Molecular dissection of dengue virus egress: involvement of the class II ARF small GTPase

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KEY MESSAGE

The transmembrane domain 2 of prM is a possible binding domain by Arf4 protein

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Four serotypes of dengue viruses 1-4 are the pathogens of dengue fever, dengue haemorrhagic fever, and dengue shock syndrome. They are estimated to cause 50-100 million cases, including 25 000 deaths, every year.[1] Despite the increased health and economic impact of dengue virus infection, there is still no specific treatment. This is due, in part, to the incomplete understanding of specific host-pathogen interactions during the life cycle of the virus in infected cells. More research into the basic biology of dengue virus is needed.

Dengue virus particles can be viewed schematically as internal and external parts. The internal part is the core structure of the virus and comprises the genome and capsid protein, which binds to the genome so that the core structure can be well organised and packaged into the external part. The internal part carries viral genetic information, stored as a long RNA molecule, and can replicate itself to form nascent viruses. The external part consists of a lipid membrane and the viral structural envelope glycoproteins that are integrated in the lipid membrane. The lipid membrane is derived from the host cell when nascent viruses are formed. Dengue viruses have two structural envelope glycoproteins, prM and E.[2]

During viral maturation, prM is cleaved by furin, a cellular protease, to form pr and M, the former being released from the mature virion, and the latter remaining on it to form a heterodimer with E protein.[3] Glycoprotein prM and E play important roles in the dengue virus life cycle. The life cycle can be divided into three stages: entry, replication, and secretion. E protein is mainly responsible for binding with a cellular receptor and then triggering viral-host membrane fusion during viral entry, whereas prM protein is required for various stages of viral secretion. The secretion process describes the assembly and subsequent transportation of virus. First, prM protein functions as a chaperone in ER to help the proper folding of E protein. Second, prM protects E protein from conformational changes that may be triggered by the acidic environment along the secretion pathway, thus promoting fusion of the nascent virion with the membrane compartment and release inside, not outside, the cell. Third, immediately before dengue virus is released from host cells, prM protein is cleaved by furin, a major processing enzyme of the secretory pathway, to form M and soluble pr proteins. This step is a critical process for the infectivity of the nascent virus and is called maturation. Although the secretion of dengue virus is a complex process, compared with viral entry and replication, little attention has been paid to understand the various stages at a molecular level.

Assembly of dengue virus occurs in the endoplasmic reticulum (ER) and is driven by the interaction between E and prM proteins. Nascent virions in the ER thus need to be transported from the ER to the Golgi apparatus, and then transported to the plasma membrane before they are finally released. As described above, prM protein performs multiple functions during the secretion process. To achieve this, prM protein needs the help of many cellular factors. Utilisation of cellular factors is a common strategy by which viruses complete their life cycle. Although such cellular factors will not be assembled into newly formed viral particles, they are really indispensable to survival of the virus. Thus, if these crucial cellular factors are not utilised by the virus, viral replication will be inhibited. Identification of these host factors and the mechanisms that govern their interaction with a virus enables design of antiviral strategies.

To identify the cellular factors and their mechanism of action, we use dengue recombinant subviral particles (RSPs), which bear several similarities to dengue virus and are a safer and convenient tool in the laboratory. Dengue RSPs are generated by cells expressing glycoprotein prME in the absence of capsid protein and RNA genome. Thus, dengue RSPs consist of only the external structure

of dengue virus and cannot cause infection because of the lack of viral genome. In previous work, we have developed a stable dengue RSP-producing cell line (HeLa-prME) using a codon-optimised dengue prME gene that greatly increases the expression level of prME proteins in mammalian cells.[4] We have shown that dengue RSPs can mimic the secretion of dengue virus, and have validated this tool to analyse trafficking and secretion of dengue virus.[4]

Using the dengue RSP-producing cell line, we have identified two cellular factors: ADP-ribosylation factor 4 and 5 (Arf4 and Arf5), and demonstrated that they are indispensible for dengue virus secretion.[5] Simultaneous depletion of Arf4 and Arf5 blocks RSP secretion for all four dengue serotypes. Intriguingly, Arf4 and Arf5 are not required for the constitutive secretion of host cells, ie the mechanism by which cells export material into the extracellular space, indicating that they are specifically required by dengue virus. Arf4 and Arf5 belong to the ADP-ribosylation factor family, of which six members have been identified and all play an important role in intracellular transportation. A predominant function of Arf proteins is that they can bind to lipid membrane, recruit some other cellular proteins, and then curve the lipid membrane to a semi-spherical or spherical structure. Such curvature of the lipid membrane is an important step for the formation of trafficking vesicles, which are the universal form of intracellular transportation from one organelle to another. It is noteworthy that bending of lipid membranes also occurs during the formation of enveloped virus including dengue virus.

