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Relative Affinity of the Human Parainfluenza Virus Type 3 Hemagglutinin-Neuraminidase for Sialic Acid Correlates with Virus-Induced Fusion Activity

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The ability of enveloped viruses to cause disease depends on their ability to enter the host cell via membrane fusion events. An understanding of these early events in infection, crucial for the design of methods of blocking infection, is needed for viruses that mediate membrane fusion at neutral pH, such as paramyxoviruses and human immunodeficiency virus. Sialic acid is the receptor for the human parainfluenza virus type 3 (HPF3) hemagglutinin-neuraminidase (HN) glycoprotein, the molecule responsible for binding of the virus to cell surfaces. In order for the fusion protein (F) of HPF3 to promote membrane fusion, the HN must interact with its receptor. In the present report, two variants of HPF3 with increased fusion-promoting phenotypes were selected and used to study the function of the HN glycoprotein in membrane fusion. Increased fusogenicity correlated with single amino acid changes in the HN protein that resulted in increased binding of the variant viruses to the sialic acid receptor. These results suggest that the avidity of binding of the HN protein to its receptor regulates the level of F protein-mediated fusion and begin to define one role of the receptor-binding protein of a paramyxovirus in the membrane fusion process.

Enveloped viruses have evolved mechanisms of protein-mediated membrane fusion that enable them to infect their hosts; these strategies have begun to shed light on membrane fusion mechanisms utilized by cells (2). The envelope of the paramyxovirus human parainfluenza virus type 3 (HPF3) contains two viral glycoproteins, the hemagglutinin-neuraminidase (HN) and the fusion protein (F). The HN glycoprotein binds to sialic acid-containing receptor molecules on the cell surface to initiate infection (24). Fusion of the viral envelope with the cell's plasma membrane is mediated by the F glycoprotein and occurs at neutral pH at the cell surface, without a requirement for an acid-mediated conformational change of the fusion molecule (8, 21, 25). This feature distinguishes paramyxoviruses from viruses, such as influenza virus, that require a low pH to mediate fusion (reviewed in reference 13). Infection results in fusion between adjacent cells, which involves the interaction of F protein, expressed on the surface of an infected cell, with the membrane of an adjacent uninfected cell.

We have reported that the HN glycoprotein is essential for cell fusion mediated by HPF3 and that interaction of HN with its receptor is required in order for F to promote membrane fusion during viral infection (18). We now report that the avidity of binding of virus to the sialic acid receptor correlates with the level of F protein-mediated fusion.

Syncytium formation by wild-type (wt) HPF3 can be prevented in a low-multiplicity infection by the addition of neuraminidase to the cells; by removing some cell surface sialic acid receptors, the neuraminidase treatment prevents the requisite interaction of the wt viral HN glycoprotein with its receptor (19). While the neuraminidase-treated infected cells do not fuse, the viral infection spreads throughout the culture, and all the cells become persistently infected (19, 20). Under these conditions, variant viruses which are able to fuse with cell membranes despite diminished receptor availability and which have a greatly increased ability to fuse cells under normal conditions are selected. These variants offer a new approach to the study of the mechanism of paramyxovirus-induced cell fusion and the role of the HN protein in this process.

MATERIALS AND METHODS

Virus and cells. CV-1 cells were grown in monolayers in Eagle's minimal essential medium supplemented with 10% fetal bovine serum. Stocks of wt HPF3 were made in CV-1 cells from virus that was plaque purified four times. Virus titer was determined by a plaque assay with CV-1 cells. Cells were infected by aspirating the medium, washing the monolayers with medium lacking serum, and then adding medium lacking serum with various amounts of wt or C variant HPF3 to the cells, as described in the text. After incubation at 37°C for 60 min, the medium was aspirated; either medium lacking serum or agar overlay medium was added. Cells were photographed through a phase-contrast microscope.

Neuraminidase treatment. Monolayer cultures of infected cells were treated with neuraminidase (from *Clostridium perfringens*; Sigma Chemical Co., St. Louis, Mo.) by washing the cells with medium lacking serum and then adding serum-free medium containing neuraminidase to the cells (19).

