Protein-protein interactions in RSV assembly: potential targets for attenuating RSV strains

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ABSTRACT

Respiratory syncytial virus (RSV) is the major respiratory pathogen of infants and children worldwide, with no effective treatment or vaccine available. Steady progress has been made in understanding the respiratory syncytial virus lifecycle and the consequences of infection, but some areas of RSV still remain poorly understood. Although many of the interactions between virus proteins that are required for efficient RSV assembly have been elucidated, many questions still remain regarding viral assembly, as well as the mechanisms of RSV budding. This review will summarise the current understanding of RSV assembly, including the various interactions between virus proteins and the involvement of cellular factors with a view to identifying possible attenuation and/or drug targets within the assembly pathway.

1. Overview

Human respiratory syncytial virus (RSV) belongs to the genus *Pneumovirinae* within the *Paramyxoviridae* family, order *Mononegavirales* [1]. Other members of this genus include bovine and caprine RSV strains, the pneumonia virus of mice and avian pneumovirus. The *Paramyxoviridae* family also includes the subfamily *Paramyxovirinae*, which includes measles, mumps and human parainfluenza viruses, and genus Respirovirus, which includes Sendai virus [1].

Like all negative-strand RNA viruses, the RSV polymerase proteins are packaged in the virion, allowing transcription and replication from the virus genome to proceed in the cytoplasm of the infected cell shortly after infection; RSV assembly occurs completely in the cytoplasm of the infected cell [1], although one of the viral components, the RSV Matrix (M) protein, is able to translocate to the host cell nucleus early in infection, presumably to modulate host transcription [2]. RSV infection of host cells is initiated when the large glycoprotein G attaches to glycosaminoglycans in cultured cells [3-5]; the receptor used in human infection in the field is still unknown. After attachment, the viral F (fusion) protein triggers fusion of cell and virus membranes, releasing the nucleocapsid into the cytoplasm where genomic RNA is transcribed and translated to give rise to individual viral proteins and replicated to form progeny genomes. The nascent genome is immediately associated with N (nucleocapsid), P (phosphoprotein) and L (large polymerase) proteins to give rise to the active ribonucleoprotein complex (vRNP) which is the site of all transcription and replication; the M2-1 protein also associates with the vRNP to assure complete transcription of genes [6, 7] (see Figure 1 for genome organisation and overview of RSV lifecycle in infected cells). The envelope glycoproteins (G, F and probably the SH (small hydrophobic)) associate with each other to form the envelope glycoprotein complex [8]. The M (matrix) protein is associated with both the nucleocapsid and envelope complexes [9, 10]. The virus particle is formed in lipid raft domains at the apical surface of the cell, where the coming together of the two complexes triggers virus budding [11-13].

2. Nucleocapsid complex

RSV infected cells have characteristic cytoplasmic inclusions that contain virion proteins N, P, L and M2-1, and dependent on the time of infection, M protein [6, 14]. These

inclusions also contain the virus genomic RNA and are probably the site of active viral transcription and replication. This is supported by data showing that purified inclusions are capable of transcription from endogenous RNA *in vitro* [14, 15]. Inclusions can be observed in infected cells from very early in infection (12h post infection [p.i.]). Later in infection (20h p.i. and later) M protein is associated with the inclusions, which then show lower transcriptase activity. Transcriptase activity can be restored in a dose dependent fashion by selectively dissociating the M protein from the complex, using antibodies [14].

