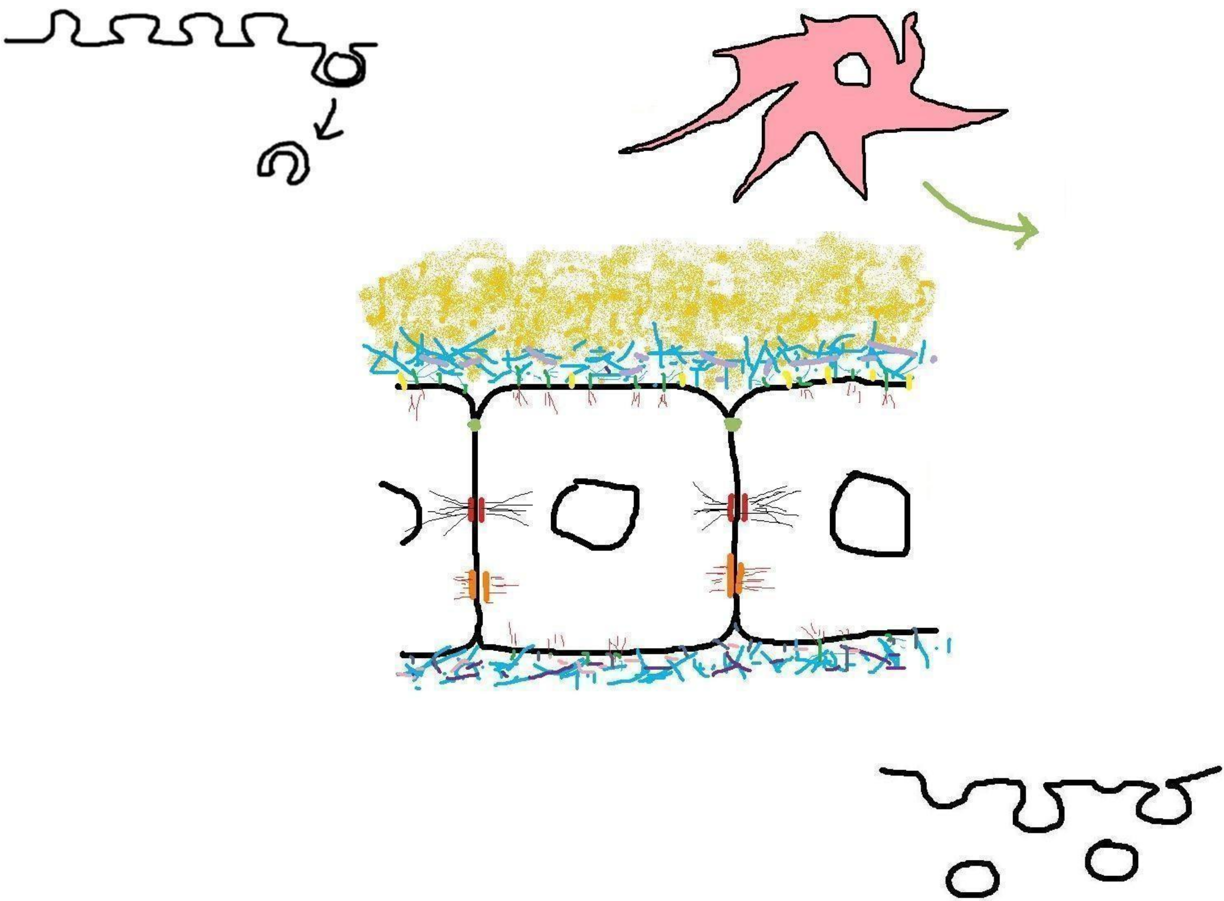
Paramyxoviruses crossing the host membrane: mechanisms for entry



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Literature Thesis Molecular & Cellular Life Sciences 03/2012 – 05/2012

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ABSTRACT

The paramyxovirus family is a group of enveloped, negative-strand RNA viruses that includes common and highly infectious human pathogens such as mumps, measles, respiratory syncytial virus, human metapneumovirus and the closely related, highly lethal zoonotic Hendra and Nipah viruses. Paramyxoviruses are unique in that they have two envelope glycoproteins that mediate virion binding and membrane fusion, instead of a single glycoprotein that fulfills both functions. The following text provides a review on how the two paramyxovirus glycoproteins, the attachment protein and fusion protein, interact with each other, and to which host-cell receptors they bind, to ensure membrane fusion and entry into the host cell. In the *Paramyxovirinae* subfamily, binding of the attachment protein to an entry receptor triggers the fusion protein, which in turn drives membrane fusion. While biochemical studies and recently solved crystal structures have shed some light on this process, the exact mechanism by which the attachment protein triggers the fusion protein upon receptor binding, remains to be elucidated for any of the *Paramyxovirinae*. In the *Pneumovirinae* subfamily on the other hand, the attachment protein is not necessary to achieve membrane fusion and the fusion protein mediates receptor binding as well as membrane fusion. The current view is that virus entry *in vivo* is a highly dynamic process that involves a complex interaction between viral glycoproteins on the one hand and host cell receptors on the other. Furthermore, individual paramyxovirus species might employ multiple entry pathways, using more than one type of receptor, relying on fusion at the plasma membrane as well as on fusion after endocytosis.

ABBREVIATIONS

CDV: canine distemper virus; **DC**: dendritic cell; **GAG:** glycosaminoglycan; **G protein**: glycoprotein; **F protein**: fusion protein; **HMPV**: human metapneumovirus; **HN**: hemagglutinin-neuraminidase; **HeV**: Hendra virus; **hPIV1, hPIV3**: human parainfluenza viruses 1 and 3; **HSPG**: heparan sulfate-containing proteoglycan; **MV**: measles virus; **NDV**: Newcastle Disease virus; **NiV**: Nipah virus; **PIV5** : parainfluenza virus 5; **RNP:** ribonucleoprotein; **RSV:** respiratory syncytial virus; **SeV**: Sendai virus.

1. PARAMYXOVIRUSES

1.1. Paramyxovirus family

Paramyxoviruses (*Paramyxoviridae*) are enveloped, negative-sense single-stranded RNA viruses belonging to the order *Mononegavirales* (Group V Baltimore classification). Members of the *Paramyxoviridae* family are generally highly infectious viruses and include many important human and animal pathogens, such as measles, mumps, human parainfluenza virus 1-4, respiratory syncytial virus and canine distemper virus. Especially measles virus and respiratory syncytial virus remain a major cause of mortality in children worldwide [Griffin, 2007; Hall *et al.*, 2009; Nair *et al.*, 2010]. Airborne paramyxoviruses target the epithelial cells lining the airways and are a major cause of respiratory tract infections. A trademark cytopathic effect caused by paramyxovirus infection is the formation of syncytia in infected epithelial and endothelial cell layers: upon infection, viral glycoproteins expressed on the cell surface promote fusion of membranes between adjacent cells to form multinucleated bodies.

Paramyxoviridae are divided into two subfamilies, mainly based on morphological characteristics and phylogenetic relations: the *Pneumovirinae* and *Paramyxovirinae* (see Fig. 1). The *Pneumovirinae* subfamily consists of the genera pneumoviruses and metapneumoviruses, including human and bovine respiratory syncytial virus (HRSV, BRSV) and human and avian metapneumovirus (HMPV, AMPV). The *Paramyxovirinae* subfamily encompasses five genera: respiroviruses [including Sendai virus (SeV) and human parainfluenza virus 1 and 3 (hPIV1, 3)], the recently emerged henipaviruses [the closely related Hendra (HeV) and Nipah (NiV) virus)], morbilliviruses [comprising measles virus (MV), canine distemper virus (CDV) and the recently globally eradicated rinderpest virus (RPV)], avulaviruses [including Newcastle disease virus (NDV) and avian parainfluenza virus (APIV1)], and rubulaviruses [including mumps virus (MuV), human parainfluenza virus 2 and 4 (hPIV2, 4) and parainfluenza virus 5 (PIV5, formerly known as SV5: simian virus 5)] [Lamb & Parks, 2007]. The *Paramyxovirinae* subfamily also contains some other recently emerged species that have not been formally classified yet, including Tioman virus, Menangle virus, Beilong virus, Mossman virus, Salem virus, Fer-de-Lance virus, Tupaia paramyxovirus and J paramyxovirus.

Most paramyxoviruses have a narrow host range and rarely cross species. However, Hendra and Nipah virus have caused zoonosis with a high mortality rate in infected humans and animals [Halpin *et al.*, 2000; Chua *et al.*, 2000].

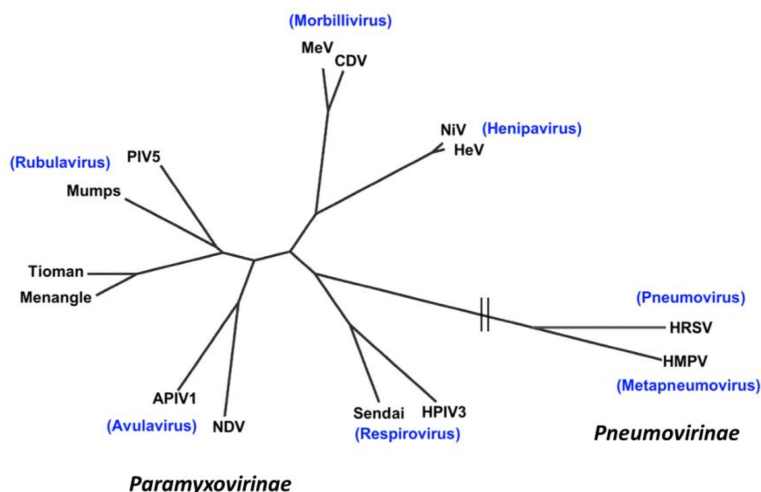

Figure 1: Phylogenetic tree of the *Paramyxoviridae* family. (from Aguilar & Lee, 2011). *Paramyxoviridae* encompass two subfamilies: *Paramyxovirinae* and *Pneumovirinae*. *Paramyxovirinae* contain five genera: respirovirus, henipavirus, morbillivirus, avulavirus and rubulavirus. *Pneumovirinae* includes the pneumovirus and metapneumovirus genera. Not all individual paramyxovirus species are depicted in this diagram. APIV1: avian parainfluenza virus 1; CDV: canine distemper virus; HeV: Hendra virus; HMPV: human metapneumovirus; HPIV3, human parainfluenza virus 3; HRSV: human respiratory syncytial virus; MeV: measles virus; NDV: Newcastle disease virus; NiV: Nipah virus; PIV5: parainfluenza virus 5. (Tree generated from a fusion protein sequence alignment using the fast minimum evolution algorithm).

1.2. Virion structure, protein composition and genome

Paramyxovirus virions are generally pleomorphic, often a mix of spherical and filamentous particles, with a size distribution ranging from 150 to 300 nm in diameter, however individual particles (from some genera) have been observed to be greater than 1 µm in some cases [Goldsmith *et al.*, 2003]. The viral envelope is a membrane bilayer with lipids derived from the host cell.

The envelope always contains at least two transmembrane proteins encoded by the viral genome: (1) an attachment protein (either hemagglutinin-neuraminidase (HN), hemagglutinin (H), or glycoprotein (G), depending on the genus), and (2) a fusion (F) protein. Both attachment and F proteins are heavily glycosylated. As their names indicate these glycoproteins mediate attachment to the host cell and subsequent membrane fusion in order to release the viral genome into the cytoplasm for replication and translation. Attachment and F proteins are thus essential for entry, and hence, infectivity of the virus. Later on in the infectious cycle, the glycoproteins play an active role in the assembly of viral proteins to specific cell membrane microdomains to form new virus particles. Because of their ability to fuse membranes at neutral pH, paramyxovirus glycoproteins expressed on the cell surface can induce the formation of syncytia (large multinucleated cells). RSV produces its G protein also in secreted form as a decoy for the immune system [Bukreyev *et al.*, 2008].

A third transmembrane protein, the small hydrophobic (SH) protein, is expressed in a few paramyxovirus species, including RSV and mumps virus. The SH protein does not seem to be important for attachment or membrane fusion, however it is thought to contribute to survival of infected host cells by inhibiting TNF-α-mediated apoptosis, and inducing host cell membrane permeability by forming hexameric pore-like structures [Takeuchi *et al.*, 1996; Bukreyev *et al.*, 1997; Wilson *et al.*, 2006; Fuentes *et al.*, 2007; Carter *et al.*, 2010].

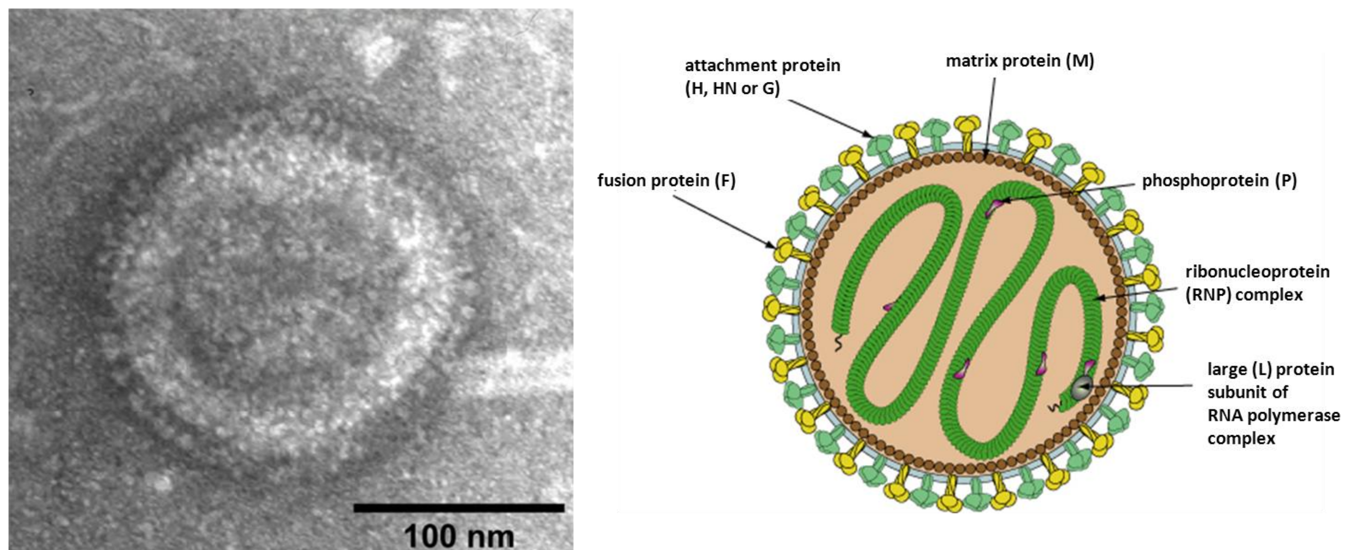

Figure 2: Paramyxovirus virion structure and protein composition. Left: Membrane glycoproteins are visible as a spiked layer in this negative staining transmission electron micrograph of a mumps virion [from Li *et al.*, 2009]. Right: General structure of a paramyxovirus virion. The six depicted proteins are encountered in all *Paramyxoviridae*. Genera differ in their attachment proteins: *Pneumovirinae* and henipavirus contain glycoprotein (G), morbillivirus contains hemagglutinin (H), while respirovirus, avulavirus and rubulavirus encode hemagglutinin-neuraminidase (HN). *Pneumorivirinae* and rubulaviruses also express short hydrophobic (SH) protein (not depicted) in their envelope. V, C, NS and M2 proteins (see text) are non-structural proteins and hence not incorporated into virus particles [adapted from ViralZone database, SIB].

