[Frontiers in Bioscience, Landmark, 18, 696-715, January 1, 2013]

Matrix proteins as centralized organizers of negative-sense RNA virions

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1. ABSTRACT

Matrix proteins are essential components of most negative-sense RNA, enveloped viruses. They serve a wide range of duties ranging from self-driven membrane budding and coordination of other viral components to modulation of viral transcription. The functional similarity between these proteins is striking, despite major differences in their structures. Whereas biochemical and structural studies have partly been hindered by the inherent aggregation properties of these proteins, their cellular functions are beginning to be understood. In this review we summarize the current knowledge on negative-sense RNA virus matrix proteins and their interactions with other viral and cellular proteins. We also discuss the similarities and differences in matrix protein functions between the different families within the negative-sense RNA viruses.

2. INTRODUCTION

Negative-sense RNA viruses are a large group of viruses that includes members capable of infecting organisms from both animals and plants. The group contains a number of significant human pathogens, such as influenza, rabies (RABV), measles (MV), ebola (EBOV) and human respiratory syncytial (HRSV) viruses. Typically the virions are heterogenous in size and shape and lack a rigid well-ordered protein capsid. Exceptions to this general scheme are the rhabdoviruses that form a bullet-shaped relatively regular particle (1, 2) and some of the bunyaviruses where a number of icosahedrally-symmetric particles can be found (3, 4). Generally the genome is contained in a helical nucleocapsid composed of nucleoprotein and the RNA genome. This nucleocapsid is enclosed in a host-derived lipid envelope along with the

RNA-dependent RNA polymerase, polymerase cofactor protein(s) and matrix protein. The membrane is spanned by one or two glycoproteins that are responsible for host cell attachment, membrane fusion and in the case of orthomyxoviruses and some paramyxoviruses also host-cell detachment. In addition some members of the negative-sense RNA viruses have an additional membrane protein, like the M2 of influenza A and the small hydrophobic protein (SH) of some of the paramyxoviruses. M2 forms a proton conducting channel responsible for acidification of the virus interior in cell entry but has recently been shown to also have an important role in membrane scission in the final stage of influenza budding (5). The role of the SH protein is less clear but it has been shown to act as an inhibitor of TNF-α signalling in infected cells for several paramyxoviruses (6-9) and has also been suggested to be a cation-selective oligomeric, ion channel in virion membranes (10, 11).

Escape of enveloped viruses from mammalian cells is an elaborate process which not only requires the viral components to interact and assemble but also relies heavily on the machinery of the host cell. Cellular machinery has a role especially in transport of the components from the cytoplasm and nucleus to the cell membranes but also in regulation of the trafficking via post-translational modification of the proteins and scission of the budding virion in the final stage of egress. The vast majority of these RNA viruses bud from the host plasma membrane, except bunyaviruses that bud from the Golgi apparatus. Many viral matrix proteins contain so called late domains (L-domain) that interact with the host cell vacuolar protein sorting pathway (VPS) components, such as VPS4. VPS4 is an ATPase belonging to the superfamily of the ATPases associated with diverse cellular functions, commonly known as AAA proteins. VPS4 functions in the last stage of VPS, when vesicles pinch off from the membrane. Inhibition of virion budding by expression of dominant negative VPS4 has been widely used as a test of virus VPS dependency in budding. The L-domains are short conserved sequence domains present in the matrix proteins of several enveloped viruses and were first identified as indispensable domains for budding of HIV, present in the HIV matrix Gag protein (12-15). Mutations in the L-domains result in defects at a late stage of virion budding, hence the name late domains. L-domains have also been found to have a role in adenovirus entry, where ubiquitinylation of an internal capsid protein by host Nedd4, a cellular HECT family E3 ubiquitin ligase, is required for microtubule-dependent trafficking of the capsid towards the nucleus (16). The ESCRT (Endosomal complex required for transport) complexes 0, I, II and III are part of the host VPS pathway and are normally involved in trafficking of proteins into late endosomes (also known as multivesicular bodies, MVB) that normally transport cargo for lysosomal degradation. The name MVB derives from the fact that most late endosomes contain intraluminal vesicles that have budded inwards to the endosome. Budding into the MVBs is topologically similar to the budding of virion outwards from the cell and therefore it is logical that many viruses have evolved mechanisms to take advantage of this host cell machinery for budding (For a review on ESCRTs, see (17)).

How do viruses coordinate assembly of the required components together at the appropriate time and place to allow formation of functional viral particles? The main actors of this molecular play are the matrix proteins. They serve multiple tasks in virion assembly and thus are the focus of this review. They have multiple interaction partners, binding to the nucleocapsid, the tails of the surface glycoproteins, cellular membranes and themselves to form helical assemblies on the nucleocapsids (1, 18, 19) or sheet-like assemblies under the membrane bilayer (19). These interactions drive the processes required for coordinated virion assembly. Many of the matrix proteins show an inherent budding ability in the absence of other viral components and upon transient expression are able to bud into virus-like particles (VLP) (20-22). The important role of the matrix proteins in budding was initially shown with vesicular stomatitis (VSV) and Sendai virus (SeV) temperature-sensitive (ts) mutants (23, 24). At the nonpermissive temperature budding was inefficient and the resulting particle morphology in the case of VSV was altered from the normal bullet shape into spherical pleomorphic particles (25). With both viruses the ts defects in budding could be complemented *in trans* by providing a WT M protein (26, 27). For SeV virus it was shown that the ts M was functional but unstable, as over-expression of the mutant M could still result in normal budding once sufficient M had accumulated (27).

Transient expression of M mutants alone and in combination with other viral components, followed later by reverse genetics have led to an improvement in our understanding of the role of M in –ssRNA virus budding through the use of engineered mutations and deletions. For RABV it was shown that a complete deletion of the M gene resulted in a five hundred thousand fold drop in virion release (28), underlining the importance of the M protein in particle release. A particularly interesting case is MV that in rare cases causes a lethal neurodegenerative disease subacute sclerosing panencephalitis (SSPE). In SSPE strains the major genetic changes occur in the M gene that has undergone hypermutation of U to C residues, which results in M protein unable to support normal budding (29). It has also been shown that *in vitro* expressed SSPE strain M proteins are defective in nucleocapsid binding (30). The effects of SSPE M has also been studied in mice using rescued viruses and it was shown that a matrix-lacking virus penetrated deeper into mice brain than other strains and a rescued SSPE strain caused a disease similar to SSPE in humans (31).

