Chapter 9
Defining the Assembleome of the Respiratory Syncytial Virus

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Abstract During respiratory syncytial virus (RSV) particle assembly, the mature RSV particles form as filamentous projections on the surface of RSV-infected cells. The RSV assembly process occurs at the / on the cell surface that is modified by a virus infection, involving a combination of several different host cell factors and cellular processes. This induces changes in the lipid composition and properties of these lipid microdomains, and the virus-induced activation of associated Rho GTPase signaling networks drives the remodeling of the underlying filamentous actin (F-actin) cytoskeleton network. The modified sites that form on the surface of the infected cells form the nexus point for RSV assembly, and in this review chapter, they are referred to as the RSV assembleome. This is to distinguish these unique membrane microdomains that are formed during virus infection from the corresponding membrane microdomains that are present at the cell surface prior to infection. In this article, an overview of the current understanding of the processes that drive the formation of the assembleome during RSV particle assembly is given.

Keywords Respiratory syncytial virus · Virus assembly · Rac-1 protein · Lovastatin · Lipid raft · F-actin · Rho GTPase

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S. Vijayakrishnan et al. (eds.), *Virus Infected Cells*, Subcellular Biochemistry 106,
https://doi.org/10.1007/978-3-031-40086-5_9

Respiratory Syncytial Virus Infection Is a Global Health Problem

Respiratory syncytial virus (RSV) infections produce a range of clinical manifestations in humans, from relatively mild disease in the upper airway, to more severe infections that are associated with lower airway infection that can lead to viral pneumonia. RSV is a leading cause of viral pneumonia in young children worldwide, and lower respiratory tract infections in neonates and young children less than 5 years of age can be fatal. The virus is responsible for high levels of global infant morbidity and mortality, with many infant deaths occurring in mid and low-income counties (Nair et al. 2010; Li et al. 2022; Shi et al. 2017). Although young children are the traditional high-risk group for severe RSV infection, the elderly are also increasingly being recognized as an additional high-risk group that is prone to severe RSV infection (Bosco et al. 2021; Thompson et al. 2003). Reinfections with RSV continue throughout life, and the general clinical scenario has been worsened by the lack of a licensed vaccine to immunize young children, and other high-risk groups and the limited availability of cost-effective antiviral drugs. An improved understanding of both the human immunology associated with RSV infection and the biology of the RSV will aid in the development of new antiviral strategies to prevent and treat RSV infection. In this context, understanding the molecular events that lead to RSV particle assembly will aid in vaccine development and facilitate the development of new drugs that prevent RSV infection by, for example, blocking virus transmission. In this article, we review the current understanding of the RSV assembly process and the role that host cell factors play in this process.

The Genetic Structure of RSV

The RSV is grouped with the *Pneumoviridae* family of viruses, and this family is divided into two subgenera, the *Orthopneumovirus* (e.g., human RSV) and the *Metapneomovirus* (e.g., human metapneumovirus). The RSV genome (vRNA) consists of a single-stranded RNA molecule of negative sense that is approximately 15 kDa in size. The RSV vRNA contains 10 virus genes that are arranged contiguously along the length of the vRNA, with the gene order from the 3′ start being 3′-NS1-NS2-N-P-M-SH-F-G-M2-L-5′ (Fig. 9.1a). Each gene in the vRNA is separated from the adjacent genes by genetic regulatory elements referred to as intergenic regions. The individual virus proteins that are known to be expressed from these genes can be grouped based on their location within mature virus particles and the role that they play during virus replication.

The SH, F, and G genes encode for the three virus integral membrane proteins, the small hydrophobic (SH) protein, the fusion (F) protein, and the attachment (G) protein, respectively. The G protein mediates cell attachment, while the F protein mediates membrane fusion during cell entry. The SH protein is not essential for virus