The inner lining of the viral envelope is formed by the highly abundant matrix (M) proteins, that provide the structural basis to all *Paramyxoviridae* virus particles. M proteins bind to the inner leaflet of the cell membrane and interact with the cytoplasmic tails of the attachment and fusion glycoproteins as well as with the encapsidated RNA core. Furthermore, for many paramyxoviruses, M proteins directly or indirectly recruit host cell proteins involved in membrane budding. Because of these properties M proteins play a central role in the assembly and release of new virus particles from infected cells.

The paramyxovirus negative-sense, single-stranded RNA genome is non-segmented, between 15 and 19 kB in size, and typically contains only 6-10 genes, depending on the genus (see Fig. 3). The overall genome structure and gene order is conserved between *Paramyxoviridae*. The RNA genome is fully covered with nucleocapsid (N) protein. The encapsidated RNA forms a helical, tubular ribonucleoprotein (RNP) complex that is coiled up within the virus particle (see Fig. 2). The most important function of RNA encapsidation is to prevent detection of the viral RNA and subsequent degradation by the host cell upon infection. N proteins also interact with M proteins to allow packaging of the genome into new virus particles.

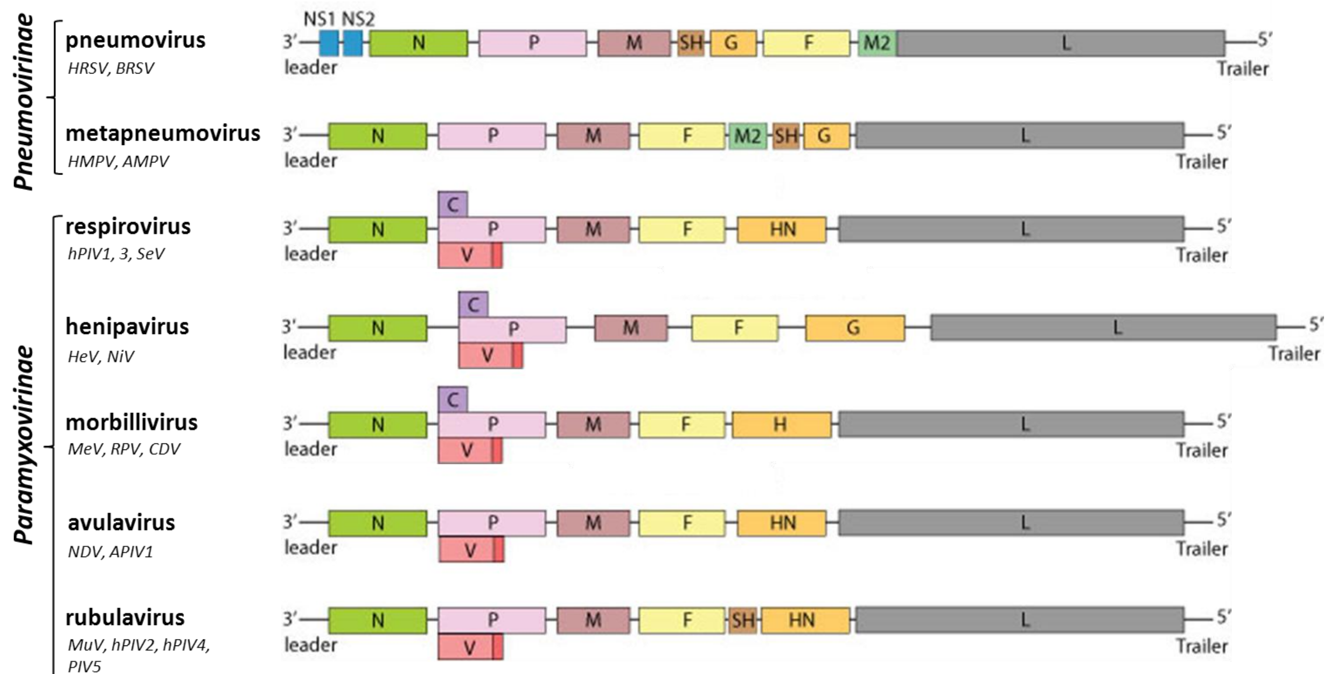

Figure 3: *Paramyxoviridae* genomes. The genome of paramyxoviruses is a 15 to 19 kB non-segmented negative-strand RNA that contains 6-10 genes, encoding a maximum of 11 proteins (in the case of RSV). The overall genome structure and gene order is conserved. Most genes have only one protein product, except for the P/V/C gene in *Paramyxovirinae*. Genera differ in their attachment glycoproteins: *Pneumovirinae* and henipaviruses contain glycoprotein (G), morbilliviruses contains hemagglutinin (H), while respiroviruses, avulaviruses and rubulaviruses encode hemagglutinin-neuraminidase (HN). The *Pneumovirinae* subfamily and the rubulaviruses encode a third transmembrane protein, short hydrophobic (SH) protein. (After ViralZone database, SIB).

Since the paramyxovirus genome is negative-sense RNA it cannot be directly translated into proteins by the ribosomes of the host cell. Therefore paramyxovirus particles (as all other *Mononegavirales* virions) contain at least one RNA-dependent RNA polymerase complex to produce translatable mRNA segments from their negative-sense genome. The polymerase complex is associated with the RNP complex and consists of the so-called large (L) protein subunit and a few phosphoproteins (P proteins).

Paramyxovirus RNA transcription and replication follow the general *Mononegavirales* model. P proteins – probably in a tetrameric configuration – direct binding of L protein to the 3' leader region of the encapsidated genome, which acts as an exclusive promoter for transcription [reviewed in Lamb & Parks, 2007]. The polymerase complex then sequentially transcribes the genes towards the 5' trailer region. However, not every gene is transcribed to the same extent: highly conserved start and stop signals within the intergenic sequences play a role in determining the probability of whether the polymerase complex dissociates from the template after releasing an mRNA, or whether it starts transcribing the next gene [mechanism first described by Whelan & Wertz, 2002]. If the polymerase complex dissociates, transcription can only start again at the 3' end. Hence genes closer to the 3' end are more abundantly transcribed than genes closer to the 5' end. This mechanism ensures the production of larger amounts of mRNA encoding structural proteins (like N and M protein) that are needed in large quantities for virion production.

The polymerase complex not only produces mRNA segments but also replicates the viral genome in a two-step process: first it creates full-length positive-sense RNA copies (antigenomes), and then it uses these as a template to create new negative-sense RNA molecules (genomes) that are incorporated into the viral offspring [Lamb & Parks, 2007]. Whether the polymerase complex favors RNA transcription (i.e. production of mRNA segments) or replication (i.e. production of full-length positive-sense genomes) is dependent on the concentration of N proteins. Replication only starts when the concentration of N protein is high enough to encapsidate newly synthesized antigenomes and genomes, thereby preventing their destruction. Thus, production of mRNAs happens earlier in the infectious cycle than genome replication.

Paramyxovirus genomes usually encode one or more accessory proteins that are not incorporated into budding virus particles. In general these non-structural proteins are not essential for virus replication in cell culture, but they increase viral yield and are required for virus survival *in vivo*. Accessory proteins are often multifunctional: typical functions include regulating RNA synthesis, assisting in particle assembly and, most importantly, manipulating host cell signal transduction pathways to attenuate anti-viral responses [reviewed in Lamb & Parks, 2007; Fontana *et al.*, 2008; Ramachandran & Horvath, 2009].

In the *Paramyxovirinae* subfamily the P(/V/C) gene not only encodes the phosphoprotein but also produces (1) an accessory V protein through co-transcriptional mRNA editing, and (2) in some genera additional proteins called C, W, X or Y from overlapping open reading frames (ORFs) [reviewed in Lamb & Parks, 2007] (see Fig. 3). V proteins attenuate host antiviral and immune responses through interference with type I&II interferon (IFN)-mediated signaling pathways at multiple steps [reviewed in Fontana *et al.*, 2008], and they counteract host cell cycle progression and apoptosis [Lin & Lamb, 2000; Sun *et al.*, 2004]. While many other enveloped viruses have developed mechanisms to inhibit IFN-mediated pathways as well, paramyxovirus V proteins are unique in that they also directly target IFN-responsive STAT proteins. The JAK/STAT pathway is a major IFN-induced gene transcriptional activation pathway that leads to the production of several antiviral and immunomodulatory proteins [reviewed in Gough *et al.*, 2008; Stark & Darnell, 2012]. However, the mechanisms by which paramyxovirus V proteins interfere with STAT proteins differ greatly between *Paramyxovirinae* genera [reviewed in Ramachandran & Horvath, 2009]. Like V proteins, C proteins counteract the immune response and are involved in the regulation of RNA synthesis. For a number of paramyxoviruses, C proteins have been shown to inhibit viral genome replication, thereby avoiding RIG-I-induced IFN production [reviewed in Fontana *et al.*, 2008].

The *Pneumovirinae* P gene does not produce accessory V or C proteins. However the *Pneumovirinae* subfamily genome encodes other accessory proteins that have similar functions, like the M2 gene products and the NS1 and NS2 proteins. The *Pneumovirinae* M2 gene produces the M2-1 and M2-2 proteins from overlapping ORFs. Both proteins are thought to regulate RNA synthesis: M2-1 enhances the processivity of transcription [Collins *et al.*, 1996; Hardy & Wertz, 1998], while the M2-2 protein appears to regulate the switch from mRNA to antigenome synthesis [Bermingham & Collins, 1999]. The genome of RSV encodes two unique non-structural (NS) proteins, NS1 and NS2, that are, like the V proteins from the *Paramyxovirinae* subfamily, involved in inhibition of the IFN-mediated antiviral responses [Spann *et al.*, 2004; Hastie *et al.*, 2012]. The NS1 and NS2 proteins target the pathways leading to IFN-β upregulation as well as IFN-induced JAK/STAT pathways at multiple steps [reviewed in Oshansky *et al.*, 2009; Collins & Melero, 2011].

2. PARAMYXOVIRUS ENTRY MECHANISMS

2.1. Attachment proteins mediate virion binding

Paramyxovirus attachment to a host cell is usually mediated by interactions between virus-expressed attachment proteins and virus-specific receptors on the cell surface. All paramyxovirus attachment proteins are type II integral membrane proteins: a C-terminal extraviral/extracellular domain, a single transmembrane stretch and an intraviral/cytosolic N-terminus.

Paramyxovirus attachment proteins are divided into three groups: (1) hemagglutinin-neuraminidases (HN proteins), which are expressed by respiroviruses, avulaviruses and rubulaviruses; (2) hemagglutinins (H proteins), expressed by morbilliviruses; and (3) glycoproteins (G proteins), expressed by henipaviruses and the members of the *Pneumovirinae* subfamily (i.e. RSV and HMPV). The attachment protein nomenclature is based on certain shared phenotypic properties rather than on similarity in amino acid sequence or 3D structure: HN and H proteins agglutinate erythrocytes in a sialic acid-dependent and –independent manner respectively, where HN proteins also possess neuraminidase activity. G proteins lack both hemagglutination and neuraminidase properties.

Results obtained from biochemical studies and recently published crystal structures indicate that *Paramyxovirinae* attachment proteins reside as homotetramers (dimers of disulfide-linked dimers) in the viral/cellular membrane [Bossart *et al.*, 2005; Paal *et al.*, 2009; Brindley *et al.*, 2010; Santiago *et al.*, 2010; Hashiguchi *et al.*, 2007, 2011; Yuan *et al.*, 2011; Bose *et al.*, 2011; Xu *et al.*, 2012(a); recently reviewed in Lee & Akyol-Ataman, 2011; Plemper *et al.*, 2011; Chang & Dutch, 2012].

What type of entry receptor a paramyxovirus attachment protein interacts with is largely dependent on whether it has neuraminidase activity or not. *Paramyxovirinae* genera containing HN proteins interact with sialylated receptors (i.e. membrane glycoproteins or glycolipids with terminal sialic acids) that are omnipresent on the cell surface. The neuraminidase moiety in HN protein can cleave the same terminal sialic acid and is likely important for virus exit as cleavage might prevent aggregation of newly-formed virus particles at the surface of the infected cell. Attachment proteins of the other *Paramyxovirinae* genera (i.e. henipavirus and morbillivirus, expressing a G and H protein respectively) do not bind to sialic acid but interact with proteinaceous receptors for entry (described in paragraphs 3.3 and 3.4).

Compared to *Paramyxovirinae* attachment proteins, *Pneumovirinae* attachment (G) proteins are much shorter (between 230 and 300 aa, compared to ~600 for *Paramyxovirinae*) and characterized by (1) a high sequence variability between subgroups [Johnson *et al.*, 1987; Bastien *et al.*, 2004] and (2) extensive glycosylation, containing several N- and many O-linked glycosylation sites (i.e. S/T-residues), a characteristic shared with mucin-like proteins secreted by epithelial cells. Other than that, the RSV and HMPV G proteins are different in size and have no sequence homology [Van den Hoogen *et al.*, 2002].

