Spring-Loaded Model Revisited: Paramyxovirus Fusion Requires Engagement of a Receptor Binding Protein beyond Initial Triggering of the Fusion Protein[▽]

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During paramyxovirus entry into a host cell, receptor engagement by a specialized binding protein triggers conformational changes in the adjacent fusion protein (F), leading to fusion between the viral and cell membranes. According to the existing paradigm of paramyxovirus membrane fusion, the initial activation of F by the receptor binding protein sets off a spring-loaded mechanism whereby the F protein progresses independently through the subsequent steps in the fusion process, ending in membrane merger. For human parainfluenza virus type 3 (HPIV3), the receptor binding protein (hemagglutinin-neuraminidase [HN]) has three functions: receptor binding, receptor cleaving, and activating F. We report that continuous receptor engagement by HN activates F to advance through the series of structural rearrangements required for fusion. In contrast to the prevailing model, the role of HN-receptor engagement in the fusion process is required beyond an initiating step, i.e., it is still required even after the insertion of the fusion peptide into the target cell membrane, enabling F to mediate membrane merger. We also report that for Nipah virus, whose receptor binding protein has no receptor-cleaving activity, the continuous stimulation of the F protein by a receptor-engaged binding protein is key for fusion. We suggest a general model for paramyxovirus fusion activation in which receptor engagement plays an active role in F activation, and the continued engagement of the receptor binding protein is essential to F protein function until the onset of membrane merger. This model has broad implications for the mechanism of paramyxovirus fusion and for strategies to prevent viral entry.

The entry of enveloped viruses into host cells requires the fusion of the viral and cell membranes. Viral fusion is driven by specialized fusion proteins that bring the viral and host membranes in close apposition to form a fusion pore (16, 23, 66, 73, 75). The trigger that initiates a series of conformational changes in the fusion (F) protein leading to membrane merger differs depending on the pathway that the virus uses to enter the cell and thus whether fusion occurs at the surface at neutral pH or in the endosome. For paramyxoviruses, the F proteins are activated when the adjacent receptor binding protein binds to a sialic acid-containing receptor, initiating the fusion process (58). Once activation occurs, the F protein undergoes a coordinated series of conformational changes that culminates in an extremely stable form of the protein that brings the two membranes together, promoting membrane fusion (30, 42). Two heptad repeat (HR) regions that are initially at opposite ends of the F protein (N-terminal heptad repeat [HRN] adjacent to the fusion peptide and C-terminal heptad repeat [HRC] immediately preceding the transmembrane domain) are brought together in its final stable form. The nature of the series of conformational changes that permit F to mediate membrane fusion and the role of the receptor binding protein of the

paramyxoviruses in this process have been subjects of recent interest (14, 29, 32).

Paramyxoviruses possess envelope proteins that provide a binding function and, depending on the specific paramyxovirus family member, also may possess receptor-cleaving (neuraminidase) activity. Paramyxovirus receptor binding proteins thus far studied, with the possible exception of that of respiratory syncytial virus (RSV), also possess a third, critical function: they activate the F protein to mediate the merger of the viral envelope with the host cell membrane. For the human parainfluenza viruses (HPIV), the envelope protein (hemagglutinin-neuraminidase [HN]) contains both receptor binding and receptor-cleaving (neuraminidase) activities. When it is receptor bound, HN activates F to initiate the conformational changes that lead to fusion (56, 58). For the parainfluenza viruses as well as other HN-containing paramyxoviruses, this one molecule thus carries out three different but critical activities at specific points in the process of viral entry: receptor binding, receptor cleaving (neuraminidase) to prevent interaction between sialic acid and HN on the same virion surface (55), and fusion activation. The efficiency of F activation by HN critically influences the degree of fusion mediated by F and the extent of viral entry (54, 58). The three functions of HN, binding, fusion activation, and neuraminidase, are in a specific balance that ultimately determines the outcome of infection (56). A clear mechanistic understanding of how these activities are regulated is key for understanding viral entry and designing strategies to block infection (42).

The precise mechanism by which HN activates F has eluded

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simple explanation (11, 32). Current models for HN-F interaction postulate that either HN and F interact in the absence of receptor and receptor engagement leads to separation of HN and F (30), or that HN-F interaction occurs only upon receptor binding (34) and that HN triggers F to proceed through fusion via a spring-loaded mechanism (14, 23, 29, 32, 45, 65). Here, we pose a third possibility in which the activation of fusion requires the engagement of the receptor binding protein beyond the initial triggering of the F protein. Using a new set of strategies, we have dissected and experimentally manipulated this series of events, and we propose a change to the existing paradigm.

For the dissection of the intermediate steps in fusion, we have used peptides derived from the HRN and HRC regions of the F protein, because these peptides can interact with fusion intermediates of F (5, 31, 36, 62, 77, 79). For the paramyxoviruses, these F-derived peptides have been shown to interact with the fusion protein intermediate that exists after the activation of the F protein and the insertion of the fusion peptide into the target cell. This extended “prehairpin” intermediate bridges the viral and cell membranes, and its refolding into a 6-helix bundle (6HB) is a key driver of membrane fusion (64). Standard peptide inhibitors derived from the HRC region of F are believed to bind the HRN domain of the prehairpin (extended) intermediate and prevent the formation of the 6HB (31, 59). Once the F protein proceeds beyond the stage of the prehairpin intermediate to the 6HB, these peptide inhibitors are ineffective. In agreement with this model, we have shown that peptides derived from the HRC domain of HPIV3 F are effective inhibitors of both HPIV3 and henipavirus infection (59). Furthermore, the efficacy of inhibition depends on both the strength of the peptide-F protein interaction and the kinetics of fusion, which determine the time window of access to the target sequence (51, 60). We recently reported, however, that when the same HRC peptides are localized to the target host cell membrane by a cholesterol tag (59, 61) and also are engineered to interact more strongly with the HRN target domain, they interact with F prior to the insertion of the fusion peptide into the target cell membrane. This occurs not only for mutant uncleaved F, as shown in this paper, but also for fully functional, cleaved F protein (59). These cholesterol-conjugated peptides thus capture an earlier stage in the F activation process than do the standard unmodified HRC peptides (59). The transient intermediate stage of F activation targeted by the conjugated peptides is dependent upon HN-receptor interaction, but it occurs before the fusion peptide inserts into the target cell membrane. In fact, even unprocessed precursor F proteins, in which the fusion peptide is buried and cannot insert into the target membrane, can be activated upon HN-receptor engagement to expose the site for HRC peptide interaction (59). Thus, in addition to the standard HRC-derived peptides that bind to F only after F activation and insertion into the target membrane (46, 60, 64), and as detailed in our recent publication (59), we have available peptides that target an earlier stage in fusion activation prior to the insertion of the fusion peptide.

In the present study, we have used these peptides for mechanistic studies of the F activation/fusion process. We are able to differentiate the steps in F activation, since cholesterol-conjugated peptides capture the transient intermediate prior

to F insertion, while standard peptides capture the extended intermediate with the fusion peptide inserted into the target cell. These studies led us to propose a new model for the role of HN during fusion activation. Our model differs from the prevailing paradigm, which proposes that after an initial triggering event by HN, F proceeds unaided to the series of conformational changes leading to fusion via a spring-loaded mechanism (14, 29, 32, 63). We propose that this new paradigm applies broadly to paramyxoviruses.

