1.** Analysis of the cell-surface expression of mutant proteins by flow cytometry. Cells transfected with 1 μg DNA per 35 mm plate were processed for analysis by flow cytometry as described in Methods. The primary antibody was an anti-HN mAb. Each panel shows background (cells transfected with vector alone and incubated with both primary and secondary antibodies) and cells expressing the HN wt protein, as well as cells expressing the indicated HN mutants. The figure shows the results of one experiment. Identical results were obtained in a duplicate experiment using different preparations of mutant DNA. FL1-Height, Fluorescence gated for FITC-labelled antibody.
Table 1. Kinetic constants for the substrate inhibition model for sialidase activity and $E_A$ of wt and the HN mutants Tyr262Phe and Ser418Ala

Kinetic data corresponding to the sialidase activity of the HN protein were fitted by non-linear least-squares regression to the substrate inhibition model, as described in Methods. $E_A$ was calculated from the Arrhenius plots of data for thermal denaturation. Data are means±SEM of three determinations. $T^*$ is the temperature at which a first-order rate constant of denaturation is equal to 1 min$^{-1}$ (Kurganov et al., 1997).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>wt HN</th>
<th>Tyr262Phe</th>
<th>Ser418Ala</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$ (nmol min$^{-1}$ ml$^{-1}$)</td>
<td>767·5±39·15</td>
<td>564·6±74·99</td>
<td>921·5±72·18</td>
</tr>
<tr>
<td>$K_m$ (mM)</td>
<td>0·5±0·05</td>
<td>4·9±0·91</td>
<td>1·1±0·15</td>
</tr>
<tr>
<td>$K_i$ (mM)</td>
<td>24·7±5·52</td>
<td>28·1±13·65</td>
<td>16·2±3·92</td>
</tr>
<tr>
<td>$E_A$ (kcal mol$^{-1}$)†</td>
<td>84·8±1·9 [20·3±0·5]</td>
<td>92·6±4·0 [22·1±1·0]</td>
<td>73·9±1·6 [17·7±0·4]</td>
</tr>
<tr>
<td>$T^*$ (°C)</td>
<td>50·8±0·2</td>
<td>48·9±0·2</td>
<td>49·9±0·2</td>
</tr>
</tbody>
</table>

†Values in square brackets are kJ mol$^{-1}$.

Tyr262Phe and Ser418Ala (Table 1), as discussed below. In the current model of substrate inhibition, at high substrate concentrations, a second substrate molecule binds to the enzyme–product complex, producing a non-productive or less-productive complex and lowering the overall reaction velocity (Taylor, 2002).

The kinetic constants for the substrate inhibition model are shown in Table 1. As deduced from the $K_m$ values, the sialidase activity of the wt HN expressed in cell culture and in intact virus proved to be similar: 0·5 mM (Table 1) and 0·49 mM (Sagrera et al., 1995), respectively. Despite this, we did detect differences with respect to $K_i$, the inhibition constant for the substrate 4MU-N-acetylneuraminic acid: the $K_i$ calculated for wt HN expressed in cell cultures (24·7 mM; Table 1) was almost threefold higher than that of intact virus (8·44 mM; Sagrera et al., 1995), indicating that there was a smaller inhibition effect exerted by the second substrate molecule in the intact virus HN. This difference can be explained by considering the higher packing degree of viral proteins in the virion, where the lipid:protein ratio is 0·21 (Muñoz-Barroso et al., 1997), while at the surface of HeLa cells the density of HN is lower. It has been proposed that the NDV HN protein is a more flexible molecule than other sialidases (Crennell et al., 2000). Thus, the lower packing degree at the HeLa cell membrane may permit the protein to exclude more easily the second substrate molecule bound to the active site and to proceed faster with hydrolysis, thereby lowering substrate inhibition.