Most studies so far have focused on qualitative description of different M variants, leaving much work still to be done on the biochemistry and structure of the proteins and their assemblies. Biochemical and structural studies have been hindered by the inherent properties of M proteins, mainly by their hydrophobic nature and their tendency to oligomerize, which have made the proteins difficult to express, purify and handle. Nevertheless an M crystal structure of a representative from each of the major

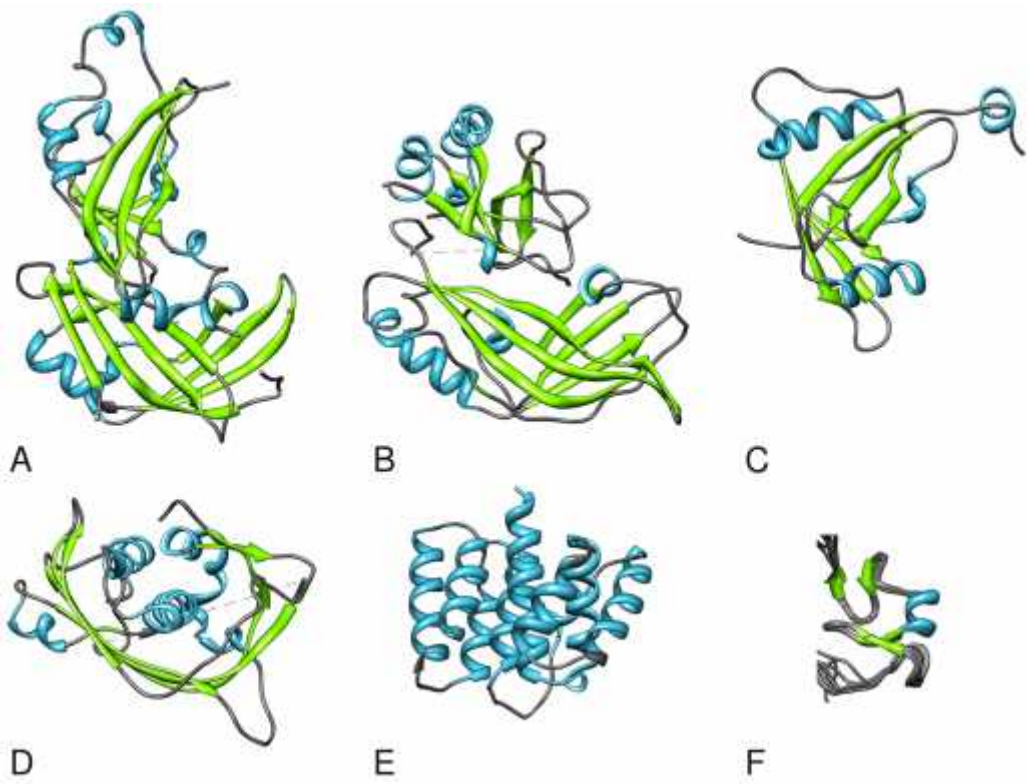

Figure 1. Comparison of matrix protein structures from different viruses. Ribbon diagram of the atomic models from (A) HRSV M (PDB 2VQP), (B) EBOV VP40 (PDB 1ES6), (C) BDV M (PDB 3F1J), (D) VSV M (PDB 2W2R), (E) influenza A M1 (PDB 1EA3) and (F) LASV Z (PDB 2KO5) (32-34, 36, 41, 121). For LASV Z the structured core domain between amino acids 26-79, is shown. The full length protein is 99 amino acids long and the termini are unstructured. The models were rendered in Chimera (221).

–ssRNA virus families has now been solved (32-37) except for the arena- and bunyaviruses which do not have a matrix protein *per se* (Figure 1). Arenaviruses, however, have a multifunctional protein known as the small really interesting new gene (RING) finger protein (Z) serving similar functions to matrix (38-40). A solution structure of the Lassa virus (LASV) Z has been published (41). The structure of Z is completely different to the matrix proteins of other –ssRNA viruses, but also differs greatly from other RING structures (41) (Figure 1). The structures of EBOV VP40 and HRSV M share a similar global fold composed of two domains separated by a linker region and consisting mainly of β-sheets, whereas Borna disease virus (BDV) M is smaller, composed of only domain that corresponds best to the N-terminal domains of EBOV VP40 and HRSV M. VSV M is mainly β-sheet (33), but its topology is unrelated to that of EBOV VP40, HRSV M and BDV M. Influenza M1 structure is totally different from the others, being completely α-helical (35). Thus, regardless of the similar functions of the matrix proteins, the structures and amino acid sequences are diverse.

The multimerization of M proteins into sheets or helices has been reported for several viruses of the –ssRNA group (1, 18, 19, 42-45) and the helical assembly seems to have a role in formation of filamentous particles, but has also been found inside spherical particles surrounding the nucleocapsid in MV (18). Currently it is unclear if the role of the oligomerization of M is to gather the viral components efficiently to the membrane or if the oligomerization has a role in providing the force for budding, or both.

This review focuses on the role of the matrix proteins in –ssRNA virion budding for each family separately and highlights recent advancements in this topic. Also budding-relevant M interactions with other viral proteins and host factors are discussed. For more detailed information on the different families, there are several recent reviews available (46-50).

3. ROLE OF MATRIX PROTEIN IN VIRUS ASSEMBLY AND BUDDING

3.1. Paramyxoviruses

Paramyxoviruses form of a large group of viruses with pleomorphic morphology. The viral particles are generally spherical and large, the diameter of MV for example can be up to 600 nm, possibly even larger (18). Some viruses of the family have also been observed to form filamentous virions with a length of up to 10 µm (51-53). The matrix gene sequences within the family are quite varied in sequence and length, for example HRSV M is 25 kDa whereas MV M is 37 kDa and the amino acid

sequences of the two do not produce a reasonable alignment. The only member of the family for which an M protein crystal structure has been solved is HRSV (34). The structure is composed of two, mainly β-sheet containing, domains that are connected by an unstructured linker region. The protein has an extensive positively-charged surface that is suggested to interact with negatively-charged phospholipid head groups in the membrane. The fold and the charged surface are conserved in both EBOV VP40 (RMSD 3.7 Å) and BDV (32, 36). Although HRSV M crystallized as a monomer, it is likely that the protein exists in a multimeric state within virions at least in filamentous HRSV particles as the protein is capable of forming helical assemblies on artificial membranes (45) and there is also evidence of a helical protein assembly under the membrane of filamentous HRSV particles from electron microscopy of freeze-fractured purified virus (51). Three dimensional ultrastructure descriptions of paramyxoviruses from electron cryotomography are currently limited to SeV (54) and MV (18) in which the M protein seems to have slightly different roles. MV matrix was shown by sub-tomographic averaging and immuno-capture electron microscopy to be predominantly wrapped around the helical nucleocapsid forming a helix itself. The nucleocapsid is a so called one-start helix, whereas the M-helix is a five-start helix, which indicates some flexibility in the way the nucleocapsid and M interact. In SeV virus such helical assemblies were not reported from electron cryotomography and M apparently partially lines the inner leaflet of the lipid bilayer. It has, however, also been shown for SeV by *in vitro* assembly and electron microscopy that isolated M is capable of forming sheets and helices (44, 55) that might reflect how the protein is organized under the viral membrane.

