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博 士 論 文

登革熱第二型病毒膜蛋白藉由胺基酸 K51 及 K241 與人類 Ubc9 蛋白產生交互作用

The Dengue virus type II envelope protein interacts with human Ubc9, a SUMO-conjugating E2 enzyme, via K51 and K241 amino acids

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摘要

登革熱為一經蚊蟲叮咬而感染人類之病毒，在全球暖化之氣候影響之下，其威脅性已不再侷限於熱帶/亞熱帶而成為全球性之流行疾病。登革熱病毒之外套膜蛋白屬於結構性蛋白，在感染宿主細胞時扮演很重要之角色，同時也是主要抗原之一。Chapter I 利用重組蛋白技術表現登革熱第二型病毒 strain PL046 之外套膜蛋白，該重組蛋白不包含 C 端之疏水性結構(transmembrane domain)，而以一段 S peptide 取代作為純化之用。在 *E. coli* 表現之結果雖然形成 inclusion body，但經過重新疊合後，此重組蛋白之結構未受影響。在病毒感染的同時加入重組膜蛋白能有效抑制病毒斑(plaque)之形成，顯示該重組膜蛋白經由與病毒顆粒本身之膜蛋白競爭，減少病毒入侵宿主細胞之機會。Chapter II 研究病毒膜蛋白與宿主細胞內蛋白之交互作用，藉由 Functional Yeast Array 篩選 500 個人類蛋白是否與登革熱第二型病毒膜蛋白發生交互作用。篩選的結果找出 5 個人類蛋白，其中一個為 Ubc9。利用 co-precipitation assay 可再次驗證 Ubc9 與 DV2E 之蛋白質交互作用，點突變結果顯示 Ubc9 可能以 K51 與 K241 作為與 DV2E 作用之位置。以共軛焦顯微鏡觀察於 BHK-21 細胞內表現 DV2E-GFP 及 Flag-Ubc9 兩種蛋白質之位置，發現當共同表現 DV2E-GFP 及 Flag-Ubc9 時，DV2E-EGFP 的位置由原本分布於細胞質範圍逐漸往細胞核趨近。另外，若於 BHK-21 細胞內大量表現 Ubc9 能減少病毒感染之病毒斑(plaque)之形成，上述結果顯示在登革熱病毒感染時，Ubc9 可能扮演一重要之角色。

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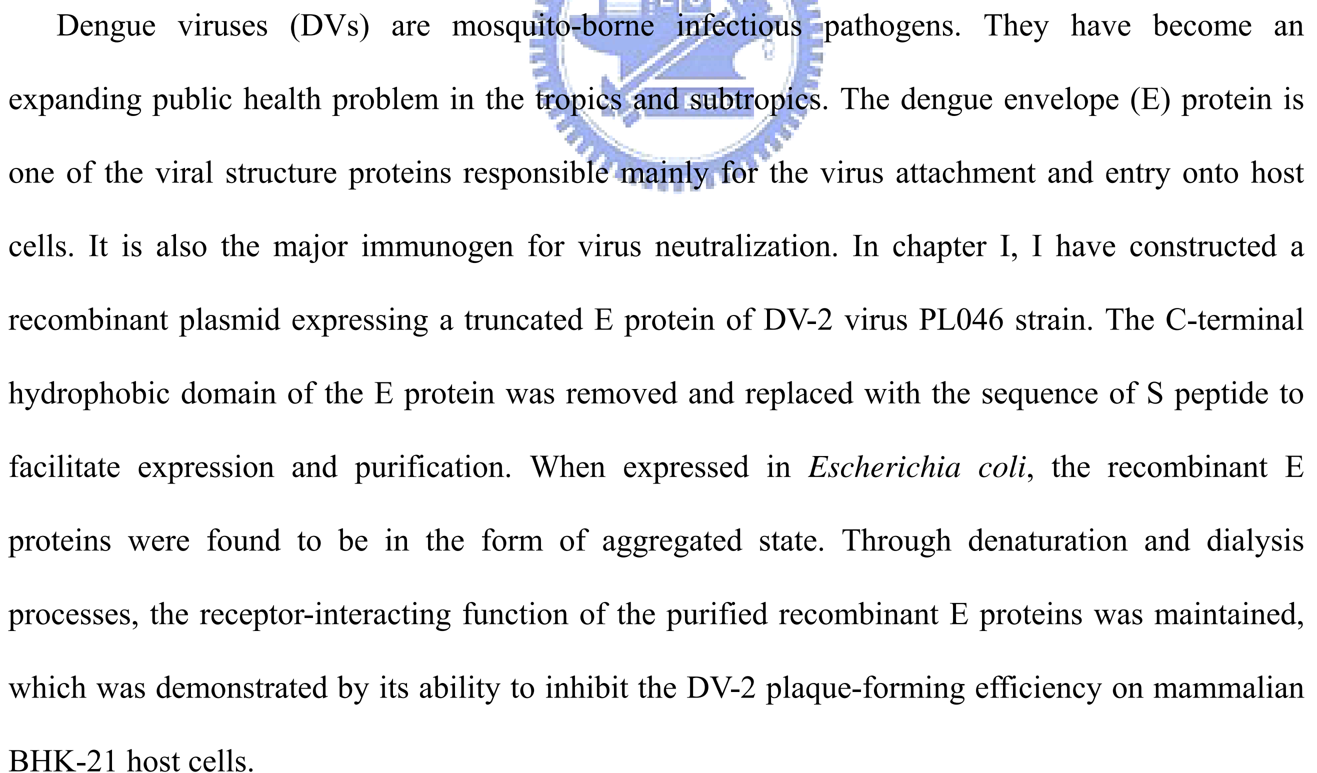
Department of Biological Science and Technology
National Chiao Tung University

ABSTRACT



Dengue viruses (DVs) are mosquito-borne infectious pathogens. They have become an expanding public health problem in the tropics and subtropics. The dengue envelope (E) protein is one of the viral structure proteins responsible mainly for the virus attachment and entry onto host cells. It is also the major immunogen for virus neutralization. In chapter I, I have constructed a recombinant plasmid expressing a truncated E protein of DV-2 virus PL046 strain. The C-terminal hydrophobic domain of the E protein was removed and replaced with the sequence of S peptide to facilitate expression and purification. When expressed in *Escherichia coli*, the recombinant E proteins were found to be in the form of aggregated state. Through denaturation and dialysis processes, the receptor-interacting function of the purified recombinant E proteins was maintained, which was demonstrated by its ability to inhibit the DV-2 plaque-forming efficiency on mammalian BHK-21 host cells.

In chapter II, to identify the human cellular proteins interacting with the envelope protein of dengue virus serotype 2 inside host cells, I have performed a screening with the yeast

two-hybrid-based “Functional Yeast Array”. Interestingly, the Small Ubiquitin-like Modifier-1 Conjugating Enzyme 9 protein, modulating cellular processes such as those regulating signal transduction and cell growth, was one of the candidates interacting with the dengue virus envelope protein. With co-precipitation assay, it is demonstrated that the dengue envelope protein indeed could interact directly with the Ubc9 protein. Site-directed mutagenesis has demonstrated that Ubc9 might interact with the E protein via amino acid residues K51 and K241. Furthermore, immunofluorescence microscopy has shown that the DV2E-EGFP proteins tended to progress toward the nucleus membrane and co-localized with Flag-Ubc9 proteins around the nucleus membrane in the cytoplasm side, and DV2E-EGFP also shifted the distribution of Flag-Ubc9 from evenly in the nucleus toward concentrating around the nuclear membrane in the nucleic side. In addition, over-expression of Ubc9 could reduce the plaque formation of the dengue virus in mammalian cells. This is the first report that DV envelope proteins can interact with the protein of sumoylation system and Ubc9 may involve in the host defense system to prevent virus infection.

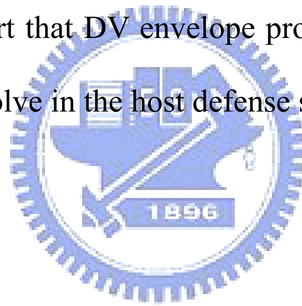


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Table of Contents

Abstract (Chinese)……………………………………………………………………… i
Abstract (English)……………………………………………………………………… ii
Acknowledgement……………………………………………………………………… iv
Table of Contents……………………………………………………………………… v
List of Figures………………………………………………………………………… vi
List of Tables………………………………………………………………………… viii
Abbreviations and Symbols…………………………………………………………… ix

General introduction………………………………………………………………… 1
1.1 The history and resurgence as a global public health problem………………… 1
1.2 Clinical spectrum of dengue infection…………………………………………… 2
1.3 Transmission and infection with dengue virus…………………………………… 3
1.4 Epidemiology………………………………………………………………………… 4
1.5 Molecular biology of dengue viruses……………………………………………… 5
1.6 Dengue virus replication…………………………………………………………… 11
1.7 Viral pathogenesis………………………………………………………………… 12

Thesis Objectives…………………………………………………………………… 14

Chapter I
Blocking the dengue virus 2 infections on BHK-21 cells with purified recombinant dengue virus 2 E protein expressed in *Escherichia coli*

Introduction…………………………………………………………………………… 15
Methods and Materials………………………………………………………………… 17
Results………………………………………………………………………………… 20
Discussion……………………………………………………………………………… 23

Chapter II
The Type 2 Dengue Virus Envelope Protein Interacts with Small Ubiquitin-like Modifier-1(SUMO-1) Conjugating Enzyme 9 (Ubc9)

Introduction…………………………………………………………………………… 31
Methods and Materials………………………………………………………………… 34
Results………………………………………………………………………………… 43
Discussion……………………………………………………………………………… 50

Reference…………………………………………………………………………… 67
Appendix
The map of pET-32c
The sequence of S-tag fragment

List of Figures

Figure 1.1 World distribution of dengue viruses and their mosquito vector, *Aedes aegypti*, in 2005 2

Figure 1.2 Transmission of dengue virus by *Aedes aegypti* 4

Figure 1.3 The distribution of dengue confirmed cases in Taiwan (1987-2006)............ 5

Figure 1.4 The genome organization of dengue virus 6

Figure 1.5 Intracellular life cycle of dengue virus 12

Figure 2.1 Construction of pStag9 plasmid.. 27

Figure 2.2 Detection of recombinant EStag9 protein expression. *E. coli* cells were sonicated and followed by centrifugation before resolution by 12 % SDS–PAGE 28

Figure 2.3 Competitive blocking assay 29

Figure 2.4 Quantitative representation of dosage-dependent competitive blocking assay of viral infection by the purified recombinant EStag9 proteins (●) and BSA (○) 30

Figure 3.1 Schematic presentation of DV2E-related constructs 56

Figure 3.2 The scheme of Function Yeast Array 57

Figure 3.3 Yeast two-hybrid analysis to assess the protein-protein interaction between Ubc9 and DV2E.. 58

Figure 3.4 Detection of DV2E protein with anti-EStag9 polyclonal antibody 59

Figure 3.5 Ubc9 interacting with DV2E *in vitro* 60

Figure 3.6 β-gal activity assay to quantify the interaction between DV2E and Ubc9 in yeast cells 62

Figure 3.7 Double mutations on DV2E abolishing the interaction between the DV2E and Ubc9 …………………………………………………………………………... 63
Figure 3.8 Subcellular localization of DV2E and Ubc9 by immunofluorescence ……….. 64
Figure 3.9 Determination of the Effect of Ubc9 over-expression on the propagation of dengue virus type 2 by plaque formation assay ………………………………. 65

List of Tables

Table 2.1 Comparison of the DV-2 E sequence (1–402 amino acids) variations between Taiwan local strain PL046 and New Guinea-C strain (Accession No. M29095)…………………………………………………………………. 26

Table 3.1 Primers used for mutagenesis ……………………………………………….. 53

Table 3.2 Comparison of traditional Yeast Two-Hybrid and Functional Yeast Array…... 54

Table 3.3 The candidates of interaction sites between Ubc9 and DV2E ………………. 55

Abbreviations and Symbols

DF	: Dengue fever
DHF	: Dengue hemorrhagic fever (DF/DHF)
DSS	: Dengue shock syndrome
DEN	: Dengue virus
DV2E	: dengue-2 virus envelope
CBA	: Competitive Blocking Assay
SUMO	: Small Ubiquitin-like Modifier
Ubc9	: SUMO conjugating E2 enzyme

General Introduction

1.1 The history and resurgence as a global public health problem

Dengue fever and dengue hemorrhagic fever (DF/DHF) are caused by the dengue viruses, which belong to the genus *Flavivirus*, family Flaviviridae. There are four serotype (DEN-1, DEN-2, DEN-3 and DEN-4), not only all of which can cause DF/DHF but also have similar natural histories, including humans as the primary vertebrate host and *Aedes* mosquitoes of the subgenus *Stegomyia* as the primary mosquito vectors. Since the 1950s, dengue has been endemic in Southeast Asia, where DHF/DSS was first recognized. Patients of DHF have been reported sporadically since 1780 in the Philadelphia epidemic [Rush, 1789]. Significant numbers of cases of hemorrhagic disease were associated with several subsequent epidemics, including Charter Towers, Australia, in 1897, Beirut in 1910, Taiwan in 1916, Greece in 1928, and Taiwan in 1931 [Hare, 1898; Koizumi et al., 1916; Copanaris, 1928; Akashi, 1932; Halstead and Papaevangelou, 1980]. Today, DHF/DSS remains one of the 10 leading causes of hospitalization and is the leading cause of childhood mortality in several Asian countries (World Health Organization, 1997).

The reasons for global emergence of DF/DHF as a major public health problem in the waning years of the twentieth century are complex and not fully understood. However, the reasarch of Gubler have identified several important factors [Gubler and Trend, 1994]. First, the susceptible individuals living in urban areas provided a pool of reinfection by *Ae. aegypti*, and the subsequently occurred epidemics of dengue also provided increased opportunity for the viruses to move between countries, both within and out of the region. Second, in most dengue-endemic countries of the world the control of mosquito was ineffective [Gubler, 1989; Newton and Reiter, 1992]. Third, major global demographics have changed by the concurrent uncontrolled population growth and unplanned urbanization. The environment provides ideal habitats for the vector mosquito. A fourth factor for emergence of DF/DHF is the increased

traveling by airplanes. It is providing the opportunities for transporting dengue viruses between population centers of the tropics, and resulting in a constant exchange of dengue viruses and other pathogens.

In 2005, dengue is the most important mosquito-borne viral disease affecting humans; its global distribution is comparable to that of malaria, and an estimated 2.5 billion people live in areas at risk for epidemic transmission (Figure 1.1) (CDC, 2007). Each year, tens of millions of cases of DF occur and, depending on the year, up to hundreds of thousands of cases of DHF. The case-fatality rate of DHF in most countries is about 5%, but this can be reduced to less than 1% with proper treatment. Most fatal cases are among children and young adults (CDC, 2007).

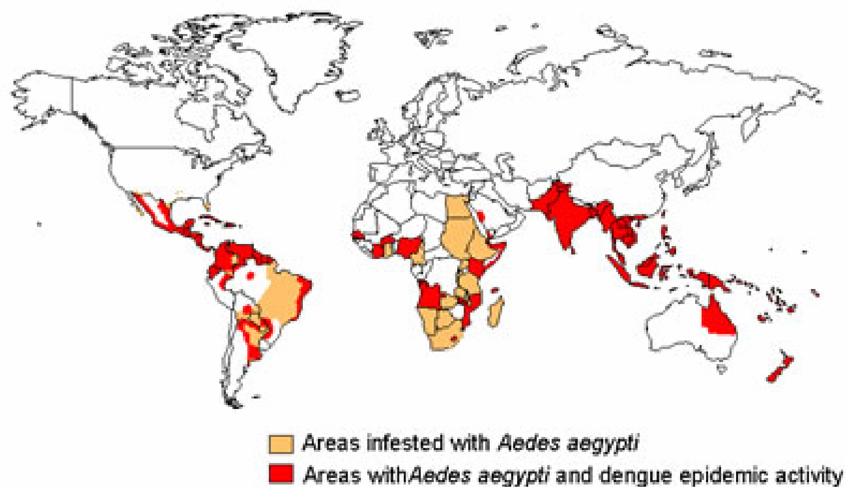


Figure 1.1 World distribution of dengue viruses and their mosquito vector, *Aedes aegypti*, in 2005 (CDC, 2007).

1.2 Clinical spectrum of dengue infection

The patients with dengue virus infection showed various clinical symptoms from no

significant illness, mild fever to life-threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The classic form of Dengue fever is a non-fatal febrile illness for older children and adults of about 5 to 7 days duration associated with sudden onset, extreme malaise, and pain of the muscles, back, limbs and eyes; rash is common as are other non-specfic constitutional symptoms such as nausea, vomiting and headache [George and Lum, 1997]. Manifestations of severe dengue include frank hemorrhage leading to shock through blood loss (dengue fever with hemorrhage), sudden increased vascular permeability leading to intravascular hypovolemia with or without frank hemorrhage, and severe encephalopathy with hepatitis. The classification of severe dengue has been complicated by the variation in clinical picture, for which the underlying pathophysiology may be different [George and Lum, 1997].

Patients with DHF may have fever lasting 2 to 7 days and a variety of nonspecific signs and symptoms, of which the most common manifestations are skin hemorrhages such as petechiae, purpura, or ecchymoses, but may also include epistaxis, bleeding gums, hematemesis, and melena. DHF patients develop thrombocytopenia and hemoconcentration, the latter as a result of the leakage of plasma from the vascular compartment, and the condition of these patients may rapidly evolve into dengue shock syndrome (DSS), which, if not immediately corrected, can lead to profound shock and death. Advanced warning signs of DSS include severe abdominal pain, protracted vomiting, marked change in temperature (from fever to hypothermia), or change in mental status (irritability or obtundation). Early signs of DSS include restlessness, cold clammy skin, rapid weak pulse, and narrowing of pulse pressure and/or hypotension. DHF/DSS can occur in children and adults, and the fatality rates among those with DSS may be as high as 44%. (CDC Division of Vector-Borne Infectious Disease, DVBID).

1.3 Transmission and infection with dengue virus

Dengue virus is transmitted by *Aedes mosquitoes* and causes the diseases mostly in tropical and subtropical regions worldwide. A number of *Aedes (Stegomyia*) mosquito species may act as

vectors including *Ae. aegypti, Ae. albopictus, Ae. polynesiensis*, and some members of *Ae. scutellaris* group. From a public health standpoint, the urban endemic/epidemic cycle is the most important transmission cycle. The viruses are maintained in an *Ae. aegypti*-human-*Ae. aegypti* cycle, with periodic epidemic occurring at 3 to 5 year intervals. Humans are infected with dengue virus by the bite of an infective *Aedes* mosquito [Gubler et al., 1979]. Adult *Ae. aegypti* mosquitoes are unobtrusive, prefer to rest indoors and feed on humans during daylight hours. The female mosquitoes often disrupt the feeding process at slightest movement and return to the same or a different person to continue feeding moments later. The *Ae. aegypti* females may thus feed on several persons during a single blood meal and transmit dengue virus to multiple persons within a short period of time [Gubler et al., 1979; Gubler et al., 1981; Gubler et al., 1984]. This behavior makes *Ae. aegypti* an efficient epidemic vector (Figure 1.2).