a. Formation of cytoplasmic inclusions

N and P proteins are necessary and sufficient for the formation of cytoplasmic inclusions; as when transiently co-expressed in mammalian cells, N and P form inclusions reminiscent of RSV infected cells [6]. These inclusions also contain RNA of both polarities showing that N, and/or P, has inherent RNA binding properties. Numerous studies have shown that N binds to RNA non-specifically [1]. N protein alone can form “nucleocapsid-like” structures even when expressed in bacteria which non-specifically incorporate bacterial RNA [16]. In RSV infected cells, P protein binds to the nascent N protein giving it the required specificity for viral RNA and placing it on the viral RNA-L complex (Figure 2, step 1,2,3). The P protein is also a polymerase cofactor, stabilizing the L protein. The domains of N that are required for N-P interaction are less clear, with seemingly large tracts throughout the protein being required [17]. The C-terminus of P is required for interaction with N [17]. Yeast two-hybrid analysis shows that the C-terminus of P is important in forming heterodimers with N [18], with changes in two residues in this region of P (E176 and G172) resulting in a temperature-sensitive phenotype due to reduced interaction with N [19]. Phosphorylation of P has been shown to be dispensable for most of its assembly functions, e.g., P-P, P-N and P-M2-1 interactions in cell-free and cell culture systems [20, 21]. Phosphorylation of P may be important, however, for its functions in transcriptase activity and in the stabilization of L protein [22, 23]. P forms oligomers in cell free systems, and in cultured cells as demonstrated in pull down experiments, but whether this is an artefact of overexpression or essential for assembly is not known [21, 24]. The M2-1 protein probably associates with the nucleocapsid via its interactions with P and/or viral RNA [25] (Figure 2, step 4). The same region of M2-1 binds both P and RNA in cell free systems, but it is not clear which interaction dominates in infected cells. M2-1-P interaction is required for M2-1 association with the nucleocapsids. Although N, P and L are sufficient to mediate viral replication; RSV transcription also requires M2-1 [26]. M2-1 oligomerises to form tetramers, which appears to be important for its functions in transcription; interestingly, oligomerisation has no effect

on its interaction with P or RNA [25]. In another study, M2-1-P interaction was shown to be important for optimal polymerase function [27]. At later times in infection, M associates with nucleocapsids [14] and inhibits RSV transcriptase activity (Figure 2, step 5,6). By analogy to other related viruses in *Mononegavirales*, M association may also result in a tightly coiled nucleocapsid in preparation for packaging with the envelope glycoprotein complex [28]. This inference is based on studies showing that removal of M from virions results in collapse of internal structure and a very loose, extended nucleocapsid [29]; and by a study showing that N protein, which is accessible to antibodies in nucleocapsids without M, becomes inaccessible when M is present [30]. Using cells cotransfected to express various combinations of N, P, L, M2-1 and M, it was shown that M only colocalised to cytoplasmic inclusions that also contained M2-1; M association was almost certainly via its interaction with M2-1 dependent on M's N terminus [9].

b. Role of host factors in nucleocapsid activity

Studies using in vitro reconstituted nucleocapsid, mini-genome systems or nucleocapsids purified from RSV-infected cells have shown that optimal transcriptional activity of the nucleocapsid also requires cellular proteins [31, 32]. Subsequent studies identified actin as one of the specific cellular factors required [31]; nucleocapsids purified from RSV infected cells always contain some actin [14]. The role of the actin-binding protein profilin in nucleocapsid activity is less clear; although it enhances transcriptase activity in cell-free systems [33], profilin knockdown in cell lines has no effect on RSV replication or transcriptase activity [34]. The possibility that profilin's role can be performed by other cellular factors has not been investigated.

c. Movement to site of virus budding

Assembly and budding of RSV takes place at the apical cell surface from lipid raft-enriched domains (Figure 2, step c, d) and all components (nucleocapsid, envelope complex) must move to the assembly site prior to budding. Nucleocapsids probably use the microfilament network to reach RSV assembly sites (Figure 2, step 6). Although a complex containing N, P, M2-1 and M as well as a complex of N, P and M2-1 can bind polymerised actin, only the M-containing complex is anchored onto the microfilament network [30]. In the same study, N and M were both shown to interact with soluble, monomeric actin. Electron microscopic studies have shown the presence of a network of microfilaments associated with cytoplasmic inclusions in RSV-infected cells and the presence of inclusions close to cell surface assembly sites as well as in the cytoplasm

[35], supporting the idea that nucleocapsids may utilise the microfilament network to reach sites of assembly at the cell surface.

