Late stages of the influenza A virus replication cycle—a tight interplay between virus and host

Marie O. Pohl, Caroline Lanz and Silke Stertz

Institute of Medical Virology, University of Zurich, 8057 Zurich, Switzerland

After successful infection and replication of its genome in the nucleus of the host cell, influenza A virus faces several challenges before newly assembled viral particles can bud off from the plasma membrane, giving rise to a new infectious virus. The viral ribonucleoprotein (vRNP) complexes need to exit from the nucleus and be transported to the virus assembly sites at the plasma membrane. Moreover, they need to be bundled to ensure the incorporation of precisely one of each of the eight viral genome segments into newly formed viral particles. Similarly, viral envelope glycoproteins and other viral structural proteins need to be targeted to virus assembly sites for viral particles to form and bud off from the plasma membrane. During all these steps influenza A virus heavily relies on a tight interplay with its host, exploiting host-cell proteins for its own purposes. In this review, we summarize current knowledge on late stages of the influenza virus replication cycle, focusing on the role of host-cell proteins involved in this process.

Introduction

Influenza A virus (IAV) is the causative agent of a febrile illness in humans, commonly referred to as ‘the flu’. IAV causes seasonal epidemics and sporadic pandemics, imposing a huge burden on human health and economy.

IAV are enveloped viruses belonging to the family of Orthomyxoviridae, whose members are characterized by a single-stranded segmented RNA genome of negative polarity (Palese & Shaw, 2007). Unlike most other RNA viruses, orthomyxoviruses replicate in the nucleus of the infected cell (Cros & Palese, 2003). While nuclear replication confers several advantages, such as access to the cellular splicing machinery, the virus faces the challenge of overcoming the nuclear envelope. Due to its small genome size of 13.5 kb, IAV relies heavily on cellular factors to complete its life cycle. In this review we focus on late stages of infection and describe the interplay between the virus and its host in the process of vRNP nuclear export, transport of viral proteins to the assembly site, genome packaging, as well as budding and release of virions.

Transport of viral components to the assembly sites

vRNP transport

After successful transcription and replication of the viral genome the so-called viral ribonucleoprotein (vRNP) complexes that consist of the viral RNA (vRNA) which is encapsidated by the nucleoprotein (NP) and bound by the viral polymerase complex (Fig. 1) need to be exported from the nucleus and shuttled to the cell surface in order to be packaged into budding virions. For specific export, vRNPs need to be recognized in the nucleus and discriminated from other RNA species. Discrimination of cRNA and vRNA takes place in the nucleus and only vRNPs are exported into the cytoplasm (Tchatalbachev et al., 2001). Likely, the viral polymerase adopts different conformations depending on whether it is associated with cRNA or vRNA, which might facilitate recognition of vRNPs as cargo for nuclear export (Tchatalbachev et al., 2001; Gerber et al., 2014). Furthermore, it has recently been suggested that vRNPs are not exported individually as proposed earlier (Chou et al., 2013), but instead as complexes consisting of two or more genome segments (Lakdawala et al., 2014). Nevertheless, export of fully assembled sets of vRNPs has not been observed yet. Export of newly assembled vRNPs takes place through the nuclear pore complex (NPC), which is an active process, dependent on the help of cellular factors, so-called exportins (Pemberton et al., 1998). Exportins recognize nuclear export sequences (NES) present in to-be-exported proteins and facilitate their transit through the NPC into the cytoplasm.

Studies indicate that the viral nuclear export protein (NEP) plays a critical role during vRNP export: viruses that lack NEP are not viable and interfering with the nuclear localization of NEP reduces viral growth (O’Neill et al., 1998; Neumann et al., 2000). Furthermore, microinjection of antibodies targeting NEP into IAV-infected cells specifically inhibits vRNP export (O’Neill et al., 1998). The amino acid sequence of NEP is highly conserved between different influenza virus strains, especially with regard to the C-terminal α-helix (Paterson & Fodor, 2012). Within the C-
terminal domain of NEP, the residue W78 has been identified to be important for the interaction with the nuclear localization signal (NLS) of matrix protein 1 (M1) (Akarsu et al., 2003). M1 is known to be required for export and the NEP–M1 interaction appears to be critical for vRNP transport (Martin & Helenius, 1991; Akarsu et al., 2003; Shimizu et al., 2011). Introducing anti-M1 antibodies into IAV-infected cells prevents shuttling of M1 into the nucleus and leads to nuclear retention of NP (Martin & Helenius, 1991). Thus, both viral proteins, M1 and NEP, appear to play important roles during vRNP export. Their late synthesis during the viral life cycle, mediated by suboptimal splicing in case of NEP, may be a regulatory mechanism to time, and then promote transport of vRNPs from the nucleus to the budding zones (Chua et al., 2013; Hutchinson & Fodor, 2013). In addition, vRNP export by NEP and M1 is also regulated by a switch in the post-translational modification pattern of M1: Early in infection M1 is ubiquitinated at lysine 242. M1 then becomes sumoylated at the same lysine during late stages, which seems to protect it from...

Fig. 1. vRNP export. vRNP export is primarily CRM1-dependent, occurs through the NPC, and is largely dependent on M1 and NEP. In this scenario, M1 and NEP connect the vRNP to the CRM1 export machinery according to the ‘daisy-chain’ or an alternative model. Other cellular factors such as YB-1, hCLE and Hsc70 also associate with the to-be-exported vRNP within the nucleus. vRNP export appears to be positively influenced by MAPK- and caspase signalling. AIMP2 promotes sumoylation of M1 which enhances vRNP export. In the cytoplasm, the vRNPs accumulate at the MTOC and associate with cellular factors such as HRB and STAU1. These factors, together with YB-1, facilitate the release from the CRM1 export machinery and mediate trafficking of vRNPs towards the cell surface.
proteasomal degradation and thereby leads to increased M1 levels and supports vRNP export (Wu et al., 2011). NEP has also been identified to be sumoylated at late stages of infection (Domingues et al., 2015), and interestingly, the switch in M1 modification from ubiquitination to sumoylation is promoted by the tumour suppressor AIMP2, which in turn binds NEP and is stabilized by its interaction with NEP (Gao et al., 2015).