Isolation of HPF3 variants. Monolayer cultures of CV-1 cells in 60-mm dishes were infected with HPF3 and treated with 0.1 U of *C. perfringens* neuraminidase as described previously (19). Supernatant fluid from these infected cultures was collected and used in plaque assays. Large plaques were picked and plaque purified twice, and a single plaque was used to infect each CV-1 cell monolayer for preparation of stocks of variant viruses.

Protein analysis. ^{35}S-radiolabeled virus proteins were prepared as previously described (17). At 3, 6, or 9 h after infection with either wt or C variant HPF3, CV-1 cell monolayers were cultured for 15 min in methionine-free minimal

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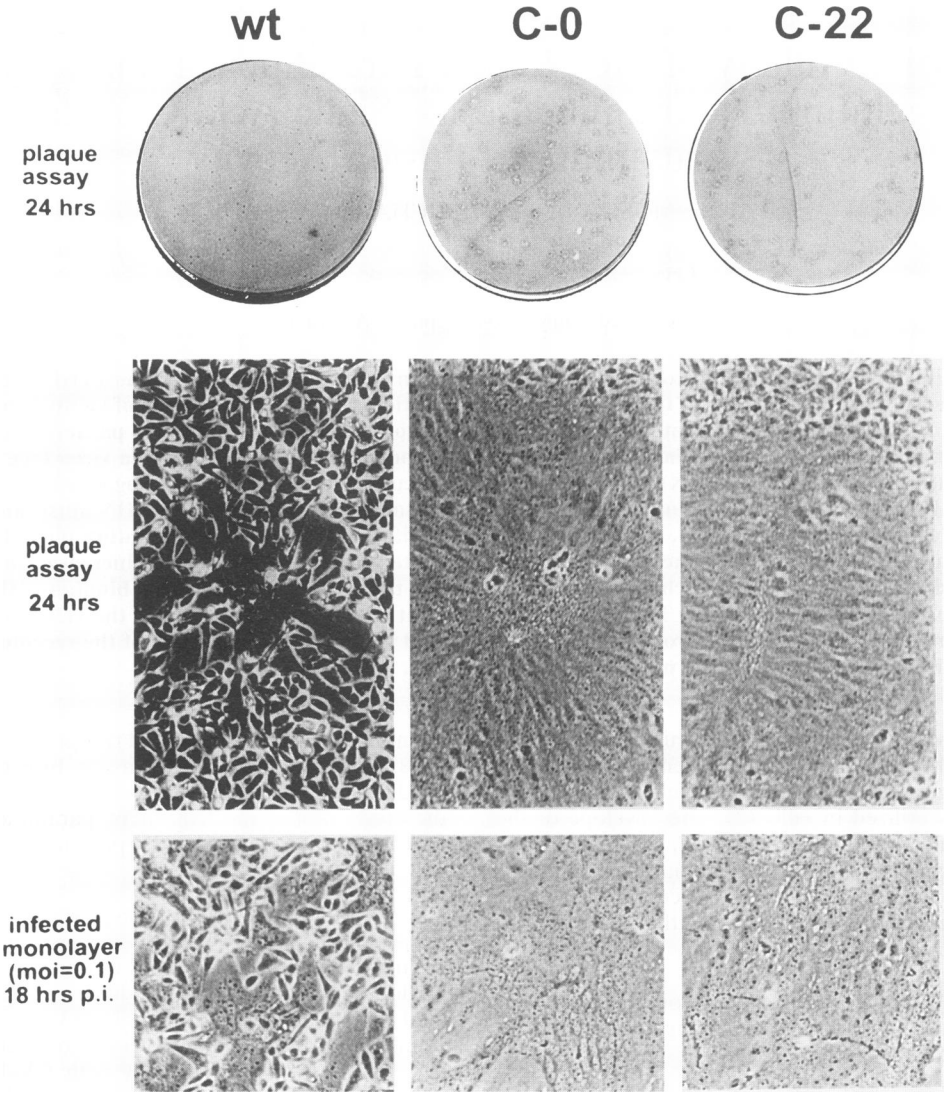

