Published in final edited form as:
Future Microbiol. 2013 January ; 8: 123–131. doi:10.2217/fmb.12.132.

Molecular mechanisms driving respiratory syncytial virus assembly

Fyza Y Shaikh[1,2,3] and **James E Crowe Jr**[*,1,2,3]

[1]Department of Pathology, Microbiology & Immunology, Vanderbilt University Medical Center, Nashville, TN 37232, USA

[2]Department of Pediatrics, Vanderbilt University Medical Center, Nashville, TN 37232, USA

[3]The Vanderbilt Vaccine Center; Vanderbilt University Medical Center, Nashville, TN 37232, USA

Abstract

Respiratory syncytial virus is a single-stranded RNA virus in the *Paramyxoviridae* family that preferentially assembles and buds from the apical surface of polarized epithelial cells, forming filamentous structures that contain both viral proteins and the genomic RNA. Recent studies have described both viral and host factors that are involved in ribonucleoprotein assembly and trafficking of viral proteins to the cell surface. At the cell surface, viral proteins assemble into filaments that probably require interactions between viral proteins, host proteins and the cell membrane. Finally, a membrane scission event must occur to release the free virion. This article will review the recent literature describing the mechanisms that drive respiratory syncytial virus assembly and budding.

Keywords

fusion proteins; paramyxovirus; respiratory syncytial viruses; virus assembly; virus filaments

Respiratory syncytial virus

Respiratory syncytial virus (RSV) is a leading worldwide cause of serious viral lower respiratory tract illness in infants and the elderly [1,2]. RSV is estimated to cause 30 million lower respiratory tract infections each year worldwide, resulting in more than 3 million hospitalizations [3]. Although mortality is less than 1% among admitted infants, infants with chronic lung disease, congenital heart disease or marked prematurity are at a higher risk of increased length of hospitalization and increased mortality [4,5]. RSV is also an important cause of lower respiratory tract infections in adults with cardiopulmonary disease, severe combined immunodeficiency or following transplantation [3,6,7].

RSV is a member of the *Paramyxoviridae* family, subfamily *Pneumovirinae,* with a single-stranded, negative-sense RNA genome encoding 11 proteins. RSV infects the apical surface of polarized lung epithelial cells [8,9]. Infection begins with attachment of the virion to the cell membrane, a process that is mediated by the fusion (F) protein and glycoprotein (G). Low-affinity interaction with cellular glycosaminoglycans, particularly heparin sulfate

[*]Author for correspondence: james.crowe@vanderbilt.edu.

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.
No writing assistance was utilized in the production of this manuscript.

[10,11], are thought to stabilize the virion on the cell surface so that F protein can mediate fusion. The F protein is sufficient for fusion *in vitro*, although cellular proteins also play a role [12–14]. Recently, nucleolin has been identified as a functional receptor for RSV through interaction with the F protein [15]. However, the exact mechanism by which these multiple interactions mediate RSV attachment and fusion is still unknown. Following fusion, the viral genome and vRNA-dependent RNA polymerase, consisting of nucleoprotein (N), phosphoprotein (P), large (L) polymerase and matrix protein 2 open reading frame 1 (M2-1), are released into the cytoplasm. The viral polymerase begins making viral transcripts for each viral protein, which is followed by translation on host ribosomal machinery [7]. These viral proteins accumulate in the cytoplasm in discrete aggregates that could be properly termed cytoplasmic inclusions, but also have been referred to in some paramyxovirus studies as inclusion bodies, a term more conventionally used in bacterial studies [16]. These viral cytoplasmic inclusions contain RSV matrix (M), N, P, L polymerase, M2-1, matrix protein 2 open reading frame 2 (M2-2) and the vRNA [16–20]. Upon accumulation of viral proteins, the viral polymerase switches from transcription to viral replication, a process thought to be controlled by M2-2 [5]. It is suspected that the ribonucleoprotein (RNP) complexes form in the inclusions and then traffic to the apical membrane, where they meet with F, G and the short hydrophobic protein that arrive from the Golgi apparatus through the secretory pathway [9,21]. RSV also encodes two nonstructural proteins, NS1 and NS2, which inhibit interferon induction, interferon signaling and apoptosis [7].

RSV proteins and vRNA assemble into virus filaments at the apical cell surface [22], but the mechanism of assembly is still poorly understood. These filaments are thought to contribute to cell–cell spread of the virus and are morphologically similar to the filamentous form of virions seen in electron microscopy studies of viruses produced in polarized cells [19]. Live imaging has shown these structures to be dynamic, with rotational and directional movement [23,24]. Membrane scission is thought to occur in a Vps4-independent manner [19], resulting in pleomorphic particles ranging from 150 to 250 nm in diameter for spherical forms and up to 10 μm in length for filamentous forms [3,25].

Currently, therapy is limited to supportive care. Passively administered antibody (palivizumab; Synagis®) is effective for prophylaxis, but is not therapeutic during infection [4]. Owing to the high morbidity and mortality associated with RSV infection and the lack of available therapies, developing a RSV vaccine is of high priority. However, RSV presents a unique challenge for vaccine development owing to numerous obstacles, including age of first infection, immune evasion and the legacy of a failed clinical trial using a formalin-inactivated whole-virus vaccine [5]. Immunization of children with the formalin-inactivated RSV vaccine candidate caused exacerbated disease, with increased rates of hospitalization and mortality during subsequent naturally acquired infection [3,7,26,27]. Current vaccine development efforts for pediatric populations are largely focused on the identification of appropriate live-attenuated strains, produced by either serial passage in low temperatures, chemical mutagenesis or generated using recombinant virus systems [7,10,11]. Live-attenuated vaccines, however, often have limited immunogenicity in older subjects owing to partial immunity following multiple previous infections with RSV [28]. Vaccine strategies in these populations include subunit vaccines, virus-like particle (VLP) vaccines and replication-competent or defective gene-base vectors [3,7]. While many empiric vaccine strategies are being explored, further insights into the biology of the virus and its interaction with the host may contribute to vaccine development or targeted therapeutics.

