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Modes of Paramyxovirus Fusion: a Henipavirus perspective

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Abstract

Henipavirus is a new genus of paramyxovirus that uses protein-based receptors (EphrinB2 and EphrinB3) for virus entry. Paramyxovirus entry requires the coordinated action of the fusion (F) and attachment viral envelope glycoproteins. Receptor binding to the attachment protein triggers F to undergo a conformational cascade that results in membrane fusion. The accumulation of structural and functional studies on many paramyxoviral fusion and attachment proteins, including recent structures of Nipah and Hendra virus G bound and unbound to cognate ephrinB receptors, indicate that henipavirus entry and fusion differs mechanistically from paramyxoviruses that use glycan-based receptors.

Nipah and Hendra virus: a different kind of paramyxovirus

Nipah (NiV) and Hendra (HeV) viruses are the deadliest human pathogens within the *Paramyxoviridae* family, which include human and animal pathogens of global biomedical importance. Their genetic distinctiveness warranted designation as a new genus (*Henipavirus*) within the *Paramyxovirinae* subfamily (Box 1). NiV and HeV were first isolated in the 1990s during fatal outbreaks amongst pigs (NiV), thoroughbred horses (HeV), and humans who came in contact with the infected animals (reviewed in [1]). NiV and HeV infections cause respiratory and encephalitic illness, and NiV's mortality rate in humans can exceed 70%. The natural reservoir appears to be bat species found in South East Asia and Australia. Horses and pigs serve as amplifying reservoirs for Hendra and Nipah virus, respectively, but direct bat to human and *human-to-human* transmission can occur [2]. NiV and HeV are the only paramyxoviruses classified as BSL4 pathogens due to their extreme pathogenicity and bioterrorism potential [1]. Henipaviruses (HNVs) use highly conserved protein receptors that account for their broad species tropism, a rare feature amongst *Paramyxovirinae* members. The clear correlation between receptor usage, cellular tropism, and end-organ pathology that results in the morbidity and mortality found in henipavirus infections makes the study of henipavirus entry particularly illuminative [3, 4].

Paramyxovirus entry and fusion requires the coordinated action of both the fusion and attachment envelope glycoproteins [5, 6]. Paramyxoviral fusion proteins are formed in a metastable state. They are activated by endogenous proteases that cleave the precursor F_0 protein into disulfide-linked F_1/F_2 subunits. Once activated, they are poised to undergo a series of conformational changes that results in membrane fusion. This fusion protein cascade is itself triggered by receptor-induced signals from the attachment protein.

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Paramyxoviral attachment proteins vary in the kinds of receptors they bind, although they all appear to have a flexible stalk region connected to a globular head domain that adopts a common six-bladed beta-propeller fold [7–13]. Many paramyxoviruses have attachment proteins with hemagglutinin and neuraminidase (HN) activity. These attachment proteins bind sialic acid receptors present on surface glycoproteins or gangliosides; hemagglutination being a convenient way of assaying sialic acid binding activity due to the abundance of sialic acids on red blood cells (RBCs). The same active site that binds sialic acid is thought to contain the neuraminidase activity [10] that cleaves itself off from cell surface receptors facilitating viral release. Some paramyxoviruses like the measles virus (MeV) have attachment proteins (H) that retain hemagglutinin but not neuraminidase activity. However, MeV-H's hemagglutinin activity is qualitatively distinct from HN's (Box 2). Finally, the attachment glycoproteins (G) of henipaviruses have neither hemagglutinin nor neuraminidase activity. Henipavirus-G uses ephrinB2 [14, 15] and ephrinB3 [16] as receptors, which are expressed on endothelial cells and neurons; the tropism for these two cell types largely accounts for the hallmark pathogenic events (microvascular endothelial syncytia and neuronal dysfunction) associated with Nipah and Hendra virus infections [3, 4, 17].

Receptor binding to the attachment protein triggers F to undergo the dramatic conformational cascade that results in membrane fusion. The nature of this triggering signal is not well characterized and may indeed be different between HN, H and G. As the general structural and functional features of paramyxovirus entry and fusion have been recently reviewed [5, 18, 19], this review will focus on the unique features of henipavirus entry mechanisms. The trove of structural and functional studies on many paramyxoviral fusion and attachment proteins, including recent structures of Nipah and Hendra virus G bound and unbound to cognate ephrinB receptors [12, 13, 20, 21], indicate that henipavirus entry and fusion differs mechanistically from other paramyxoviruses. Differences can be illuminating precisely because they illustrate similarities as well. We hope that highlighting the differences in henipavirus entry will facilitate a better understanding of paramyxovirus entry in general. Table 1 summarizes the unique features of henipavirus envelope glycoproteins and will serve as a point of reference for this review.

Henipavirus G (attachment) protein

Henipavirus-G oligomerize as dimers of disulfide linked dimers (HeV-G) [22, 23], consistent with the tetrameric architecture common to other paramyxoviral attachment proteins [5]. However, structural alignment of attachment protein dimers from HeV-G, MeV-H and hPIV3-HN reveals some surprising distinctions (Fig. 1). While the globular head of all attachment proteins have six-bladed propeller structures, the size and location of the receptor-binding site on HN, H and G can differ substantially. The receptor-binding sites on HeV-G and PIV3-HN extend from the central core of the six-bladed beta-propeller and the respective receptor-binding footprints are located on the plane parallel to the blades of the beta-propeller (Fig. 1a and 1b). In contrast, the receptor-binding surface for MeV-H maps to the “side” of the beta-propeller barrel betweens blades 4 and 5 [7, 8, 24] (Fig. 1b and 1c).