The *in vivo* receptor binding properties of the *Pneumovirinae* G proteins are not fully understood. Although several G protein-receptor interactions have been described in cell culture experiments (see paragraph 3.3), many *in vitro* and *in vivo* studies have shown these are not necessary to achieve membrane fusion and virus entry into the host cell. Cold passaged or engineered G protein deletion mutants can still replicate efficiently in cell cultures [Karron *et al.*, 1997; Techaarpornkul *et al.*, 2001, 2002; Teng *et al.*, 2001; Teng and Collins, 2002; Biacchesi *et al.*, 2004, 2005; Chang *et al.*, 2012]. However, several studies have demonstrated that HMPV and RSV G proteins enhance replication and virus survival *in vivo* [Karron *et al.*, 1997; Teng *et al.*, 2001; Biacchesi et al., 2005]. Recent studies have shown that in *Pneumovirinae* the F protein itself also interacts with host cell receptors and that these interactions are crucial for virus entry (discussed in later paragraphs).

Apart from its involvement in attachment, RSV G protein has been shown to inhibit activation of certain toll-like receptors (TLRs) in monocytes, thereby suppressing innate immune responses [Polack *et al.*, 2005; reviewed in Klein Klouwenberg *et al.*, 2009]. Furthermore, RSV G is also expressed in secreted form due to a second translational initiation codon [reviewed in Collins & Melero, 2011]. This secreted form may serve as a decoy for the host immune system [Bukreyev *et al.*, 2008].

Although it has been established that the G protein is not necessary to achieve membrane fusion, up till now no wild-type RSV strain has been isolated that does not contain a G protein. Indeed, differences in fitness between RSV strains are to a large part attributed to differences in G protein gene sequence, yielding differences in protein structure, glycosylation pattern and hence differences in receptor interactions [Zlateva *et al.*, 2005; Parveen *et al.*, 2006]. In what part the apparent necessity of the G protein for *in vivo* infectivity can be attributed to its attachment function or its involvement in immune evasion and immunomodulation remains to be elucidated. Recently a mutant RSV strain lacking a large portion of the G protein extraviral domain was detected only in immunocompromised children in South Africa, suggesting that for RSV *in vivo* infection the immunomodulating property of the G protein is more important [Venter *et al.*, 2011].

In the last decade, crystal structures have been obtained for a number of attachment protein (partial) extraviral domains of the *Paramyxovirinae* subfamily: either in monomeric, dimeric or tetrameric form, unliganded, or in complex with a receptor or inhibitor [reviewed in Iorio *et al.*, 2009; Plemper *et al.*, 2011; Chang & Dutch, 2012]. From these structures it has become clear that the attachment protein extraviral domain has the shape of a membrane-distal globular or cuboidal head on a membrane-proximal stalk. The head-on-a-stalk structure of the monomer is reflected in the mushroom-like protrusions seen in EM micrographs that allegedly represent the physiological attachment protein homotetramers. The monomeric globular head domains mediate receptor binding and contain a highly conserved six-bladed β-sheet propeller structure characteristic for neuraminidases (and first identified in influenza NA protein [Varghese *et al.*, 1983]). Only in HN proteins the central pocket of the β-propeller fold still possesses a sialic acid binding site and neuraminidase activity (NA-site) [Crennell *et al.*, 2000; Iorio *et al.*, 2001; Yuan *et al.*, 2005]. In MV H and henipavirus G proteins this pocket is still present but has lost its sialic acid binding capacity and neuraminidase function due to point mutations [Colf *et al.*, 2007, Hashiguchi *et al.*, 2007, Bowden *et al.*, 2008, Xu *et al.*, 2008]. Crystal structures suggest that sialic acid binding and NA activity occur in different conformation in NDV, drastically changing the HN dimer interface, while no such changes occur in hPIV3 and PIV5 [Crennell *et al.*, 2000; Lawrence *et al.*, 2004; Yuan *et al.*, 2005]. In NDV HN a second sialic acid binding site lacking neuraminidase activity was identified which is formed by residues from two monomers at the membrane-distal dimer interface upon sialic acid binding to the NA-site [Zaitsev *et al.*, 2004; Porotto *et al.*, 2006]. Although postulated to play a role in activation of the fusion mechanism in hPIV3 HN, this second sialic acid binding site was not crystallographically confirmed for hPIV3 and PIV5 HN proteins [Lawrence *et al.*, 2004; Yuan *et al.*, 2005; Porotto *et al.*, 2007].

There appear to be great differences in binding site locations among the attachment proteins that have lost the capacity of binding to sialic acid-containing receptors, which is reflected in the different proteinaceous receptors they recognize. While the henipavirus G protein receptor binding sites (ephrins B2/B3 – see paragraph 3.3) map to the top of each monomeric globular domain and partially overlap with the sialic acid binding pockets in HN proteins, the currently known MV H protein receptor binding sites (CD46, SLAM and nectin-4 – see paragraph 3.3) are located much farther away from the dimer interface towards the lateral sides of each β-propeller fold [Colf *et al.*, 2007; Bowden *et al.*, 2008; Xu *et al.*, 2008, 2012; Santiago *et al.*, 2010; Hashiguchi *et al.*, 2011; Zhang *et al.*, 2013]. Also, the overall crystal structure of the MV H globular head domains is more cube-like compared to the more globular structure assumed by the head domains of HN protein and henipavirus G proteins [Colf *et al.*, 2007; Hashiguchi *et al.*, 2007; Crennell *et al.*, 2000; Lawrence *et al.*, 2004; Bowden *et al.*, 2008; Xu *et al.*, 2008]. These findings are not only consistent with the notion that morbilliviruses and henipavirus have adapted independently to proteinaceous receptors, but also suggests that morbillivirus H protein is an evolutionary outgroup in relation to HN proteins and henipavirus G protein [Bowden *et al.*, 2008].

Recently obtained crystal structures of the NDV and PIV5 HN protein homotetrameric ectodomains containing a part of the stalk region show that it forms a tetrameric coiled-coil bundle (4HB) [Yuan *et al.*, 2011; Bose *et al.*, 2011].

2.2. Membrane fusion is achieved through refolding of F protein

After attachment of a virus particle to the host cell, fusion of the viral membrane with the plasma membrane releases the genomic content into the host cell cytoplasm for replication and translation. The process of membrane fusion requires energy. In paramyxoviruses as well as other enveloped viruses, this energy is provided by virus-expressed fusion proteins.

Paramyxovirus fusion (F) proteins are type I integral membrane proteins: a large N-terminal extraviral/extracellular domain, a single transmembrane stretch and a small intraviral/cytosolic C-terminus. F proteins belong (together with other viral fusion proteins such as influenza HA, Ebola GP and HIV Env) to the class I viral fusion proteins and are characterized by an internal cleavage site adjacent to a stretch of 20-25 hydrophobic amino acids, the fusion peptide, followed by a linker region that contains 2 heptad repeat regions (HRA and HRB; see Fig. 4A). Apart from that, class I fusion proteins from different virus families do not have any significant sequence similarity, but exhibit a similar production and membrane fusion mechanism [Lamb & Parks, 2007]. Also among paramyxovirus geni F protein sequences vary substantially, although S-S bridge-forming cysteine residues are often conserved. Among *Pneumovirinae* the F protein is more conserved (~33-38% amino acid sequence identity) compared to *Paramyxovirinae* (10-18%) [Van den Hoogen *et al.*, 2002].

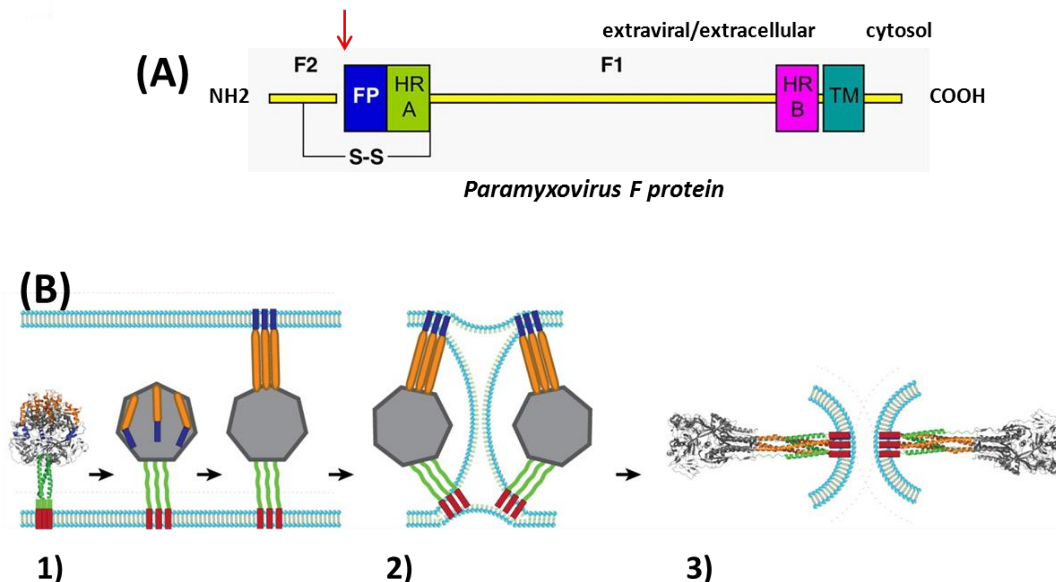

Figure 4: F protein refolding drives membrane fusion. (A) Linear schematic drawing of a cleaved paramyxovirus F protein monomer with its conserved features: depicted are the N-terminal F2-chain, the cleavage site (red arrow), and the F_1-chain containing the fusion peptide (FP), two heptad repeat regions (HRA and HRB) and a transmembrane region (TM). The F_1- and F_2-chain are linked by a disulfide bridge. (B) Refolding of F protein drives membrane fusion. 1) After the F protein trimer in its proteolytically activated pre-fusion state is triggered, the coiled-coil HRB domain *(green)* melts, and HRA *(orange)* refolds into a trimeric coiled-coil, leading to the projection of the fusion peptide *(blue)* towards the host cell membrane to form the so-called pre-hairpin intermediate. 2) During further refolding of the pre-hairpin intermediate the TM domain *(red)* and the fusion peptide move towards each other. Somewhere along this step membrane hemi-fusion is hypothesized to occur. It is likely that the concerted action of multiple F proteins is necessary for membrane fusion. 3) The post-fusion stable 6HB corresponds to fused viral and host cell membranes (Schematic from Chang & Dutch, 2012).

F proteins are synthesized as non-fusogenic precursors (called F_0) and form homotrimers in the cell membrane/viral envelope [reviewed in Morrison, 2003; Lamb & Jardetzky, 2007; Plemper, 2011]. Analysis of the crystal structure of the PIV5 F_0 complex revealed a membrane-distal large globular head domain connected to a membrane-proximal three-helix coiled-coil domain [Yin *et al.*, 2006].

Only after proteolytic cleavage F proteins become fusogenic: a cellular protease cleaves the F_0 precursor into a metastable F_1-F_2 heterodimer linked by disulfide bonds (forming a trimeric 'pre-fusion complex'). Proteolytic cleavage yields a new hydrophobic N-terminal domain of F_1 (the 'fusion peptide') that lies buried within the mature F protein [reviewed in White *et al.*, 2008]. For most

paramyxoviruses F_0 is cleaved by a furin protease during transport through the *trans*-Golgi network [Lamb & Parks, 2007]. Exceptions are HMPV and Sendai virus F_0, which are cleaved by tissue-specific extracellular proteases and Henipavirus F_0 which is activated through cleavage by cathepsin-L and/or –B in acidic, endosomal compartments [Meulendyke *et al.*, 2005; Pager & Dutch, 2005; Pager *et al.*, 2006; Diederich *et al.*, 2005, 2008, 2012]. Furthermore, RSV F protein is unique in that it has two furin cleavage sites instead of one, and cleavage at both sites is necessary for fusogenic activity [Gonzalez-Reyes *et al.*, 2001; Zimmer *et al.*, 2001(b)]. Despite differences in proteolytic activation and transport route, paramyxovirus F proteins (with the exception of RSV F – see next paragraph) are activated before being incorporated into budding virus particles [Diederich *et al.*, 2008; Krzyzaniak *et al.*, 2013].

Paramyxovirus F proteins mediate membrane fusion through a conserved, irreversible conformational change from a meta-stable high-energy state (i.e. the proteolytically activated F_1-F_2 pre-fusion complex) towards a stable low-energy state (the post-fusion complex) [reviewed in Morrison, 2003; Lamb & Jardetzky, 2007; Plemper, 2011]. This F protein refolding mechanism is directly or indirectly (i.e. through the attachment protein or pH-mediated) triggered after binding to the target cell (discussed in paragraph 2.3). Biochemical studies as well as crystal structures obtained from influenza HA, HIV gp41 and several paramyxovirus F protein soluble domains in precursor form as well as in proteolytically activated pre- and post-fusion states have generated insight in the F protein structural rearrangements leading to membrane fusion [Bullough *et al.*, 1994; Weissenhorn *et al.*, 1997; Zhao *et al.*, 2000; Yin *et al.*, 2005, 2006; Connolly *et al.*, 2006; Swanson *et al.*, 2011; Porotto *et al.*, 2011; McLellan *et al.*, 2011, 2013; Welch *et al.*, 2012; Chan *et al.*, 2012; Wen *et al.*, 2012]. First, the HRAs from each monomer rearrange into a trimeric coiled-coil and the hydrophobic fusion peptides are harpooned into the target cell membrane, forming a so-called pre-hairpin intermediate structure (see Fig. 4B). The pre-hairpin intermediate then somehow refolds into the stable post-fusion structure that is mainly characterized by the formation of a stable six-helical bundle (6HB) composed of the helical heptad repeats A and B (HRA and HRB) from each monomer [Zhao *et al.*, 2000]. Since in the post-fusion structure the fusion peptides and the transmembrane regions of the F_1 chains are in the same membrane, it is thought that the transition from the pre-hairpin intermediate into the stable post-fusion structure provides the energy (1) to pull the viral and host cell membranes into close proximity, and (2) to allow the energetically unfavorable fusion of the membrane-phospholipid bilayers, ultimately resulting in the formation of a fusion pore [reviewed in Colman & Lawrence, 2003; Lamb & Parks, 2007; Lamb & Jardetzky, 2007]. Exactly how the biophysical mechanism of membrane bilayer fusion and subsequent formation of the fusion pore works, and how it is correlated with refolding of F protein is uncertain [Plemper, 2011]. It is likely that simultaneous refolding of a small cluster of F protein homotrimers is necessary to create a fusion pore [Dutch *et al.*, 1998]. In other enveloped viruses estimates of how many trimeric fusion protein complexes are necessary to create a fusion pore vary between a single complex and over a dozen complexes [Roche & Gaudin, 2002; Yang *et al.*, 2005].