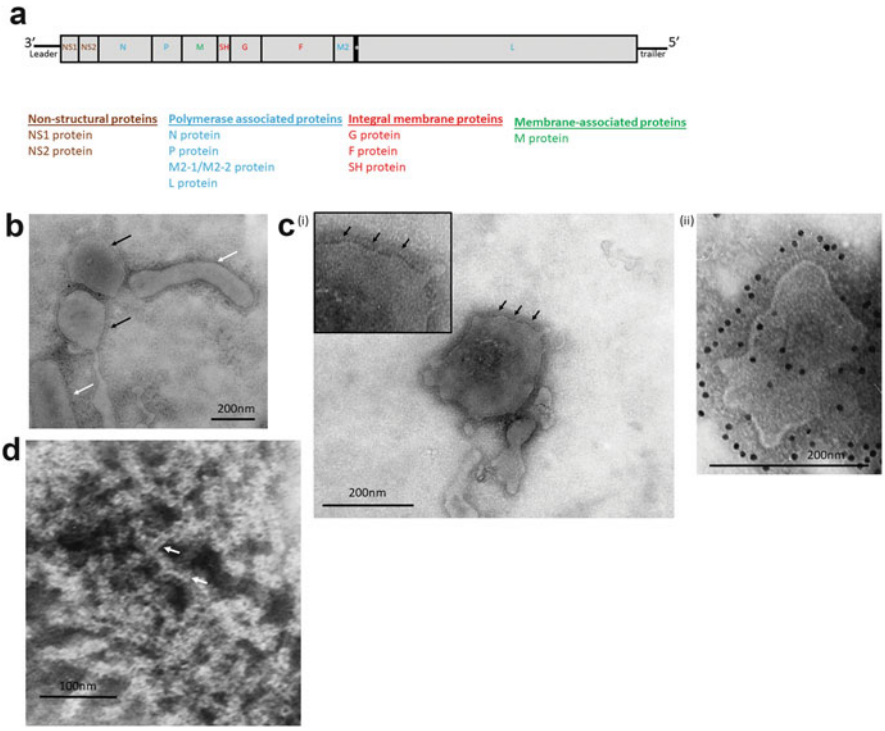

Fig. 9.1 Organization of the respiratory syncytial virus (RSV) genome. (**a**) The virus genome (vRNA) from the beginning at the 3′ leader region to the 5′ trailer region is shown. The location of the genes that express the nonstructural proteins (brown), the proteins that form the ribonucleoprotein (RNP) complex (light blue), the membrane-associated protein (green), and the virus integral membrane proteins (red) are highlighted in the vRNA. The overlap between the M2 and L genes is also highlighted (*). The individual genes are drawn approximately to scale. Examination of purified RSV particles by transmission electron microscopy (**b**) reveals the appearance of pleomorphic-shaped RSV particles. The round (black arrows) and the more filamentous shaped (white arrows) virus particles are highlighted. (**c**) (**i**) At higher magnification, the location of the virus spike proteins (highlighted by black arrows) that protrude from the virus envelope. Inset is an enlarged image showing these structures. (**ii**) after immunostaining with anti-G. The black spots are the presence of 10 nm colloidal gold which indicates the location of the bound anti-G on the virus particles. (**d**) The appearance of the RNP (highlighted by white arrows) that are released from disrupted RSV particles that are present in a virus preparation

infection, and it is believed to play a role in immune evasion during the early stages of infection. Examination of cell-free RSV particles using electron microscopy to image negative-stained virus particles shows them to have a typical pleomorphic morphology, with a diameter ranging from 150 nm to upwards of 300 nm (Fig. 9.1b). The RSV particles are surrounded by a lipid envelope that is derived from the host cell in which the virus integral membrane proteins are inserted. Closer inspection of these particles by electron microscopy reveals that the G and F glycoproteins protrude from the virus envelope, and collectively they are referred to as the virus

spike proteins (Fig. 9.1c). The M gene encodes the matrix (M) protein, and although itis not an integral virus membrane protein, it associates with the inner surface of the virus envelope (Money et al. 2009). It is therefore not detected when electron microscopy is used to image negative-stained intact virus particles.

The N, L, P, and M2 genes encode the nuclear (N) protein, the large (L) protein, the phospho (P) protein, and the M2-1 protein, respectively. The M2 gene contains two open reading frames (ORF), one ORF that encodes the M2-1 protein, and another ORF called M2-2 is predicted to encode for the M2-2 protein. The N, L, P, and M2-1 proteins and the vRNA form into a larger distinct helical structure called the virus nucleocapsid (NC) (Cao et al. 2020; Gilman et al. 2019; Decool et al. 2021). The N protein is the RNA binding protein that coats the vRNA, and the polymerase activity resides in the L protein. The P protein interacts directly with the L protein and functions as a co-factor to facilitate polymerase activity, and the precise binding sites have been identified using structural analysis of the L protein (Gilman et al. 2019). The M2-1 protein also functions as part of the polymerase complex, and its role in facilitating virus gene transcription is established (Fearns and Collins 1999). The fully functional RSV NC requires the presence of all these different factors to achieve a full activity in virus-infected cells. The virus NC can also be detected in virus preparations using electron microscopy once the virus particles have been disrupted to reveal the internal structures, and under these conditions, the NC appear as smaller but distinct helical arrays (Fig. 9.1d).

The RSV vRNA also contains the NS1 and NS2 genes that encode two nonstructural proteins called the NS1 and NS2 proteins, respectively, and these proteins are thought to play a role in immune evasion (Sedeyn et al. 2019). While there is some similarity in the gene order and encoded virus proteins in the RSV and human metapneumovirus (HMPV) genomes, there are also some distinct differences in the genome structure between the two viruses. In the HMPV genome the NS1 and NS2 genes are absent, and there is a genetic rearrangement in the position of the M2 gene to give 3′-N-P-M-F-M2-SH-G-L-5′(van den Hoogen et al. 2002). However, the HMPV expresses a set of structural proteins with similar properties to those displayed by the RSV structural proteins, and these HMPV proteins play analogous roles in the HMPV replication cycle.

RSV Particle Morphogenesis at the Assembleome

The virus membrane-associated proteins are trafficked to the sites of RSV particle assembly at the plasma membrane, and their presence is an important determinant of the architecture of the mature RSV particles. In addition, these sites consist of an array of specific cellular factors that are required for RSV assembly and they also contribute to the formation of the virus envelope. The available evidence (discussed in this review) suggests that virus infection modifies the preexisting cell membrane microdomains that are ultimately used during RSV assembly, and it is proposed that these changes create unique membrane domains that are used for virus

assembly. In order to distinguish these modified cell surface membrane sites in virus-infected cells from the corresponding sites in non-infected cells, in this review, the sites of RSV assembly that form during RSV infection are referred to as the RSV assembleome. The RSV assembleome can be considered to be analogous to the budozone that has been described in influenza virus-infected cells during influenza virus assembly (Schmitt and Lamb 2005).

Interactions between the Virus Membrane Structural Proteins at the Assembleome

The G protein is a type II integral membrane protein, and its primary role is mediating the attachment of the virus to the host cell. The G protein is initially expressed as a polypeptide chain that is subsequently extensively modified by N-linked and O-linked glycosylation in the C-terminus ectodomain (Collins 1990; Collins and Mottet 1992). While the cytoplasmic N-terminus and the transmembrane domain are highly conserved between circulating viruses, the C-terminal ectodomain contains a region of greater sequence variability. The ectodomain contains two mucin-like domains that are separated by a substructure called the cysteine noose, which is a short sequence stabilized by two intra-chain disulfide bonds (Langedijk et al. 1996; 1998). The cell receptor binding sites for heparin sulfate (HS) (Feldman et al. 1999; Hallak et al. 2000a, b) and the CX3CR1 chemokine fractalkine receptor (Jeong et al. 2015; Johnson et al. 2015; Tripp et al. 2001) are located in the ectodomain, and these binding sites are conserved in non-tissue culture adapted circulating RSV stains (Kumaria et al. 2011).