Transient transfection of cells with M gene-containing vectors has yielded valuable information on what viral components are required for VLPs to bud from the cell membrane. For several of the paramyxoviruses expression of M alone is sufficient for VLP formation (22, 56-61) but some require nucleoprotein (NP) and one of the glycoproteins for efficient VLP production (62, 63). Further evidence for M protein's central role in paramyxovirus budding comes from early studies with ts mutants and recombinant viruses lacking the M gene. These studies demonstrated that a stable pool of M is required for virion budding as ts mutants at the nonpermissive temperature and M-lacking viruses showed severe defects in infectious particle production (27, 31). It has been shown that preventing M accumulation in SeV infection by siRNA, reduces virion budding 50-100 fold (64) and that a mutation of valine 101 to alanine, a residue conserved in 13 out of 14 paramyxoviral sequences compared, prevents accumulation of M leading to 10-100 fold drop in virion release without affecting other M functions (58). Newcastle disease virus (NDV) M is even capable of deforming membranes and budding when purified M is incubated with phospholipid bilayer vesicles (65), which highlights the self-standing role of M polymerization in membrane deformation, just as has been demonstrated for I-BAR motif proteins (66). However, unlike in the I-BAR proteins, there is still a lack of understanding of the exact nature of the interactions of M with lipid which drive the process of deformation. Infection with HRSV lacking M expression was shown to result in formation of filamentous protrusions on the cell surface, but these protrusions were much shorter than those seen with WT virus infected cells (67). This led the authors to suggest that M is not required for initiation of the viral filament, but is needed for maturation of the filaments to full length virions. Although these experiments indicate that M is one of the factors important for budding, it has been observed that the expression of just the SeV F (60), MV F (22) and Nipah virus (NiV) F and G (68) glycoproteins alone result in budding VLPs. However, it is also important to remember that VLP release upon transfection of viral components does not necessarily correspond to what is required for budding of virions from infected cells. Influenza A hemagglutinin (HA), for example, drives VLP release when expressed alone, but during infection other viral proteins, like M2, are required (69). In addition, budding *per se* does not ensure the incorporation of the genome into the particles, hence matrix is central here as it interacts with both the genome-containing nucleocapsid and the membrane.

Several of the paramyxoviruses undergo polarized budding in epithelial cells and bud preferentially from the apical membrane. Viruses for which this has been demonstrated include HRSV (70), MV (71), human parainfluenza virus 3 (HPIV3) (72), parainfluenza virus 5 (PIV5) and SeV (73). HRSV fusion protein (F) has a sorting signal to the apical membrane, but a virus lacking all the surface glycoproteins still buds from the apical membrane (74). MV glycoproteins instead are not preferentially translocated to the apical membrane when expressed without other viral components, but are taken to the apical side in virus infection (75). Furthermore, epithelial cells infected with a virus lacking the M gene do not show polarized distribution of the surface glycoproteins which demonstrates the importance of M in glycoprotein apical targeting (76). Thus M seems to define the polarity of budding at least for MV, and probably also for HRSV. In NiV infection of polarized epithelial cells the distribution of the viral surface glycoproteins differ to that of F or G transfected cells (77). Infected cells show a bipolar distribution, whereas in transfected cells the majority of the glycoproteins are sorted to the basolateral membrane. Whether the difference is due to M or some other viral component has yet to be studied.

In addition to a preference for budding from one side of the epithelial cell, many of the paramyxoviruses tend to bud from lipid raft microdomains. Lipid rafts are cell membrane domains that are enriched in sphingolipid, cholesterol and proteins. They have multiple roles in membrane signalling and trafficking (reviewed in (78)) and serve also as a budding platform for many viruses. Characteristic of lipid rafts is their resistance to solubilisation by cold non-ionic detergents and therefore they have been referred to as detergent-resistant membranes (DRM), although detergent resistance of a membrane fraction is not necessarily a guarantee that it is composed solely of lipid raft (78, 79). The main factors for determining the lipid raft localization for paramyxoviruses

vary. MV structural proteins are enriched in lipid rafts in virus infection (80, 81). When M is expressed alone it is partly taken into lipid rafts, but enrichment is enhanced by coexpression of F (22). F expressed alone localizes into lipid rafts but the hemagglutinin (H) does not. When F and H are expressed together, H also localizes into lipid rafts, which reflects the interactions between the two proteins (80). Since M functions as a carrier of the nucleocapsid (82) to cell membranes and M is efficiently localized to lipid rafts when F is present, it could be the lipid raft association and the interaction between F and M that finally merges the virion components coming through the ER-Golgi route to the components that are taken to the cell membrane by other, currently poorly characterized means. For SeV the structural proteins F, HN, M, N and P are all at least partially present in lipid rafts upon infection (83). Expression of F or HN is however required for M targeting to lipid rafts (84). In HRSV infected cells M is found partly in lipid rafts and using recombinant M from Vaccinia vectors it was shown that M does not associate with lipid rafts unless F is present (85).

