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Influence of the Human Parainfluenza Virus 3 Attachment Protein's Neuraminidase Activity on Its Capacity To Activate the Fusion Protein

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In order to examine functions of the hemagglutinin-neuraminidase (HN) protein that quantitatively influence fusion promotion, human parainfluenza virus 3 (HPIV3) variants with alterations in HN were studied. The variant HNs have mutations that affect either receptor binding avidity, neuraminidase activity, or fusion protein (F) activation. Neuraminidase activity was regulated by manipulation of temperature and pH. F activation was assessed by quantitating the irreversible binding of target erythrocytes (RBC) to HN/F-coexpressing cells in the presence of 4-GU-DANA (zanamivir) to release target cells bound only by HN-receptor interactions; the remaining, irreversibly bound target cells are retained via the fusion protein. In cells coexpressing wild-type (wt) or variant HNs with wt F, the fusion promotion capacity of HN was distinguished from target cell binding by measuring changes with time in the amounts of target RBC that were (i) reversibly bound by HN-receptor interaction (released only upon the addition of 4-GU-DANA), (ii) released by HN′s neuraminidase, and (iii) irreversibly bound by F-insertion or fusion (F triggered). For wt HN, lowering the pH (to approach the optimum for HPIV3 neuraminidase) decreased F triggering via release of HN from its receptor. An HN variant with increased receptor binding avidity had F-triggering efficiency like that of wt HN at pH 8.0, but this efficiency was not decreased by lowering the pH to 5.7, which suggested that the variant HN′s higher receptor binding activity counterbalanced the receptor dissociation promoted by increased neuraminidase activity. To dissect the specific contribution of neuraminidase to triggering, two variant HNs that are triggering-defective due to a mutation in the HN stalk were evaluated. One of these variants has, in addition, a mutation in the globular head that renders it neuraminidase dead, while the HN with the stalk mutation alone has 30% of wt neuraminidase. While the variant without neuraminidase activity triggered F effectively at 37°C irrespective of pH, the variant possessing effective neuraminidase activity completely failed to activate F at pH 5.7 and was capable of only minimal triggering activity even at pH 8.0. These results demonstrate that neuraminidase activity impacts the extent of HPIV3-mediated fusion by releasing HN from contact with receptor. Any particular HN′s competence to promote F-mediated fusion depends on the balance between its inherent F-triggering efficacy and its receptor-attachment regulatory functions (binding and receptor cleavage).

Human parainfluenza virus 3 (HPIV3), like other paramyxoviruses, possesses two envelope proteins directly involved in viral entry and cytopathology. Hemagglutinin-neuraminidase (HN), by binding to sialic acid-containing cellular surface receptors, brings the viral envelope in proximity to the plasma membrane. Fusion protein (F) is the active mediator of fusion between the viral and cell membranes, which leads to release of the viral nucleocapsid into the cytoplasm. In order to mediate fusion, F must be present in its cleaved state and then undergo a second activation step to assume its fusion-ready conformation. It has been shown that HN plays an essential role in the fusion process, and one proposed role of HN is to drive the final conformational change in F that renders it fusion active (30). Fusion of the virus with the target cell, which permits synthesis of viral macromolecules and expression of viral envelope proteins on the host cell's surface, is followed by two processes that lead to the spread of infection and HPIV3 cytopathogenesis. One is the assembly and budding of new virions that then infect distant as well as neighboring cells. Second, by virtue of HN and F expressed on its surface, an infected cell can fuse with adjacent uninfected cells, forming syncytia. In the spread of infection via budding, HN′s receptor-destroying neuraminidase activity plays the essential role of preventing progeny virions from remaining aggregated on the infected cell's surface rather than spreading to additional cells (29). For syncytium formation, no direct role has yet been found for HN′s neuraminidase activity, although the magnitude of the receptor-cleaving activity modulates the rate of formation and size of syncytia (11, 29).

The present study dissects the contribution of each of three factors—receptor avidity, F triggering, and receptor cleavage—to HN′s effect on fusogenicity. Mutations in HPIV3 HN and Newcastle disease virus (NDV) HN that affect either receptor binding, neuraminidase activity, or HN′s ability to interact with F result in variants that have altered fusogenicity (5, 7, 11, 16, 22, 23, 30, 34, 38). The extent to which variant fusogenicity stems from alteration in HN′s attachment to target cell receptors, or alteration in its ability to induce F to assume fusion-active conformation, or both is not clear. The assay that we have used to quantitate the ability of HN to

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induce F to assume its fusion-active conformation (F triggering) is based on the finding that cells that coexpress HN and F may either retain receptor-bearing target cells bound to them via their sialic acid-containing receptors in a reversibly bound state (from which they may be released by neuraminidase or 4-GU-DANA [zanamivir]), release the bound target cells, or retain the cells irreversibly via F insertion or fusion. Using this assay, we have measured the F-triggering potential of wild-type (wt) and variant HN molecules (30). We have now extended this assay to monitor the fate of the target cells (erythrocytes [RBC]) after they interact with HN/F-coexpressing cells. We quantitated not only those RBC that were irreversibly bound via F insertion, indicating that F had been induced to assume its fusion-ready conformation, but also those RBC that stayed reversibly bound to the cells and those released from the HN/F-coexpressing cells by HN′s neuraminidase activity. This method allows a precise analysis of the relationship between F activation and receptor binding and release (receptor cleaving). We quantified HN′s F-triggering potential using wt and several variant HN molecules that are characterized by neuraminidase deficiency or increased receptor binding affinity. Since no HN variants of HPIV3 that have increased neuraminidase activity and wt avidity have yet been identified, lowering the pH to approach HPIV3 neuraminidase's optimum level was used as a means of enhancing its activity. Using the assay method that accounts for the outcome of each HN-receptor interaction, with our HN variants as the experimental tools, we assessed the contributions made by receptor binding and neuraminidase activity to binding and fusion with target cells, and separated these contributions from that of the inherent F-triggering efficacy of HN. This assessment allowed us to differentiate between HN′s various functions and explore the pathway that leads to the activation of F.

We previously noted that the relationship between HN′s receptor binding and receptor cleavage activities determines the rate of dissociation of HN from its receptor-bound state at physiological temperatures (23, 30). In the process of developing the methods that allow us to assess the three functions of HN quantitatively, we studied the effect of temperature on the release of target cells bound to cells that express HN alone (30). When HN-expressing cells with target RBC bound to them at 4°C are transferred to higher temperatures, target RBC are released as a result of HN′s neuraminidase activity. The extent of this release depends on the magnitude of that particular HN′s neuraminidase activity relative to its receptor binding avidity (23, 30). For cells that coexpress HN and F at temperatures that permit receptor-cleaving neuraminidase activity, we proposed that F activation would depend, in part, on properties of HN that modulate the availability of target cell receptors. We hypothesized that under conditions that permit all three HN functions, both neuraminidase activity and receptor binding activity would impact receptor availability and thereby the efficiency of the third function, F triggering. The results suggest that each of the three discrete properties of HN can independently affect HN′s ability to complement F in mediating fusion.