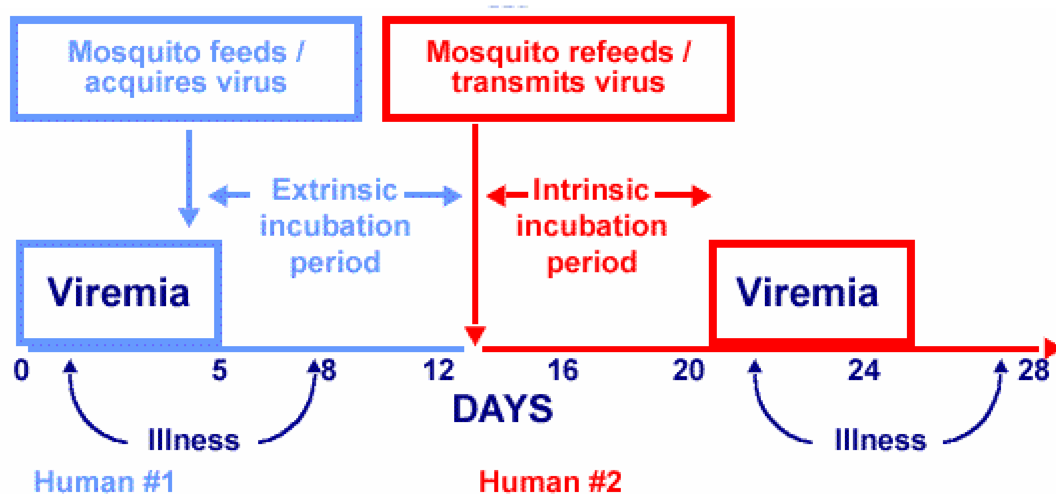


Figure 1.2 Transmission of dengue virus by *Aedes aegypti*. (CDC, 2007)

1.4 Epidemiology

The dengue fever may occur endemically or as epidemics. Within countries the virus spreads along transporation routes; between countries it appears first in seaports or airport cities. Epidemic dengue is most often described in setting where most or all of the population are non-immune. Outbreaks are often explosive with a majority of patients being older children and adults. Attack rates may be high, sometimes 80 to 90 percent, but more commonly, 40 to 50

percent of the population [Halstead, 1997]. A dengue epidemic requires the presence of the vector mosquito (*Aedes aegypti*), the virus, and a large number of susceptible human hosts. Outbreaks may be explosive or progressive, depending on the density and susceptibility of the vector, the strain of dengue virus, the immune level in the human population, and the amount of vector-human contact.

The dengue fever at Taiwan had several epidemics between 1901 and 1987. According the statistics of Centers for Disease Control R.O.C., there was outbreak at Kaohsiung County in 1987. Since them, the infections of dengue virus serotypes 1 to 4 have also been reported, and the imported cases have increased in recent years. It is suggested that Taiwan now has become a high dangerous region of dengue infection.

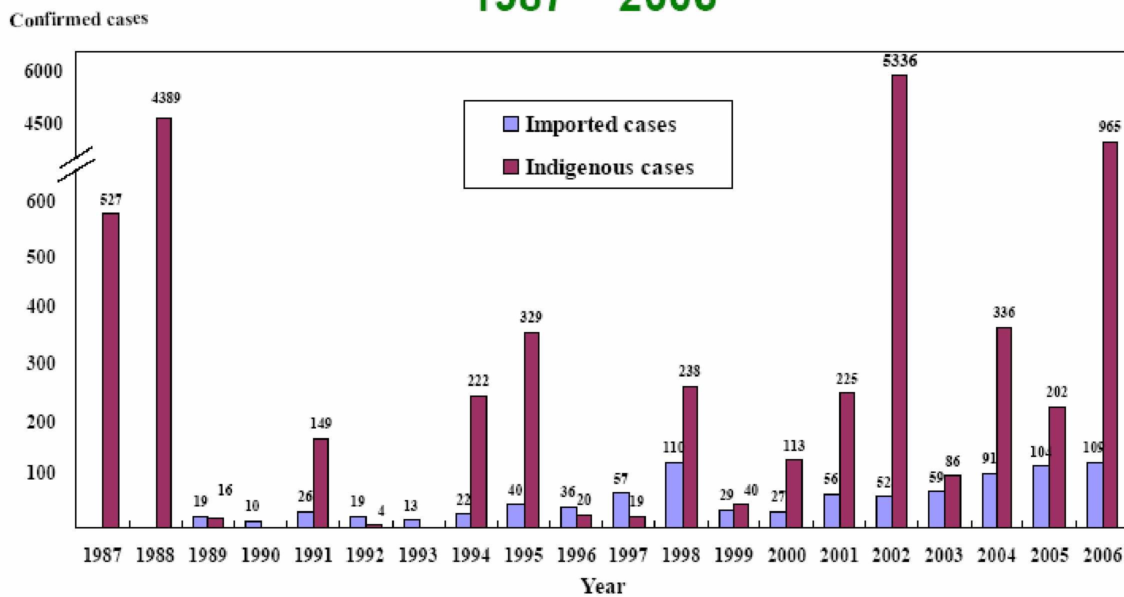


Figure 1.3 The distribution of dengue confirmed cases in Taiwan (1987-2006) (Centers for Disease Control R.O.C.)

1.5 Molecular biology of dengue viruses

Dengue virus type 2 (DEN-2), a member of the family *Flaviviridae*, contains a

single-stranded RNA genome of 10,723 nucleotides (New Guinea C strain), having a type 1 cap at the 5' end, but lacking a poly(A) tract at the 3' end [Westaway et al., 1985; Rice et al., 1986; Brinton, 1986.; Westaway, 1987; Chambers et al., 1990]. The genomic RNA is of positive-strand polarity, having a single open reading frame that encodes a polyprotein of 3,391 amino acids, which is processed into three structural and at least seven nonstructural proteins so far identified. The gene order is 5'-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4BNS5-3', where C, prM, and E are the structural proteins and NS1 through NS5 represent the nonstructural proteins. The processing of the polyprotein precursor occurs cotranslationally as well as posttranslationally and is performed by either the host signalase in association with the membranes of the endoplasmic reticulum or the viral protease(s) [Chambers et al., 1990].

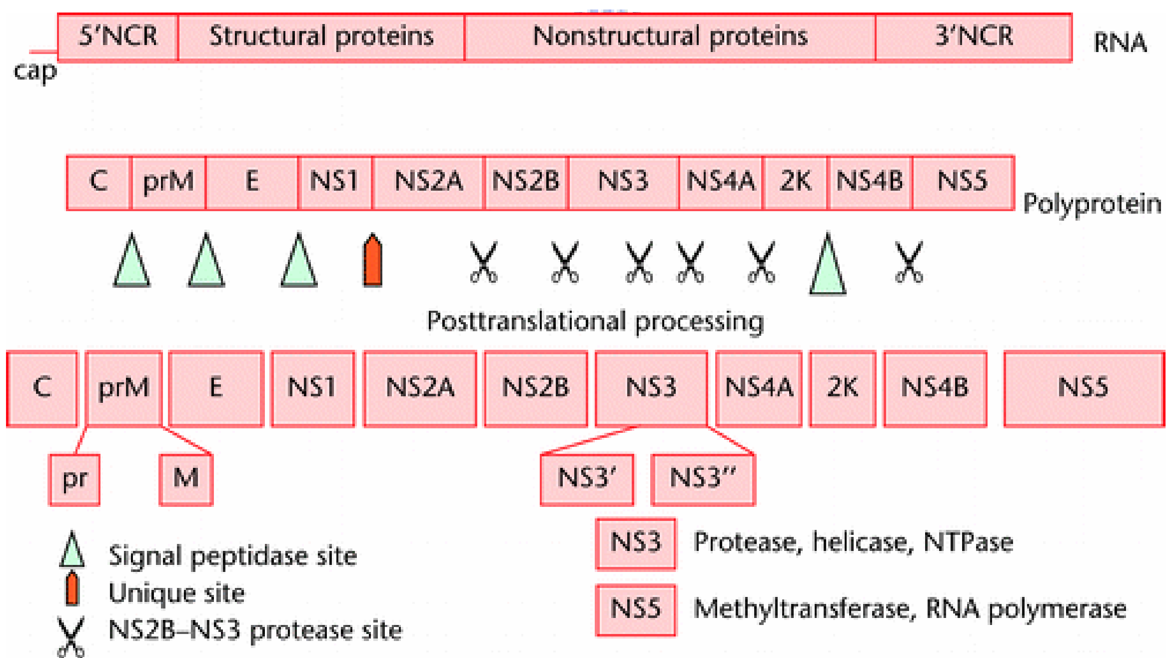


Figure 1.4 The genome organization of dengue virus. (Gubler, D. J., Centers for Disease Control and Prevention, Fort Collins, Colorado, USA)

1.5.1 Capsid (C) protein

The dimeric capsid or core protein is one of DEN structural proteins, it is essential in virus

assembly to ensure specific encapsidation of the viral genome. The mature form of DEN C protein is a highly basic protein of 12 kDa after removal of the C-terminal hydrophobic signal sequence. When the polyprotein is processed, the function of C-terminal region for capsid protein is serving as a signal sequence, the capsid protein then anchors into the ER membrane and thus translocates prM into the lumen of the endoplasmatic reticulum. Subsequently, this signal sequence is cleaved by the host cell signalases liberating the N-terminus of prM whereas C remains closely associated with the ER membrane promoting viral assembly. The membrane-associated capsid protein mediates the viral assembly by coordinated interaction with the E-prM heterodimer in the ER. The immune viral particles containing C protein and genomic RNA that forms as the nucleocapsid (NC) are then budding into the lumen of the ER [Wang et al., 2002]. It was reported that the capsid protein is also found in the nucleus and can possibly interact with hnRNP K, suggesting that C protein may play a role in regulation of the dengue lifecycle by controlling apoptosis [Chang et al., 2001].

1.5.2 Envelope (E) protein

The virus attachment and entry are mainly dependent on the envelope (E) protein, the major glycoprotein on the flavivirus particle [Chambers et al., 1990; Monath and Henize, 1996]**.** The E protein forms an oligomer with the small membrane (M) protein and constitutes most of the accessible virion surface [Lee et al., 2000]. This reflects that the E protein is essential for membrane fusion and mediates the binding to host, it is also the primary antigen that inducing protective immunity and the major antigen for virus neutralization [Rice et al., 1996; Roehrig, 1997]. Therefore, the protein directly affects the host range, cellular tropism and in part, the virulence of DEN virus [Monath and Henize, 1996].

Based on the crystallography data of the tick-borne encephalitis flavivirus E protein, Rey et al. [Rey et al., 1995] noted that each E-protein monomer is folded into three distinct structural domains, domain I, II, and III. The domain I in the central structure of E protein is the antigenic

domain that carries the N glycosylation site. Structural domain II of the E protein is suggested to be responsible for pH-dependent fusion of the viral E protein and the endosomal membrane during uncoating. Structural domain III is reported to play the important role for flavivirus binding to host cells [Rey et al., 1995]. The structural domain III of E protein contains an immunoglobulin-like constant domain and is postulated to form the receptor-binding site for the virus particles.

1.5.3 Membrane (M) and pre-membrane (prM) protein

The flavivirus particle consists of a nucleocapsid core, which is surrounded by an ER-derived lipid bilayer containing E and prM/M, the structural proteins that were synthesized as a polyprotein [Mukhopadhyay et al., 2005]. The prM will be processed to the mature M protein late in secretion in the *trans* Golgi compartment by furin [Stadler et al., 1997]. Maturation of processed from prM to M protein is necessary to expose the E receptor binding domain and thus for virus infectivity [Heinz and Allison, 2003]. The prM is suggested to protect the E protein from pH-induced reorganization and premature fusion during the secretion of E proein [Guirakhoo et al., 1991; Guirakhoo et al., 1992; Zhang et al., 2003], and the prM protein is possibly to serve as a chaperone for proper E folding and assembly [Heinz and Allison, 2003]. It was reported that regulated efficiency of cleavage of prM/M is important for viral replication [Keelapang et al., 2004].

1.5.4 Non-structural 1(NS1) protein

NS1 is the first non-structural protein in the DEN polyprotein, following E protein and preceding the NS2A protein. It is a 46 kD glycoprotein, with two glycosylated asparagines, and 12 cysteines that form 6 disulfide bridges. Although it does not contain a hydrophobic membrane spanning region, the NS1 is translocated into the lumen of the ER during translation, where it dimerizes and stays membrane associated. The double stranded RNA corroborating evidence

showed that NS1 is essential for viral replication by an unknown mechanism [Muylaert et al., 1997; Flamand et al., 1999; Lindenbach and Rice, 1999]. Additionally, when the NS1 is exported through the Golgi excretory pathway, NS1 can be detected both outside the plasma membrane of infected cells and anchored into the membrane by a glycosyl-phosphatidylinositol (GPI). It has been suggested that the antibodies against NS1 in a DEN infected patient may bind to the NS1 protein both on the cell surface and activate GPI-mediated signaling in the infected cell. The binding of anti-NS1 antibodies is possibly enhancing viral replication or disease pathology [Jacobs et al., 2000]. NS1 is also excreting in a soluble hexameric form from mammalian cells but not from mosquito cells [Flamand et al., 1999]. Soluble NS1 is found in the blood of DEN infected patients [Young et al., 2000; Alcon et al., 2002] and the NS1 blood levels is reported to correlate with disease severity [Libraty et al., 2002; Avirutnan et al., 2006]. Soluble NS1 in the blood is suggested to contribute to DEN pathology by activating complement [Avirutnan et al., 2006], inducing auto-immune antibodies [Lin et al., 2003] or accumulating in hepatocytes in the liver [Alcon-LePoder et al., 2005]. Vaccination studies used to target and reduce free NS1 circulating in the blood have shown different results, some studies showed protection against an intracerebral challenge with DEN [Costa et al., 2005], but other studies did not show protection [Timofeef et al., 2004; Calvert et al., 2006].

1.5.5 NS3 protein

NS3 is a 67 to 70-kDa protein of 618 to 623 amino acids that is highly conserved among flaviviruses. The NS3 is proposed to have two functions in viral replication: serine protease and helicase. A region near the N-terminus of NS3 exhibits sequence and structural homology to the active domain of trypsin related serine protease [Bazan and Fletterick, 1989]. In combination with NA2B, it is required for proteolytic processing at the dibasic site of many viral proteins. The C-terminus of flaviviruses NS3 is suggested to be involved in several functions including RNA helicase [Gorbalenya et al., 1989], RNA-stimulated NTPase activity [Wengler and Wengler,

1991; Wallner et al., 1993], and the capping and methylation [Wengler and Wengler, 1993].

1.5.6 NS2A, NS2B, NS4A, and NS4B protein

NS2A, NS2B, NA4A, and NS4B are small non-structural proteins. All four proteins are poorly conserved in sequence but exhibit conserved hydrophobicity profiles among flaviviruses. The evidence suggesting that they are membrane-associated proteins (Chambers T. J., 1990). The functions of these four proteins remain largely undefined. NS2A (18 to 22-kDa of 218 to 231 amino acids) is reported to be required for the C-terminal processing of NS1 [Flagout and Lai, 1989]. NS2B (13 to 15-kDa of 130 to 132 amino acids) is suggested to be involved in the protease function of the NS2B-NS3 complex and essential for protease activity of the complex [Flagout et al., 1993]. The functions for NS4A (16.0 to 16.4-kDa of 149 to 150 amino acids) and NS4B (27 to 28-kDa of 248 to 256 amino acids) have not yet been identified. They may be involved in membrane localization of NS3-NS5 replication complex via protein-protein interaction, since the NS3-NS5 complex is reported to weakly associate with the membrane in spite of its hydrophilic characteristic [Chambers et al., 1990; Wengler et al., 1990].

1.5.7 NS5 protein

The NS5 protein consists of at least three important enzymatic functions which are essential for viral propagation in flaviviruses [Khromykh et al., 1998; Hanley et al., 2002]. The N-terminal region of NS5 protein represents the active domain of S-adenosyl-L-methionine dependent methyltransferase (SAM)(amino acids 1-320), which possess the methyl transferase and guanylyl transferase activities responsible for capping and methylating at the positive strand genomic RNA on its 5’ terminus [Egloff et al., 2002]. The C-terminal domain encodes the RNA dependent RNA polymerase (residues 420-900) responsible for synthesizing the double stranded replicative intermediate RNA template and also plus(+) strand RNA genomic RNA [Bartholomeusz and Thompson, 1999; Egloff et al., 2002; Nomaguchi et al., 2003]. The RNA dependent RNA

polymerase activity of this domain has been demonstrated for several other flaviviruses including West Nile virus, Kunjin virus, Hepatitis C viruses (HCV) and BVDV [Tan et al., 1996; Khromykh et al., 1998; Steffens et al., 1999; Guyatt et al., 2001]. The RNA dependent RNA polymerase has an essential GDD motif. In Flavivirus, it is shown that the mutations on this motif would result the virus non-replicative [Khromykh et al., 1998; Ranjith-Kumar et al., 2001].

1.6 Dengue virus replication

The intracellular replication cycles of the flaviviruses are very similar. Infection with the dengue virus is introduced into the host by the mosquito. The virus enters the permissive host cell via receptor-mediated endocytosis (RME), and fusion of viral and vesicular membranes allows the nucleocapsid entrying into cytoplasm and uncoating the viral genome. The translation of positive-strand viral RNA (vRNA) and viral proteins then begin and the structural protein capsid or core (C), premembrane (prM), and envelope (E) proteins, along with viral RNA, are assembled into progeny virions, and the virions are transported through the Golgi compartment and secreted.

It is generally accepted that DEN gains entry to its target cell by receptor-mediated endocytosis (RME). A number of different mammalian cell receptors have been proposed, including heparan sulfate [Chen et al., 1997; Hilgard and Stockert, 2000; Germi et al., 2002; Lin et al., 2002a], heat shock protein 70 (Hsp70) and Hsp90 [Valle et al., 2005], GRP78/BiP [Jindadamrongwech et al., 2004], CD14 [Chen et al., 1999], and 37-kDa/67-kDa high affinity laminin receptor [Thepparit and Smith, 2004], as well as DC-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin (DC-SIGN) [Navarro-Sanchez et al., 2003; Tassaneetrithep et al., 2003; Lozach et al., 2005] and liver/lymph node-specific ICAM-3-grabbing nonintegrin [Tassaneetrithep et al., 2003]. Although it is suggested that the mononuclear phagocyte lineage cells like monocytes, macrophages, and dendric cells are the primary targets *in vivo* [Jessie et al., 2004], DEN has been reported to be capable of infecting

numerous human cells, including dendritic cells (DCs), monocytes/macrophages, B cells, T cells, endothelial cells, hepatocytes, and neuronal cells, as well as a number of cell lines used for viral propagation *in vitro* [Anderson, 2003].

