รายงานวิจัยฉบับสมบูรณ์

โครงการการวิเคราะห์องค์ประกอบของ dengue replication complex และ vesicle ที่มาจากเซลล์ยุงลาย C6/36

โดย ดร.บรรพท ศิริเดชาดิลก

เดือน ปี ที่เสร็จโครงการ
1 พ.ค. 2556

สัญญาเลขที่ P-10-10633

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สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ (สวทช.)

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และศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ
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Abstract

Project Code : 10633-10-P

Project Title : Compositional analysis of the dengue replication complex and vesicle derived from mosquito cell C6/36

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Viral RNA replication complex is a protein complex that was built inside infected cells for the purpose of multiplication of viral genome to be packaged into progeny virus particles. Despite being essential machinery for virus replication and adaptation, our understanding of how it functions is limited because of the lack of a purified and functional complex for biochemical and structural characterization. Here, we present the results from our effort to apply the purification scheme used to enrich the dengue replication complex from infected mammalian cells with the complex from infected mosquito cells. The comparison between the two complexes should not only suggest the essential host factors that are required for the function of the replication complex but also indicate host-specific factors that could underline different cellular physiology that the virus must handle to replicate its genome.

Keywords : dengue, viral RNA replication

บทคัดย่อ (ภาษาไทย)

รหัสโครงการ: 10633-10-P

ชื่อโครงการ: การวิเคราะห์องค์ประกอบของ dengue replication complex และ vesicle ที่มาจากเซลล์ยุงลาย C6/36

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ระยะเวลาโครงการ: 1 พ.ค. 2553 – 1 พ.ค. 2556 (ขยายเวลา)

Viral RNA replication complex เป็น protein complex ที่ถูกสร้างภายในเซลล์ที่ติดเชื้อด้วยไวรัส โดย complex นี้มีหน้าที่สำคัญในการเพิ่มจำนวนของสารพันธุกรรมของไวรัสเพื่อนำไปประกอบเป็นอนุภาคไวรัสที่สามารถไปติดเซลล์อื่น ๆต่อไปได้ แม้ว่า viral RNA replication complex จะมีบทบาทสำคัญต่อการเพิ่มจำนวนและการปรับตัวของไวรัส แต่ความเข้าใจในกลไกการทำงานของ complex ก็ยังมีอยู่อย่างจำกัดเนื่องจากปัจจุบันเรายังไม่มีวิธีการที่จะแยก complex เหล่านี้ออกจากเซลล์ที่ติดเชื้อเพื่อทำการศึกษาและวิเคราะห์ด้วยเทคนิคต่าง ๆ ก่อนหน้านี้ทางคณะผู้วิจัยได้พัฒนาวิธีการที่จะแยก complex ของไวรัสเด็งกี่ออกจาก mammalian cells เนื่องจากไวรัสเด็งกี่สามารถเพิ่มจำนวนได้ทั้งใน mammalian cells และเซลล์ยุง การเปรียบเทียบองค์ประกอบของ complex ที่ได้จากเซลล์ทั้งสองชนิดจะบ่งบอกถึงองค์ประกอบของเซลล์เจ้าบ้านที่สำคัญต่อการทำงานของ complex และ องค์ประกอบที่จำเพาะต่อ host แต่ละชนิด ในรายงานฉบับนี้ทางคณะได้นำเสนอถึงผลที่ได้จากการนำวิธีการแยกที่พัฒนาใน mammalian cells มาใช้ในการแยก complex ออกจากเซลล์ยุง

คำหลัก : ไวรัสเด็งกี่ การจำลองสาย RNA ของไวรัส

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า)
   ยังไม่มีในขณะนี้

2. การนำผลงานวิจัยไปใช้ประโยชน์
   - เชิงพาณิชย์ (มีการนำไปผลิต/ขาย/ก่อให้เกิดรายได้ หรือมีการนำไปประยุกต์ใช้โดยภาคธุรกิจ/บุคคลทั่วไป) **:ยังไม่มีในขณะนี้**
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   - เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง)**:ยังไม่มีในขณะนี้**
   - เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่):**ยังไม่มีในขณะนี้**
3. อื่นๆ (เช่น หนังสือ การจดสิทธิบัตร)
   :ยังไม่มีในขณะนี้