The F protein is a type III integral membrane fusion protein that mediates the fusion of the virus envelope and cell membrane during virus entry into the host cell. The mature F protein exists as a homotrimer, and every single chain within the trimer is initially expressed as an inactive precursor (F0). The F0 is subsequently cleaved by furin into the F1 and F2 subunits (Anderson et al. 1992b) as it transits through the Golgi complex, and the available evidence suggests that the F0 protein is cleaved at two furin cleavage sites (Sugrue et al. 2001; Zimmer et al. 2001; González-Reyes et al. 2001). The F1 subunit contains the fusion peptide and heptad repeat (HR) regions, which play pivotal roles in mediating the membrane fusion process during cell entry of the virus (McLellan et al. 2013). The F1 and F2 subunits are covalently attached by inter-chain disulfide bridges (Scheid and Choppin 1977), and both subunits undergo N-linked glycosylation (Leemans et al. 2019; Rixon et al. 2002; McDonald and Sugrue 2007; Olmsted et al. 1986).

In RSV-infected cells, the SH protein is expressed as a small pentameric transmembrane protein (Collins and Mottet 1993; Rixon et al. 2005), and the presence of nonglycosylated, N-linked glycosylated and polylactosaminoglycan-modified SH protein species can be detected (Anderson et al. 1992a; Olmsted and Collins 1989). Although the functional significance of the different SH protein species is

still currently unclear, the SH protein does not appear to be required for establishing a virus infection in permissive cell lines (Bukreyev et al. 1997). A role for the SH protein viroporin activity in mediating immune evasion during the early stages of infection has been proposed (Triantafilou et al. 2013; Russell et al. 2015; Gan et al. 2008; Bukreyev et al. 1997).

The virus membrane-associated proteins can be trafficked to the plasma membrane independently of each other, but the existence of larger protein complexes involving these proteins within the virus envelope is now becoming established. Evidence suggests that the F protein interacts with the G protein to form a larger protein complex on the surface of infected cells (Low et al. 2008). This protein complex is also detected on purified virus particles (Ravi, Iyer, and Sugrue, unpublished observations) and in VLPs that are formed by recombinant expression of the F and G proteins (Ravi et al. 2021). It has recently been suggested that the interaction between the F and G proteins may stabilize the F protein in its prefusion form, which may have implications for RSV vaccine design (Cullen et al. 2022). The molecular cues that lead to the conversion of the RSV F protein from its prefusion conformation into the fusogenic conformation during cell entry are currently unclear. In several other paramyxoviruses, the conversion of the corresponding fusion protein into its fusogenic form is initiated by the engagement of the attachment protein with its cell receptor (reviewed in (Azarm and Lee 2020; Navaratnarajah et al. 2020)). The identification of a larger protein complex involving the RSV F and G proteins on infectious virus particles suggests that a similar mechanism may also be employed during RSV cell entry, although this possibility will require further investigation.

An early study provided evidence for a functional association between F, G, and SH proteins, suggesting the existence of a single protein complex involving all three virus proteins that were relevant to membrane fusion (Heminway et al. 1994). There have been no subsequent studies describing this protein complex, suggesting that if such a protein complex exists in RSV-infected cells, it may only exist transiently. Although the SH protein localizes at lipid raft microdomains in the Golgi complex (Olmsted and Collins 1989; Rixon et al. 2004), a stable protein complex involving the SH and G proteins on the surface of RSV-infected cells has been described (Rixon et al. 2005; Low et al. 2008). The functional significance of this interaction is currently unclear, but the presence of this stable complex on the surface of infected cells and the presence of low levels of the SH protein in the tissue culture supernatant of RSV-infected cells (Olmsted and Collins 1989; Low et al. 2008) had suggested that the SH protein may be incorporated into the virus envelope. Recent direct evidence has demonstrated that the SH protein is transported into the lipid envelope of mature virus particles that form on virus-infected cells (Huong et al. 2023).

The M protein undergoes dimerization during RSV infection (Bajorek et al. 2014; Förster et al. 2015), and although it is not an integral membrane protein, this peripheral membrane protein interacts with the inner surface of the virus envelope (Money et al. 2009). The M protein is thought to play an important role in virus particle assembly, but during the early stages of the virus replication cycle, the M protein is also transported to the nucleus of infected cells. The functional significance of the nuclear localization of the M protein is less well defined, but it has been

postulated to play an additional role in controlling host gene expression (Li et al. 2021). The M protein interacts with the virus envelope at its inner surface (Money et al. 2009), and an interaction between the M protein and the N-terminal cytoplasmic of the G protein domain has also been described (Ghildyal et al. 2005). The M protein also interacts with the NC via the M2-1 protein, suggesting that the M protein may inhibit the activities associated with the virus polymerase complex (Ghildyal et al. 2002; Li et al. 2008). In the context of RSV particle assembly, since the NC is located beneath the virus envelope in virus particles, in the mature virus particle the M protein may act as a stable link between the virus envelope (via the G protein) and the underlying virus NC (via the M2-1 protein).

RSV Particles Form as Filamentous Structures at the Assembleome on Infected Cells

The infectious RSV particles form as distinct filamentous projections on the surface of RSV-infected cells (Roberts et al. 1995; Jeffree et al. 2003), and these filamentous projections are referred to as virus filaments (Fig. 9.2a). In RSV-infected cells, the nucleocapsids accumulate in the cytoplasm, and they form part of a second distinct structure called the cytoplasmic inclusion body (Fig. 9.2a). Although several virus proteins are located in these structures, the co-expression of the N and P proteins appears to be the minimum requirement for inclusion body formation (García et al. 1993). The inclusion bodies can be detected in close proximity to the location where the virus filaments form (Fig. 9.2b and c), and current evidence suggests that nucleocapsids are trafficked from the inclusion bodies to the site of RSV assembly (Santangelo et al. 2006; Santangelo and Bao 2007). This paradigm in nucleocapsid packaging is supported by the spatial proximity of the virus filaments and cytoplasmic inclusion bodies in virus-infected cells (Radhakrishnan et al. 2010). It has been proposed that the virus utilizes molecular motors to achieve the short-range cellular movement from the inclusion bodies to the site of virus assembly, where the virus filaments form (Santangelo and Bao 2007).