3. Envelope glycoprotein complex

RSV has three envelope glycoproteins; these are G, the attachment glycoprotein, F, the fusion protein and SH, the small hydrophobic protein of unknown function [1]. RSV G is a type II membrane protein and the major attachment protein of RSV [36]. Recent studies have shown that RSV lacking the G gene can replicate efficiently in cell culture, implying that RSV has an auxiliary attachment function, probably involving F [37-39]. Genetically engineered RSV lacking the entire G gene replicates efficiently in some cell lines but not in others, and is attenuated in BALB/c mice [39]. Thus, although G is not essential for replication in some cell lines, it is required for full infectivity and *in vivo* pathogenicity, and a largely intact G gene has been found to be present in all RSV field isolates analysed to date. Several groups have shown that deletion of the SH gene has minimal effects on the infectivity and replication of RSV [4, 40]; substitution of the SH gene with other heterologous genes in the context of an attenuating deletion is currently a favoured vaccine strategy [41]. F protein is a disulfide linked type I glycoprotein and essential for formation of infectious virus [4, 42].

a. Formation of the envelope glycoprotein complex

As observed in other paramyxoviruses, it is proposed that the coalescence of RSV components at plasma membrane budding sites is facilitated by interactions between the cytoplasmic domains of the envelope glycoproteins and M. This is supported by the observation that M associates with cellular membranes by itself, but that the nature of that association is modified in the presence of F [11]. Colocalisation studies have shown that the initial interaction of M with F and G occurs in the intracellular membranes (Figure 2, step a, b). M, G and F bind to membranes when expressed alone, but only F is sorted into detergent insoluble lipid raft domains. It appears F is key to the correct positioning of the envelope glycoprotein complex at the sites of virus budding. Coimmunoprecipitation studies have shown a complex containing F and G at the plasma membrane as well as one with G and SH; a complex containing all three proteins could not be isolated [43]. It is not clear whether the interaction between F and G is direct or via their association with M. Co-localisation studies in cells infected with a recombinant RSV lacking G suggest that F and M can interact directly; a direct interaction between M and the cytoplasmic domain of

G has also been shown [10]. The motif in F responsible for interacting with M is unknown; the M domains interacting with either F or G are also unknown.

i. G (attachment) glycoprotein

The G or large glycoprotein is the main attachment protein of RSV; virus lacking G is able to infect cells in culture but is highly attenuated *in vivo* (also see above). Although the cellular receptor for field isolates of RSV still unknown, in cell culture, G glycoprotein mediates attachment via binding to cell surface glycosaminoglycans [5]. G protein is translated as a 32 kDa protein which acquires N-linked and O-linked glycosylation as it passes through the cellular glycoprotein secretory pathway, growing to 85-90 kDa in size. G can be present as a membrane bound full length protein or truncated "soluble" form that is released extracellularly. The soluble form can arise by alternative initiation at an internal AUG or by cleavage immediately C-terminal to the membrane anchor by an as yet unidentified protease [44].

ii. F (fusion) protein

F plays a central role during virus infection by mediating fusion of the virus and host cell membranes, and in cell-to-cell spread of the virus, via fusion of neighbouring plasma membranes, forming the large epithelial cell syncytia seen in cell culture as well as in the lungs of infants dying of RSV infection, the property for which this virus is named. The rapid destruction of ciliated epithelial cells lining the airways ultimately causes the symptoms characteristic of the infection. F protein is synthesised as an inactive precursor F0 (75 kDa), which undergoes proteolytic cleavage by furin in the trans-Golgi compartment, generating mature, active form of the protein consisting of two disulfide linked subunits, F1 (55 kDa) and F2 (20 kDa). The mature F1+F2 protein is inserted into the host cell membrane and interacts with M and G.