Introduction

Viral genome replication is an important aspect of a viral replication in its natural life cycle. It is a multi-step process that the viral proteins and hijacked host-cell machinery participate to multiply the viral genomic RNA/DNA. In the case of virtually all positive-sense RNA viruses, the RNA genome replication involves the alterations of the membrane structure of the host cells, resulting in the formation of the membrane compartments that the viruses use as a platform for replication(1). As a group member of positive-sense RNA viruses, flaviviruses, such as dengue viruses, induce unique membrane structure consisting of replication vesicles packed in a bag of membrane, called vesicle packets (VP), in the cytoplasm of host cells during its infection(2). Dengue virus replicates in both human cells, contributing to the pathogenesis of dengue disease, and mosquito cells, contributing to the spreading of the viruses by mosquitoes. In spite of the critical role of viral RNA replication in infection and spreading, very little is known of how the replication machinery is derived and functions. Various studies have suggested that host proteins are essential to the function of dengue replication machinery. By targeting these host proteins, one may be able to effectively contain the viral replication. Yet, the identities of these proteins in the viral RNA replication machinery are not comprehensive and definite because there has not been a way to isolate the viral RNA replication machinery for in vitro characterization. In our ongoing research, we have found an immunoaffinity purification method capable of recognizing dengue replication vesicle/complex from dengue-infected mammalian cells. In this proposal, we propose to extend the affinity purification to isolate dengue replication vesicle/complex from infected mosquito cells. The isolated replication vesicle/complex will then be characterized to determine its molecular composition. We expect that the result will identify the host factors from the mosquito cell that constitutes the dengue replication vesicle/complex. While we expect that there will be host factors that are shared by the replication complex/vesicle from mammalian host and mosquito host, there could be factors that are specific to only either mammalian or mosquito hosts. This information will pave the way for future functional studies of the host factors' contributions to dengue replication in mosquito. The knowledge of the host factors' contributions and interactions with viral nonstructural proteins in the replicating complex/vesicle could help in designing vaccine candidates with limited spreading capabilities by mosquitoes.

Results

Dengue replication complexes reside in heavy-membrane fraction of cytosol.

Preliminary crude fractionation of the cytosol from dengue-infected mosquito C6/36 cells show that the replicase activity exclusively resides in the heavy-membrane fraction as found to be the case for the cytosol from dengue-infected mammalian cells. Similarly, the replication vesicles from

dengue-infected C6/36 cells are extractable from heavy-membrane fraction by TritonX-100 but can be dissolved by deoxycholate (figure 1). These results suggest that the replication vesicle/complex derived from infected C6/36 should have similar properties to those derived from Vero cells and that the purification strategy developed in the mammalian system should be applicable to study the replication vesicle/complex derived from C6/36 cells.

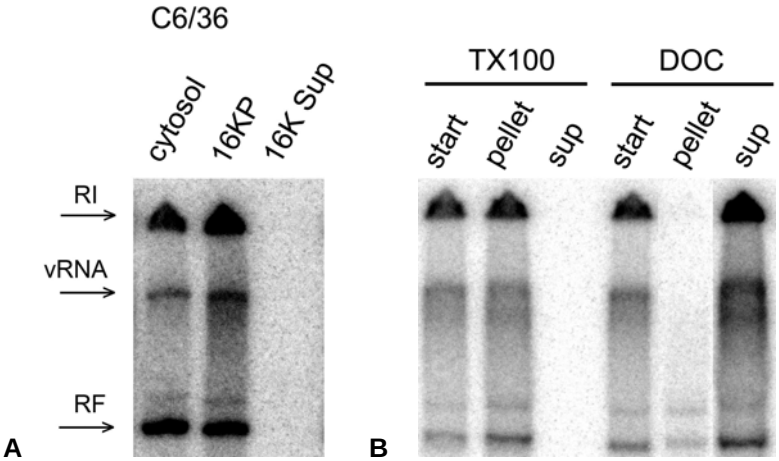


Figure 1: *Heavy-membrane fraction from C6/36 cytosol* ***A*** *The in vitro RdRp activity of the cytosol, heavy-membrane fraction (16KP) and supernatant.* ***B*** *The ultracentrifugation of the TritonX-100-treated and deoxycholate-treated 16KP that have been P^{32}-labeled by in vitro RdRp assay.*