NEP contains two NES in its N-terminal domain and is known to interact with the cellular β–exportin chromosome maintenance region 1 (CRM1) (O’Neill et al., 1998; Neumann et al., 2000; Iwatsuki-Horimoto et al., 2004; Huang et al., 2013). Indeed, studies confirm that vRNP export is largely dependent on CRM1 and its cofactor Ran-GTP as blocking the CRM1–operated export pathway by leptomycin B largely abrogates vRNP export in IAV-infected cells (Fukuda et al., 1997; Elton et al., 2001; Ma et al., 2001; Watanabe et al., 2001). It has been proposed that M1 and NEP function as adaptors to bridge the association of a vRNP complex with the CRM1 export machinery: In this scenario, M1 associates with vRNPs through an interaction with NP or through binding directly to the vRNA (Elster et al., 1997; Ye et al., 1999; Baudin et al., 2001; Noton et al., 2007). In addition, M1 interacts with NEP (Yasuda et al., 1993; Akarsu et al., 2003; Shimizu et al., 2011). NEP in turn is recognized by CRM1–Ran–GTP through its NES [however, binding of NEP to CRM1 in the absence of Ran–GTP occurs independently of the NES (Neumann et al., 2000)], thereby enabling the CRM1–dependent transport of the vRNP export complex through the NPC (Fig. 1) (Paterson & Fodor, 2012). This model, referred to as the ‘daisy-chain model’, was recently challenged by a study which reported that the interaction between M1 and the vRNPs is dependent on the presence of NEP. Via its C-terminal domain, NEP interacts not only with M1 but also with the polymerase to provide an additional binding site and support the M1–vRNP association (Brunotte et al., 2014). These data argue for an export complex in which the vRNP is bound by both M1 and NEP, and highlight again the requirement of NEP for CRM1-dependent export.

Of note, other studies suggest that other viral proteins play substantial roles in vRNP export. For example, inhibition of the CRM1 export machinery by leptomycin B caused perinuclear accumulation of NP, but did not affect the localization of NEP or M1 (Elton et al., 2001). NP encodes three NES, one of which is recognized by CRM1 (Yu et al., 2012), while the other two are CRM1-independent and might enable vRNP export independently of M1 and NEP. Also, M1 has previously been proposed as master regulator of vRNP export (Whittaker et al., 1996) and was able to mediate vRNP export in the absence of NEP (Bui et al., 2000). M1 also possesses an NES, which is not CRM1-specific. Mutations in this NES impair nuclear export of M1 and NP (Cao et al., 2012). These data suggest that vRNP export is not entirely dependent on NEP and CRM1 and indicate that CRM1-independent transit routes exist.

Cellular factors have also been shown to influence vRNP export in IAV-infected cells (Fig. 1). For example, heat shock cognate 70 (Hsc70) binds to the C-terminal domain of M1 (Watanabe et al., 2006) and, more weakly, to NEP with which it competes for binding to M1 (Watanabe et al., 2014a). Due to its NES (Tsukahara & Maru, 2004), Hsc70 was suggested to promote CRM1–dependent nuclear export of M1-bound vRNPs (Watanabe et al., 2008). Furthermore, Y-box-binding protein 1 (YB-1) was recently shown to associate with the vRNP export complex. vRNPs are bound in the nucleus by YB-1, where it accompanies the viral gene segments during their transport through the NPC (Kawaguchi et al., 2012). YB-1 appears not to be involved in vRNP export directly but instead exerts its function in the cytoplasm where it plays an important role in the transport of vRNPs to the apical cell surface (Kawaguchi et al., 2012). Another example is the mitogen-activated protein kinase (MAPK)-dependent signalling pathway, which stimulates NEP-dependent vRNP export (Pleschka et al., 2001). Furthermore, inhibition of caspase 3 leads to retention of vRNPs in the nucleus and it was suggested that IAV-induced caspase 3 activation increases the diffusion capacity of the NPC, thereby promoting vRNP export (Wurzer et al., 2003). Both, MAPK- and caspase 3-dependent pathways are activated during late phases of infection and appear to act independently to stimulate viral trafficking.