Structural phylogeny analysis indicates that HNV-G is more closely related to HN than H [12]. HN is thought to be the ancestral attachment protein as HN-bearing paramyxoviruses are more widespread and can be found in lower vertebrates like reptiles. However, the greater structural divergence of MeV-H from HN than HNV-G implies that henipavirus-G independently evolved the ability to use protein-based receptors *after* MeV-H's own switch to using protein-based receptors. The relative angle of dimer association also supports a greater kinship between hPIV3-HN and HeV-G [21]. In Fig. 1b, the right monomer of

hPIV3-HN needs to rotate about 31° to match the orientation of HeV-G, while MeV-H will have to rotate 63° away from the direction of HN's rotation to match HeV-G. Thus, the relative angle of dimer association between MeV-H and PIV3-HN is more than 90° underscoring their greater divergence from each other than to HeV-G (Fig. 1b).

The receptor interacting surface in HeV-G is much larger than MeV-H and PIV3-HN (Fig. 1b, Table 1) and may account for the unusually high affinity between Henipavirus-G and ephrinB receptors [16]. On the other hand, the dimer interface area in HeV-G and MeV-H is almost half of most HN proteins (800–1000 $Å^2$ vs 1,800 $Å^2$)[21] (Fig. 1d and Table 1). Perhaps, the strength of dimer interface interactions is important for HN function but less so for MeV-H and HeV-G. Indeed, engineering disulfide bonds to stabilize dimer interface interactions in NDV-HN enhances fusion [25] while the same strategy inhibits fusion for MeV-H [26]. Thus, the mode of receptor binding, the relative angle of dimer association, the dimer interface area, and the variable effects of disulfide bond stabilization of dimer head domains on fusion, already suggest mechanistic differences early on in the fusion cascade between glycan- versus protein-receptor using paramyxoviruses.

Receptor-induced conformational change

The notion of receptor-induced conformational change in paramyxoviral attachment proteins is a matter of some controversy and is likely to be different between HN, H, and G (Table 1). For HN, receptor binding likely does not induce a significant conformation change. Crystal structures between the receptor bound or unbound hPIV3 [27] and PIV5 [10] HNs are almost identical. For NDV HN, the initial structure suggested that receptor binding induces a dramatic change in NDV-HN's dimer conformation [28]. However, subsequent studies called into question the biological relevance of this change as disulfide bonds engineered to preemptively lock NDV HN in the post-receptor binding conformation, enhance, rather than inhibit fusion[25]. For some HNs (e.g. NDV and hPIV3) [11, 29, 30] [31] but not others (PIV5) [10], there is evidence to indicate that initial receptor binding may modulate formation of a second sialic acid binding site between the HN dimer interface. Regardless of whether a second binding site model is generalizable to all HN-bearing paramyxoviruses, HN needs to maintain both receptor binding and neuraminidase activity—a unique constrain not imposed on H and G, which further suggests that the fusion promoting activities of protein-based receptors can evolve under a different set of selective pressures.

For MeV-H, the crystal structures of MeV-H bound or unbound to the first two short consensus repeat domains (SCR1-2) of CD46 do not show a dramatic conformational change (r.m.s. deviation of 1.33 Å over 791 equivalent residues) [24]. However, Cattaneo and colleagues have suggested that receptor binding to MeV-H can rotate the MeV-H dimer association angle with respect to the vertical stalk axis, and that this re-alignment of the dimer heads triggers the underlying F protein by releasing the “clamp” on metastable F [26]. In support of this model, engineered disulfide bonds between the obliquely oriented MeV-H dimer heads (Fig. 1) that prevented rotation around the stalk axis compromised fusion without affecting receptor binding. Thus, some movement of the MeV-H head domains post-receptor binding seems to be required for F triggering. These kinds of changes would not be seen in crystal structures of soluble envelope-receptor complexes as they do not take into consideration the molecular tension imposed by membrane anchoring of both receptor and envelope proteins.

The evidence for receptor-induced conformational change is perhaps the strongest for henipavirus-G. The crystal structures of receptor bound and unbound forms of HeV-G and NiV-G indicate significant deviations (r.m.s. >3 Å) in certain loops between the beta-sheets

in blades 1 and 6 [12, 13, 21] as they move to accommodate the incoming ephrinB receptor. These conformational changes are more than what is seen for receptor binding to HN and H, but still do not seem particularly dramatic. However, the absence of the stalk domain, implicated by other biophysical methods in the ephrinB2-induced conformational changes that are critical for F-triggering [32], limits our interpretation of these soluble receptor-envelope complexes.