Finally, to investigate further the appropriateness of our expression system, we studied the $E_A$ of the sialidase activity (Sagrera et al., 1995), which may be correlated with the rigidity of the protein active site: the higher the value of $E_A$, the more energy is required for catalysis. Our data indicated that this parameter was very similar in wt HN expressed at the cell surface [84·8 kcal mol$^{-1}$ (20·25 kJ mol$^{-1}$); Table 1] and in HN in the membrane of intact viruses [84·2 kcal mol$^{-1}$ (20·11 kJ mol$^{-1}$); Sagrera et al., 1995]. In conclusion, the expression of the protein at the Hela cell surface with the gene expression system used by us appears to be a good model to study HN sialidase properties.

To elucidate the involvement of certain conserved residues in the HN globular head in its three associated activities, we examined the effect of mutations in residues at the catalytic site that are largely conserved across the family Paramyxoviridae. Based on the crystal structure of the NDV HN protein (Crennell et al., 2000), a total of nine individual conservative and non-conservative substitutions were performed for amino acids from the active site that are highly conserved in different NDV strains: Glu258, Tyr262, Tyr317 and Ser418 (thought to be involved in substrate recognition) and Arg498, a member of the catalytic Arg triad (Fig. 2). The following substitutions were constructed: Glu258Asp, Tyr262Phe, Tyr262Ser, Tyr317Phe, Tyr317Ser, Tyr317Ala, Ser418Ala, Arg498Lys and Arg498Gln. All nine mutant proteins were detected at the cell surface (Fig. 1) and the number of positive fluorescent cells was similar for all the mutants (Table 2).

The activities of sialidase, receptor binding and fusion promotion were assayed for the mutant proteins (Table 2). Since the Tyr262Phe and Ser418Ala mutants were the only ones to exhibit 50 % or more of the wt activity, we were able to study certain other parameters as discussed below.

The importance of the Arg triad at the sialidase active site was confirmed when mutagenesis was carried out on Arg498. When this residue was replaced by another basic residue such as Lys, whose side-chain is shorter in length, most of the sialidase activity disappeared (Table 2). This suggested that, although a basic residue is needed, a closer contact between that residue and the substrate is necessary. It was also interesting that the same result was found for other residues believed to be important for substrate recognition (Crennell et al., 2000; Watson et al., 2003), namely Tyr262, Glu258 and Ser418. When Tyr262 (a polar, neutral residue) was mutated to Ser (also polar and neutral but much shorter in length), almost all the sialidase activity was lost (Table 2). However, when mutated to Phe (similar
in length but non-polar), most of the sialidase activity was retained, also suggesting that the aromatic ring might be important. When Glu258 (acidic) was mutated to Asp (acidic but shorter), again most of the activity was lost. When Ser418 (polar, neutral) was changed to Ala (non-polar, but similar in size), most of the sialidase activity was retained. These results suggested that the hydroxyl group in Tyr and Ser is irrelevant for catalysis. However, the kinetic parameters of the sialidase activity for Tyr262Phe and Tyr317Phe (Table 1) suggested that the mutated enzymes were much less effective (their $K_m$ was much higher than that of the wt), supporting the idea that hydroxyl groups do play an important role in the active site.

When Ser418Ala, this mutant showed a lower $K_i$ for substrate recognition, lower Sialidase HAd Fusion-promotion activities of each mutant were calculated as described in Methods and expressed as a percentage of wt NDV HN. Cell-surface expression was determined by FACS analysis and is also expressed as a percentage of wt NDV. Data were normalized to the cell-surface expression of wt NDV HN. Sialidase inhibitor 2-deoxy-2,3-dehydro-$N$-acetylneuraminic acid.