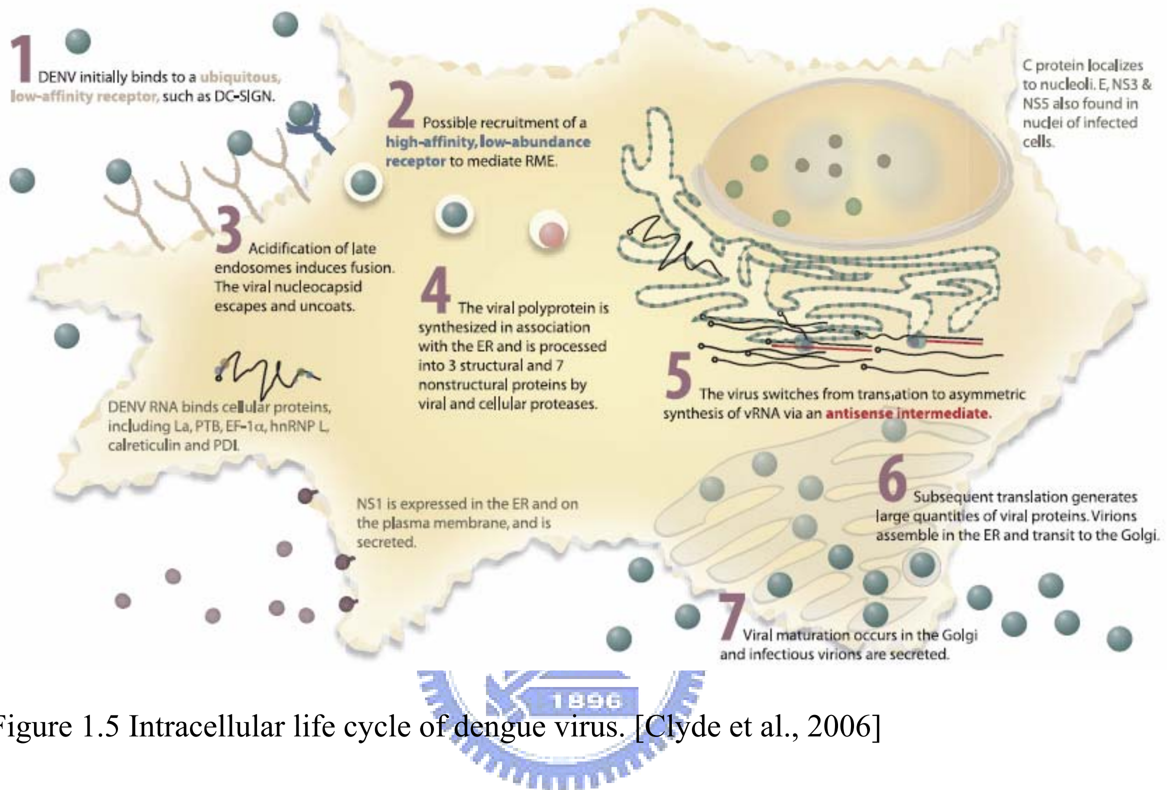


Figure 1.5 Intracellular life cycle of dengue virus. [Clyde et al., 2006]

1.7 Viral pathogenesis

The pathogenesis of dengue fever is not understood completely, and the sites of dengue replication in humans have not been well characterized. The main pathophysiologic features of DHF/DSS are (1) increased vascular permeability resulting in loss of plasma from the vascular compartment, leading to hemoconcentration and shock; and (2) a disorder of the homeostasis involving vascular changes, thrombocytopenia, and coagulopathy (WHO, 1997). Several hypotheses have attempted to explain DHF/DSS findings, but the lack of an animal model for DHF/DSS has made it difficult to clarify the steps involved in DHF/DSS pathogenesis. There is no treatment for these diseases and immunization may provide a relastic approach for controlling dengue infection [Jimenez and Lopes da Fonseca, 2001]. Although considerable research has

been directed towards the development of a safe, effective DEN vaccine since the middle of 20th century, no approved product is presently available. Several approaches have been taken to the expression of recombinant dengue E protein to develop as the vaccine candidate; unfortunately there is no effective vaccine for DEN available in spite of years of effort to develop live attenuated, inactivated whole virion and subunit vaccines based on *E. coli*, vaccinia virus and baculovirus expression system.

Thesis Objectives

Dengue viruses have reemerged as an increasingly important public health threat. Unfortunately, there is still no effective commercial DEN vaccine available in spite of years of effort to develop live attenuated, inactivated whole virion vaccines. Moreover, the exact internalization and trafficking pathways taken by flavivirus to gain access into host cells still remains unclear. Among the structural proteins, the envelope protein of flaviviruses has been demonstrated to play a crucial role in mediating virus-host cellular receptors interaction [Heinz et al., 1994; Helenius, 1995; Rey et al., 1995; Crill et al., 2001; Thullier et al., 2001], and it is also the primary antigen that induces protective immunity and hence the major antigen for virus neutralization. Therefore, the goals of thesis aimed to:

1. Expression of recombination E protein in order to test for the functionality of the receptor binding region of the truncated DV2E and to provide a potentially economic antigen source for subunit vaccine, and study the function of E protein.
2. Investigate the pathogenesis of dengue virus through the protein-protein interaction between host cellular protein and DV2E.

Chapter I

Blocking the dengue virus 2 infections on BHK-21 cells with purified recombinant dengue virus 2 E protein expressed in *Escherichia coli*

Introduction

Dengue viruses are human pathogens that have reemerged as an increasingly important public health threat. Currently, there is only supportive treatment for those patients who infected by DEN. The treatments such as immunization are being developed in the hope of providing a realistic approach for controlling dengue infection [Jimenez and Lopes da Fonseca, 2001]. Unfortunately, there is still no effective commercial DEN vaccine available in spite of years of effort to develop live attenuated, inactivated whole virion vaccines.

Among the structural proteins, the E protein is the major structural protein on the surface of the mature dengue virions. The E protein forms oligomers with the small membrane (M) protein and constitutes most of the accessible virion surface [Lee et al., 2000]. This reflects the fact that the E protein is essential for membrane fusion and mediates binding to host cells. E protein is consisted of 495 amino acids with a molecular weight of about 60 kDa. It has been suggested that E protein has the major role responsible for host cell attachment and entry [Chambers et al., 1990; Monath and Henize, 1996]. It is also the primary antigen that induces protective immunity and hence the major antigen for virus neutralization. Therefore, the protein directly affects the host range, cellular tropism, and in part, the virulence of DVs [Monath and Henize, 1996].

Several approaches, based on *Escherichia coli*, vaccinia virus, Pichia yeast, and baculovirus expression systems [Delenda et al., 1994; Staropoli et al., 1996; Staropoli et al., 1997; Wei et al., 2003], have been taken to achieve the expression of recombinant DV E protein for the

development of subunit vaccine candidates, but the yields of recombinant E protein have been low and/or the procedures were not economically sound [Delenda et al., 1994; Staropoli et al., 1996; Staropoli et al., 1997; Wei et al., 2003]. In order to study the structure and function of the E protein and to provide a potentially economic antigen source for subunit vaccine development, it is set out to express E proteins in *E. coli* using expression vector pcDNA3 to produce recombinant proteins fused with in vivo expression tag sequences for purposes of identification and purification.

Here is report the functional expression of a recombinant E protein of DEN-2 virus strain PL046. This recombinant E protein has a C-terminal deletion of the hydrophobic region and a C-terminal addition of the S-tag peptide sequence that allows the application of a simple purification procedure by S-protein–agarose and antibody detection. Initially, majority of the expressed recombinant E proteins were in the form of inclusion body. The inclusion body could then be denatured by urea for isolation and purification procedures. After the removal of urea using dialysis treatment, the purified proteins still retained the receptor-binding function demonstrated by the ability of competitive blocking wild-type virus infection on host cell BHK-21.

Methods and Materials

Cells and viruses

The dengue-2 virus strain PL0146 is a gift from Dr. Yi-Ling Lin (IBMS, Academia Sinica).

BHK-21 cell were cultured at 37°C, 5% CO_2 in MEM medium (Gibco 41500-034) supplemented with 0.22% of sodium bicarbonate and 10% of fetal bovine serum (FBS)(Gobco).

C6/36 cells were growth at 28°C in MEM medium (Gibco 41500-034) supplemented with 0.11% of sodium bicarbonate and 10% of FBS.

E. coli BL21 (DE3) were used for plasmid replication and expression in bacteria.

Construction of plasmids

The cDNA fragment containing the DV2 E gene was kindly provided by Dr. Yi-Ling Lin (IBMS, Academia Sinica, Taipei). The E gene without the sequence of the C-terminal 93 amino acids was then cloned into the expression vector pcDNA3 and named pTru11E3. Two restriction sites were introduced to flank the truncated E gene sequence by PCR. The 5' primer (5' TTTCTCGAGGACAATGCGTTGCATAGG 3') introduced a 5' end *Xho*I site, while the 3' primer (5' AAATCTAGACTCAAGCATTTGGCCGATAGA 3') introduced an *Xba*I site. The amplified fragments were digested with *Xho*I and *Xba*I enzymes and introduced into the pcDNA3 expression vector also restricted with *Xho*I and *Xba*I enzymes. To introduce the S tag sequence into pTru11E3, additional PCR was employed. The S sequence of pET-30a vector (Novagen) was amplified by PCR. The 3' primer (5' TTTGGGCCCACTACGTGAACCATCACC 3') contained *Apa*I site and the 5' primer (5' TTTTCTAGACTGGTGCCACGCGGTTCT 3') contained *Xba*I site. There is a stop codon before the sequence of 3' primer. The amplified fragments containing the S sequence were then restricted with *Xba*I and *Apa*I and introduced into pTru11E3. The resulting plasmid, pStag9, contains the N-terminal 402 amino acid residues of DV2 E protein (nucleotides 937–2124 of

DV-2 genome) with an in-frame S tag fused at C-terminus as shown in Fig. 2.1. A thrombin cleavage site along with spacer sequences has also been included and situated between E gene and S tag sequence.

Expression and purification of recombinant protein

Escherichia coli BL21(DE3) were transformed with pStag9 and grown in LB broth at 37 ^{o}C until OD600 reached 0.4. After additional 3 h incubation, the cells were harvested and centrifuged for 10 min at 5000 rpm. The pellets were resuspended with 1mM Tris–HCl (pH 8.0) containing 1mM PMSF and sonicated. The centrifuged cell lysates were re-suspended with inclusion body solubilization buffer (50mM Tris–HCl, pH 8.0, 1mM EDTA, 100mM NaCl, 8M urea, and 1mM PMSF) before incubation for 1 hour on ice. Removal of the urea was achieved by dialysis overnight in dialysis buffer (20mM Tris–HCl, pH 7.0, 150mM NaCl, and 0.1% Triton X-100).

Purification of recombinant proteins was performed by affinity purification using S-protein agarose (Novagen) following the instruction provided by the manufacturer. The S-protein agarose was washed with 10 volumes of binding/wash buffer before the sample was applied to the S-protein agarose. This was then followed by washing the agarose with 10 volumes of binding/wash buffer. The elution buffer (20mM Tris–HCl, pH 7.0, 150mM NaCl, 0.1% Triton X-100, and 3M magnesium chloride) was applied to elute the bound proteins. The eluted fractions were analyzed by SDS–PAGE. The collected proteins, named EStag9, were then subjected to dialysis treatment to remove the salt and urea. The concentrations of the purified EStag9 proteins were determined by Protein Assay Kit (Bio-Rad).

Western blot

Samples were subjected to a 12% SDS polyacrylamide gel and electrophoretically transferred onto nitrocellulose membranes (PROTRAN, Schleicher & Schuell). The membranes were then

washed in TBS buffer (Tris-buffered saline, 10mM Tris, pH 8.0 and 150mM NaCl) containing 5% non-fat dried milk at room temperature before the first antibody was added to the reaction mixture. The first antibody was either rabbit anti-DV2 E domain III polyclonal antibody (provided by Dr. Wen Chang IMB, Academia Sinica, Taipei) or anti-S tag antibodies conjugated with horseradish peroxidase protein (HRP). The reaction was then incubated overnight at 4 ^{o}C. The membranes were then washed three times in TBST buffer (Tris buffered saline, 10mM Tris, pH 8.0, 150mM NaCl, and 0.05% Tween 20) before the addition of horseradish peroxidase-conjugated goat anti-rabbit IgG or anti-mouse IgG (Chemicon international) in 1:10,000 dilutions as the second antibody when required. The reaction was performed in TBS buffer containing 5% non-fat dried milk and incubated at room temperature for 1 hour. The membranes were then washed with TBST buffer and the proteins were detected by the LumiGLO system (Kirkegaard and Perry Laboratories).

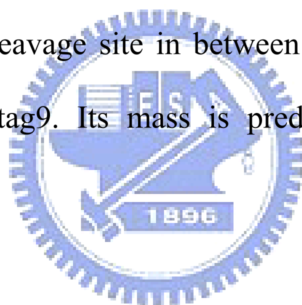
Competitive Blocking Assay (CBA)

BHK-21 cells were passaged at $4x10^5$ cells per well in 6-well plates and incubated at 37 ^{o}C with 5% CO_2 for 48 h. Serial dilution of the purified EStag9 proteins or BSA in MEM without FBS was added to 0.5 ml of DV-2 PL046 strain in the amount of 80–100 pfu/well. The mixtures were mixed gently and added onto the BHK-21 cells in 6-well plates and then incubated at 37°C with 5% CO_2 for 1 h. After aspiring the supernatant, 1:1 mixture of MEM and 2% methylcellulose were added to the well and incubated at 37°C with 5% CO_2 for 5 days. The medium was aspired before the cells were fixed with 3.7% formaldehyde. After 30 min, the solution was removed and the cells were stained with 1% crystal violet in 3.7% formaldehyde. The plates were washed with 3.7% formaldehyde before the plaque numbers were scored.

Results

Construction of the recombinant DV-2 E gene

The first 1206 nucleotides of the DV-2 (strain PL046) E gene coding region from the cDNA clone (provided by Dr. Yi-Ling Lin, IBMS, Academia Sinica, Taipei) were amplified by PCR. Restriction enzyme sites of *Xho*I and *Xba*I were introduced separately into the 50 and 30 of the PCR product to facilitate subsequent cloning into pcDNA3, an *E. coli*/mammalian expression shuttle vector. The S tag region of pET-30a was then subcloned into the *Xba*I and *Apa*I sites of the recombinant plasmid and was in frame with the sequence of the truncated E gene sequence. The resulting plasmid was named pStag9 **(Fig. 2.1)**. The encoded recombinant protein is predicted to have 402 amino acids from E gene and a C-terminal S tag peptide and spacer sequence along with a thrombin cleavage site in between. This created a recombinant protein with 482 amino acids named EStag9. Its mass is predicted to be 53 kDa if there is no modification on the protein.

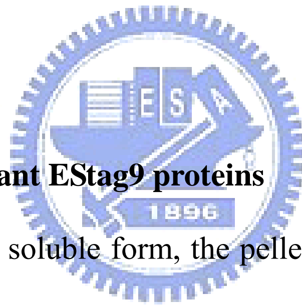


Sequence analysis of the recombinant E gene

The recombinant expression clone of the DV2 E gene of strain PL046 was subjected to sequence analysis. There are nine variations on the cDNA sequence in comparison to the DV2 strain New Guinea C (Accession No. M29095). As shown in Table 2.1, the mutation on nucleotide 1255 (amino acid 107) is a silent mutation, the rest are missense mutations. Those mutations are located at amino acid residues 47, 53, 55, 71, 107, 116, 402, 454, and 474 of DV-2 E proteins. According to the reported crystal structure [Rey et al., 1995; Modis et al., 2003], residue 47 is in domain I, residues 53, 55, 71, 107, and 116 in domain II, and the rest in the transmembrane domain.

Production and purification of recombinant DV-2 E protein in *E. coli* cells

The pStag9 construct was transformed into *E. coli* strain *BL21(DE3)*. The total cellular proteins from *E. coli* transformants were analyzed by SDS–PAGE with Coomassie blue staining (Fig. 2.2A) or by Western blot (Figs. 2.2B–D) using antibody against the S-tag sequence or against domain III of E proteins (provided by Dr. Wen Chang, IMB, Academia Sinica, Taipei). As shown in Fig. 2.2, panel A, a dominant band corresponding to the predicted size of the recombinant protein can be detected in the pellet from cells transformed with the pStag9 but not from cells transformed with the control vector pcDNA3 alone. No dominant band was detected in the supernatant of cell lysates (Fig. 2.2). Hence, the expressed proteins were in the form of inclusion bodies in *E. coli* cells.



Solubilization of the recombinant EStag9 proteins

In order to obtain the protein in soluble form, the pellets of cell lysates containing inclusion body were denatured in 8M urea following sonication treatments to the *E. coli* cells. After the purification with S-protein agarose, the EStag9 proteins then underwent dialysis to removed urea. The S-protein agarose (Novagen) can bind the 15 amino acid S tag peptides fused in frame at the C-terminus of EStag9. After elution, elutes in fraction of 0.5 ml were analyzed by SDS–PAGE and stained with Coomassie blue and assessed by Western blotting with anti-E domain III polyclonal antibodies. The resulted products were quantitated by Protein Assay Kit (Bio-Rad). The overall yield is 3 μg purified protein for 100 milliliter of *E. coli* culture.

The competitive blocking assay assessing the biological function of EStag9 proteins

To assess whether the purified EStag9 proteins still have the biological function to bind host

cells, the purified proteins were serial diluted and mixed with virions of DV2 PL046 strain. If the purified EStag9 can still bind host cells, it will compete with the DV2 virions for host cell surface receptors. This shall reduce the frequency of BHK-21 cells infected by wild-type virions and shall result in the reduction of the number of plaques formed. Since every plaque represents an infection event, the number of plaques in an assay plate indicates the numbers of successful virion infection events. As shown in Figs. 2.3 and 2.4, the plaque number was significantly reduced by the addition of the purified EStag9 proteins to the virions. Addition of BSA did not exhibit the same effect. Fig. 2.4 shows that the number of plaques formed on BHK-21 cells decreased as the amount of EStag9 proteins increased. This indicated that the purified EStag9 proteins could effectively block the plaque formation by competing with DV2 virions for the infection of host cells. As shown in Fig. 2.4, the addition of 3 μg of EStag9 proteins inhibited more than 50% PFU (plaque forming unit) compared to no Estag9 proteins addition. Addition of equal amount of BSA reduced 20% of the PFU under conditions tested. Addition of 10 μg of EStag9 protein reduced the PFU down to less than 10% of that of wild type, while the addition of BSA still reduced only 20%. This result indicates that the receptor binding function of the purified recombinant E protein is still retained.

Discussion

Analysis of the sequence of the cDNA clone of DV2 E gene (strain PL046) showed that there are nine single nucleotide variations compared with the DV-2 strain New Guinea C (Accession No. M29095). Those variations may arise from strain diversity or the process of cDNA construction. The crystal structure of the first 394 residues of DV2 E protein homodimer has been solved [Modis et al., 2003] and multiple lines of evidence indicated that the structure of E protein is conserved across the *Flaviviridae* [Rey et al., 1995]. According to their results, the flavivirus E protein can be divided into three distinct structural domains besides the transmembrane region. For DV E protein, domain I consists of amino acid residues 1–51, 132–192, and 280–295. Amino acid residues 52–131 and 193–279 are domain II and amino acid residues 296–394 are domain III. Hence, the mutation on residue 47 is in the domain I. Mutations on residues 53, 55, 71, 107, and 116 are in domain II. The last three are in the transmembrane domain at residues 402, 454, and 474. There is no variant in the domain III region, which is characterized by an immunoglobulin-like structure, and has been hypothesized to be the receptor-binding domain of the E proteins [Rey et al., 1995; Roehrig et al., 1998]. According to the model of Rey et al. [Rey et al., 1995], those mutations in each region seem to cluster together in space. Among those mutations, the one at residue 107 is a silent mutation, which is part of the fusion peptide structure [Modis et al., 2003]. The region from amino acids 100 to 108 between the anti-parallel strands c and d of domain II in TBE virus is almost completely conserved among Flaviviruses [Allison et al., 2001]. The cd loop region has been suggested to be directly involved in the interaction with target membrane during fusion and has been hypothesized to function as an internal fusion peptide at low pH [Allison et al., 2001]. The function of fusion peptide is critical to the successful infection and propagation of virions, hence, any mutation affecting its function will have detrimental effect. Therefore, it is not surprising to find a silent mutation at this location.