MATERIALS AND METHODS

Cells, media, and virus. HeLa-CD4-LTR-β-gal cells and HeLa-Tat cells were obtained through the AIDS Research and Reference Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health. HeLa cell lines and CV-1 (African green monkey kidney) cells were maintained in Eagle's minimal essential medium supplemented with L-glutamine, antibiotics, and 10% fetal bovine serum (Sigma) in 5% CO_2. 293T (human kidney epithelial) cells were grown in Dulbecco's modified Eagle's medium (Cellgro; Mediatech) supplemented with 10% fetal bovine serum and antibiotics in 5% CO_2. For assays of cells in air at different pHs, the above medium was replaced by a CO_2-independent medium (Invitrogen), which was adjusted according to the manufacturer's instructions to the desired pHs (28) through the addition of HCl or NaOH. HPIV3 wt virus stock was prepared by infecting CV-1 cell monolayers at a multiplicity of infection of 0.1 as described previously (15). Variant virus stocks were made in CV-1 cell monolayers from virus that was plaque purified three times (29). Viral titers were determined by plaque assay in CV-1 cells as described previously (15).

Chemicals. 4-GU-DANA (4-guanidino-Neu5Ac2en) was prepared from RELENZA Rotadisks (5 mg of zanamivir with lactose). A 50 mM stock solution was prepared by dissolving each 5-mg blister capsule in 285 μl of serum-free medium. Stock solutions were stored at −20°C.

HN and F constructs. Mutagenized HN cDNAs were digested with EcoRI and BamHI and ligated into the digested pEGFP-C3 mammalian expression vector (BD Biosciences Clontech, Palo Alto, Calif.). The pUC19 vector (BD Biosciences Clontech) containing a wild-type F cDNA insert was used as the template DNA for PCR amplification of the wild-type F cDNA with forward primer XhoI/F/pCAGGS.MCS (5′-CCCTCGAGGACCATGCCAACCTCAATACTGC) and reverse primer BamHI/F/pCAGGS.MCS (5′-CCCGGATCCTTTGTTTGTTAATACATATGG), which were designed for ligation into the pCAGGS.MCS expression vector (24). Positive clones were sent for sequencing to verify the mutations and to ensure that no additional alterations had been introduced, as described previously (23).

Transient expression of F and HN genes. Transfections were performed according to the PolyFect transfection reagent protocol (QIAGEN, Valencia, Calif.). Briefly, 293T cell monolayers were seeded into poly-D-lysine-coated 24-well plates (Becton Dickinson Labware) at a density of 3×10^5 cells/well. A transfection mixture containing F and HN DNA (F:HN ratio of 3:2) was added to the cells, followed by the addition of 15 mU of *Clostridium perfringens* neuraminidase (Sigma N-2876) to prevent cell-to-cell fusion (30) and to allow the neuraminidase-dead variant HN to bind cells at 4°C (29). Cells were used for experiments 24 to 36 h later.

Quantification of cell surface expression of HN and F by ELISA. The quantification of cell surface expression was performed essentially as described previously (3, 4) with the following modifications (23, 30). Briefly, transfected 293T cells (in 24-well plates) were washed with phosphate-buffered saline (PBS) after incubation at 37°C, fixed with 2% formaldehyde in PBS, blocked with 3% bovine serum albumin (BSA) in PBS, and reacted with a mixture of anti-HPIV3 HN or F monoclonal antibodies (180 μl/well, 1:1,000 dilution, in PBS containing 3% BSA), supplied by Judy Beeler (World Health Organization Repository). The cells were left at room temperature for 30 min before being washed three times with PBS (300 μl/well). Horseradish peroxidase-conjugated anti-mouse immunoglobulin G (Pierce) was then added to the cells (180 μl/well, 1:10,000 dilution, in PBS containing 3% BSA) and incubated for 30 min at room temperature. The cells were washed three times with PBS (300 μl/well) followed by incubation for 30 min with substrate (200 μl of 3,3′,5,5′-tetramethylbenzidine [Pierce]/well). Absorbance measurements for 200-μl aliquots from each culture well mixed with 50 μl of H_2SO_4 were taken at 450 nm with an enzyme-linked immunosorbent assay (ELISA) reader (Power Wave X equipped with KC4 Kineticalc for Windows, version 2.7; BIO-TEK Instruments, Winooski, Vt.). These measurements were carried out before each experiment to ensure that the expression levels of the proteins in these preparations were within 10% of one another.

Reporter gene assay for cell fusion. HeLa-CD4-LTR-βgal cells, persistently infected with HPIV-3, were prepared and their fusion with HeLa-Tat cells was quantified, as described previously (15). Briefly, 1 day after plating of the persistently infected HeLa-CD4-LTR-βgal cells in 96-well plates, the cell monolayers were rinsed and HeLa-Tat cells were added in media buffered to the indicated pHs. After 6 h at 37°C, β-galactosidase (β-Gal) activity of the lysed cells was determined as described previously (15, 25).

Assay for F activation. Monolayers of 293T cells transiently expressing F and wt or mutant HNs (or HNs alone as a control) were washed three times and incubated with 1% RBC suspensions at pH 7.3 for 30 min at 4°C. After rinsing to remove unbound RBC, the plates were incubated at 20 or 37°C with the simultaneous addition of medium buffered to the indicated pHs and prewarmed to either 20 or 37°C. At the indicated time points during incubation at these temperatures, the plates were rocked and the liquid phase was collected in V-bottom tubes for measurement of released RBC. The monolayers were then