Both HeV-G [22] and NiV-G [32] exhibit receptor-induced monoclonal antibody epitopes that are better exposed upon ephrinB2 binding. The monoclonal antibodies (mAb), derived from different species, and generated via different methods, cross-react with the other henipavirus-G that was not the immunogen [32, 33], suggesting they target a conserved functional epitope. The receptor binding enhanced (RBE) epitope on NiV-G has been more fully characterized for Mab45 [32]. Its salient properties are: (1) Mab45's RBE epitope is temperature-dependent, (2) Mab45 exhibits neutralization activity without competing for receptor binding, (3) Mab45's complex RBE epitope maps to the base of the head domain, extends into the predicted dimer interface area (B6S4 to B1S1), but also requires an intact stalk domain, and (4) RBE epitope mutants are competent to bind receptor, but are fusion-deficient. Interestingly, in the majority of the structures, the regions of greatest flexibility coincide with residues that when mutated give rise to the most fusion-defective Mab45 RBE epitope mutants (~ aa205–211). This region is also predicted to be in or near the dimer interface (B6S4-B1S1) [12, 13, 20, 21]. Thus, receptor-induced F protein triggering may be a functional correlate of this region's plasticity, although it is unclear whether the physical region is directly involved in F triggering.

Abundant evidence implicates the stalk domain in self-oligomerization and the homotypic F interactions that lead to F triggering, properties that are critical to fusion promotion [34]. Electron micrographs of the full PIV5 HN ectodomain clearly showed flexible stalk structures linking globular head structures in multiple oligomeric forms [35]. However, the inherent flexibility of the stalk domain also limits our efforts to characterize its structure by traditional crystallographic methods [10].

Attachment and fusion protein interactions

Paramyxovirus entry and fusion is a molecular choreography requiring cognate interactions between protein partners (HN/H/G and F) in which motion and form must both be carefully regulated. The fusion protein is a metastable protein that is triggered, at the right time, and in the right place, to undergo an irreversible conformational cascade that results in merger of envelope and receptor containing membranes. Fusion protein triggering is regulated by receptor-induced allosteric signals from the attachment proteins. The nature of this signal induced by receptor binding, and how this is transmitted in a process that triggers F, is an area of intense investigation and also a matter of great debate. HN, H, and G differ in kinds of receptors they use, their mode of receptor binding and dimer association, and the kind of conformational change that is induced upon receptor binding. Thus, F triggering may occur via different mechanisms between glycan- and protein-receptor using paramyxoviruses. Several recent reviews have contrasted the prevailing models of paramyxovirus fusion [19, 34, 36], variously called the “dissociation” versus “association ” model, or more recently, the “clamp” versus “provocateur” model [37]. The *clamp* versus *provocateur* nomenclature provides an evocative imagery that better clarifies the key distinctions between models for F triggering. In the clamp model, the attachment protein stabilizes the metastable F protein, and receptor binding induces the attachment protein to release F from its “clamp” thus triggering the fusion cascade. The provocateur model posits that upon receptor binding, the attachment protein actively transduces an allosteric signal that destabilizes F, “provoking” F to undergo the fusion cascade.

HN-F interactions

The strongest evidence suggest that HN-F do not interact until they reach the cell surface and that F-HN interactions induced by receptor binding is the key parameter that triggers the F-mediated fusion cascade. For many paramyxoviruses, there is little convincing evidence that HN-F interact in the absence of receptor [34, 38]. In **NDV HN**, point mutations that destroy sialic acid binding and neuraminidase activity but do not affect conformational integrity, also abolish HN's ability to co-immunoprecipitate with F[39], indicating that receptor binding triggers HN-F interactions of high enough avidity to detect by co-immunoprecipitation. In addition, HN stalk mutations that decrease fusion also decrease the avidity of HN-F interactions. Thus, fusogenicty is *positively* correlated with F-HN avidity[40]. This was inadvertently confirmed for PIV5, where HN and F engineered with spilt fluorescence tags in their cytoplasmic termini, were unexpectedly hyperfusogenic because the intrinsic affinity of the spilt fluorescent tags for each other was higher than that of HN for F [37]. By various measures, Lamb and colleagues also provided evidence that the key role of HN in F triggering is to destabilize F upon receptor binding [37]. Overall, these data are consistent with the *provacateur* model for HN-bearing paramyxoviruses.

For **MeV and henipaviruses**, the preponderance of evidence is more compatible with the *clamp* model. For **MeV**, the same stalk mutations in H that compromise fusion efficiency in NDV-HN lead to increased F-H avidity [41]. Conversely, ctyoplasmic tags that inadvertently weakened H-F avidity resulted in increased fusion [42]. Taken together, H-F avidity is *negatively* correlated with fusogenicity. Also, intracellular retention of H or F resulted in convincing co-retention of the other [43] unlike the negative results obtained for hPIV3 and PIV5 [38]. These results suggest that H-F hetero-oligomerize before they get to the surface. Furthermore, H-F can be easily co-immunoprecipitated in the presence or absence receptor using receptor-negative cells or receptor-binding site mutants [42, 43]. Thus, MeV-H and F clearly interacts before receptor binding, which suggest a different mode of regulating fusion triggering compared to HN-F.