The larger virus filaments that form on the surface of infected cells can be clearly distinguished from the shorter microvilli that are present on the surface of non-infected cells. This can be seen by using high-resolution immuno-scanning electron microscopy (I-SEM) to examine the surface topology of non-infected and RSV-infected cells that allow detection of the immunolabelled virus filaments (Fig. 9.3a). Although these virus filaments do not conform to the pleomorphic morphology exhibited by cell-free RSV particles, there is now a general acceptance that these filamentous structures are mature infectious virus particles. The virus filaments are observed on almost all cell types that are permissive to RSV infection, including primary cell types and tissues that are representative of the upper airway (Jumat et al. 2015). Analysis of virus-infected cells using I-SEM has revealed a high density of G protein incorporation into the virus filament, and the distribution of the

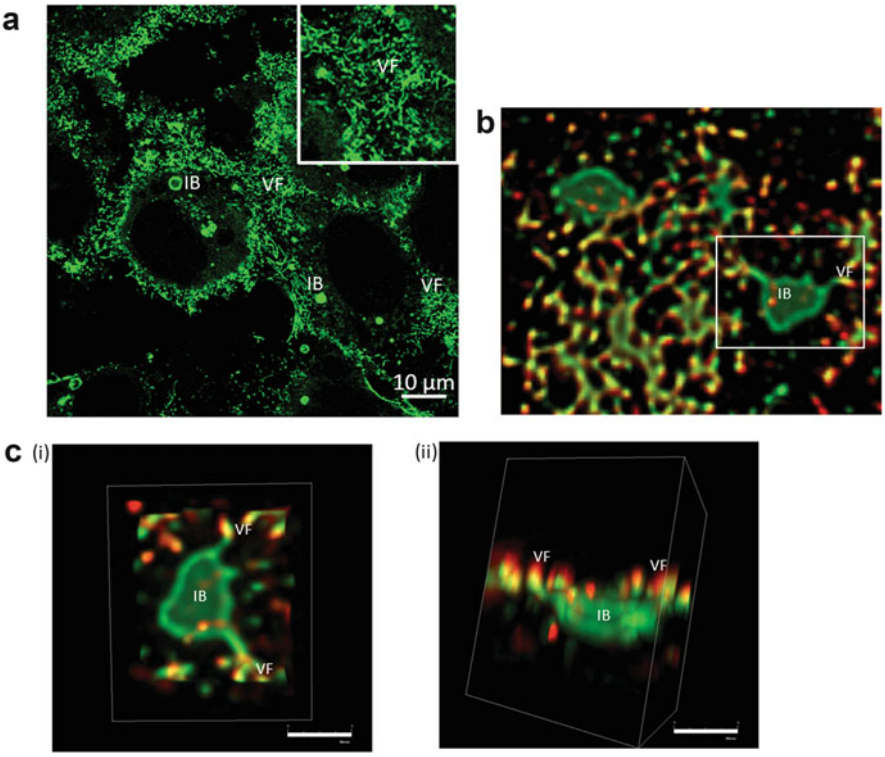

Fig. 9.2 The presence of inclusion bodies and virus filaments in respiratory syncytial virus (RSV)-infected cells. (**a**) At 20 hrs post-infection RSV-infected cells were labeled using an anti-N antibody. These cells were examined in a Zeiss LSM 510 scanning confocal microscope to visualize the virus filament (VF) and cytoplasmic inclusion body (IB). Inset is an enlarged segment of the image showing the presence of the virus filaments. (**b** and **c**) RSV-infected cells were labeled using antibodies to the P protein (green) and F protein (red) and an image series was obtained from the same cell at different focal planes in the Z-axis using confocal microscopy, and the data were processed into three-dimensional representations. (**b**) It is a low-magnification image showing a region near the surface of the cell viewed from above the cell. The IB highlighted by the open white box is viewed at higher magnification from (**c** (**i**)) above and (**c** (**ii**)) in cross-section. While only the IB is stained with anti-P, the VF is labeled by both anti-P and anti-F, and co-staining is shown by the yellow staining

G protein staining has suggested that the G protein is closely packed along the length of the virus filament (Fig. 9.3b). This is supported by more recent evidence employing high-resolution imaging to examine the spatial distribution of the virus glycoproteins in the envelope of the virus filaments (Conley et al. 2022). In cells infected with the closely related HMPV, the infectious virus particles also form on the surface of infected cells with a similar filamentous morphology (Jumat et al. 2014). This suggests a similar mechanism of virus particle assembly for both RSV and HMPV, and further suggests that virus filament formation may be a common feature of the virus assembly process in the *Pneumoviridae* family of viruses.