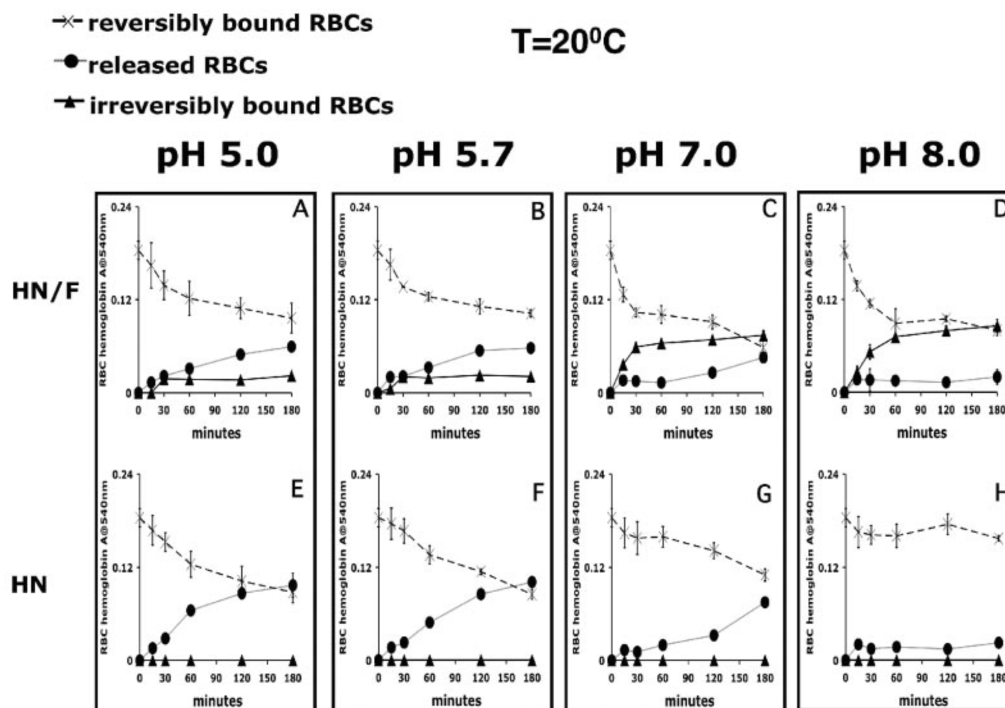

FIG. 1. F activation by wt HN at 20°C: quantitation of the amount of RBC that are reversibly bound, released, and irreversibly bound via F triggering. For the F activation assay, monolayers of cells coexpressing wt F and HN (or expressing HN alone as the control) with RBC bound to them at 4°C were transferred to 20°C, and medium was replaced by medium buffered to pH 5.0, 5.7, 7.0, or 8.0. After the indicated times of incubation, RBC that were (i) reversibly bound by HN-receptor interaction, (ii) released by HN′s neuraminidase, and (iii) irreversibly bound by F triggering were quantified. The ordinate values are means (± standard deviations [SD]) of results for three wells. At pHs 5.0 and 5.7, more RBC are released than at pHs 7.0 or 8.0 (more receptor is released from contact with HN) and less F triggering occurs.

incubated at 4°C with 180 μl of 10 mM 4-GU-DANA at pH 7.3 for 10 min. The liquid phase was collected in V-bottom 96-well plates for measurement of reversibly bound RBC. The cells were then lysed in 180 μl of 0.2% Triton X-100–PBS and were transferred to flat-bottom 96-well plates for quantification of the pool of irreversibly bound and fused RBC, referred to as triggered RBC. This term reflects the fact that in these RBC, F activation, or triggering of the F protein, leads to irreversible binding. The amount of RBC in each of the above three compartments was determined by measurement of absorption at 540 nm with an ELISA reader (Power Wave X equipped with KC4 Kineticalc for Windows, version 2.7; BIO-TEK Instruments).

Assay for infected cell-RBC fusion. HeLa cells seeded in 96-well plates were infected with wt or variant virus at a multiplicity of infection of 20 in 100 μl of PM (Eagle's minimal essential medium supplemented with L-glutamine and antibiotics). To prevent fusion between cells, 25 mU of *C. perfringens* neuraminidase (Sigma N-2876) in 100 μl of PM was added 60 min after infection. After 24 h, the plates were washed twice with CO_2-independent medium (pH 7.3) and this medium, buffered to the indicated pHs, was then added (200 μl per well), followed by the addition of 1% RBC suspension in medium at the same pHs (20 μl per well). After centrifugation at 1,400 rpm for 5 min in a Beckman G5-6R centrifuge to ensure RBC seeding, the plates were transferred to 37°C for 60 min. The medium was aspirated, the wells were rinsed with medium at pH 7.3, and 50 μl of 10 mM 4-GU-DANA was added to each well in order to release all reversibly bound RBC. The monolayers were then rinsed with medium at pH 7.3, the cells were lysed with 150 μl of 0.2% Triton X-100–PBS/well, and the irreversibly bound RBC were quantified as described above.

RESULTS

F triggering in the presence of HN under conditions that promote receptor-HN detachment. The assay that we developed for comparing the F-activating potential of wt and variant HNs makes use of the fact that 4-GU-DANA releases all reversibly bound RBC from HN/F-coexpressing cells (30). The extent to which RBC remain bound in the presence of 4-GU-DANA provides a measure of the F-activating potential of the different HNs. To analyze the relationship between F activation and receptor binding and release, at several times during incubation of HN/F-coexpressing cells with target RBC we measured the amounts of target RBC that were (i) reversibly bound by HN-receptor interaction (released only upon the addition of 4-GU-DANA), (ii) released by HN′s neuraminidase, and (iii) irreversibly bound by F triggering. We reasoned that, since HN′s F triggering depends on its contact with receptor, conditions that promote the release of reversibly bound receptor-bearing RBC would limit the amount of available receptor molecules for HN and thereby decrease F triggering.

For wt HN, we hypothesized that lowering the pH (to approach the optimum for HPIV3 HN′s neuraminidase, pH 4.7) (1) would decrease F triggering by releasing HN from its receptor and thus releasing RBC from the cells; the first set of experiments test this idea. For the experiment whose results are shown in Fig. 1, cells coexpressing wt F and HN were allowed to bind RBC at 4°C. The cells were then transferred to 20°C at a range of pHs: 5.0, 5.7, 7.0, or 8.0. This temperature (20°C) was chosen because preliminary studies of the temperature dependence of wt HN′s F activation revealed that at 20°C, F activation was readily detectable, and the effect of pH on HNs that possess wt neuraminidase activity was maximized (data not shown). At sequential time points, the amount of reversibly bound RBC (retained by HN-receptor interaction and released only upon addition of 4-GU-DANA) was com-