In our model, upon receptor engagement, HN activates F to advance through a series of transient intermediates that precede (but do not depend upon) the insertion of the fusion peptide into the target cells. Continuous receptor engagement by HN is required for the evolution of these transient intermediates; in particular, receptor engagement still is required after the insertion of the fusion peptide into the target cell membrane to enable F to fold into its final low-energy conformation and drive membrane merger. Engagement by the receptor binding protein seems to be similarly required for paramyxoviruses that lack receptor-destroying activity and whose receptor is not sialic acid based, e.g., Nipah virus (NiV). This makes it unlikely that for paramyxoviruses in general, receptor engagement leads to release of F from structural constraints imposed by HN, enabling F to complete fusion by itself (29, 30). In fact, we show that the impact of HN on fusion is eliminated only when the HN-receptor interaction is severed.

MATERIALS AND METHODS

Antibodies. Anti-HRC antibodies were custom generated in rabbits by Invitrogen using the previously described HPIV HRC sequence (52).

Viral glycoprotein constructs. Mutagenized HPIV3 HN cDNAs were digested with *SacI* and *BamHI* and ligated into digested pCAGGS mammalian expression vectors as previously described (44). Newcastle disease virus (NDV) Australia-Victoria wild-type (WT) HN in pCAGGS was obtained from Ronald Iorio. The genes of influenza hemagglutinin (HA) and NiV WT G and F proteins were commercially codon optimized and subcloned into the mammalian expression vector pCAGGS using *EcoRI* or *XhoI* and *BglIII*. Chimeric glycoprotein Nipah G (from amino acids [aa] 1 to 188) and NDV HN (from aa 124 to 571) were cloned in pCAGGS.

Transient expression of HN and F genes in cells. 293T (human kidney epithelial) cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Mediatech; Cellgro) supplemented with 10% fetal bovine serum and antibiotics in 5% CO₂. Transfections were performed according to the Lipofectamine 2000 protocols of the manufacturer (Invitrogen).

Assays for F activation, receptor retention, and receptor release. Monolayers of 293T cells transiently expressing cleavage site mutant F (F-csm) and WT or mutant HNs were washed and incubated with 1% red blood cell (RBC) suspensions at pH 7.5 for 30 min at 4°C. After rinsing to remove unbound RBCs, 1 or 20 μ M of peptide was added, the plates were placed at 37°C for the indicated time, zanamivir was added at a final concentration of 10 mM, and the plates were left at 37°C until the end of the last time point. Plates then were rocked, and the liquid phase was collected in V-bottomed tubes for the measurement of released RBCs. The monolayers then were incubated at 4°C with 200 ml of RBC lysis solution; the lysis of unfused RBCs with NH₄Cl removes the RBCs whose membranes have not fused with HN/F-coexpressing cells. The liquid phase was collected in V-bottomed 96-well plates for the measurement of reversibly bound RBCs. The cells then were lysed in 200 μ l 0.2% Triton X-100-phosphate-buffered saline (PBS) and were transferred to flat-bottomed 96-well plates for the quantification of the pool of fused RBCs. The amount of RBCs in each of the three compartments described above was determined by the measurement of absorption at 405 nm.

Peptide synthesis. All peptides were produced by standard 9-fluorenylmethoxy carbonyl (Fmoc) solid-phase methods. The cholesterol moiety was attached to the peptide via a chemoselective reaction between the thiol group of an extra

cysteine residue, added C terminally to the sequence, and a bromoacetyl derivative of cholesterol, as previously described (28, 59, 61).

Protease K differential cleavage for F activation. 293T cells in biocoated 6-well plates (BD) were cotransfected with 4.0 μ g of DNA/well of the N terminus of Venus yellow fluorescent protein (YFP) (designated HN-N-Venus) and the C terminus of Venus YFP (F-csm-C-Venus) in an HN/F ratio of 3:1. At 3.5 h posttransfection, the medium was replaced with DMEM containing 1 mM zanamivir to block HN-receptor interaction and then incubated at 37°C. Twenty-four hours posttransfection, the cells were washed with cold Opti-MEM (Invitrogen) and the medium was replaced with Opti-MEM containing 1 mM zanamivir and 100 μ M cycloheximide (to prevent *de novo* protein synthesis), and then the cells were incubated at 37°C for 1 h for the maturation and synchronization of the glycoproteins. For the wells in which fusion was permitted, the cells were treated at 4°C in Opti-MEM with 100 μ M cycloheximide and 1 mM zanamivir with tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (15 μ g/well) for 15 min. This treatment generates fusion-competent F-csm. After 15 min, the TPCK-treated trypsin was neutralized by the addition of fetal bovine serum (FBS) to a final concentration of 10%, and the cells were washed on ice with cold Opti-MEM, and then Opti-MEM with 100 μ M cycloheximide with or without zanamivir was added and the plates transferred to 37°C for 1 h. After 1 h, the plates were transferred to 4°C, the medium was aspirated, and the cells were lysed with 0.5 ml/well of lysis buffer (33, 34, 39) plus protease inhibitor (11697498001; Roche) for 15 min on ice. The cell lysates were transferred to 1.5-ml tubes and centrifuged at 13,200 rpm at 4°C for 15 min. The supernatant fluid was collected, and 30 μ l of agarose beads containing anti-green fluorescent protein (GFP) antibodies against full-length GFP (SC9996AC; Santa Cruz Biotechnology) was added and mixed for 12 h at 4°C. The following day, the beads were washed three times with 500 μ l of DH buffer, resuspended in 1.3 ml of DH buffer, and aliquoted into 1.5-ml tubes (400 μ l each).

The agarose beads were spun down, and after removing the supernatant fluid, 30- μ l volumes of different dilutions of protease K were added. The agarose beads then were transferred to 22.5°C for 1.5 h, 10 μ l of 4 $\times$ SDS-Laemmli buffer was added, and the solutions were incubated at 95°C for 10 min. The samples were run for 2 h at 120 V on a 4 to 20% gradient gel, and then the samples were transferred to a polyvinylidene difluoride (PVDF) membrane with a Bio-Rad transblot cell overnight at 40 V and 4°C. The PVDF membrane was blocked with 5% nonfat dry milk-0.1% Na₂S₂O₃ in PBS for 5 min, and the proteins were detected with anti-HPIV3 HRC (a custom product from Invitrogen) at 1:1,000 in 5% nonfat dry milk-0.1% Na₂S₂O₃ for 1 h at room temperature (RT). After four washes with PBS 0.1%-Tween 20, the membranes were incubated with protein G-horse-radish peroxidase (HRP)-conjugated (1:5,000) Pierce in 0.1% PBS-Tween 20 for 1 h at RT, followed by four washes (three washes in 0.1% PBS-Tween 20 and a final wash in Mqh20) and detection with tetramethyl benzidine (TMB) (W4121; Promega).

Images of the membranes were taken with a Kodak2000MM image station.