Based on amino acid sequence identity, six Arfs are grouped into three classes: class I (Arf1-3), class II (Arf4, 5), and class III (Arf6). Arf4 and Arf5 are mainly distributed in the peri-nuclear region. Arf4 participates in the transport of rhodopsin from the Golgi apparatus to the plasma membrane by binding to a VxPx motif at the C terminus of rhodopsin. Arf proteins are hijacked by various viruses for their life cycle. For example, Arf1 protein is utilised by viruses such as HIV and HCV for virus assembly or replication, whereas Arf6 protein is hijacked by viruses such as coxsackievirus and HIV for virus entry.

We designed experiments to investigate the molecular mechanism by which Arf4 and Arf5 affect dengue virus secretion. One important technique used is co-immunoprecipitation. Cells producing RSP are broken and the resulting lysate is incubated with beads covered with an antibody that specifically recognises prM protein. The prM protein and other proteins binding with prM attach to the bead through the bridge of anti-prM antibody and can be precipitated by centrifugation. This approach enables extraction of the cell molecules that can interact with the viral protein under study. Arf4 and Arf5 proteins and prM protein bind with each other in RSP-producing cells. This means that dengue prM protein may recruit Arf4 or Arf5, and that this interaction in turn facilitates virus secretion. Dengue prM protein has been reported to help the correct folding of E protein in ER and then protect it from fusing within the host cell before progeny viruses are released. Here, we demonstrated another important role of prM during the secretion process: recruiting Arf4 or Arf5, two crucial factors for intracellular trafficking, to the lipid membrane to facilitate virus secretion.

As mentioned above, Arf4 can recognise a VxPx (where X stands for any amino acid) motif that is found at the C terminus of four serotypes of dengue virus prM proteins. To study whether VxPx in prM is also recognised by Arf4 or Arf5, we have used molecular cloning techniques to generate mutant prME genes in which VxPx motif is either deleted (prME-DVxPx) or substituted (prME-AxAx) and then use transfection reagents to deliver these mutants as well as wild type prME to mammalian cells. We found that substitutions inside this motif (V161A and P163A) did not reduce the expression or secretion of RSP. Taken together, our results indicate that although VxPx at the C terminus of prM affects the expression level of the protein, it is not the sequence recognised by Arf4 or Arf5.

In further experiments, we used another technique called glutathione S-transferase (GST) pull-down assay to gain insight into the molecular basis of the interaction between prM and Arf proteins. The principle of GST pull-down is similar to that of co-immunoprecipitation. In this assay, instead of using an anti-prM antibody to co-immunoprecipitate interacting molecules, some parts of prM protein are fused to the GST gene and then immobilised at the surface of beads coated with glutathione, which interacts with high affinity with GST. Using the GST pull-down assay, we extended our previous observations and demonstrated that the four amino acid peptides VxPx or AxAx are not sufficient to pull down class II Arf protein. The efficient pull-down of Arf4 protein requires the additional presence of the second transmembrane domain of prM, suggesting that Arf4 might interact with this region of prM protein.

The different binding ability to Arf protein by rhodopsin VxPx and prM VxPx might be explained by considering their different topology. Whereas the VxPx motif of rhodopsin is located at the cytosolic side of the intracellular membrane compartment in which it is inserted and Arf protein is able to recognise it for interaction, the same sequence in prM is exposed to the luminal side of those compartments and theoretically inaccessible to Arf protein. The transmembrane domain of prM, which spans the lipid membrane, is therefore more likely

to interact with class II Arfs. Further experiments are needed to test the role of this transmembrane domain for prM interaction with Arf4 and Arf5.

In the second part, we focused on KDELRs. KDELRs (KDELR1-3) are seven-transmembrane proteins that regularly cycle between the ER and Golgi apparatus. Depletion of Arf4 and Arf5 blocks not only the secretion of RSP, but also the transportation of KDELR. The depletion of Arf4 and Arf5 results in a concentration of KDELR around the Golgi apparatus and a decrease of KDELR in the ER compartment, suggesting that in the absence of Arf4 and Arf5, KDELR cannot exit the Golgi apparatus and therefore accumulates in that organelle. Co-immunoprecipitation experiments found that prME proteins also interact with KDELR. Because KDELR shuttling between the ER and Golgi apparatus requires both Arf4 and Arf5, we postulate a novel mechanism of dengue virus secretion: dengue RSP are formed in the ER where they bind to KDELR as a means to be transported from the ER to the Golgi apparatus. RSPs and KDELRs dissociate in the Golgi apparatus by an unknown mechanism, and KDELR is retrieved back to the ER in a class II Arf dependent manner. In this model, the transportation of dengue virus from the ER to Golgi apparatus requires not only the presence of KDELR but also the normal cycle of KDELR, which is subject to Arf4/Arf5 availability. This model incorporates the experimental results in a testable model and we will design experiments to study this hypothesis in future.

Although the mechanism by which Arf4 and Arf5 are involved remains to be studied, our findings shed new light on a molecular mechanism used by dengue viruses during the late stages of the replication cycle and demonstrate a novel role for prM protein that could represent a novel therapeutic target. More basic knowledge of the life cycle of the dengue virus is needed in order to devise better strategies to treat this disease and reduce its burden worldwide.

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