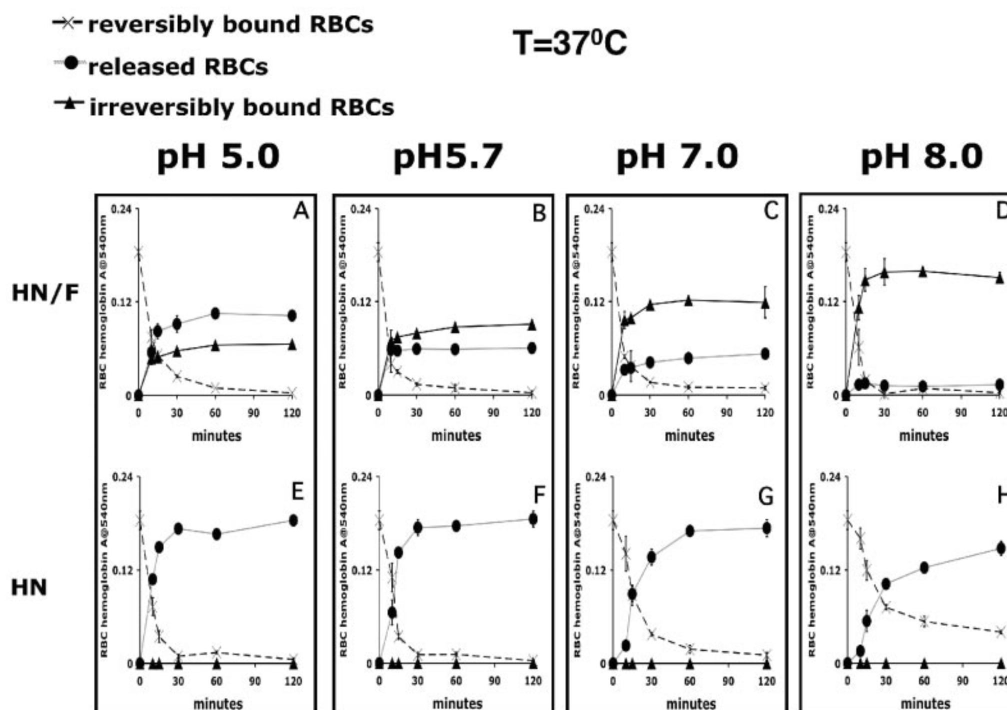

FIG. 2. F activation by wt HN at 37°C: quantitation of the amount of RBC that are reversibly bound, released, and irreversibly bound via F triggering. For the F activation assay, monolayers of cells coexpressing wt F and HN (or expressing HN alone as a control) with RBC bound to them at 4°C were transferred to 37°C, and medium was replaced by medium buffered to pH 5.0, 5.7, 7.0, or 8.0. After the indicated times of incubation, RBC that were (i) reversibly bound by HN-receptor interaction, (ii) released by HN′s neuraminidase, and (iii) irreversibly bound by F triggering were quantified. The ordinate values are means (± SD) of results for three wells. At 37°C, the activation of F is more rapid than at 20°C, and despite the more active neuraminidase and the enhanced release of RBC from the HN/F-expressing cells, more F triggering occurs.

pared with the amounts of released RBC (released by HN′s neuraminidase prior to the addition of 4-GU-DANA) and of triggered RBC (those retained irreversibly by F insertion or fusion and not released by 4-GU-DANA). The graphs in Fig. 1 (top row, cells coexpressing F and HN) show the amount of RBC in each pool—reversibly bound, released, and triggered cells—at each time after transfer to 20°C. The same procedures were carried out by using cells expressing only HN. Figure 1 (bottom row) shows the amount of RBC in each pool for the cells expressing only HN and demonstrates that there is no irreversible binding in the absence of F, confirming the requirement of F for the irreversible binding of RBC.

F triggering by wt HN at pHs 7.0 and 8.0 (Fig. 1C and D) increased during the entire 180-min incubation at 20°C as reflected by the triggered RBC pool. RBC release remained minimal at pH 8, and significant at pH 7.0 only at the 180-min time point. The decrease in reversibly bound RBC is accounted for entirely by the increase in triggered RBC. In contrast, at the lower pHs of 5.0 and 5.7 there was considerable release of RBC after the first 30 min, allowing for very little triggering after this time point. These results show that in cells coexpressing F and wt HN, the proportion of RBC that are released from the HN/F-expressing cells increases as the pH is decreased. More receptors are released from contact with HN, and less F triggering occurs.

We next hypothesized that at the physiological temperature of 37°C, F activation would be more efficient; however, neuraminidase would also be more active at each pH. Therefore, the relationship between the two activities would determine the extent of triggering. Figure 2 shows that at 37°C, activation of F is more rapid and effective than at 20°C. Triggering (irreversible RBC attachment) occurs rapidly and is maximal by 15 to 30 min at pH 7.0 or 8.0, so that at these pHs, the majority of the RBC become irreversibly bound. At the lower pHs (5.0 and 5.7), which are more conducive to neuraminidase activity, F triggering by wt HN was limited by substantial RBC release, and less RBC were irreversibly bound than at the higher pHs. However, in comparison to the results at 20°C, triggering was still far more effective even at these low pHs. As the pH is increased towards 7.0 at 37°C, the triggered pool overtakes the reversibly bound pool, while at 20°C the slow triggering process had left many reversibly bound RBC, which were still attached to HN due to low neuraminidase activity. Thus, at the physiological temperature of 37°C, despite the more active neuraminidase and the enhanced release of RBC from the HN/F-expressing cells, more F triggering occurs.

As a control for the requirement for F in the process of irreversible binding and in order to assess the effect of neuraminidase, which is more active at 37°C, on the reversibly bound and released RBC pools, the same procedures were carried out using cells expressing only HN. Figure 2 (bottom row) shows the amount of RBC in each pool for the cells expressing only HN and demonstrates that there is no irreversible binding in the absence of F, confirming the requirement of F for the irreversible binding of RBC at this temperature. In addition, at 37°C, the differences between the neuraminidase

TABLE 1. Features of HPIV3 HN variants[a]

HPIV3 HN variant[b]	HN mutation	Relative avidity	NA activity compared to wt (%)	F-triggering activity
wt	None	wt	100	wt
C0	T193A	High	100	wt
C28a	P111S/D216N	wt	<1	Defective
P111S	P111S	wt	30	Defective

[a] See references 23 and 30.
[b] C0 and C28a exist as viruses.

activities at various pHs are reduced, thereby reducing the differences in release from the receptor. Even at pH 8.0, at a temperature of 37°C, there is considerable release of RBC and a corresponding decline in the reversibly bound pool, compared to the same pH at a temperature of 20°C (Fig. 1, bottom row), where release was minimal. Thus, it is even more striking that the increased F-triggering efficiency at 37°C (Fig. 2, top row) could override the effect of the enhanced neuraminidase activity and thereby cause irreversible retention of an even larger pool of RBC.