RNP assembly & protein trafficking

To produce progeny virions, RSV must coordinate assembly between proteins translated in the cytoplasm, glycoproteins that have trafficked to the cell surface through the secretory

pathway and the newly synthesized genomic RNA. RNP assembly is thought to occur within the viral cytoplasmic inclusions, since the viral polymerase proteins and genomic RNA are found to colocalize at these structures [16]. However, it is unknown how the RNP complex traffics to the cell surface to meet with the other viral proteins at the cell surface. Cytoplasmic inclusions have been described to originate in close proximity to filaments forming at the cell surface in some cases [17,29], and actin has been implicated in the connection between cytoplasmic inclusions and the cell surface, potentially providing a means for transport of viral proteins to the cell surface [18,29]. It is logical to think that a physical relationship between cytoplasmic inclusions and filaments is critical for viral protein trafficking.

The M protein has also been implicated in viral protein trafficking. When the M protein is not present during infection, N and P proteins accumulate in viral cytoplasmic inclusions and viral filaments do not form, suggesting that M may be responsible for trafficking the RNP complex to the cell surface [30]. This hypothesis is consistent with the observation of M protein colocalization with N protein in cytoplasmic inclusions and coprecipitation with RNP complexes [31,32]. The RSV M protein associates with the plasma membrane [22,33] and specifically sorts into detergent-resistant membranes (DRMs) when the F protein is present [34]. According to the crystal structure, the M protein may interact with phospholipids in the host membrane using a large positively charged surface that spans its N and C terminal domains [35]. Interestingly, specific residues in the cytoplasmic domain of G affect M colocalization with G [36]. However, G is not required for the generation of progeny virus, and M can localize with F proteins in the absence of G [34]. Therefore, it seems probable that M proteins associate with F and G proteins independently [37]. Furthermore, F and G proteins form a complex at the cell surface that is detectable by immunoprecipitation [38], which probably enhances virion assembly.

In addition, the apical recycling endosome (ARE) is implicated in RSV protein trafficking and membrane scission. The ARE is a network of endosomes in polarized epithelial cells that help maintain the composition of apical and basolateral membranes [39]. Rab proteins, which are small Ras-related GTPases, help regulate this network, and motor proteins and adapters play a role in trafficking and cargo specificity, respectively [40]. Myosin Vb is an actin motor protein that binds Rab11 and Rab11 family interaction proteins (FIPs) that confer cargo specificity for Rab11 endosomes [40]. Dominant-negative inhibitors of myosin Vb or Rab11–FIP1 disrupt viral filament formation and result in diminished infectious viral progeny [9]. By contrast, disruption of Rab11–FIP2 function causes an increase in cell-associated virus and longer viral filaments on the surface of infected cells, suggesting a defect in membrane scission to produce free virions [19]. Although these studies show that ARE proteins are involved in RSV assembly and budding, a direct interaction between ARE and viral proteins has not been demonstrated. Therefore, the mechanism by which the ARE functions in RSV assembly and budding remains undetermined.

A number of heat shock proteins are also associated with viral structures. HSP90 is associated with viral filaments and cytoplasmic inclusions, as shown by immunofluorescence. Inhibition of HSP90 leads to loss of viral filament formation and reduced viral spread *in vitro* [41]. Furthermore, HSP70 and HSC70 colocalize with RSV cytoplasmic inclusions, and mass spectrometry studies suggest they are physically associated with budded particles [41]. However, the mechanisms by which these proteins affect viral assembly are currently unknown.

Cytoplasmic tail of the F protein of RSV & other paramyxoviruses

The RSV F protein is a trimeric type I integral membrane protein that traffics though the cellular secretory pathway. It is responsible for viral fusion with the host cell membrane, as well as the characteristic syncytia produced in RSV-infected cultured cell lines. The F protein has an endoplasmic reticulum signal sequence, a large ectodomain that mediates fusion, a 23-amino acid transmembrane domain and 24-amino acid cytoplasmic tail (CT). Unlike other paramyxovirus F proteins, RSV F protein does not require other viral proteins to fuse membranes [7]. Although the fusion function of the F protein has been well characterized, the F protein also plays a role in viral assembly. The F protein traffics to the apical surface in the absence of other viral proteins or vRNA. The CT is completely dispensable for apical trafficking [42]. Deletion of the CT leads to a 100–1000-fold decrease in viral titer in a multicycle growth assay and results in a virus that is incapable of assembling into filaments [43]. Deletion of the CT, however, does not affect the capacity of the F protein to mediate cell–cell fusion, suggesting that the CT is critical for assembly into filaments [43,44]. In fact, filament formation and incorporation of internal virion proteins into VLPs appears to depend on a single phenylalanine residue in the F protein CT [18].

Coordination of RSV assembly by the F protein CT is consistent with many other studies citing paramyxovirus glycoprotein CTs as crucial to both assembly and budding [45]. For RSV and human metapneumovirus, both members of the *Pneumovirinae* subfamily, G is dispensable for viral replication *in vitro* [46,47]. Although the F protein CT may be sufficient for generation of progeny virions, optimal incorporation of viral proteins and RNA may require both F and G proteins, since residues in the RSV G CT are thought to be important for interactions with M proteins [36]. However, for the *Paramyxoviridae* subfamily, the role of glycoprotein CTs varies with each virus [45]. The F protein CT is necessary for Sendai virus assembly. Newcastle disease virus F protein and HN glycoproteins interact with different internal viral proteins (M and N, respectively). By contrast, the F protein and HN CTs of parainfluenza virus (PIV) 5 serve somewhat redundant functions. The function of measles virus F protein, and its interaction with M proteins, is complex. Mutations in the measles F protein tail reduce assembly and budding, but may increase fusion competence [48]. Modulation of the interaction of the measles H protein with M proteins also affects the growth of measles virus, but does so in a cell-type dependent manner [49]. The lack of a common theme for the role of CT domains in paramyxoviruses may simply indicate that the specific mechanisms of viral assembly remain undetermined, and further investigation into the role of the RSV F protein CTs may contribute to general knowledge regarding glycoprotein CT-mediated viral assembly for paramyxoviruses.