Following arrival in the cytoplasm, the vRNPs are transported to the cell surface for packaging into progeny virions. Even though diffusion of vRNPs towards the cell membrane has been reported (Babcock et al., 2004; Amorim et al., 2011), vRNP trafficking is generally believed to occur via Rab11-and microtubule-dependent vesicular transport (Bruce et al., 2010; Eisfeld et al., 2011a; Momose et al., 2011). Once in the cytosol, vRNPs have been shown to associate with the microtubule-organizing centre (MTOC) (Momose et al., 2007; Amorim et al., 2011; Kawaguchi et al., 2012). Four cellular factors have been shown to be involved in these early steps of vRNP transport to the cell surface: the human immunodeficiency virus Rev-binding protein (HRB), Staufen 1 (STAU1), YB-1 and hCLE/C14orf166. HRB interacts with NEP after vRNP export in the perinuclear region late in infection (O’Neill et al., 1998; Eisfeld et al., 2011b). Due to its GTPase activating protein (GAP) domain, HRB was proposed to mediate release of vRNPs from the CRM1–Ran–GTP export complex after its transit through the NPC (Eisfeld et al., 2011b). This has not been proven yet, but siRNA-mediated knockdown of HRB results in retention of vRNPs in the perinuclear region (Eisfeld et al., 2011b). This indicates that HRB is required to promote apical shuttling of vRNPs in the cytoplasm. STAU1 is a cellular factor involved in mRNA transport, for example it has been implicated in transport of mRNAs to the site of their translation at the rough endoplasmic reticulum, and has been shown to bind to a number of viral proteins (e.g. NS1, NP and M1) as well as to viral mRNA and vRNA (Falcon et al., 1999; Marion et al., 1999; Shapira et al., 2009; Watanabe et al., 2014b). STAU1 co-localizes with vRNPs in the cytoplasm. Knockdown of STAU1 expression does not affect viral replication, but
reduces the amount of viral particles released from infected cells indicating a role for STA1 in vRNP transport (de Lucas et al., 2010). Furthermore, YB-1, which associates with vRNPs already in the nucleus, mediates the interaction of progeny vRNPs with microtubules (Kawaguchi et al., 2012). Thus, YB-1 is required for vRNP accumulation at the MTOC and mediates, potentially in concert with HRB, the transfer of vRNPs to the endosomal vesicular trafficking system for subsequent transport to the plasma membrane. hCLE, a cellular transcription factor, associates with vRNPs in the nucleus where it promotes viral polymerase and Pol II activity (Huart et al., 2001; Rodriguez et al., 2011). Recently, it was shown that hCLE interacts with vRNPs in the cytoplasm where it co-localizes with Rab11, PA and NP (Rodriguez-Frandsen et al., 2016). Interestingly, hCLE appears to remain attached to vRNPs during further routing of the viral genome and is even incorporated into budding viral particles (Rodriguez-Frandsen et al., 2016). However, the function of hCLE binding to vRNPs during vRNP transport and packaging remains to be determined.

The Rab11-dependent recycling endosomal pathway plays an important role in vRNP trafficking from the MTOC to the cell surface and virion assembly (Fig. 2) (Bruce et al., 2010; Jo et al., 2010; Amorim et al., 2011; Eiffeld et al., 2011a; Momose et al., 2011; Avilov et al., 2012b). Recycling endosomes transport endocytosed material from the plasma membrane to apical or perinuclear recycling endosomes and shuttle cargo back to the cell surface. Rab11 is a marker for recycling endosomes that mediates vesicle transport along cytoskeletal structures through interactions with downstream adaptor and effector proteins (Bruce et al., 2012). Live-cell imaging using tagged vRNPs confirmed that exported vRNPs localize to Rab11-positive vesicles through a direct interaction of PB2 and active Rab11 (Amorim et al., 2011; Avilov et al., 2012b). Concordantly, knockdown of Rab11a abrogates apical vRNP localization in infected cells and leads to retention of the vRNPs in the perinuclear region (Eiffeld et al., 2011a). In the presence of Rab11, transport occurs through intermittently directed movements of vRNPs, which are dependent on an intact microtubule network (Amorim et al., 2011; Avilov et al., 2012a). Indeed, destabilization of microtubules using nocodazole has been shown to disrupt vRNP accumulation at the MTOC as well as shuttling of vRNPs to the plasma membrane (Momose et al., 2007; Amorim et al., 2011; Eiffeld et al., 2011a). Following transport, the vRNPs reside in patches adjacent to the plasma membrane (Eiffeld et al., 2011a; Chou et al., 2013; Hutchinson & Fodor, 2013). The genome segments then move to the budding zones for incorporation into virions. Interestingly, vRNPs appear to dissociate from Rab11 for insertion into the budzone (Eiffeld et al., 2011a) and Rab11 has not been detected in progeny virions (Shaw et al., 2008; Hutchinson et al., 2014). However, mechanistically it is not clear how vRNPs are moved from Rab11-positive submembranous patches into the budding area on the apical plasma membrane.

### Transport of viral envelope proteins

The structural proteins haemagglutinin (HA), neuraminidase (NA) and matrix protein 2 (M2) are synthesized and folded in the rough endoplasmic reticulum (ER) and then transported to the plasma membrane through the secretory pathway (Fig. 2) (Doms et al., 1993). Both, HA and NA are glycosylated along their route through the ER and Golgi towards the apical cell surface (Deom & Schulze, 1985). HA is synthesized as a precursor, HA0, which requires post-translational processing by proteases into HA1 and HA2, in order to gain fusion activity. Proteolytic cleavage of HA0 takes place either in the trans-Golgi network (TGN) or at the cell surface. Cleavage is mediated by a variety of cellular proteases depending on the cell type as well as the type (mono- versus polybasic cleavage site) and sequence of the cleavage site present in HA [reviewed in (Bottcher-Friebertshäuser et al., 2013)]. Within the TGN, M2 is critical to ensure conformational stability of HA: Through its ion channel activity, M2 regulates the pH-balance between the cytoplasmic and trans-Golgi compartment which is important to prevent premature pH-induced changes in the HA conformation (Ciampor et al., 1992; Grambas & Hay, 1992; Sakaguchi et al., 1996). Indeed, the pH stability of HA and the activity of the ion function of M2 were found to be inversely correlated in a study by Grambas & Hay (1992).