2.3. Paramyxoviruses differ in the mechanism of F protein triggering

Since F protein refolding is an irreversible process, it is important that its triggering is regulated in a spatio-temporal context. While the F protein refolding mechanism itself is conserved, different paramyxoviruses employ different mechanisms for F protein triggering.

In the *Paramyxovirinae* subfamily, F protein refolding is commonly thought to be triggered upon binding of the attachment protein to an entry receptor. Multiple studies have demonstrated that the attachment protein is necessary for entry of these viruses, since cell-cell fusion events occur only upon expression of a functional receptor for the attachment protein, and upon co-expression of the attachment and F protein, but not upon expression of the F protein alone [Moscona & Peluso,1991*;* Hu *et al.*, 1992; Yao *et al.*, 1997]. If the hypothesis that receptor binding by the attachment protein

triggers refolding of the F protein is correct, (an at least temporal) interaction between the two glycoproteins is required to transduce the receptor binding signal to the fusion protein. Transfection of heterotypic glycoprotein pairs revealed a virus-specific interaction between attachment protein and fusion protein: when an attachment protein is co-expressed with a heterotypic fusion protein (i.e. from another *Paramyxovirinae* species), no syncytia are formed, except in some cases where the glycoproteins are from closely related species (e.g. Hendra and Nipah, MV and CDV) [Hu *et al.*, 1992; Yao *et al.*, 1997; Lee *et al.*, 2008; Bossart *et al.*, 2002]. The distribution of the attachment and fusion functions over two glycoproteins is in contrast with other enveloped viruses such as influenza virus, Ebola virus and vesicular stomatitis virus (VSV) in which a single glycoprotein mediates both attachment and fusion, and glycoprotein refolding is either triggered by receptor binding, or pH lowering (endocytosis) depending on the type of virus [Lamb & Parks, 2007].

In *Paramyxovirinae*, the interactions between the glycoproteins that are necessary to instigate membrane fusion are predicted to occur primarily at the head region of F protein [Lee *et al.*, 2008; Paal *et al.*, 2009; Tsurudome *et al.*, 2011]. Transfection of HN protein chimeras composed of regions from different viral HN proteins showed that HN proteins interact with F protein through their stalk regions [Deng *et al.*, 1995; Tanabayashi & Compans, 1996; Tsurudome *et al.*, 1995; Porotto *et al.*, 2012]. Chimera, mutagenesis and crystallographic studies identified F protein interacting domains in the stalk regions of hPIV3, NDV and PIV5 HN and MV H [Deng *et al.*, 1999; Porotto *et al.*, 2003; Melanson & Iorio, 2004, 2006; Corey & Iorio, 2007; Lee *et al.*, 2008; Bishop *et al.*, 2008; Paal *et al.*, 2009; Bose *et al.*, 2011; Yuan *et al.*, 2011]. Residues that affect interaction with F protein have also been found in the globular head domain and TM regions [McGinnes *et al.*, 1993, Bousse *et al.*, 1994; Aguilar *et al.*, 2009; Mirza *et al.*, 2011].

In the last decade multiple functional, biochemical and crystallographic studies have been performed to illuminate (changes in) the interactions between the *Paramyxovirinae* glycoproteins (induced by receptor binding) that finally lead to triggering of the F protein, and subsequent membrane fusion [recently reviewed in Lamb & Jardetzky, 2007; White *et al.*, 2008; Iorio *et al.*, 2009; Smith *et al.*, 2009; Plemper *et al.*, 2011; Aguilar & Lee, 2011; Chang & Dutch, 2012].

Intracellular retention of hPIV3 and PIV5 HN or F protein obtained through modification with an ER-localization signal did not affect transport of its homotypic HN or F protein towards the plasma membrane, indicating that HN-F complexes are not formed in the ER or Golgi [Paterson *et al.*, 1997]. For hPIV3 and NDV HN proteins with mutations in the F-interacting stalk domain a direct relationship was found between fusion promoting activity and the amount of HN-F complexes detected by co-immunoprecipitation at the cell surface of HN-F co-transfected cells: mutated HN proteins formed less HN-F complexes and induced less syncytia formation [Porotto *et al.*, 2003; Melanson & Iorio, 2004, 2006]. Moreover, the extent of induced membrane fusion was found to be directly proportional to the strength of the HN-F interaction (i.e. the amount of detected HN-F complexes). Also, for NDV, HN-F complexes were only detected in the presence of sialic acid receptors, and receptor binding-deficient mutants (i.e. with mutations in the NA active site) showed less interaction between the glycoproteins [Iorio *et al.*, 2001; Melanson & Iorio, 2004; Li *et al.*, 2004].

The above described experimental findings formed the basis of an “association” or “provocateur” model for F protein activation for *Paramyxovirinae* that bind to sialic acid-containing receptors (see Fig. 5A). In short: receptor binding triggers a series of spatial/structural rearrangements in the HN protein that leads to formation of the HN-F complex through interactions in the HN stalk region, i.e. the attachment protein acts as a molecular scaffold. This interaction then triggers refolding of the F protein towards it low-energy conformation.

A set of mutagenesis and co-IP studies on transfected MV and henipavirus glycoproteins, similar to those performed in sialic-acid binding *Paramyxovirinae*, resulted in a different view on F protein activation in *Paramyxovirinae* binding to proteinaceous receptors. A MV construct with a modified H protein showed increased fusogenicity, despite a weakened interaction between the glycoproteins at the plasma membrane (i.e. less H-F complexes detected by co-immunoprecipitation compared to WT) [Plemper *et al.*, 2002]. In another study, mutations induced in the MV H protein stalk, corresponding to those introduced in NDV [Melanson & Iorio, 2004], had the opposite effect on the formation of hetero-oligomers. Mutated H proteins that were almost completely deficient in inducing membrane fusion, were all detected to a higher extent in complex with F at the cell surface than wild-type H protein, suggesting that the induced mutations in the MV H stalk strengthen rather than weaken the H-

F interaction [Corey & Iorio, 2007]. In both studies, the extent of fusion was shown to be inversely proportional to the amount of H-F complex formation. A transfection study with receptor-binding deficient MV H protein mutants also showed increased H-F interaction [Corey & Iorio, 2009]. Similar results were obtained for Hendra and Nipah G: (glycosylation) mutations in G or F that decreased membrane fusion increased the avidity of the G-F interaction [Aguilar *et al.*, 2006; Bishop *et al.*, 2007; Xu *et al.*, 2012(a)]. Some mutations in Hendra and Nipah G also decreased G-F interaction (and increased membrane fusion – maintaining the inverse relationship) [Aguilar *et al.*, 2006; Bishop *et al.*, 2007], while in MV H no such mutations were identified.

Intracellular retention of MV H or F resulted in ER-retained H-F hetero-oligomers, suggesting that MV H and F are incorporated as a complex into budding virions [Plemper *et al.*, 2001]. In a similar study Hendra G and F were not detected as ER-retained complexes [Whitman *et al.*, 2007, 2009]. However, henipavirus F_0 protein differs from other paramyxovirus F proteins in that it is activated through cleavage by cathepsin-L and/or –B in acidic, endosomal compartments [Pager & Dutch, 2005; Pager *et al.*, 2006; Diederich *et al.*, 2005, 2008, 2012]. This activation is required for incorporation into budding virus particles, suggesting a more complex transport route for henipavirus F proteins [Diederich *et al.*, 2008]. Therefore, it may very well be possible that henipavirus G and F associate at a later stadium [Whitman *et al.*, 2007].

The above described experimental findings formed the basis of a “dissociation” or “clamp” model for F protein activation by MV H and henipavirus G in *Paramyxovirinae* species (see Fig. 5B). In short: in the viral envelope, the fusion protein trimer is locked into a meta-stable high energy conformation through complex formation with the attachment protein tetramer. Interaction with a host cell receptor induces some structural rearrangements in the attachment protein that result in the dissociation of the attachment protein/F protein-complex. Dissociation from the attachment protein triggers refolding of the F protein towards its stable low-energy conformation.

However, for MV and henipavirus, the inverse correlation between syncytium formation and amount of H-F complexes as detected by co-IP upon transfection of a mutant attachment protein does not provide definitive evidence for a dissocation model (i.e. release of a “loaded spring”). It might be possible that mutations induced in the stalk region prevent fusion by the inability to transfer the receptor binding signal towards the F protein, rather than by preventing dissociation (i.e. keeping the F protein ‘clamped’). The observed increase in mutant H-F complex detection by co-IP can also be explained because of an increased avidity between the mutant H protein and F protein compared to WT H-F.

A recent study using mutant hPIV3 HN proteins and peptides that inhibit F protein refolding at different stages, indicates that a continuous HN-receptor interaction, rather than a single triggering event (after which F protein refolding proceeds unaided), is necessary to guide the F protein through a series of transient refolding intermediates [Porotto *et al.*, 2011]. HN-receptor interaction was required even beyond insertion of the fusion peptide into the host cell membrane. In a follow-up study by the same group it was demonstrated that attachment protein chimeras with an NDV HN globular head domain and an hPIV3 HN or Nipah G stalk region also require a continuous receptor engagement to guide the homotypic F protein (i.e. from hPIV3 or Nipah) through the refolding process [Porotto *et al.*, 2012] Based on these studies, Porotto *et al.* argue against the whole “association/dissociation” paradigm and propose an F protein triggering mechanism that is conserved among all *Paramyxovirinae* (including the ones binding to proteinaceous receptors). This model is in fact an extension of the “association” model where, instead of a single triggering event, a continuous interaction between receptor and attachment protein, and hence between attachment and F protein is necessary to induce membrane fusion. If this were the case, HN-F and H/G-F complexes only differ in their association characteristics (and possibly stoichiometry) prior to receptor binding.

Using a chimeric attachment protein with a MV stalk region in the experimental setup of Porotto *et al.* should shed some light on this matter.

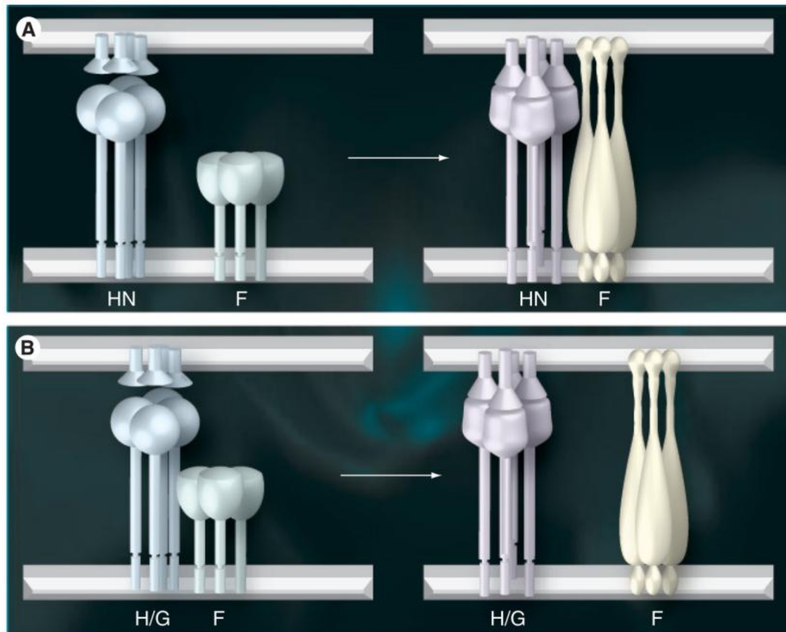

Figure 5: Proposed *Paramyxovirinae* membrane fusion mechanisms. *Paramyxovirinae* attachment proteins interact with F proteins through their stalk region. There are two models for F protein activation: (A) Association or provocateur model: receptor binding results in a rearrangement in the HN tetramer that induces interaction with the F protein such that the latter is triggered. Continuous interaction between the glycoproteins may be required during the fusion process. (B) Dissociation or clamp model: the glycoproteins reside as a complex in the viral envelope, which might be necessary to keep F protein in its metastable pre-fusion state. Receptor binding results in a structural rearrangement in the H/G tetramer that is relayed towards the F protein, causing it to dissociate and refold. In *Pneumovirinae* the F protein can be triggered in the absence of an attachment protein. (Picture from Iorio *et al.*, 2009).

Recent research on *Paramyxovirinae* glycoproteins has focused on the conformational and spatial rearrangements of the attachment protein homotetramers underlying the above described mechanisms of F protein triggering upon receptor binding [reviewed in Plemper *et al.*, 2011].

Crystal structures of the MV H tetrameric head domains in complex with its receptors CD46 and SLAM suggest that receptor binding induces a spatial reorganization in the homotetramer rather than conformational changes in the individual head domains, a mechanism earlier proposed for hPIV5 HN [Santiago *et al.*, 2010; Hashiguchi *et al.*, 2011; Yuan *et al.*, 2005, 2008]. Co-crystals of WT MV H head domains and SLAM spontaneously assembled into a tetrameric form with a distinctively different spatial configuration compared to co-crystals with a H variant harboring a point mutation, corresponding to a spatial reorganization of the non-covalent dimers relative to each other [Hashiguchi *et al.*, 2011]. The researchers hypothesized that these two distinct configurations may represent the receptor-bound pre-fusion H tetramer before F protein triggering and the receptor-bound post-fusion H tetramer after F protein triggering, respectively. The transition between the configurations (i.e. the reorganization of the dimer-dimer interface upon receptor binding) then would open up the 4HB into two dimeric stalk domains, triggering the conformational changes in F protein. Recently, this hypothesis was substantiated by the finding that mutations in the dimer-dimer interface of the H head domain led to less efficient fusion protein triggering [Nakashima *et al.*, 2013].

A recently solved crystal structure of Hendra G protein in complex with Ephrin B2 indicates that receptor binding induces small changes in the binding interface (not observed in MV) that are relayed towards the dimer interface, resulting in dissociation of the dimers and F protein triggering [Xu *et al.*, 2012(a)]. Various point mutations of G protein residues at the binding interface had surprisingly little effect on receptor binding, however they significantly inhibited viral entry, and the mutated G proteins showed increased association with F protein by co-immunoprecipitation (possibly in agreement with a "dissociation" model of F triggering). This suggests that the induced mutations prevent receptor binding-induced conformational changes and dimer dissocation and demonstrate that receptor binding and F protein triggering can be uncoupled, as shown in earlier *Paramyxovirinae* mutagenesis studies [Bishop *et al.*, 2007; Corey & Iorio, 2009; Porotto *et al.*, 2011, 2012; Mirza *et al.*, 2011].