Viral assembly at the plasma membrane

Localization of viral proteins to the cell surface, however, is not sufficient for viral assembly into filaments. In order for filaments to form, viral proteins must deform the host cell membrane outward to initiate bud formation and then elongate the bud through the incorporation of additional membrane to create long filaments. To form a free particle, a membrane scission event must occur to release the viral particle from the cell membrane. All of these processes are energy intensive and require coordination of surface proteins and nucleocapsids containing RNA and viral proteins [50]. Previous work in the field has shown F, M, N and P proteins to be the minimum requirements for passage of a minigenome construct, indicating that these four proteins are the minimum requirements for packing vRNA into cell-free particles and for formation of filaments [18,51]. Many other paramyxoviruses also require both internal and membrane proteins for budding. For example, PIV5 and mumps both require M, nucleoprotein and F. However, other

paramyxoviruses require only the M protein for efficient VLP formation, such as PIV1, Sendai virus and measles virus [45]. For RSV, since both internal and surface proteins are required, assembly into filaments probably involves coordination of both surface and internal proteins in order to initiate bud formation and elongate filaments.

These data suggest a model for RSV assembly in which viral polymerase proteins produce nascent genomic RNA in cytoplasmic inclusions that may be spatially related to the cell surface and viral filaments by the actin cytoskeleton. The viral genome, N, P, L polymerase and M2 form the RNP complex, which can associate with M proteins. The M protein is also capable of associating with the plasma membrane, F protein and G at the cell surface, and in so doing, may be able to transport the RNP complex to that site. The specific role of the actin cytoskeleton in this process has not been well described. At the cell surface, the phenylalanine residue in the F protein CT is critical for filament formation, possibly through direct protein–protein interactions, protein–lipid interactions or for conformation of the F protein CT in a trimeric complex. Live imaging of vRNA or fluorescently tagged viral and cellular proteins may help elucidate the real-time dynamics of viral protein trafficking to the cell surface, while biochemical studies may help determine potential interactions.

Virus budding & membrane scission

The final step in viral assembly and budding involves a membrane scission event to sever the assembled viral particle from the host cell membrane. Many viruses accomplish this task by using host machinery for multivesicular body formation [52]. Multivesicular body formation depends on endosomal sorting complex required for transport (ESCRT) proteins. These proteins utilize a common ATPase, Vps4, to perform membrane scission during vesicle formation. Using dominant-negative inhibitors of Vps4, the pathway can be disrupted, resulting in vesicles that remain tethered to the original membrane rather than separating [52]. The use of ESCRT proteins in viral budding has been well characterized for many viruses, including HIV and other paramyxoviruses [45,50]. However, RSV budding is unaffected by inhibition of Vps4, suggesting that RSV uses a novel mechanism to accomplish membrane scission [19]. Furthermore, there is evidence that budding for a number of viruses is ESCRT independent. Although the mechanism for ESCRT independence is unknown for many viruses, influenza appears to use a viral protein-mediated mechanism to accomplish membrane scission through the use of an amphipathic helix in the CT of the M2 protein [53]. While it is probable that host proteins play some role in viral budding, viral proteins may be sufficient to perform membrane scission to produce free virions. Exploration of other mechanisms of cellular exocytosis may yield some insight into viral mechanisms of egress in the future.

RSV interactions with the host membrane

The formation of RSV particles at the cell surface also requires viral interactions with the host cell lipid membrane. The plasma membrane contains regions where lipid and protein composition differ from the rest of the plasma membrane. These microdomains can serve as platforms for cell signaling, protein trafficking and other cellular functions. Lipid rafts, a type of lipid microdomain, are cholesterol- and sphingolipid-enriched regions that often function to concentrate proteins within defined regions for a functional purpose [54]. Lipid rafts can function as assembly and budding platforms for viruses, including HIV-1, Ebola virus and influenza virus [45,55].

Many membrane microdomain studies focus on cholesterol dependence and detergent insolubility that results in DRMs, also termed lipid rafts. Cholesterol-depletion studies indicate that cholesterol is required for viral filament formation, but is dispensable for cytoplasmic inclusions [16,56]. Inhibiting the synthesis of raft-associated PIP_3 impairs the

formation of progeny virus [56]. Furthermore, RSV assembles into filaments at lipid microdomains that are rich in the lipid raft ganglioside GM1 [57], and ultrastructural analysis indicates that GM1 incorporates into viral filaments [58]. RSV proteins localize with lipid microdomain-specific dyes and raft-associated proteins, such as caveolin-1, CD44 and RhoA [59]. Specifically, the RSV F protein associates with DRMs in the absence of other viral proteins through an unidentified domain in the extracellular portion of the protein [60] and localizes with raft-associated CD55 and CD59 [57]. Disruption of RhoA signaling using a *Clostridium botulinum* exotoxin C3 results in relocalization of F protein from DRMs to nonlipid raft structures [59].

Internal virion proteins also localize to lipid microdomains. RSV M proteins associate with membranes by themselves; however, in the presence of the F protein, M protein has been shown to sort into DRMs using Triton™ X-100 insolubility assays [34] and flotation gradients [33]. Finally, the viral polymerase complex associates with lipid raft proteins, with M2-1 demonstrating a greater degree of partitioning into these lipid structures [61]. These studies indicate that viral proteins localize to specific lipid microdomains that may facilitate viral assembly. Additionally, the concentration of the various RSV proteins at specific membrane domains may induce a chain of reactions that affect association of all these components, as well as the curvature of the plasma membrane. The specific mechanisms by which lipid interactions aid viral assembly, however, have not been completely defined.