β -Gal complementation-based fusion assay. We adapted an assay (41) that detects early stages of fusion activation, since the readout does not depend on downstream transactivation events. We used this assay for experiments in which the greater range of detection of this assay was necessary. The assay is based on the alpha complementation of β -galactosidase (β -Gal); the β -Gal protein lacking the N-terminal 85 residues (omega peptide) is expressed from one plasmid, and the N-terminal 85 residues (alpha peptide) are expressed from a second plasmid. Cell fusion leads to complementation, and β -galactosidase is quantitated using the Galacto-Star (Applied Biosystems) chemiluminescent reporter gene assay system. Receptor-bearing cells expressing the omega peptide are mixed with viral glycoproteins coexpressing cells that also express the alpha peptide at various temperatures and at specific time points. Fusion is stopped by lysing the cells with lysis buffer.

RESULTS

Role of HN in initiating the activation of HPIV3 F. To investigate the earliest steps in F activation, we took advantage of our recent finding that cholesterol-conjugated peptides anchored in a target cell membrane can interact with an uncleaved F (F0) molecule that is in the early stages of activation. An uncleaved F molecule cannot form a stable bridge between membranes because its fusion peptide is not available for insertion into the target cell. However, if the cholesterol-conjugated peptide is anchored in the target cell (e.g., an erythrocyte

[RBC]) and the uncleaved F is expressed on a separate cell, the interaction between the anchored peptide and F can form a bridge between the cells, thereby mediating HN-independent attachment. To measure HN-independent attachment after the activation of F, HN can be prevented from binding its receptor with the receptor mimic drug zanamivir (54). The F peptide bridge requires the activation of the uncleaved F0 protein by the HN protein to expose its HRN regions, enabling capture by the cholesterol-tagged peptide (59). These results provided the first evidence for the conformational flexibility of the complete, membrane-bound uncleaved F0 protein under physiological conditions (59).

Here, we assess the role of HN in driving F to this stage by analyzing WT HN and two mutant HN molecules for their effect on the steps in F activation. The HN mutant D216R, fully functional in F activation, is devoid of neuraminidase activity (49). For that reason, once D216R HN is bound to its receptor, it retains this association unless detached by zanamivir. This mutant HN ensures constant HN-receptor engagement but can be reversed by the addition of zanamivir (49). When this deactivating mutation in the neuraminidase is combined with a stalk mutation (P111S) that confers a defect in F triggering (58), it provides an HN that constantly engages the receptor but is impaired in F activation (58). We coexpressed each of these HNs with a cleavage site mutant F (F-csm); this F cannot expose its fusion peptide and therefore cannot insert into the target cell to complete membrane merger (33, 34, 39). We assessed the impact of each HN on the activation of F-csm. The readout for early F activation by HN in each case is the retention of the peptide-bearing RBC by the HN/F-expressing cell, indicating interaction between peptide and an early-stage-activated intermediate of F. For this experiment, we used the cholesterol-conjugated peptide that has optimal interaction with the F-csm: a 36-residue peptide with the VIKI sequence and a 4-polyethylene glycol (PEG) unit linker, designated VIKI-PEG₄-chol (59).

To assess F activation due to these three HN molecules in the experiment shown in Fig. 1, cells coexpressing HPIV3 F-csm and HPIV3 WT HN, D216R HN, or D216R/P111S HPIV3 HN were allowed to bind RBCs at 4°C. The cells then were washed, and medium containing VIKI-PEG₄-chol (1 μ M) or no peptide was added at 4°C. The cells were transferred to 37°C to permit F activation, and at the indicated time points zanamivir was added to detach HN from its receptor (22, 58). At each of these time points we determined the amounts of target RBCs that (i) were released into the medium by zanamivir, indicating that they were attached only via HN (circles); (ii) were bound but had not fused (squares), indicating that a conformational change in F occurred to allow interaction with cholesterol-conjugated peptide; or (iii) had undergone fusion (triangles), indicating that the F activation process proceeded past the transitional intermediate to membrane merger. As expected, no fusion was mediated by F-csm. In the absence of peptide (Fig. 1A), all of the RBCs were released by zanamivir, indicating that binding was HN dependent at all time points. Figure 1B shows the results for the coexpression of F-csm with each of the HN molecules in the presence of VIKI-PEG₄-chol (cholesterol-conjugated) peptide. For WT HN coexpressed with F-csm, minimal peptide bridging is observed (the WT HN neuraminidase likely severs HN-receptor interaction before F

FIG. 1. Role of HN in initiating the activation of F. If HN is constitutively receptor bound, unprocessed F is activated to the transient intermediate captured by cholesterol-conjugated peptides. Monolayers of cells coexpressing HN (WT HN, D216R HN, or D216R/P111S HN) and unprocessed F (cleavage site mutant) were allowed to bind to receptor-bearing RBCs at 4°C. Upon transfer to 37°C, medium containing no peptide (A) or 1 μ M VIKI-PEG₄-chol peptide (B) was added. Zanamivir was added to disengage HN from its receptor at the times indicated, and the values on the y axis reflect the quantitation of RBCs that were reversibly bound by HN-receptor interaction (circles), irreversibly bound (squares), or fused (triangles). The values are means ($\pm$ standard deviations) of results from three experiments.

activation occurs). In the case of D216R HN, which continuously engages receptor, there is a progressive increase in the amounts of RBCs that are irreversibly bound and not detached by zanamivir, indicating that many F-csms were at the stage of the early intermediate. However, in the presence of triggering-defective P111S/D216R HN, the rise in irreversibly bound RBCs (reflecting the exposure of F's transient intermediate and peptide bridging) is very limited. This result demonstrates that, even with constant receptor engagement by HN, the F activation property of HN is necessary to trigger F.

For the triggering-competent D216R HN, after transfer to 37°C the irreversible binding of RBCs reaches 80% at 120 min, showing that if HN is constitutively receptor bound, the uncleaved F protein attains a transitional state in which it interacts with the cholesterol-anchored peptide and forms a bridge between cells. For the triggering-defective D216R/P111S HN, however, irreversibly bound RBCs are observed only after 60 min, and even after 120 min there is less than 5% bridging, indicating minimal F activation. HN-receptor engagement is an absolute requirement in this process; when receptor binding is blocked by zanamivir at time zero, there is no F activation with any HN molecule. As a control for the requirement for the membrane insertion of the peptide in the observed bridging effect, we carried out parallel experiments with standard peptide (no cholesterol) of the same sequence; these peptides do not attach the RBCs to the HN/F-coexpressing cells, and the results are identical to those without peptide (Fig. 1A) for all HN variants (data not shown).