As opposed to members of the *Paramyxoviridae* subfamily, where attachment protein binding to a receptor triggers the F protein, in the *Pneumovirinae* (RSV, HMPV) many studies have shown that the G protein is not necessary to achieve membrane fusion and entry into the host cell. Transfection of RSV and HMPV F protein alone results in the formation of syncytia in cell culture [Heminway *et al.*, 1994; Schowalter *et al.*, 2006; Herfst *et al.*, 2008]. Cold passaged or engineered RSV and HMPV strains lacking a functional G protein can replicate efficiently in cell cultures and are still infectious *in vivo* [Karron *et al.*, 1997; Kahn *et al.*, 1999; Techaarpornkul *et al.*, 2001, 2002; Teng *et al.*, 2001; Teng and Collins, 2002; Biacchesi *et al.*, 2004, 2005]. Recent studies have described multiple direct

interactions between the RSV and HMPV F proteins and host cell receptors (discussed in paragraphs 3.3 and 3.4). Also, some HMPV strains have been described that require low pH for F protein triggering [Schowalter *et al.*, 2006, 2009; Herfst *et al.*, 2008]. Mutagenesis studies suggest that protonation of His435 in the membrane-proximal region of the F protein head domain is important for this dependence on low pH [Schowalter *et al.*, 2009; Mas *et al.*, 2011; Chang *et al.*, 2012]. A recent study demonstrated that the unique, second proteolytic cleavage step of RSV F protein occurs only after virus particle internalization (discussed in paragraph 2.5) and is crucial for virus infection [Krzyzaniak *et al.*, 2013]. Interestingly, earlier, a chimeric SeV F protein with the RSV cleavage sites had been shown to be capable of producing syncytia in the absence of HN protein, and, in another study, recombinant SeV expressing a double cleavage site mutant F protein was significantly less dependent on interaction of HN with sialic acid, suggesting that the second proteolytic cleavage is indeed a crucial determinant for RSV F protein triggering [Rawling *et al.*, 2008, 2011; Zimmer *et al.*, 2005].

The experimental findings described above suggest that in the *Pneumovirinae* subfamily interactions between the G and F protein are not required to trigger the latter, and the F protein itself mediates both attachment and fusion. F protein refolding is then triggered either through (1) direct interaction of the F protein with an entry receptor; (2) in the case of RSV, a second proteolytic cleavage step after interaction of the F protein with an internalization receptor; or, (3) a pH-dependent mechanism – in the case of some HMPV strains (see also paragraph 2.5) – or a yet to be identified mechanism, after interaction of the F protein with an internalization receptor. The G protein appears to have a role mainly in trapping of virus particles (or perhaps more specifically, in facilitating interaction of F protein with an entry receptor) and – especially in the case of RSV – immunomodulation [Bao *et al.*, 2008; Bukreyev *et al.*, 2008; reviewed in Collins & Melero, 2011].

Interestingly, it has been shown that for some sialic acid-binding *Paramyxovirinae* membrane fusion can also be triggered in the absence of the attachment protein, although at a dramatically lower rate [Leyrer *et al.*, 1998; Dutch *et al.*, 1998; Ito *et al.*, 2009]. For these viruses, significant membrane fusion can be achieved by raising the temperature [Paterson *et al.*, 2000; Wharton *et al.*, 2000]. These findings suggest that in *Paramyxovirinae* interaction with the attachment protein lowers the energy barrier(s) for F protein refolding.

It is not known what structural characteristics of the F proteins of RSV and HMPV underlie their ability to mediate membrane fusion without assistance of the attachment protein.

2.4. Role of glycosylation in receptor binding and immune evasion

The paramyxovirus attachment and fusion proteins are both heavily glycosylated in the ER and Golgi of the host cell. Hence, the glycosylation pattern is both species and tissue-specific. N-linked glycans on envelope proteins of paramyxoviruses and other virus families have been shown to play a role in a multitude of processes, such as efficient folding and transport, attachment to cell surface receptors, and prevention of recognition by neutralizing antibodies [reviewed in Vigerust & Shepherd, 2007]. This automatically implies that the extent and pattern of glycoprotein glycosylation contribute to the differences in infectivity between individual virus strains.

Multiple studies have been done to investigate the importance of glycosylation in paramyxoviruses, with N-glycosylation of the F protein in particular [Bagai & Lamb, 1995: Zimmer *et al.*, 2001; von Messling & Cattaneo, 2003; Aguilar *et al.*, 2006; Samal *et al.*, 2012]. Removal of specific F protein N-glycans in PIV5 and NDV was shown to have deleterious effects on folding, transport, and F-protein mediated membrane fusion [Bagai & Lamb, 1995; von Messling & Cattaneo, 2003], confirming the importance of N-glycosylation.

Surprisingly, in Nipah virus N-deglycosylation of the F protein was shown to enhance membrane fusion, while having little effect on F_0 processing and cell surface expression [Aguilar *et al.*, 2006]. However, deglycosylated F protein also provided less protection from antibody neutralization, likely due to an increase in epitope exposure. This suggests that in Nipah virus the N-glycans on F protein

contribute to a more efficient immune evasion at the cost of membrane fusion efficiency. Recently, also N-glycans on the Nipah G protein have been shown to protect against antibody neutralization, while modulating fusion and viral entry [Biering *et al.*, 2012]. This trade-off touches a common theme in the virus' infection strategy: exposed receptor binding epitopes increase infectivity but at the same time provide a target for neutralizing antibodies. Recently, the N- and O-glycan composition of Hendra G protein expressed in two different cell lines has been characterized through MS analysis and a difference was found in site occupancy rather than in glycan composition [Colgrave *et al.*, 2012].

Both N- and O-linked glycans on the highly glycosylated RSV G protein have been shown to have a large impact on virus infectivity in cell culture [Lambert, 1988; Garcia-Beato *et al.*, 1996]. However, these N- and O-linked glycosylation sites are situated in the two hypervariable regions in the ectodomain and hence are poorly conserved [Johnson *et al.*, 1987]. Also, the type and amount of glycosylation of cell culture-grown virus has been shown to be cell type-specific [Garcia-Beato *et al.*, 1996; Rawling & Melero, 2007]. Therefore, it is perhaps not surprising that different studies using different cell types found different molecular weight shifts upon RSV G protein deglycosylation: from ~90kD or ~180kD to ~32kD [Wertz *et al.*, 1989; Kwilas *et al.*, 2009]. Differences in extent and pattern of glycosylation between individual wild type strains are likely to reflect differences in infectivity, pathogenicity and virus survival *in vivo* [Rawling & Melero, 2007].

2.5. Entry routes: fusogenic vs. endocytic

Many enveloped viruses, such as influenza virus, Ebola and vesicular stomatitis virus (VSV) are endocytosed after attachment to an internalization receptor [Lamb & Parks, 2007]. Lowering of the endosomal pH to a certain threshold then triggers fusion of the viral envelope with the endosomal membrane. For these viruses, membrane fusion and genome release thus occur somewhere along the endocytic pathway.

Paramyxoviruses have generally been thought to enter at the plasma membrane, i.e. to employ a 'fusogenic' entry mechanism: receptor binding at the PM directly or indirectly – through the attachment protein – triggers refolding of the fusion protein. This was mainly because for many paramyxoviruses infection in cells had been shown to be efficient at neutral pH, indicating that these viruses do not rely on endocytic pathways [Nagai *et al.*, 1983; Srinivasakumar *et al.*, 1991; Kahn *et al.*, 1999; Bissonnette *et al.*, 2006; Aguilar *et al.*, 2009; Lamb & Parks, 2007]. Also, paramyxovirus glycoproteins expressed at the plasma membrane of infected cells induce formation of syncytia, further indicating that their membrane fusion capabilities are pH-independent. However, direct evidence that paramyxoviruses enter (solely) at the PM has not been obtained. Moreover, a low pH does not inhibit the infection of a number of paramyxoviruses, whereas fusion activity of RSV and NDV was shown to be enhanced in acidic environments [Srinivasakumar *et al.*, 1991; San Román *et al.*, 1999; Bissonnette *et al.*, 2006; Cantin *et al.*, 2007]. Interestingly, membrane fusion is in fact dependent on low pH for some HMPV strains [Schowalter *et al.*, 2006; Herfst *et al.*, 2008]. Furthermore, infection with a low pH-dependent HMPV strain was significantly reduced by treatment with inhibitors of clathrin-mediated endocytosis [Schowalter *et al.*, 2009].

Recent studies have found evidence that at least a few other paramyxoviruses (partially) rely on endocytic pathways [Cantin *et al.*, 2007; Kolokoltsov *et al.*, 2007; Gutierrez-Ortega *et al.*, 2008; Diederich *et al.*, 2008; Pernet *et al.*, 2009; San Juan-Vergara *et al.*, 2011; Krzyzaniak *et al.*, 2013]. NDV virions were shown to co-localize with early endosomal markers [Cantin *et al.*, 2007]. Nipah infection was significantly decreased by chemical inhibitors of endocytic and macropinocytic pathways [Diederich *et al.*, 2008; Pernet *et al.*, 2009]. While in one study siRNA inhibition of clathrin light chain and some other components of the clathrin-mediated endocytic pathway greatly reduced RSV infection [Kolokoltsov *et al.*, 2007], in another recent study RSV endocytosis was reported to be independent of clathrin and dynamin but rather showed characteristics of macropinocytosis [Krzyzaniak *et al.*, 2013]. Here, the requirement for endocytosis was the unique, second proteolytic cleavage step of the RSV F protein, which was shown to occur only after virus particle internalization in an endosomal compartment that ultimately resulted in complete membrane fusion [Krzyzaniak *et al.*, 2013]. Furthermore, RSV infection induced transient rearrangements in the actin cytoskeleton.

Recently, RSV infection was shown to be dependent on cholesterol and Pak1 in NHBE cells, and RSV particles co-localized with a marker for cholesterol- and sphingolipid-rich microdomains (more commonly called 'lipid rafts' or 'detergent-resistant membranes' (DRMs)) [San Juan-Vergara *et al.*, 2011].These findings suggest that RSV docks to lipid rafts, and provide further evidence that RSV infection induces cytoskeletal rearrangements, followed by endocytosis.

In general, it is plausible to assume that the location of membrane fusion is not only dependent on the stimuli required for F protein triggering, but also linked to the fate of its attachment factors and entry receptors upon ligand binding. If, as it seems for most paramyxoviruses, a low pH is not required for F protein triggering, then it depends on the speed of receptor recycling (i.e. endocytosis) compared to the speed of membrane fusion (i.e. triggering of a sufficient amount of F proteins to create a fusion pore) whether the latter occurs predominantly at the PM or someway along the endocytic pathway. The picture would become even more blurred if a virus uses more than one type of receptor, each with its own membrane distribution and transport characteristics.

As it is likely that multiple receptor interactions are necessary to trigger enough fusion proteins to create a fusion pore, it could be possible that clathrin/caveolin-mediated mechanisms are intimately linked to membrane redistribution of a sufficient amount of receptors towards the site of infection (see paragraph 3.4). Furthermore, in certain cases it could be possible that interaction between attachment factors and entry receptors is needed, and that this interaction is also dependent on/connected with the formation of clathrin- or caveolin-coated pits. On the other hand, the importance of lipid rafts/DRMs could be in facilitating a platform with high receptor concentration and minimal lateral movement, thereby increasing the chance of binding to receptors and interaction with immunomodulatory molecules.

3. HOST CELL RECEPTORS

3.1. Introduction

Interactions between the viral glycoproteins and their host cell receptors are non-covalent in nature and their strength depends on the affinity of the glycoprotein for the receptor. The higher the avidity (i.e. the receptor affinity multiplied by the number of glycoprotein-receptor interactions) the higher the chance that virion binding is irreversible. Host cell receptors can be subdivided into so-called "attachment factors" and "entry receptors" based on their role in virus infection. Attachment factors merely bind viral glycoproteins, leading to an accumulation of virus particles at the cell surface. Entry receptors on the other hand not only bind viral glycoproteins, but also trigger membrane fusion. The presence of an attachment factor on a cell type that expresses an entry receptor (i.e. a virus-permissive cell type) usually increases its virus-susceptibility, as trapping of virus particles increases the chance of binding to an entry receptor.

Viruses differ in the set of receptors with which they interact. Since host species differ in their repertoire of cell surface receptors, viruses differ in their host range ("host tropism"). The receptor pattern also varies between cell types within a host, which explains in a large part the characteristic cellular tropism of each virus. This does not exclude the possibility that –as is often the case– different viruses attach to and infect the same cell type through interactions with different receptors, or that a virus might enter a particular cell type through interactions with more than one type of receptor.

During infection *in vivo*, viruses typically attach to and enter different cell types dependent on the stage of infection, usually employing interactions with different receptors. Many viruses first infect epithelial cells that are encountered at their site of entry. Many airborne viruses target polarized epithelial cells lining the airways, while enteric viruses infect polarized epithelial cells lining the gastroenteric tract. The dissemination route of a virus that infects epithelial cells also depends on polarized assembly, budding and release from these cell types [reviewed in Harrison *et al.*, 2010].

Apart from epithelial cells, other common early viral targets are immune cells, such as dendritic cells (DCs) that patrol epithelial tissues in contact with the external environment. While DCs are highly specialized in the recognition and uptake of pathogens and subsequent activation of both innate and adaptive immune responses against these pathogens, many viruses exploit and specifically rely on the mechanisms that DCs have developed against them. After uptake by DCs these viruses can somehow avoid lysosomal degradation, thereby preventing antigen-presentation on the DC surface and an effective T cell-mediated immune response. Furthermore, viruses take advantage of binding to and uptake by DCs to spread across the epithelium to other tissues, where viral replication is sustained. After prolonged replication in susceptible tissues viruses finally target organs from where they can spread to other hosts. Typical late target tissues include lung epithelial cells – this time entered from the basolateral side – and salivary glands.

In the last decades, the identification of host cell entry receptors and attachment factors for various paramyxovirus species has shed light on their tropism and dissemination routes *in vivo* [reviewed in Backovic & Rey, 2012].

