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Review

Influenza virus assembly and budding

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ABSTRACT

Influenza A virus causes seasonal epidemics, sporadic pandemics and is a significant global health burden. Influenza virus is an enveloped virus that contains a segmented negative strand RNA genome. Assembly and budding of progeny influenza virions is a complex, multi-step process that occurs in lipid raft domains on the apical membrane of infected cells. The viral proteins hemagglutinin (HA) and neuraminidase (NA) are targeted to lipid rafts, causing the coalescence and enlargement of the raft domains. This clustering of HA and NA may cause a deformation of the membrane and the initiation of the virus budding event. M1 is then thought to bind to the cytoplasmic tails of HA and NA where it can then polymerize and form the interior structure of the emerging virion. M1, bound to the cytoplasmic tails of HA and NA, additionally serves as a docking site for the recruitment of the viral RNPs and may mediate the recruitment of M2 to the site of virus budding. M2 initially stabilizes the site of budding, possibly enabling the polymerization of the matrix protein and the formation of filamentous virions. Subsequently, M2 is able to alter membrane curvature at the neck of the budding virus, causing membrane scission and the release of the progeny virion. This review investigates the latest research on influenza virus budding in an attempt to provide a step-by-step analysis of the assembly and budding processes for influenza viruses.

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Introduction

Influenza is a major cause of morbidity and mortality around the world. It is estimated that infection with seasonal strains of influenza virus results in the death of over 50,000 people per year (Thompson et al., 2003). In addition to the seasonal strains, pandemic strains of influenza virus are capable of circling the globe rapidly, causing severe disease, such as that caused by the 1918 pandemic strain that resulted in over 50 million deaths during a two year period. Pandemic strains, such as the currently circulating 2009 H1N1 strain that has infected over 22 million people (C.D.C., 2009), arise from the reassortment of the eight different genome RNA segments of two strains of influenza virus within a single host cell.

The eight, negative-sense, RNA segments of the influenza virus genome encode 11 different proteins, of which 8 are packaged into the infectious, enveloped, virion (Palese and Shaw, 2007). On the viral surface are the two main antigenic determinants of the virus, the spike glycoproteins; hemagglutinin (HA) and neuraminidase (NA). HA mediates viral entry into cells and has receptor binding and membrane fusion activity. NA mediates enzymatic cleavage of the viral receptor at late stages of infection, allowing for the release of progeny virions. A third integral membrane protein, M2, is a multi-functional, proton-selective, ion channel which has roles both in virus entry as well as in assembly and budding. Inside the viral envelope, the matrix protein (M1) provides structure to the virion and bridges interactions between the viral lipid membrane and the ribonucleoprotein (RNP) core. The RNP

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core is composed of the RNA polymerase complex proteins, PB1, PB2 and PA, and the nucleocapsid protein (NP) which mediates binding and packaging of the viral genome. During virus replication three other proteins are expressed that are not incorporated into the mature virion. Non-structural protein 1 (NS1) is a multi-functional protein with a major role in evasion of the host immune system. NS2 (NEP) plays a crucial role in mediating the export of viral RNPs from the cell nucleus during replication. Additionally, many strains of influenza virus, but not all, express a protein designated PB1-F2 which is transcribed from a second reading frame in PB1. The PB1-F2 protein is involved in the induction of host-cell apoptosis.

The influenza virion is pleiomorphic, forming spherical virions that are ~100 nm in diameter as well as filamentous virions that are ~100 nm in diameter but reaching over 20 μm in length. Whereas several laboratory strains produce solely spherical virions it is thought that *in vivo* human infection produces predominantly filamentous virions (Chu et al., 1949; Kilbourne and Murphy, 1960). Indeed, samples isolated directly from the human upper respiratory tract show mainly filamentous forms of influenza virus (Chu et al., 1949; Kilbourne and Murphy, 1960). Additionally, analysis of lung sections from fatal cases of the 2009 H1N1 pandemic strain show the presence of filamentous virions (Nakajima et al., 2010) and passage of the 2009 H1N1 strain in tissue culture shows that the virus retains the ability to form filamentous virions during repeated passage (Itoh et al., 2009; Neumann et al., 2009).

Filamentous forms of influenza virus have been noted in the literature for many years (Ada et al., 1958; Burnet and Lind, 1957; Choppin, 1963; Choppin et al., 1961; Choppin et al., 1960; Chu et al., 1949; Kilbourne and Murphy, 1960; Morgan et al., 1956), although the functional significance of these filamentous virions has never been ascertained. Recent work has shown that both the filamentous and the spherical forms of influenza virus contain only one copy of the viral genome (Calder et al., 2010; Noda et al., 2006; Rossman et al., 2010a). The virions appear to contain similar ratios of most viral proteins and appear to be comparably infectious (Roberts et al., 1998). Interestingly, serial passage of filamentous isolates of influenza virus in eggs causes a loss of the filamentous morphology (Choppin et al., 1960). Indeed, the two most widely used laboratory spherical strains, A/WSN/33 and A/PR/8, have been passaged extensively in eggs. Thus, the loss of filament-forming ability may be an adaptation to growth in eggs and not specifically applicable to human infection, although it should be noted that even infections with filamentous strains produce both filamentous as well as spherical virions. It is not clear if certain infection conditions will select for one form of the virus over the other, thus research into both the filamentous as well as the spherical forms of influenza virus is necessary.