iii. SH (small hydrophobic) glycoprotein

SH is a type II transmembrane protein of 64 (or 65) amino acids. It is found in several forms in infected cells, SH_0, SH_g, SH_p and SH_t. SH_0 is the 7.5 kDa non-glycosylated form and is the most common form expressed. SH_g is the N-linked glycosylated form (15–19 kDa) which is a precursor to SH_p; the latter (21–40 kDa) is a polylactosaminoglycan modified form of the protein. SH_t is a truncated, 4.8 kDa protein generated by translation initiation at in internal AUG [45]. Although some studies have suggested a role for SH in viral fusion [8, 38, 46] probably via its "viroporin"-like function [47, 48], RSV lacking the SH gene is viable, able to cause syncytium formation, and grows at a similar rate to wild

type in cell culture; it is attenuated *in vivo*, however, perhaps due to impairment of the reported anti-apoptotic functions of SH [40, 49]. Interactions of SH with other virus proteins and the mechanism(s) of its incorporation into the virion are not known.

b. Additional roles of M protein

As alluded to above, it should not be overlooked that additional to its key functions in assembly, M plays an important role in RSV infection in the nucleus. In particular, a nuclear targeting signal within M is able to be recognised by a specific member of the importin superfamily of cellular nuclear transporters – importin β1 [50] – which is believed to transport M into the nucleus early in infection [2, 50]. The role of nuclear M appears to be dual; to inhibit host cell transcription, and at the same time, to sequester M from RSV transcription occurring in the cytoplasm [14]; inactivation by mutation of the nuclear import signal in the context of RSV itself reduces virus production markedly [51], underlying the importance of RSV nuclear import to viral infection. Later in infection M traffics back to the cytoplasm to initiate/participate in virion assembly. Export from the nucleus is through the action of a distinct importin superfamily member – the exportin CRM1 – which recognises a specific nuclear export signal within M [51]. Inactivation by mutation of M's nuclear export signal in the context of RSV results in a non-viable RSV, while inhibition of nuclear export using the specific CRM1 inhibitor leptomycin B reduces RSV virus production over 20-fold [51], emphasising the critical importance of M nuclear export to the RSV life cycle. The switch between nuclear import and export of M, critical to its role in both compartments, is presumed to be through post-translational modulation, possibly phosphorylation [52]. Clearly, trafficking of M between the nuclear and cytoplasmic compartments is a key aspect of its multiple functions in infected cells/viral infection.

4. Movement to the plasma membrane

F and G utilise the cellular glycoprotein maturation pathway and can be seen associated with ER and Golgi. Inhibition of the glycoprotein secretory pathway with Brefeldin A results in retention of F and G in the ER and loss of cell surface viral filaments; in contrast, the localisation of M is not affected by Brefeldin A. There is no information on the movement of SH protein through the cell. F protein interacts with the small GTPase RhoA via aa 146-155 within the F fusion peptide and aa 67-100 in RhoA. Interestingly, upregulation of RhoA expression appears to facilitate RSV-induced syncytium formation [53]. Given the functions of RhoA in formation of actin stress fibers, its interaction with F

may have implications for the movement of the envelope glycoprotein complex to the plasma membrane. Many viruses use the cellular cytoskeleton to move their components through the cell to areas of virus assembly and budding. Sucrose gradient flotation assays in the presence of detergents show that M protein is associated with the actin cytoskeleton. An important role for actin in RSV assembly (see also above) is also suggested by the fact that actin expression is induced in RSV infection, that purified RSV virions contain actin, and that treatment with chemical inhibitors of actin polymerisation results in decreased virus production [11, 12, 30, 31]. Thus, actin may be involved in RSV budding as well facilitating movement of nucleocapsid and envelope glycoproteins to the cell surface.