FIG. 1. Fusion properties of HPF3 variants. CV-1 cell cultures and HPF3 were grown as described in Materials and Methods. Confluent cell monolayers were infected with either wt HPF3 or C variant HPF3 at an MOI of 0.1 PFU/cell. For observation of plaques, overlay medium was prepared and added after a 90-min adsorption period. The photographs show the plaque size and morphology and the extent of CPE in a monolayer at 1 day postinfection (p.i.).

essential medium and then for 1 h in methionine-free minimal essential medium containing [^{35}S]methionine (50 μCi/ml). Mock-infected cell proteins were prepared in parallel with the infected cell proteins at each time point.

Assays of viral neuraminidase. Neuraminidase activities, substrate specificities, and pH optima were determined for wt HPF3 and C variants by the thiobarbituric acid assay (14). 2,3- and 2,6-neuraminyllactose substrates were purchased from Sigma Chemical Co.

Hemagglutination inhibition assays. Hemagglutination inhibition assays were performed at 4°C (22) with fetal calf serum (FCS) or bovine fetuin as the competitor. Four hemagglutination units of virus in 50 μl was added to each dilution of FCS (in 50 μl of phosphate-buffered saline [PBS]) or to dilutions of fetuin in the wells of a microtiter plate. Fifty microliters of a 0.5% suspension of human erythrocytes in PBS was then added to each well. The plates were photographed 90 min after the addition of erythrocytes.

Sequence analysis. The F and HN genes of C variant viruses were sequenced after reverse transcription-polymerase chain reaction (PCR) amplification of each gene, either by direct sequencing of the PCR product or by cloning into pUC19 and subsequent dideoxy sequencing of plasmid DNA. Total RNA from infected cells was prepared as described previously (17). The primers used for the reverse transcriptase reaction were complementary to the 3′-terminal 19 nucleotides of the F gene mRNA sequence (5) and the 3′ 32 nucleotides of the HN gene mRNA sequence (27). The upstream primers used for PCR amplification corresponded to the 5′-terminal 15 nucleotides of the F gene mRNA sequence and the 5′-terminal 16 nucleotides of the HN mRNA sequence. Primers for sequencing were synthesized to be complementary to the F or HN gene