RSV & the host cytoskeleton

Manipulation of the plasma membrane probably also involves a variety of host proteins that function at the cell surface. Many cellular structures extending outward from the cell surface depend on actin polymerization (e.g., microvilli, filapodia, lamellipodia and membrane ruffles) [62]. Lipid microdomains have also been linked to the cortical actin network [63]. In fact, actin is involved in multiple aspects of the RSV life cycle, but its specific role in RSV assembly is not well understood. Gross disruption of the cytoskeleton using inhibitors of polymerization and depolymerization reduces viral replication [64,65]. Both actin and profilin, an actin modulatory protein, are required for optimal RSV replication without the need for actin polymerization [66–69]. Consistent with these results, β-actin and other actin-related proteins colocalize with RSV F protein at the cell surface and have been found in a variety of sucrose gradient-purified RSV preparations by immunoblot analysis and mass spectrometry [41,66,70]. Therefore, while the role of actin has been well characterized for RSV transcription, the function of actin in RSV assembly and budding remains unclear.

In contrast to β-actin incorporation into viral particles, filamentous actin is found at the base of viral filaments, consistent with its role as part of the cortical actin network [62]; however, it is absent from the filaments themselves [18,29]. In addition, cytoplasmic inclusions are often localized near the plasma membrane (near viral filaments), and actin is associated with viral cytoplasmic inclusions [29]. These data support the hypothesis that filamentous actin may serve a structural role in viral filament formation, but it does not form the structure of the filament itself. Other actin-associated proteins, however, colocalize with viral filaments. Filamin A and caveolin-1 associate with viral filaments by immunofluorescence and in budded particles by mass spectrometry [41,71]. Actin interacts with caveolin-1, a lipid raft protein, and filamin A interacts with caveolin-1, suggesting another link between actin-associated proteins, lipid rafts and RSV assembly [41]. However, other cytoskeletal proteins, such as ezrin, moesin and tubulin, are excluded from viral filaments [19,65]. Exclusion and inclusion of certain cellular proteins from viral filaments also suggests some type of specific sorting of viral and host proteins into filaments, a process that likely is highly regulated and probably involves interactions among viral proteins, host proteins and lipid microdomains.

Finally, disruption of actin-related proteins or actin-associated signaling pathways affects protein trafficking or filament formation, respectively. RSV infection results in activation of RhoA, a kinase involved in actin cytoskeletal rearrangement [72]. Inhibition of RhoA results in a reduced number of filaments that also exhibit a blunted shape with a shift to more spherical particle morphology, although the total number of progeny is unaffected [73]. Treatment of RSV-infected cells with a PI3K inhibitor or Rac GTPase, a downstream effector of PI3K, prevents formation of viral filaments, but does not affect virus protein expression or trafficking of viral glycoproteins to the cell surface [29]. These data suggest a role for actin signaling in viral assembly.

Future perspective

Although both viral and host determinants of RSV assembly and budding have been described, there are many questions that remain unanswered. First, direct protein interactions between viral glycoproteins and internal proteins need to be further characterized, as these interactions are probably critical for virus assembly. The M protein has been shown to interact with G using a plate assay [36] and has been implicated as an interacting partner of both F and G proteins [34]. However, it has been difficult to show direct protein interactions of viral glycoproteins with internal virion proteins. Previous work for RSV and other paramyxoviruses has been largely limited to VLP assays, colocalization studies by indirect immunofluorescence and association with lipid microdomains. It is probable that a significant limitation of showing a direct interaction by immunoprecipitation is due to a weak or transient interaction. In addition, these interactions may require the presence of viral protein complexes, viral protein oligomers or the host membrane. Development of the methods needed to determine the nature of G interactions with internal virion proteins would contribute significantly to our knowledge of the viral determinants of RSV assembly and may be applicable to other viral glycoproteins.

Although viral assembly into filaments may be driven largely by direct viral protein–protein interactions, it is not possible to exclude a role for host factors. Viral assembly and budding occurs at specific lipid microdomains, but most viral lipid studies have centered on detergent insolubility. To determine what role lipids play in RSV assembly, it may be necessary to determine what types of lipids are incorporated into filaments and virions. Furthermore, the protein interactions between viral and host proteins may need to be considered in the context of the membrane, especially since both F and G proteins have small cytoplasmic domains. Insight into the lipids that RSV selectively incorporates into virions may help determine the specific microdomains at which viral assembly occurs. Furthermore, as lipid microdomains continue to become better defined, the localization of viral proteins and viral assembly at specific locations in the plasma membrane may become better determined.

In addition, live imaging of virus-infected cells may help answer questions regarding the kinetics of virus assembly and budding. Live-cell imaging is often limited by poor resolution and photobleaching; however, recent advances have produced fluorescent proteins that can be imaged for longer periods of time with greater resolution [74]. By labeling vRNA or viral proteins, the kinetics of viral protein and genome trafficking to the plasma membrane can be determined [75]. At the cell surface, the kinetics of filamentous assembly and virion budding can also be assessed. Live-cell imaging may also lead to information regarding the mechanism by which RSV performs the final membrane scission step.

Currently, RSV budding is thought to be independent of host cell exocytic mechanisms involving ESCRT proteins. It is plausible that viral proteins are capable of mediating membrane scission, such as the mechanism described for the influenza M2 protein in which an amphipathic helix mediates membrane scission [45,53]. However, these helices have not

been identified in RSV proteins. Studies involving giant unilaminar vesicles may be able to decipher whether viral proteins are sufficient for budding [53,55]. Further study of host cell exocytosis mechanisms may help determine if RSV relies on host machinery to bud from infected cells. Moreover, these studies may be able to answer how the F or M proteins biophysically drive filament formation. Recent data suggest that the F and G proteins can induce initial membrane curvature [30], which may be due to interactions with lipids, other viral or host proteins, or the biophysical properties of the proteins [76]. Elongation of viral filaments may require protein–protein interactions, as well as oligomerization of viral proteins, as has been demonstrated for other viruses [45,77]. Furthermore, the effect of various lipid compositions could be tested in such a system. These studies may elucidate the biophysical mechanisms by which F protein and other viral proteins drive bud initiation, elongation and scission.