5. Association with the host cell membrane

Each of the envelope glycoproteins as well as M can associate with cell membranes when expressed alone [11]. In RSV-infected cells, M protein colocalises with G and F at the cell surface and the virus filaments that protrude out of the cell; all three proteins can be found in the detergent insoluble lipid raft fraction of the membrane, along with nucleocapsids [12] (Figure 2, step d). The minimal requirements for formation of helper-dependent RSV infectious particles are the nucleocapsid, M and F [42] implying that these three components need to come together at the cell surface prior to budding. Intracellular retention of F by inhibition of the glycoprotein secretory pathway results in M being present in the detergent soluble, non-lipid raft fraction of the membrane, suggesting that F protein determines the localisation of the envelope glycoprotein complex at the budding site [11].

6. Formation of the budding virion

a. Role of host cell factors

RSV buds from the lipid raft domains of the plasma membrane [11, 12, 54]. Analyses of the membranes from RSV-infected cells shows that all components of the nucleocapsid (N, P, L, M, M2-1) and envelope glycoprotein (M, F, G) complexes are present in the detergent insoluble lipid raft fraction [12, 13, 54]. As has been discussed above, F is able to associate with lipid rafts when expressed alone. These observations lend themselves to a model wherein the envelope glycoprotein complex is directed to the lipid rafts by F;

the nucleocapsid complex, now associated with M moves to the cell surface by using the actin network and is pulled into the lipid rafts by largely ionic interactions of M. Chemical inhibition of phosphoinositide-3-kinase (PI3K) pathways interferes with virus filament formation with no effect on virus replication, further supporting a role for lipid rafts as PI3K signalling pathways are associated with lipid rafts [55, 56]. The RhoA-actin pathway is also implicated in virus budding as the small GTPase and actin regulator RhoA is found close to the "neck" of the virus filaments as shown in electron micrographs [35] and inhibition of the F-RhoA interaction results in decreased virus production. Profilin has also been implicated in RSV budding as there is decrease in progeny virus when profilin is knocked down using siRNA [34]. Interestingly, profilin and RhoA are both implicated in the formation of stress fibres that are characteristic of RSV infection. RSV assembly and budding take place at the apical membrane in polarized cells and the proteins associated with apical recycling endosome (ARE, distinguished by the presence of the small GTPase Rab11a) appear to have a role in RSV budding [57]. A number of Rab11-interacting proteins have been identified including myosin Vb (MVb) and the Rab11 family interacting proteins (Rab11-FIPs). Expression of a dominant negative form of either MVb or Rab-FIP2 in polarized cells blocks RSV budding, causing retention of assembled viruses on infected cells as observed by decreased length of virus filaments by electron microscopy. Neither the ESCRT (endosomal sorting complex required for transport) nor ubiquitin machinery, both of which are used by many enveloped viruses, appear to play a role in RSV budding [57].

b. Role of viral proteins

F is emerging as the main driver of RSV budding [13, 43, 54], just as M appears to play the key role in bringing together the various other virus proteins for assembly [9, 10, 14]. Given its role in related viruses, M may also have an important role in virus budding by inducing the curvature of the cell membrane which is essential to the initiation of the bud [28, 58]; when expressed alone, M induces filaments on the cell surface similar to the filaments observed in RSV-infected cells (Ghildyal, Teng, unpublished data) and M can be detected in the culture supernatant (Teng, Tran, unpublished data). F interaction with the actin polymerisation pathway via RhoA is important for the formation of the long virus filaments; but whether it directs formation or stabilises the filaments is not clear. M and F probably work together to direct budding from the lipid raft domains of the plasma membrane. The mechanisms underlying fission of the buds to release the virus are still unknown but may be facilitated by M-F/G interaction and SH in a similar manner to that described for the release of influenza virus [59].