Paramyxoviruses vary in the extent to which they utilize cellular pathways in budding. Although many of the interactions between viral and host cell components remains elusive, some of the paramyxoviruses have been shown to be dependent on the host ESCRT machinery for budding (63, 86) whereas others are ESCRT-independent (87, 88). PIV5 virions and mumps virus (MuV) VLPs have been shown to be dependent on the ESCRT machinery in budding as budding was inhibited by expression of dominant negative VPS4A (63, 86). HRSV and MV budding was not blocked in similar experiments (87, 88) and thus they do not require ESCRT for exit. HRSV requires instead a functional FIP2, an apical recycling endosome-associated protein for release from the host cell (88). The L-domain sequences identified so far for paramyxoviruses are different from the canonical sequences (P[T/S]AP, PPxY, YxxL) and it is possible that the budding pathway, despite requiring some common components with for example retroviruses, is not identical to that of viruses with canonical L-domain sequences. The crucial amino acid sequence for PIV5 is FPIV and FPVI for (MuV). The importance of ESCRT and the viral L-domain motifs involved for SeV, NiV, and human metapneumovirus (HMPV) are contradictory. SeV was first reported to interact with AIP1/ALIX, a component linking ESCRT-1 and ESCRT-3, both with M and an accessory C-protein (89, 90). Also, dominant negative VPS4A was reported to inhibit budding of SeV (90). Yet Gosselin-Grenet *et al.* concluded that SeV budding is not at all dependent on AIP1/ALIX or VPS4A (91). A later study, where M-deficient SeV was recovered and found to be mutated at the YLDL corresponding position, again demonstrated the importance of the YLDL motif and AIP1/ALIX in budding (92). For NiV it has been shown that the YMYL motif on M protein is required for VLP budding and that the motif can complement the budding defect of an L-domain mutant of EBOV VP40 (61) leading the authors to present YMYL as a potential L-domain. In a later study it was shown that NiV VLP budding also requires the YPLGVG sequence and that expression of dominant-negative VPS4 proteins has no effect on budding (93). Hence these authors concluded that NiV did not require ESCRT. Thus, the matter of ESCRT dependency in NiV is still unclear. In (HMPV) M a canonical L-domain sequence YAGL was found to be important for budding but could not be changed to the classical PPPY and YPDL L-domains from moloney murine leukemia virus and equine infectious anemia virus nor was it affected by expression of dominant-negative VPS4 (94). The YAGL sequence did not act as an L-domain but instead it is important for higher-order oligomer formation and the normal filamentous morphology of the virion. It is important to note that often the experiments to determine ESCRT-dependency have only been done in one or two cell lines and if the cell lines used in different studies are not the same, the results may not be comparable. Ubiquitinylation also possibly has some role in the budding of PIV5, as M was found to be ubiquitinylated in transfected cells, and mutations of the target lysines to arginine resulted in a six-fold reduction in the maximum titer of the budded particles (95). Treatment of WT-virus infected cells with the proteasome inhibitor MG-132 reduced budding correspondingly and M ubiquitinylation was not detected (86, 95). NiV M can also be ubiquitinylated and the ubiquitinylation is required for retention of M in the cytoplasm (96). Ubiquitin depletion by the protease inhibitor bortezomib blocked budding and resulted in M accumulation in the nucleus. Currently it is a mystery why M of NiV and some other paramyxoviruses undergo nuclear-cytoplasmic shuttling as replication is thought to happen exclusively in the cytoplasm. In addition to ubiquitinylation PIV5 has been shown to be phosphorylated and this phosphorylation is required for interaction with the host cell 14-3-3 protein. This interaction inhibits budding possibly by keeping M away from budding sites (97). M phosphorylation has been reported also for SeV, NDV and MuV matrix proteins but the significance of this modification has yet to be determined (98-101). Phosphorylation of MV M has been reported, but not confirmed (102-104).

Recently, using live-cell fluorescent imaging and nocodazole treatment of infected cells, it was found that SeV nucleocapsid transport to the cell surface is dependent on microtubules and nucleocapsids are transported along microtubules in a directional fashion (105). In addition, nucleocapsids colocalized with RAB11A and transferrin, both indicative of involvement of recycling endosomes in the process. Thus, it seems that at least some of the paramyxoviruses utilize cellular vesicular trafficking along microtubules in the transport of nucleocapsids to the budding sites. It is still unknown, whether this is mediated by the matrix protein, but is likely to be so, as M is required for nucleocapsid transport to the cell surface in several paramyxoviruses including SeV (82, 106).

In addition to the demonstrated roles of M in budding and incorporation of other viral components to the virions, it has been shown to have a role in repressing viral genome transcription for at least MV and HRSV (82, 107, 108). This effect is presumably a consequence of M binding to the nucleocapsid and making it transcriptionally

inactive preceding the transport to budding sites. For MV this could be mechanistically explained by the formation of an M helix around the nucleocapsid preventing the polymerase complex from contacting the RNA (18). In contrast, siRNA suppression of SeV M did not have any demonstrable effect on viral replication or transcription (64).

3.2. Orthomyxoviruses

Orthomyxoviruses comprise a small family of viruses of which the best known members are the influenza viruses. There are three members in the influenza group, (influenza A, B and C), all capable of causing disease in humans. Of the three species influenza A is the most rapidly evolving and is responsible for the majority of epidemics caused by influenza viruses. Influenza A virions are pleomorphic and form mainly spherical particles of approximately 100 nm in diameter or filamentous particles with similar diameter but up to 20 um in length (19, 109, 110). In this respect the influenza A particles are relatively similar to the paramyxoviruses, except that the variance in the size and shape of the nonfilamentous particles is greater in the paramyxoviruses. Whereas at least some of the paramyxoviruses can accommodate multiple copies of the nonsegmented genome (111), influenza A virions probably contain only one copy of the genome that is composed of 8 segments (112), which is reflected also in the generally smaller size of the influenza particles compared to the paramyxoviral particles (18). For influenza A it is thought that the clinically more relevant form of the virion is filamentous as samples isolated from infected patients are predominantly filamentous shape (113, 114) such as the 2009 pandemic H1N1 (115).

Influenza virus budding was originally reported to be driven by the M1 protein as expression of M1 from T7 RNA polymerase-expressing Vaccinia virus infected cells transfected with M1 gene carrying plasmids resulted in M1 release in to the culture supernatant (116). These results were later questioned by another study reporting that HA plays the main role in budding and that M1 expression alone does not result in VLP budding (117). M1 is, however, required for transfer of a reporter GFP gene in the VLPs to target cells. So M1 has a clear role in incorporating the genetic material into budding VLPs but is unlikely to be solely responsible for driving VLP budding. Interestingly, however, it is M1 that defines the filamentous virion morphology. The requirement for at least M2 (69) for budding in addition to HA during infection underlines the fact that virion budding is a complex interplay between several viral components. Again it is obvious that results from VLP budding studies are not always conclusive about what happens in virion budding. In viral budding HA also seems to be dispensable and the initiation of budding can be taken over by other viral proteins when HA is mutated or deleted. The resulting particles are defective, however, and it is unknown whether this is only because of the indispensable functions of HA in cell attachment and entry or also because of other possible defects (118). Although the protein(s) responsible for bud initiation in infection is still uncertain, the role of M2 has been shown to be in mediating an ESCRT-independent membrane scission in the final stage of virion budding (5). It was also shown that influenza does not require the ESCRT pathway components VPS4 or VPS28 for budding, further solidifying the proof of influenza independency of ESCRT in budding (119, 120).