HA and NA contain apical sorting signals in their transmembrane domains (TMDs) (Kundu et al., 1996; Lin et al., 1998; Barman & Nayak, 2000), targeting them for transport to the cell surface after synthesis in the ER. The coat protein I (COPI) complex, which is involved in vesicle transport of cargo between the Golgi and ER, was recently shown to be involved in apical targeting of the viral structural proteins HA, NA and M2 (Sun et al., 2013). In addition, the Rho GTPase Cdc42 was suggested to promote apical transport of NA (Wang et al., 2012). It is believed that both, HA and NA are associated to sphingolipid-cholesterol-rich membrane patches (lipid raft microdomains) already during their transport through the TGN as well as after insertion into the apical membrane (Scheiffele et al., 1997; Simons & Ikonen, 1997; Barman & Nayak, 2000). The TMD of the viral glycoproteins and their cytoplasmic tail (CT) were both shown to be required for lipid raft association (Barman & Nayak, 2000; Zhang et al., 2000b; Chen et al., 2005). Co-expression of HA and NA led to their accumulation in lipid rafts and accelerated their transport to the cell surface, which could indicate that clustering of lipid rafts contributes to HA and NA shuttling (Ohkura et al., 2014). This is in line with previous studies that argue for a requirement of lipid rafts for apical targeting of HA and NA (Scheiffele et al., 1997; Keller & Simons, 1998; Ohkura et al., 2014). The dependency on lipid raft association for apical targeting might be virus strain-dependent, as other studies report only a weak delay of glycoprotein transport upon mutating the TMD or CT (Simpson & Lamb, 1992; Zhang et al., 2000b; Takeda et al., 2003).
Fig. 2. Apical transport of viral components. vRNPs are transported in a microtubule-dependent manner on Rab11-positive recycling endosomes from the MTOC towards the cell surface. vRNPs associate with GTP-bound Rab11 through interactions with PB2. On these vesicles, vRNP sorting and bundling is believed to occur before complete genome sets are incorporated into budding virions. hCLE associates with vRNPs in the nucleus, remains attached to vRNPs during cytoplasmic trafficking and is incorporated into virions. Structural viral proteins, such as HA, NA and M2 are synthesized in the ER and are transported to the budding sites through the secretory pathway with the help of cellular factors such as UBR4. HA and NA are associated with lipid raft structures from which M2 is largely excluded. HA maturation is mediated through cleavage by several cellular proteases which are present in the trans-Golgi network, at the cell surface or the extracellular space. Other structural viral components (M1 and NEP) travel to the budding sites along with vRNPs or might be recruited by cellular factors such as the F1Fo-ATPase which binds to NEP.
M2 is excluded from these lipid raft microdomains (Leser & Lamb, 2005) and an apical sorting signal in M2 has not been identified. Nevertheless, in infected cells M2 is also targeted to the apical membrane in an actin-dependent manner where it clusters with HA (Hughey et al., 1992; Thaa et al., 2010). A recent study that combined a meta-analysis of genome-wide RNAi screens with a screen for interaction partners of the different viral proteins identified the ubiquitin ligase UBR4 (ubiquitin N-recognin domain-containing E3 ligase 4) as a required host factor for IAV and an interaction partner of M2 (Tripathi et al., 2015). Interestingly, UBR4 positively influenced apical routing not only of M2 but also of the viral glycoproteins. Knockdown of UBR4 reduced M2 expression levels and viral particle production indicating that UBR4 might be required to protect M2 from degradation and to ensure delivery of viral components to the budding zones at the cell surface (Tripathi et al., 2015).

Despite M2 being co-transported with HA and NA, Rab11 was also shown to be involved in apical delivery of M2 (Rossman et al., 2010b). This could either indicate that the transport of M2 is at least partially mediated by the Rab11-dependent endosomal pathway or alternatively, Rab11 could affect M2 endocytosis and thereby impact the plasma membrane levels of M2.

**Transport of M1 and NEP**

M1 does not contain an apical localization signal. However, due to its ability to associate with lipids, vRNPs and the other structural viral proteins, M1 might be co-transported together with other viral components to the cell surface. In fact, transport of the viral gene segments and M2 through Rab11-containing vesicles might be linked by M1, which binds to both, M2 and vRNPs (Noton et al., 2007; Chen et al., 2008). It has been suggested that M1 stays attached to vRNPs after export, which on one hand prevents re-import of vRNPs into the nucleus (Martin & Helenius, 1991) and on the other hand promotes association of vRNPs with other viral components through interaction with M2 during vesicular shuttling and after apical delivery (Eisfeld et al., 2015).

Even though the majority of NEP is localized in the nucleus and cytoplasm during late stages of the viral life cycle, small amounts of NEP have also been detected at the apical plasma membrane (Carrasco et al., 2004; Gorai et al., 2012). This is in line with earlier studies showing that NEP is incorporated into virions (Richardson & Akkina, 1991; Shaw et al., 2008). It is not clear how NEP transport to the cell surface occurs, but NEP could piggy-back on vRNPs through its association with M1 (Yasuda et al., 1993). In support of this model, NEP, M1 and NP were shown to co-localize at the apical plasma membrane (Carrasco et al., 2004).

**Genome assembly and packaging**

It is currently believed that packaging of vRNPs into virus particles is a highly regulated process rather than a random one. In order to generate infectious virions, the distinct genome segments need to be sorted, bundled and inserted into budzones at the plasma membrane. Several studies suggest that sorting of vRNPs does not occur in the nucleus or during budding, but rather during cytoplasmic transport of the genome segments towards the cell surface (Takizawa et al., 2010; Chou et al., 2013; Lakdawala et al., 2014). In addition, neither HA nor M2 are required for co-localization of gene segments of different identities (Chou et al., 2013) which indicates that vRNP sorting takes place prior to arrival at the budzone. It is not clear whether vRNPs are sorted following the delivery of vRNPs from the MTOC to Rab11-containing vesicles, during transport, or upon accumulation of vRNPs in Rab11-positive patches in the apical periphery (Eisfeld et al., 2011a; Chou et al., 2013). Nevertheless, Rab11-positive membranes may serve as a platform for the gathering of the distinct genome segments (often described as bundling), which could allow reassortment as well as packaging of vRNPs into packaging-ready bundles, which are then incorporated into budding particles (Eisfeld et al., 2015).