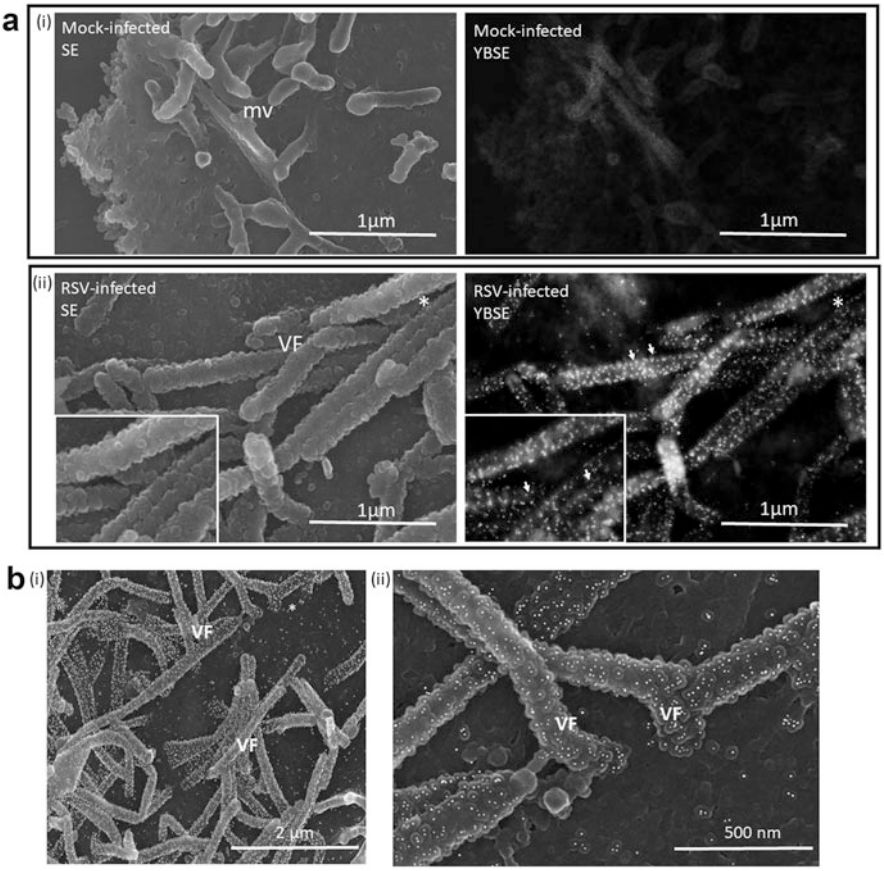

Fig. 9.3 The Surface topology of respiratory syncytial virus (RSV)-infected cells revealed by immune-scanning electron microscopy. (**a**) (**i**) Mock-infected cells (non-infected cells) and (**ii**) RSV-infected cells were labeled with an antibody against the G protein and a second antibody conjugated to 10 nm colloidal gold. The cells were then examined using a Hitachi F4700 scanning electron microscope. The surface topology of the cell surface was imaged using a secondary emitter (SE) detector, and the presence of the bound antibody (and gold particles) using the YAG back scatter (YBSE) detector. The colloidal gold is visualized as small white spots in the images (highlighted by short white arrows) obtained using the YBSE detector. Inset in (**a**) (**ii**) image is an enlarged segment of the image taken obtained from the region indicated by *. (**b**) (**i**) Image was taken from RSV-infected cells where the images using the SE and YBSE detectors in the same field of view are superimposed. (**ii**) is a higher magnification image taken from the area (*) highlighted in (**i**). The microvilli (mv) and virus filaments (VF) are indicated

A low multiplicity of infection model was previously used to examine RSV multiple cycle infection in several permissive cell lines, and using this infection model, two distinct phases during multiple cycle RSV infection were identified (Huong et al. 2016). An earlier phase involved direct and localized cell-to-cell virus transmission in the cell monolayer, during which the virus infectivity remained

exclusively cell-associated. A second phase was identified at the later stages of infection, during which the localized transmission continued, but it was characterized by virus-induced changes in cell physiology that correlated with the appearance of low levels of cell-free virus infectivity. Experimental conditions that inhibited virus filament formation led to an inhibition of both this localized virus transmission and the levels of recoverable virus infectivity, providing evidence that the virus filaments played a direct role in mediating this localized cell-to-cell transmission. This phenomenon was also observed when using the human nasal epithelial cell (hNEC) model to examine RSV transmission, where the virus infectivity was largely cell-associated. Low levels of cell-free infectivity that were shed into the overlying mucus in the hNEC model were associated with changes in the physiology of the infected ciliated cells at the later stages of infection (Huong et al. 2018).

Warning: Processing-instruction not allowed here!!!The Assembleome Forms at Lipid Raft Microdomains on the Surface of Infected Cells

Specialized lipid domains that are referred to as lipid raft microdomains are present in the plasma membrane of many different cell types. Lipid raft microdomains are characterized by the increased concentration of specific lipid classes, which include cholesterol, sphingolipids such as sphingomyelin, and glycosphingolipid GM1. These lipid classes concentrate in the plasma membrane, and they provide localized stabilized membrane microdomains within the more mobile and fluid bulk lipid membrane (Sezgin et al. 2017). Specific proteins are also known to be trafficked into the lipid raft domains, and several of these raft-associated proteins play a role in mediating cell signaling events in the cell, for example, Rho GTPases. Since viruses are large multicomponent biological structures, these lipid raft microdomains are ideal platforms where the different cellular activities that are associated with the formation of viruses can be orchestrated. They thus provide ideal cellular platforms to drive the process of virus particle assembly for an array of different viruses, including RSV.

Several early studies employing high-resolution I-SEM revealed that the virus glycoproteins were trafficked into the virus filaments as they form at the cell surface (e.g., Jeffree et al. 2003). These data provided direct evidence that the virus structural proteins that ultimately were associated with the virus envelope were trafficked to specific microdomains on the plasma membrane. Early studies using imaging demonstrated that RSV particle assembly occurred at sites on the cell that were enriched in the established lipid raft marker protein caveolin-1 (cav-1), and that the cav-1 protein was incorporated into the virus filaments on the surface of virus-infected cells (Brown et al. 2002a). This was followed by the demonstration that the sites of RSV particle assembly could be identified using fluorescence probes that allow the detection of raft-associated lipid classes (Brown et al. 2002b; McCurdy

and Graham 2003; Jeffree et al. 2003). Lipid raft microdomains can be experientially distinguished from the bulk lipid membrane based on their insolubility in nonionic detergents such as Triton X100 at 4 °C, giving rise to an operational definition of lipid rafts as detergent-resistant membranes (DRM). The imaging analayses were further supported by using this biochemical approach that demonstrated that in RSV-infected cells the virus structural proteins were found to partition into the DRM fraction (Brown et al. 2004; McCurdy and Graham 2003; McDonald et al. 2004; Marty et al. 2004). RSV particle assembly is now established to occur at lipid raft microdomains on the surface of virus-infected cells. In this context, drugs that solubilize membrane-associated cholesterol and destabilize lipid raft microdomains also lead to the inhibition of RSV particle assembly (Yeo et al. 2009). However, a detailed comparison of the lipid composition of lipid rafts isolated from non-infected and RSV-infected cells indicated that virus infection-induced changes in their lipid composition (Yeo et al. 2009). These changes in the lipid composition would be expected to alter the biological properties of these lipid raft microdomains in virus-infected cells.