The M1 of influenza A is a 252 amino acid 28 kDa protein that is composed of α-helices connected by flexible loops (35, 121, 122). The C-terminus (residues 165-252) of the protein has been refractory to crystallization, but solution studies have shown it to be mainly flexible with some α-helical content (121, 123). As influenza enters the host cell via endocytosis and requires acidification of the virion and acid-mediated activation of the fusion machinery, the structure of M1 has been solved in both neutral (121, 122) and acidic pH (35). From these structures it is clear that acidic pH does not result in considerable changes in the fold of the N-terminal fragment of M1 (residues 1-164). Concomitantly, the structural change upon acidification has been suggested to occur in the linker region that contains a zinc-binding domain (124). The structure of this linker region was studied in neutral and acidic pH using circular dichroism, Raman and absorption spectroscopy and it was shown that upon acidification a bound zinc ion is released from the peptide and the structure changes from a partially unfolded conformation to an α-helix-rich conformation. It has also been shown that intact influenza virions contain zinc but only a fraction (4% - 20% depending on the strain) of M1 has zinc bound to it (125). Furthermore, from electron cryotomographic studies it is evident that the M1 layer undergoes a major change as a whole upon a pH drop from neutral to pH 4.9 (19, 109). At pH 4.9 the M1 layer is no longer visible close to the membrane but instead it forms a large aggregate together with the RNPs in the virion interior. What is the order of events in entry, fusion before M1 aggregation or vice versa is still unclear from current electron cryotomographic studies. If M1 falls off from the membrane before fusion, a distinct M1-RNP uncoating step would be required. An analogous uncoating problem is present in measles virus where the M is already detached from the membrane and bound to nucleocapsid before binding the host cell (18).

The influenza A M1 protein is thought to be monomeric and soluble in the cytoplasm (126) and undergoes nuclear-cytoplasmic shuttling mediating the export of RNPs from the nucleus (127, 128). M1 is also capable of inhibiting the RNA transcriptase complex in a minigenome system and binds directly to RNA (129, 130). M1 can oligomerize at the membrane to form helical structures lining the inside of the viral membrane in filamentous virions (19). The only post-translational modification identified so far on M1 is phosphorylation, mainly on serines, but the functional significance of such modifications is not yet known (131, 132). The phosphorylation can be executed by the host phosphokinase C (133). M1 has been shown to bind to the cytoplasmic tails of both the HA and NA and it has been suggested that this interaction ensures the incorporation of M1 into virions (134). M1 also binds the RNP (135) and is possibly required in mediating the transport of RNPs to the budding

sites as it is required to retain RNPs in the cytoplasm after export from the nucleus following replication (127). Recently it was reported that cellular RAB11A has a crucial role in transport of RNPs to the plasma membrane (136), but whether the interaction between RAB11A and the RNPs is direct or mediated by M1, remains currently unknown. Thus, similarly to the paramyxoviruses, the M1 matrix protein serves as a bridge between the membrane, HA, NA and the RNP in gathering all the required viral components into a functioning virion (137, 138). However, influenza A M1 does not seem to have intrinsic membrane targeting properties but is directed to membranes via the interaction with the envelope proteins (139). It has been suggested that the sequence of events in budding could occur in the following order: first, HA and NA cluster into lipid raft domains and initiate a bud, second, M1 together with RNP is recruited to the membrane where M1 multimerizes causing elongation of the bud and incorporation of the RNP to the budding tip, last, M2 located at the edge of the budding lipid raft interacts with M1 and cleaves off the virion from the cell membrane by insertion of an amphipathic helix at the lipid phase boundary (47). Thus, compared to paramyxoviruses, influenza A budding seems to be a slightly more complicated event, where counteracting membrane-curving forces balance each other to allow for optimally timed budding and scission to include all the essential components of an infectious virion.

3.3. Filoviruses

Filoviruses comprise a group of pathogenic human and non-human primate viruses that cause hemorragic fever. The family contains two genera, Ebolaviruses and Marburg viruses. The virions are predominantly filamentous in shape and contain a helically arranged nucleocapsid inside a membrane tubule covered by the VP40 matrix protein on the inner side of the membrane (42, 43). Conversely to the paramyxoviruses and the orthomyxoviruses there is only one glycoprotein spike, GP, on the membrane. In common with many of the paramyxoviruses the matrix protein is mainly responsible for virion budding and is also able to drive release of VLPs when expressed on its own (20, 140, 141). VLP budding is, however, enhanced by coexpression of GP and NP indicating that also other viral proteins have a role in efficient budding (140, 142). In MARV infection most of the GP is sorted to the apical side of polarized epithelial cells, but budding occurs exclusively from the basolateral side (143). Therefore it is likely that other viral proteins than GP determine the budding site.

The crystal structure of the monomeric EBOV VP40 shows that it is folded in two related β sandwich domains that are connected by a flexible linker similar to HRSV M (32, 34). VP40 has a tendency to self-associate into hexamers and octamers formed from dimers (144). The hexameric form has been associated with membrane binding (145), whereas the octameric form shows specific RNA binding properties (146) and is important in replication (147). VP40 was found to be monomeric in the cytosol, but hexameric upon binding to membranes containing negatively-charged lipids. The domain responsible for inducing hexamerization was mapped to the C-terminus of the protein (145, 148). It has also been shown that oligomerization of VP40 is important for EBOV VLP budding and that it is required for repression of transcription, but not for repression of viral genome replication (149). VP40 is also required for nucleocapsid transport to the cell surface and it binds directly to NP (150, 151). EBOV VP40 also has a tendency to localize to lipid rafts (152).

As with other –ssRNA viruses the means by which VP40 and the nucleocapsid are transported to the budding sites is incompletely characterized. Obviously cytoskeletal proteins are good candidates for having a role in this. For filoviruses roles for both tubulin and actin have been described (153, 154). EBOV VP40 was shown to colocalize and immunoprecipitate with tubulin and a direct interaction between the two was shown *in vitro*. In addition, *in vitro* studies demonstrated VP40-induced tubulin polymerization (153). Actin was detected inside MARV particles and actin depolymerization by Cytochalasin D significantly diminished VP40 VLP budding (154). Tubulin depolymerization by Nocodazole did not influence VP40 budding (154) and MARV VP40 does not contain a region corresponding to the tubulin binding region of EBOV VP40 (153), so the two viruses probably differ in their requirements for cytoskeletal proteins in virion assembly. In addition to actin and tubulin, a role for COPII vesicular transport system in filovirus budding has been demonstrated (155). VP40 was found to interact directly with Sec24c, part of the COPII complex responsible for ER-Golgi vesicle transport. Interfering with the function of COPII components inhibited the budding of VLPs and the essential VP40 amino acids for Sec24c interaction were required for plasma membrane accumulation of VP40. As noted in the article (155), COPII vesicles are not normally transported to the plasma membrane so the link between COPII and vesicles and plasma membrane is still undetermined. As COPII vesicles transport is microtubule-dependent (156) and VP40 binds to and polymerizes microtubules (153), it is possible that VP40 could alter the routing of the COPII vesicles to the plasma membrane. This is supported by the fact that VP40 expression changes the location of Sec24c and Sec24a to the plasma membrane (155). In addition to the demonstrated role of COPII vesicles in EBOV budding, COPI vesicles have also shown to have some role in that (157).