7. RSV assembly as a target for attenuation

The development of vaccine candidates deficient in assembly has thus far focussed on the envelope glycoproteins, with a recombinant RSV lacking G one of the first candidates [37]. Cold-passaged, G-less RSV was viable, able to spread from cell to cell in culture, and found to be poorly infectious in humans, but overly attenuated and hence not viable as a vaccine candidate [37]. F has been a focus for development of anti-RSV therapeutics and is discussed in detail in another article in this issue (Costello et al). Thus far, there are no vaccine or drug candidates that target RSV assembly. Recent advances in our understanding of the functions of M and the availability of its structure as a monomer [60] should lend to the production of vaccine candidates that are attenuated due to mutations in M. As M is not the major antigenic determinant of RSV, such a virus should induce the required immune response. The cellular factors important for RSV assembly (as described above) are also viable therapeutic targets as are the interaction of RSV proteins with these factors. Cell culture studies have shown that targeting the cellular factors required for RSV assembly such as RhoA and profilin, can significantly reduce RSV titre. Similarly, a small molecule that could inhibit F-RhoA interaction should result in reduced virus budding as has been shown in cell culture [35].

In summary, improved understanding of the molecular interactions between RSV proteins, and between RSV and host cell factors during RSV assembly is central to the generation of better attenuated vaccine candidates. Continued basic and applied research on RSV assembly remains the best way forward to tackle RSV infection, which still accounts for more deaths each year than influenza [61], and hence is a major problem world-wide for human health.

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FIGURE LEGENDS

Figure 1 - Genome organisation and infectious cycle of human RSV.

A. The sequence of genes from 3' to 5' end is shown. The genome is flanked by a 3' Leader (Le) sequence and a 5' Trailer (Tr) sequence. Each gene is flanked by conserved gene start and gene end sequences (N gene end and P gene start are shown) separated by a variable intergenic sequence (IGS). NS – nonstructural, N – nucleocapsid, P – phosphoprotein, M – matrix, SH – small hydrophobic, G – glycoprotein, F – fusion, L – large polymerase.

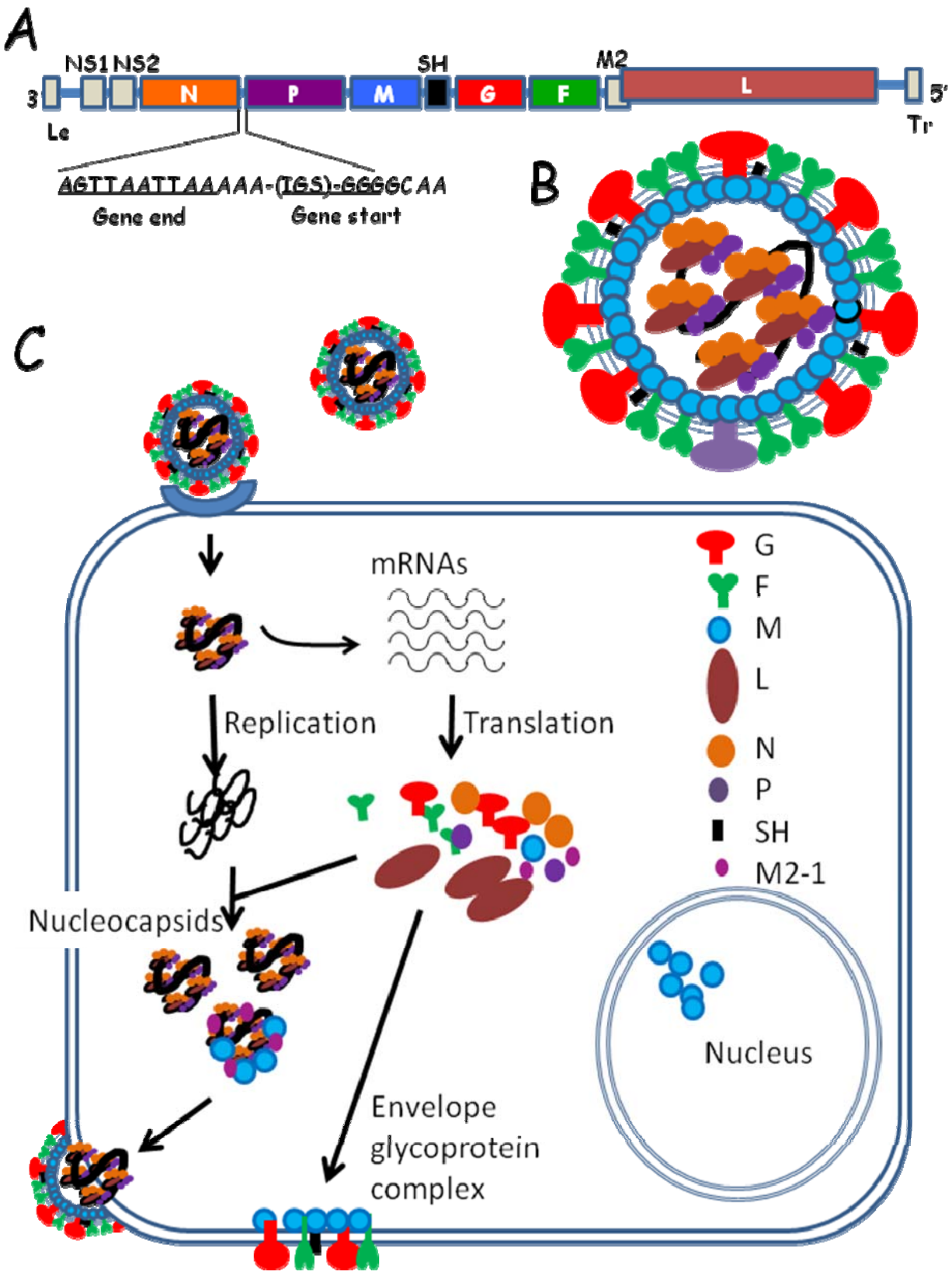
B. Schematic diagram of the virion showing the genome and component structural proteins.