Complex isolation by chromatography

We have used the method developed to isolate active dengue replication complex from infected mammalian cells to enrich the complex from infected C6/36 cells. We found that the buffer conditions used for the purification of the complex derived from mammalian cells can still maintain the activity of the complex derived from C6/36 cells (data not shown). We have used an anion-exchange and anti-dsRNA immunoaffinity to isolate the active dengue replication complex, with the yield of 10-15% activity recovered in the final elution (figure 1). To test the specificity of the purification procedure, we compared the protein profiles in the final elution between mock-infected and dengue-infected cytosol. Mock-infected and dengue-infected 16KP of the same amount and the same protein concentration were treated with the same purification procedure. 1D SDS-PAGE analysis of the final elution indicates that a set of proteins were enriched from dengue-infected samples (figure 2). Viral proteins such as NS5 (viral RNA-dependent RNA polymerase), NS3 (viral helicase), and NS1 proteins can be specifically observed in the final elution of the dengue-infected sample. Nevertheless, some of the proteins found in final elution of the dengue-infected samples were also observed in the mock samples but with significantly lesser amount in the final elution of mock samples.

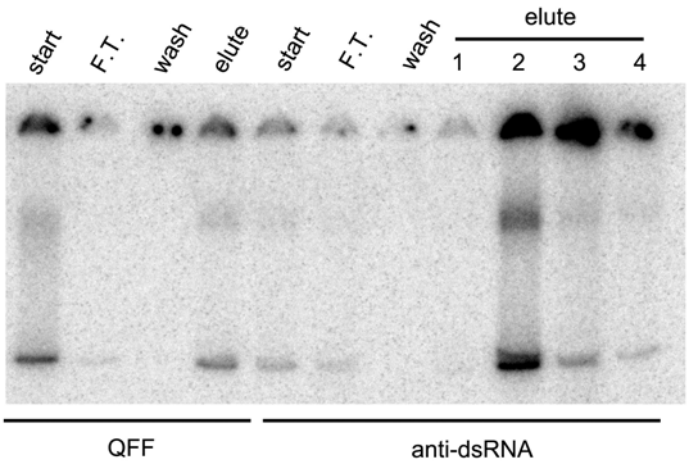


Figure 2: *The result from in vitro RdRp assay of the fractions from anion-exchange and anti-dsRNA immunoaffinity chromatography. F.T. = flow through.*

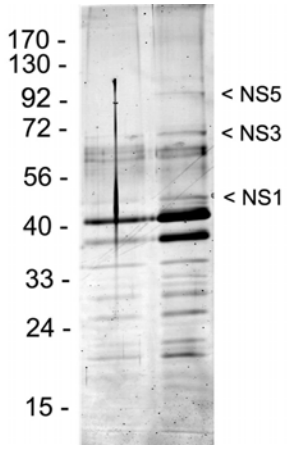


Figure 3: *SDS-PAGE analysis of the final elution from anti-dsRNA immunoaffinity chromatography. The numbers on the left indicate the molecular weights of the standard proteins on the gel. The left lane is the final elution of the mock-infected sample while the right lane is the final elution of the dengue-infected sample. The proteins were visualized by SYPRO-RUBY staining.*