Altered protease sensitivity confirms structural changes in uncleaved HPIV3 F0 after activation is initiated by HN. Al-

tered protease sensitivity upon fusion activation has been demonstrated for a number of viral fusion proteins (3, 13, 15, 20, 21, 67). Influenza HA, in its native conformation, is resistant to digestion by protease K, but in its acid-induced conformation it is digested to a specific, protease-resistant product. The two species are readily resolved by PAGE and detected by immunoblot analysis, providing a biochemical assay for the conformational change in HA (7). We modified this approach to determine whether the protease sensitivity of F0 (F-csm) is altered upon HN-receptor engagement, indicating a structural rearrangement despite the absence of furin cleavage. This was expected, since a conformational change in F is necessary to expose the target domain for the HRC cholesterol-tagged peptide. For this purpose it was important to show that it is HN-receptor engagement, and not simply HN-F association, that leads to the structural rearrangement of F. This is to distinguish between the effects of HN-receptor engagement and the effects of HN-F association on the structural rearrangements of F. To address this distinction clearly, we took advantage of the recent observation that the addition of complementary halves of yellow fluorescent protein (YFP) to the cytoplasmic tails of PIV5 HN and F caused the tagged HN and F molecules to be brought together by YFP fluorophore reconstitution (10), a process termed bimolecular fluorescence constitution (BIFC) (4, 10, 26, 27, 35, 68), enabling efficient coimmunoprecipitation. For PIV5, the binding and neuraminidase functions of HN were similar to those of the untagged protein but fusion efficiency was augmented, a result attributed to stronger HN-F interaction (10). Since tagging permitted the efficient coimmunoprecipitation of a homogeneous population of HN-F com-

plexes, we used this tactic to generate a homogenous population of HN-F pairs, which then could be subjected to timed receptor engagement. We prepared HPIV3 WT HN constructs with the N terminus of Venus YFP (HN-N-Venus) and HPIV3 F-csm with the C terminus of Venus YFP (F-csm-C-Venus). Similarly to PIV5, we found that for HPIV3 the tagged proteins caused increased fusion compared to that of untagged HN/F pairs (data not shown).

To determine the role of HN-receptor engagement on the activation of F, we generated a homogenous population of HN/F pairs prior to receptor engagement, all at the same stage of activation, by expressing HN/F in the presence of zanamivir and then removing the zanamivir and examining the conformational change in F by its sensitivity to protease K. We transfected 293T cell monolayers with HN-N-Venus and F-csm-C-Venus and incubated the monolayers for 18 h in medium containing zanamivir, which we have shown to inhibit both HN-receptor interaction and neuraminidase activity (22). This step allowed for the formation of HN-F complexes in the absence of HN-receptor interaction, generating a homogeneous population of HN-F pairs linked together by their cytoplasmic tails. To synchronize and mature the HN-F complexes before allowing HN-receptor interaction, the cells were cycloheximide treated (47) starting 1 h before the experiment and throughout the experiment. After 1 h, zanamivir was removed to allow receptor binding, and the cells were placed at 37°C, permitting HN-receptor interaction and downstream effects on F.

As controls, parallel sets of experiments were carried out either in the constant presence of zanamivir, to prevent HN-receptor engagement, or with TPCK-treated trypsin treatment, to allow the cleavage of F-csm and allow fusion to proceed. After an incubation period of 2 h (the time point at which the TPCK-treated trypsin control cell monolayer was completely fused), the cells were transferred to 4°C and lysed. The cell lysate was immunoprecipitated by anti-GFP antibodies to capture HN-F complexes, either with or without prior receptor interaction (designated R+ and R−, respectively). The HN-F complexes were treated with protease K at the indicated range of concentrations to detect a change in conformation (Fig. 2). Figure 2A shows a representative Western blot using an anti-HRC peptide antibody to recognize F-csm-C-Venus. In the HN-F complexes that had experienced HN-receptor interaction (R+), protease K treatment led to more extensive degradation of F0 than that in the samples that had no HN-receptor interaction (R−). In the controls without protease K digestion, F0 levels are similar for the samples with and without receptor interaction, as expected. This analysis shows that the F-csm complexed with receptor-engaged HN is more susceptible to protease K degradation than F-csm complexed with non-receptor-engaged HN, suggesting that only upon receptor binding does HN induce a conformational change in F.

We obtained essentially the same biochemical evidence for the activation of processed F (F1; cells were trypsin treated before allowing HN-receptor engagement) in the presence of receptor-engaged HN (Fig. 2B). Figure 2C shows that in the absence of HN, no differential protease K degradation was observed with or without zanamivir. Taken together, these results indicate that HN-receptor interaction is critical, causing a structural rearrangement in the associated uncleaved F0.

FIG. 2. Structural rearrangements in activated, unprocessed F are detected by altered protease sensitivity. Monolayers of cells coexpressing F and WT HN (A and B) or F alone (C) were incubated for 60 min in medium either without zanamivir (receptor engaged; R+) or with zanamivir to disengage HN from its receptor (R−). The cells were lysed, and the envelope glycoproteins were immunoprecipitated and then incubated in the presence of the indicated concentrations of protease K. (A) Representative Western blot showing F proteolysis detected by polyclonal anti-F HRC antibodies. F0 (precursor) protein is indicated. (B) Cells were trypsin treated to process F0 and allow fusion. The representative Western blot shows F proteolysis detected by polyclonal anti-F HRC antibodies. F0 and F1 proteins are indicated. (C) Representative Western blot showing F proteolysis, in the absence of HN, detected by polyclonal anti-F HRC antibodies. F0 (precursor) protein is indicated.

Therefore, the structural alterations indicative of F activation occur specifically as a result of HN-receptor engagement. HN-F interaction is insufficient to trigger F. We hypothesize that this structural change exposes the sites (likely the HRN domains) targeted by the cholesterol-conjugated peptides.

Continued activation of HPIV3 F by receptor-bound HN is necessary even after HPIV3 fusion peptide insertion: HN is required for progression to membrane merger. The experiments described in Fig. 1 and 2 using F-csm show that HN-

receptor engagement drives the initial activation of F to the early intermediate stage.

The use of cholesterol-conjugated peptides in the experiments described above permitted us to assess the effect of HN on the progression of F to the early transitional intermediate stage (59). The interaction of these cholesterol-conjugated peptides with F is dependent on HN-receptor engagement. While we have shown that these peptides interact with F before fusion peptide insertion into the target membrane (59), standard fusion inhibitory peptides (without cholesterol conjugation) inhibit fusion by preventing the refolding steps of F after the extended intermediate stage, i.e., after HN has activated F triggering and after the fusion peptide has inserted into the target cell membrane but before membrane merger (29, 63, 64). In fact, the ability of standard HRC peptides to inhibit fusion has been used as an indication that F activation and insertion into the target membrane has occurred already (46, 63). At this stage, the F trimer is presumed to be bound by inhibitory peptide and is unable to proceed through fusion (23, 29, 63). To determine the timing during which HN is actively involved in the fusion process and whether HN-receptor engagement is required beyond the stage of F fusion peptide insertion, we asked whether activation by receptor-engaged HN can overcome the fusion-inhibitory effect of the standard inhibitory peptides. For this purpose, we used these peptides in the assay described above (54, 58). The experimental flow chart is shown in Fig. 3.

Cells coexpressing WT-cleaved HPIV3 F and D216R HN (receptor-engaged/full trigger function) were allowed to bind RBCs at 4°C for 30 min. The cells then were washed, and medium alone or medium containing standard peptide (20 μ M) was added. The cells were transferred to 37°C for 30 min, a temperature and period of time that should result in complete fusion and complete F triggering (49, 54, 56). The cells then were washed and covered with medium alone or medium containing 10 mM zanamivir to disengage HN from its receptor (25, 43, 44, 53–55, 57, 58) for 30 min at either 4 or 37°C. The readout for the progress of F activation by HN to the point of fusion peptide insertion in each case is the irreversible retention of the RBC by the HN/F-expressing cell via interaction with native F. The readout for the progress of F activation by HN, moving F through the intermediate steps toward fusion, is the merger of the RBCs with the target cells. After the 30-min incubation at 4 or 37°C, we collected the medium and lysed the RBCs to quantitate the bound RBC population. Finally, the HN/F-bearing cells were lysed for the quantitation of the fused RBC population.