For **henipaviruses**, G-F avidity is also *negatively* cor related with fusogenicity [44–46]. Like MeV-F/H, henipavirus F and G can be co-immunoprecipitated in the absence or presence of receptor [23, 32, 44–46]. For both HeV-G and NiV-G, the receptor induced conformational change in G can be linked to fusion triggering. In HeV-G's case, the evidence is indirect: stalk region mutations were found that abrogated fusion, but were otherwise wild-type with respect to oligomeric status, ability to bind ephrinB2/3, and co-immunoprecipitation with F [22]. Intriguingly, many of these mutants bound better to a Mab with a receptor induced epitope [22]. This suggests that G itself is metastable and that the receptor induced conformational change needs to be carefully regulated, as premature transition to a post-receptor binding conformation abolishes G's ability to trigger F. Importantly, receptor binding to NiV-G can induce NiV-F dissociation, and fusion-defective mutants that lack the receptor induced conformational change (e.g. Mab45 RBE epitope mutants) can still bind receptor but fail to dissociate from F [32]. Thus, F dissociation from G is a key parameter that governs F triggering upon receptor binding. Altogether the evidence suggests that henipavirus F-G are in a pre-formed complex that is dissociated upon receptor binding, which is more consistent with the “clamp” model.

Henipavirus F (fusion) protein processing

Paramyxovirus F proteins are synthesized as inactive precursors (F_0) that need to be cleaved by cellular proteases into disulfide linked subunits ($F_1 + F_2$). Cleavage generates a metastable protein that is now “activated” and poised to undergo the conformational fusion cascade when triggered by cognate signals from the receptor-bound attachment protein. Most *Paramyxovirinae* either have a monobasic cleavage site activated by secreted trypsin-

like proteases in the respiratory tract (e.g. Sendai) or a dibasic site recognized by furin-like proteases in the *medial* and *trans*- Golgi (e.g. Mumps and Measles virus)[19]. Henipavirus-F has an apparent monobasic cleavage site (VGDV-K/R_{109}) that is neither cleaved by trypsin[47] nor furin[48]. Instead, henipavirus-F traffics to the cell surface as F_0 and requires subsequent endocytosis[49, 50] to be cleaved by capthesin L[51, 52] in acidic endosomes before being recycled back to the cell surface as activated F_1/F_2. Thus, henipavirus-F has a processing requirement unique amongst paramyxoviruses.

These complex trafficking and processing requirements of F impose multiple constraints on where and when productive F/G complexes may form. Interestingly, HeV-G traffics through to the medial and trans Golgi(~120 min)[53] much slower compared either to other HNs (15–30 min for NDV[54], PIV5[55] and hPIV3[38]) or to HeV-F itself (30 min)[53]. This indicates HeV-F and G are unlikely to form complexes on their way to the surface. Since F maturation/cleavage requires endocytosis from the cell surface and recycling, G's slower trafficking may enhance the chances of associating with cleaved fusion-competent F at the cell surface. Indeed, cell surface biotinylation assays indicate that mature cleaved F does not start accumulating appreciably until 2 hours post-synthesis and peak at about 6 hours, similar to the kinetics of G surface accumulation[53]. Thus, the disparate trafficking rate between F–G compared to F-HN may have evolved to synchronize cell surface F–G interactions that take into account the added time needed for F processing.

Henipavirus F (fusion) protein structure

Henipavirus-F proteins have conserved structural features common to all paramyxoviral fusion proteins. The functional unit is a homotrimer, and each monomer has N- (HR1) and C- (HR2) terminal heptad repeats, and a cleavage site immediately N-terminal to the fusion peptide. Fig. 2 shows a schematic of these conserved features in relation to the three identifiable domains that can be tracked during the fusion cascade[56]. The structural transitions from the pre to post fusion forms of paramyxovirus F are summarized in Fig. 2 b–d and detailed in recent reviews[5, 18, 57]. The pre-fusion form (Fig. 2b) has a short stalk and globular head that resembles a “lollipop” while the post-fusion form is more elongated and shaped like a “golf tee” (Fig. 2d). Critically, the fusion cascade progresses through a transitional structure known as the pre-hairpin intermediate (PHI) (Fig. 2c).

Henipavirus-F follows the general features of this fusion cascade (Fig. 2b–d). The 6-HB core of henipavirus-F has been crystallized[58], and HR2 region derived peptides block henipavirus entry and fusion[45, 58–60], presumably by binding to the HR1 trimer core in the PHI and preventing 6-HB formation. This model predicts that the stability of HR2/HR1 interhelical interactions, and the transition time to 6HB formation may affect the sensitivity to HR2 peptide inhibition. Indeed, heterotypic HR2 peptides from hPIV3 inhibit henipavirus entry better than homotypic henipavirus HR2 peptides by virtue of the HR2 hPIV3 peptide forming more stable 6-helix bundles with the henipavirus HR1 trimer core[60, 61]. Interestingly, time of addition experiments indicate that henipavirus entry remains susceptible to this HR2 peptide inhibition for a longer time following F triggering than hPIV3 itself. This suggests the kinetics of hPIV3 F triggering is faster than that for henipavirus F[62]. Indeed, low affinity HN-sialic acid interactions (where the probability of falling off the receptor is high) likely selects for rapid F triggering upon receptor binding, whereas high affinity H/G protein receptor interactions (where the measured off rate is exceedingly slow) may trigger F at a more leisurely pace (Table 1).