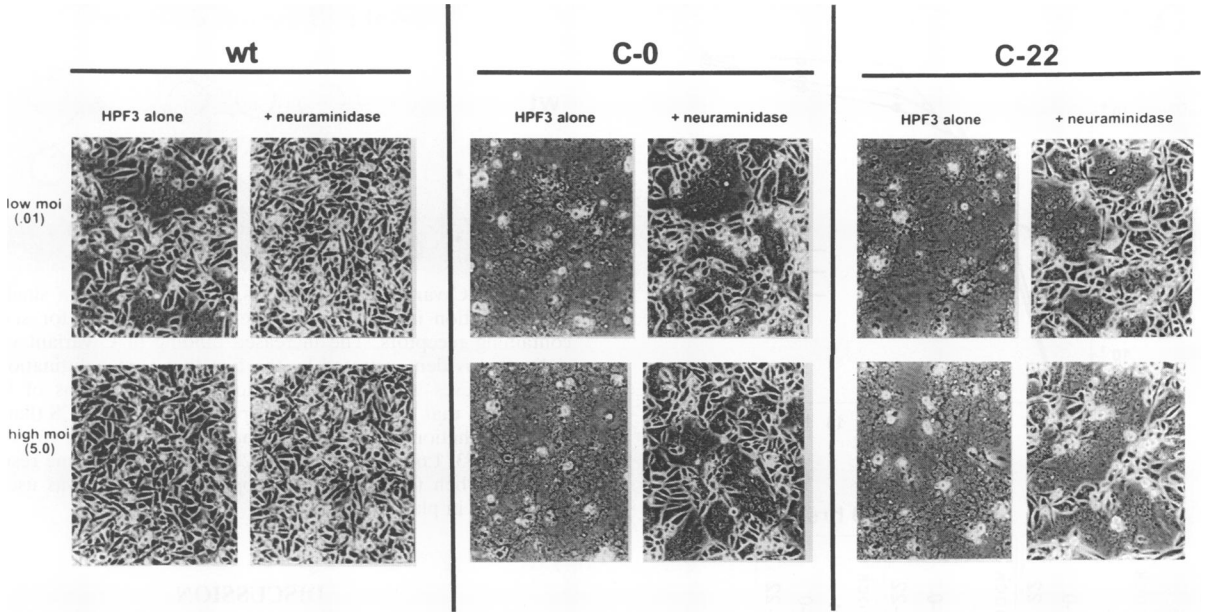

FIG. 2. Effect of MOI and of neuraminidase addition on CPE produced by HPF3 variants. Monolayer cultures of CV-1 cells were infected with wt or C variant HPF3 at either a low MOI (0.01 PFU/cell) or a high MOI (5 PFU/cell). For each virus, the cells were either infected with virus alone or received 0.1 U of *C. perfringens* neuraminidase after viral infection, as described in Materials and Methods. The photographs show CPE 1 day after infection. In cells infected with C variant HPF3, infection at a high MOI did not block fusion; the CPE in these cells is equivalent to that in those infected at a low MOI. In addition, in cells infected with variant HPF3, exogenous neuraminidase addition did not prevent cell fusion.

sequence at 200-bp intervals. Sequence alterations were confirmed by repeating at least twice the RNA isolation from infected cells, reverse transcription-PCR amplification, and sequencing of the altered region.

RESULTS

To further our understanding of the role of the HN glycoprotein in membrane fusion, we isolated and characterized two highly fusogenic variants of HPF3. Figure 1 shows the dramatic difference in plaque size and in cytopathic effect (CPE) on a cell monolayer between the wt and two HPF3 variants, C-0 and C-22. The HPF3 variants are highly fusogenic by several criteria: plaques (consisting of fused cells) form more rapidly and are much larger than wt plaques, and infection of a monolayer results in much more rapid and widespread cell fusion. These variants were found to have single amino acid changes in their HN glycoproteins and normal F proteins (see below).

For wt HPF3, the degree of cell fusion that occurs in a monolayer of infected cells depends on the multiplicity of infection (MOI); as the MOI increases, the extent of syncytium formation decreases (17, 19). We have shown that this effect is caused by the viral neuraminidase removing sialic acid residues from the target cell surface in proportion to the size of the viral inoculum (19). Larger inocula thus result in cleavage of a higher percentage of sialic acid residues, blocking cell fusion by wt virus. Figure 2 shows that the sialic acid receptor requirements for variant (C) HPF3-mediated cell fusion differ from those for wt HPF3-mediated cell fusion. For the HPF3 variants, cell fusion is not blocked by either high-MOI infection or exogenous neuraminidase treatment. These variants differ from the wt in their interaction with sialic acid-containing receptors during infection; the variants escape the inhibitory effect of neuraminidase on cell fusion and are able to fuse efficiently under conditions of decreased receptor availability.

We determined whether the variant fusion phenotype could be explained by altered growth characteristics of the virus. Figure 3 shows that the growth characteristics of the variant viruses do not differ from those of the wt. The rate of viral replication (Fig. 3A) and the rate of viral protein synthesis (Fig. 3B) are both similar in wt- and variant-infected cells. Figure 3B also shows that the relative amount of HN in wt- and variant-infected cells is similar, as determined by comparing the relative densities of the bands corresponding to HN and NP in each lane. Moreover, polyacrylamide gel electrophoresis analysis of virus particles released from wt- and variant-infected cells shows that the ratio of HN to NP in wt and variant virus particles is similar, as assessed by laser densitometry scanning of the Coomassie-stained gel (data not shown), suggesting that the incorporation of HN into virus particles is similar in the wt and variant viruses.