**Table 2. Functional profiles of putative sialidase active-site mutants**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Cell-surface expression</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sialidase</td>
</tr>
<tr>
<td>Glu258Asp</td>
<td>140.1 ± 21.5</td>
<td>8.4 ± 4.1</td>
</tr>
<tr>
<td>Tyr262Phe</td>
<td>111.5 ± 9.7</td>
<td>64.0 ± 1.7</td>
</tr>
<tr>
<td>Tyr262Ser</td>
<td>127.5 ± 20.2</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td>Tyr317Phe</td>
<td>114.1 ± 11.2</td>
<td>10.4 ± 5.5</td>
</tr>
<tr>
<td>Tyr317Ser</td>
<td>120.5 ± 15.3</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>Tyr317Ala</td>
<td>112.9 ± 8.1</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>Ser418Ala</td>
<td>114.1 ± 3.7</td>
<td>89.7 ± 9.4</td>
</tr>
<tr>
<td>Arg498Gln</td>
<td>170.1 ± 9.8</td>
<td>4.9 ± 3.2</td>
</tr>
<tr>
<td>Arg498Lys</td>
<td>139.7 ± 16.4</td>
<td>10.9 ± 3.7</td>
</tr>
</tbody>
</table>

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*Fig. 2.* Three-dimensional location of the mutated amino acid residues in the HN active site in relation to the sialic acid-binding site. Data were obtained from the coordinates deposited in the Protein Data Bank with accession number 1e8u (HN hexagonal crystal form complexed with sialic acid at high pH; Crennell *et al.*, 2000). Neu5Ac2en, Sialidase inhibitor 2-deoxy-2,3-dehydro-$N$-acetylneuraminic acid.
non-polar residues play an important role in thermodynamic parameters of the protein such as $\Delta C_p$, which influences the protein $E_A$. The presence of more non-polar residues in hydrophobic cores may decrease their stability (Loladze et al., 2001). It is thus possible to speculate that Ala at position 418 might have a different orientation from that of Ser and, since interactions with water could then not be established, the non-ordered segment would be more disordered or in a less stable state. By contrast, mutant Tyr262Phe, located in a helical segment ($\alpha_1$), proved to be more stable than the wt. This discrepancy may be due to the fact that this helix is an ordered structure and also that it is located in a more hydrophilic area of the protein. Since Phe is a more hydrophobic residue than Ala, it could render the helix segment more stable, thereby making the active site more rigid or leading it into a more stable state.

Based on mAb-binding data and the sequencing of escape mutants, many references in the literature favour two separate sites for HAd and sialidase activities (Ray & Compan, 1986; Gorman et al., 1990; Mirza et al., 1994; Iorio et al., 2001). Also, studying the influence of some lipids on both activities, our group (Muñoz-Barroso et al., 1997) observed a differential effect, also supporting the existence of two different sites. In addition, using a 1,1-bis(4-anilino)naphthalene-5,5-disulfonic acid assay, we have recently shown that gangliosides, acting as receptor mimics, did not compete with N-acetylmuraminic acid for binding (Ferreira et al., 2004), although both provoked a conformational change in the HN protein. Nevertheless, early papers suggested that a single site could be responsible for both functions (Scheid & Choppin, 1974; Merz et al., 1981), whereas other studies have suggested the close proximity of the two sites (Thompson & Portner, 1987). In this sense, based on their X-ray data of the globular head of the HN protein (lacking the first 123 residues of the N-terminal portion of the polypeptide), Crennell et al. (2000) suggested the existence of a unique, interchangeable site with two states, a sialic acid-binding state and a catalytic state.