There is no variation in the domain III region. Monoclonal antibody recognizing the domain III could neutralize target cell infection [Thullier et al., 1999] and could strongly block virus adsorption [Crill et al., 2001]. This suggests that interactions between this region and target cells could mediate virus entry [Thullier et al., 2001]. Mutations in this region may affect the entry and/or infection of the virus. Hence, it is not surprising that no found variations in this area. Previous reports have shown that DV E proteins expressed in *E. coli* form inclusion bodies [Mason et al., 1990; Sugrue et al., 1997], which makes the recovery of the recombinant protein difficult. Even after the removal of the transmembrane domain, it was still largely insoluble in the bacterial cytoplasm [Sugrue et al., 1997]. This is consistent with our data that the expressed EStag9 proteins were presented in the precipitated fraction of cell lysate (Fig. 2.2). It is a possibility that over-expression of the E protein in *E. coli* facilitates the formation of inclusion body. Here, I have overcome this issue by denaturing the cell lysates containing protein inclusion bodies in 8M urea, and then refolded the proteins by dialysis to remove urea. I have tried to express the full length E gene in *E. coli* at the beginning, but no protein production was detected (data not shown). Truncation of the C-terminal hydrophobic region allows the over expression of the E protein in *E. coli*. To facilitate the purification process, a 15-amino-acid S-tag peptide was fused to the C-terminal of the truncated recombinant E proteins, which allow affinity purification by S-protein agarose (Novagen). The recombinant peptide also contains a thrombin cleavage site between the truncated E protein and S-tag. Therefore, the S-tag can be removed easily by digestion treatment to the recombinant proteins with biotinylated thrombin and then capture the cleaved S-tag with streptavidin agarose. This procedure allows the recovery of the purified recombinant proteins rapidly and easily.

The key issue of expressing the recombinant E protein in *E. coli* is whether the function and structure could be retained. Most studies used monoclonal antibodies to recognize the structural epitopes to determine the structural integrity [Staropoli et al., 1996; Staropoli et al., 1997; Kelly et al., 2000]. By using competitive assay on viral infections, it was directly tested the relevant

biological function of the purified EStag9 proteins. It have shown that the recombinant EStag9 proteins, when mixed with the dengue virions, could inhibit plaque formations of host cells caused by dengue viral infections up to greater than 90%, presumably by competing with wild type virions for host cell viral receptor binding. This suggests that purified and dialyzed EStag9 proteins have retained the structure, at least partially in the region possessing the functions for cell surface binding and entry, hence maintain the key function of our concern. Interestingly enough, BSA could also reduce 20% of PFU regardless of the amount of proteins added under our experimental conditions. Expressing proteins in *E. coli* is still the most economic and convenient method to produce large amount of viral proteins for research and application purposes. However, formation of inclusion body has always been a common issue. Here is reported that by removal of the denaturing urea in dialysis, the purified recombinant E proteins could still compete with wild type viral particles for infection on host cells.

Nucleotide position	Nucleotide changes		Amino acid changes
	M29095	PL0146	(M29095 → PL0146)
1075	G	A	E 47 K
1093	C	T	P 53 S
1099	A	G	T 55 A
1149	T	A	D 71 E
1255	C	T	L 107 L
1282	T	C	C 116 R
2140	A	C	I 402 L
2297	T	C	I 454 T
2356	T	C	S 474 P

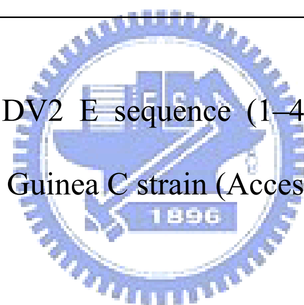


Table 2.1 Comparison of the DV2 E sequence (1–402 amino acids) variations between Taiwan local strain PL046 and New Guinea C strain (Accession No. M29095)

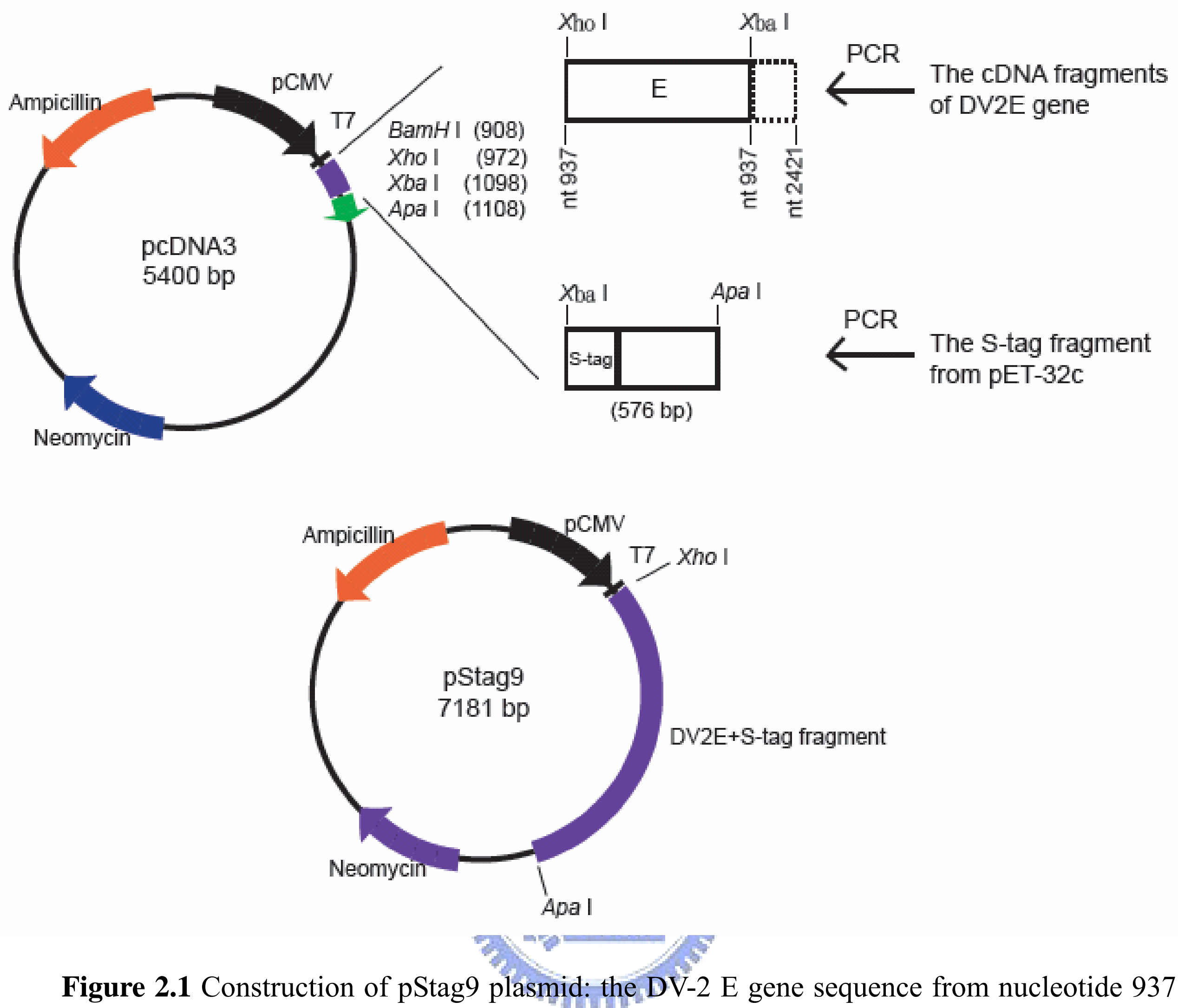


Figure 2.1 Construction of pStag9 plasmid: the DV-2 E gene sequence from nucleotide 937 to 2142 (full-length E gene is from 937 to 2421) was amplified by PCR and inserted to pcDNA3 expression vector with the addition of an *Xho*I site and ATG at the 5' end and an XbaI site at the 3' end. This truncated E sequence was then fused at the 3' an S-tag fragment amplified from the pET-30c (appendix 1 and 2) and cloned into the *Xba*I and *Apa*I sites introduced by PCR onto the S-tag fragment.

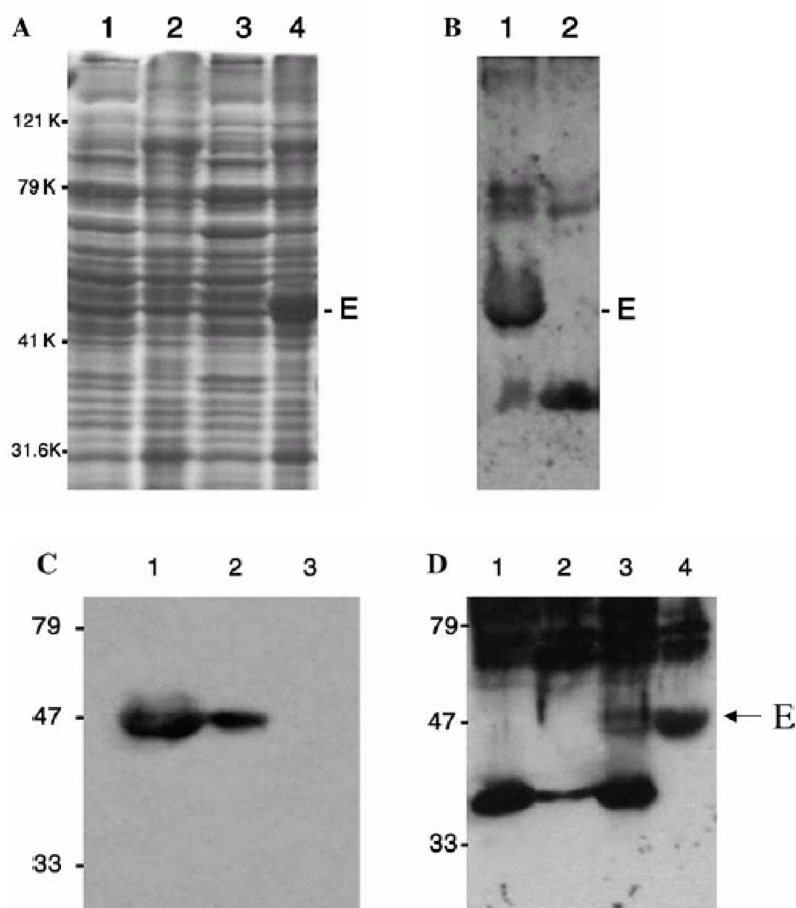


Figure 2.2 Detection of recombinant EStag9 protein expression. *E. coli* cells were sonicated and followed by centrifugation before resolution by 12% SDS–PAGE. The detection of protein expression was by (A) SDS–PAGE and with Coomassie blue staining. Total proteins were from supernatant (lanes 1 and 3) and pellet (lanes 2 and 4). Lanes 1 and 2 were mocks and lanes 3 and 4 were lysates from *E. coli* transformed with pStag9. (B) Western blotting with an anti-DV2 domain III antibody (provided by Dr. Wen Chang IMB, Academia Sinica, Taipei). Lane 1, *E. coli* lysates from cells transformed with pStag9; lane 2, mock. (C) Western blotting with an anti-S tag antibody. Lane 1, cell lysates of *E. coli* transformed with pStag9; lane 2, proteins purified by S-protein agarose from *E. coli* transformed with pStag9; and lane 3, mock. (D) Western blotting with an anti-DV2 domain III antibody. Lane 1, mock; lane 2, the supernatant of *E. coli* lysates from cells transformed with pStag9; lane 3, total *E. coli* cell lysates of cells transformed with pStag9; and lane 4, proteins purified from *E. coli* cells transformed with pStag9. The bands corresponding to EStag9 are indicated by E or an arrow by the side.

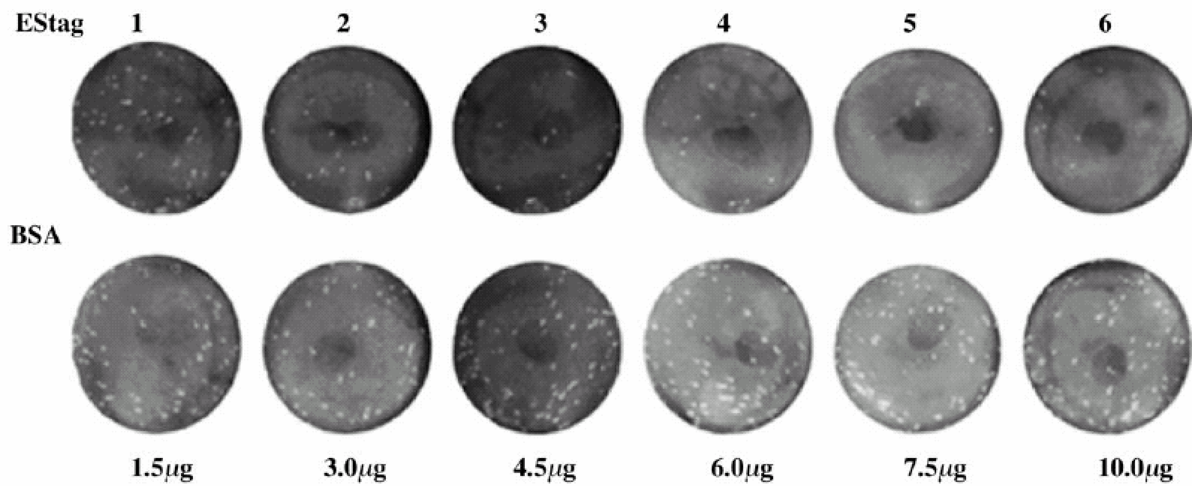


Figure 2.3 Competitive blocking assay. Purified EStag9 proteins and BSA were serial diluted with medium without FBS and mixed with DV-2 virions before the addition to BHK-21 cells growing in 6-well plates. After absorption for 1 h at 37 ^{o}C with 5% CO_2; the media were aspired and the cells overlaid with agarose. After incubation for 5 days at 37 ^{o}C, 5% CO_2, cells were fixed and stained with crystal violet before the plaque numbers were scored.

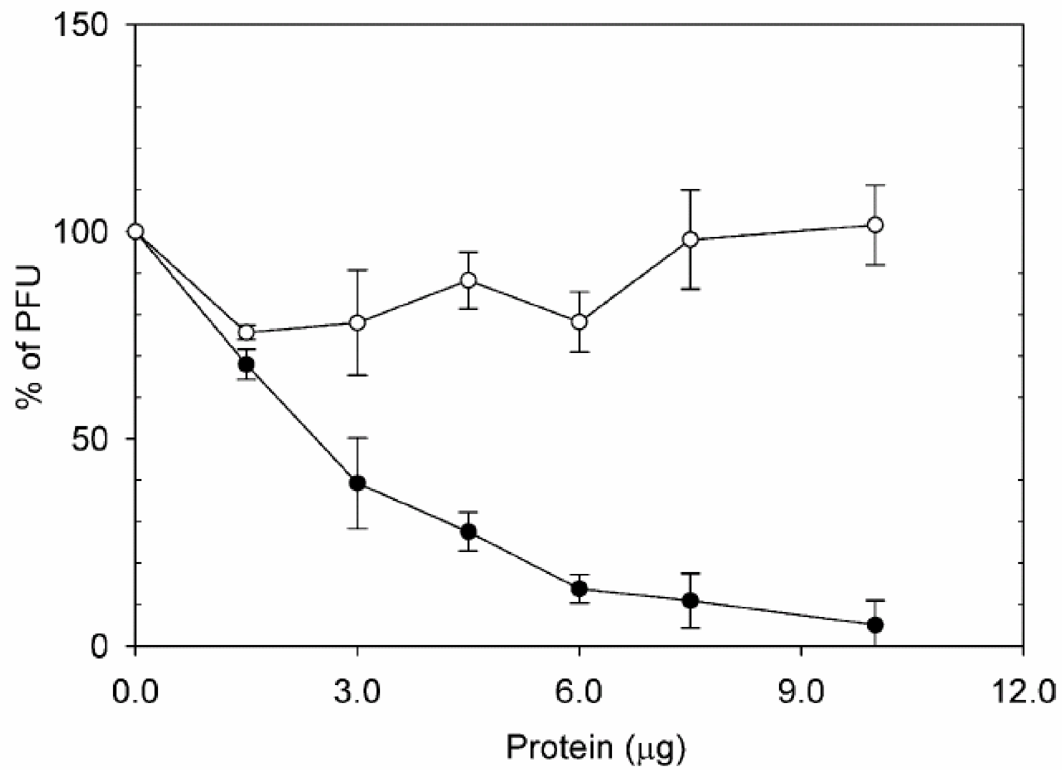


Figure 2.4 Quantitative representation of dosage-dependent competitive blocking assay of viral infection by the purified recombinant EStag9 proteins (●) and BSA (○). Proteins were serial diluted with medium without FBS and mixture with DV2 strain PL046.

Chapter II

The Type 2 Dengue Virus Envelope Protein Interacts with Small Ubiquitin-like Modifier-1(SUMO-1) Conjugating Enzyme 9 (Ubc9)

Introduction

Yeast two-hybrid technology

The yeast two hybrid (Y2H) system, first developed by Stan Fields and coworkers [Bartel et al., 1993a; Bartel et al., 1993b; Fields, 1993; Fields and Sternglanz, 1994], is a powerful technique for identifying novel protein-protein interactions [Fields et al., 1989]. The system involves the expression of chimeric proteins and their subsequent interactions within the yeast cells. The interaction is detected by the expression of a reporter gene that changes the phenotype of the recipient yeast cell. Unlike the *in vitro* biochemical methods, Y2H can detect *in vivo* interactions. Moreover, neither protein purification nor antibody production is required for this methodology [Topcu and Borden, 2000].

The Y2H system provides a sensitive method to detect relatively weak and transient protein-protein interactions. Such interactions may not be biochemically detectable, but may be critical for the proper functioning of complex biological systems [Guarente et al., 1993; Estojak et al., 1995]. It also allows one to screen a library of activation domain fusions or preys for the binding partners of one's favorite protein expressed as a DNA binding domain fusion or bait, and it can be used to pinpoint protein regions mediating the interactions [Ito et al., 2002].

Sumolyation

Small Ubiquitin-like Modifier (SUMO) is a ubiquitin-related protein that covalently binds to

other proteins. Conjugation of ubiquitin and ubiquitin-related proteins (Ublps) to cellular target proteins is involved in many aspects of eukaryotic gene expression by regulating the signaling for degradation and/or modifying the functions of target proteins [Vashavsky, 1997; Hershko and Ciechanover, 1998; Melchior, 2000; Yeh et al., 2000; Hay, 2001; Muller et al., 2001; Schwartz and Hochstrasser, 2003; Seeler and Dejean, 2003]. Unlike ubiquitin, conjugation of SUMO does not typically lead to degradation of the substrate and instead it causes alterations in function or changes in intracellular localization [Wilson and Rangasamy, 2001]. Three SUMO paralogues have been reported in mammalian cells and they are known as SUMO-1, -2, and -3 [Lapenta et al., 1997; Kamitani et al., 1998].