The F and HN genes of two fusogenic variants, C-0 and C-22, were sequenced. The variants have a wt F gene sequence. Each variant has one point mutation in the HN gene corresponding to a single amino acid change in the HN glycoprotein. An A to G mutation in variant C-0 converts threonine 193 to an alanine, and a T to G mutation in variant C-22 converts histidine 552 to a glutamine. Hemagglutination inhibition assays (Fig. 4) revealed that the viruses containing variant HN glycoproteins show increased binding to the sialic acid receptor. The increased fusogenic activity of the variant viruses altered in HN thus results from increased binding of these variants to sialic acid-containing receptors. This finding suggests that a key component of HN's function in promoting fusion is its avidity of binding to sialic acid-containing receptors.

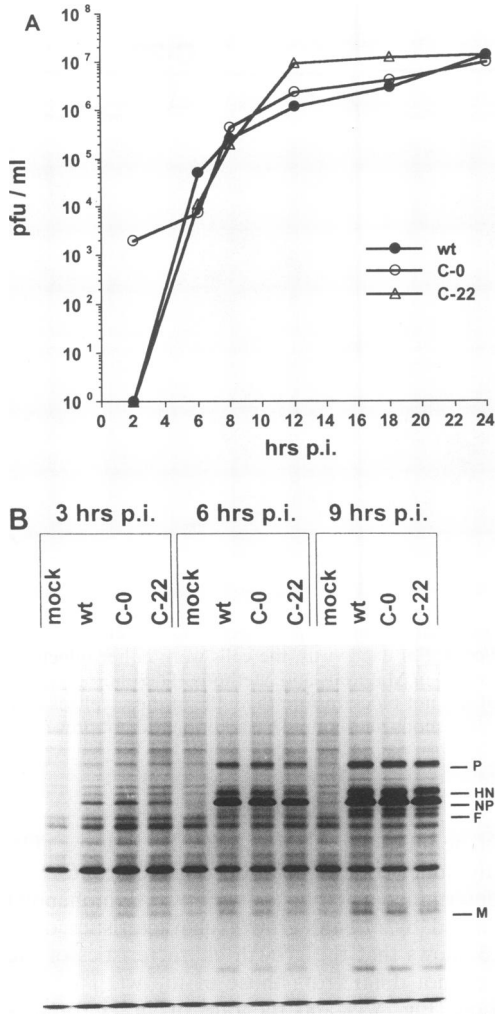

FIG. 3. Growth characteristics of C variants compared with those of wt HPF3. Monolayer cultures of CV-1 cells were infected with wt or C variant HPF3 at a high MOI (5 PFU/cell); these conditions of infection were chosen so that every cell would be infected at the time of the initial infection and growth parameters would not be influenced by fusogenicity. The rate of viral replication was determined by a standard plaque assay on virus released from infected cells and was similar in wt- and C variant-infected cells. The rate of viral protein synthesis was determined by metabolic labeling of infected cells and was similar in wt- and C variant-infected cells at 3, 6, and 9 h postinfection (p.i.).

We determined whether, in addition to altering virus binding, the mutations in the variant HNs affected neuraminidase enzymatic properties. The neuraminidase activity levels, substrate specificities, and pH optima of the HPF3 variants are not significantly different from those of the wt (data not shown). Each neuraminidase has an approximately 10 to 1 preference for the 2-3 sialic acid linkage over the 2-6 linkage, and each enzyme's pH optimum is 4.7. Neuraminidase activity, measured with *n*-acetylneuraminyllactose as the substrate, was unaffected by the amino acid substitutions in HN. The acidic pH optimum for HPF3 neuraminidase is in agreement with the pH optima reported for other paramyxovirus neuraminidases (14) and is intriguing in light of the fact that virus entry into and exit from cells, two postulated sites of neuraminidase action, occur at the cell surface at physiological pH.