The presence of raft-associated cellular proteins such as the cav-1 protein in the envelope of RSV particles suggested that the lipid envelope of RSV can be considered to have raft-like properties. This suggestion was supported by the detailed analysis of the lipid profile of isolated purified RSV particles which has revealed a high level of lipid classes that are associated with the formation of lipid raft membrane domains were also present in the virus envelope (Chan, Wenk and Sugrue, unpublished observations). An additional consequence of the use of lipid raft microdomains during RSV assembly is that specific raft-associated cell factors that have biological activities of clinical relevance may also be incorporated into the virus envelope, and which may potentially impart important functional properties to the virus envelope. In this context, the raft-associated CD55 and CD59 complement regulatory proteins were both detected in the envelope of mature virus particles (Brown et al. 2004). It was originally proposed that their presence may have a clinical context, since the presence of these complement regulatory proteins in the virus envelope could potentially impact on how the viruses interact with the complement system. This suggestion is supported by more recent research that has confirmed the association of CD55 protein with mature RSV particles, and that has assessed the consequence of virus-associated CD55 protein in relation to opsonization by the complement system (Kuppan et al. 2021).

Although differential detergent solubility at 4 °C is a robust operational definition to identify proteins that are associated with lipid raft microdomains, this can refer to a broad range of different types of lipid raft microdomain that are only defined by their detergent solubility properties. Consequently, in many cases the specific type of lipid raft microdomain under investigation that is isolated using detergent treatment remains uncharacterized. Caveolae are established as a specific class of lipid raft microdomain that plays a variety of different roles in cell homeostasis. Although several cellular proteins are involved in the formation of the caveolae coat complex (Ludwig et al. 2013), the cav-1 protein is a major structural determinant of caveolae. Therefore, in the earlier observations that described the presence of the cav-1 protein

in the envelope of virus filaments it was unclear if the cav-1 protein was specifically recruited to the site of RSV assembly, or if the presence of cav-1 protein at these sites indicated the involvement of caveolae in the virus assembly process. More recent work has identified other caveolae-associated proteins (e.g., the cavins) at the site of RSV assembly, which confirmed the involvement of caveolae in the RSV assembly process (Ludwig et al. 2017). The distribution of the cav-1 protein in the virus particle was determined using advanced imaging techniques, and its presence in the virus envelope involved an interaction with the RSV G protein. At present the effect of cav-1 protein and the various cavin proteins in the virus envelope is unclear, but since these proteins are major structural determinants of the caveolae, it would be expected that their presence could impart specific biological properties to the RSV envelope (e.g., an increase in membrane rigidity). Although evidence for the involvement of lipid raft microdomains in the assembly of infectious HMPV particles has been presented (Jumat et al. 2014), it is currently unclear if caveolae are also involved in the HMPV assembly process.

Recombinant expression of combinations of the virus membrane-associated proteins is sufficient to lead to the formation of virus-like particles (VLPs) that display similar morphological features to the virus particles that form on RSV-infected cells. VLPs that resemble virus filaments can be detected on cells expressing only the recombinant G protein (Ravi et al. 2021), suggesting that the G protein may play an important role in virus particle assembly. The recombinant G protein is trafficked to lipid raft membrane domains that contain both the actin and filamin-1 proteins, host cell factors that are shown to be present in virus filaments isolated from infected cells (Radhakrishnan et al. 2010). This provides evidence that the virus glycoproteins contain inherent trafficking signals that allow their transport to the site of virus assembly. Although these trafficking signals have not been clearly defined, the F and G proteins are both modified by palmitoylation (Arumugham et al. 1989; Collins and Mottet 1992), and the addition of palmitoyl moieties to these proteins may facilitate their interaction with lipid raft microdomains. Evidence also suggests that an interaction between the M protein and the RSV glycoproteins may traffic the M protein into the DRM fraction (Henderson et al. 2002). This further suggests that during virus infection, the trafficking of the M protein to the assembleome may involve an interaction between the M protein and the virus glycoproteins (e.g. the G protein). In this context, stable interactions between the G protein and the other virus membrane-associated proteins and cell proteins at the assembleome may stabilize their interaction within the lipid envelope of the virus filaments.

Filamentous Actin (F-Actin) Remodeling at Lipid Raft Microdomains at the Assembleome Drives Virus Filament Formation

Microvilli and similar cell surface projections are stabilized by the filamentous form of actin (F-actin) that forms into F-actin bundles, and on the surface of non-infected cells, the microvilli are the dominant topographical feature. However, on the surface of RSV-infected cells, there is a reduction in the levels of the microvilli, and the virus filaments become the dominant surface feature detected (Jeffree et al. 2003). Indeed, on RSV-infected cells the microvilli appear to be largely replaced by the virus filaments, and since the microvilli are stabilized by F-actin bundles, this change in surface topology is consistent with virus infection leading to remodeling of the cell surface of infected cells. It is currently unclear what effect the trafficking of the virus membrane proteins to the sites of virus assembly has on the biological properties of the microvilli and other surface projections that are initially present on the cell surface. However, virus-induced changes in cell surface topology suggest that the biological properties of the sites on the cell membrane that are used for virus particle assembly are modified via structural changes to the underlying the F-actin network. Early studies on RSV-infected cells have implicated the cortical cytoskeleton as playing a role in the morphogenesis of the mature RSV particles (Ulloa et al. 1998; Burke et al. 1998). The role of F-actin in RSV morphogenesis was supported by high-resolution imaging that has demonstrated a role for the F-actin network in the process of virus assembly (Jeffree et al. 2007), and in this context, the M protein has also been shown to directly interact with actin in RSV-infected cells (Shahriari et al. 2018). In addition, actin and several actin-binding proteins have been detected in purified virus preparations and virus filaments, and among these was the actin-binding protein filamin-1(Radhakrishnan et al. 2010; Ravi et al. 2021). The filamin-1 protein is an established actin-binding protein that interacts with the F-actin, and the filamin-1 protein is also able to cross-link F-actin bundles and facilitate F-actin remodeling (Popowicz et al. 2006). Collectively these observations by several groups have provided robust evidence for the role of the F-actin network in the morphogenesis of RSV particles.