It is believed that conjugation and de-conjugation of SUMO to other proteins happens in a process similar to ubiquitination, which involves an E1 activating enzyme, an E2 conjugating enzyme, and an E3 target specificity enzyme. An inactive SUMO is converted to its active form by removal of its last four amino acids to expose at the C-terminus two essential glycine residues, which then form a thioester bond with a cysteine of the SUMO-activating E1 enzyme (SAE1-SAE2). Consequently, it was transferred to a conjugating E2 enzyme (Ubc9) and finally passed to the ε-amino group of specific lysine residues on the target proteins [Jensen et al., 2004]. Some evidence has suggested that Ubc9 can itself bind specifically to substrates presenting a consensus SUMO modification motif, ψKxE (ψ represent the hydrophobic resides, K is lysine, x is any residue, and E is glutamic acid). Recently, structural analysis has revealed that Ubc9 can recognize this sequence directly [Hay, 2001; Benier-Villamor et al., 2002; Lin et al., 2002b; Lin et al., 2002c; Tatham et al., 2003]. Ubc9 binding may play an important role in substrate recognition as well as in substrate modification [Sampson et al., 2001]. In recent years, growing numbers of viral proteins have been found to conjugate with SUMO-1 [Muller and Dejean, 1999; Hofmann et al., 2000; Rangasamy and Wilson, 2000a; Rangasamy et al., 2000b; Adamson and Kenney et al., 2001; Ahn et al., 2001; Endter et al., 2001; Xu et al., 2001; Gravel et al., 2002; Saitoh et al., 2002; Spengler et al., 2002; Lethbridge et al., 2003]. There are currently six known

sumoylated viral proteins, distributed among three DNA viral families: Adenoviridae, Papillomaviridae, and Hepesviridae [Rangasamy and Wilson, 2000a; Rangasamy et al., 2000b; Endter et al., 2001; Gravel et al., 2002; Saitoh et al., 2002; Lethbridge et al., 2003]. All six viral proteins are products of early genes with important regulatory roles in viral transcription or replication. In addition, some other viral proteins have been shown to interact directly with components of the sumoylation system. For examples, the Vaccinia Virus early protein E3L and the Tula Hantavirus Nucleocapsid protein (TULV-N) both interact with SUMO-1 [Rogan and Heaphy, 2000; Kaukinen et al., 2003], while the Mason-Pfizer Monkey Virus (MPMV) Gag protein interacts with Ubc9 [Weldon et al., 2003]. The Hantaan Virus Nucleocapsid protein (HTNV-N) interacts with both SUMO-1 and Ubc9 [Maeda et al., 2003], and the Epstein Barr Virus nuclear antigen 3C (ENBA-3C) interacts with SUMO-1 and SUMO-3 [Lin et al., 2002b].

In this study, I have identified Ubc9 as one host protein interacting with DV2E through the yeast-two-hybrid-based “Functional Yeast Array” (Level Biotechnology Inc., Taipei, Taiwan,). It was also demonstrated that DV2E could interact with Ubc9 in *in vitro* pull down assay. Furthermore, the mutagenesis result showed that residues K51 and K241 on DV2E were critical for the interaction with Ubc9. It was observed that DV2E-EGFP affected the distribution of Flag-Ubc9, making it concentrate toward the nuclear membrane instead of evenly distribute predominantly in whole nucleus. And these two proteins were co-localized near the cytoplasmic side of the nuclear membrane. In addition, over-expression of Ubc9 could reduce DV2 infection in BHK-21 cells in plaque assay, suggesting that Ubc9 may interfere with dengue viral propagation such as involving in the host defense system to prevent virus infection.

Methods and Materials

Cells and viruses

BHK-21 cells were cultured at 37°C, 5%CO2 in MEM medium supplemented with 0.22% of sodium bicarbonate and 10% of FBS. C6/36 cells were grown at 28°C in MEM medium supplemented with 0.11% of sodium bicarbonate and 10% of FBS.

Mammalian 293 cells were cultured at 37°C with 5% CO2 in MEM medium (Gibco) supplemented with 4mM of L-glutamine, 1.5g/L of sodium bicarbonate, 4.5g/L of glucose, and 10% of fetal bovine serum (FBS) (Gibco).

C6/36 cells were growth at 28°C in MEM medium (Gibco 41500-034) supplemented with 0.11% of sodium bicarbonate and 10% of FBS.

Saccharomyces cerevisiae yeast strain LY001 and LY002 used for screening were provided by Level Biotechnology Inc., Hsi-Chih, Taipei, Taiwan. Strain L40 (*MATa ade2 his3 leu2 trp1 LYS2::lexAop-HIS3 URA3::lexAop-lacZ)* was used for assessing the result of Functional Yeast Array by yeast-two-hybrid.

E. coli BL21 (DE3) were used for plasmid replication and expression in bacteria.

Construction of plasmids

All the DV2E-related constructs in this study were shown in Fig. 3.1.

A recombinant plasmid containing E protein sequence, pStag9, was used as the template to amplify the DV2E DNA fragment by PCR. The forward strand primer, 5' TTTCTCGAGGACAATGCGTTGCATAGG 3', introduced a 5' end *Xho* I site and the reverse strand primer, 5' AAAGGTACCCTAAAGCATTTGGCCGATAGA 3', introduced a stop codon and a *Kpn* I site. The amplified fragments were digested with *Xho* I and *Kpn* I and ligated to pLB1.0 vector (Level Biotechnology Inc., Hsi-Chih, Taipei, Taiwan) at the *Xho* I and *Kpn* I sites. The resulted plasmid encoding a recombinant protein contained the Lex A sequence at the

N-terminus alone with the N-terminal 88% of the DV2E protein (nucleotides 937~2142 of DV 2 annotated according to NGC strain) and was named pLexA-DV2E, which can express the DV2E in LY002 yeast cells. The DNA fragment containing DV2E sequence from pLexA-DV2E was introduced into the pBTM116 vector at the restriction sites *Xho* I and *Kpn* I to construct pBTM-D2E. For the yeast-two-hybrid, the pACT2-SUMO1 and pACT2-Ubc9 were used as the preys while the pBTM116-MST3 and pBTM116-DAXX were used as the negative and positive baits, respectively. The plasmid pStag9 was again used as template to construct the pDV2E-EGFP, containing the sequence encoding E protein with in-frame fusion of the EGFP at the C-terminal end. The forward strand primer, 5' TTTCTCGAGGACAATGCGTTGCATAGG 3', introduced a 5' end *Xho* I site and the reverse strand primer, 5' AAAGGTACCCAAGCATTTGGCCGATAGAA 3', introduced a *Kpn* I site to the DV2E sequence amplified by PCR. The amplified DV2E fragments were digested with *Xho* I and *Kpn* I, and ligated to pEGFP-N2 vector (BD Biosciences) in frame with the sequence of EGFP to generate pDV2E-EGFP. The resulted construct expresses a protein containing the N-terminal 88% of the DV2E protein with the EGFP sequence.

The cDNA of E gene was then cloned into expression vector pcDNA3 by PCR amplification, and the construct was named pDEΔ94, which also containing the sequence of the last 66 amino acids of prM and the truncated E gene. The positive strand primer 5' TTTCTCGAGTGGGAATGGGACTGGAGA 3' introduced a 5' end *Xho* I site and the negative strand primer 5' AAATCTAGACTCAAGCATTTGGCCGATAGA 3' introduced an *Xba* I site. The amplified fragments were digested with *Xho* I and *Xba* I site, and were introduced into pcDNA3 vector. To introduce a His tag sequence, two primers: 5' TTTGGGCCCACTACGTGAACCATCACC 3' containing *Apa* I site, and 5' TTTTCTAGACTGGTGCCACGCGGTTCT 3' containing *Xba* I site were used for PCR amplification using pET-30b vector (Novagen) as template to obtain fragment containing the His tag sequence. The PCR products were then restricted with *Xba* I and *Apa* I site and introduced

into pDEΔ94. The resulted plasmid pDEΔ94-pta15 contains the 3’ end of prM (nucleotides 734~936) and the N-terminal 88 % of DV2E (nucleotide 937-2142) protein along with a His-tag at C-terminus (nucleotides 937~2142). The pACT2-SUMO1, pACT2-Ubc9, pBTM116-MST3, pBTM116-DAXX, and pFlag-Ubc9, and pGEX-4T-Ubc9 were from the collection of Dr. Hsiu-Ming Shih (IBMS, Academia Sinica, Taipei, Taiwan). The pACT2 carries a *GAL4* activation domain and a *LEU* marker while pBTM116 contains a DNA-binding domain and a *TRP* marker. The pFlag-Ubc9 carries a FLAG tag fused to Ubc9 gene in pCMV-Tag2a vector, and can express the fusion protein in mammalian cells. The pGEX-4T-Ubc9 carries the GST-Ubc9 fusion protein and can over-express it in *E. coli*.

Rapid Screening by Functional Yeast Array

The Functional Yeast Array system (Level Biotechnology Inc., Hsi-Chih, Taipei, Taiwan) is derived from the yeast-two hybrid technology. The procedure is a rapid, high-through-put screening based on the automated 96-well plate liquid handling. This system contains about 1200 full-length cDNA of known human genes, each fused to the VP16 trans-activation domain, as the preys. Those genes are divided into 5 different groups known as modules according to their functions. These five modules are Apoptosis, Cell Interaction, Transcription Factor, Cancer Related, and Signal Transduction. The plasmids containing the preys have special design to restrict the growth of cells to reduce the false positive (Level Biotechnology Inc.) [Gietz et al., 2002; Ito et al., 2002; Knudsen et al., 2002; Mouradian, 2002; Ranish et al., 2003]. The Functional Yeast Array screening utilizes a recombinant LexA-DV2E construct which contains the DV2E gene fragment cloned into pLB-1.0 vector (Level Biotechnology Inc.) as the bait to against module preys. For this study, it was chosen three sets of modules, Apoptosis, Cell Interaction and Signal Transduction, totally about 500 individual clones, as preys for the procedure. As shown in Fig. 3.2, the Trp^- Lys^- LY001 yeast strain (Level Biotechnology Inc.) expressing LexA-DV2E was mated with the Leu^- LY002 yeast (Level Biotechnology Inc.)

expressing different preys separately in 96-well plates. After overnight incubation, the resulting yeast cells were cultured in -Trp/-Ura/-Leu/-Lys (-TULK) medium for selection of diploid cells and culture in -Trp/-Ura/-Leu/-Lys/-His (-TULKH) medium for screening protein-protein interaction. When bait proteins interacted specifically with prey proteins, it could drive both *HIS3* and *lacZ* genes to express, and the diploid yeast cells could grow on –Trp/-Ura/-Leu/-Lys/-His medium. To assess the expression of X-gal activity, yeast clones shown positive in growth were lysed with zymolase and vortexed with glass beads before incubated at 30°C for 48 hours in the medium containing 80 μM of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal).

Protein expression and purification

To express Ubc9-GST proteins, the recombinant plasmid pGEX-4T-Ubc9 was transformed into the *E. coli* strain *BL21 (DE3)*. The transformants were inoculated in LB and grown at 37°C until the A600 researched 0.6 and 0.1mM IPTG was added before continuing incubation for 3 more hours. Then the culture was centrifuged and the cell pellet was resuspended in 50 mM Tris-HCl (pH7.5) with the addition of 1mM dithiothreitol prior to sonication. The lysates were centrifuged at 8000 rpm at 4°C for 15 minutes to remove the cellular debris. Then, the supernatant was added to glutathione-Sepharose 4B column (Amersham Biosciences). After washed with 10 volume of 1X ice-cold PBS, the GST-fusion proteins were eluted competitively with 10 mM reduced glutathione in 50mM Tris-HCl (pH7.5). The eluted GST-Ubc9 proteins were then dialyzed against 50mM Tris-HCl (pH7.5) with 1mM dithiothreitol and concentrated using 10-kDa molecular mass cut-off microconcentrators (Millipore).

To express EStag9 (protein product of pStag9), DEΔ94-pta15 (protein product of pDEΔ94-pta15), and priB-His proteins, corresponding plasmids were transformed separately into *E. coli* BL21(*DE3*) and the transformants were grown in LB broth at 37°C until the A600 researched 0.4. The cells were then harvested after additional 3-hour incubation. The pellets were

resuspended with 1mM Tris-HCl (pH8.0) containing 1mM PMSF. After sonication, the centrifuged cell lysates were resuspended with inclusion-body solubilization buffer (50mM Tris-HCl pH8.0, 1mM EDTA, 100mM NaCl, 8 M urea, 1mM PMSF) and incubated on ice for 1 hour.

For the EStag9, the urea was removed by dialysis overnight in the S-protein Agarose (Novagen) binding/wash buffer (20mM Tris-HCl pH7.0, 150mM NaCl, 0.1 % Triton X-100). And the affinity purification of the recombinant EStag protein was performed with the S-protein Agarose (Novagen). The S-protein Agarose was washed with 10 volume of the binding/wash buffer before the samples were applied. Next, the mixture was washed again with 10 volume of the binding/wash buffer again. The elution buffer (20mM Tris-HCl pH7.0, 150mM NaCl, 0.1 % Triton X-100, 3M magnesium chloride) was applied and the eluted fractions were subjected to SDS-PAGE to identify the protein. The collected protein was then dialyzed to concentrate and to remove the salt. The concentration of the purified Estag9 protein was determined by Protein Assay kit (Bio-Rad).

For the DEΔ94-pta15 and the priB-His proteins, the urea was removed by dialysis overnight with Ni-HisTrap binding buffer (50mM Tris-HCl pH9.0, 10mM imidazole, 0.1 % Triton X-100). Purification of the His-tagged DV2E protein, DEΔ94-pta15, was with Ni-HiTrap column (GE Healthcare) affinity binding. The HiTrap column was washed with 10 volume of binding/wash buffer (50mM Tris-HCl pH9.0, 10mM imidazole, 0.1 % Triton X-100, 1mM dithiothreitol). After the sample was applied, the column was washed with 10 volume of binding/wash buffer. Then, elution buffer (50mM Tris-HCl pH9.0, 500mM imidazole, 0.1 % Triton X-100, 1mM dithiothreitol) was used to elute the protein, which was then collected in fractions. The fractions were then analyzed with SDS-PAGE to determine the fractions containing the protein. The collected proteins were then dialyzed against 50mM Tris-HCl (pH9.0) with 1mM dithiothreitol and concentrated using 10-kDa molecular mass cut-off microconcentrators (Millipore).

Pull down assay

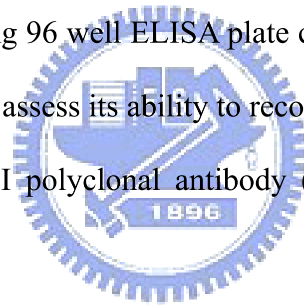
For GST pull down assay with the DV2E, the purified GST or GST-Ubc9 proteins were pre-incubated at room temperature with 30 μl of glutathione-Sepharose 4B (GE 250 Healthcare) at the concentration of ~1 mg/ml. Mammalian 293 cells were transiently transfected with pDV2E-EGFP in a 10cm culture dish. After 48 hours, the cells were harvested and washed 3 times with 1X PBS. The pellets were resuspended with 1 ml of ice-cold 1X RIPA buffer (Upstate) containing cocktail mixture (Calbiochem) and gently mixed on orbital shaker at $4^{o}C$ for 30 minutes to lyse the cells. After centrifugation, the supernatants of cell lysates were then incubated with the GST-Ubc9 bound to the glutathione-Sepharose 4B, at room temperature. After tumbling overnight, the beads were collected by centrifugation, and washed three times in 1 ml of 1X PBS. The washed pellets were then re-suspended in SDS sample buffer and subjected to further analyses by Western blotting. ^{35}S-methionine-labeled DV2E-EGFP proteins were produced with the TNT reticulocyte lysate system (Promega) according to instruction provided by the manufacturer. ^{35}S-labeled DV2E-EGFP proteins were then incubated with GST or GST-Ubc9 agarose beads in 300 μl of binding buffer (50mM Tris-HCl pH7.5 , 1mM DTT) for 2 hours. The mixtures were then washed with the binding buffer 3 times and analyzed by SDS-PAGE and autoradiography.

For His-tag pull down assay with Ubc9, priB-His and DEΔ94-pta15 proteins were pre-incubated separately at room temperature with 30 μl of Ni-HiTrap agarose (GE Healthcare). Mammalian 293 cells were transiently transfected with pCMV-Tag2a or pFlag-Ubc9 in a 10cm culture dish. After 48 hours, the cells were harvested and washed 3 times with 1X PBS. The pellets were resuspended with 1 ml of ice-cold 1X RIPA buffer (Upstate) containing cocktail mixture (Calbiochem) and gently mixed at 4 ^{o}C for 1 hour to lyse the cells. After centrifugation, the supernatants of cell lysates were then incubated with the His-tag fusion protein or

DEΔ94-pta15 proteins bound to the Ni-HiTrap agarose at 4°C. After tumbling for 2 hours, the beads were collected by centrifugation and washed three times in 1 ml of 1X binding/wash buffer (50mM Tris-HCl pH9.0, 10mM imidazole, 0.1 % Triton X-100, 1mM dithiothreitol). The washed pellets were then re-suspended in SDS sample buffer and subjected to further analyses by Western blotting.

Production of the Anti-EStag9 Polyclonal antibody, C001P

At 7-day intervals, ICR mice were immunized 4 times subcutaneously with 15-30 μg of the purified recombinant EStag protein, emulsified in Freund's complete adjuvant for the first three times, and in Freund's incomplete adjuvant for the last immunization. Mouse sera were acquired 4 days after the last immunization, and then assessed by the solid phase enzyme-linked immunosorbent assay (ELISA) using 96 well ELISA plate coated with EStag proteins. Sera were also tested by Western blot assay to assess its ability to recognize the EStag protein by comparing with rabbit anti-DV2 E domain III polyclonal antibody (provided by Dr. Wen Chang, IMB, Academia Sinica, Taipei, Taiwan).



Gel Electrophoresis and Western Blot Analysis

The samples were heated at 100°C for 5 minutes before subjected to resolve by 12% of SDS-PAGE and the resultants were transferred onto nitrocellulose membranes (Schleicher & Schuell). The membrane was then blocked in PBS buffer containing 5% non-fat dried milk. Proper antibody was used as the first antibody at 1: 3000 dilution. Those antibodies were the mouse anti-EStag9 polyclonal antibodies, the mouse anti-GST antibody (Upstate), the rabbit anti-Flag polyclonal antibody (Sigma), and the anti-His antibody conjugated horseradish peroxidase. The reaction was incubated at 4°C overnight. After washed three times in PBST buffer (PBS, 0.05% Tween-20), the membrane was then incubated with horseradish peroxidase-conjugated anti-mouse IgG (Chemicon) or anti-rabbit IgG (Chemicon) in PBS buffer

containing 5% non-fat dried milk. Incubated at room temperature for 1 hour, the membrane was then washed and processed by the chemoiluminescence detection system (Upstate).

Analysis of the Interaction between DV2E and Ubc9

A series of point mutations were introduced into the DV2E gene by the PCR-based site-directed mutagenesis procedure. PCRs were performed using *pfu* polymerase (Fermentas). Primers 1 and 2 (Table 3.1) were used to introduce mutations to pBTM-D2E to form pBTM-D2E-K51R; primers 3 and 4 (Table 3.1) to pBTM-D2E-K241R. For the introduction of double mutations, pBTM-K51R-K241R was based on pBTM-D2E-K51R and primers 3 and 4. The conditions of performing mutagenesis followed the instruction of QuikChange Site-Directed Mutagenesis kit (Stratagene).

