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The type 2 dengue virus envelope protein interacts with small ubiquitin-like modifier-1 (SUMO-1) conjugating enzyme 9 (Ubc9)

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Abstract

Dengue viruses are mosquito-borne flaviviruses and may cause the life-threatening dengue hemorrhagic fever and dengue shock syndrome. Its envelope protein is responsible mainly for the virus attachment and entry to host cells. To identify the human cellular proteins interacting with the envelope protein of dengue virus serotype 2 inside host cells, we 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, we have demonstrated that it 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 nuclear membrane and co-localized with Flag-Ubc9 proteins around the nuclear membrane in the cytoplasmic 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 propagation.

Introduction

Dengue viruses (DVs) are members of *Flavivirus* and are transmitted by *Aedes* mosquitoes. Patients with DV infections show various clinical symptoms from no significant illness, mild fever, to life-threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The infection caused by DVs is increasingly an important public health threat, especially in tropical and subtropical areas. Currently, there is no effective treatment for these diseases. Although it has been suggested that immunization may provide a realistic approach for controlling DV infections [1], no approved commercial product is presently available, after considerable research has been directed toward the development of a safe, effective DV vaccine over the past 50 years.

Conventionally, DVs are categorized into four serotypes, 1–4. Their genomes contain a single positive-stranded RNA encoding an 11-kb open-reading-frame translated into a single polyprotein. After processed by the proteases of the virus and the host, the polyprotein produces three structural proteins- capsid (C), precursor membrane (preM) protein and envelope (E) protein, and seven nonstructural proteins- NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 [2].

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The viral attachment and entry are mainly dependent upon the E protein, the major glycoprotein on the flaviviral particles [3, 4]. The E protein forms an oligomer with the M protein and constitutes most of the accessible virion surface [5]. This reflects the fact that the E protein mediates attachment to the host and is essential for membrane fusion during the stage of viral entry. It is also the primary antigen inducing protective immunity and the major antigen for virus neutralization. Therefore, the E protein directly affects the host range, the cellular tropism and in part, the virulence of the DVs [4].

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 [6–13]. 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 [14]. Three SUMO paralogues have been reported in mammalian cells and they are known as SUMO-1, -2, and -3 [15, 16]. 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 [17]. 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 [6, 18–21]. Ubc9 binding may play an important role in substrate recognition as well as in substrate modification [22].

In recent years, growing numbers of viral proteins have been found to conjugate with SUMO-1 [23–34]. There are currently six known sumoylated viral proteins, distributed among three DNA viral families: Adenoviridae, Papillomaviridae, and Hepesviridae [25, 26, 28, 30–32]. 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 [35, 36], while the Mason-Pfizer Monkey Virus (MPMV) Gag protein interacts with Ubc9 [37]. The Hantaan Virus Nucleocapsid protein (HTNV-N) interacts with both SUMO-1 and Ubc9 [38], and the Epstein Barr Virus nuclear antigen 3C (ENBA-3C) interacts with SUMO-1 and SUMO-3 [19].

In this study, we 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 distribute evenly and predominantly in the 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 propagation.

Methods

Materials

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. BHK-21 and 293 cells were used to express genes in mammalian cells.

Cell culture

Mammalian 293 cells were cultured at 37 °C with 5% CO_2 in MEM medium (Gibco) supplemented with 4 mM of L-glutamine, 1.5 g/l of sodium bicarbonate, 4.5 g/l of glucose, and 10% of fetal bovine serum (FBS) (Gibco). BHK-21 cells were cultured at 37 °C, 5% CO_2 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.

Construction of plasmids

A recombinant plasmid containing E protein sequence, pStag9, [39] 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′ TTCTCGAGGACAATGCGTTGCATAGG 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.