Finally, the cell substrates that are used for RSV studies could be improved. Many studies have been conducted with the HEp-2 epithelial cell line, which is contaminated with HeLa cells and adenovirus sequences, or other transformed cell lines. Polarized cell line studies probably better represent the morphology of cells in the airway, but these are also artificial. Studies in primary human airway cells in culture, tissue sections in culture or even live animal or human airway studies are desirable. However, the technical challenges to performing high-resolution live imaging and complex biochemical studies in such systems are currently formidable.

Acknowledgments

The authors would like to thank T Utley, M Lindquist, A Lifland and P Santangelo for helpful discussions.

Financial & competing interests disclosure

FY Shaikh was supported by the Vanderbilt Medical Scientist Training Program NIGMS/NIH T32 GM007347. Respiratory syncytial virus research in the Crowe laboratory has been supported by a grant from the March of Dimes (to JE Crowe), Burroughs Wellcome Fund Clinical Scientist Award in Translation Research (to JE Crowe) and NIH grant R01 GM 094198.

References

Papers of special note have been highlighted as:

- of interest

1. Falsey AR, Hennessey PA, Formica MA, Cox C, Walsh EE. Respiratory syncytial virus infection in elderly and high-risk adults. N. Engl. J. Med. 2005; 352(17):1749–1759. [PubMed: 15858184]
2. Hall CB, Weinberg GA, Iwane MK, et al. The burden of respiratory syncytial virus infection in young children. N. Engl. J. Med. 2009; 360(6):588–598. [PubMed: 19196675]
3. Graham BS. Biological challenges and technological opportunities for respiratory syncytial virus vaccine development. Immunol. Rev. 2011; 239(1):149–166. [PubMed: 21198670]
4. Krilov L. Respiratory syncytial virus: update on treatment and prevention. Expert Rev. Anti Infect. Ther. 2011; 9(1):27–32. [PubMed: 21171875]
5. Knipe, DM.; Howley, PM., editors. Fields Virology Fifth Edition. Lippincott Williams & Wilkins; PA, USA: 2007.
6. Falsey AR. Respiratory syncytial virus infection in adults. Semin. Respir. Crit. Care Med. 2007; 28(2):171–181. [PubMed: 17458771]
7. Collins PL, Melero JA. Progress in understanding and controlling respiratory syncytial virus: still crazy after all these years. Virus Res. 2011; 162(1–2):80–99. [PubMed: 21963675]

8. Zhang L, Peeples ME, Boucher RC, Collins PL, Pickles RJ. Respiratory syncytial virus infection of human airway epithelial cells is polarized, specific to ciliated cells, and without obvious cytopathology. J. Virol. 2002; 76(11):5654–5666. [PubMed: 11991994]
9. Brock SC, Goldenring JR, Crowe JE. Apical recycling systems regulate directional budding of respiratory syncytial virus from polarized epithelial cells. Proc. Natl Acad. Sci. USA. 2003; 100(25):15143–15148. [PubMed: 14630951]
10. Levine S, Klaiber-Franco R, Paradiso PR. Demonstration that glycoprotein G is the attachment protein of respiratory syncytial virus. J. Gen. Virol. 1987; 68(Pt 9):2521–2524. [PubMed: 3655746]
11. Feldman SA, Audet S, Beeler JA. The fusion glycoprotein of human respiratory syncytial virus facilitates virus attachment and infectivity via an interaction with cellular heparin sulfate. J. Virol. 2000; 74(14):6442–6447. [PubMed: 10864656]
12. Behera AK, Matsuse H, Kumar M, Kong X, Lockey RF, Mohapatra SS. Blocking intercellular adhesion molecule-1 on human epithelial cells decreases respiratory syncytial virus infection. Biochem. Biophys. Res. Commun. 2001; 280(1):188–195. [PubMed: 11162498]
13. Malhotra R, Ward M, Bright H, et al. Isolation and characterization of potential respiratory syncytial virus receptoRs) on epithelial cells. Microbes Infect. 2003; 5(2):123–133. [PubMed: 12650770]
14. Tripp RA, Jones LP, Haynes LM, Zheng H, Murphy PM, Anderson LJ. CX3C chemokine mimicry by respiratory syncytial virus G glycoprotein. Nat. Immunol. 2001; 2(8):732–738. [PubMed: 11477410]
15. Tayyari F, Marchant D, Moraes TJ, Duan W, Mastrangelo P, Hegele RG. Identification of nucleolin as a cellular receptor for human respiratory syncytial virus. Nat. Med. 2011; 17(9):1132–1135. [PubMed: 21841784]
16. Lindquist ME, Lifland AW, Utley TJ, Santangelo PJ, Crowe JE. Respiratory syncytial virus induces host RNA stress granules to facilitate viral replication. J. Virol. 2010; 84(23):12274–12284. [PubMed: 20844027]
17. Li D, Jans DA, Bardin PG, Meanger J, Mills J, Ghildyal R. Association of respiratory syncytial virus M protein with viral nucleocapsids is mediated by the M2-1 protein. J. Virol. 2008; 82(17): 8863–8870. [PubMed: 18579594]
18. Shaikh FY, Cox RG, Lifland AW, et al. A critical phenylalanine residue in the respiratory syncytial virus fusion protein cytoplasmic tail mediates assembly of internal viral proteins into viral filaments and particles. MBio. 2012; 3(1):e00270–e00311. [PubMed: 22318318]
19. Utley TJ, Ducharme NA, Varthakavi V, et al. Respiratory syncytial virus uses a Vps4-independent budding mechanism controlled by Rab11–FIP2. Proc. Natl Acad. Sci. USA. 2008; 105(29):10209–10214. [PubMed: 18621683] ■ Investigation into the role of the apical recycling endosome in virus budding and demonstration that respiratory syncytial virus (RSV) budding is ESCRT independent.
20. Brown G, Rixon HWM, Steel J, et al. Evidence for an association between heat shock protein 70 and the respiratory syncytial virus polymerase complex within lipid-raft membranes during virus infection. Virology. 2005; 338(1):69–80. [PubMed: 15936795]
21. Batonick M, Wertz GW. Requirements for human respiratory syncytial virus glycoproteins in assembly and egress from infected cells. Adv. Virol. 2011; 2011:343408. [PubMed: 21931576]
22. Roberts SR, Compans RW, Wertz GW. Respiratory syncytial virus matures at the apical surfaces of polarized epithelial cells. J. Virol. 1995; 69(4):2667–2673. [PubMed: 7884920]
23. Bächi T. Direct observation of the budding and fusion of an enveloped virus by video microscopy of viable cells. J. Cell Biol. 1988; 107(5):1689–1695. [PubMed: 3182934]
24. Santangelo PJ, Bao G. Dynamics of filamentous viral RNPs prior to egress. Nucleic Acids Res. 2007; 35(11):3602–3611. [PubMed: 17485480] ■ Live imaging of viral filaments using probes to RSV genomic RNA.
25. Bächi T, Howe C. Morphogenesis and ultrastructure of respiratory syncytial virus. J. Virol. 1973; 12(5):1173–1180. [PubMed: 4128827]