The bar graphs in Fig. 3 show the percentage of RBCs that were bound only via HN-receptor interaction (reversibly bound; gray), were bound by fusion peptide insertion (irreversibly bound; white), or had undergone fusion (fused; black). After the opportunity for HN to bind its receptor (R+) and activate F in the presence or absence of peptide at 37°C, cells were washed and placed at 4°C in the absence or presence of zanamivir to disengage HN from its receptor (R–). For each graph, the conditions of temperature and receptor engagement (R+) or disengagement (R–) are noted at the top. At 4°C, with or without receptor engagement, the populations remain essentially static at the end of the 30-min activation period. In the presence of peptide, the cells are mostly irreversibly re-

FIG. 3. Role of HN after F has inserted the fusion peptide into the target cell: progression to membrane merger in the presence of constitutive receptor engagement. If HN is constitutively receptor bound, processed F is activated to the stage of fusion peptide insertion captured by nonconjugated peptides and then proceeds to membrane merger. As shown in the experimental time line, monolayers of cells coexpressing D216R HN and processed F were allowed to bind to receptor-bearing RBCs at 4°C. Unbound RBCs were washed away, and cells were incubated with standard HRC peptides (20 μ M) at 37°C for 30 min. The plates were washed to remove unbound peptide and incubated without (A and C) or with (B and D) zanamivir for 30 min at either 4 (A and B) or 37°C (C and D). The values on the y axis reflect the quantitation of RBCs that were reversibly bound by HN-receptor interaction (gray), irreversibly bound (white), or fused (black). The values are means ($\pm$ standard deviations) of results from triplicate samples and are representative of the experiment repeated at least four times.

tained via F insertion and do not proceed to fusion, since the standard peptide inhibits this stage of F activation. In the absence of peptide, after the incubation at 37°C, the cells are mostly fused, since F activation was not inhibited.

In parallel, after the opportunity for HN to bind its receptor-activated F at 37°C in the presence or absence of peptide, cells were washed and replaced at 37°C (instead of 4°C) in the absence or presence of zanamivir to disengage HN from its receptor. Surprisingly, at this temperature, continued HN engagement causes fusion to proceed even though F had been halted by peptide (Fig. 3A and B). The population of fused RBCs reaches almost 100% after 30 min, as virtually all the irreversibly bound RBCs proceed to fusion. This stands in contrast to results shown in Fig. 3D, where zanamivir disrupts HN-receptor engagement and cells remain in an irreversibly bound state with the fusion peptide inserted without fusion, indicating that the inhibitory effect of preincubation with peptides and peptide blockade is not overcome. Note the contrast between the graph in Fig. 3C and its control graph in Fig. 3A, in which the 4°C condition prevented further progress of F activation (fusion) despite HN-receptor engagement, since this temperature does not permit structural rearrangement. The

FIG. 4. Role of HN after F has inserted the fusion peptide into the target cell: progression to membrane merger without constitutive receptor engagement. If HN is not constitutively receptor bound, processed F is activated to the stage of fusion peptide insertion captured but does not proceed to fusion. As shown in the experimental time line in Fig. 3, monolayers of cells coexpressing WT HN and processed F were allowed to bind to receptor-bearing RBCs at 4°C. Unbound RBCs were washed away, and cells were incubated with standard HRC peptides (20 μ M) at 37°C for 30 min. The plates were washed to remove unbound peptide and incubated without (A and C) or with (B and D) zanamivir for 30 min at either 4 (A and B) or 37°C (C and D). The values on the y axis reflect the quantitation of RBCs that were reversibly bound by HN-receptor interaction (gray), irreversibly bound (white), or fused (black). The values are means ($\pm$ standard deviations) of results from triplicate samples and are representative of the experiment repeated at least four times.

use of D216R HN, which is constitutively receptor engaged and is fully active in triggering, permits us to observe the constant action of HN on the process and dissect the process by timed interruptions.

Fig. 3C and D, which show the same samples under identical conditions but with and without HN-receptor engagement, demonstrate that if HN continues to engage the receptor, it continues to activate F and overcomes the peptide blockade at the transitional intermediate state. Interestingly, F does not spontaneously recover from the peptide blockade, indicating that the transitional intermediate stage of F is effectively blocked by the peptide unless HN activation persists. The results of these experiments show that HN continues to play a role in the activation of F at the prehairpin intermediate stage, where the fusion peptide is inserted in the target cell membrane, extending to the onset of the fusion phase.

The same experiment as that shown in Fig. 3, but using WT HN instead, does not permit these observations, most likely because the neuraminidase activity of HN leads to the destruction of and release from its receptor, and the RBCs remain irreversibly bound after F insertion but do not proceed to fusion (Fig. 4).

The key to the strategy used in the experiments shown in Fig. 3 and 4 is the protocol for achieving the synchronization of the F proteins, namely, the activation of all available F proteins during the 30 min before the start of the second incubation, so

that F activity occurring after that time results only from the continued progress of those activated F proteins and not from newly activated F proteins. In the case of Nipah virus F protein, it has been shown that after 30 min at 37°C the F activation is maximal, as measured by the maximal binding of HRC peptides to the activated F (1). The kinetics of the activation of HPIV3 F protein have been shown to be faster than those of Nipah F (60), so this should not be a problem. To directly address the question of whether all of the F proteins are activated by 30 min at 37°C, we carried out a time course experiment. Cells coexpressing WT-cleaved HPIV3 F and D216R HN (receptor-engaged/full trigger function) were allowed to bind RBCs at 4°C for 30 min. The cells then were washed, and medium alone (supplemented with cycloheximide) or containing standard peptide (20 μ M) was added. The cells were transferred to 37°C. At times beginning 15 min after transfer, cells containing peptide were washed, media were replaced, and the samples were returned to incubation at 37°C. After 180 min, we collected the medium from all the wells, namely, those that contained no peptide as well as those that initially contained peptide but were washed to remove peptide at 0, 15, 30, 45, 60, 90, or 120 min, and we lysed the RBCs to quantitate the irreversibly bound RBC population. The HN/F-bearing cells were lysed for the quantitation of the fused RBC population. If all F proteins in a well had been fully activated by a specific time point, then after washing unbound peptide at that time point no additional F would be activated and peptide would be bound to all of the activated F proteins. Only if HN can stimulate F to proceed to fusion despite the presence of peptide, as was the case for HN D216R, will fusion be detected. If all of the F proteins had not yet been activated by a specific time point, then after the wash new proteins would be activated and mediate fusion. However, in the latter case the amount of fusion should be progressively less in each set of cells that had longer and longer incubations with peptide inhibitors, since fewer and fewer F proteins would remain available for activation after the wash. Regardless of the length of time of incubation with peptide, the amount of subsequent fusion is the same (Fig. 5). These data suggest that our results do not reflect new F proteins undergoing the early stages of F activation, but rather that all of the available F proteins had been activated, and that the similar fusion in all wells corresponded to those F proteins proceeding to mediate fusion.