For filamentous virus, in addition to unknown methods of transmission and pathogenesis, the mechanism by which the filaments enter cells is unknown. Research into spherical virions has demonstrated that influenza virus enters cells by receptor-mediated endocytosis. HA, on the surface of the virus, binds to sialic acid moieties on surface exposed glycoproteins and glycolipids, triggering endocytosis of the virus. The mechanism by which influenza virus triggers endocytosis is unknown; however, recent data suggest that spherical virus particles binding to cells may trigger the activation of receptor tyrosine kinases, such as the epidermal growth factor receptor (Eierhoff et al., 2010), causing cellular signaling that results in the *de novo* formation of clathrin coated pits (Rust et al., 2004) and the subsequent uptake of influenza virions. For filamentous virions, however, entry may require a different mechanism as the viral filaments are too large to fit in a standard clathrin-coated pit. Studies on the entry mechanism of the filamentous Ebola virus have shown that the virus enters cells via macropinocytosis (Nanbo et al., 2010; Saeed et al., 2010). Thus, we speculate that filamentous influenza virions may also utilize macropinocytosis. This would provide an explanation for previous data that showed that influenza viruses can utilize, an as-yet-undefined, non-clathrin, non-caveolin entry pathway (Rust et al., 2004; Sieczkarski and Whittaker, 2002).

Nonetheless, it has been shown that the entry of both forms of influenza virus requires endosomal acidification (Sieczkarski and Whittaker, 2005). The low pH environment of the endosome is necessary, both for triggering HA-mediated fusion of the viral–endosomal membranes and for activating the M2 ion channel. The M2 proton-selective ion channel, on endosomal acidification, mediates proton conduction into the virion core, causing dissociation of the RNP core from the M1 protein, which allows for its subsequent import into the nucleus and the start of viral replication (reviewed in Helenius, 1992; Lamb and Pinto, 2005; Pinto and Lamb, 2006; Skehel, 1992). Upon replication of the viral genome and expression of the viral proteins, assembly begins at lipid raft domains of the apical plasma membrane, leading to the production of the next generation of influenza virions.

HA, lipid rafts and the initiation of virus budding

Influenza virus, both spherical as well as filamentous forms, utilize lipid raft domains in the plasma membrane of infected cells as sites of virus assembly and budding (Chen et al., 2007; Chen et al., 2005; Leser and Lamb, 2005; Takeda et al., 2003). Lipid rafts are variably-sized, cholesterol and sphingolipid-enriched, regions of the plasma membrane (Brown and Rose, 1992). Lipid rafts concentrate proteins within defined regions of the plasma membrane thus serving as functional domains (reviewed in Lingwood and Simons, 2010). Alternatively, expression of raft-associated proteins, such as the influenza virus HA protein, can cause a coalescence of lipid raft domains, forming a 'barge of rafts' or the viral 'budozone' (Schmitt and Lamb, 2005). Lipid rafts facilitate the budding of several different viruses, including HIV-1, Ebola virus and influenza virus (Brown and Rose, 1992; Cary and Cooper, 2000; Simons and Ikonen, 1997; Simons and Toomre, 2000; Suomalainen, 2002). For influenza virus, HA and NA are intrinsically associated with lipid raft domains, whereas the M2 protein is excluded from these domains (Chen et al., 2007; Leser and Lamb, 2005; Rossman et al., 2010a; Takeda et al., 2003). HA is a homotrimeric glycoprotein containing a 529 residue ectodomain that mediates viral attachment and entry. The HA 27 residue transmembrane (TM) domain, in conjunction with three palmitoylated cysteine residues (one located in the TM domain and two in the 10 amino acid cytoplasmic tail), mediates lipid raft association (Chen et al., 2005; Scheiffele et al., 1997; Takeda et al., 2003; Zhang et al., 2000b). The lipid raft association of HA is essential for efficient viral replication, as mutations within the HA TM domain that eliminate raft association significantly attenuate viral replication (Chen et al., 2005). Additionally, recent data has shown that influenza virus infection induces the expression of the, interferon-inducible, anti-viral protein, viperin (Wang et al., 2007). Expression of viperin causes a destabilization and reduction in lipid raft domains, leading to attenuation of influenza virus replication (Wang et al., 2007). Thus, it is possible that in virus-infected cells targeting HA to lipid raft domains serves to concentrate HA, allowing for the initiation of virus budding and this is antagonized by viperin-mediated disruption of the lipid raft.

The exact mechanism of virus bud initiation is not currently known. However, in a virus-like particle (VLP) system, wild-type (wt), raft-associated, HA protein buds from cells in vesicles that resemble virions, without requiring the expression of any other viral proteins (Chen et al., 2007). Thus, HA appears to possess the ability to alter membrane curvature, which in conjunction with lipid raft-mediated concentration effects may enable the initiation of virus budding. In the VLP system, plasmid-based expression of HA in 293T cells, leads to the alteration of membrane curvature, the completion of budding and the release of a virus-like particle. Similarly, single-protein expression of NA or M2 lead to the release of VLPs, albeit at