26. Chin J, Magoffin RL, Shearer LA, Schieble JH, Lennette EH. Field evaluation of a respiratory syncytial virus vaccine and a trivalent parainfluenza virus vaccine in a pediatric population. Am. J. Epidemiol. 1969; 89(4):449–463. [PubMed: 4305200]
27. Kapikian AZ, Mitchell RH, Chanock RM, Shvedoff RA, Stewart CE. An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. Am. J. Epidemiol. 1969; 89(4):405–421. [PubMed: 4305197]
28. Gonzalez IM, Karron RA, Eichelberger M, et al. Evaluation of the live attenuated cpts 248/404 RSV vaccine in combination with a subunit RSV vaccine (PFP-2) in healthy young and older adults. Vaccine. 2000; 18(17):1763–1772. [PubMed: 10699324]
29. Jeffree CE, Brown G, Aitken J, Su-Yin DY, Tan B-H, Sugrue RJ. Ultrastructural analysis of the interaction between F-actin and respiratory syncytial virus during virus assembly. Virology. 2007; 369(2):309–323. [PubMed: 17825340]
30. Mitra R, Baviskar P, Duncan-Decocq RR, Patel D, Oomens AGP. The human respiratory syncytial virus matrix protein is required for maturation of viral filaments. J. Virol. 2012; 86(8):4432–4443. [PubMed: 22318136]
31. Ghildyal R, Mills J, Murray M, Vardaxis N, Meanger J. Respiratory syncytial virus matrix protein associates with nucleocapsids in infected cells. J. Gen. Virol. 2002; 83(Pt 4):753–757. [PubMed: 11907323]
32. Ulloa L, Serra R, Asenjo A, Villanueva N. Interactions between cellular actin and human respiratory syncytial virus (HRSV). Virus Res. 1998; 53(1):13–25. [PubMed: 9617766]
33. Marty A, Meanger J, Mills J, Shields B, Ghildyal R. Association of matrix protein of respiratory syncytial virus with the host cell membrane of infected cells. Arch. Virol. 2004; 149(1):199–210. [PubMed: 14689285]
34. Henderson G, Murray J, Yeo RP. Sorting of the respiratory syncytial virus matrix protein into detergent-resistant structures is dependent on cell-surface expression of the glycoproteins. Virology. 2002; 300(2):244–254. [PubMed: 12350355]
35. Money VA, McPhee HK, Mosely JA, Sanderson JM, Yeo RP. Surface features of a *Mononegavirales* matrix protein indicate sites of membrane interaction. Proc. Natl Acad. Sci. USA. 2009; 106(11):4441–4446. [PubMed: 19251668]
36. Ghildyal R, Li D, Peroulis I, et al. Interaction between the respiratory syncytial virus G glycoprotein cytoplasmic domain and the matrix protein. J. Gen. Virol. 2005; 86(Pt 7):1879–1884. [PubMed: 15958665]
37. Ghildyal R, Ho A, Jans DA. Central role of the respiratory syncytial virus matrix protein in infection. FEMS Microbiol. Rev. 2006; 30(5):692–705. [PubMed: 16911040]
38. Low K-W, Tan T, Ng K, Tan B-H, Sugrue RJ. The RSV F and G glycoproteins interact to form a complex on the surface of infected cells. Biochem. Biophys. Res. Commun. 2008; 366(2):308–313. [PubMed: 18036342]
39. Mostov KE, Verges M, Altschuler Y. Membrane traffic in polarized epithelial cells. Curr. Opin. Cell Biol. 2000; 12(4):483–490. [PubMed: 10873817]
40. Goldenring JR, Smith J, Vaughan HD, Cameron P, Hawkins W, Navarre J. Rab11 is an apically located small GTP-binding protein in epithelial tissues. Am. J. Physiol. 1996; 270(3 Pt 1):G515–G525. [PubMed: 8638719]
41. Radhakrishnan A, Yeo D, Brown G, et al. Protein analysis of purified respiratory syncytial virus particles reveals an important role for heat shock protein 90 in virus particle assembly. Mol. Cell Proteomics. 2010; 9(9):1829–1848. [PubMed: 20530633] ▪ Demonstration that a number of host proteins may play a role during RSV assembly.
42. Brock SC, Heck JM, McGraw PA, Crowe JE. The transmembrane domain of the respiratory syncytial virus F protein is an orientationindependent apical plasma membrane sorting sequence. J. Virol. 2005; 79(19):12528–12535. [PubMed: 16160180]
43. Oomens AGP, Bevis KP, Wertz GW. The cytoplasmic tail of the human respiratory syncytial virus F protein plays critical roles in cellular localization of the F protein and infectious progeny production. J. Virol. 2006; 80(21):10465–10477. [PubMed: 16928754] ▪ Evidence that the fusion protein cytoplasmic tail is critical for virus assembly into filaments.