The role of HN in fusion after the stage of F insertion into the target cell is specific and not simply to provide membrane attachment. We sought to further characterize the role of HN in membrane fusion after F peptide insertion, i.e., whether HN plays a specific role after F peptide insertion or merely serves to keep the two membranes in close apposition. The thermal activation of membrane-bound F protein has been shown for Sendai virus (74), and PIV5 F in its soluble, pretriggered state can be induced to change conformation by heat (11). We adapted an assay for HN-independent F fusion that uses influenza HA as a nonspecific binding protein (64) to ask whether HPIV3 F is activated by heat and whether F alone mediates fusion over time in the presence of HRC peptide inhibitors. Cells coexpressing WT-cleaved HPIV3 F and influenza HA were allowed to bind RBCs at 4°C for 30 min. The cells then were washed and transferred to 25, 37, or 45°C for 60 or 120 min. After this incubation, the populations of released,

FIG. 5. Time course of F activation: timed exposure to nonconjugated peptides shows that progress through fusion does not depend on a population of unactivated F proteins. Monolayers of cells coexpressing D216R HN and processed F were allowed to bind to receptor-bearing RBCs at 4°C. Unbound RBCs were washed, and standard HRC peptides (20 μ M) were added at 37°C for the indicated times. The plates were washed to remove unbound peptide and incubated at 37°C for a total of 180 min. The values on the y axis reflect the quantitation of RBCs that were fused. The values are means ($\pm$ standard deviations) of results from triplicate samples and are representative of the experiment repeated at least four times.

irreversibly bound, and fused RBCs were quantitated. The percentage of RBCs that underwent fusion after 60 (white) or 120 min (black) is shown in Fig. 6. For those cells coexpressing influenza HA and HPIV3 F, fusion occurs more efficiently at the higher temperature of 45°C, achieving 100% fusion by 1 h (Fig. 6, white bars), as opposed to 50% fusion by 2 h (Fig. 6, black bars) at the lower temperature of 37°C. These results indicate that HPIV3 F fusion activity is caused by heat activation. Cells transfected with HA alone or HPIV3 F-csm alone did not show any fusion, confirming that the observed fusion was mediated by HPIV3 F but required the attachment function, in this case from HA (data not shown). Note that for cells coexpressing D216R HN and F, the 60-min incubation resulted in complete fusion even at 25°C (data not shown.) These re-

FIG. 6. HPIV3 F activation by heat. Monolayers of cells coexpressing uncleaved influenza HA and cleaved F were allowed to bind to receptor-bearing RBCs at 4°C and then transferred to the indicated temperature for 60 (white) or 120 min (black). The values on the y axis reflect the quantitation of the percentage of RBCs that were fused. The values are means ($\pm$ standard deviations) of results from three experiments.

FIG. 7. Role of HN in fusion after the stage of F insertion into the target cell is specific. Monolayers of cells coexpressing influenza HA and processed F were allowed to bind to receptor-bearing RBCs at 4°C. Unbound RBCs were washed, and standard HRC peptides (20 μ M) or medium was added at 45°C. Cells were either washed after 60 min and incubated until 120 min without peptide or continuously incubated with constant peptide. The values on the y axis reflect the quantitation of RBCs that were fused. The values are means ($\pm$ standard deviations) of results from triplicate samples and are representative of the experiment repeated at least four times.

sults indicated that while HN decreases the energy of fusion activation, F can be activated by heat. This finding enabled us to address the specific role of HN.

We next asked whether thermal energy activation alone activates F over time in the presence of HRC peptides, or if, as we proposed, HN is specifically required to overcome the inhibitory peptide effect. Cells coexpressing WT HPIV3 F and influenza HA were allowed to bind RBCs at 4°C for 30 min and were washed, and medium with or without the standard peptide was added. The cells were transferred to 45°C for 60 min, a period of time that results in complete F activation (Fig. 6), and then were washed and incubated in medium alone for an additional 60 min at 45°C. The populations of irreversibly bound and fused RBCs then were quantitated. Figure 6 shows the percentage of RBCs that had undergone fusion and shows that even at 45°C, the inhibitory effect of the peptides is not reversible (Fig. 7). Without the addition of peptide, cells fuse, but with peptide present for the full 120 min or just for the first 60 min, there is virtually no fusion, indicating that the peptide effect is not overcome by heat. This is in contrast to the experiments shown in Fig. 3 and 5, where the continuous HN-receptor interaction enables F-mediated fusion in the presence of HRC peptides. HA, which is constitutively receptor engaged but does not activate HPIV3 F, did not mediate fusion in the presence of HRC peptides, indicating that simple tethering is insufficient to mediate fusion in the presence of the peptide. This experiment supports the notion that fusion in the presence of peptides seen in Fig. 3 is due to the ongoing interaction between receptor-bound HN and the F protein, even after F insertion into the target cell.

Role of receptor engagement in fusion activation of Nipah F: constant receptor engagement is required for fusion promotion. We asked whether the lack of viral receptor-destroying activity (neuraminidase) in our experimental HPIV3 HN could account for our results if neuraminidase is involved in the entry

process. We have shown that the balance between the three functions of HN determines the outcome of infection (56), and it is likely that the lack of neuraminidase of the D216R HN skews the balance toward receptor binding, so that the ongoing activation of F by receptor-bound HN becomes observable. To ask if the role of the receptor binding protein in F activation beyond triggering is generally relevant, we tried the converse experiment. We studied the fusion machinery of Nipah virus, a paramyxovirus that lacks receptor-destroying activity and binds its non-sialic acid receptor tightly, but we skewed the balance in the opposite direction by replacing the globular head of the Nipah attachment protein G with the receptor-destroying globular head of Newcastle disease virus (NDV) HN. We thus determined what happens when a constitutively bound attachment protein becomes easily detachable. In this scenario, constitutive receptor engagement is achieved by activating NDV HN site II, thus separating the effect of neuraminidase itself from the effect of receptor detachment.

The Nipah attachment protein G binds a proteinaceous receptor, EFNB2, with high affinity (32) and has no receptor-cleaving activity. By using a chimeric protein composed of the stalk domain from the Nipah G protein and the globular domain of NDV HN, we established a system in which receptor engagement can be experimentally modulated with zanamivir, while the F activation function contained in the stalk region (32) is specific for Nipah virus. The globular domain of Nipah G starts at residue 189, therefore we incorporated the G stalk up to and including residue 188. The advantage of using the globular head of NDV HN (starting at NDV residue 124) is that the engagement of NDV HN binding site I by zanamivir leads to the activation of binding site II (38, 53). The second binding site binds receptor more avidly but lacks receptor-cleaving activity, and therefore the zanamivir-treated NDV HN is constitutively receptor engaged (53). This experimental design allows us to modulate receptor engagement, since we can add zanamivir to ensure receptor engagement (blocking site I and activating site II) or withhold zanamivir to ensure receptor disengagement mediated by site I neuraminidase activity.