C. RSV lifecycle in infected cells. RSV infects the target cell via the attachment of G protein to its receptor, followed by fusion of the virus and cellular membranes and release of the negative genome into the cytoplasm. The genome is transcribed into individual mRNAs that are translated into vial proteins. Early in infection, M protein is localised to the nucleus. The genome is replicated into new genomes that associate with N, P, L and M2-1 proteins to form nucleocapsids; M protein associates with nucleocapsids late in infection. Concurrently, envelope glycoproteins mature through the ER-golgi secretory pathway, associate with M protein and form envelope glycoprotein complexes at the cell membrane. The glycoprotein complex and nucleocapsids come together and the virus buds out of the cell. The key to the various structures is indicated on the right in C and refers to diagrams in both Figures 1 and 2.

Figure 2 – Schematic model for the sequential events in RSV assembly.

The sequence of events in the formation of the nucleocapsid complex (step 1 to 6) and the envelope glycoprotein complex (step a-c) are shown, along with the final assembly of the virion at the cell membrane (steps 7, d). P associates with N (2) and directs it to the L-RNA complex (3). M2-1 is recruited to the complex via its interactions with P (4). M associates with the nucleocapsid complex only in the presence of M2-1 (5), inhibits transcriptase activity and, probably induces tighter coiling of the genomic RNA (6) to facilitate packaging. M associates with F (a) and with G (b) in intracellular membranes. M, F, G come together at lipid rafts (facilitated by F) (c). The nucleocapsid and the envelope glycoprotein complexes move to the cell surface, maybe facilitated by microfilaments, where they associate via M-M interactions (7, d) and the new virion buds out from lipid rafts.