44. Branigan PJ, Day ND, Liu C, et al. The cytoplasmic domain of the F protein of human respiratory syncytial virus is not required for cell fusion. J. Gen. Virol. 2006; 87(Pt 2):395–398. [PubMed: 16432027]
45. Harrison MS, Sakaguchi T, Schmitt AP. Paramyxovirus assembly and budding: building particles that transmit infections. Int. J. Biochem. Cell Biol. 2010; 42(9):1416–1429. [PubMed: 20398786] ■ Comprehensive review of paramyxovirus assembly and budding.
46. Teng MN, Whitehead SS, Collins PL. Contribution of the respiratory syncytial virus G glycoprotein and its secreted and membrane-bound forms to virus replication *in vitro* and *in vivo*. Virology. 2001; 289(2):283–296. [PubMed: 11689051]
47. Biacchesi S, Skiadopoulos MH, Yang L, et al. Recombinant human metapneumovirus lacking the small hydrophobic SH and/or attachment G glycoprotein: deletion of G yields a promising vaccine candidate. J. Virol. 2004; 78(23):12877–12887. [PubMed: 15542640]
48. Cathomen T, Naim HY, Cattaneo R. Measles viruses with altered envelope protein cytoplasmic tails gain cell fusion competence. J. Virol. 1998; 72(2):1224–1234. [PubMed: 9445022]
49. Tahara M, Takeda M, Yanagi Y. Altered interaction of the matrix protein with the cytoplasmic tail of hemagglutinin modulates measles virus growth by affecting virus assembly and cell–cell fusion. J. Virol. 2007; 81(13):6827–6836. [PubMed: 17442724]
50. Chen BJ, Lamb RA. Mechanisms for enveloped virus budding: can some viruses do without an ESCRT? Virology. 2008; 372(2):221–232. [PubMed: 18063004]
51. Teng MN, Collins PL. Identification of the respiratory syncytial virus proteins required for formation and passage of helper-dependent infectious particles. J. Virol. 1998; 72(7):5707–5716. [PubMed: 9621029] ■ Identification of the minimum requirements for passage of an RSV minigenome.
52. Hurley JH, Boura E, Carlson L-A, Różycki B. Membrane budding. Cell. 2010; 143(6):875–887. [PubMed: 21145455]
53. Rossman JS, Jing X, Leser GP, Lamb RA. Influenza virus M2 protein mediates ESCRT-independent membrane scission. Cell. 2010; 142(6):902–913. [PubMed: 20850012] ■ Demonstration of an endosomal sorting complex required for transport-independent mechanisms for virus budding.
54. Brown DA, Rose JK. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell. 1992; 68(3):533–544. [PubMed: 1531449]
55. Rossman JS, Lamb RA. Influenza virus assembly and budding. Virology. 2011; 411(2):229–236. [PubMed: 21237476]
56. Yeo DS-Y, Chan R, Brown G, et al. Evidence that selective changes in the lipid composition of raft-membranes occur during respiratory syncytial virus infection. Virology. 2009; 386(1):168–182. [PubMed: 19178924]
57. Brown G, Jeffree CE, McDonald T, Rixon HWM, Aitken JD, Sugrue RJ. Analysis of the interaction between respiratory syncytial virus and lipid-rafts in Hep2 cells during infection. Virology. 2004; 327(2):175–185. [PubMed: 15351205]
58. Jeffree CE, Rixon HWM, Brown G, Aitken J, Sugrue RJ. Distribution of the attachment (G) glycoprotein and GM1 within the envelope of mature respiratory syncytial virus filaments revealed using field emission scanning electron microscopy. Virology. 2003; 306(2):254–267. [PubMed: 12642099]
59. McCurdy LH, Graham BS. Role of plasma membrane lipid microdomains in respiratory syncytial virus filament formation. J. Virol. 2003; 77(3):1747–1756. [PubMed: 12525608]
60. Fleming EH, Kolokoltsov AA, Davey RA, Nichols JE, Roberts NJ. Respiratory syncytial virus F envelope protein associates with lipid rafts without a requirement for other virus proteins. J. Virol. 2006; 80(24):12160–12170. [PubMed: 17005642]
61. McDonald TP, Pitt AR, Brown G, Rixon HWM, Sugrue RJ. Evidence that the respiratory syncytial virus polymerase complex associates with lipid rafts in virus-infected cells: a proteomic analysis. Virology. 2004; 330(1):147–157. [PubMed: 15527841]
62. Taylor MP, Koyuncu OO, Enquist LW. Subversion of the actin cytoskeleton during viral infection. Nat. Rev. Microbiol. 2011; 9(6):427–439. [PubMed: 21522191]