3.2. CAMs, PRRs and GAGs: common themes in paramyxovirus entry

A conserved feature in the entry of many other enveloped viruses seems to be the interaction with molecules that mediate intercellular adhesion and/or are a part of cell-cell and cell-ECM adhesion structures such as tight junctions, adherens junctions, desmosomes and focal adhesion spots [reviewed

in Delorme-Axford & Coyne, 2011.]. Cell adhesion molecules (CAMs) include members of the integrin, cadherin and selectin superfamilies, all of which are Ca^{2+}-dependent, and members of the immunoglobulin superfamily, which are Ca^{2+}-independent. Other molecules that play a role in cell-cell and cell-ECM interaction are membrane-bound proteoglycans such as syndecans, and certain membrane-bound mucins. Especially integrins are used as entry/internalization receptors by many viruses such as hantaviruses, rotaviruses, human herpesvirus 8, West Nile virus, human cytomegalovirus, reovirus, Kaposi's sarcoma-associated herpes virus and human echovirus 1 [Gavrilovskaya *et al.*, 1998; Guerrero *et al.*, 2000; Graham *et al.*, 2005; Wang *et al.*, 2003; Chu *et al.*, 2004; Feire *et al.*, 2004; Maginnis *et al.*, 2006; Garrigues *et al.*, 2008; Jokinen *et al.*, 2010].

It is no coincidence that many viruses have evolved affinity for CAMs, since they benefit from interaction with CAMs in multiple ways. First, interaction with viral glycoproteins may unravel cell-cell adhesion structures between adjacent epithelial cells, thereby increasing the available membrane area for entry and exit of virus particles [reviewed in Delorme-Axford & Coyne, 2011]. This is reflected by the observation that many paramyxovirus infections of epithelial tissues are not only characterized by syncytium formation, but also by disruption of the epithelial architecture and shedding of epithelial cells [Kuiken *et al.*, 2004; Hamelin *et al.*, 2006; Papenburg *et al.*, 2010]. For some viruses it has been demonstrated that disruption of cell-cell adhesion structures occurs after infection through the action of non-structural proteins [reviewed in Delorme-Axford & Coyne, 2011].

Second, many CAMs have cytosolic domains that are connected to the cytoskeleton through a network of linking proteins. Ligand binding then activates remodeling of the cytoskeleton, often initiating endocytosis and hence uptake of receptor-bound virus particles [reviewed in Delorme-Axford & Coyne, 2011]. Furthermore, activation of certain CAMs through ligand-binding initiates a signaling cascade leading to modulation of the immune response.

Hence, another common theme in virus infection, although in most cases not directly enhancing virus entry, is interaction with molecules that have immunomodulatory signaling properties that are exploited in favor of virus survival. Apart from CAMs with signaling properties, these molecules are often pattern recognition receptors (PRRs).

Before reaching their receptors on the surface of epithelial cells, viruses have to penetrate a thick gel-like mucus layer formed by mucins and other gel-forming glycoproteins. Many enveloped viruses, including some paramyxoviruses, have been shown to bind to glycosaminoglycans (GAGs) in cell cultures [Feldman *et al.*, 1999; Fujita *et al.*, 2007; Terao-Muto *et al.*, 2008]. GAGs are long unbranched chains of repeating disaccharide units that are usually coupled to a protein core, forming so-called proteoglycans. Secreted and membrane bound proteoglycans are ubiquitously expressed and populate a significant portion of the extracellular matrix and cell surface of most mammalian cell types [reviewed in Varki *et al.*, 2009 (chapter 16)].

3.3. Entry receptors

Paramyxovirinae genera containing HN proteins interact with sialylated receptors (i.e. membrane glycoproteins or glycolipids with terminal sialic acids) for entry into the host cell. The affinity of HN proteins for varying sialic acid containing molecules differs among HN-expressing viruses, likely contributing to the differences in their pathogenicity [reviewed in Villar & Barroso, 2006]. Apart from interacting with sialylated receptors through HN protein, Sendai virus is unique in that it has also been described to interact with the asialoglycoprotein receptor (ASGP-R) through interaction with the F protein [Bitzer *et al.*, 1997; Leyrer *et al.*, 1998]. *Paramyxovirinae* genera containing H or G proteins bind proteinaceous entry receptors.

The first MV entry receptor, the complement activation gene cluster regulatory protein CD46, was identified in the early '90s using attenuated vaccine and laboratory strains [Naniche *et al.*, 1993; Dorig *et al.*, 1993; Manchester *et al.*, 1994]. CD46 is expressed in virtually all nucleated cells including respiratory epithelial and immune cells, the major target cells of MV. CD46 was later also confirmed as an entry receptor for some clinical MV isolates [Manchester *et al.*, 2000]. Several *in vitro* studies have described the interaction between MV H protein and CD46, its signaling leading to several MV

pathogenic effects [reviewed in Sato *et al.*, 2012]. However, several virulent B cell line-isolated MV strains display such low affinities for CD46 that it was discarded as an important *in vivo* entry receptor.

The cellular entry receptor through which MV and other morbilliviruses infect immune cells is signaling lymphocyte activation molecule (SLAM, also known as CD150), first identified in a cDNA screen for MV [Tatsuo *et al.*, 2000, 2001]. SLAM is a type I transmembrane protein with two extracellular Ig-like domains, V and C2, and is exclusively expressed on immune cells. SLAM interacts with another SLAM molecule on an adjacent immune cell and is involved in many regulatory pathways of the innate and acquired immune responses [reviewed in Veillette *et al.*, 2007]. The V domain mediates SLAM-SLAM and SLAM-morbillivirus interactions. Several studies have demonstrated the importance of SLAM as an *in vivo* MV entry receptor [Leonard *et al.*, 2010]..

Recently two research groups independently identified adherens junction protein nectin-4 as the basolateral epithelial entry receptor for MV [Mühlebach *et al.*, 2011; Noyce *et al.*, 2011]. The existence of a basolateral epithelial MV entry receptor had already been demonstrated from cell culture and *in vivo* studies [Tahara *et al.*, 2008; Leonard *et al.*, 2008]. Nectin-4 is an adherens junction protein that belongs to the immunoglobulin superfamily and its expression is localized to polarized epithelial cells [reviewed in Rikitake *et al.*, 2012]. Nectin-4 interacts with MV H protein through its V domain [Mühlebach *et al.*, 2011].

The identification of SLAM and nectin-4 as the MV entry receptors for infection of, respectively, immune cells and polarized epithelial cells, has finally revealed the MV dissemination route [recently reviewed in Sato *et al.*, 2012]. Immune cells present in the inner lining of the airways, such as immature DCs and macrophages are the main initial targets of MV infection [Tatsuo *et al.*, 2000; Leonard *et al.*, 2008, 2010; Ferreira *et al.*, 2010]. These immune cells, especially alveolar DCs, facilitate virus spread to local lymph nodes where other immune cells are infected and extensive virus replication occurs [Von Messling *et al.*, 2006; de Swart *et al.*, 2007; Lemon *et al.*, 2011]. This leads to viremia and hence spreading to secondary sites of infection, which, apart from lymphoid organs, include a variety of other tissues and organs [de Swart *et al.*, 2007; Griffin, 2007]. Basolateral infection of the airway epithelial cells through interaction with nectin-4, followed by shedding from the apical side is then the main route to escape from the host organism. Since MV is also capable of SLAM and nectin-4 independent entry into many cell types with low infectivity, it is likely that there also exist ubiquitously expressed low-affinity receptors for MV H.

Henipavirus (Hendra and Nipah) G proteins interact with class B ephrins (ephrinB2 and B3) [Bonaparte *et al.*, 2005; Negrete *et al.*, 2005, 2006; Bishop *et al.*, 2007; reviewed in Steffen *et al.*, 2011; Xu *et al.*, 2012(b)]. EphrinB2 and ephrinB3 are tyrosine kinase receptors that are highly expressed on the cell surface of blood vessel endothelial cells and neurons, a tissue distribution that is consistent with the major complications caused by henipavirus infections. EphrinB2 and ephrinB3 play a fundamental role in cell-cell signaling, especially during angiogenesis and development of the CNS [reviewed in Pasquale, 2008]. EphrinB2 and B3 are highly conserved among different species, which may explain the broad host range of henipavirus. Hendra has lower affinity for its receptors than Nipah due to a less hydrophobic G protein-receptor interface [Bossart *et al.*, 2007; Xu *et al.*, 2012(a)].

Although many RSV glycoprotein-receptor interactions have been described in cell culture experiments, it is still unclear what interactions are crucial for host cell entry *in vivo*. This is in a large part due to the complex nature and ambiguous role of the RSV G protein (see paragraph 3.1). A recent study searching for candidate RSV entry receptors described an interaction between RSV F protein and nucleolin at the apical cell surface in cell culture [Tayyari *et al.*, 2011]. Another research group had earlier identified nucleolin as an interaction partner of fucoidan, a polysaccharide composed mainly of fucose sulphate found in algae that inhibits RSV infection in cell culture [Malhotra *et al.*, 2003]. However, no competition or expression assays were done to confirm RSV interaction with nucleolin [Malhotra *et al.*, 2003]. Other studies have confirmed that nucleolin, an abundant protein involved in many processes in the nucleus [reviewed in Mongelard & Bouvet, 2007], is also expressed at the plasma membrane [Hovanessian *et al.*, 2000; Losfeld *et al.*, 2009]. Nucleolin had previously also been shown to play a role in hPIV3 infection [Bose *et al.*, 2004]. Nucleolin-transfection of non-permissive cells led to RSV susceptibility, while RNAi-mediated knock-down of lung nucleolin was associated with a significant reduction of RSV infection *in vivo* [Tayyari *et al.*, 2011]. These findings

strongly suggest that nucleolin mediates RSV entry through a direct interaction with F protein. However, one might question whether nucleolin is an important entry receptor for RSV *in vivo* as nucleolin is expressed in many tissues, while RSV has a very restricted tropism [Zhang *et al.*, 2002].

$\alpha_v\beta_1$-integrin was identified as a functional entry receptor in cell culture for HMPV through interaction with F protein [Cseke *et al.*, 2009]. Many ECM-resident integrin-binding proteins have a specific amino acid sequence, such as RGD or KGE/DGE in their integrin-interacting domain. The researchers observed that an RGD sequence is also found in the F protein of all currently known HMPV isolates, but not in its closest relative, the HRSV F protein, or any other paramyxovirus F protein. This observation led the researchers to hypothesize that HMPV might use an integrin as entry receptor. The divalent cation chelator EDTA was found to inhibit HMPV but not HRSV infectivity in cell culture, suggesting that only the former uses a Ca^{2+} or Mg^{2+}-dependent CAM as entry receptor. RGD, but not RGE-containing short peptides reduced HMPV but not HRSV infectivity. Next, antibodies against various integrins were tested and an antibody specific for $\alpha_v\beta_1$-integrin inhibited HMPV infectivity. SiRNA reduction of $\alpha_v\beta_1$-integrin expression in permissive cells as well as transfection of $\alpha_v\beta_1$-integrin in non-permissive cells confirmed its role as an HMPV entry receptor in cell culture. Vice versa, a recombinant HMPV F protein could bind the cells, whereas a mutant recombinant HMPV F protein (RGE instead of RGD) could not. In a later study by the same research group, other RGD-binding integrins, specifically $\alpha_5\beta_1$-integrin and other α_v-integrin heterodimers, were identified as receptors for HMPV F (see Table 1) [Cox *et al.*, 2012]. Antibody blocking of individual integrins led to a significant reduction in virus infectivity in a dose dependent manner up to >90% when all RGD-binding integrins were blocked [Cox *et al.*, 2012]. However, upon complete blocking, virus attachment was only reduced ~40%, comparable to the reduction observed on blocking individual integrins, suggesting that integrin-mediated attachment is saturable [Cox *et al.*, 2012]. Residual HMPV attachment is mediated by attachment factors, such as heparan sulfate containing proteoglycans (HSPGs – see paragraph 3.4), which were demonstrated to be crucial for efficient infection of CHO cells through interaction with F protein [Chang *et al.*, 2012].

While interaction of the HMPV F protein with RGD-binding integrins seems crucial for efficient HMPV infectivity in cell culture, it is still uncertain whether binding directly triggers refolding of HMPV F (i.e. RGD-binding integrins are true entry receptors, as proposed by Cseke *et al.*, 2009), or whether RGD-binding integrins function as internalization receptors (after HSPG- and integrin-mediated attachment) and F protein triggering and subsequent membrane fusion require other factors as well. These factors might be a low pH (as has been demonstrated for some laboratory strains), and/or likely another proteinaceous (and trypsin- and proteinase K-sensitive) receptor [Schowalter et al., 2006, 2009; Chang *et al.*, 2012].

3.4. Attachment factors

From recent studies it has become apparent that some attachment factors do not just trap virus particles, but can further promote virus infection by interacting with, or inducing membrane redistribution of an entry receptor.

An attachment factor that recently has been shown to exhibit these characteristics is the C-type lectin DC-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN, also known as CD209). DC-SIGN is an important PRR expressed by certain types of DCs and macrophages. DC-SIGN is used as an attachment factor (and in some cases entry receptor) by many enveloped viruses including HIV-1 [Geijtenbeek *et al.*, 2000], Influenza A viruses [Londrigan *et al.*, 2011; Hillaire *et al.*, 2013], Ebola virus [Alvarez *et al.*, 2002; Marzi *et al.*, 2007], Marburg virus, SARS coronavirus [Marzi *et al.*, 2004; Yang *et al.*, 2004], some bunyaviruses [Lozach *et al.*, 2011] and the paramyxoviruses MV [de Witte *et al.*, 2006] and RSV [Johnson *et al.*, 2011]. DC-SIGN has a broad substrate specificity and has multiple functions including regulation of adhesion through interaction with integrins (i.e. DC migration), establishing DC–T cell adhesion for T cell activation, and as a PRR that recognizes high-mannose glycans thereby initiating endocytosis of bound pathogens [recently reviewed in Svajger *et al.*, 2010; Garcia-Vallejo & Van Kooyk, 2013]. As such, DC-SIGN was shown to play an important role in the DC-mediated HIV-1 *trans*-infection of T cells [Kwon *et al.*, 2002]. It has also become clear

that DC-SIGN has immunomodulatory and other properties that are exploited – in various manners and to various degrees – by many pathogens [reviewed in Van Kooyk & Geijtenbeek, 2003]. DC-SIGN was established as a MV attachment factor in DCs, binding both F and H glycoproteins, probably through interaction with their N-linked glycans [de Witte *et al.*, 2006].