Although the target residues chosen in the present work have been assigned to the active site of the sialidase activity of HN (Crennell et al., 2000), it is not known whether they might affect the other two protein activities, HAd and fusion promotion. Therefore, to analyse the possible involvement of the mutant residues in these activities, we also evaluated HAd activity and fusion promotion. Our results indicated that the mutations performed at the sialidase active site modified the HAd activity to different degrees (Table 2): in four of the nine mutants characterized, Glu258Asp, Tyr317Phe, Arg498Gln and Arg498Lys, sialidase activity was practically abolished (5–10% that of the wt), while significant HAd activity was detected (67–94% that of the wt); in two of the mutants, Tyr262Phe and Ser418Ala, both activities were slightly reduced or even increased; and, in three of the constructions, Tyr262Ser, Tyr317Ser and Tyr317Ala, both sialidase and HAd activities were strongly decreased. Thus, according to our data, Glu258 and Arg498 play a key role in sialidase activity but are less critical for receptor binding, whereas, as discussed above, Tyr317 is critical for catalysis but only its aromatic ring is essential for the HAd activity. The above results, however, do not allow us either to confirm or reject the topological coincidence of the sialidase and HAd sites. Despite this, assuming the single-site hypothesis, our results lead us to pose the following question: why is sialidase activity reduced to a greater extent than HAd activity? The answer could be that catalysis requires more precise interactions between the substrate and the active site than carbohydrate recognition (the binding site). However, if there is a single site, what triggers the switch from binding to catalytic activity? With a single site, after virus binding, sialidase activity would release the sialic acid residue from the receptor and this would detach the virus from the cell, preventing membrane fusion and viral entry. Thus, there could be two different and closely located sites, sharing a domain affected by the mutations and influencing the two sites to different extents. When some of the major anchor points, such as Arg498 or Glu258, were mutated, the resulting proteins lost their sialidase activity, whereas receptor-binding activity persisted (Table 2). Another possibility, which does not exclude the above, is that these mutations might alter the mechanism that allows the switch between the two states in favour of the HAd state.

Our data support the results of previous recent site-directed mutagenesis studies on conserved residues belonging to the active site of the sialidase protein (Iorio et al., 2001; Connaris et al., 2002). Connaris et al. (2002) characterized 19 mutants, including Glu258Asp, Tyr317Phe and Arg498Gln, obtaining similar results to ours concerning the loss of sialidase activity. However, our findings for Arg498Gln differ from those of Connaris et al. (2002) in the sense that we obtained a significantly higher cell-surface expression and HAd activity. With regard to fusion-promotion activity, their three mutants showed 80% of that of the wt (Connaris et al., 2002), while we detected levels of activity of 0–20% (Table 2). These differences may be due to the different NDV strains and/or the different cell types used for expression. In fact, we observed that the cell line used affects HN expression (data not shown).

All the mutations studied in the present work reduced the fusion-promotion activity of the protein. Therefore, our results suggest that the globular head of the HN protein could in some way be involved in fusion promotion. Much of the work generating HN chimeras or different mutants has indicated that the stalk region, as well as the globular head of the HN protein, are involved in the specific HN–F interactions necessary for fusion promotion (Bousse et al., 1994, 1995; Deng et al., 1995; Tsurudome et al., 1995, 1998; Tanabayashi & Compan, 1996). Recently, Takimoto et al. (2002) suggested that the hydrophobic area at the surface of the HN globular head must play an important role in fusion promotion. It is possible that, if its receptor recognition activity is modified, the HN protein would not interact.
properly with the target cell and hence it would not be possible for the F protein to undergo the conformational change required to trigger membrane fusion. This could be the case for mutants in which both HAd activity and fusion promotion were similarly affected, such as Tyr262Ser, Tyr317Ser or Tyr317Ala (Table 2). Nevertheless, the mutant Tyr317Phe showed an HAd activity that was similar to or slightly lower than that of the wt, while fusion promotion was substantially reduced. Tyr317 is located in the β-strand of residues 311–320 and is in close contact with sialic acid in the active site. Since its hydroxyl group is not present in Phe, the mutant may have lost its spatial arrangement in this domain, leading to structural changes in HN in such a way would modify the capacity of HN to interact with the F protein to promote fusion. In this sense, our thermal stability analyses revealed that mutant Tyr262Phe was more stable than the wt (Table 1), implying that structural changes in the protein were possible.

In conclusion, we have shown that some residues that mediate HN sialidase activity are not critical for its receptor-binding capability. It remains to be determined why the loss of sialidase activity also resulted in a loss of fusion-promotion activity. Changes in the overall structure of the protein might modify regions involved in HN–F interactions leading to fusion.

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