To analyze the interaction between DV2E and Ubc9, various "baits" such as pBTM-D2E, pBTM-D2E-K51R, pBTM-D2E-K241R, , pBTM-K51R-K241R, and pBTM116-MST3 were independently paired and cotransformed with either the "prey", pACT2-Ubc9, or the pACT2 vector, into the yeast strain *L40 (MATa ade2 his3 leu2 trp1 LYS2::lexAop-HIS3 URA3::lexAop-lacZ)* by the lithium acetate method [Gietz et al., 1992]. The yeast cells were grown at 30 ^{o}C overnight until the A600 reached 1.3. The pellets were lysed with zymolase and incubated at 37 ^{o}C for 2 hours before centrifugation, then the activity of X-gal in the supernatants were quantitated using the Reporter Assay Kit –β gal (Toyobo) and detected with luminometer to determine the interactions between bait and prey proteins, which drove the *lacZ* genes.

Immunofluorescence Microscopy

BHK-21 cells were passaged onto chamberslide (Lab-Tek, Nagle NUNC) and cultured for 1 day in 5% CO_2 at 37°C. This was followed by transient transfection of pFlag-Ubc9 or pDV2E-EGFP or both. After 48 hours, cells were fixed in 4% paraformadehyde in PBS for 15

minutes at room temperature, and then incubated in 0.5% Triton X-100 in 1X PBS for 5 minutes. After washed three times with 1X PBS for 5 minutes each, the cells were blocked with 1% BSA in 1X PBS for 30 minutes. Then the cells were incubated with Anti-FLAG M2 monoclonal antibody (Sigma) overnight. After washed three times (5 minutes each) with 1X PBS, the cells were incubated with anti-mouse IgG-TRITC (Sigma) for 1 hour at room temperature.

Nuclei were visualized by DAPI (4', 6'-diamidino-2-phenylindole) staining for an additional 1 hour. The stained cells were analyzed with either Leica TCS NT confocal microscope or Olympus Fluoview BX51 confocal microscope.

Plaque Assay

BHK-21 cells were passaged to a density of $2x10^5$ cells/ml in 6-well plates and incubated 5% CO_2 at 37°C for 24 hours before the transfection of the pFlag-Ubc9 or pCMV-Tag2a with the Lipofectamine 2000 (Invitrogen) according to the manufacturer. Twenty-four hours after transfection, the cells were infected with DV2 virus PL046 strain. After gentle mixing, the plates were incubated at 37 °C with 5% CO_2 for 2 hours to allow viral absorptions. The supernatant in the plates were discarded and replaced by 1:1 mixture of MEM medium and 2% methylcellulose.

These plates were then incubated at 37°C with 5% CO2 for 5~6 days. The medium was then aspired to allow the fixation of cells with 3.7% formaldehyde and stained with 1 % crystal violet in 3.7% formaldehyde. The plates were washed before the plaques were scored.

Results

Rapid screening with Functional Yeast Array

Although the yeast-two hybrid technique is a straightforward approaches to study protein-protein interaction, it still has some drawbacks in screening such as false positive, tedious workload and time-consuming. Here, the Functional Yeast Array system (LEVEL Biotechnology Inc.), a new improved yeast-two hybrid (YTH) strategy was introduced. It allows researchers to quickly get access of information of protein-protein interactions by combining yeast-two hybrid with 96 well plate screening process.

Currently, the system has generated about 1,200 yeast individual human full-length ORF clones as preys which fused to the transactivation domain and an antibiotic gene. The resulting yeast cells can grow on the antibiotic selective medium, which ensures the expression of the prey proteins in yeast. These clones have been arranged into 96-well plate and carry on the yeast two-hybrid screen via mating strategy with automatic liquid handling system to reduce the screening time. The 1200 individual clones is classified to five different categories, including apoptosis, transcriptional factors, cancer-related, signal transduction, and cell-cell interaction. Therefore, the "Functional Yeast Array" should facilitate the discovery of research by rapidly identifying protein-protein interactions (Table 3.2).

To identify possible host cellular proteins interacting with the DV2E, I have constructed the N-terminal 402 amino acids of the E protein as the "bait" to perform screening with the "Functional Yeast Array" (Level Biotechnology Inc.). Five positive clones were identified, including serine protease, one adherent protein, one mitochondrial related protein, one scaffold protein, and SUMO conjugating E2 enzyme (Ubc9) protein.

Ubc9 Interacting with DV2E in the Yeast-Two-Hybrid Analysis

To identify possible host cellular proteins interacting with the DV2E, the N-terminal 402 amino acids of the E protein was used to construct into the "bait" to perform screening on the "Functional Yeast Array" (Level Biotechnology Inc.). The results revealed five positive clones and one of them encodes Ubc9. The Ubc9 protein is known to be an enzyme functioning to conjugate the SUMO-1 to the target substrate. Thus, to determine whether the DV2E can interact with SUMO-1 as well as Ubc9, the *L40* cells were transformed with different sets of plasmids and the growth of cells were determined on different media. As expected, all transformants were able to grow on -Trp/-Ura/-Leu/-Lys (-TULK) medium (Fig. 3.3A). The DAXX protein, known to interact with Ubc9 and SUMO-1 [Lin et al., 2004; Chang et al., 2005], was used as the positive control whereas the MST3 protein was the negative as described previously [Chang et al., 2005]. Thus, cells co-transformed with pBTM-DAXX and either pACT2-Ubc9 or pACT2-SUMO-1 were able to grow on -Trp/-Ura/-Leu/-Lys/-His (-TULKH) selective medium (Fig. 3.3B). In contrast, cells co-transformed with pBTM-MST3 and either pACT2-Ubc9 or pACT2-SUMO-1 failed to grow on -Trp/-Ura/-Leu/-Lys/-His (-TULKH) selective medium. Interestingly, in the experimental set of DV2E contained cells on –TULKH medium, the DV2E pairing with Ubc9 grew very well and the pair of DV2E and SUMO-1 failed to grow. This result suggests that DV2E interacts with Ubc9 but not SUMO-1 and this is also the first report that DV proteins can interact with the protein of sumoylation system.

Ubc9 Interacting with DV2E in vitro

To determine if DV2E directly interacts with Ubc9, an anti-DV2E antibody was generated to perform the pull down assay. To obtain the anti-DV2E antibody, the plasmid Stag9 were transformed into *E. coli BL21* cells and the EStag9 protein was purified as described in the

section of material and method. And then the purified proteins were used for the immunization of mice. After harvest, the mouse sera were used for western blot on both the *E. coli* cell lysates containing the EStag9 protein and the purified EStag9 protein. The anti-EStag9 polyclonal antibody could recognize both the EStag9 protein in the cell lysate (Fig. 3.4A, lane 3; Fig. 3.4C, lane 1) and the purified EStag9 (Fig. 3.4A, lane 4; Fig. 3.4C, lane 2). In the lysate of cells containing no Estag9-expressing plasmid, there was no detectable band at the corresponding position (Fig. 3.4A, lane 1-2; Fig. 3.4C, lane 3). To further confirm the specificity of the polyclonal antibody, BHK-21 cells were infected with DV2 strain PL046, and the cell lysate was analyzed with western blotting by the anti-Estag9 polyclonal antibody. The result showed that the polyclonal antibody recognized a band at the position of about 65kDa (Fig. 3.4B, indicated by arrow), which was correspond to the expected molecular mass of the dengue virus E protein after it was processed and glycosylated [Wei et al., 2003]. For further validation of the protein-protein interaction between Ubc9 and DV2E proteins, the pGST-Ubc9 was transformed into *E. coli* for expression and purification. To conduct *in vitro* pull down assays, the GST-Ubc9 proteins were immobilized to GST-Sepharose 4B beads before incubating with the supernatant of the cell lysates from pDV2E-EGFP-transfected 293 mammalian cells. The reaction mixtures were analyzed with western blotting using anti-DV2E antibody. The DV2E-EGFP could be precipitated down by GST-Ubc9 and detected by the DV2E specific antibody (Fig. 3.5A, indicated by an arrow), whereas DV2E-EGFP was not precipitated by GST (Fig. 3.5A, right panel), suggesting that DV2E-EGFP was specific for Ubc9 but not GST. These results demonstrated that DV2E directly interacts with Ubc9 *in vitro* and consistent with the result of the yeast-two-hybrid analysis. To further verify this interaction, *in vitro* transcriptional and translational lysates containing ^{35}S-methionine-labeled DV2E-EGFP proteins were incubated with the immobilized GST-Ubc9 or GST protein to perform the pull down assay. The resulted reaction mixture was analyzed by SDS-PAGE and detected by autoradiography. As shown in Fig. 3.5B, the *in vitro* translated DV2E-EGFP proteins were again precipitated by GST-Ubc9 but not

GST. The translation products appear as triplets following separation by SDS-PAGE, possibly due to initiation of translation at internal start sites or protein processing and modification.

To further assessing the specific interaction between the DV2E and Ubc9, pDEΔ94-pta15 was transformed into *E. coli* for expression and purification of the DV2E-His protein, and the *E. coli* His tag fusion protein priB-His was used as the control [Zavitz et al., 1991]. To conduct the *in vitro* pull down assays, the DEΔ94-pta15 or the control pri-B-His proteins were immobilized separately to Ni-HiTrap agarose before incubating with the supernatant of the cell lysates from pFlag-Ubc9 or pCMV-Tag2a transfected 293 mammalian cells. The reaction mixtures were analyzed with western blotting using anti-Flag antibody and anti-His-HRP. As shown in **Fig.** 3.5C, The Flag-Ubc9 could be precipitated down by DEΔ94-pta15 and detected by anti-Flag antibody (Fig. 3.5C, indicated by an arrow), while the Flag-Ubc9 was not precipitated by the control His fusion protein (Fig. 3.5C, right panel), and the pCMV-Tag2a control also showed that the interaction was not with the Flag sequence. These results demonstrated that DV2E indeed directly interacts with Ubc9 in *in vitro* condition and reflected the result of the yeast two-hybrid analysis.

The Interaction Sites of the DV2E with Ubc9

To assess the direct interaction between DV2E and Ubc9, the potential sumoylation consensus motif of DV2E were analyzed gene by SUMOplot^{TM} (ABGENE), this system is based on two criteria: the first is the direct amino acid match to the observed SUMO consensus sequence that binds Ubc9; and the second is the substitution of the consensus amino acid residues with other residues exhibiting similar hydrophobicity. The system recommended three high probability binding sites, residues 51, 241, and 393 (Table 3.3) among seven potential candidates. Next, according to the X-ray crystallography [Rey et al., 1995] envelope structure of the tick-borne encephalitis (TBE) virus, which also belongs to *Flaviviridae* family, the E protein

consists of three separate domains, the central domain I, the dimerization domain II, and the domain III responsible for the receptor binding and endosomal uptake during viral infection [Rey et al., 1995; Modis et al., 2004]. Sequence alignment and structural modeling revealed that on DV2E, residue 393 located too close to the transmembrane domain and therefore, due to stereo hindrance, it should not have the opportunity to interact with Ubc9 during viral infection. Hence, it was ranked to be the less likely candidate. By site-directed mutagenesis, the amino acids at position 51 and 241 were changed from K to R either individually or in pair. This has generated 3 constructs: pBTM-D2E-K51R, pBTM-D2E- K241R, and pBTM- K51R-K241R. The mutation sites of these constructs are shown in Table 3.3.

To determine the effects of the mutated residues on DV2E, It were co-transformed pACT2-Ubc9 with plasmid carrying different alleles of DV2E in *Saccharomyces cerevisiae L40* cells. Firstly, the interaction between Ubc9 with different alleles of DV2E was determined by β-gal activity assay. As shown in Figure 3.6, the Ubc9 can interact with the wild-type allele DV2E and induce the expression of *lacZ* (columns 7 and 8). Single mutations on either K51 or K241 significantly reduced the β-gal activity (columns 3-6). Furthermore, mutations on both residues further reduced the β-gal activity (columns 1-2).

If the β-gal activity represents the strength of interactions between Ubc9 and different alleles of DV2E, it would be able to detect similar effects from each mutated allele on the growth of cells on –TULKH selective medium using the same interaction to drive the *His3* reporter. The results of cell growth on selective medium are shown in Figure 3.7. There were less cells expressing DV2E with single mutation on either K51 or K241 than the cells expressing the wild-type DV2E on the selective medium (Fig. 3.7), which is consistent with the quantitative β-gal activity assay (Fig. 3.6). Furthermore, double mutation on both K51 and K241 completely abolished the interactions since cells expressing Ubc9 alone with DV2E allele containing both mutations failed to grow on the selective medium (Fig. 3.7). Thus, these results suggest that both K51 and K241 are required for the interactions between Ubc9 and DV2E.

Subcellular localization of Ubc9 and DV2E in immunofluorescence microscopy

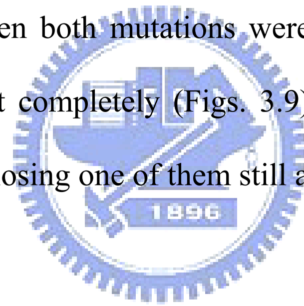
Since DV2E and Ubc9 can be co-precipitated *in vitro*, it is possible that both proteins may be co-localized *in vivo*. Thus, to further examine the protein-protein interaction of the DV2E and Ubc9 in mammalian cells, it has transiently expressed the DV2E-EGFP and Flag-Ubc9 together in BHK-21 cells for 24 and 48 hours, and then examined the cells using confocal microscopy. As shown in Figure 3.8, the expression of DV2E-EGFP was present predominantly within cytoplasm (3.8A, 3.8D; green fluorescence) 24 hours and 48 hours after the transfection. The Flag-Ubc9 distributed predominantly in the cytoplasm after 24 hours (3.8B; red fluorescence) has moved predominantly into nucleus after 48 hours (3.8A-E). Comparing Fig. 3.8C and 3.8F shows that in the presence of pDV2E-EGFP, some Flag-Ubc9 proteins remained in the cytoplasm and co-localized around the nucleus membrane with the DV2E-EGFP protein 48 hours after the transfection (the yellowish patches indicated by arrows in 3.8F). In addition, the number of nuclear foci appeared in the cells transfected with pFlag-Ubc9 were lower then those that co-transfected pFlag-Ubc9 and pD2E-EGFP. Meanwhile, the nuclear foci distributed toward the nuclear membrane rather than evenly in whole nucleus (Fig. 3.8G-I)), suggesting that the function of Ubc9 may have been changed by the over-expression of pD2E-EGFP. However, the mechanism and purpose will need further investigation.

Over-expression of Ubc9 Interfering with the DV2 Virus Production

To assess whether the Ubc9 may affect the propagation of DV2 virus, the pFlag-Ubc9 was transfected into BHK-21 cells cultured in 6-well plates. After incubation for 48 hours, the transfected cells were infected with DV2 virus strain PL046. Additional 48 hours later, one well of the transfected cells were harvested to determine the expression of proteins (Fig. 3.9B). As

shown in Figure 3.9A, there is about 60% reduction in plaque formation with the transiently over-expressed Ubc9 compared to the mock transfection. And transfection of the negative control pCMV-Tag2a did not affect the plaque formation. Neither the plaque size nor the morphology has significantly changed by the transfection of either pFlag-Ubc9 or pCMV-Tag2. The result indicated that expression of Ubc9 could affect the propagation or infection of DV2 virus on BHK-21 cells.

Previous studies have shown that a large number of proteins were revealed to interact with Ubc9 in the yeast-two-hybrid system [Jensen et al., 2004]. In this study it has shown that DV2E proteins, the E protein of DV type 2, also interacted with the sumoylation system via the Ubc9. This is the first report of such observation for DVs. The mutagenesis result indicated that mutations on E protein at the K51 and K241 separately could reduce the interaction with Ubc9 about 50~80% (Figs. 3.9), but when both mutations were introduced together, the interaction between DV2E and Ubc9 was lost completely (Figs. 3.9). Therefore, the interaction between them is mediated by both residues, losing one of them still allowed partial activity.



Discussion

Ubc9 is a protein that covalently conjugates SUMO-1 to the substrate proteins to alter the subcellular localization [Muller and Dejean, 1999; Melchior, 2000] or the stability of the proteins [Buschmann et al., 2000], as well as DNA replication and repair [Matunis et al., 1996; Mao et al., 2000]. The Ubc9 is known to mediate other proteins to interact with SUMO, some of them have turned out to be substrates for sumolyation, while the other might have been regulated by Ubc9 via mechanisms that do not involve covalent modification [Huggins et al., 1999; Poukka et al., 1999; Xu et al., 2000]. Here described the discovery using Functional Yeast Array that DV2E interacts with Ubc9 and assessing the observation further with *in vitro* pull down assay, confocal microscopy, and site-directed mutagenesis. It is interesting that DV2E can interact directly with Ubc9, but not with SUMO-1 (Fig. 3.3). This is consistent with several reports revealing that Ubc9 can recognize the substrate protein directly [Hay, 2001; Benier-Villamor et al., 2002; Lin et al., 2002b; Lin et al., 2002c; Tatham et al., 2003]. Ubc9 binding may play an important role in substrate recognition as well as in substrate modification [Sampson et al., 2001].

There are both direct and indirect evidences that the presence of viruses can alter the sumoylation status of host cellular proteins [Adamson and Kenney et al., 1999; Muller and Dejean, 1999; Hofmann et al., 2000; Ahn et al., 2001; Xu et al., 2001; Gravel et al., 2002; Saitoh et al., 2002; Spengler et al., 2002; Kaukinen et al., 2003]. Therefore, it is possible that the DV2E protein may sequester the SUMO-1 via direct interaction with Ubc9 with the consequence of influencing SUMO-1, which leads to the onset of pathogenesis or host cell defense system. It has been suggested that such modulation of critical host proteins may be important for inhibiting cellular defense mechanisms or for promoting an intracellular state that is supportive of viral reproduction [Wilson and Rangasamy, 2001]. For example, viral proteins could utilize the SUMO modifying machinery to become activated for targeting specific sites in the cell or to compete with cellular substrates as part of the metabolic changes in favor of viral replication

[Jensen et al., 2004]. On the other hand, it is also a possibility that modification of viral proteins by the sumoylation is part of a cellular antiviral mechanism [Jensen et al., 2004], which is consistent with our data that expression of Ubc9 reduces the plaque formation (Fig. 3.9). Of course, it is possible that this reduction of plaque formation was caused by the combined effort of the transfected Ubc9 and the indigenous one. If this is the case, then, it is a quantitative effect rather than a qualitative effect. Other well-defined effects imposed by sumoylation on certain targets are to increase the stability of the protein [Saitoh et al., 2002], to enhance transcriptional activity [Gill et al., 2003; Verger et al., 2003], to enhance or to interfere with the enzyme activity in the sumoylation system, to bind specific host substrates, and to block or stimulate their sumoylation [Bell et al., 2000; Adamson and Kenney et al., 2001; Merezak et al., 2002].