Henipavirus F glycosylation

Paramyxovirinae F proteins vary in the number (3–6), location, and function attributed to their N-linked glycans[45]. Despite this variability, *some* of the extant N-glycans for a given

F protein is required for proper folding, transport or processing as multiple N-glycan removal generally compromise F function. Surprisingly, henipavirus-F is unusually resistant to removal of N- glycans. There are four utilized N-linked glycosylation sites in henipavirus-F: the F2 and F3, and the F4 and F5 sites in the F_2 and $_1$ subunits, respectively [45, 63, 64](Fig. 2a). Removal of every N-glycan (except F4), singly or in combination, results in hyperfusogenicity with no overt effects on folding, processing, and cell surface expression[45], suggesting that N-glycans at these sites intrinsically limit F function.

Fig. 3 shows the location of the F3 and F5 N-glycan sites on the modeled pre- and post-fusion forms of Nipah-F. Only a few paramyxoviruses have N-glycans at homologous sites, but their removal results in contrasting phenotypes, which suggest mechanistic differences in how F promotes fusion. For example, removal of the F3 N-glycan (or its homolog) results in hyperfusogenicity in both NiV and SeV that are not mechanistically equivalent. This N-glycan removal led to increased F cleavage in SeV[65], but had no effect on NiV-F processing[45, 63, 64]. Thus, the F3 N-glycan limits NiV fusion in a different way. Fig. 3a shows the F3 N-glycan site in pre-fusion F where its proximity to the fusion peptide and the HR1 segments (yellow) (Fig. 3a, z_0 may interfere with structural transitions to the PHI.

The F5 N-glycan site is located in the HR-2 region close to the base of the globular head region in the pre-fusion F (Fig. 3). Although SV5 and NDV also have N-glycans in the HR-2 region, their removal results defective folding[66] or reduced fusogenicity[67]. This further suggests that henipavirus F may be triggered differently from other paramyxovirus F or undergo structural transitions that are not limited by parameters that guide other F protein transitions. Increased fusogenicity associated with most N-glycan losses in NiV-F is also correlated with increased sensitivity to antibody neutralization suggesting that opposing selective pressures may have lead to maintenance of N-glycan sites that limits fusion.

Concluding remarks

An accumulating body of data indicates that Henipavirus entry differs in many respects from other paramyxoviruses. Although henipavirus fusion and attachment proteins share common structural features with other paramyxoviral envelope glycoproteins, they differ in their mode of receptor interaction and how the quaternary structure of the fusion and attachment protein complex translates receptor binding into a series of conformational changes that result in membrane fusion (Table 1). For glycan-using paramyxoviruses, the abundance and ubiquity of sialic acid bearing receptors and the need to regulate receptor binding and cleavage activity on the attachment protein, likely imposes a different set of constraints on the optimal way HN can evolve to trigger F upon receptor binding. Henipaviruses also have complex F processing and trafficking requirements that may impact on where, when and how ephrinB-receptor binding to G induces F triggering and the fusion cascade. The totality of evidence suggests that glycan binding to HN triggers F differently from protein-receptor binding to morbillivirus-H or henipavirus-G.

Despite our increased understanding of the paramyxovirus fusion process, *how* receptor binding regulates the fusion and attachment glycoprotein interactions that result in membrane fusion remains an ill-defined process. Potential roadblocks include the lack of structural information on any full-length paramyxovirus attachment protein (i.e. how the stalk domain is organized in relation to the globular head domain), and on F–G (H/HN) complexes either as soluble ectodomains or in situ on virions. The inherent flexibility of the stalk domain may necessitate strategies that can stabilize or select for particular conformers. A careful comparison on the kinetics of fusion triggering using complementary but distinct assays may further refine the most applicable models to use in describing receptor-mediated F triggering for henipaviruses. Potential differences between how glycan-using (HN) and

protein-using (H or G) paramyxoviruses regulate the fusion cascade may serve to highlight the outstanding questions in the field (see box), the answers to which may illuminate virus entry mechanisms in general.

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Outstanding Questions

- What are the cognate interacting regions between henipavirus-F and –G, and if they differ from F-HN or F-H interacting regions in other *Paramyxovirinae*?
- What is the structural organization of the stalk domain? How does receptor-binding change the conformation of full-length G (or at least the ectodomain of G)?
- How can we obtain the structures of the F-G complex? What kinds of stabilization or “homogenization” strategies do we need?
- What is the stoichiometry of F-G interactions required for productive fusion? How many F-trimers and G dimers/tetramers act co-ordinately to form a fusion pore?
- What is the organization of envelope spikes on virions? Does spike density or arrangement differ between HN, H and G bearing paramyxoviruses?
- What are the key parameters that govern the kinetics of F triggering? How do they differ between glycan- and protein-using paramyxoviruses?