MARV VP40 has been shown to be associated with MVB membranes (158) and to employ the retrograde late endosomal route for its transport to the plasma membrane (159). It has also been shown that expression of VP40 with GP redistributes GP from the trans-Golgi network to the MVB indicating that the MVBs is the location where VP40 and GP meet for assembly into virions (160). It is currently still unclear how the findings with EBOV and MARV on the involvement of cellular vesicular trafficking in budding come together and to what extent the two viruses make use of the same cellular functions.

Recent electron cryotomographic studies on EBOV and MARV indicate that the nucleoprotein is organized into a rigid helix inside the filamentous virions together with VP24 and VP35 (42, 43). VP40 was found to be required for condensation of the NP helix in EBOV VLPs, but it was not organized around the nucleocapsid in a regular manner. In MARV some order was found within the VP40 layer but again it was not organized into a rigid helix around the nucleocapsid. The lack of shared symmetry between the nucleocapsid and the matrix layer despite their close interactions is similar to what has been found in MV (18), but differs from the rhabdoviruses where M and nucleocapsid are arranged in two concentric helices with the same symmetry (1).

VP40 of filoviruses contain L-domains that are associated with the involvement of the host ESCRT -machinery in budding of the virions. The L-domain of EBOV VP40 consists of two overlapping late sequence motifs PTAP and PPXY near its N-terminus. The PTAP motif was shown to be crucial for VLP budding via binding to the host Tsg101, a component of the ESCRT-I complex (161). Later it was shown that the PTAP and PPEY are L-domains contributing to efficient budding (162). The PPEY domain in oligomeric VP40 was shown to bind Nedd4 and the PTAP domain to bind Tsg1 irrespective of the oligomeric state of VP40 (163). VP40 could also be ubiquitinylated *in vitro* by yeast Rsp5, a Nedd4 homologue (164). It is, however, in general unclear how important the ubiquitinylation of the M proteins is for budding. In fact, it was recently shown with a lysine-free retroviral Gag that ubiquitinylation of one of the interacting components needed to recruit ESCRT is required, but it seems to be irrelevant, whether it is a viral protein or one of the essential cellular proteins (165). Despite the proven importance of L-domains in budding, it has been shown that recombinant viruses with mutations in the late domains are still capable of replicating in cell culture albeit with approximately 10-fold attenuation (166), so other viral proteins might be able to compensate for the lack of functional L-domain in VP40. MARV only has the PPXY domain and in its case an interaction with Tsg101 is mediated by that motif (167). Confusingly, MARV VP40 was found to contain other non-late domain amino acid residues that were important for VP40 VLP budding and the role of PPXY-Tsg101 interaction was hypothesized to be dispensable. Also, the NP was found to contain a PSAP motif that was shown to bind Tsg101 and to have an important role in NP-mediated enhancement in budding efficiency (168, 169). Thus, other viral proteins than VP40, can also help to recruit the ESCRT-machinery. Recently, the tyrosine in the PPEY domain of EBOV was found to be phosphorylated by the c-Abl1 tyrosine kinase independently of Nedd4. Budding was inhibited up to 10^4-fold when c-Abl1 was downregulated by siRNAs or inhibited by nilotinib, an inhibitor of the Abl-family (170). Although tempting to draw a direct line between VP40 phosphorylation and budding, it is also known that c-Abl is involved in varied cellular processes including F-actin regulation (171), so inhibiting c-Abl expression could affect other budding-relevant cellular processes. A direct link is supported, however, by the fact that mutating the phosphorylated tyrosine on VP40 to alanine reduced VLP budding by 84 % (170). MARV VP40 has also been found to be phosphorylated (172). Mutation of the four major phosphorylation acceptor tyrosines to phenylalanine did not affect VLP budding or the cellular distribution of VP40, but inhibited incorporation of nucleocapsids into the VLPs.

3.4. Rhabdoviruses

Rhabdoviruses are a large group of viruses infecting organisms within animals and plants. They are typically bullet-shaped and have an unusually well-organized structure compared to other –ssRNA viruses. The most well studied members of the family are VSV and RABV belonging to the genera *Vesiculovirus* and *Lyssavirus*, respectively. VSV is the prototype of the family and its biology is one of the best known within the *Mononegavirales* whereas RABV is the most significant human pathogen in the family. Rhabdoviruses replicate in the cytoplasm and typically the genome contains only five genes. Similarly to the filoviruses, rhabdoviruses have only one surface glycoprotein, G, on their lipid envelope that is responsible for host cell attachment and fusion.

The regular shape of VSV has allowed single particle cryo-EM analysis of the internal structure (1) revealing the helical arrangement of the matrix protein around the helical nucleocapsid. Although the ectodomains of the G protein were not resolved probably due to the flexibility of the ectodomains or inconsistent symmetry in organization, the tails of G seemed to be touching the underlining matrix helix. In addition, direct interactions between M and NP were evident in the structure. Conversely to the filoviruses and MV, the matrix layer was found to follow the symmetry of the nucleocapsid, or most likely vice versa as it is known that M is required for condensation of nucleocapsid into the form found in virions (173, 174). EBOV VP40 has a similar function in condensing the nucleocapsid (42), whereas in MV no such condensation was found (18). This could be due to the fact that the MV and paramyxoviral nucleocapsids in general can form helical structures when expressed alone in insect cells (175-177). Purified VSV M has a typical matrix protein behaviour of aggregation in physiological salt conditions, but its aggregation can be prevented by infected or uninfected cell cytosol (178) and the majority of the M inside infected and transfected cells is in a soluble form (179, 180).

Crystal structures of the full length VSV and Lagos bat virus (LBV) matrix proteins show that the structures share a common fold even though no sequence homology could be identified (37). The structures are different from all the other available M structures of –ssRNA viruses (32, 34-36), but it is intriguing how many functions all these proteins share despite the apparent lack of homology: membrane, glycoprotein and nucleocapsid binding and self-assembly into ordered structures, to name a few. In the crystal the VSV and LBV M monomers were packed into a linear array through the insertion of a short loop from one monomer in to the hydrophobic cavity of its neighbour (37). This led the authors to propose that the packing might reflect the M-M interaction within the

virions. The subsequent cryo-EM map fitted with the crystal structure (1) revealed that the intermolecular M-M interactions are due to these two regions, but the packing in the virion is helical with the loop insertion occurring between subsequent turns of the M helix. These two articles provide a beautiful example of how two structure determination methods can complement each other and lead to a detailed understanding of the structure of very large complexes, such as viruses. Further investigation of this loop region with mutants confirmed its importance in virus assembly and also demonstrated its role in translation as viruses with mutations in the loop showed reduced viral protein synthesis but unchanged replication or transcription (181). RABV M instead has been shown to regulate the transition from transcription to replication and this function can be abolished by a single R58G mutation (182, 183). The interaction of M with the lipid membrane is mainly electrostatic as purified M binds only to liposomes that have negatively-charged phospholipids in them and binding can be prevented with 500 mM NaCl (184). Additionally, characterization of the viral membrane shows that it is enriched in negatively-charged lipids (2) and in the M structure the main regions contributing to membrane binding are positively-charged (33).

