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Alterations of membrane curvature during influenza virus budding

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Key Words
Influenza virus, membrane curvature, membrane scission, budding, assembly, M2 protein

Abbreviations: AH, amphipathic helix; CT, cytoplasmic tail; HA, hemagglutinin; M1, matrix protein 1; M2, matrix protein 2; NA, neuraminidase; NGC, negative Gaussian curvature; PGC, positive Gaussian curvature; RNP, ribonucleoprotein; TMD, transmembrane domain; VLP, virus-like particle; vRNA, viral RNA

Abstract

Influenza A virus belongs to the *Orthomyxoviridae* family. It is an enveloped virus that contains a segmented, negative sense RNA genome. Influenza A viruses cause annual epidemics, occasional major pandemics, are a major cause of morbidity and mortality worldwide and have a significant financial impact on society. Assembly and budding of new viral particles is a complex and multistep process involving several host and viral factors. Influenza viruses use lipid raft domains in the apical plasma membrane of polarized epithelial cells as sites of budding. Two viral glycoproteins, hemagglutinin and neuraminidase concentrate in lipid rafts, causing alterations in membrane curvature and initiation of the budding process. The matrix protein 1 (M1), which forms the inner structure of the virion, is then recruited to the site followed by incorporation of the viral ribonucleoproteins and the matrix protein 2 (M2). M1 can alter membrane curvature and progress budding whereas lipid raft-associated M2 stabilizes the site of budding, allowing for proper assembly of the virion. In the later stages of budding, M2 is localized to the neck of the budding virion at the lipid phase boundary where it causes negative membrane curvature leading to scission and virion release.

Introduction to the influenza virus

Influenza A virus causes annual epidemics, occasional pandemics and is a major cause of morbidity and mortality worldwide. The most severe influenza pandemic was a “Spanish” flu outbreak at the end of the First World War, between 1918 and 1919. In one year influenza virus caused the death of approximately 50 million people around the world, which was greater than the total number of war casualties [1]. The first pandemic in the 21st century arose in 2009 and was called “Swine” flu due to the origin of the virus. The “Swine” flu outbreak was associated with a low pathogenicity; however in the USA alone over 22 million people were infected [2]. Each influenza outbreak has an impact on society, not only in terms of mortality and morbidity but also in financial and economical terms.

Influenza A virus is an enveloped virus with a negative-sense, single-stranded RNA genome and belongs to the *Orthomyxoviridae* family. The viral envelope is a host derived lipid membrane, which anchors three transmembrane-domain (TMD) containing proteins: hemagglutinin (HA), neuraminidase (NA) and matrix protein 2 (M2). HA is the most abundant envelope protein and has receptor binding and fusion activity whilst NA is a receptor-destroying enzyme involved in viral release. M2 is a 97 amino acid, tetrameric, TMD-containing protein, which is involved in both virus entry as well as assembly and budding. The TMD has ion channel activity which is essential for virus entry [3, 4] and the first 17 amino acids of the cytoplasmic tail (CT) form an amphipathic helix (AH) that is essential for membrane budding [4, 5]. On the inside of the viral envelope there is a layer of matrix protein 1 (M1), which is the most abundant structural protein of the influenza virus. M1 interacts with cytoplasmic tails of the surface glycoproteins [6, 7], with the ribonucleoprotein complexes (RNPs) [8] and plays a role in nuclear export, virus assembly and budding. The viral core contains eight RNPs each of which consist of a viral RNA (vRNA) segment bound to the nucleoprotein (NP) and associated with the three polymerase proteins: polymerase basic 1, polymerase basic 2 and polymerase acid [9].

Initiation of virus budding

Influenza viruses assemble and bud from lipid raft domains in the apical plasma membrane of polarized epithelial cells [10-12]. Lipid rafts are ordered microdomains in the plasma membrane, enriched in sphingolipids and cholesterol [13]. The two viral glycoproteins, HA and NA, concentrate in, cluster and enlarge lipid raft domains, causing the initiation of virus budding [10-12]. In contrast, the third viral envelope protein, M2, is normally excluded from lipid rafts but can be recruited to the periphery during the process of virus budding, which may explain its low incorporation into virus particles [10-12].