Although abundant DC-SIGN expression in cells non-permissive for MV did not confer any susceptibility, both MV attachment to and infection of immature DCs were blocked in the presence of DC-SIGN inhibitors, suggesting an important role for DC-SIGN in promoting MV interaction with its entry receptor SLAM (CD150). SLAM+ DCs are among the first cell types to be infected by MV, although the entry receptor is not abundantly present at the surface of immature DCs [de Witte *et al.*, 2006].

A recent study illuminated the mechanism by which MV binding to DC-SIGN led to redistribution of its entry receptor SLAM from intracellular storage compartments to ceramide-enriched domains at the DC surface to enhance virus uptake [Avota *et al.*, 2011]. MV binding to DC-SIGN was shown to lead to rapid activation of neutral and acid sphingomyelinases (SMAses). Upon activation, these SMAses convert sphingomyelin into ceramides leading to formation of ceramide-enriched membrane platforms, which are thought to play a role in various cellular stress responses, the support of membrane fusion and clustering of membrane receptors and their signalosome components [reviewed in Zeidan & Hannun, 2010; Stancevic & Kolesnick, 2010]. MV binding to DC-SIGN was shown to lead to redistribution of SLAM and acid SMAse to the plasma membrane, whereas in immature DCs SLAM was found to be mainly localized at acid SMAse-containing intracellular storage vesicles [Avota *et al.*, 2011]. DC-SIGN-induced SMAse activation was found to be an essential step in enhancing MV entry in DCs as well as DC-SIGN signaling: first, pharmacological inhibition of acid SMAse significantly reduced MV infection. Second, inhibition of acid SMAse prior to DC-SIGN activation counterfeited (1) ceramide enrichment at the surface, (2) SLAM surface recruitment as well as (3) DC-SIGN-mediated signaling via Raf-1 and the MAP kinases ERK1 and ERK2 (and hence modulation of TLR signaling). Activation of SMAses through DC-SIGN (or another PRR) leading to clustering of entry receptors in ceramide-enriched domains at the cell surface might also be an important entry mechanism for other enveloped viruses infecting DCs [Avota *et al.*, 2011].

DC-SIGN was also identified as a RSV attachment factor in DCs, and although the DC-SIGN-G protein interaction was shown to suppress DC activation, it appears not to be enhancing RSV entry [Johnson *et al.*, 2011]. This might suggest that either the RSV entry receptor for DCs is already present on the surface of immature DCs, or RSV interacts with another attachment factor to achieve a similar redistribution of its internalization receptor.

Intercellular adhesion molecule-1 (ICAM-1, also known as CD54 on immune cells) has been identified as a receptor that enhances RSV entry into HEp-2 cells, through interaction with the F protein [Behera *et al.*, 2001]. ICAM-1 is *in vivo* mainly expressed on endothelial cells and immune cells, but also on (respiratory) epithelial cells and is known as an entry receptor for rhinovirus [Greve *et al.*, 1989]. However, whether ICAM-1 plays a role as an internalization receptor and/or attachment factor for RSV infection *in vivo* is not known. RSV G protein has been shown to interact with annexin II on epithelial cells and L-selectin on immune cells, however these interactions are not essential for entry [Malhotra *et al.*, 2003]. Annexin II has previously been shown to be a cofactor in HIV-1 infection of macrophages [Ryzhova *et al.*, 2006]. Other described attachment factors for RSV are RhoA [Pastey *et al.*, 1999, 2000], CX3CR1 (fractalkine receptor), on immune cells [Tripp *et al.*, 2001, Zhang *et al.*, 2010, Choi *et al.*, 2012], and several TLRs [Rudd *et al.*, 2005, Kurt-Jones *et al.*, 2000, Haynes *et al.*, 2001; Marr *et al.*, 2012]. While interactions with CX3CR1 and TLRs are important because they modulate the host immune response [Zhang *et al.*, 2010; Johnson *et al.*, 2012; reviewed in Klein Klouwenberg *et al.*, 2009] they do not seem to enhance RSV entry.

Both RSV and HMPV G and F protein have been shown to bind independently to the GAGs heparin and heparan sulfate, an interaction that is necessary for efficient infection of cell cultures [Krusat & Streckert, 1997; Feldman *et al.*, 1999, 2000; Hallak *et al.*, 2000(a); Techaarpornkul *et al.*, 2001; Barretto *et al.*, 2003; Shields *et al.*, 2003; Escribano-Romero *et al.*, 2004; Crim *et al.*, 2007; Thammawat *et al.*, 2008; Chang *et al.*, 2012; Adamson *et al.*, 2012]. Also, in one study, heparin inhibited the initial tissue culture passage of primary isolates from both RSV subgroups, indicating that RSV-GAG interactions may also be important during *in vivo* infection [Teng *et al.*, 2001].

Heparin/heparan sulfate-binding proteins generally interact through electrostatic interactions between the negatively charged sulfate groups on the GAG and positively charged amino acids within the protein's heparin binding domain (HBD). However, RSV GAG-interaction has been shown to be dependent on iduronic acid, GAG chain length, and degree and type of sulfation, suggesting polyvalence (i.e. more than one protein-glycan interaction) rather than a simple charge interaction between the glycoproteins and the sulfate groups [Hallak *et al.*, 2000(a), 2000(b); Martinez & Melero, 2000; Techaarpornkul *et al.*, 2002].

While it has been established that glycoproteins of *Pneumovirinae* interact with GAGs for entry into cell cultures, the relevance of GAG-binding for *in vivo* infection has been questioned. RSV has been shown to infect cells devoid of GAGs, and recombinant RSVΔG was less dependent on GAGs for infection and entry [Techaarpornkul *et al.*, 2002]. Furthermore, a mutant RSV strain lacking the central conserved domain and cysteine noose (including the putative HBD) of the G protein had little effect on replication *in vitro* and in the respiratory tract of mice, suggesting that GAG interaction – at least through G protein – is not necessary for efficient replication *in vivo* [Teng *et al.*, 2001; Teng & Collins, 2002]. Recently, glycoprotein interaction with GAGs has been claimed to be an *in vitro* artifact [Villenave *et al.*, 2011, 2012, recently reviewed in Villenave *et al.*, 2013].

Although there is controversy over the use of GAGs as *Pneumovirinae* attachment factors *in vivo*, it might still be possible that specific heparan-sulfate containing proteoglycans (HSPGs; protein-glycan interaction) interact with the HBDs in the F protein [Chang *et al.*, 2012]. Some HSPGs, notably syndecans, are known to interact with members of the integrin family [reviewed in Roper *et al.*, 2012], leading to the hypothesis that these could be more than just generic, particle trapping factors. Hence, in the case of HMPV, attachment to a specific syndecan could be necessary for efficient virus entry.

Other candidate *Pneumovirinae* attachment factors for infection *in vivo* might be certain lectins (glycan-protein interaction) that interact with glycans on either the G or F protein, or both. This is supported by studies that show that both N- and O- deglycosylation of G protein has a major impact on virus infectivity in cell culture [Lambert, 1988; Garcia-Beato *et al.*, 1996].

Just like the *Pneumovirinae*, MV and CDV were also shown to bind to GAGs and infection of several SLAM-negative cell lines was inhibited by soluble heparin in a dose-dependent manner [Fujita *et al.*, 2007; Terao-Muto *et al.*, 2008]. This suggests that, although probably not acting as the low affinity entry receptors on SLAM-negative cells, the abundant expression of GAGs guarantees enough attachment for infection. Interaction in MV is dependent only on H protein, while in CDV both H and F protein interact with heparin.

Subfamily	Genus	Species	Attachment & entry interactions			
			Attachment factor	Entry/internalization receptor	Viral ligand	Interaction type
Pneumovirinae	pneumoviruses	HRSV	**glycosaminoglycans (GAGs)**		G and **F**	*glycan - protein*
			RhoA (small cellular GTPase)		**F**	*protein - protein*
			TLR3			*protein - glycan*
			TLR4		**F**	*protein - glycan*
			CD14		**F**	*protein - glycan*
			ICAM-1		**F**	*protein - protein*
			L-selectin		**G**	*protein - glycan*
			annexin II		**G**	*protein - glycan*
			CX3CR1 (fractalkine receptor)		**G**	*protein - protein*
			DC-SIGN, LC-SIGN		**G**	*protein - glycan*
				nucleolin (?)	**F**	*protein - glycan (?)*
				ICAM-1 (?)	**F**	*protein - protein*
	metapneumoviruses	HMPV	**glycosaminoglycans (GAGs)**		G and **F**	*glycan - protein*
				RGD-binding integrins	**F**	*protein - protein*
Paramyxovirinae	respiroviruses	hPIV1		**sialic acids**	**HN**	*glycan - protein*
		hPIV3	**nucleolin**			
				sialic acids	**HN**	*glycan - protein*
		SeV (mPIV1)		**sialic acids**	**HN**	*glycan - protein*
				ASGR2 [?]	**F**	*protein - glycan*
	henipaviruses	Nipah		**ephrin B2**	**G**	*protein -protein*
				ephrin B3	**G**	*protein -protein*
		Hendra		**ephrin B2**	**G**	*protein -protein*
				ephrin B3	**G**	*protein -protein*
	morbilliviruses	measles	**glycosaminoglycans (GAGs)**		H and **F**	*glycan - protein*
			DC-SIGN		H and **F**	*protein - glycan*
				SLAM (CD150)	**H**	*protein -protein*
				CD46	**H**	*protein -protein*
				nectin-4	**H**	*protein -protein*
	avulaviruses	NDV		**sialic acids**	**HN**	*glycan - protein*
	rubulaviruses	mumps		**sialic acids**	**HN**	*glycan - protein*
		hPIV2		**sialic acids**	**HN**	*glycan - protein*
		hPIV4		**sialic acids**	**HN**	*glycan - protein*
		PIV5 (SV5)		**sialic acids**	**HN**	*glycan - protein*

Table 1: Paramyxovirus receptors. Some currently known entry/internalization receptors and attachment factors.

4. DISCUSSION

In the last two decades numerous studies have been performed to illuminate paramyxovirus entry. Mechanistic models of attachment protein-mediated triggering of F protein have been proposed, largely based on crystal structures and functional (i.e. mutagenesis, biochemical) studies in cell culture. Although X-ray crystallography is an indispensable tool to study viral glycoprotein mediated entry mechanisms on a structural level up till now no crystal structures are available of intact *Paramyxovirinae* fusion complexes. Hence the current crystallographic data provide only limited information about the spatial organization of the functional fusion complexes and therefore they must be integrated with the information obtained from mutational and biochemical studies.

The mechanisms of membrane fusion have been studied almost exclusively in glycoprotein-transfected cells, with detection of syncytia formation as a read-out system for fusion promoting activity. These transfection studies have led to insights into how paramyxoviral glycoproteins trigger membrane fusion, but they have to be viewed with some skepticism as syncytia formation is not the same as virus particle fusion, that is, the glycoproteins might behave differently in the cell membrane compared to the viral envelope.

It is well established that in the *Paramyxovirinae* subfamily membrane fusion is instigated after receptor binding to the attachment protein, while in the *Pneumovirinae* subfamily the F protein can mediate membrane fusion on its own, and is triggered either directly after receptor binding or after

endocytosis, followed by proteolytic cleavage (in the case of RSV), low pH (in the case of some HMPV strains), or a yet to be identified mechanism.

Regarding the membrane fusion mechanism in *Paramyxovirinae*, the current dogma tells that receptor binding induces a spatial rearrangement in the head region of the attachment protein homotetramer (possibly a rearrangement of the dimer-dimer interface) which alters the configuration of the stalk domain and in turn triggers refolding of the F protein, either through association or dissociation of the fusion complex. However, the detailed structure and stoichiometry of the fusion hetero-oligomer in the viral membrane as well as the cascade of spatial reorganizations that transduces the receptor binding signal from the attachment protein globular head to its stalk region and finally to the F protein remains to be elucidated for any of the paramyxoviruses. Also, the exact series of conformational changes that underlie F protein refolding (both in *Paramyxovirinae* and *Pneumovirinae*) are not known. High resolution cryo-electron tomography of native fusion complexes might be useful in combination with XRC and functional studies to address these questions.

A detailed understanding of the structure of paramyxovirus glycoproteins has important clinical applications. In this light, the recently solved structure of the RSV F protein in its pre-fusion conformation has revealed a major antigenic site that is recognized by recently isolated murine and human antibodies that are substantially more potent in inhibiting RSV infection in cell culture than the prophylactic antibodies palivizumab and motavizumab, which recognize different epitopes and bind the post-fusion RSV F protein [McLellan *et al.*, 2013; McLellan *et al.*, 2011; Kwakkenbos *et al.*, 2010; Swanson *et al.*, 2011]. A RSV F protein stabilized in its pre-fusion conformation would therefore be a promising vaccine antigen, or at least elicit new prophylactic antibodies. Also, the recently solved structure of MV H protein in complex with nectin-4 revealed a hydrophobic pocket that is involved in binding to all currently identified MV receptors (CD46, SLAM and nectin-4), hence representing a target for anti-viral drugs [Zhang *et al.*, 2013]. Furthermore, identification of conserved epitopes that might become exposed during the transition of the fusion complex from its pre- to post-fusion state could also lead to the development of new, and possibly more effective, anti-viral drugs [recently reviewed in Aguilar & Lee, 2011]. Also, paramyxovirus glycoprotein-mediated membrane fusion mechanisms may be useful in vectors for targeted oncolysis as well as therapeutic gene delivery because they can be triggered by a wide array of cell surface receptors [reviewed in Russell & Peng, 2009; Cattaneo, 2010].

N- and O-linked glycans on the paramyxovirus attachment and fusion proteins have been shown to be important for virus entry since incorporation of glycosylation sites or glycosidase treatment have large effects on protein folding, immune evasion and virus infectivity in cell cultures. However, research on the exact glycosylation pattern of paramyxovirus glycoproteins, its cell-type dependence and its specific effect on virus entry has only just begun. Deciphering the glycosylation pattern – both in site occupancy as well as glycan composition – of attachment and fusion proteins and its effect on virus entry is an important future research goal and may assist in the development of glycan-targeted therapeutic intervention strategies.