Problems that impede further analysis

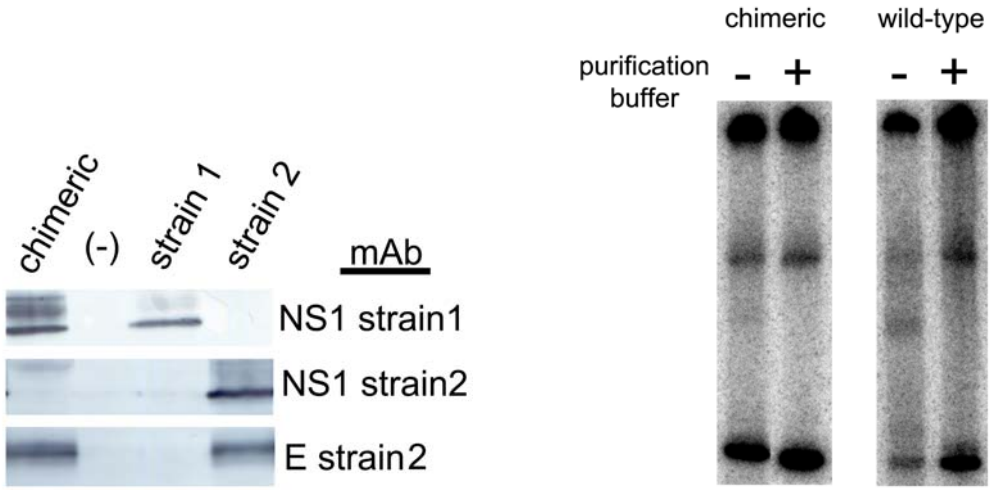
While some enrichment of dengue NS5 and NS3 has been achieved, the purification still needs improvement for proteomic analysis. In addition, during subsequent purification, inconsistency in virus culture has gotten worse. To go forward, two things have to be achieved:

1. A second affinity to clean up the purification
2. A consistent virus culture that can support preparation of large amount of lysate.

The complex purification from mammalian cells (vero cells) also encountered similar issues. The results presented in this section were obtained to address both the mammalian and the mosquito systems.

A second affinity to clean up the purification

An anti-NS1 antibody was found to be able to recognize NS1 on the replication complex. However, the antibody does not bind to the NS1 of the dengue strain suitable for the purification procedure. Therefore, to utilize the antibody for the purification, the NS1 gene on the strain used for purification must be swapped for the NS1 recognizable by the antibody. We have built the chimeric virus and tested the antigencity of NS1 by Western blot (figure 4a). The replication complex of the virus can also tolerate the buffer condition used for purification (figure 4b).

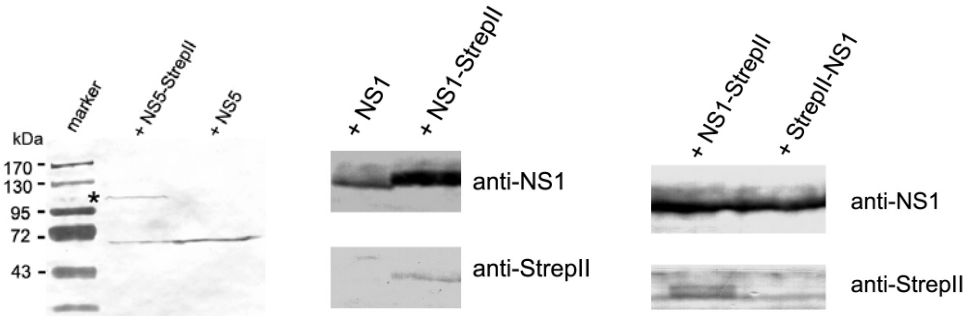


a) b)

Figure 4*: Antigenicity of NS1 of the chimeric virus as probed by Western Blot of infected cell lysate (a) and the resistance to the purification buffer of its replication complex (b)*

In addition to using NS1 from different strain, NS5 and NS1 were chosen for tagging with Strep-II affinity tag. Strep-II tag is suitable for our purpose for two reasons. First, the tag is a small, eight-amino-acids peptide(Trp-Ser-His-Pro-Gln-Phe-Glu- Lys) that has been adopted for highly specific pull-down in many protein-protein interaction studies. Second, the buffer conditions used in our purification method do not interfere with the binding and the elution of Strep-II tag. NS5 was chosen for tagging as it can be conveniently tagged in the C-terminus and transferred onto the virus genome. Functionally, NS5 is also the replicase protein. NS1 was chosen based on our preliminary results which show that the protein is an accessible component of the complex. We have constructed NS5-StrepII (C-term tag), StrepII-NS1 (N-term tag), and NS1-StrepII (C-term tag) on mammalian expression vectors (verified by DNA sequencing). NS5-StrepII and NS1-StrepII can be expressed in 293T cells and detected by both strep-tactin-HRP and anti-NS1 (figure 5a and 5b). However, StrepII-NS1 cannot be detected by strep-tactin-HRP but by an anti-NS1 when expressed in 293T cells (figure 5c). We suspect that the

surrounding amino acids around the N-terminus of NS1 could interfere with the binding of strep-tactin to the affinity tag.