Figure 1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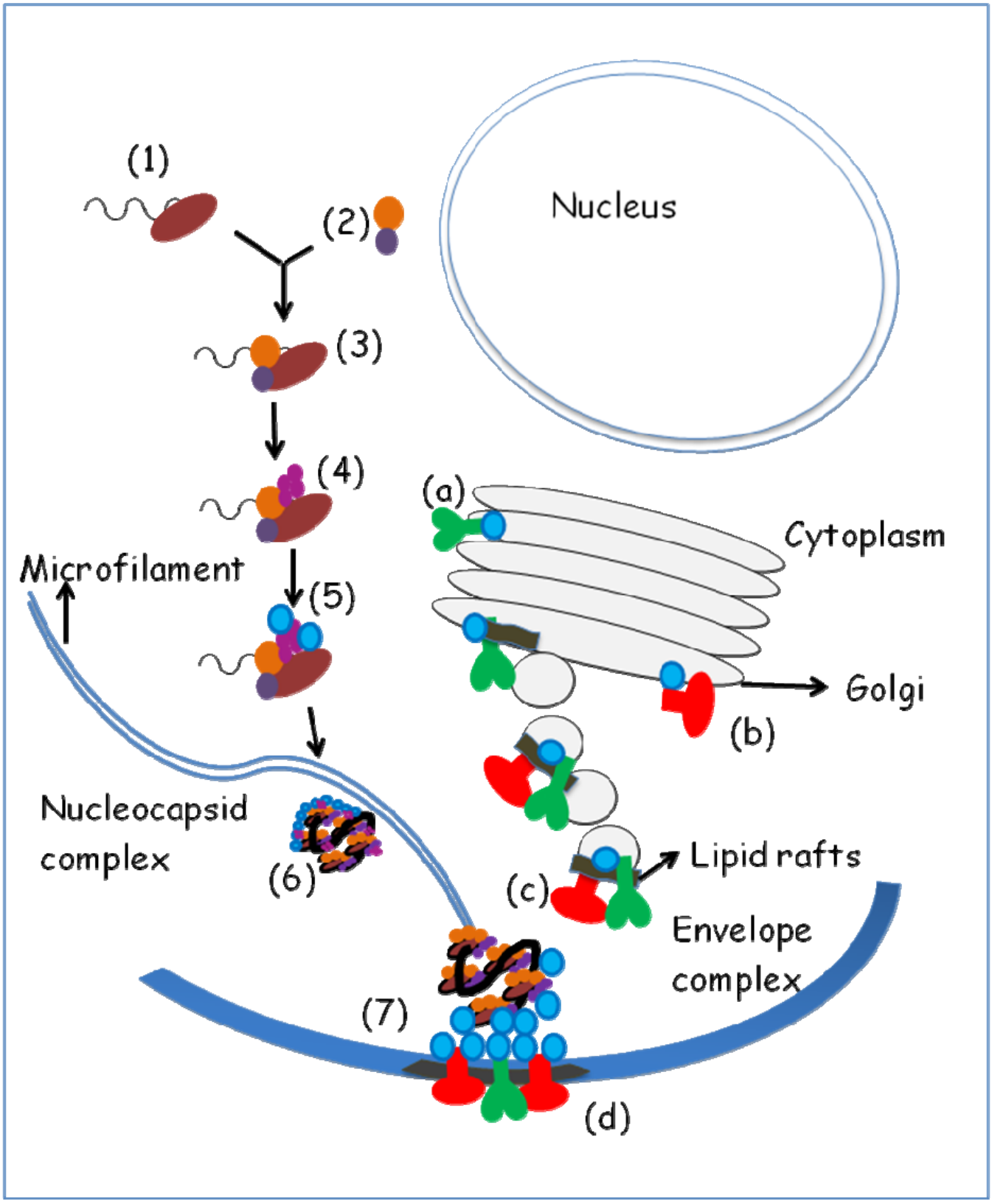
SH
M2-1

Figure 2


(1)
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Cytoplasm
Golgi
(b)
(c)
Lipid rafts
Envelope
complex
(d)
Microfilament
Nucleocapsid
complex