F triggering by variant HNs: the effects of receptor avidity and neuraminidase. To further address the question of how F triggering is regulated by HN′s opportunity to remain in contact with target cell receptors (with the target cell receptors represented by reversibly bound RBC), we next studied variants of HN. We analyzed variants with either increased affinity for receptors or with decreased neuraminidase activity. Table 1 provides for convenience a summary of the sequence alterations and properties of the variant HN molecules and indicates which of the variant HNs exist in infectious viruses. To assess the impact of receptor avidity on triggering under conditions that favor neuraminidase, we studied cells coexpressing wt F with a variant HN that we had characterized previously, C0 (22, 23). This HN contains a single mutation (T193A) which confers higher avidity for receptor, while the neuraminidase activity of this variant HN is wt. We determined the effect of conditions conducive to receptor dissociation (release of reversibly bound RBC) on this high-avidity HN′s ability to trigger F. For these experiments, we carried out the assays for F triggering at 37°C in order to enable comparisons between several informative HN variants, some of which are defective in F triggering. Basing the design of this comparison on the results in Fig. 1 and 2, we carried out the studies at two widely separated pHs, 5.7 and 8.0. Figure 3A and B show that at 37°C at pH 8.0, F triggering by C0 (high-avidity) HN and wt HN proceeded rapidly and at the same rate and was virtually complete by 15 min. The reversibly bound RBC pool decreases as the triggered pool increases, and virtually no RBC are released at pH 8.0. While the C0 HN has higher receptor avidity than the wt, it behaves similarly to the wt HN in terms of the reversibly bound RBC, released RBC, and triggering at pH 8.0; for both C0 and the wt, the amount of triggered RBC was greater than that of released RBC. At pH 5.7, which is con-

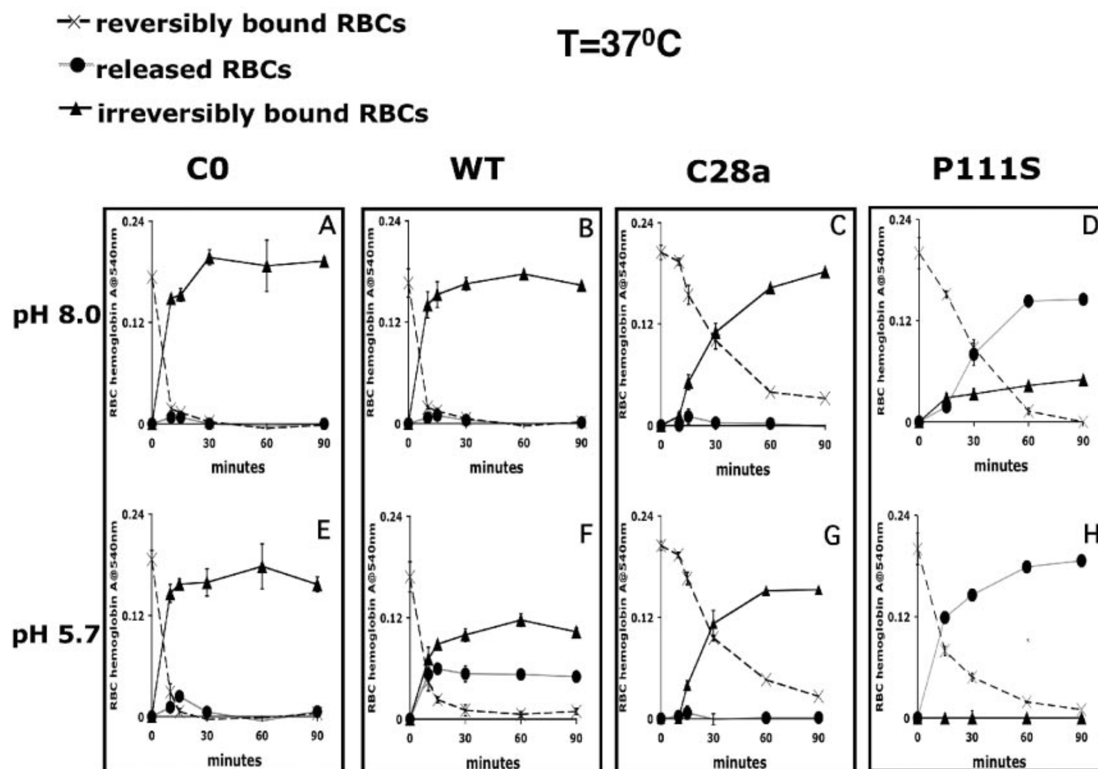

FIG. 3. Comparison of F activation by wt HN and by variant HNs with increased receptor avidity or reduced or undetectable neuraminidase activity. These triggering assays were carried out at 37°C as described in Materials and Methods (see also legend to Fig. 1.) The ordinate values for the amount of RBC that are (i) reversibly bound by HN-receptor interaction, (ii) released by HN′s neuraminidase, and (iii) irreversibly bound by F triggering are means (± SD) of results for three wells.

ducive to neuraminidase activity, F triggering by wt HN was limited by substantial RBC release. For C0 HN, however, F triggering remained as high, and RBC release remained as low, at pH 5.7 as at pH 8.0, suggesting that the higher receptor avidity counterbalances the effect of increased neuraminidase at pH 5.7. These results support the conclusion that inherent triggering efficacy of C0 HN is not higher than that of wt HN and that it is the increased receptor binding avidity of this HN that accounts for the enhanced fusion capability of the C0 HPIV3 variant (22).

To determine specifically whether the effect of pH on wt HN′s F triggering is due to cleavage of receptors by neuraminidase, we used the neuraminidase-dead C28a HN variant that we have previously characterized (29, 30). This HN variant has three features that are relevant to its use here. First, the HN possesses a mutation in the stalk region (P111S) that confers a specific defect in triggering the homologous F protein, with a resulting delay (compared to wt HN) in F triggering (30). Second, the HN is devoid of neuraminidase activity due to the combined effect of the P111S mutation and a second mutation (D216N) in the globular head enzyme active site (29, 30). Finally, the HN′s receptor binding avidity is wt (30). The triggering defect, initially characterized at room temperature (30), is also striking at 37°C, a feature that makes it most useful for our studies in this section. Figure 3C shows that the C28a HN is delayed in triggering F, compared to the wt at 37°C (Fig. 3B, triggered RBC pool). At the 15-min time point for the cells coexpressing C28a HN and wt F, few of the RBC are irreversibly bound, and even by 30 min only half of the RBC are in the triggered pool. However, since virtually no RBC are released in the absence of neuraminidase activity by this HN, the triggering catches up, and by 60 to 90 min it reaches the level of wt HN. This result serves to emphasize the fact that this C28a HN′s F-triggering defect is not absolute; while triggering is delayed, it is not dead, and given enough time it catches up to wt levels. Most important from the point of view of the present study is that the rate and extent of F triggering by C28a HN are the same at both pH 5.7 and at pH 8.0. The C28a HN releases virtually no RBC, due to its neuraminidase deficiency, and it is therefore unaffected by the altered pH. This result provides evidence that it is, in fact, the enhancement of the neuraminidase activity of wt HN at pH 5.7 that accounts for the diminished F triggering by wt HN at this lower pH.