In mammalian cells, Ubc9 has been found in the nucleus but it is particularly prominent on the cytoplasmic side of the nuclear pore fibrils [Lee et al., 1998; Saitoh et al., 1998; Yeh et al., 2000; Saitoh et al., 2002; Zhang et al., 2002]. Therefore, it is thought that Ubc9 controls intracellular targeting of substrate proteins either directly through protein interactions or indirectly via sumoylation [Yeh et al., 2000; Kurtzman et al., 2001]. The idea that Ubc9 itself may have involved directly in viral replication cycle has been suggested from the study of retroviral replication. Two viral proteins, the nucleocapsid from hantaviruses and a retroviral Gag protein, are reported to co-localize with Ubc9 [Kaukinen et al., 2003; Maeda et al., 2003; Weldon et al., 2003]. First, the Hantaan virus nucleocapsid protein (HTNV-NP) has been reported to interact with Ubc9 and SUMO-1, and these interactions have been postulated to play a critical role for the assembly of viral nucleocapsids by targeting HTNV-NP to the perinuclear region, the place where Hantaan viral replication and assembly occur [Maeda et al., 2003]. Second, Weldon et al. [Weldon et al., 2003] have identified Ubc9 as a cellular interacting partner for the Mason-Pfizer monkey virus Gag protein (MPMV-Gag). MPMV is the prototypical betaretrovirus, the MPMV Gag polyproteins assemble into immature procapsids in the cytoplasm, near the nuclear membrane. Co-localization experiments performed by co-transfection of a Myc-tagged

Ubc9 and MPMV-Gag have showed that over-expression of Ubc9 led to the co-localization of Myc-Ubc9 with MPMV-Gag in the cytoplasm near the nuclear membrane as well as within the nucleus [Weldon et al., 2003]. This is consistent with our observation that when the DV2E-EGFP and Flag-Ubc9 were co-expressed transiently, they were co-localized near the nuclear membrane on the cytoplasmic side (Fig. 3.8), and DV2E-EGFP also effected the distribution of pFlag-Ubc9 from predominantly in nucleus to near the nuclear membrane (Fig. 3.8) at 48 hours after transfection. Furthermore, the plaque assay has showed that over-expression of Ubc9 would interfere with the number of the plaque formed (Fig. 3.9). This is also consistent with the idea that Ubc9 itself may have played a direct and novel role in the DV propagation and/or pathogenesis. However, not all the Ubc9 and the DV2E were co-localized, which may be due to the abundance of both DV2E-EGFP and Flag-Ubc9 and the presence of endogenous Ubc9. It is also possible that since Ubc9 protein possesses multiple functions and complicate interaction with other proteins, only portion of the Ubc9 is required to interact with the DV2E. Whether the endogenous Ubc9 is influenced by DV2E will require further investigation.

In conclusion, the investivagetion have found that Ubc9 can interact with the E protein of Dengue virus type 2 via the residues K51 and K241 on DV2E. Co-transfection of DV2E-EGFP and Flag-Ubc9 in mammalian cells showed Ubc9 and DV2E co-localized near the nuclear membrane and the result of plaque assay revealed that over-expression of Flag-Ubc9 could reduce the propagation of virus. The exact role of the DV2E-Ubc9 interaction in viral propagation, pathogenesis, and cell physiology will require further study.

Primer Name	Sequence	Mutation position (Nucleotide)
1	5’ AACAGAAGCCAGACAATCTGCCGCTCTAAG 3’	151
2	5’ CTTAGAGCGGCAGATTGTCTGGCTTCTGTT 3’	151
3	5’ TTGGTCACTTTCAAGAATCCCCATGCGAAG 3’	721
4	5’ CTTCGCATGGGGATTCTTGAAAGTGACCAA 3’	721

Table 3.1 Primers used for mutagenesis. The mutation positions are indicated by underline.

	Yeast Two-Hybrid	Functional Yeast Array
Screen source	cDNA library	1200 clones of known human genes
Gene category	Depends on the source of cDNA (e.g. Brain, Liver, Kidney… etc.)，the quality of cDNA will influence the screen result	Specific selected 1200 clones, and category to 5 modules by the function of genes (Apoptosis, Cancer-Related, Cell Interaction, Transcription Factor, Signal Transduction)
Gene expression	The screen clones which come from cDNA library are not to guarantee the full length and correct expression	The specific design to make sure the 1200 clones will be full length and expressing correctly
Operation	Cotransformation of the bait and cDNA library, and spread all the clones in agar plate, the positive candidates will needed to be advance sequenced and examined to eliminate false positive and redundant clones	The 1200 known clones are array at 96 well plate and culture on liquid handling to reduce the time, no advance sequencing, no redundant clones
Quality control	Not provide	Functional Yeast Array provide the positive control, negative control, and a known bait gene as internal control
Screening time	Two months	Only 5 days
Total operation time	At least 6 months (including bait construction, cDNA library preparation, protein expression, 3-AT test, screening, isolation of cDNA clones, and elimination of false positive)	6 to 8 weeks(about 2 months) (including bait construction, protein, protein expression, 3-AT test, and screening)

Table 3.2 Comparison of traditional Yeast Two-Hybrid and Functional Yeast Array

Name of constructs	Mutation Position (Amino acid)	Sumoylation consensus motif (ΨK*X*E)
pBTM-D2E- K51R	K51	LIETEAKQPATLRK
pBTM-D2E- K241R	K241	ETLVTFKNPHAKKQ
pBTM-K51R-K241R	K51 and K241	LIETEAKQPATLRK LKLNWFKKGSSIGQ

* The mutation sites are indicates by underline.

Table 3.3 The candidates of interaction sites between Ubc9 and DV2E.

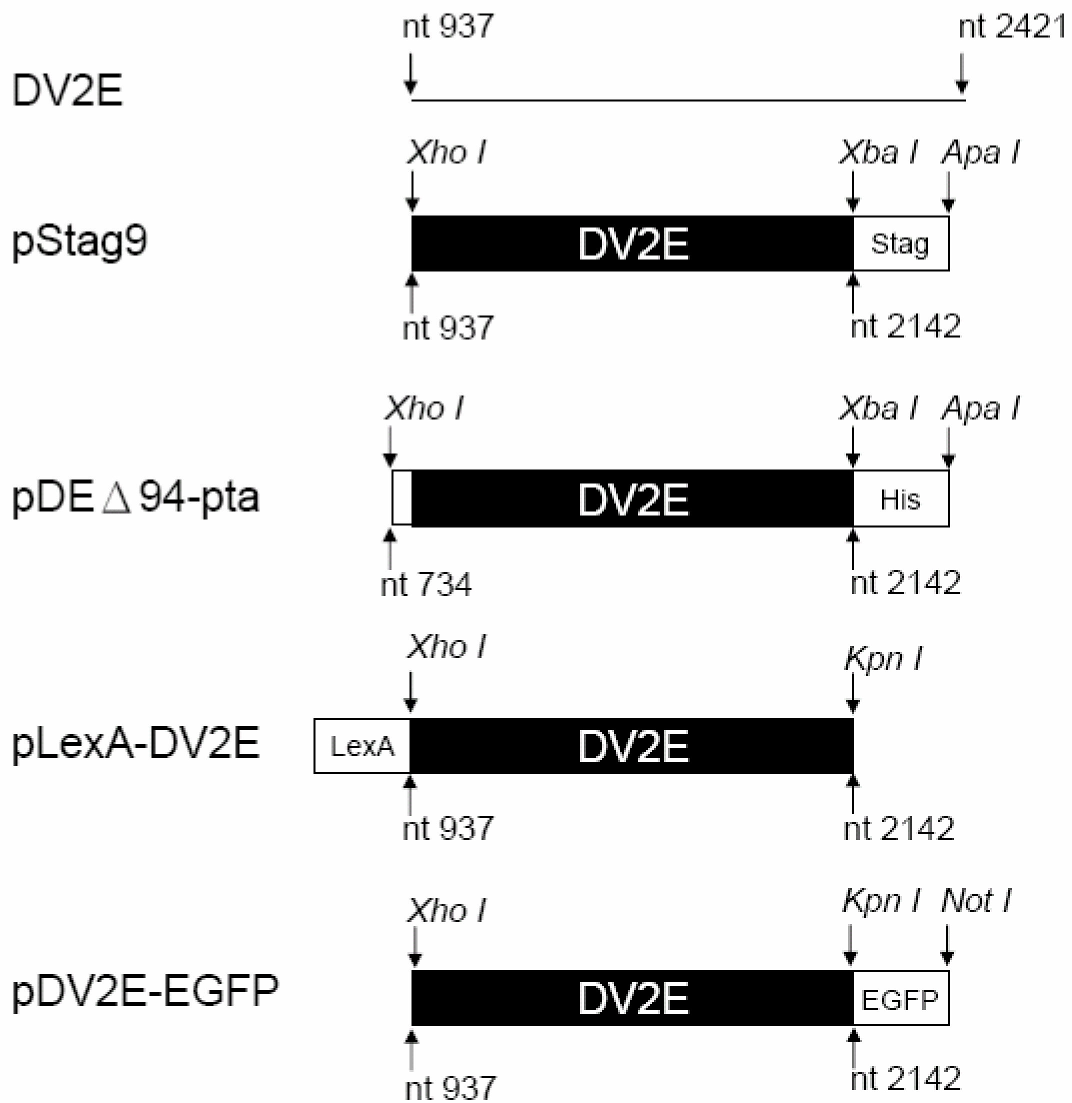


Figure 3.1 Schematic presentation of DV2E-related constructs. The nucleotide sequence is denoted according to the DV2 genome sequence of NGC strain. DV2E: the whole ORF of DV2 E gene. The N-terminal DV2 E gene sequence is cloned into different vectors and fused in frame with in-vivo expression tags. pStag9 and pDED94-pta15 are pcDNA3-based with S tag from the pET-30a and His tag from pET-30b, respectively. pLexA-DV2E is pLB1.0 vector-based, which carries a LexA sequence. pDV2E-EGFP is pEGFP-N2 based and carries the EGFP sequence. Related restriction sites on the constructs are indicated by arrow.

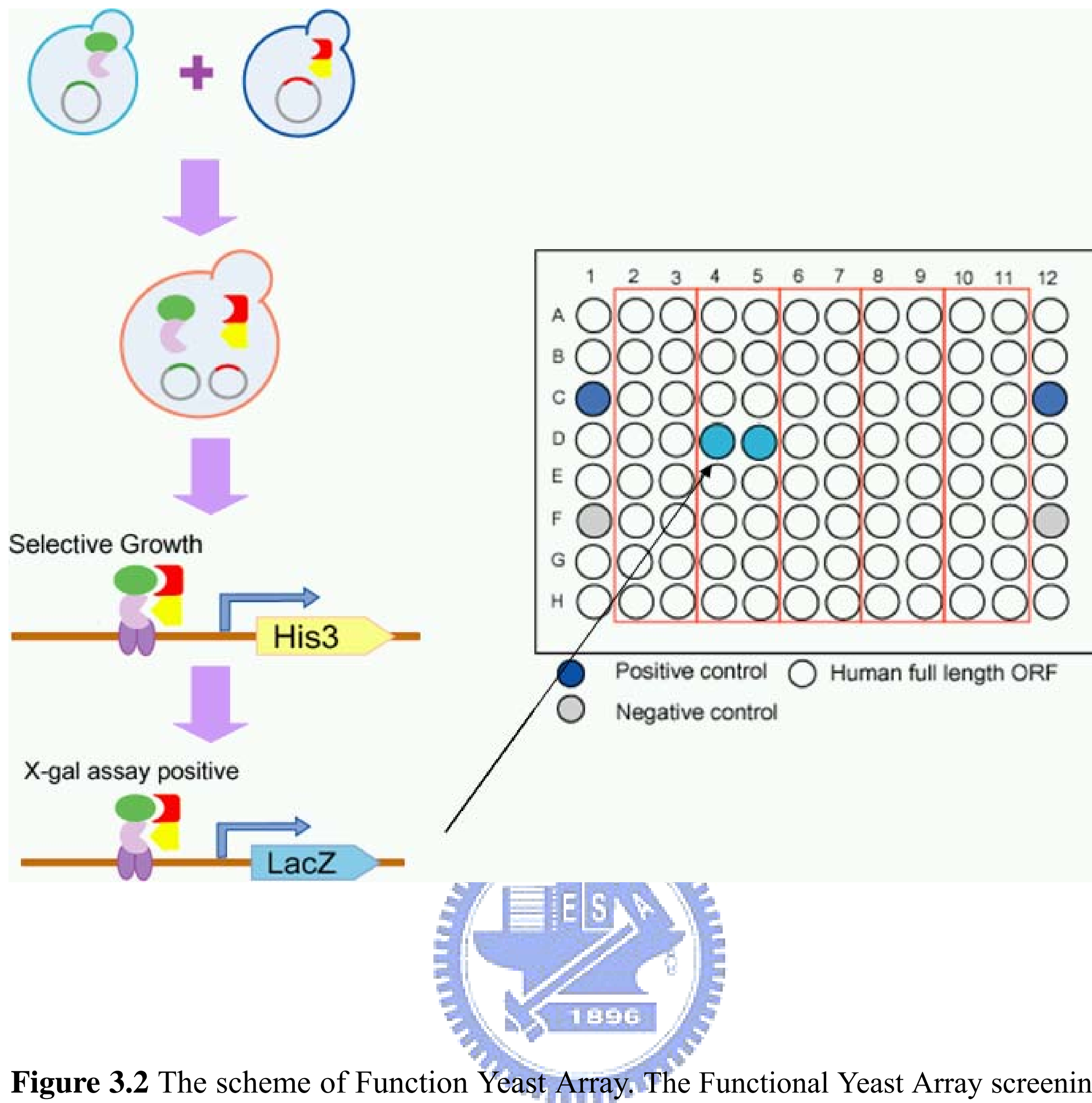


Figure 3.2 The scheme of Function Yeast Array. The Functional Yeast Array screening utilizes a target gene as the bait to against module preys. Briefly, the yeast strain expressing bait gene was mated with each yeast strain expressing different preys separately in 96-well plates. After overnight incubation, the resulting yeast cells were cultured in growth selection medium for diploid cells, and at the same time the mating yeast cells were cultured in another selection medium for screening protein-protein interaction. When bait proteins interacted specifically with prey proteins, it could drive both *HIS3* and *lacZ* genes to express, and the yeast cells could grow on growth selection medium and could detected blue color by assessing the expression of X-gal activity.

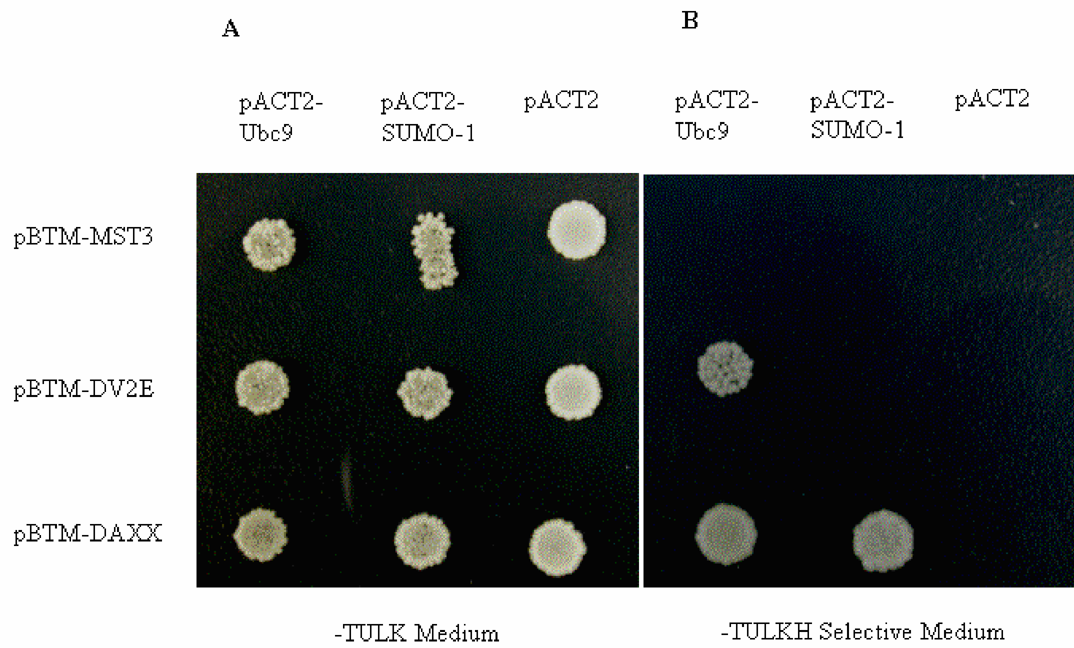


Figure 3.3 Yeast two-hybrid analysis to assess the protein-protein interaction between Ubc9 and DV2E. *Saccharomyces cerevisiae L40* was co-transformed with a pBTM-based plasmid encoding the protein as 'bait" and a pACT2-based plasmid encoding the protein as "prey". The co-transformation were grown on -TULK plate (A) and selected on -TULKH plate (B). The gene sequences carried are indicated as in the names of the plasmids.

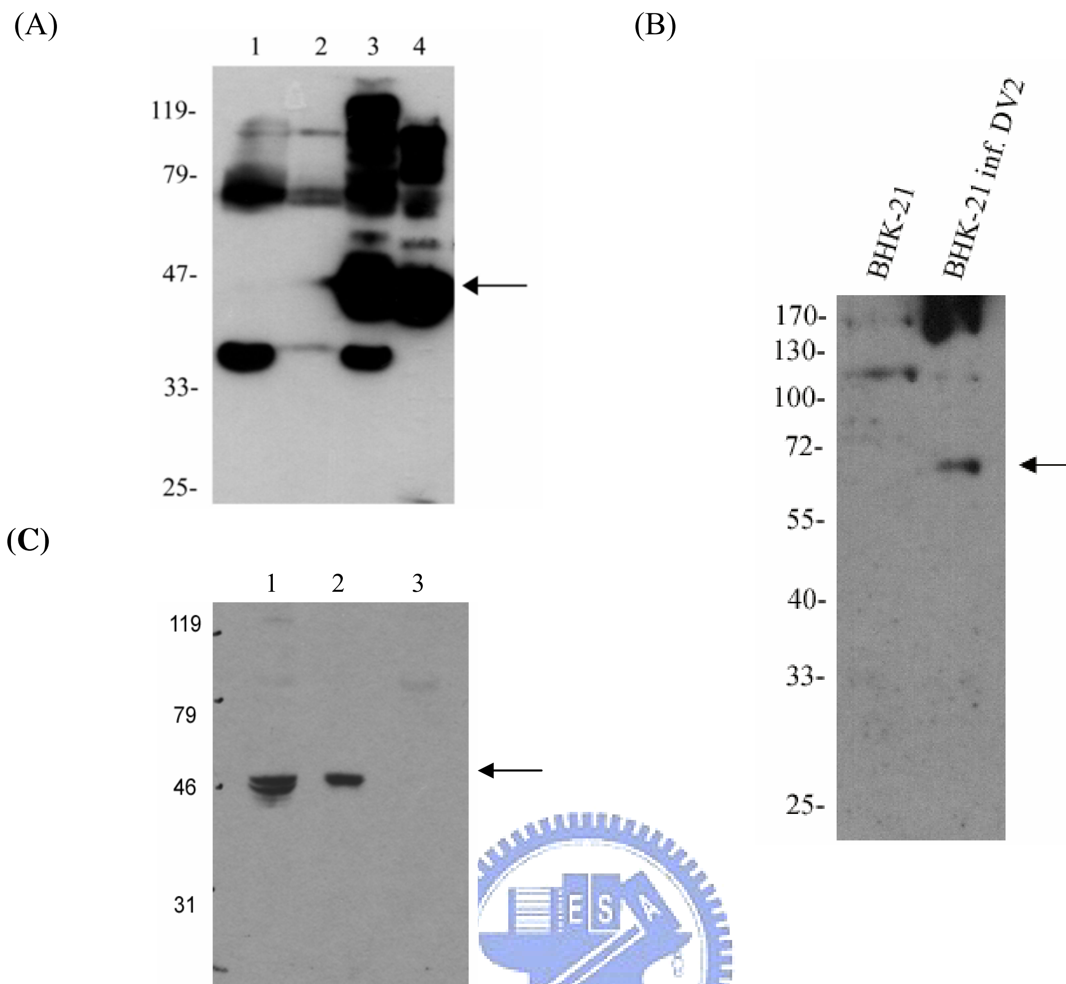


Figure 3.4 Detection of DV2E protein with anti-EStag9 polyclonal antibody. (A). Western blotting. Lane 1, *E. coli* lysates from cells transformed with pcDNA3 vector; Lane 2, the supernatant of *E. coli* lysates from cells transformed with pStag9; Lane 3, *E. coli* lysates from cells transformed with pStag9; Lane 4, proteins purified from *E. coli* cells transformed with pcStag9. (B) Western blotting on DV2 PL046 strain. BHK-21 cells were cultured in 10 cm dish and infected with DV2 PL046 strain, the cells then lysed with RIPA and loaded 20 μl of supernatant in SDS-PAGE. BHK-21: BHK-21 cells lysed with RIPA; BHK-21 inf. DV2: BHK-21 cells infected with DV2 PL046 strain and lysed with RIPA. The arrow indicates the position of dengue virus envelope protein with proper processing and glycosylation. (C). Western blotting with short expose. Lane 1, *E. coli* lysates from cells transformed with pStag9; Lane 2, purified recombinant EStag9 proteins; Lane 3, *E. coli* lysates from cells transformed with pcDNA3 vector. The arrow indicates the EStag9.