In the last decade, host cell attachment factors and entry receptors have been identified for a number of paramyxovirus species, which has shed more light on the dissemination route and pathogenicity of these paramyxoviruses in their respective hosts. Glycoprotein-receptor interactions are complex and involve protein-protein, protein-glycan (and glycan-protein) and glycan-glycan interactions.

The identification of host cell receptors is usually done through biochemical experiments in cell culture, however genetics based approaches have also been used for receptor identification, such as transfection of a cDNA library into a non-susceptible cell line followed by virus incubation. In recent years alternative, faster methods for receptor identification have become available thanks to the rapid progress in the genomics, proteomics and bioinformatics fields: genome-wide microarray analyses have made it possible to compare differences in membrane protein gene transcription between virus-permissive and non-permissive cell-lines, yielding a short-list of candidate receptors (i.e. membrane proteins that are strongly expressed in virus-permissive cells compared to non-permissive cells) in a high-throughput manner. Recent methods for identifying receptors (and other host factors involved in viral infection) are reviewed by Hsu and Spindler, 2012.

For several reasons, it is important to be aware that receptors identified in *in vitro* experiments are not automatically (important) *in vivo* receptors. First, the receptor pattern expressed in immortal cell

lines usually differs from that of the target cells in the host organism. Second, – although not documented for all paramyxoviruses – due to their high mutation rate virus strains grown in cell culture are prone to develop adaptations (including receptor-binding adaptations) that enhance infection of that specific cell culture. It has been shown that many RNA virus laboratory strains (including paramyxoviral strains) are selected for their affinity to cell surface glycosaminoglycans (GAGs) during passage in cell culture [Hallak *et al.*, 2007]. Third, culture-grown virus strains acquire (reversible) cell-type specific modifications, such as the lipid content of their envelopes and the amount and pattern of glycosylation [Garcia-Beato *et al.*, 1996; Rawling & Melero, 2007], which might lead to certain protein-glycan receptor-binding interactions that do not occur, or have a marginal effect in *in vivo* infection. Therefore, since cell culture experiments do not give definitive answers, *in vivo* studies in transgenic animals using wild-type virus or primary isolates (i.e. patient samples) are decisive to address the importance of a receptor in *in vivo* infection. However, virus survival, dissemination and clinical manifestations of infection are often very different in transgenic animals compared to the original host, for instance if the expressed receptor is dependent on a species-specific co-receptor or if the virus interacts with species-specific immunomodulatory receptors. Other than that, the importance of protein-glycan interactions in *in vivo* infection is very hard to validate, since it is impossible to obtain mutant animals with specific glycosylation deficits, and also the cell surface glycosylation pattern varies between species. Strategies to characterize interactions between viral glycoproteins and cell surface glycans (from an influenza HA viewpoint) are reviewed in Shriver *et al.*, 2009.

While some important entry receptors of the protein-binding *Paramyxovirinae* appear to have been found, it is still unclear what receptor interactions are crucial for *Pneumovirinae*, and particularly, RSV infection *in vivo*. It might be that interaction with an attachment factor, or a co-receptor specifically expressed on the apical side of ciliated airway epithelial cells is required for efficient RSV particle trapping, and hence entry.

It might also be possible that some of these attachment factors not merely trap RSV virions, but actively promote entry either by directly interacting or co-localizing with an entry (or internalization) receptor, or by regulating membrane redistribution of the entry receptor, as shown for MV. It is possible, perhaps even likely, that RSV entry is driven by multiple receptor interactions that facilitate RSV attachment and entry into epithelial cells *in vivo*.

5. SUMMARY FOR LAYMEN

Virus particles (also called ‘virions’) can be viewed as little packages that are specialized in the transmission of their content, that is, their genetic material, from one cell to another and from one host organism to another. Viruses are obligate parasites: they are dependent on the molecules of the infected cell to replicate their genetic material and to produce new virus particles.

Based on their morphology (that is, how they look like from the outside), viruses can be divided into two groups: the ones with a ‘coat’ made up out of proteins, and the ones that have a lipid membrane wrapped around their protein coat (called ‘enveloped viruses’). Paramyxoviruses belong to the enveloped viruses. The replication cycle of enveloped viruses (and hence that of paramyxoviruses) will be described hereafter (– the paramyxovirus life cycle is illustrated in Fig. 6). To infect a cell, viruses first have to cross the physical barrier of the plasma membrane that surrounds each cell. For enveloped viruses this means that the viral envelope has to fuse with the membrane of the host cell. Virus particles (including paramyxoviruses) have specialized proteins on their surface that bind to receptors on the host cell membrane to achieve fusion of the viral envelope with the host cell membrane. Membrane fusion allows release of viral genetic material into the cytoplasm where it is replicated and viral structural proteins are produced. These newly produced genomes and viral structural proteins in turn hijack cellular transport mechanisms to assemble at the inside of the host cell membrane, and to start a budding process, which allows exit of the newly-formed virus particles. The viral envelope thus consists of lipids from the host cell membrane.

A cell infected with one virus particle can produce thousands of new virions that in turn can infect other cells or another host. Furthermore, to increase the amount of progeny, many viral genomes encode non-structural proteins that manipulate anti-viral immune responses. On an organismal level, one can say that the fate of a virus, as well as the consequences for its host, are dependent on the sum of the functions of all of the host cell molecules with which the virus interacts, most notably those involved in (1) virus attachment and entry, (2) virus assembly and budding, and (3) immunomodulation. The most successful and hence most common viruses achieve maximum viral offspring and spread before eradication by the host's immune system, with minimal deleterious effects on the host's fitness. This literature thesis (i.e. the preceding chapters) tries to give an overview of the research that has been done to illuminate the way paramyxoviruses enter their host cells.

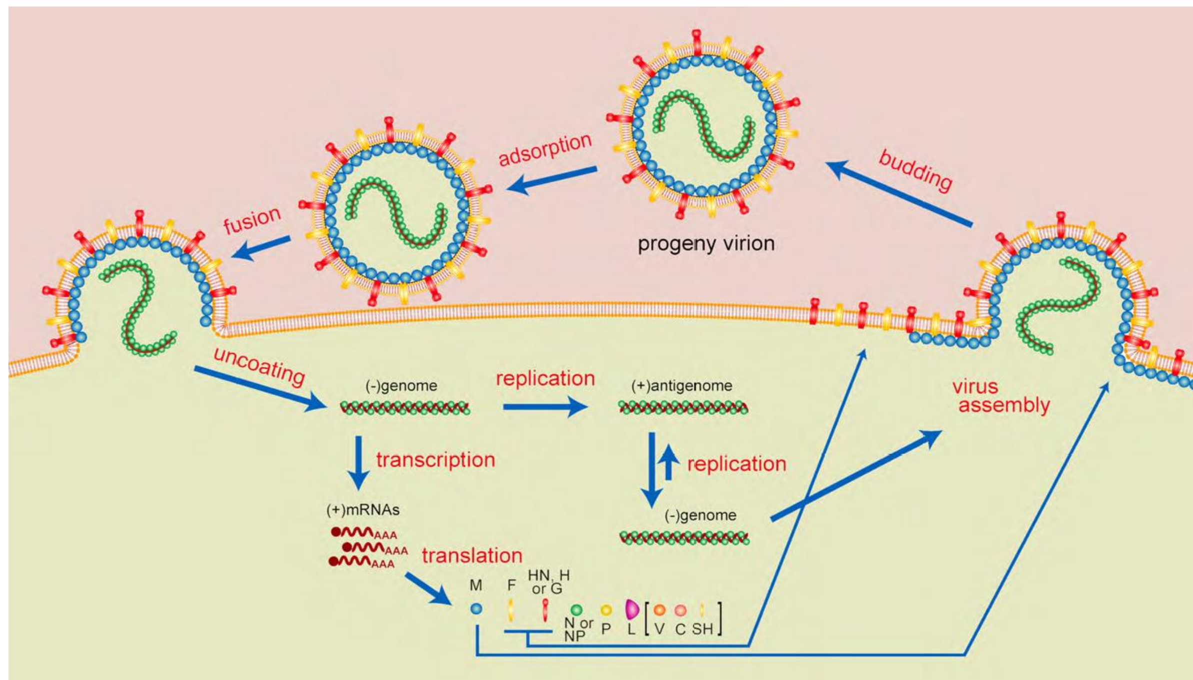

Figure 6: Schematic illustration of the paramyxovirus life cycle. Infection starts with attachment of the virus particle to the host cell. After attachment, fusion of the viral membrane with the plasma membrane releases the viral genome into the host cell cytoplasm for replication and production of viral structural proteins. Following their synthesis, newly-made viral proteins and genomes assemble on selected sites of the plasma membrane. Finally, the buds pinch off from the membrane and new virus particles are released. The viral membrane thus consists of host cell-derived lipids with viral transmembrane glycoproteins. (after Harrison et al., 2010).

The first step in the viral life cycle is gaining entry into the host cell. Paramyxoviruses have two proteins on their envelope that play a role in the entry process: the attachment protein and the fusion protein (together named 'fusion complex'). For a virus particle to enter the cell, their membranes should merge. This membrane fusion does not happen spontaneously and therefore costs energy. Fusion proteins in the viral membrane provide this energy: based on their molecular structure, they can be viewed as a kind of loaded springs that are released when a virus particle binds to the host cell. The energy that comes free by releasing the spring ('refolding') pulls the membranes of the virus and the host cell into proximity and ultimately ensures their fusion.

Paramyxoviruses differ in the way in which releasing of the spring ('refolding of the fusion protein') is triggered: in the *Paramyxovirinae* subfamily binding of the attachment protein to a receptor on the host cell triggers refolding of the fusion protein, while in the *Pneumovirinae* subfamily the attachment protein is not necessary. The fusion protein can mediate both receptor binding and membrane fusion by itself. The attachment protein of the *Pneumovirinae* is thought to be more important for modulating the immune response of the host cell, rather than for helping the virus to gain entry into the host cell. Nevertheless, in all cases, binding to a receptor on the host cell is instigates the

whole membrane fusion process. This binding to a specific receptor ensures that the fusion protein does not release its energy prematurely (which would render a virion not infectious).

Because in the *Paramyxovirinae* the receptor binding and fusion functions are divided over two proteins, there has to be some interaction, some 'communication' between them upon receptor engagement by the attachment protein to transduce the signal towards the fusion protein. Based on experimental research of the past two decades, there are currently two popular models that describe this interaction (see Fig. 5). In the first model, called the 'association' or 'provocateur' model, it can either be that the attachment protein and fusion protein are separated from each other prior to receptor binding, or that they are already associated, however, in any case, receptor binding induces an interaction between the two to trigger the fusion protein to refold. In the second model, called the 'dissociation' or 'clamp' model, the attachment protein functions as a clamp that keeps the fusion protein from triggering prematurely, and receptor binding then results in dissociation of the complex and releasing of the fusion protein. While a few years ago it seemed that these association and dissociation models fitted the *Paramyxovirinae* that bind to specific sugar-coated receptors or proteinaceous receptors respectively, nowadays it is not sure anymore whether this holds true. It might be the case that all *Paramyxovirinae* employ a quite similar mechanism of fusion protein triggering, with only a difference in the status (i.e. separate, together, stoichiometry…) of the fusion complex prior to receptor binding among the individual species.

Current research on the entry of paramyxoviruses focuses on the exact molecular structure of the fusion complex and the structural changes therein induced by receptor binding, and the (possible) differences in these mechanisms between the individual paramyxovirus species. However, detailed knowledge about the molecular structure of the fusion complex is in many cases still lacking, and would definitely be fruitful for the development of more efficient vaccine strategies, therapeutic antibodies and anti-viral drugs.

Because the attachment proteins of different viruses have different molecular structures, viruses differ in the set of receptors on the host cell with which they can interact, like different keys for different locks. Since host species differ in their repertoire of cell surface receptors, this explains why viruses differ in their host range (this phenomenon is called 'host tropism'). The receptor pattern also varies between cell types within a host, which explains in a large part the characteristic 'cellular tropism' of each virus. During infection, viruses typically attach to and enter different cell types dependent on the stage of infection, usually employing interactions with different receptors. Viruses first infect the cells that are encountered at their site of entry, such as the cells lining the lungs, respiratory tract and intestines or the immune cells that patrol these tissues. The route of infection that a virus follows through the body (which is called the 'dissemination route') often not only depends on the receptors with which it interacts to enter a specific cell type, but also on the fact that they sometimes exit a certain cell type from only one side (called 'polarized' assembly, budding and release). After replication in susceptible tissues viruses finally target organs from where they can spread to other hosts. Typical late target tissues include the cells lining the airways and salivary glands.

All viral receptors have a very specific role in the normal functioning of the cell: they bind either a substance in the body or another receptor of another cell. Binding of their natural partner ('ligand') leads to all kinds of signaling towards the interior of the cell, with outcomes depending on the type of receptor, for instance an enhanced or decreased production of a certain protein or hormone.

It is actually quite logical that viruses not just bind some random receptor, but that they have often evolved to recognizing receptors that have beneficial effects for their own survival. One can imagine for instance: receptors that ensure that the virus gets taken up by the cell ('endocytosis'); receptors that redistribute other viral receptors towards the virus particle; receptors that suppress the immune response; or receptors that signal to loosen up the tight contacts between cells, so that more membrane space becomes available for entering and leaving ('budding') of virus particles. To summarize the above, viruses exploit the natural functioning and response mechanisms of the host cell (and organism) in numerous ways. Also paramyxoviruses have been shown to manipulate host cell anti-viral defense mechanisms in their own favor.

Understanding the dissemination route of a virus is only possible when it is known with what receptors, and hence what cell types, the viral attachment and fusion proteins interact to gain entry into the cell. In the last decades the discovery of receptors for measles virus and henipavirus has shed light

on their behavior in the body. However, for the important airway pathogens respiratory syncytial virus (RSV) and the related, recently discovered, human metapneumovirus (HMPV) that infect millions of infants world-wide it is still unclear what receptor interactions are crucial for infection.

6. ACKNOWLEDGEMENTS

I would like to thank: my thesis supervisor Dr. Frank Coenjaerts for his help on the manuscript and his patience during the long period I worked (and did not work) on this thesis; Dr. Maaike Ressing for reviewing the manuscript; Dr. Jos Koeckhoven for his counceling and advice; and last but not least Drs. Carlijn Mertens and Ing. Frans Mertens for all the support they have given me.

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