Box 1 Paramyxovirus phylogeny

The paramyxoviruses (*Paramyxoviridae* family) are membrane enveloped, singled-stranded, negative-sense RNA viruses. They are classified into 7 genera belonging to two subfamilies. Until the turn of the new millennia, *Respirovirus, Morbillivirus*, and *Rubulavirus* were the three main genera that comprise the *Paramyxovirinae* subfamily. They generally contain three structural and three non-structural genes. The fusion and attachment envelope glycoproteins are two of the three structural proteins required for virus entry and fusion. Hendra and Nipah viruses have the same gene organization but have substantially longer genomes (~18 kb vs <15 kb) and sequence divergence to merit classification into a new genus named *Henipavirus*. The designation of a new Henipavirus genus also led to a re-classification of a group of avian paramyxoviruses into their own *Avulavirus* genus (they were formerly part of the Rubulavirus genus). Presently, 5 distinct genera comprise the *Paramyxovirinae* subfamily. *Metapneumovirus* and *Pneumovirus* are more distantly related, have several additional non-structural genes, and form a separate *Pneumovirinae* subfamily. In addition, while both subfamilies have fusion and attachment proteins, the *Pneumovirinae* do not appear to require the attachment protein for virus entry and replication. Thus, we will restrict our review on henipavirus entry to comparisons with other members of the *Paramyxovirinae* subfamily.

The majority of paramyxoviruses cause some sort of respiratory disease although henipaviruses can cause severe encephalitis in addition, while morbilliviruses (Measles in humans, Canine Distemper Virus in dogs, Rinderpest virus in cattle/swine) can cause immunosuppression, CNS disease, and gasteroenteritis. Respiroviruses (Human parainfluenza 1, 3) and Rubulaviruses (HPIV2, 4 and Mumps virus) can cause significant morbidity in pediatric populations. Mumps is a well-known childhood disease that can lead to swelling of the salivary glands and testicles, and can even proceed to frank meningitis in susceptible unvaccinated individuals. Epidemics of Newcastle disease virus and other virulent strains of avian paramyxoviruses (Avulavirus) can have a significant economic impact in the poultry industry.

Box 2 Paramyxovirus attachment protein nomenclature

All paramyxoviruses have fusion and attachment proteins, although deletion of the attachment proteins in *Pneumovirinae* does not appear to affect viral replication. Amongst the *Paramyxovirinae*, only the morbilliviruses and henipaviruses use protein-based receptors for entry. Respirovirus, Rubulavirus and Avulavirus use sialic acids on cell surface glycoproteins or gangliosides as receptors and their attachment proteins have hemaggultinin and neuraminidase (HN) activity. The neuraminidase activity is essential for cleaving off the sialic acid receptor and releasing virions from the producer cell during viral replication.

Morbillivirus (MV) attachment proteins retain hemagglutinin, but lack neuraminidase activity and are designated as H. This terminology is unfortunate as it conveys the impression that MV-H's ability to agglutinate red blood cells (RBCs) is due to some residual sialic acid binding activity. This is not true as all seven essential sialic acid binding site residues in HN are missing in MV-H [8] and sialidase treatment *enhances* rather than inhibit infection as they do for glycan-receptor using *Paramyxovirinae* [71]. Measles virus (MeV), a prototypical morbillivirus that naturally infects only humans, uses protein-based receptors such as CD46 and/or SLAM [69]. Curiously, MeV-H can only agglutinate RBCs from certain primates, but not humans [71]. Non-human primates (e.g. African green monkeys) express high levels of CD46 on their RBCs, whereas CD46 in humans is expressed in all nucleated cells except RBCs (hence, the exception). Specific mutations in the CD46 promoter region may have accumulated in the human lineage that resulted in the lost of erythroid specific expression.

Henipavirus (HNV) attachment proteins have neither hemagglutinin nor neuraminidase activity, and are designated as G (for glycoprotein). HNV-G uses ephrinB2 (EFNB2) and/or ephrinB3 (EFNB3) as entry receptors. Henipavirus G proteins bind ephrinB receptors with extremely high (picomolar) affinity (EFNB2>EFNB3)[16], but in contrast to morbilliviruses that exhibit species-specific interactions with their protein receptors, henipaviruses can use EFNB2 and EFNB3 from a multitude of animal species[68]. Astonishingly, even zebrafish ephrinB2 can be used for viral entry (unpublished observations).

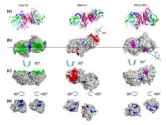

Figure 1. Relative positions of the ligand binding sites and dimer interfaces of the Hendra virus (HeV-G), Measles virus (MeV-H) and Parainfluenza virus 3 (PIV3-HN) attachment glycoproteins

(a) Cartoon representations of HeV-G, MV-H and PIV3-HN (PDB accession no. 2X9M, 3INB, and 1V2N, respectively) in their dimeric form are shown with the left monomer in each case anchored in the same orientation. The central axis of the six-bladed beta-propeller in each left monomer is pointing towards the reader, and each blade is color coded as indicated (b1;red, b2;blue, b3;green, b4;cyan, b5;gray, b6;magenta). (**b**) Surface representation of the cartoon structures are shown. The ligand binding residues are highlighted in green (ephrinB2 on HeV-G), red (CD46 on MV-H), and magenta (sialic acid on PIV3-HN). Residues involved in monomer-monomer interactions at the dimer interface are shown in blue and may not be fully visible in this view. According to the analysis performed by Bowden et al[21], relative to the angle of association between HeV-G dimers (set at 0°), the angle of association between MeV-H and PIV3-HN dimers is 63° and 31°, respectively. In other words, if the left monomer for all three attachment proteins is anchored to match the orientation of HeV-G, then the right monomer of MV-H would have to rotate 63° away from the reader into the page in order for it to line up with the right monomer of HeV-G. In contrast, the right monomer of PIV3-HN would have to rotate 31° towards the reader in order for the same alignment to be achieved. **(c)** Dimers in panel B are rotated 90° towards the reader so that the ephrinB2 binding site on HeV-G is now facing upwards towards the direction of the target cell. **(d)** To better visualize the dimer interacting surface the monomers in each dimer are separated with a motion akin to opening a pair of French windows from the outside. That is, each monomer is rotated 90° towards the reader. The dimer interacting residues colored in blue are now more easily visualized and the approximate area buried in the dimer interfaces are circled. The dimer-interface areas were calculated using PISA as 876.2 $Å^2$, 1075 $Å^2$, 1780.2 $Å^2$ for HeV-G, MV-H and PIV3-HN, respectively.