Previous studies, using temperature sensitive mutants, have shown that HA is not required for virus assembly and budding from infected cells [14]. Subsequently, studies of NA-deficient influenza viruses showed that the mutant virus assembles normally, though the budded virions are retained as large aggregates on the cell surface due to the absence of NA enzymatic activity [15]. This suggests that NA is not required for virus assembly and budding, but is essential for subsequent virion release. In contrast, research using a virus-like particle (VLP) system, in which membrane budding is assessed following transfection and expression of individual viral proteins, has shown that, in presence of exogenous

neuraminidase, HA VLPs can be released from cells without need for expression of any other viral protein [10]. These results suggest that HA might be sufficient to induce positive Gaussian curvature (PGC) of the membrane and initiate the budding process. Nevertheless HA is not sufficient to complete *in vivo* budding in virus-infected cells, which may be prevented by assembly of other viral proteins, such as interactions of M1 with the CT of HA and recruitment of the vRNPs (reviewed in [16]). Correspondingly, expression of NA [17] and M2 [10] alone also caused release of VLPs, but neither are sufficient to complete *in vivo* budding. Moreover, co-expression of all three viral envelope proteins increased VLP release [10], which suggests that each viral envelope protein may play a role in altering membrane curvature and initiating virus budding. Whilst each viral envelope protein can cause budding in a VLP system, all appear to be required for efficient virus budding. The reasons for the difference between *in vitro* VLP budding and *in vivo* virus budding are not known.

Assembly of the virion

The next step in the budding process is assembly of the virion. Newly synthesised RNP complexes and M1 are transported from the nucleus to the assembly site, following recruitment of M2 [16]. M1 is the most abundant protein among all viral particle components. It acts as a "bridge" between viral envelope proteins and the viral core; therefore it interacts with most of the viral proteins. Previous studies have shown that M1 is a major driving force in influenza budding and it is essential for VLP formation and when expressed alone assembles into VLPs [18]. However, recent studies suggest that M1 by itself fails to form VLPs and the interactions of M1 with the viral envelope proteins is essential for M1 plasma membrane localization and its incorporate into virions [19]. It has been shown that M1 interacts with the CT and TMD of HA and NA and that both glycoproteins support the lipid raft association of M1 [7, 20]. Thus, M1 may also be able to alter membrane curvature during the progression of virus budding provided HA and NA have already initiated the budding event. Once properly targeted to the plasma membrane, M1 interacts with the inside layer of the lipid bilayer [21], which could cause bending of the plasma membrane, generating PGC and progressing viral bud formation [16]. This suggests that during virus budding, HA, NA and M1 all cooperate to alter membrane curvature and promote virion assembly.

Later in the assembly process, the M2 protein is incorporated into the site of virus budding. When expressed alone, M2 is not associated with lipid rafts; however, when expressed with other viral proteins it is recruited to the raft periphery [5]. It is possible that the M2 interaction with M1 or HA alters M2 localization and facilitates M2 incorporation into virions [22]. In addition, it has been shown that the AH of M2 is able to insert into membranes and alter membrane curvature in a cholesterol dependent manner, a process that is necessary for membrane scission [23]. M2 causes a strong induction of negative Gaussian curvature (NGC) [23, 24] when cholesterol is below 17 molar %; however, in high levels of cholesterol the AH may not insert as deeply into the membrane, limiting the extent of membrane curvature [16, 23, 25]. Viral budding domains are enriched in cholesterol [13] and thus, the localization of M2 at these domains will prevent the strong elicitation NGC that could result in premature membrane scission.