For successful genome packaging, vRNPs need to be discriminated from other viral and cellular RNAs. In addition, the distinct genome segments are required to be identified and bundled in order to ensure packaging of a complete genome set. Many studies have reported that the information for these processes lies within the vRNA sequence: vRNP-specific genome sorting and bundling signals have been identified in the vRNAs (Hutchinson et al., 2010; Gerber et al., 2014). Concordantly, two vRNA-like molecules derived from the same gene segment but encoding different reporter genes were shown to compete for incorporation into virions (Inagaki et al., 2012). In addition, the idea of packaging signals present in the vRNAs was strengthened by the finding that vRNA-derived defective interfering vRNAs containing deletions compete with their parental vRNAs for insertion into virions (Duhaut & McCauley, 1996; Odagiri & Tashiro, 1997; Duhaut & Dimmock, 2002). To date, many signals required for packaging have been mapped to different regions of the vRNA such as the highly conserved 3′ and 5′ UTR, the terminal part of the coding regions as well as the central part of the vRNA (Fujii et al., 2003, 2009; Watanabe et al., 2003; Dos Santos Alfonso et al., 2005; Liang et al., 2005; Muramoto et al., 2006; Gog et al., 2007; Marsh et al., 2007, 2008; Hutchinson et al., 2008, 2009; Ozawa et al., 2009; Wise et al., 2011; Gavazzi et al., 2013). These studies show that some packaging signals are universal for all vRNPs, while others appear to be segment- or even virus strain-specific. Such variations in packaging requirements between strains can lead to genomic incompatibilities and incomplete packaging. Indeed, reassortment occurs more frequently between closely related virus strains compared to more
distantly related viruses (Essere et al., 2013; Marshall et al., 2013).

For genome bundling, interactions between the different vRNPs are thought to be of importance. In fact, recent studies favor a model of selective and hierarchical bundling of genome segments into supramolecular complexes prior to incorporation into virions (Gerber et al., 2014). The organization of vRNPs within virions predicts direct connections between the individual gene segments: vRNPs are organized in a so-called ‘7+1’ pattern in which seven vRNPs are localized around one central segment (Harris et al., 2006; Noda et al., 2006; Fournier et al., 2012b; Noda et al., 2012). Indeed, a linear organization network between vRNPs has been described (Fournier et al., 2012a, b). Mutations in packaging signals of one segment can affect the incorporation of other segments, which further supports the existence of interactions between individual vRNPs (Marsh et al., 2007; Hutchinson et al., 2008; Marsh et al., 2008; Hutchinson et al., 2009; Fournier et al., 2012b). Interestingly, interaction networks of vRNPs have been visualized through 3D electron tomography and revealed the presence of a transition zone at the tip of the budding virion which indicates that vRNPs are incorporated as a supramolecular complex in which the gene segments directly interact with each other (Fournier et al., 2012a, b). Furthermore, differences in vRNP interaction between virus strains were demonstrated (Gavazzi et al., 2013), suggesting that sequences required for genome bundling might evolve independently in distantly related virus strains (Gerber et al., 2014).

The 7+1 organization of the IAV genome is observed particularly in budding virions (Fournier et al., 2012a, b; Noda et al., 2012) and it is believed that one central gene segment mediates structural assembly of the vRNP bundle through interactions with the surrounding segments (Hutchinson et al., 2010; Gerber et al., 2014). In line with this model, many studies have shown that certain gene segments are of a higher regulatory order and more strongly affect genome bundling compared to other segments (Muramoto et al., 2006; Marsh et al., 2007, 2008; Hutchinson et al., 2008, 2009; Gao et al., 2012). This is supported by the finding that two to three vRNPs of different identities have been found to assemble already before nuclear export (Lakdawala et al., 2014). Thus, for successful incorporation of all eight genome segments, a two-step bundling process is suggested, in which vRNPs of high hierarchical order form a complex first within the nucleus. Following export, the other vRNPs are added to the vRNP bundle in a second step, which could potentially take place during transport on Rab-11 platforms in recycling endosomes (Gerber et al., 2014).

The efficiency and accuracy of vRNP packaging is still under debate: Some studies found that packaging of eight different segments is possible and that packaging of more than eight segments is uncommon (Noda et al., 2006; Chou et al., 2012). These data are indicative of efficient, regulated inclusion of eight distinct genome segments into virions.

However, this has been questioned by the finding that at low MOI (multiplicity of infection) most infected cells were reported to lack expression of at least one major viral protein (Brooke et al., 2013). This could potentially indicate that frequently genome segments are missing but it could also be explained by incorporation of damaged or mutated segments.

**Budding and release of new virions**

Different morphologies, ranging from spherical particles to long filaments, have been described for influenza virions (Palese & Shaw, 2007). Importantly, the particle morphology seems to change when adapting influenza viruses to tissue culture conditions: Laboratory-adapted strains typically produce mostly spherical particles with a diameter of about 100 nm as well as virions of pleomorphic shape (Fig. 3). Clinical isolates of influenza viruses in contrast display a filamentous morphology and their virions can be >1 μm in length (Mosley & Wyckoff, 1946; Choppin, 1963). In line with this difference between primary isolates and laboratory strains, a recent study found that upon passing a laboratory-adapted, spherical virus strain in guinea pigs filamentous morphology was selected for. In contrast, passing virus in embryonated chicken eggs, a method routinely used to grow virus in the laboratory, favoured spherical particles (Seladi-Schulman et al., 2013). While the different shapes of virions have been described structurally in detail, only little is known about the differences in assembly and budding for spherical versus filamentous viruses. It has been demonstrated that not only M1 has a major influence on the shape of the particle but also HA, NA, M2 and NP have been suggested to impact virus morphology (Bourmakina & Garcia-Sastre, 2003; Ellemann & Barclay, 2004; Rossman et al., 2010a; Bialas et al., 2014; Chlanda et al., 2015). Given that, most up-to-date studies on assembly and egress of influenza virus have been performed with laboratory-adapted spherical virions, this review will focus on budding of spherical virions.