The M protein is the budding directing factor in rhabdoviruses as with most of the other –ssRNA viruses. Importantly, a mutant RABV lacking the gene coding for M was found to bud $5x10^5$ fold less efficiently than the wild-type virus (28). Similarly to the filoviruses and some of the paramyxoviruses the M of rhabdoviruses can cause budding of M-containing vesicles when expressed alone (21, 185). Also as with filoviruses, the G has an assisting role in egress as coexpression of G with M enhances the efficiency of vesicle release by about 10- to 30-fold in VSV and RABV (186, 187). The concerted action of M and G has been pictured as a "push-and-pull" –type of event, where M pushes and G pulls the particle to bud (188). Using ts-mutants, it was also shown that WT M could complement ts-M at the nonpermissive temperature restoring the bullet shape to budded particles when in the absence of WT M budded particles were spherical or pleomorphic (26).

Similarly to the filoviruses and some paramyxoviruses, both VSV and RABV contain L-domains in their M protein N termini. VSV has a PPPY motif at amino acids 24-27 and RABV has a corresponding motif PPEY at positions 35-38 (189-191). VSV has in addition a PSAP motif at amino acids 37-40, which was first found to be dispensable and VSV budding was found to be independent of VPS4A and TSG101 as shown by overexpression of an ATPase deficient VPS4A and depletion of TSG101 by siRNA (192, 193). Later, using recombinant cell lines with accurately controlled expression of mutant VPS4A and VPS4B, it was reported that VSV budding is VPS4 dependent (194). Recently slightly contrasting results about the importance of the PSAP motif in budding were also published (195). In this article it was shown that the PSAP binds to TSG101 in yeast two hybrid screens and mutated PSAP affects budding in some cell lines. Thus it is still unclear how the PPPY and PSAP motifs affect VSV budding. VSV M has also been reported to be phosphorylated, but again, as with most of the other reported –ssRNA virus M phosphorylations, the functional significance is unknown and mutation of the major phosphorylation sites on VSV M had no detectable effect on budding (196).

Similarly to EBOV VP40, RABV in addition to the PPEY motif has another potential overlapping L-domain YVPL (162, 164). Whereas the PPEY domain has been found to be important for interaction with Nedd4 and for the consequent ubiquitinylation required for efficient budding (189, 190, 194, 197), the YVPL motif has yet to be assigned a function.

Another interesting finding was recently reported, where a direct interaction between VSV M and cellular dynamin was demonstrated (198). This interaction was found to be crucial at a late stage of the viral life cycle and the interaction could be abolished by a single leucine to alanine mutation at position 4 in the N-terminal largely unstructured region of M. By interacting with dynamin, the VSV M also affects host clathrin-dependent endocytosis most likely by hijacking the dynamin normally used in endosome transport from the cell membrane (198).

3.5. Borna disease virus

Borna disease virus comprises its own family of *Bornaviridae* differing from other families of the *Mononegavirales* mainly by its exceptionally small genome of 8.9 kb, nuclear replication and transcription and usage of the host RNA splicing machinery (199). BDV infects mainly farm animals such as horses and sheep, but also cats, dogs and zoo animals. BDV is mainly neurotropic and manifests itself in varied neurological disorders such as alterations in behaviour, disturbances in fertility, ataxia and blindness leading finally to paralysis and death (200). In humans BDV has been associated with a wide range of psychiatric disorders (199), but the matter remains controversial. The associations have been mainly determined based on immunoreactivity of patient serum to BDV components or detection of BDV components in patient samples, mainly from peripheral blood mononuclear cells.

BDV biology is much more superficially characterized than that of the other families discussed above, but nevertheless the matrix protein has been successfully crystallized and the structure solved (36). Therefore it is also of interest to discuss BDV M here in comparison to the M structures of other –ssRNA viruses. The size of the BDV M (16 kDa) is significantly smaller than that of other –ssRNA viruses (~25 – 40 kDa). Although initially described as a glycoprotein (201), the BDV M was later shown to be a nonglycosylated membrane binding protein that oligomerizes readily in solution forming mainly tetramers (202, 203). BDV M has been shown to colocalize with the RNP in infected cells, but unusually to –ssRNA viruses it binds the phosphoprotein instead of the nucleoprotein in the RNP complex (204). Later it was shown that M also binds directly to ssRNA oligonucleotides of approximately 16

nucleotides or more in length (36). Many other –ssRNA virus M proteins have also been shown to bind RNA directly (137, 146, 205, 206). Despite binding to the RNP, the BDV M does not to inhibit the activity of the polymerase (204), which is in contrast to many other –ssRNA viruses, like VSV (207), RABV (183), influenza (130), and MV (208) where M represses transcription by the viral polymerase.

In line with the experiments done for BDV M in solution, it also crystallized as a tetramer (36). Although there is no apparent amino acid sequence similarity between BDV M and EBOV VP40 or HRSV M, the structure of BDV M resembles closely the two domains of EBOV VP40 and HRSV M. Yet, as it consists of only one such domain some of its functions are likely to be different. The single domain, however, carries out many of the typical M functions like oligomerization, lipid membrane affinity and RNP binding. Whether or not BDV M is capable of budding when expressed alone, has yet to be studied. If BDV possesses budding capability, the budding possibly involves the host ESCRT machinery as a putative L-domain YVEL is present in the BDV M N-terminal sequence and it is exposed to solvent in the crystal structure.

3.6. Arena and bunyaviruses

Arenaviruses comprise a genus of about 20 viruses and includes some human pathogens but most of the viruses use rodents as hosts. The genus is further divided to two serogroups, the old world and the new world arenaviruses Lymphocytic choriomeningitis virus (LCMV) being the type species and representative of the old world arenaviruses and Tacaribe virus (TACV) of the new world arenaviruses. The new world arenaviruses are further separated into three clades A,B and C. Arenaviruses have a bisegmented genome composed of the large (L) and small (S) segments with sizes of 7.3 kb and 3.5 kb, respectively. Arenaviruses have a specially organized genomic structure in the sense that both L and S are ambisense and code for two proteins, each in different direction. Many of the human pathogenic arenaviruses cause severe diseases, mainly hemorrhagic fever. Such viruses include LASV, Junin, Machupo and Guanarito viruses.