Membrane scission and the completion of budding

The final step in virus budding is membrane scission, which takes place at the neck of the budding virion. Recent results examining influenza virus budding and release show that the M2 protein is responsible for membrane scission [23]. During budding, M2 localizes to the boundary between lipid-ordered (raft) and lipid-disordered domains. Viral proteins clustered in lipid raft budding domains are progressively incorporated into the virion. As M2 is located at the periphery of the budding domain, this will concentrate M2 at the neck of budding virus at the boundary between the lipid-ordered virus and the lipid-disordered bulk plasma membrane. In this lower cholesterol environment, the M2 AH may insert deeper into the membrane and cause further alterations in membrane curvature. Strong induction of NGC in the neck of budding virion may constrict the membrane neck below 10 nm, enabling spontaneous membrane scission to occur and triggering the release of new influenza viruses [23]. These studies also showed that mutation of the AH of M2 blocks membrane scission and release of the virus. Mutant virions were not able to complete budding and displayed a "beads on a string" morphology indicative of a scission defect [23].

Recent studies, using solid-state NMR spectroscopy confirmed that M2 amphipathic helix alters membrane curvature [26]. The extent of M2-induced membrane curvature was affected by membrane cholesterol [25, 26], which is consistent with previous observations of full-length protein in large unilamellar vesicles [23]. It was also shown that the M2 AH binds to lipid domains with high radii of curvature such as would be found at the neck of the budding virus and that this binding causes significant disruption of lipid head group packing [26]. Additionally, research using small angle X-ray scattering has confirmed that M2 is able to generate NGC in lipid membranes [24]. Consistent with previous results, reduction in the hydrophobicity of the AH by amino acid substitution resulted in inhibition of M2's ability to alter membrane curvature [24]. Although the M2 AH was necessary and sufficient to generate NGC, the presence of the M2 TMD enhanced the M2 AH-induction of NGC, suggesting that the TMD may affect AH activity, facilitating alteration of membrane curvature [24]. It has been shown, that mutation of the CT of the M2 protein causes a significant reduction in budding efficiency, but does not completely inhibit virus release [22, 27]. This suggests that other cellular factors, such as Rab11 [28], may be involved in the budding process or may orchestrate a redundant scission mechanism.

Summary

Influenza virus assembly and budding are complex, multi-step processes involving many host and viral factors. The exact model of assembly and budding has not been elucidated despite many years of research. One of the main difficulties has been the presence of conflicting results derived from comparing VLP budding and virus budding. It has been shown that viral envelope proteins and the M1 protein can cause VLP budding when expressed alone [10, 17, 18, 23]. In contrast, the expression of all viral proteins is essential for efficient virus budding from infected cells [14, 15]. The reasons for the difference between VLP and virus budding are not fully understood; however, it is probable that in infected cells the presence of each additional viral protein may sequentially alter protein localization, interactions and membrane curvature, causing significant differences in the process of

assembly and budding. It is not clear what the major driving force is for influenza virus assembly and budding.

An overall model of influenza virus assembly and budding suggest that several virus proteins mediate the process, rather than a single one. HA and NA are transported to lipid raft domains, where they concentrate. This may cause alteration of membrane curvature and initiation of the budding event. M1 may then be recruited to the site by interacting with the cytoplasmic tails of HA and NA. Membrane bound M1 can polymerize and form the inner structure of the virion, excluding host proteins from the budding site. Additionally, M1 may alter membrane curvature to progress the budding event; however, M1-mediated recruitment of vRNPs or M2 may temporally block completion of budding process. Recruitment of M2 to the lipid-ordered budding site may cause stabilization necessary to allow for proper assembly of the virion before the completion of membrane scission. Following assembly, M2 will be localized to the neck of budding virion, at the lipid phase boundary, where insertion of the M2 AH can cause NGC, leading to scission and virion release [23]. It has been proposed that M2 AH-alternations of membrane curvature may enable membrane scission through the alteration of line tension between the two lipid phases and through lipid packing defects caused by insertion of the AH into the inner leaflet of the membrane bilayer [23]. Recent studies, have confirmed that the AH of the M2 protein alters membrane curvature in a manner that is affected by the specific lipid environment [24, 26], however the exact molecular mechanisms that regulate the alteration of membrane curvature during influenza virus assembly and budding remain unclear.

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