While the absence of neuraminidase activity in C28a HN is the combined effect of its two amino acid alterations, P111S and D216N, the delayed F-triggering potential of the C28a-HN is attributable to the P111S alteration alone (30). It was thus useful to compare the doubly mutated C28a HN with an HN containing only the P111S mutation and therefore possessing the same triggering defect but an effective neuraminidase (30% of wt) along with wt receptor avidity (30). In triggering assays we previously performed at room temperature (30), the P111S triggering defect resulted in equivalent delays in accumulation of irreversibly bound RBC for cells expressing either P111S HN alone or the double mutant C28a HN (P111S/D216N). In the present study, we hypothesized that at 37°C, while triggering may be faster, P111S HN′s neuraminidase activity (which is 30% of wt) would have a negative effect on F triggering when compared to the neuraminidase-dead C28a HN (P111S/D216N) variant. In the experiment whose results are shown in Fig. 3D and H (contrast with panels C and G), we tested this hypothesis by comparing triggering at both pHs by P111S HN (triggering defect and neuraminidase active) and C28a HN (triggering defect and neuraminidase dead). At pH 8 (37°C), F triggering by P111S HN is low (compared to that achieved by either C28a HN or wt HN) because even at this pH, the residual neuraminidase activity of the P111S HN promotes RBC release. Over time, this HN (triggering defect and neuraminidase active) releases many of the reversibly bound RBC, and unlike C28a HN (triggering defect and neuraminidase dead), it cannot catch up in terms of triggering by 60 to 90 min. Triggering is thus low but significant. At pH 5.7, however, P111S HN rapidly released all reversibly bound RBC before triggering could occur, thus abolishing triggering. This result is in remarkable contrast to that with the cells expressing C28a HN, which, due to its neuraminidase deficiency, releases virtually no RBC and is unaffected by the altered pH, activating F equally at pHs 5.7 and 8.0 (see Fig. 3C and G). Note also that the P111S HN, though possessing only 30% of wt HN′s neuraminidase activity, allows more extensive RBC release at pH 5.7 than does wt HN (compare panels H to F, released RBC pool). It is likely that this occurred because the defective F triggering results in a larger pool of reversibly bound RBC available for release. Thus, the comparison between the doubly mutated C28a HN (triggering defect and neuraminidase dead) and the HN containing only the P111S mutation (triggering defect and neuraminidase active) revealed the key effect of neuraminidase. Under identical conditions, the presence of an effective neuraminidase led to less opportunity for HN to remain in contact with receptor and therefore less opportunity for the slowly triggering HN to catch up.

F/HN-mediated cell fusion under conditions that promote receptor-HN detachment: the effect of receptor avidity on infected cells. To determine whether fusion in the context of virus-infected cells is modulated by pH and therefore neuraminidase activity, we used a reporter gene fusion assay that quantitates fusion of uninfected HeLa cells constitutively expressing human immunodeficiency virus-Tat with HeLa cells persistently infected with wt HPIV3 that express β-Gal under the control of the human immunodeficiency virus LTR (15). The assay rests on the fact that persistently infected cells do not fuse with one another, due to the receptor-destroying activity of the expressed wt HN molecules. Only upon addition of uninfected cells with intact receptors for HN does cell fusion occur (15, 21). Thus, enhanced cleavage of receptors on the uninfected target cells by HN′s neuraminidase at pHs below neutral during this assay might inhibit fusion. In fact, we found that lowering the pH of the medium from 7.5 to 6.7 caused almost complete inhibition of fusion (Fig. 4); even at pH 7.0, fusion was inhibited by over 50%. While low pH can damage a variety of cellular functions involved in membrane fusion, the results from the next experiments (results shown in Fig. 5 and 6; see below) indicate that stimulation of neuraminidase activity is likely to account for the striking inhibition of fusion caused by small downward deviations from physiological pH.

In order to assess the impact of receptor avidity on the effect of pH in cells, we designed a new application of the F-triggering assay used for Fig. 1 to 3. In the experiment whose results are shown in Fig. 5, RBC were bound to cells infected with wt or C0 (HN with high receptor avidity) HPIV3 in media buff-

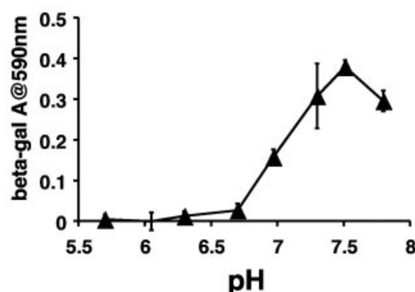

FIG. 4. Effect of pH on fusion mediated by HeLa cells persistently infected with HPIV3. The reporter gene (β-Gal) assay for fusion is described in Materials and Methods. The ordinate values, referring to β-Gal activity after 6 h of incubation at the indicated pHs (abscissa), are means (± SD) of results for five wells.

ered to different pHs at 37°C. For each pH, we measured the amount of triggered RBC (those retained irreversibly by F-insertion or fusion and not released by 4-GU-DANA) after 60 min of incubation. At pHs close to neutral, wt and C0 (HN with high receptor avidity) HPIV3-infected cells showed identical F activation at 37°C, and therefore identical amounts of irreversibly bound RBC. However, at the lower pHs of 6.2 and 5.7, the inhibitory effect of lower pH on triggering was much less for C0 than for wt HPIV3-infected cells. This result suggests that the effects seen on cell fusion are not a nonspecific effect of low pH on the cells but rather related to the individual HNs. The higher receptor binding avidity of the C0 HN can balance the effect of increased neuraminidase on fusion and in this way minimize the negative effect of decreased pH on F triggering.

The cell fusion ability of the neuraminidase-dead HN variant virus is unaffected by pH. The effect of pH on cell fusion during plaque formation in HPIV3-infected cells was also assessed microscopically. We compared plaque formation at pHs 8.0 and 6.7 in cells infected with wt, high-avidity C0, and neuraminidase-dead C28a variant HPIV3 (Fig. 6). Lowering the pH to 6.7 completely inhibited cell fusion and consequent plaque formation mediated by the wt virus. However, no such effect was apparent for C0 variant-infected cells, suggesting that the enhanced neuraminidase activity at pH 6.7 was counterbalanced or minimized by the higher receptor binding avidity of C0-HN. For cells infected with C28a virus, the plaque size was smaller, due to the F-triggering defect of this variant (30), but was completely unaffected by pH. Since C28a HN is devoid of neuraminidase activity and has wt receptor binding avidity, these results provide evidence that the negative effect of lowering the pH on fusion promotion by wt HN is not due to effects on the cells themselves but is fully attributable to increased neuraminidase activity. C28a, which is without neuraminidase activity, is thus unaffected by the altered pH.