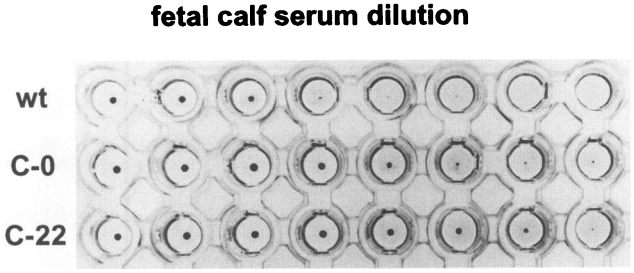

FIG. 4. C variant HPF3 viruses, each containing a single amino acid alteration in HN, have higher relative affinity for sialic acid-containing receptors. The increased binding of C variant viruses to sialic acid is demonstrated by the fact that hemagglutination by the variant viruses was inhibited by lower concentrations of fetal calf serum than that by the wt; the endpoint dilution of FCS that blocked hemagglutination by 4 hemagglutination units of each virus was 1:16 for wt HPF3, 1:64 for C-0, and 1:128 for C-22. The same results were obtained when fetuin (a sialoglycoprotein in FCS) was used as the competitor in place of FCS.

DISCUSSION

These studies begin to define the role of the receptor-binding protein of a paramyxovirus in the fusion process. The experiments assign a fusion helper function to the HPF3 hemagglutinin-neuraminidase, localize this function on the hemagglutinin-neuraminidase molecule, and reveal an essential function of the protein in the mechanism of membrane fusion. The interaction of HN with its receptor is one key determinant of the level of fusion protein-mediated membrane fusion. Importantly, these conclusions were reached in the context of natural viral infection, without introducing artifacts associated with heterologous viral expression systems.

The HPF3 variants that we describe were found to have single amino acid alterations in their HN glycoproteins and wt F proteins. This suggests that increased fusogenicity was determined by an alteration in the HN protein, providing evidence of the importance of HN in HPF3-mediated membrane fusion during natural virus infection. The locations of the amino acid changes responsible for the altered fusion phenotype provide clues about the site of one fusion-promoting function of the paramyxovirus HN glycoprotein.

While our results show that the attachment function of the HPF3 HN is an important component of HN's contribution to F protein-mediated fusion, this attachment function is clearly not the only way in which HN contributes to the fusion process; ample experimental evidence indicates that simple attachment is not sufficient for promoting subsequent fusion. In our previous experiments on the fusion of uninfected cells with cells persistently infected with HPF3, agglutination of the infected and uninfected cells by lectins did not substitute for the interaction of HPF3 HN with neuraminic acid in promoting cell fusion (18). Hu and coworkers showed that coexpression of HN and F from HPF3 resulted in cell fusion but that expression of HPF3 F with HPF2 HN did not, indicating that the heterologous HN attachment function is not sufficient to promote fusion (10). Horvath and coworkers found that the attachment function of simian virus 5 HN could not substitute for Newcastle disease virus (NDV) HN in promoting fusion by the NDV F protein (9), and Morrison and coworkers demonstrated that neither the attachment function of influenza virus hemagglutinin (16) nor that of Sendai virus HN (26) could substitute for NDV HN in promoting fusion mediated by the NDV F protein. In addition, a deletion mutant of the NDV

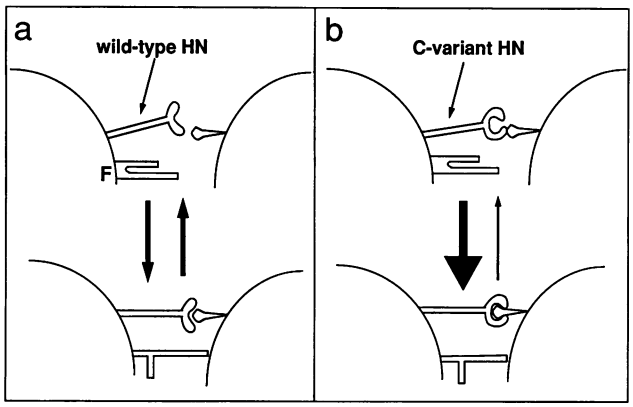

FIG. 5. Schematic diagram of possible interactions of (a) wt and (b) C variant HN with sialic acid receptors and of the proposed outcome of each type of interaction. One possible relationship of HN-receptor interaction to F protein-mediated membrane fusion and a possible effect of HN-receptor interaction on F protein conformation are diagrammed. For details, see the text.