Arenaviruses encode a small 11 kDa RING finger protein which encompasses similar functions to the matrix proteins of other –ssRNA viruses, but shares no homology with them and is much smaller in size. Importantly, the Z protein can drive release of Z-containing particles when expressed alone, is present in viral particles, binds to membranes and contains L-domains important in budding (38, 209). It also interacts with the nucleoprotein N to incorporate the nucleocapsid into budding VLPs (210). This interaction and thus inclusion of N, but also of the glycoprotein complex GPc (formed by proteolytical cleavage of the GP precursor into a stable signal peptide (SSP), GP1 and GP2), into budding VLPs can be abolished by a single point mutation L79A (210) which is thus likely to be critically involved in the interactions. Z proteins of LCMV, TACV and LASV have all been shown to exhibit repression of both transcription and replication of minigenomes (211-213), also a common feature among the matrix proteins. Uniquely within the matrix proteins of –ssRNA viruses, the Z protein of LASV and LCMV are myristoylated and this modification has been shown to be crucial for both Z-mediated VLP budding (214) and for an interaction between Z and GPc (215).

Arenaviral Z proteins contain a variety of different L-domains. Most of the viruses characterized so far, contain the PT/SAP domain, but many contain also the PPPY domain and a few contain a composite of the two, similar to EBOV VP40 (46). These L-domains are positioned in the structurally disordered C-terminal end of the Z. In addition all the characterized Z proteins have an YxxL motif in the RING domain, but it is unknown whether this acts as an L-domain. For TACV Z the YxxL motif was shown not to function as an L-domain, but was required for interaction with NP (40, 216). The same was found for the ASAP motif that replaces the canonical PT/SAP domain of other arenaviruses (40, 216). TACV appears to differ from LASV and LCMV in that it does not require Tsg101 for budding (40, 217).Based on experiments using siRNA and expression of dominant negative mutants, all the three require, however, VPS4A/B (217). Thus, it seems that these three viruses all require the final VPS4A/B dependent step of the MVB pathway for budding, but differ in the need for at least one upstream component, Tsg101. For Mopeia virus an interaction between Z and NP was demonstrated to be mediated by AIP1/ALIX and this interaction was required for NP incorporation into budding particles (218). Such a function of AIP1/ALIX has yet to be shown for any other virus.

Bunyaviruses form a large family of viruses with five genera containing several human pathogens, like La Crosse, Oropouche, Hantaan, Sin nombre, Rift valley fever and Crimean-Congo hemorrhagic fever viruses. Similarly to Arenaviruses and Orthomyxoviruses the Bunyaviruses have a segmented genome, but it is tripartite. Bunyaviruses have unique way of escaping the host cell in that: firstly, they bud into the Golgi apparatus instead of the plasma membrane and secondly, they do not have a protein that would correspond to the matrix protein of other –ssRNA viruses. Instead the main viral determinants of budding seem to be the glycoproteins G(N) and G(C) and more specifically their cytoplasmic tails (219). Due to these reasons the budding process of bunyaviruses is likely to be quite different from the other –ssRNA viruses and it will not be further covered in this review. See (220) for a recent review on bunyavirus structure.

4. SUMMARY AND PERSPECTIVES

Matrix is a key coordinator of virus assembly within the negative-strand RNA viruses. The common roles seen are in self-assembly, membrane binding, budding and interaction with the RNP and glycoproteins. However, what emerges from this survey of the current literature is the wide variety of interactions with host cell proteins that are required in budding and transport of the components to the correct points of assembly. Directly related to the interactions with host cell proteins, are the various post-

translational modifications, like ubiquitinylation, phosphorylation and myristoylation of the viral matrix proteins. All of the viruses covered in this review rely on the host cell machinery during their life cycle within the host cell. In the literature on –ssRNA virus budding, plenty of emphasis has been put on the MVB pathway involving the ESCRT complexes, which many viruses require for exit from the cell. However, in addition to ESCRT, many other cellular proteins and protein complexes, like actin and microtubules are required for transport of the viral complexes to the assembly sites. Within this bountiful variety, we can see glimpses of as yet little understood cellular mechanisms, such as COPII cycling to the plasma membrane which can be explored further by using these intriguing viruses as tools.

Despite the progress in our understanding of –ssRNA budding and the components involved, many gaps remain. What is the driving force of the bud on the plasma membrane? How is the virion pinched off from the plasma membrane? How is matrix self-assembly executed and controlled? Where do matrix and the nucleocapsid first meet inside the cell? How are the nucleocapsid and the matrix transported to the budding sites? How much of the observed virion pleomorphism is caused by tissue culture or sample preparation artefacts? These and many other open questions will continue to motivate present and future studies on these important viruses. The promise lies in combining a number of complementary techniques, like systematic screening of required cellular proteins using siRNA screens and finding new protein-protein interactions with mass spectrometry-based techniques and then verifying the significance and meaning of the hits with cell biological and biochemical tools in combination with structural studies. In structural studies the combination of light microscopy, electron tomography and atomic resolution techniques will aid us in understanding the detailed interaction and function of virus-host molecular complexes. Electron cryotomography combined with subtomographic averaging will be crucial in understanding the last steps of budding close to the cell surface. Similar approaches will also be of value in studying the cell entry of these viruses.

Although not emphasized in this review, many of the negative-strand RNA viruses are significant pathogens and thus understanding in detail the mechanisms they use for replication is crucial for developing targeted drugs against them. Finding common cellular proteins required by several viruses might allow development of broad spectrum antiviral drugs that could inhibit the interaction of the viral proteins with their cellular counterparts.

5. ACKNOWLEDGEMENTS

LL is a fellow of the Viikki Doctoral Programme in Molecular Biosciences. This work was supported by the Academy of Finland grant 1139178 to SJB.

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Abbreviations: BDV: Borna disease virus; cryo-EM: cryo-electron microscopy; DRM: detergent-resistant membrane; EBOV: Ebola virus; ESCRT: endosomal complex required for transport; HIV: human immunodeficiency virus; HMPV: human metapneumovirus; HPIV3: human parainfluenza virus 3; HRSV: human respiratory syncytial virus; LASV: Lassa virus; LBV: Lagos bat virus; LCMV: lymphocytic choriomeningitis virus; MARV: Marburg virus; MuV: mumps virus; MV: measles virus; MVB: multivesicular body; NDV: Newcastle disease virus; NiV: Nipah virus; PIV5: parainfluenza virus 5; RABV: rabies virus; RMSD: root-mean-square deviation; RNP: ribonucleoprotein; SeV: Sendai virus; siRNA: small interfering RNA; SSPE: subacute sclerosing panencephalitis; TACV: Tacaribe virus; ts: temperature-sensitive; VLP: virus-like particle; VPS: vacuolar protein sorting; VSV: vesicular stomatitis virus; WT: wild-type

Key Words: Matrix, Budding, Negative-strand, Paramyxovirus, Respiratory syncytial virus, Influenza, Rhabdovirus, VSV, Rabies, BDV, Arenavirus, LASV, L-domain, Review

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