Budding is a dynamic multistep process, which includes clustering of viral components at the apical cell surface, bud initiation, bud outgrowth, incorporation of the viral genome and scission of the bud in order to release the progeny virion. Studies on human bronchoeotraechal epithelial (HTBE) cells, a model for the human airway epithelium, revealed that virus budding occurs preferentially at the tips of epithelial microvilli (Kolesnikova et al., 2013). It can be speculated that the lipid and/or protein composition of the membrane at these tips is particularly suitable for virus budding, but this hypothesis has not been analyzed yet. IAV envelopes are enriched for sphingolipids and cholesterol compared to the average lipid composition of the plasma membrane (Gerl et al., 2012), supporting the idea of lipid raft-dependent virus budding.

Generally, all structural components of the virus need to be transported to the apical cell surface and clustered in lipid raft
domains to initiate the budding process. The accumulation of HA and NA at lipid raft microdomains in the plasma membrane results in the formation of larger functional raft domains for assembly and budding, the so-called budzone (Schmitt & Lamb, 2005; Rossman & Lamb, 2011). The trigger for bud formation is not known but most likely accumulation of viral glycoproteins starts the budding process. Studies investigating the budding of virus-like particles (VLPs) indicate that individual overexpression of HA, NA or M2 can mediate VLP formation (Chen et al., 2007; Lai et al., 2010). However, during virus infection HA is not sufficient to complete budding and therefore VLP budding appears not to mimic IAV budding accurately (Nayak et al., 2009; Rossman & Lamb, 2011). A recent study showed that the generation of VLPs which morphologically resemble budding viral particles, requires at least the expression of either HA or NA together with M1 and M2 (Chlanda et al., 2015).

Besides the glycoproteins, also M1 plays a critical role during budding. M1 is believed to interact with the plasma membrane and HA, NA, M2 and NEP as well as with vRNPs (Yasuda et al., 1993; Ali et al., 2000; Baudin et al., 2001; Noton et al., 2007; Chen et al., 2008), which could be required for reciprocal recruitment and organization of the viral components in the budzone. M1 can form oligomers (Zhang et al., 2012) and interactions of M1 with the plasma membrane have been shown to be required for multimerization (Hilsch et al., 2014). Formation of M1 oligomers below the plasma membrane provides structure and sturdiness to viral particles (Harris et al., 2001; Calder et al., 2010) and could in addition be required for bud elongation (Rossman & Lamb, 2011). Furthermore, the interaction of M1 with the CT of HA and NA was shown to be of particular importance for virion morphogenesis (Jin et al., 1997; Barman et al., 2004).