HN retained its attachment function while losing its ability to promote fusion by the NDV F protein (26); this mutant confirms that HN's attachment function is not sufficient for fusion promotion but does not contradict the notion that attachment is one critical determinant of HN's ability to promote fusion. It is most likely that several steps, and several sites on the HN molecule, are involved in HN's complex role in fusion and that fusion promotion does not reside in only one site but requires several sequential events (see below). HN does not provide nonspecific attachment but rather plays a specific role in which attachment is a critical first step and determines the degree of subsequent fusion.

Information about the functional site(s) of the paramyxovirus HN has been lacking because the crystal structure of the molecule has not been determined. Recently, Colman et al. carried out a structural comparison between influenza virus neuraminidase and several paramyxovirus HNs (4). Since the crystal structure of neuraminidase had been determined and the functional sites mapped (3), this comparison allowed predictions about the three-dimensional structure and potential functional sites on the paramyxovirus HN. From these comparisons, the T193A and H552Q changes we identified in the C variant HN molecules are located in predicted active-site regions. T-193 is contained within a highly conserved sequence motif in HN that can be mapped to an active-site region in neuraminidase, corresponding to residues 118 to 121 (neuraminidase sequence numbering corresponds to the N2 sequence). In light of the fact that the T193A change promotes binding of virus to receptor without altering neuraminidase activity, it is significant that, of the four highly conserved regions of HN that correspond to regions on the active site of neuraminidase, this site is the only one to appear in the hemagglutinin protein of morbilliviruses, an attachment protein without neuraminidase activity. This further implicates the T-193 region in the attachment process. Interestingly, both mumps virus (28) and NDV (11) variants with decreased neuraminidase activity are altered in the residue corresponding to T-193; in these cases, this amino acid change affects an alternate aspect of HN-substrate interaction.

The H552Q change in C-22 HN corresponds to a position three amino acids away from an absolutely conserved residue in HN and neuraminidase (neuraminidase residue E-425), which, as determined from a comparison with bacterial neuraminidases, is a "framework" residue outside the active site, defined by Colman et al. as a residue that does not directly contact the substrate but interacts with functional residues to maintain the conformation needed for binding and catalysis (4).

Binding of HN to a receptor brings the viral membrane or infected-cell membrane near the target cell membrane; the efficiency of this process is a function of the affinity of HN for the receptor. Binding of HN to a receptor is thought to be low affinity, by analogy to the influenza virus hemagglutinin (6); in the equilibrium between the bound and unbound state, a variant HN with a higher relative affinity for the receptor would spend a higher proportion of time bound to the receptor. Figure 5 illustrates one hypothetical model of wt and variant HN function and of the potential interaction of HN with F during fusion. Specific interaction between HN and F has been proposed (10), and the possibility that attachment of HN to the receptor leads to activation of F has been suggested previously (26). If F functions only when an adjacent HN is bound to the receptor, then it is feasible that the variant HN allows the F protein more time in which to induce fusion. It is also likely that for any given receptor density, a higher proportion of the receptors are utilized by the variant viruses. We have postulated that cell fusion by HPF3 requires more sialic acid-containing receptors than are required for virus entry and spread; multiple points of contact may be required in order for two cell membranes to fuse, while fewer points of contact may be required for entry of a single virus particle (19). The behavior of the variants described here supports this model; increased virus binding allows more efficient use of the available receptors. It is possible that the fusion mechanism is similar to that proposed for human immunodeficiency virus, which also fuses cells at neutral pH. In the case of human immunodeficiency virus, it has been proposed that the binding of gp120-gp41 to cell surface CD4 receptors leads to a conformational change in this envelope glycoprotein, exposing epitopes required for fusion (1, 7, 12, 15, 23). By analogy, it is possible that binding of HN to a sialic acid-containing receptor activates a conformational change in an adjacent F glycoprotein, permitting fusion to occur.

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