DISCUSSION

Insight into the mechanism of membrane fusion promoted by paramyxoviruses has been furthered recently by studies of the molecular properties of the two envelope proteins, F and HN. The models proposed for the involvement of HN in the process differ in detail, but it has become clear that in addition to receptor binding, HN plays an essential role in activating F to assume its fusion-ready conformation. Insertion of the fusion peptide region of F into the target cell membrane after this activation step is the key event leading to membrane fusion. Efficiency of F triggering by HN should therefore be an important variable influencing the extent of fusion mediated by F; thus, different HNs could vary in their ability to trigger the homotypic wt F.

Several HN variants of HPIV3, NDV, and mumps virus are associated with altered fusogenicity as judged by the rate of syncytium formation or plaque enlargement during viral infection (4, 11, 12, 18, 22, 30, 43, 44). Our previous work (11, 20–22) taken together with the work of others (3, 18, 19, 32, 36, 37, 43, 44) favored the hypothesis that the neuraminidase activity of paramyxovirus HN proteins may modulate the level of receptors available to interact with the viral glycoproteins, thus influencing the degree of cell fusion. Study of an HPIV3 variant with decreased neuraminidase provided evidence that neuraminidase determines whether infection in cell culture will be cytopathic or persistent, by modulating the degree of cell fusion (11). For mumps virus, a comparison of naturally occurring strains revealed that those with high fusion activity possessed low levels of neuraminidase, while those with higher neuraminidase were less fusogenic (18); neuraminidase-deficient mumps variants were more fusogenic than their high-neuraminidase parents (43, 44). Such findings with several virus systems established the relationship between neuraminidase activity and fusogenicity as assessed by rate of syncytium formation or plaque enlargement; however, these methods do not distinguish between the effects of a given HN′s receptor binding avidity and its F-triggering capacity on fusogenicity. The present study aimed to determine how the individual properties of HN regulate the fusion process.

Our previous studies showing that the severely restricted release of neuraminidase-dead variant viruses can be overcome equally by either inhibition of HN-receptor binding with 4-GU-DANA or by exogenous neuraminidase pointed to the requisite balance between receptor binding and neuraminidase activity in regulating multicycle replication (29). Wagner et al., using FPV recombinants (41), provided direct evidence that

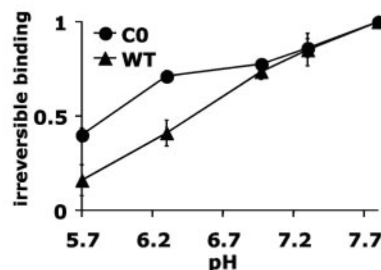

FIG. 5. Irreversible binding of RBC to cells infected with wt HPIV3 or high-avidity variant C0 HPIV3. Infected cells with RBC prebound at 4°C were incubated in media at the indicated pHs (abscissa) at 37°C for 60 min. The RBC that are irreversibly bound via F triggering were quantified as described for the assay for F activation in Materials and Methods. The extent of binding (ordinate) at each pH value (abscissa) is expressed relative to that of the irreversible binding at pH 8.0 (the amount of RBC irreversibly bound to the infected cells at pH 8.0). Each ordinate value is a mean (± SD) of results for five wells.

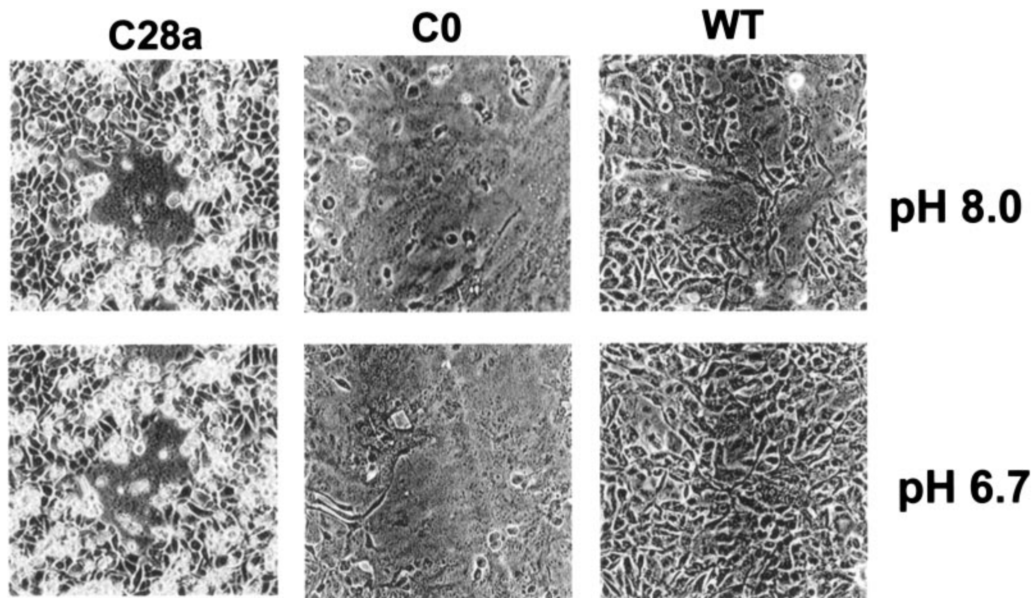

FIG. 6. Virus plaques in cell monolayers infected with wt HPIV3, variant C0, or variant C28a. CV1 cells (5×10^5) were infected with 100 PFU of wt, C0, and C28a and overlaid with 0.5% agarose at the indicated pHs following a 90-min adsorption period. Photographs were taken 36 h postinfection.

virion release depends on the balance between receptor binding and receptor cleaving (40). In the present study, we provide the first quantitative evidence for the significance of this balance in a step of the viral life cycle other than release and show that the match between binding and cleaving functions exerts a direct effect on F triggering and, thus, viral fusion. The high-avidity HPIV3 HN variant C0 was of use in assessing the variable of receptor avidity. The inherent triggering efficacy, as well as the neuraminidase activity of this high-avidity HN, was the same as for wt HN. However, while stimulation of neuraminidase at low pH caused receptor shortage and decreased F activation for wt HN, the increased receptor binding avidity of C0 counteracted this effect. To dissect the specific contribution of neuraminidase to triggering, we evaluated two variant HNs that are triggering defective due to a mutation in the HN stalk (P111S). One of these (C28a; P111S/D216N) has, in addition, a mutation in the globular head (D216N) that renders it neuraminidase dead, while the HN with the P111S stalk mutation alone has 30% of the wt's neuraminidase. Given these two HNs with the same triggering defect (conferred by the stalk mutation at P111S), only the HN with a coexisting absence of neuraminidase due to a second mutation (C28a; P111S/D216N) retains F-triggering capacity and fusion promotion at conditions conducive to neuraminidase activity. The HN that has residual neuraminidase along with its triggering defect (P111S HN) fails to retain receptor contact long enough for triggering to occur.