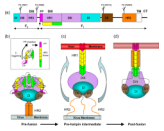

Figure 2. Schematic representation of the Nipah virus fusion (NiV-F) protein and major structural transitions during the fusion cascade

(a) Linear schematic of NiV-F. Domains I, II, and III are colored cyan, brown, and purple, respectively. The fusion peptide (FP) is a stretch of ~25 residues (magenta box) immediately C-terminal of the F cleavage site (upright arrow). Cathepsin L cleaves F into disulfide linked F_1 and F_2 subunits. The N- (HR1) and C- (HR2) terminal heptad repeats are both in the F_1 subunit. HR1 is located in the DIII domain and the HR2 is close to the transmembrane region. A third heptad repeat (HR3) is found in the F_2 subunit, and forms part of the DIII core that helps to stabilize the HR1 segments in the pre-fusion form. The relative positions of the 4 utilized N-linked glycosylation sites (F2–F5) are indicated by branched stick figures. Schematic of the pre-fusion (**b**), pre-hairpin intermediate (**c**), and postfusion form of F (**d**) adapted from Russell et al [57]. The color scheme for the major structural features are as indicated in (a). In the pre-fusion form (**b**), the individual segments of HR1 (yellow and green) are in a variety of secondary structures tucked into stable elements of the DIII core at the top of the trimer. For clarity, the boxed insert shows an isolated HR1 region undergoing F-triggering. Receptor induced allosteric signals from the attachment protein induces release of the fusion peptide (FP, magenta), which then inserts itself into the host cell membrane. FP insertion facilitates re-folding of the disparate HR1 segments into an extended alpha helix. The fusion cascade now progresses through a transitional form known as the pre-hairpin intermediate (PHI) shown in (**c**). Re-folding of the HR1 segments forms an N-terminal trimeric coiled coil that now anchors F to the host cell membrane. The HR2 alpha-helices (orange) that form the stalk of the pre-fusion structure disassemble, and translocate 180° to pack against the interstices of the HR1 trimer in an anti-parallel fashion. Packing of the HR2 segments onto the trimeric HR1 core forms a six-helix bundle (6-HB) structure, which necessarily brings the viral membrane in close apposition to the host cell membrane (not shown). Formation of the stable 6-HB structure drives merger of the virus and cell membranes that results in membrane fusion. The post-fusion structure of F with the 6-HB is shown in (**d**).

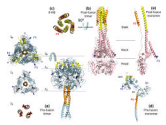

Figure 3. Structural models for the pre- and post- fusion forms of Nipah virus F

Models were generated using 3D-PSSM (http://www.sbg.bio.ic.ac.uk) based on the pre- and post-fusion PIV5-F [56] and hPIV3-F [9] structures, respectively (**a**) Trimeric model of the pre-fusion form of NiV-F. HR1 and HR2 sequences that eventually fold into the six-helix bundle in the post-fusion form are colored in yellow and orange, respectively. The F3 (Asn 99) and F5 (Asn 464) N-glycan sites are indicated by blue (F3) and red (F5) ball and stick figures. To better appreciate the location of the F3 and F5 N-glycans with respect to conserved features of the trimer (HR1 and HR2 regions), the trimer is rotated 90° towards the reader such that the reader is looking down the three-fold axis of the F protein from the perspective of the target cell membrane. This top-on view is shown at three different *z* levels represented by the dotted lines. From the top of the molecule (z_0 view), the labeled F3 N-glycan site can be seen in close proximity to the cleavage site (asterisk) and the fusion peptide loop (colored black). The HR1 region (yellow) is nicely tucked into a cradle at the vertices of the trimer. For clarity, portions above the dotted lines (distal to viral membrane) at the z_1 and z_2 levels have been removed. (**b**) Trimeric model of the post-fusion form where the head, neck, and stalk domains are indicated. (c) The six-helix-bundle (6-HB) is shown from the perspective of the central three-fold axis just N-terminal (membrane distal) to the HR-2 region. For comparisons, the monomer of both the pre and post-fusion form are shown in (**d**) and (**e**), respectively. The HR1 and HR2 regions and the F3 and F5 N-glycan sites are colored as in the pre-fusion structure.