a) b) c)

Figure 5*: Expression of StrepII-tagged-NS5 and NS1 of dengue 4 in 293T cells. a) Western blot of 293T-lysate expressing NS5-StrepII and NS5 alone. The asterisk marks the band that corresponds to the molecular weight of NS5-StrepII. b) Western blot of 293T-lysate expressing NS1-StrepII and NS1 alone. The top panel shows the blot probed with anti-NS1 antibody that is specific to dengue NS1. The bottom panel shows the blot probed with strep-tactin-HRP. c) Western blot of 293T-lysate expressing NS1-StrepII and StrepII-NS1.*

We incorporate the NS5-StrepII and NS1-StrepII onto the virus genome. Unfortunately, none of the recombinant viruses with intact StrepII tag could be recovered.

A consistent virus culture that can support the preparation of large amount of lysate.

The suitable virus strain for purification has a drawback that it cannot be consistently produced in high titer to support infection at high MOI, a condition required for producing lysate with large mount of replication complex. Therefore, we also initiated a search for mutations that can enhance the virus production and replication in cell culture system. The search involved virus passaging, virus isolation, and sequence analyses of different virus strains. We have incorporated these mutations into both the original and the chimeric viruses to test the utility of the mutations in increasing the titer. So far, we have identified the mutations that can increase the original titer by approximately two logs.

Summary and future work

Our continuous efforts to purify the dengue replication complex from both mammalian (funded by BIOTEC grants) and mosquito host cells (funded by TRF grant), we have achieved the following:

1. *Activity and Function:* We have found the way to maintain the activity of the dengue replication complex during purification.
2. *Purification:* We have identified key affinities that can be used to purify the complex from infected cells.

3. *Starting material:* We have found the way to manufacture the starting lysate with large amount of replication complex.

We now have the suitable system that could allow us to purify the complex in suitable amount to do proteomic and functional studies. We are now producing the lysate in large amount for purification.

Material and methods

Virus and cell culture

Dengue viruses were cultured from C6/36 cell line. The infection was performed at 28°C for 4-6 hours before supplementing cells with L15+1.5% fetal bovine serum (FBS) with TPB. The virus was either concentrated by PEG precipitation or stored frozen at -70°C until further use. Vero cells were maintained in MEM+10% FBS at 37°C under 5% CO_2 and 100% humidity. Infection of vero cells were carried out by inoculating vero culture with virus (from virus culture in C6/36). The infection was done at 37°C for 3 hours. After the infection period, the cells were washed with 1X PBS twice before supplemented with MEM+2%FBS. The infected cells were maintained for 24-27 hours before being harvested for lysate preparation.

Preparation of cell lysate, cytosol, and heavy-membrane

The lysate, the cytosol, and the heavy-membrane fraction from infected vero cells were prepared according to Chu&Westaway (1987)(3) and Uchil et.al (2003)(4).

In vitro RNA-dependent RNA polymerase (RdRp) assay

In vitro RNA-dependent RNA polymerase assay was performed according to the established protocol by Uchil et.al (2003) and Chu et.al (1987). GTP-alpha-P^{32} was used for labeling.

Chromatography

Flow-through, wash, and elute fractions were tested for in vitro RdRp activity and compared with the activity in the starting material (detergent-solubilized heavy membrane). The resin for anti-dsRNA column was generated by CNBr coupling of the monoclonal antibodies with activated Sepharose-CNBr resin (GE healthcare) according to the manufacturer protocol.

Tagging of strepII on NS5 and NS1

NS5 and NS1 were derived from PCR amplification using DV4H241 cDNA as the template. The PCR is then ligated onto the mammalian expression vector pcDNA3.1 (+) Hygro with StrepII-tag on it. The plasmid constructs with the correct sequence were then transfected into 293T cells using either lipofectamine reagent or PEI method. Two days after transfection, the cells were washed twice with cold 1XPBS before harvesting with SDS-PAGE reducing sample buffer.

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