The role of the F-actin network in regulating and orchestrating the clustering of lipid raft microdomains has been established (Chichili and Rodgers 2007). In the context of RSV particle assembly, the analysis of the lipid composition of lipid raft preparations isolated from RSV-infected cells has demonstrated that RSV infection led to the presence of increased levels of the signaling lipid phosphatidylinositol 4, 5-bisphosphate (PIP2) in the lipid raft membranes (Yeo et al. 2009). This event was consistent with earlier observations that RSV infection was associated with increased levels of phosphatidylinositol 3 kinase (PI3K) expression and PI3K activation during RSV infection (Thomas et al. 2002; Jeffree et al. 2007). The enzyme PI3K phosphorylates the substrate PIP2 to generate the product phosphatidylinositol-3, 4, 5-triphosphate (PIP3), and the presence of both PIP2 and PIP3 in inclusion bodies in RSV-infected cells suggests that these signalling

lipids may have a role RSV particle assembly (Yeo et al. 2009). The cortical F-actin network can be remodeled by Rho GTPases, and the first evidence for the involvement of an activated rho GTPase in RSV particle assembly was the activation of the rhoA protein during RSV infection (Gower et al. 2005; Gower et al. 2001; McCurdy and Graham 2003). More recently, the rac1 protein has been shown to also play a major role in RSV particle assembly in virus-infected cells (Ravi et al. 2021; Malhi et al. 2021). Since the PI3K protein signaling pathway activates cellular proteins that in turn regulate the activity of the rac1 protein (Campa et al. 2015), this has suggested the contribution of a signaling pathway during RSV particle assembly that involves the PI3K and rac1 proteins. While the interaction between the F protein and the rhoA protein has been described (Gower et al. 2001), it is currently unclear if the rac1 protein similarly interacts with one or more virus proteins at the assembleome, and this will require further investigation.

Although the rac1 and rhoA proteins are distinct proteins, there is likely to be a degree of crosstalk between their respective signaling networks in the cell. Since inhibiting the activation of either protein leads to impaired RSV particle assembly (Gower et al. 2005; Jeffree et al. 2007), it is possible that the rhoA and rac1 proteins may be part of a larger signaling network that ultimately leads to RSV particle assembly, via the activation of common or related downstream effectors. Currently, the downstream effectors of these signaling networks that are activated during RSV assembly are still uncertain. Changes in F-actin staining and the altered membrane distribution of the filamin-1 protein during RSV infection support the role of F-actin remodeling in virus particle assembly (Ludwig et al. 2017; Ravi et al. 2021). The filamin-1 protein plays a role in the formation of the RSV particle architecture (Radhakrishnan et al. 2010), and a role for rac-1 protein in inducing F-actin remodeling by the filamin-1 protein has been described using other experimental systems (Bellanger et al. 2000; Ramírez-Ramírez et al. 2020). It is, therefore, possible that the rac-1 protein signaling pathway may also play a similar role by inducing structural changes in the F-actin network via filamin-1 protein. The actin-related protein 2/3 (Arp2/3) complex also plays an important role in mediating actin polymerization (Papalazarou and Machesky 2021), and the Arp2/3 complex facilitates RSV spread during the localized cell-to-cell transmission (Mehedi et al. 2016). The Arp2/3 complex is activated by upstream effectors such as the WAVE Regulatory Complex (WRC) (Papalazarou and Machesky 2021), and the WRC is in turn activated by the rac1 protein (Chen et al. 2017). This is consistent with the observations that inhibition of rac1 protein activation also inhibits localized RSV transmission in virus-infected cell monolayers (Huong et al. 2016). However, it remains to be ascertained by experimentation if during RSV infection the filamin-1 protein and the Arp2/3 complex function as downstream effectors of the activated rac1 protein during virus filament formation and virus transmission.

Trafficking of the rac1 and rhoA Proteins to the Assembleome Is Required for Virus Filament Formation

Although the mevalonate pathway is established as a key metabolic pathway in the regulation of cholesterol biosynthesis, this metabolic pathway is also involved in the cellular process that provides the building blocks to make the isoprenoids that are used in protein prenylation. During protein prenylation, specific cytosolic proteins undergo a post-translation modification, whereby a small lipid molecule (e.g., geranylgeranyl isoprenoid) is attached to a c-terminal cysteine of the target protein via established consensus sequence motifs (reviewed in (Wang and Casey 2016)). The attachment of the lipid molecule to these normally cytosolic proteins provides a lipid anchor that allows their interaction with cellular membranes. The enzyme 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR) plays a role in regulating the activity of the mevalonate pathway, and the HMGCR protein can be inhibited using statin-based drugs such as lovastatin. As a consequence, these statin-based drugs inhibit both cholesterol biosynthesis and the formation of the lipid precursors that are involved in protein prenylation. Analysis of the changes in the host cell transcriptome that occur during the early stage of RSV infection indicated that prior to virus filament formation, there was an increased expression of several enzymes in the mevalonate pathway, including the HMGCR (Yeo et al. 2009). This indicated that prior to virus particle formation, virus infection induced the early increased expression of the mevalonate pathway, and since the HMGCR activity is transcriptionally regulated, this also suggested increases in the HMCGR activity. In this context, RSV filament formation is inhibited by lovastatin in a cholesterol-independent manner, suggesting that the involvement of the HMGCR in particle assembly was not related to cholesterol biosynthesis (Ravi et al. 2013; Gower and Graham 2001). Since lipid raft microdomains are composed of cholesterol, this also suggested that the inhibitory effect of lovastatin was not related to the destabilization of these membrane microdomains. This inhibition, however, could be reversed by providing exogenously added geranylgeranyl pyrophosphate (GGPP) to the cells, which is one of the isoprenoids that is generated via the mevalonate pathway (Malhi et al. 2021). This suggested that the antiviral effects of statins were related to the inhibition of the isoprenoid formation, and in this context, the membrane association of activated rhoA and rac1 proteins is mediated by protein prenylation (Wang and Casey 2016). Recent evidence has demonstrated that statins inhibit rac1 protein prenylation in RSV-infected cells (Malhi et al. 2021), suggesting that lovastatin, and other similar statin drugs, may inhibit virus filament formation by blocking the trafficking of the activated Rho GTPases to the cell membranes at the site of RSV particle assembly. Furthermore, lovastatin treatment inhibited localized RSV transmission (Gower and Graham 2001; Ravi et al. 2013), suggesting that F-actin remodeling via the membrane trafficking of Rho GTPases also plays a role in facilitating localized RSV transmission. Collectively, these data provide a functional link between RSV-induced changes in the expression of at least one cellular