Table 1

Unique features of henipavirus fusion (F) and attachment (G) envelope glycoproteins

	Receptor interactions	Henipavirus	Ref	Other *Paramyxovirinae*[a]	Ref
1	Unusually high affinity for receptor(s)	NiV-G/EFNB2, Kd=0.06 nM NiV-G/EFNB3, Kd=0.58 nM	[16]	MeV-H/CD46, Kd=2.2 μM HN/sialic acid[b], Kd=~ 0.5 to 5 mM	[8]
2	Receptor highly conserved amongst species	Henipavirus-G can bind to EFNB2/EFNB3 from human, horse, pig, dog, cat, bat and mouse	[68]	MeV-H cannot use CD46/SLAM in non-primates; HN can bind to sialic acid bearing glycoproteins or gangliosides	[69] [70]
3	Receptor interacting surface is larger	Henipavirus-G/EFNB2, ~1,300 Å^2	[12, 13]	MeV-H/CD46,~1,000 Å^2 hPIV3/HN, ~230 Å^2	[8] [9]
4	Conformational change upon receptor binding	Significant deviations (> 3 Å) in certain loops (B6S2-B6S3, B1H1-B1S1,B1S2-B1S3) between bound and unbound Henipavirus-G	[13, 21]	No significant change noted between CD46 bound and unbound MeV-H (r.m.s. deviation 1.33 Å)	[24]
		Receptor induced structural changes monitored by circular dichroism	[32]	No change noted in receptor bound and unbound structures of PIV5-HN and PIV3-HN	[10] [9]
		Receptor binding enhances conformational monoclonal antibody epitope: Mab45 and m106	[32] [22]		

	Attachment protein	Henipavirus	Ref	Other *Paramyxovirinae*	Ref
5	Strongly stabilized by disulfide bonds	Henipavirus-G=7 (one in each blade and one between B3 and B4)	[12, 13]	MeV-H =2 HN=4–5	[8] [9–11]
6	Smaller dimeric interface contact area	HeV-G ~880 Å	[21]	MeV-H ~1,070 Å HN ~1,800 Å (NDV, PIV3, SV5)	[21] [8] [9–11]
7	Relative dimer association angle	HeV-G(set at 0°) (see Fig.1, this paper)	[21]	MeV-H (63°) PIV3 (31°)	[21]

	Fusion protein	Henipavirus	Ref	Other *Paramyxovirinae*	Ref
8	Cleavage and activation	Requires endocytosis from cell surface and cleavage by Cathepsin L before recycling to cell surface in active form	[49–52]	Cleaved by proteases in trans-Golgi during transport to surface, or extracellular protease during transport	[19]
9	YXXL cytoplasmic motif	Required for endocytosis (and subsequent Cap L mediated cleavage)	[49, 50]	Endocytosis not required for F cleavage	
10a	Unique role of N-glycans	Single or multiple N-glycan removal results in hyperfusogenicity	[45]	Variable, but unusual for F to tolerate multiple N-glycan removal	(see text)
10b	N-glycans protect from neutralizing antibodies	Removal of N-glycans increase sensitivity to neutralizing antibodies	[45]	No reports of N-glycan effects on neutralization sensitivity	
10c	N-glycan in HR-3 region (F2 subunit) close to cleavage site	Third N-glycan (F3) site in F2 subunit is in HR-3 region, close to cleavage site; N-glycan removal does not affect cleavage but results in hyperfusogenicity	[45]	Only respirovirus (SeV and hPIV1) have N-glycan in HR-3 region; N-glycan removal (SeV) results in increased cleavage which accounts for hyperfusogenicity	[45] [65]

	Fusion protein	Henipavirus	Ref	Other *Paramyxovirinae*	Ref
10d	N-glycan in HR-2 region	Fifth N-glycan site (F5) in F_1 subunit is in HR-2 region, N-glycan removal does not affect folding or transport, but results in hyperfusogenicity	[45]	PIV5 and NDV have N-glycan in HR-2 site; N-glycan removal results in defective folding or reduced fusogenicity	[66] [67]
11	Kinetics of F triggering	Slower than hPIV3--remains susceptible to HR2 peptide inhibition for a longer time than hPIV3	[62]	hPIV3 appears faster than henipavirus—remains susceptible to HR2 peptide inhibition for a shorter time than henipavirus	[62]

	Fusion and attachment protein interactions	Henipavirus	Ref	Other *Paramyxovirinae*	Ref
12	Effects of receptor binding	F and G co-immunoprecipitate in the absence or presence of receptor	[23, 32, 44–46]	MeV-F/H co-immunoprecipitate in the presence or absence of receptor; intracellular retention of F or H can also retain the other. NDV-F/HN co-immunoprecipitate only when receptor is bound; hPIV3 and PIV5 F/HN do not co-IP well and intracellular retention of one does not affect the other	[43] [39] [38] [38]
13	Modulation of fusogenicity	Fusogenicity negatively correlates with F-G avidity: (a) hyper- and hypo- fusogenic mutants in NiV-F and (b) hypofusogenic mutants in HeV-G	[32] [46]	**MeV:** fusogenicity negatively correlates with F-H avidity **NDV:** fusogenicity positively correlates with F-HN avidity **PIV5:** inadvertently increasing F-HN avidity also increases fusogenicity	[41] [42] [40] [37]

[a] MeV=Measles virus, hPIV3=human parainfluenza virus 3, PIV5=parainfluenza virus 5 (formerly SV5), SeV=Sendai virus, hPIV1=human parainluenza virus 1, NDV=Newcastle Disease virus

[b] Measured by IC50 of entry of inhibition by sialic acid analogs or binding site antagonists.