Cells coexpressing Nipah F and the chimeric receptor binding protein (Nipah G aa 1 to 188 and NDV HN aa 124 to 571) were incubated with RBCs at 4°C for 30 min in the presence or absence of zanamivir. In the presence of zanamivir, NDV HN site I is occupied and receptor binding occurs via site II. In the absence of zanamivir, receptor binding occurs only via site I. Additional cells coexpressing Nipah F and NDV HN were treated identically. The cells then were washed, medium with or without zanamivir (2 mM) was added, and the cells were transferred to 37°C for 60 min, during which the activation of Nipah F and fusion could occur. After the 60-min incubation at 37°C, the unbound, bound, and fused RBCs were quantified. Note that in contrast to the HPIV3 experiments, the presence of zanamivir results in constant receptor engagement by binding site II on the globular head of NDV HN, whereas its absence allows for the endogenous NDV neuraminidase to release the chimeric attachment protein from the receptor. (Note that the Nipah G/F pair cannot be assessed in parallel, since G-receptor binding is constitutive.) The results reveal that, similarly to HPIV3, continued receptor engagement by G-HN causes Nipah F-mediated fusion to proceed (Fig. 8),

FIG. 8. Role of receptor engagement in fusion activation of Nipah F. Constant receptor engagement is required for fusion promotion. Monolayers of cells coexpressing Nipah F and either chimeric glycoprotein G-HN (Nipah G aa 1 to 188 and NDV HN aa 124 to 572) or NDV HN (HN) were allowed to bind to receptor-bearing RBCs at 4°C with (A) or without (B) zanamivir. Unbound RBCs were washed away, and standard medium with (A) or without (B) zanamivir to activate binding site II was added at 37°C for 120 min. The values on the y axis of each graph reflect the quantitation of RBCs that were released (gray), bound (white), or fused (black). The values are means ($\pm$ standard deviations) of results from triplicate samples and are representative of the experiment repeated at least four times.

indicating that for Nipah F a receptor-engaged binding protein promotes fusion. When the G-HN chimera is receptor bound, the population of fused RBCs reaches almost 100% after 120 min. In the absence of constant receptor engagement, G-HN does not promote fusion. NDV HN, even in the presence of constant receptor engagement, does not promote Nipah F-mediated fusion. This is as expected, since the stalk region of homologous attachment protein, but not that of the heterologous NDV HN protein, is needed to confer specific F-triggering activity (29, 32, 40). These results demonstrate that the engagement of the receptor binding protein, whether detachable by neuraminidase in the case of sialic-acid binding attachment proteins (HN) or constitutively bound (G), promotes fusion beyond the stage of F insertion. Detachment from the receptor, whether by viral neuraminidase or not, halts the process. However, the ability of the G-HN chimera to activate NiV F shows that the attachment function is provided and suffices to allow the attachment protein stalk-determined activation of F to proceed.

DISCUSSION

The relationship between HN receptor binding and the subsequent triggering of F protein, as well as the nature of the signal that is transmitted from HN to F during this process, is unknown. Previous models for HN-F interaction have suggested that either HN-F interaction occurs in the absence of

FIG. 9. Schematic representation of role of HN in sequential steps of F activation during the paramyxovirus fusion process. The use of HPIV3 HN and HPIV3 F as the model system is described in Discussion. HN engages its sialic acid receptor on the target cell (A and B) and initiates a conformational change in F (C). F (processed and native) forms an early, transient intermediate prior to the insertion of the fusion peptide into the host cell membrane (preinsertion). (D) With continued HN-receptor engagement, F proceeds to insert the fusion peptide into the target cell, reaching the late transient intermediate stage (postinsertion). (E) If the HN-receptor interaction instead is severed with zanamivir, fusion is halted at this stage. With continued HN-receptor engagement, the prehairpin intermediate proceeds to fusion (E), but if the HN-receptor interaction is severed with zanamivir (G), fusion is halted at this stage as well.

receptor (and they separate upon receptor engagement) or that the HN-F interaction occurs only upon receptor binding (29, 30, 34, 70). Our data suggest that HN continues an active role in fusion after receptor engagement, not releasing F to refold and cause fusion but actively participating in the process.

To clarify the series of events in the initial phase of fusion activation, we have built an experimental system in which we can selectively alter individual parameters in the activation process of human parainfluenza virus. The interaction between HN and the receptor is modulated with zanamivir, which for HPIV3 blocks HN-receptor interaction or reverses binding that has already occurred (54). The structural rearrangements of activated F are monitored by the use of inhibitory peptides derived from its HRC domain, which can interact with F after triggering but before fusion peptide insertion (59) or later in the process, when the fusion peptide is inserted into the target cell membrane (63).

HN molecules with specific F-triggering deficiencies or advantages have been useful in dissecting the steps in fusion activation. First, a neuraminidase-dead HN (D216R HN), which is fully functional in F activation (49), is constitutively associated with receptor molecules unless it is detached (e.g., by the receptor analog zanamivir). The use of this HN permits us to tightly control the timing of HN-receptor interaction and to ensure constant HN-receptor engagement. An HN with a stalk mutation that confers a defect in F triggering (P111S HN)

(56, 58, 72) allows us to assess the direct contributions of HN F-triggering function. Comparisons of HNs, mutated singly or in combination, to WT HN allow the analysis of each step in the involvement of HN in initiating the fusion.

Based on our results, we propose that the role of HN-receptor interaction in the fusion process is not limited to initiating the chain of structural rearrangements of F but continues to play an active role in the progress of F through its transitional states beyond the prehairpin intermediate stage, when the fusion peptide is inserted into the target cell membrane. Our model is depicted in Fig. 9. HN first engages its sialic acid receptor on the target cell (Fig. 9A and B) and, in doing so, initiates a conformational change in the processed, native F (Fig. 9C). This initial conformational change in F forms an early transient intermediate prior to the insertion of the fusion peptide into the host cell membrane. This early intermediate can be captured by cholesterol-conjugated HRC peptides (59). With continued HN-receptor engagement, F proceeds to insert the fusion peptide into the target cell, reaching the late prehairpin intermediate stage (Fig. 9D). If instead the HN-receptor interaction is severed with zanamivir, fusion is halted at the preinsertion stage (Fig. 9F). The late prehairpin intermediate can be captured by unconjugated HRC peptides, as presented in Fig. 3 and 5. With continued HN-receptor engagement, the prehairpin intermediate proceeds to fusion (Fig. 9E), while again the process is halted if the HN-receptor interaction is severed with zanamivir (Fig. 9G). The findings

described here argue against a pure spring-loaded mechanism (30, 81) where, after an initial triggering event, the activated F protein mediates fusion unaided. Instead, our data indicate a broader involvement of the attachment protein in the fusion process, a process in which receptor engagement is required for the continuous activation of F.

We have shown previously that uncleaved F can be activated (59), and here we provide biochemical evidence for the ability of uncleaved F to undergo conformational changes in the presence of HN (Fig. 2). The reported crystal structure of the uncleaved HPIV3 F appeared to be in a postfusion state (80), also indicating the activation of uncleaved F and supporting the notion that uncleaved F has flexibility. However, in our experiments, only in the presence of HN can uncleaved F undergo conformational change (Fig. 1 and 2). This is in contrast to the processed F, which can undergo activation induced by temperature alone (Fig. 6 and 7), indicating that F has the ability to progress spontaneously. We also have found that, in the absence of HN, processed F is sensitive to proteolysis (18). Taken together, these data suggest that HN protects processed F from untimely activation (18, 37), and only when it is receptor engaged does it proceed to activate F.