**Fig. 3. IAV budding.** (a) Electron micrograph of budding IAV. Viral particles can be of spherical, rod-like or filamentous shape. A549 cells were grown on 6 mm carbon-coated sapphire discs. Cells were infected with A/WSN/33 MOI=2 and 16 h post-infection, cells were fixed with 2.5 % glutaraldehyde in 0.1 M cacodylate buffer. Samples were processed for transmission electron microscopy (TEM) imaging using high pressure freezing. Images were acquired on a Phillips CM-100 TEM at the Center for Microscopy and Image Analysis of the University of Zurich. Shown are representative images (magnification: ×135 000 (upper image) and ×24 500 (lower image). (b) Cartoon. Viral particles form at budding sites on the apical cell surface. Accumulation of HA and NA on lipid rafts on the plasma membrane stimulates bud formation and outgrowth. M1 stabilizes the viral particles and might contribute to vRNP incorporation and recruitment of other viral components including M2. M2 plays an important role in scission of virions from the cell surface. For release of progeny viral particles, NA breaks interactions between HA and sialic acid-containing glycoproteins. Besides vRNP bundles and M1, also NEP and NS1 are components of IAV virions. In addition, cellular factors such as ubiquitin, hCLE, CD9, CD81, annexins or cytoskeletal components are incorporated into viral particles.
The assembled vRNP complexes accumulate in Rab11-positive submembranous patches below the plasma membrane. The importance of Rab11 for virus budding has been demonstrated: knockdown of Rab11 and its effector FIP2 delays or stalls virus budding which is probably due to defects in vRNP transport to the cell surface as discussed above (Bruce et al., 2010). Recruitment of the vRNP complex into the budzone could take place through interactions with other structural proteins that have already been inserted into the apical membrane. The CTs of HA and NA as well as that of M2 have been shown to be required for efficient genome packaging into budding virions (Zhang et al., 2000a; McCown & Pekosz, 2005; Iwatsuki-Horimoto et al., 2006). Also, the interaction between M1 and M2 is believed to be critical for the production of infectious viral particles (McCown & Pekosz, 2005; Iwatsuki-Horimoto et al., 2006; Chen et al., 2008; Grantham et al., 2010). In the absence of other viral proteins, M2 is not part of lipid raft microdomains in the plasma membrane (Zhang et al., 2000b; Leser & Lamb, 2005). However, in the presence of viral proteins, M2 is recruited to the site of budding and was shown to localize to the outer periphery of the budzone of infected cells (Rossman et al., 2010a; Rossman & Lamb, 2011). Several possibilities for the recruitment of M2 to lipid raft domains harbouring the budzone have been suggested: The amphipathic helix, which is present in the CT of M2 is required for localization of M2 at the budzone (Robert et al., 2013). M2 has been shown to interact with cholesterol directly and to associate with cellular membranes (Schroeder et al., 2005; Rossman et al., 2010a; Thaa et al., 2011), which indicates that association with cholesterol-rich domains might occur without the help of other recruitment factors. Indeed, M2 clusters with larger lipid raft domains, a process which was shown to be dependent on palmitoylation of the CT (Thaa et al., 2011). In addition, M2 is known to interact with M1 and HA (Chen et al., 2008; Thaa et al., 2010), which might further promote re-localization of M2 to the lipid raft structure of the budzone. Importantly, M2 was reported to mediate pinching of virus particles off the plasma membrane (Rossman et al., 2010b). For membrane scission, membrane curvature at the base of the budding virion needs to be induced and concordantly, M2 localizes primarily to the neck of budding virions (Rossman et al., 2010a; Roberts et al., 2013). The conserved amphipathic helix of M2 mediates completion of the budding process in a cholesterol-dependent manner through its insertion into the lipid bilayer (Rossman et al., 2010b). Indeed, the amphipathic helix was shown to be capable of inducing a strong, negative Gaussian curvature at the base of the bud which is required for scission of viral particles (Schmidt et al., 2013). Pinched off viral particles remain attached to the cell surface due to binding of HA to sialic acid-carrying proteins. For virion egress, NA cleaves off sialic acid residues on glycoproteins in proximity of the budzone (Palese et al., 1974; Griffin et al., 1983). Only few cellular factors have been identified to be required for IAV budding. For example, the tetraspan CD81 was shown to be recruited to the budzone and to be incorporated into progeny virions (Shaw et al., 2008; He et al., 2013). Knockdown of CD81 results in altered virion morphology and stalled detachment of particles from the plasma membrane indicating that CD81 might be involved in the scission process (He et al., 2013). Furthermore, the scaffolding protein receptor for protein C kinase 1 (Rack1) was shown to interact with a motif in the N-terminal part of M1 and this interaction was found to contribute to the release of virus buds from the cell surface (Demirov et al., 2012). In addition, the ATPase activity of the cellular F1Fo-ATPase was shown to be important for the egress of budding virions (Gorai et al., 2012). The F1Fo-ATPase associates with NEP and localizes to the budzone during late stages of infection (Gorai et al., 2012). While it is not yet known how the F1Fo-ATPase supports budding, it can be hypothesized that it contributes to the induction of membrane curvature. For mitochondrial F1Fo-ATPase it could be demonstrated that the polymerization of dimers of the F1Fo-ATPase induces membrane budding, leading to cristae formation in the mitochondria (Allen, 1995). It is therefore tempting to speculate that a similar function of the F1Fo-ATPase at the plasma membrane is usurped by IAV to initiate virus budding.

In summary and according to the model proposed by Rossman and Lamb (2011), initiation of budding is mediated by HA and NA which fulfill redundant functions. Together with M1, recruitment of other viral proteins and the genome results in virion assembly and bud elongation. Inclusion of M2 to the neck of budding virions results in incorporation of M2 into the virions as well as scission of the viral particles. Therefore, budding is a much more complex process than suggested by earlier VLP studies. Likely, in the context of a viral infection, various interactions between different viral components as well as support of cellular factors are required to orchestrate budding.

**Incorporation of cellular proteins into virions**

For many viruses it has been shown that in addition to viral proteins, host-cell proteins can be incorporated into virus particles [reviewed in (Cantin et al., 2005)]. It was found that most of those proteins are associated with lipid rafts, which are the budding sites for many viruses, such as IAV, human immunodeficiency virus (HIV)-1, respiratory syncytial virus (RSV) and others (Chazal & Gerlier, 2003). While some incorporated proteins have been described to be advantageous for the virus (e.g. cyclophilin A, a peptidyl prolyl isomerase incorporated into HIV-1 particles, which promotes capsid uncoating upon infection of the virus) (Franke et al., 1994; Thali et al., 1994), some have been found to decrease virus infectivity (e.g. APOBEC3G, a cytidine deaminase, which induces hypermutation of the HIV-1 genome) (Sheehy et al., 2002; Mangeat et al., 2003). However, for most of the cellular proteins incorporated into viruses, their functional significance remains to be determined.
The knowledge on cellular proteins which are incorporated into IAV virions is limited. First, a proteomics study was published in which cytoplasmic as well as envelope-associated proteins present in purified IAV were identified (Shaw et al., 2008). The identified host-cell proteins were mostly cytoskeletal proteins, annexins, glycolytic enzymes and tetraspanins, but their function for the virus (or their antiviral role) has not been elucidated yet. However, for some members of the annexin family and the tetraspanins follow-up studies have addressed this question. Annexin II has been shown to be incorporated into IAV particles and to convert plasminogen into plasmin, enabling proteolytic activation of HA in the absence of suitable cellular proteases (LeBouder et al., 2008). Recently, it was found that annexin V is upregulated and translocated to the cell surface upon IAV infection. Localizing to lipid rafts, annexin V gets incorporated into IAV particles and is associated with a decrease in $\gamma$-interferon signalling in newly infected cells, leading to an increase in viral replication (Berri et al., 2014). Intriguingly, not only annexins but also other host-cell proteins described to be incorporated into IAV have been reported to be incorporated into other enveloped viruses. Shaw et al. (2008) distinguish between highly abundant cellular proteins that may be incorporated unspecifically (e.g. $\beta$-actin or tubulin) and proteins enriched at virus budding sites, such as lipid rafts. Whether the virus chooses its budding site because of the presence of specific beneficial proteins or whether incorporated proteins just happen to be present at the virus budding site remains elusive.