metabolic pathway and the trafficking of Rho GTPases to the site of RSV particle assembly during RSV infection.

In cells that produce RSV VLPs, the rac1 protein was also been shown to be present at the lipid raft microdomains where the VLPs formed (Ravi et al. 2021). It is suggested that the presence of the rac1 protein at the lipid raft microdomains that are used during virus particle assembly may be required to modulate F-actin remodeling at these sites during the process of virus filament formation. Although the RSV VLPs resemble virus filaments, these VLPs are not the same as the virus filaments that form on infected cells. It is proposed that other aspects of RSV infection would be required to initiate cell signaling events that lead to the activation of the Rho GTPases at these sites. These signaling events would ultimately lead to the modification of these cellular locations on the plasma membrane and lead to the formation of the RSV assembleome prior to virus filament formation.

Future Perspectives

The virus filaments are a robust feature of RSV infection in permissive cells, and their presence in primary airway cells (e.g. hNEC) has reinforced their clinical relevance. Research efforts are being undertaken to understand both the structure of the virus filaments and the cellular processes that lead to their formation in RSV-infected cells. This information should facilitate the development of novel antivirus drugs that can block RSV particle assembly and prevent infection, and this information should also aid in the development of new RSV vaccine candidates, for example, VLPs. The work of several laboratories described in this review has highlighted the important role that the host cell factors play during RSV particle assembly. Several host cell factors identified at the assembleome also contribute to the formation of infectious and mature RSV particles. There have been several high-resolution structural analyses performed on cell-free RSV particles using cryo-electron microscopy (e.g., Liljeroos et al. 2013). However, in these analyses, it has been assumed that all the data that is revealed in these images of the purified virus particles is of virus origin. The research undertaken by several groups clearly indicates that host cell factors are also incorporated into the virus particles, but these non-virus factors are not featured in these high-resolution structures. It would be expected that these host cell factors will have an influence on the spatial organization of the virus proteins in the virus envelope, and consequently, may influence the architecture of the infectious mature virus particle. The challenge to understanding the complete architecture of RSV particles will be to identify and locate these host cell factors in the virus particles. This could be achieved, for example, by using a combination of high-resolution imaging and specialized labeling procedures, similar to the procedures that have been described for locating the presence of the cav-1 protein in the RSV filaments (Ludwig et al. 2017).

The collective body of evidence has established that virus filament formation involves the activation of cell signaling networks that are regulated by the rac1 and

rhoA proteins. However, these proteins are upstream members of a larger signaling network, and future work will need to focus on identifying the important downstream proteins that are acted on by these signaling pathways during RSV infection. It is possible that during virus particle morphogenesis, these downstream effectors may act on one or more virus or host cell factors, and as a consequence, the biological properties of these factors may be modified. The identification of downstream effectors that are activated by the rac1 and rhoA proteins during RSV infection will be required to obtain a complete understanding of the molecular events that lead to RSV particle assembly. It may also be possible that the identification of these down steam effectors could also be used to better understand the process of virus particle assembly in other related paramyxoviruses.

Lastly, the body of work on RSV assembly has utilized cell systems that are permissive to RSV infection. This work has provided a proof-of-concept that cell factors play important roles in the RSV assembly process, and that several of the host cell factors identified are potentially druggable. This opens the possibility of using drug repurposing to target these cell factors using established drugs with existing safety profiles. This could shorten the time to administer effective and cost-effective treatments for RSV infection, particularly in low-income counties where many RSV-related childhood deaths occur (Nair et al. 2010). The future challenges will be to determine if these host cell factors that have been identified using permissive cells can form the basis for cost-effective antivirus strategies for use in patients. For example, statin-based drugs have known safety profiles and are used by many people to regulate blood cholesterol levels, such as for example, lovastatin (Mevacor®). These drugs are also effective at inhibiting RSV infection in both permissive cells and in animal models of infection, and the mechanism of its antiviral action is now being elucidated. Such drugs could be used to treat RSV infection, and it is presumed that some of the adverse side effects associated with the long-term use of statins will not be encountered during their short time use to treat RSV infection. It will therefore be interesting to determine if these and other cost-effective drugs that block RSV assembly can be translated from the bench to the bedside to control RSV infection in human patients.

Acknowledgments We would also wish to acknowledge previous group members at the MRC Virology Unit in Glasgow and at the Nanyang Technological University in Singapore, including previous colleagues and collaborators Gaie Brown and Jim Aitkin, (Institute of Virology, Glasgow UK) and Chris Jeffree (EMCB, Biological Sciences EM Facility, University of Edinburgh UK) for light and electron microscopy imaging of RSV. In addition, we acknowledge previous funding support to RJS from the Medical Research Council (UK), National Medical Research Council (Singapore), Singapore-MIT Alliance for Research and Technology (Singapore), and the Ministry of Education (Singapore).

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