63. Lillemeier BF, Pfeiffer JR, Surviladze Z, Wilson BS, Davis MM. Plasma membrane-associated proteins are clustered into islands attached to the cytoskeleton. Proc. Natl Acad. Sci. USA. 2006; 103(50):18992–18997. [PubMed: 17146050]
64. Kallewaard NL, Bowen AL, Crowe JE. Cooperativity of actin and microtubule elements during replication of respiratory syncytial virus. Virology. 2005; 331(1):73–81. [PubMed: 15582654]
65. Shaikh FY, Utley TJ, Craven RE, et al. Respiratory syncytial virus assembles into structured filamentous virion particles independently of host cytoskeleton and related proteins. PLoS One. 2012; 7(7):e40826. [PubMed: 22808269]
66. Burke E, Dupuy L, Wall C, Barik S. Role of cellular actin in the gene expression and morphogenesis of human respiratory syncytial virus. Virology. 1998; 252(1):137–148. [PubMed: 9875324]
67. Huang YT, Romito RR, De BP, Banerjee AK. Characterization of the *in vitro* system for the synthesis of mRNA from human respiratory syncytial virus. Virology. 1993; 193(2):862–867. [PubMed: 8460489]
68. Harpen M, Barik T, Musiyenko A, Barik S. Mutational analysis reveals a noncontractile but interactive role of actin and profilin in viral RNA-dependent RNA synthesis. J. Virol. 2009; 83(21):10869–10876. [PubMed: 19710142]
69. Burke E, Mahoney NM, Almo SC, Barik S. Profilin is required for optimal actin-dependent transcription of respiratory syncytial virus genome RNA. J. Virol. 2000; 74(2):669–675. [PubMed: 10623728]
70. Fernie BF, Gerin JL. Immunochemical identification of viral and nonviral proteins of the respiratory syncytial virus virion. Infect. Immun. 1982; 37(1):243–249. [PubMed: 7107006]
71. Brown G, Aitken J, Rixon HWM, Sugrue RJ. Caveolin-1 is incorporated into mature respiratory syncytial virus particles during virus assembly on the surface of virus-infected cells. J. Gen. Virol. 2002; 83(Pt 3):611–621. [PubMed: 11842256]
72. Gower TL, Graham BS. Antiviral activity of lovastatin against respiratory syncytial virus *in vivo* and *in vitro*. Antimicrob. Agents Chemother. 2001; 45(4):1231–1237. [PubMed: 11257039]
73. Gower TL, Pastey MK, Peeples ME, et al. RhoA signaling is required for respiratory syncytial virus-induced syncytium formation and filamentous virion morphology. J. Virol. 2005; 79(9): 5326–5336. [PubMed: 15827147]
74. Ball G, Parton RM, Hamilton RS, Davis I. A cell biologist's guide to high resolution imaging. Meth. Enzymol. 2012; 504:29–55. [PubMed: 22264528]
75. Santangelo PJ, Lifland AW, Curt P, et al. Single molecule-sensitive probes for imaging RNA in live cells. Nat. Methods. 2009; 6(5):347–349. [PubMed: 19349979]
76. Callan-Jones A, Sorre B, Bassereau P. Curvature-driven lipid sorting in biomembranes. Cold Spring Harb. Perspect. Biol. 2011 (Epub ahead of print).
77. Hoenen T, Biedenkopf N, Zielecki F, et al. Oligomerization of Ebola virus VP40 is essential for particle morphogenesis and regulation of viral transcription. J. Virol. 2010; 84(14):7053–7063. [PubMed: 20463076]

Executive summary

Respiratory syncytial virus

- Respiratory syncytial virus (RSV) is a member of the *Paramyxoviridae* family and is a leading cause of serious viral lower respiratory tract illness in infants.
- RSV assembles and buds preferentially from the apical surface of polarized epithelial cells, where the viral genome and protein assemble into filamentous structures.
- Treatment is limited to supportive care. Passively administered antibody (palivizumab; Synagis®) is effective for prophylaxis, but is not therapeutic during infection. No licensed vaccine is currently available.

Ribonucleoprotein assembly & protein trafficking

- Ribonucleoprotein assembly is thought to occur within cytoplasmic inclusions, since the viral polymerase proteins and genomic RNA are found to colocalize at these structures.
- Protein trafficking from cytoplasmic inclusions to the cell surface may be mediated by the matrix (M) protein or by host components, such as actin.
- Dominant-negative inhibitors of myosin Vb or Rab11–FIP1 disrupt viral filament formation and result in diminished infectious viral progeny.
- Disruption of Rab11–FIP2 causes an increase in cell-associated virus and production of longer viral filaments on the surface of infected cells.
- HSP90 and HSP70 associate with virus cytoplasmic inclusions and either filaments or budded particles, respectively.

RSV fusion protein & paramyxovirus cytoplasmic tails

- Deletion of the fusion protein cytoplasmic tail leads to a 100–1000-fold decrease in viral titer in a multicycle growth assay and results in a virus that is incapable of assembling into filaments.
- Coordination of RSV assembly by the fusion (F) protein cytoplasmic tail is consistent with many other studies citing paramyxovirus glycoprotein cytoplasmic tails as crucial to both assembly and budding.

Viral assembly at the plasma membrane

- At the cell surface, RSV assembles into viral filaments. F and M protein, nucleoprotein and phosphoprotein are required for filament formation independent of virus infection.
- A phenylalanine residue in the cytoplasmic tail of F protein is necessary for assembly into virus-like filaments.

Viral budding & membrane scission

- RSV buds using a Vps4-independent mechanism.

RSV interactions with the host membrane

- RSV assembles and buds at specific microdomains at the cell surface.

- Lipid raft markers (i.e., GM1, caveolin-1, CD44, RhoA, CD55 and CD59) colocalize with viral proteins and filaments.
- RSV F protein associates with detergent-resistant membranes in the absence of other viral proteins, and M protein associates with detergent-resistant membranes in the presence of the F protein.
- The viral polymerase complex associates with lipid raft proteins.

RSV & the host cytoskeleton

- Gross disruption of the actin and tubulin cytoskeleton disrupts virus replication.
- Actin is required for optimal RSV genome replication, without the need for polymerization.
- Inhibition of actin polymerization does not affect viral assembly into filaments.
- Filamentous actin is excluded from virus filaments. β-actin and other actin-related proteins colocalize with RSV F proteins at the cell surface and have been found in a variety of sucrose gradient-purified RSV preparations.
- Filamin A and caveolin-1 associate with viral filaments by immunofluorescence and in budded particles by mass spectrometry.
- Disruption of actin-related proteins or actin-associated signaling pathways affects protein trafficking or filament formation, respectively.