Hutchinson et al. (2014) added to the knowledge on cellular proteins incorporated into IAV by performing proteomics studies of IAV grown on either mammalian Madin–Darby canine kidney (MDCK) and Madin–Darby bovine kidney (MDBK) cells or in embryonated chicken eggs. They detected the incorporation of a myriad of host-cell proteins into IAV, many of which overlapped with previously described proteins, namely cytoskeletal proteins, annexins, glycolytic enzymes and ubiquitin (Franke et al., 1994; Thali et al., 1994; Shaw et al., 2008). In addition, they described membrane proteins, small GTPases and other signalling proteins previously unaccounted for to be incorporated into IAV particles. Intriguingly, some host-cell proteins, such as ISG15, were uniquely found to be incorporated into IAV particles when the virus was grown on MDCK cells. Other proteins, such as the tetraspanin CD9, seemed to be functionally replaced by an avian substitute when the virus was grown in eggs. These data suggest that incorporation of certain host proteins is conserved and required across different species, whereas others might have species-specific roles.

Although some cellular proteins incorporated into IAV may be functionally irrelevant, many of them might harbour unanticipated functions and play important roles during infection, providing the virus with host factors needed at or shortly after viral entry into a susceptible target cell. In addition, as host factors stemming from one species may not be functional in another, they have the potential to determine the virus’ host range, which is of great importance for IAV that is able to infect multiple species.

**Table 1.** Selected host factors with a described function during the late stages of IAV infection

<table>
<thead>
<tr>
<th>Host factor</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exportin</td>
<td>vRNP nuclear export</td>
<td>Pemberton et al. (1998)</td>
</tr>
<tr>
<td>CRM1-Ran-GTP</td>
<td>vRNP nuclear export</td>
<td>O’Neill et al. (1998), Neumann et al. (2000), Iwatsuki-Horimoto et al. (2004), Huang et al. (2013)</td>
</tr>
<tr>
<td>MAPK pathway</td>
<td>vRNP nuclear export</td>
<td>Pleschka et al. (2001)</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>vRNP nuclear export</td>
<td>Wurzer et al. (2003)</td>
</tr>
<tr>
<td>AIMP2</td>
<td>vRNP nuclear export</td>
<td>Gao et al. (2015)</td>
</tr>
<tr>
<td>YB-1</td>
<td>vRNP trafficking</td>
<td>Kawaguchi et al. (2012)</td>
</tr>
<tr>
<td>HRB</td>
<td>vRNP trafficking</td>
<td>O’Neill et al. (1998), Eisfeld et al. (2011)</td>
</tr>
<tr>
<td>Staufen 1</td>
<td>vRNP trafficking</td>
<td>de Lucas et al. (2010)</td>
</tr>
<tr>
<td>microtubules</td>
<td>vRNP trafficking</td>
<td>Amorim et al. (2011), Avilov et al. (2012b)</td>
</tr>
<tr>
<td>Hsc70</td>
<td>vRNP trafficking</td>
<td>Watanabe et al. (2006, 2014a)</td>
</tr>
<tr>
<td>hCLE</td>
<td>vRNP trafficking</td>
<td>Rodriguez et al. (2011), Rodriguez-Frandsen et al. (2016)</td>
</tr>
<tr>
<td>Rab11</td>
<td>vRNP trafficking</td>
<td>Bruce et al. (2012), Hutchinson &amp; Fodor (2013)</td>
</tr>
<tr>
<td>actin</td>
<td>M2 trafficking</td>
<td>Rossman et al. (2010b)</td>
</tr>
<tr>
<td>UBR4</td>
<td>Trafficking of HA, NA and M2</td>
<td>Tripathi et al. (2015)</td>
</tr>
<tr>
<td>COPI</td>
<td>Trafficking of HA, NA and M2</td>
<td>Sun et al. (2013)</td>
</tr>
<tr>
<td>Cdc42</td>
<td>NA trafficking</td>
<td>Wang et al. (2012)</td>
</tr>
<tr>
<td>cellular proteases</td>
<td>HA activation</td>
<td>Bottcher-Friebertshauser et al. (2013)</td>
</tr>
<tr>
<td>F1F0 ATPase</td>
<td>Virus budding</td>
<td>Gorai et al. (2012)</td>
</tr>
<tr>
<td>CD81</td>
<td>Virus budding</td>
<td>He et al. (2013)</td>
</tr>
<tr>
<td>Rack1</td>
<td>Virus release</td>
<td>Demirov et al. (2012)</td>
</tr>
</tbody>
</table>
Conclusions

In summary, the roles of viral proteins and vRNA during late stages of the infection have been studied in detail and we have made progress in understanding the processes leading to the release of new virions. Also with regards to cellular proteins involved, several host factors have been identified and characterized for their prroviral function (Table 1). However, many open questions remain: despite tremendous progress and efforts we still only partially understand the mechanisms guiding packaging of the vRNPs. It is still unclear how M1 and NEP are transported to the assembly sites and it is not well understood how localization and assembly of the virion components at the budzone are regulated, timed and organized. Moreover, for technical reasons most studies have been performed with laboratory-adapted IAV strains which differ in their morphology from primary isolates and thus far, it is unclear how the assembly and budding processes differ between spherical and filamentous virions. It can also be assumed that many more cellular proteins are usurped by IAV for assembly and egress than currently known as hundreds of cellular factors have been identified as required for the virus but not been characterized yet (Stertz & Shaw, 2011; Tripathi et al., 2015). Future studies are expected to reveal novel insights into the tight interplay between the virus and its host cell at late stages of the infection.

References


Assembly and egress of influenza A virus