Figure 3.5

(A)

+	+	GST-Ubc9
+	−	pEGFP-N2
−	+	pDV2E-EGFP

anti-DV2E 72-

anti-GST 40- ← GST-Ubc9

+	+	+	GST
−	+	−	pEGFP-N2
+	−	+	pDV2E-EGFP

72-
55-
33-
25- ← GST

(B)

Input GST GST-Ubc9

170-
130-
100-
72-
55-
40-
33-
25-

1896 E S A

(C)

−	−	+	+	+	DEΔ94-pta15
+	−	−	+	−	pCMV-Tag2a
−	+	−	−	+	pFlag-Ubc9
1	2	3	4	5	

+	+	+	priB-His fusion protein
−	+	−	pCMV-Tag2a
−	−	+	pFlag-Ubc9
6	7	8	

anit-Flag 25-

anti-His 55- 40-

15-

Figure 3.5 Ubc9 interacting with DV2E *in vitro*. (A). GST pull down assay. GST-Ubc9 were expressed in *E. coli BL21(DE3)* and purified by GST affinity. The purified proteins were immobilized on GST-Sepharose 4B and incubated with the cell lysate of 293 expressing pDV2E-EGFP or pEGFP-N2 vector. Bound proteins were analyzed with anti-EStag9 polyclonal antibody in western blot analysis. The proteins encoded are indicated on the top of the panel and the presence or absence of them are indicated by "+" and "-", respectively. GST-Ubc9, purified GST-Ubc9 protein; GST, purified GST protein; pEGFP-N2, the lysate from cells transfected with vector alone; pDV2E-EGFP, the lysate of pDV2E-EGFP-transfected cells. Protein molecular weight markers are indicated by number and the left of the panel. Antibodies used are indicated at the far left of the panel. (B) TNT assay. In vitro translated ^{35}S-labeled DV2E protein was incubated with GST or GST-Ubc9 protein for the pull down assay. Input represents a 20% of ^{35}S-labeled DV2E proteins subjected to the GST pull down assay. (C) His-tag pull down assay. The proteins encoded are indicated on the top of the panel and their presence or absence are indicated by "+" and "-", respectively. DEΔ94-pta15, purified DEΔ94-pta15 protein; His fusion protein, the purified unrelated His-tag fusion protein; pCMV-Tag2a, the lysate from cells transfected with vector alone; pFlag-Ubc9, the lysate of pFlag-Ubc9-transfected cells. The antibodies used are indicated at the far left of the panel. Arrow indicates the band correspond to the Ubc9 protein.

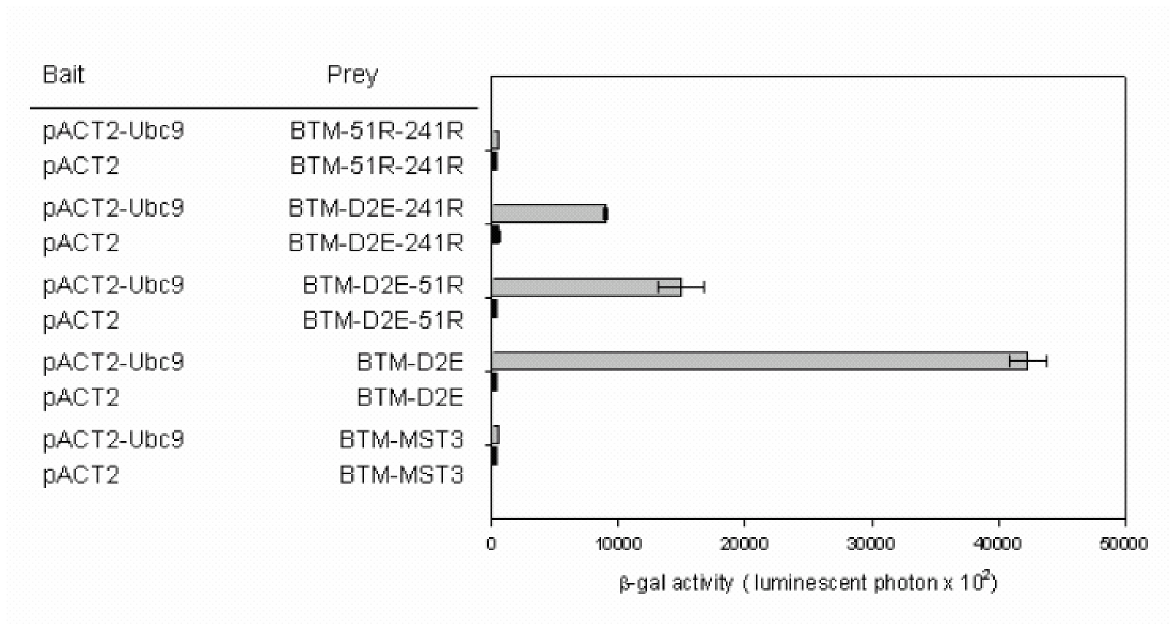


Figure 3.6 β-gal activity assay to quantify the interaction between DV2E and Ubc9 in yeast cells. *Saccharomyces cerevisiae L40* was cotransformed with constructs pBTM-D2E-K51R, pBTM-D2E-K241R, pBTM-K51R-K241R, pBTM-MST3 (negative control) separately with either pACT2-Ubc9 or pACT2 (control prey vector). The interactions between the encoded proteins are indicated by the level of β-gal activity.

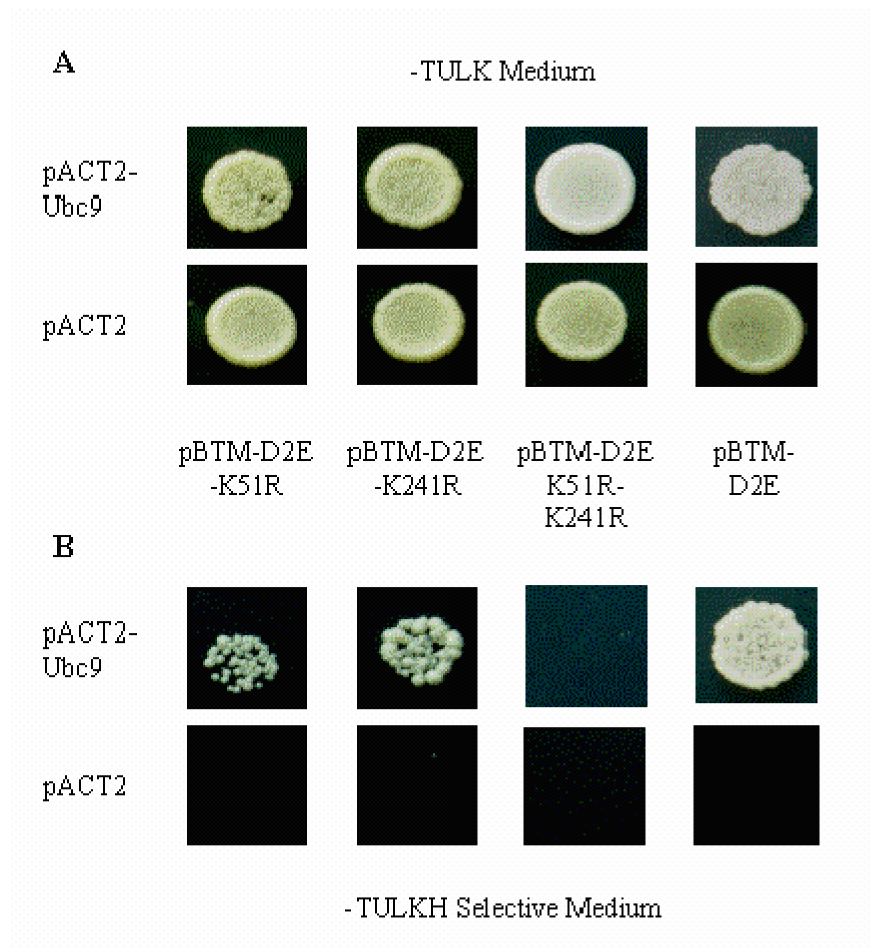


Figure 3.7 Double mutations on DV2E abolishing the interaction between the DV2E and Ubc9. *Saccharomyces cerevisiae L40* cells were co-transformed with the pBTM-based plasmids encoding the mutated or wild type DV2E proteins as the ‘bait” and the pACT2-based plasmid encoding the Ubc9 protein as the “prey”. pACT2 was used as the control prey vector and pBTM-MST3 was the negative bait control which encoded the MST3 protein known not to interact with Ubc9 protein. Mutations introduced into the sequence of DV2E are indicated as in the names.

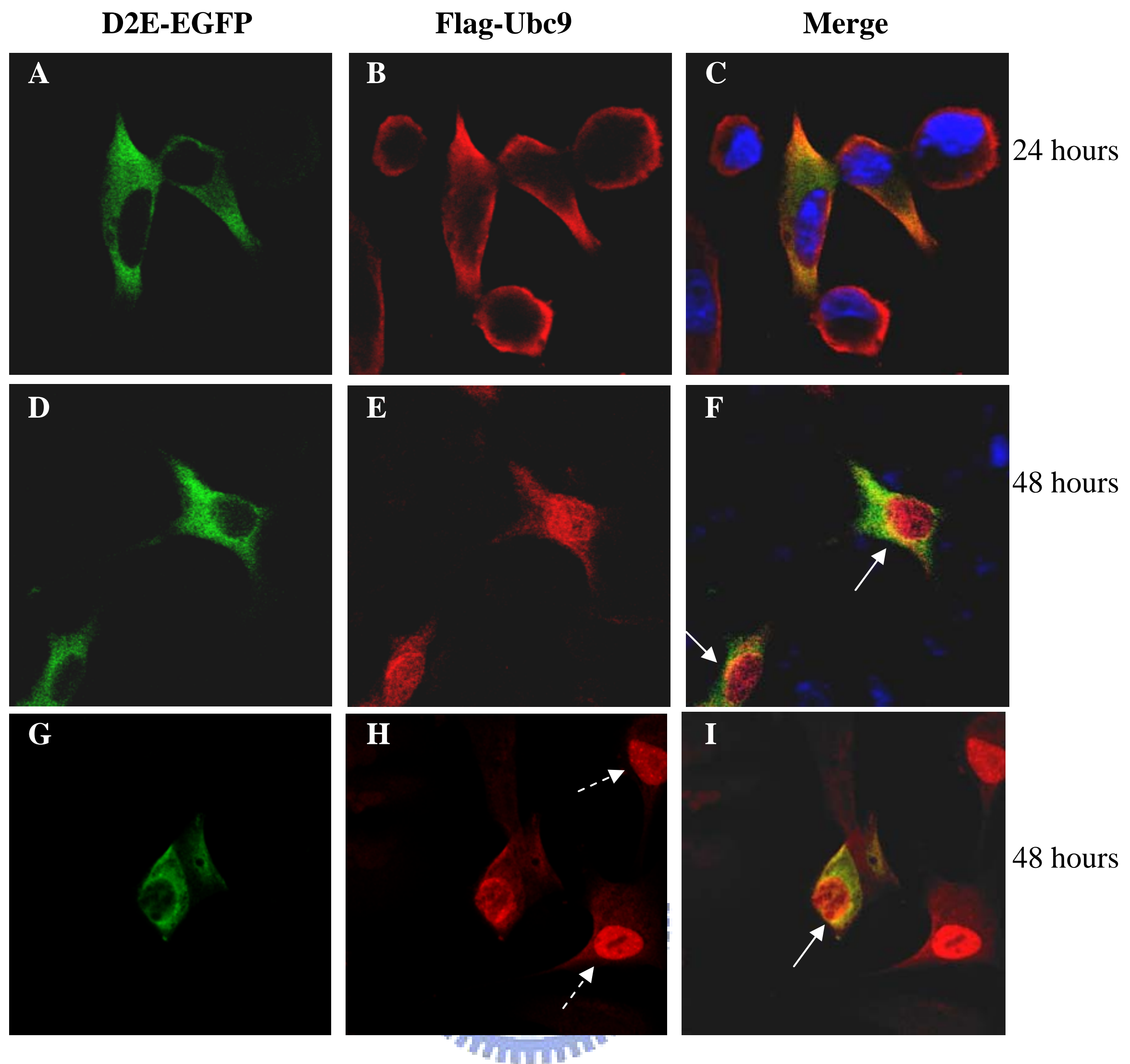
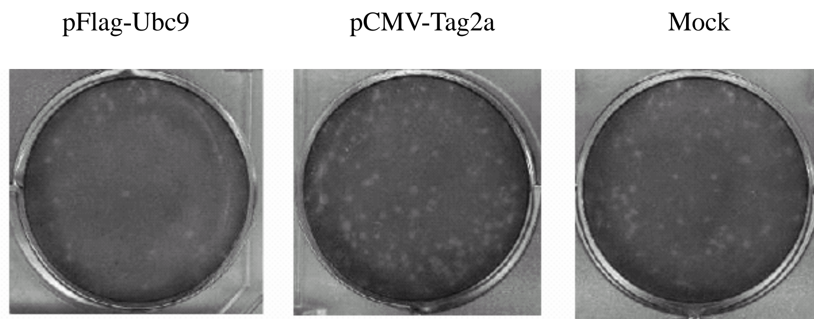


Figure 3.8 Subcellular localization of DV2E and Ubc9 by immunofluorescence. BHK-21 cells were co-transfected with pDV2E-EGFP and pFlag-Ubc9, and then were fixed at 24 and 48 hours post-transfection. Flag-Ubc9 proteins were detected with primary antibody anti-FLAG M2, and labeled with secondary antibody anti-mouse IgG-TRITC. Cell nuclei were stain with DAPI. Those cells were then visualized with confocal immunofluorescence microscopy. The subcellular localizations of DV2E-EGFP are represented by the green fluorescence (A, C, D, F, G, I) and that of Flag-Ubc9 by red (B, C, E, F, H, I). Nuclei are represented by blue (C and F) and the co-localization of DV2E-EGFP and Flag-Ubc9 proteins are shown in the merged images (C,F, I) Solid arrows indicate the location of co-localization (F, I) and the dashed arrows indicate the presence of nuclear foci (H).

(A)

	pFlag-Ubc9	pCMV-Tag2a	Mock



Plaque number	45.5±17.9	116.0±20.4	100.0

(B)

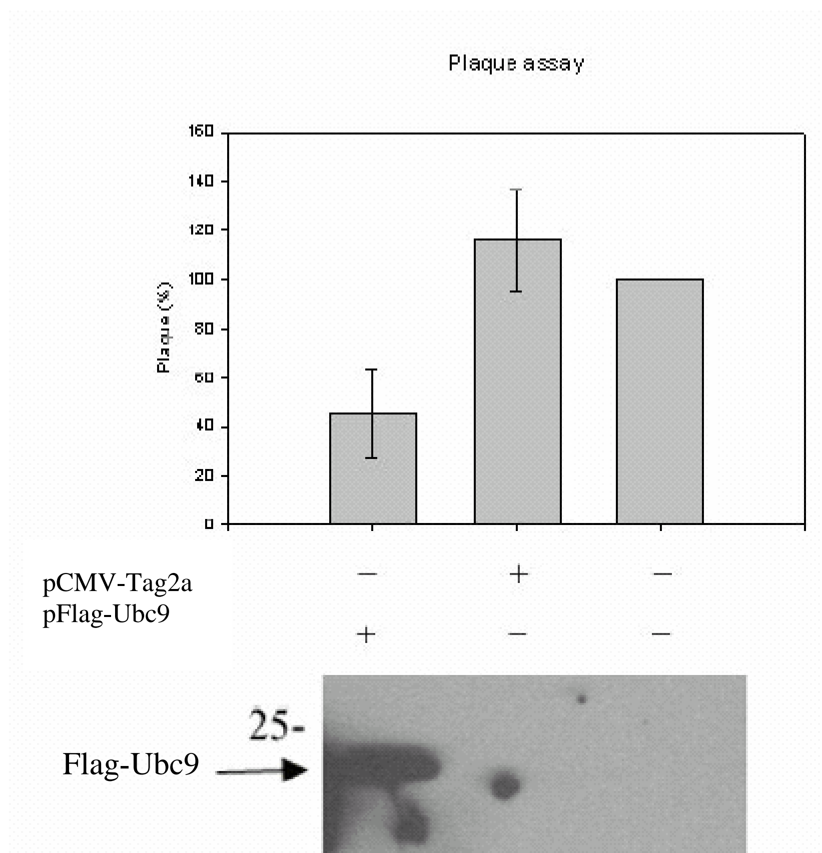


Figure 3.9 Determination of the Effect of Ubc9 over-expression on the propagation of dengue virus type 2 by plaque formation assay. BHK-21 cells were grown in 6-well plates and transfected with pFlag-Ubc9 or pCMV-Tag2a before the addition of dengue virus type 2 PL046. After absorption for 1 h at 37 ^{o}C, the media were aspired and the cells overlaid with growth medium/methyl cellulose. Then, the cultures were incubated at 37 ^{o}C with 5% CO_2 for 5 days

before the cells were fixed and stained with crystal violet. (A) The result of plaque formation assay. (B) The quantitative data of the result of plaque formation assay; the transfected constructs are indicated on the lower left of the upper panel and their presence or absence are indicated by "+" and "-", respectively. Lower panel, cells from one of the 6-well transfected with pFlag-Ubc9 or pCMV-Tag2a or neither were harvested and analyzed by western blotting with anti-Flag antibody. Arrow indicates the position of Flag-Ubc9 protein.

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