For influenza virus, the structural rearrangements that result in fusion are triggered by the acidification of the environment of the hemagglutinin (HA) receptor binding/fusion protein (7, 8, 24, 69, 76). Recent experiments indicate that for HA to undergo the correct series of sequential transitions from the prefusion to the postfusion state, at least two pH stimuli are required in proper sequence (78). Our finding that continuous HN-receptor engagement is required for parainfluenza virus fusion is consistent with the notion that the complex rearrangements of both fusion proteins require input at several stages. For the parainfluenza virus F, refolding to the final postfusion state does not appear to be guided only by the HRC-HRN complex achieving the most energetically stable conformation, because it also requires either continuous or at least sequential input from receptor-bound HN. Even after the insertion of the fusion peptide in the target membrane, receptor-bound HN still plays an essential role.

The efficacy of peptides at capturing intermediate states of the fusion process (and thereby halting the fusion process) depends on the kinetics of HN-mediated fusion activation. HN molecules with a more effective triggering function promote enhanced kinetics of activation and may even overcome the inhibitory effect of HRC peptides that interact strongly with the trimer HRN of F (60). We now show that without HN, when F is activated by temperature, peptides irreversibly block fusion. It is the continued engagement of HN that subverts peptide inhibitory activity. To explain this surprising finding, future experiments will address whether the half-life of the peptide-F complex is affected by the presence of HN.

To gain a unified view of the paramyxovirus fusion mechanism, we have investigated Nipah virus, a paramyxovirus whose G protein binds to a proteinaceous receptor and does not have receptor-cleaving activity. To assess the requirements for F activation, we paired the Nipah F protein with a chimeric G protein composed of the stalk domain from Nipah G and the globular domain of NDV, thus establishing a system in which F activation contained in the G stalk region (32) is specific for Nipah virus, while receptor engagement can be experimentally

modulated with zanamivir. For these experiments, we took advantage of previous studies on the globular head of the NDV HN, which possesses two receptor binding sites (82); site I is responsible for receptor binding and cleavage, and site II is responsible for receptor binding alone. We have shown that while site I and its activity may be blocked by zanamivir, the engagement of site I by zanamivir results in the activation of binding site II, resulting in the constitutive interaction of NDV HN with its receptor (53). With zanamivir inducing the constitutive interaction of the G-HN chimeric receptor binding protein with its receptor and the entire Nipah virus G stalk conferring triggering specificity, Nipah F was activated to mediate fusion.

Based on these results, we propose that the role of receptor engagement in the fusion process for Nipah virus also is essential to the progress of F through its transitional states. The experimental model illustrating this conclusion is depicted in Fig. 10. The chimeric protein, comprised of the stalk of Nipah virus G (which confers triggering specificity) and the globular head domain of NDV HN (which confers receptor specificity and neuraminidase activity), engages the receptor on the target cell (Fig. 10A and B) but is rapidly detached by the receptor-cleaving activity of HN site I (Fig. 10C), and the result of this transient receptor engagement is a failure to activate Nipah virus F. Thus, for Nipah F, too, the fusion process is thwarted if the receptor binding protein-receptor interaction is severed. If, however, HN site II (which lacks receptor-cleaving activity) is activated by the presence of zanamivir at site I, then lasting receptor engagement is mediated by binding at site II, as shown in Fig. 10E. With the continued engagement of receptor by the chimeric G protein, F proceeds to fusion (Fig. 10F). Our results indicate that for Nipah virus, like for HPIV3, fusion occurs only when the engagement of the chimeric G protein with the receptor is maintained throughout the process. Future work will elucidate whether the involvement of the Nipah receptor binding protein also is required beyond the insertion of the fusion peptide into the target.

Our data for Nipah virus are at variance with a recent study using a set of chimeric glycoproteins based on the stalk region of Nipah G and the globular domain of NDV HN, which were not able to promote Nipah F-mediated fusion (40). The differences are that the globular head domain was identical to the one used here, while the stalk domain was slightly different (they used Nipah G aa 1 to 182, while we used aa 1 to 188), and that we used zanamivir to maintain receptor engagement. In the earlier report, the neuraminidase activity of NDV HN site I may have disengaged HN from its receptor, and/or binding site II may have engaged. It is possible that the region of aa 182 to 188 in Nipah G is responsible for either clamping the F protein, as in a previously proposed model (2, 6, 29, 32, 40), or that these residues are required to transmit the fusion activation signal to the stalk domain.

Based on our data and the available evidence, we favor a unifying model for paramyxovirus fusion activation in which receptor engagement is required for the activation of F and is not simply responsible for unleashing F to fuse. It will be important to apply the same set of experimental strategies to additional paramyxoviruses that lack receptor-destroying activity and whose receptor is not sialic acid based. For these viruses, including measles virus, it has been suggested that the

FIG. 10. Schematic representation of role of binding site II of the G-HN chimera in F activation. The use of chimeric Nipah G/NDV HN as a receptor binding protein and Nipah F as a model system is described in Discussion. The chimeric protein, comprised of the stalk of Nipah virus G (triggering specificity) and the globular head domain of NDV HN (receptor specificity and neuraminidase activity), engages the receptor on the target cell (A and B) but is detached by HN site I receptor-cleaving activity (C) and therefore fails to activate Nipah virus F. (E) If, however, HN site II (which lacks receptor-cleaving activity) is activated by the presence of zanamivir at site I, then lasting receptor engagement is mediated by binding at site II. (F) With the continued engagement of the receptor by the chimeric G protein, F proceeds to fusion.

role of the receptor binding protein is mainly repressive (2, 6, 12, 29, 32, 40, 45, 50), and that the classical spring-loaded mechanism applies: the receptor binding proteins stabilize the metastable state of the fusion proteins prior to receptor engagement, and upon receptor binding the fusion proteins are released and proceed independently to fusion (29, 32, 40, 45). Our working hypothesis is that a receptor-engaged attachment protein is responsible for localizing the fusion complex to the site of entry, and that the physical interaction of receptor-engaged attachment protein with F is required for the critical number of F proteins to be recruited to the site of fusion pore formation. A special case is represented by respiratory syncytial virus (RSV), whose infection does not require any surface protein other than F, and whose mechanism of activation of F through the series of intermediates remains to be determined (9).

The results reported here have medical relevance for understanding the mechanism of resistance to entry/fusion inhibitors, including the peptide inhibitor in clinical use for HIV (19) and the neutralizing antibodies in development for

the influenza virus (17, 71). The current model, which informs strategies to counteract viral resistance, suggests that fusion inhibition should not be overcome by mutations in the receptor binding protein (or domain) but only by mutations in the fusion protein. We have shown instead that HN variants that are more efficient at activating F may promote partial resistance to standard peptide inhibitors (60, 61).

Future mechanistic studies will address the specific nature of HN-F interaction before and during receptor engagement by HN. We have shown that the fusion-triggering function of the paramyxovirus receptor binding protein has functional relevance in a model that mimics the natural host, and it is key for pathogenesis (48). The experiments reported here suggest that a common theme for paramyxoviruses underlies these dynamics. The pursuit of the paradigm described here should provide important insights into how paramyxoviruses achieve fusion via a common mechanism, with entry mediated by a fusion complex within which both receptor binding and fusion promotion are balanced ideally.

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