The antagonistic effect of HN′s neuraminidase activity (enhanced by lowering pH) on F activation by cells expressing wt HN and F is also evident in cells persistently infected with HPIV3. The stimulation of neuraminidase activity is likely to account for the inhibition of fusion that we observed, for several reasons. First, the fact that the high-avidity C0 variant HN′s triggering efficiency was less influenced than wt HN by pH alterations suggests that properties of HN, rather than nonspecific effects on the cells, are responsible. Second, cell fusion mediated by the neuraminidase-dead virus C28a is completely unaffected by pH variations in this pH range (Fig. 6). The observed differences for other HNs at altered pHs are thus very likely to be driven solely by alterations in neuraminidase activity as the pH is moved toward or away from the optimum. A small decrease in pH within the range of 6.4 to 7.5 raises neuraminidase activity sufficiently to interfere with receptor availability and thus with cell fusion. This finding also suggests that discrepancies between results of fusion assays on infected cells or HN/F-expressing cells may arise from small differences in the pHs of the media. Taken together, these experiments show that HN-receptor interaction, influenced by both receptor avidity and neuraminidase activity, determines the possible extent of F triggering carried out by HPIV3 HN.

The recently published three-dimensional structure of HPIV3 HN (14) has permitted us to place the amino acids altered in our HN variants into a structural context. The residues altered in the C0 high-avidity variant (T193A) and one of the two mutations responsible for neuraminidase deficiency in the C28a variant (D216N) are located in the catalytic active site of the molecule. The T193 position has been established in several paramyxoviruses as an important residue for receptor binding and catalysis (4, 6, 22, 23), and in the HPIV3 HN structure, T193 is shown to interact directly with sialic acid in the binding pocket. The third HN residue that contributes to our study, proline 111 (mutated to serine in the triggering-defective HN), forms part of a short stretch of residues in the stalk region of HN (not present in the three-dimensional crystal structure) that is conserved among the paramyxoviruses. Proline 111 is absolutely conserved among paramyxovirus HN proteins. The stalk region has been implicated as important in specific interactions with F for several paramyxoviruses (3, 7,

38, 39, 42, 46). We have now shown that, while the triggering defect that we have identified is due to a stalk mutation (P111S), it is HN-receptor interaction, influenced by receptor avidity and neuraminidase activity in the globular head of the HN molecule, that determines the possible extent of F triggering. The question of how a signal may be transmitted from the HN binding pocket to F via the stalk domain remains of foremost interest.

A different role for pH in the fusion process of another paramyxovirus was suggested recently by the finding that fusion mediated by the SER virus F coexpressed with SER HN was activated at low pH (35). In light of our data, one might expect that at low pH the neuraminidase activity of the SER HN would decrease F activation due to receptor dissociation. It is, however, possible that the short incubation times used in that study may not have sufficed for depletion of receptors by neuraminidase. Interestingly, for NDV it had been noted previously that fusion with COS-7 cells was enhanced at low pH (33), leading to the suggestion that NDV may enter via the endocytic pathway as well as at the plasma membrane at neutral pH. In our study, the effect of pH on neuraminidase was the dominant factor responsible for the outcome on HN′s receptor interaction and on the subsequent triggering of F.

Our experiments suggest that differences in neuraminidase activity and/or receptor binding activity of HNs can influence their ability to manifest their inherent F-triggering capacity and, thus, our ability to detect their relative fusion promotion potential. It therefore seems possible that the observed need for a homotypic HN to activate F (2, 3, 7, 10, 13, 39) may not be due only to specificity of the triggering function itself, but that it may also be influenced by a requirement for the correct match in terms of receptor avidity and receptor cleavage in relation to the inherent F-triggering efficiency. For any given F, perhaps several heterotypic HNs could provide activation if the variables of receptor binding avidity and/or receptor cleavage were eliminated and the inherent triggering capacity alone were assessed. While cells coexpressing SV5 F with heterologous binding proteins were conclusively shown to fuse less rapidly and extensively than do cells coexpressing SV5 F and HN (2), it is possible that measurement of fusion in the presence of some heterotypic HNs might be limited because the neuraminidase activity of these HNs allows premature target RBC release, rather than because they inherently have a lesser capacity to activate SV5 F. This consideration may be especially relevant to the several F proteins reported to have absolute requirements for the homotypic HN; it is possible that in some cases, the neuraminidase activity of the heterologous HN limited target cell attachment so that syncytium formation was reduced below the detectable level. We do not suggest that fusion specificity is entirely or even primarily determined by this match but rather that the balance between the properties of avidity and neuraminidase can influence the outcome of triggering. It would be possible to test, by using the assays presented here, whether assay conditions that modulate neuraminidase activity would allow a mismatched HN/F pair to complement each other.

For many viruses that require neuraminidase for effective propagation, this enzyme has been postulated to cleave host cell receptors for newly budded virions which might otherwise remain aggregated on the cell surface (9, 11, 27, 29). Electron microscopic studies showed that the aggregation results mainly from attachment of virus particles to each other (8, 17, 27), indicating that receptors are present on the virions. Temperature-sensitive influenza virus mutants that lack active neuraminidase at nonpermissive temperatures aggregate under these conditions, demonstrating that the viral envelopes contain receptor molecules that bind hemagglutinin on other virions (17, 26, 27). The presence of cellular receptors in the viral envelope, incorporated at the time of budding (45), needs to be taken into account in the analysis of membrane fusion and of the role of neuraminidase during viral entry. In a recent work, Russell et al. (31) identified the region in the heptad repeat B region of SV5 F that stabilizes the prefusogenic conformation of F and demonstrated that if the fusion peptide is released prematurely, i.e., before the target membrane is within reach, then no fusion occurs. We propose that such premature release of the fusion peptide could occur in virions if receptors present in their envelope remain uncleaved; HN binding these receptors would activate F, decreasing the prefusogenic F population needed for mediating fusion upon contact of HN with receptive host cells. According to this hypothesis, HN′s neuraminidase normally facilitates viral entry by destroying receptors on the viral envelope and thereby preventing indiscriminate F activation. It follows that neuraminidase deficiency (like that of the variant C28a) and consequent premature F activation in a virion may impair entry. The neuraminidase-dead HPIV3 C28a HN, however, has the additional defect of being slow in activating F; a correspondingly slow premature F activation in the virion's receptor-containing envelope would leave more prefusogenic F available for fusion upon HN′s contact with target cells. We propose that the triggering defect of C28a HN compensates for the handicap that its neuraminidase deficiency might otherwise represent for viral entry and viability and explains why such a virus could emerge in culture. Future studies will test the hypothesis that neuraminidase not only prevents the aggregation of progeny virions but by destroying sialic acid receptors on the viral envelope also plays a necessary role in virion entry into host cells.

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