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 1100 full-length cDNA of known human genes, each fused to the VP16 trans-activation domain, as the preys. Those genes are divided into five 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.) [40–44]. 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, we have chosen two sets of modules, Apoptosis and Signal Transduction, totally 500 individual clones, as preys for the procedure. Briefly, 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 cultured 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 β-gal activity, yeast clones shown positive in growth were lysed with zymolase and vortexed with glass beads before incubated at 30 °C for 48 h 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 A_{600} researched 0.6 and 0.1 mM IPTG was added before continuing incubation for three more hours. Then the culture was centrifuged and the cell pellet was resuspended in 50 mM Tris–HCl (pH7.5) with the addition of 1 mM dithiothreitol prior to sonication. The lysates were centrifuged at 8,000 rpm at 4 °C for 15 min to remove the cellular debris. Then, the supernatant was added to glutathione-Sepharose 4B column (Amersham Biosciences). After washed with 10 volume of 1× ice-cold PBS, the GST-fusion proteins were eluted competitively with 10 mM reduced glutathione in 50 mM Tris–HCl (pH7.5). The eluted GST-Ubc9 proteins were then dialyzed against 50 mM Tris–HCl (pH7.5) with 1 mM dithiothreitol and concentrated using 10-kDa molecular mass cut-off microconcentrators (Millipore).

To express EStag9 (protein product of pStag9), DEΔ94-pta15 (protein product of p DEΔ94-pta15), and priB-His proteins, corresponding plasmids were transformed separately into *E. coli* BL21(*DE3*) and the transformants were processed mainly according the previous report [39] with modification. Briefly, the transformants were grown in LB broth at 37 °C until the A_{600} researched 0.4. The cells were then harvested after additional 3-h incubation. The pellets were resuspended with 1 mM Tris–HCl (pH8.0) containing 1 mM PMSF. After sonication, the centrifuged cell lysates were resuspended with inclusion-body solubilization buffer (50 mM Tris–HCl pH8.0, 1 mM EDTA, 100 mM NaCl, 8 M urea, 1 mM PMSF) and incubated on ice for 1 h.

For the EStag9, the urea was removed by dialysis overnight in the S-protein Agarose (Novagen) binding/wash buffer (20 mM Tris–HCl pH7.0, 150 mM 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. The elution buffer (20 mM Tris–HCl pH7.0, 150 mM NaCl, 0.1% Triton X-100, 3 M 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 (50 mM Tris–HCl pH9.0, 10 mM 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 (50 mM Tris–HCl pH9.0, 10 mM imidazole, 0.1% Triton X-100, 1 mM dithiothreitol). After the sample was applied, the column was washed with 10 volume of binding/wash buffer. Then, elution buffer (50 mM Tris–HCl pH9.0, 500 mM imidazole, 0.1% Triton X-100, 1 mM 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 50 mM Tris–HCl (pH9.0) with 1 mM dithiothreitol and concentrated using 10-kDa molecular mass cut-off microconcentrators (Millipore).

Pull down assays

For GST pull down assay with DV2E, the purified GST or GST-Ubc9 proteins were pre-incubated at room temperature with 30 μl of glutathione-Sepharose 4B (GE Healthcare) at the concentration of $\sim$1 mg/ml. Mammalian 293 cells were transiently transfected with pDV2E-EGFP in a 10-cm culture dish. After 48 h, the cells were harvested and washed 3 times with 1× PBS. The pellets were resuspended with 1 ml of ice-cold 1× RIPA buffer (Upstate) containing cocktail mixture (Calbiochem) and gently mixed on orbital shaker at 4 °C for 30 min 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 1× 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 (50 mM Tris–HCl [pH7.5], 1 mM DTT) for 2 h. 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 10-cm culture dish. After 48 h, the cells were harvested and washed 3 times with 1× PBS. The pellets were resuspended with 1 ml of ice-cold 1× RIPA buffer (Upstate) containing cocktail mixture (Calbiochem) and gently mixed at 4 °C for 1 h 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 h, the beads were collected by centrifugation and washed three times in 1 ml of 1× binding/wash buffer (50 mM Tris–HCl pH9.0, 10 mM imidazole, 0.1% Triton X-100, 1 mM 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-DV-2 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 min before being 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 h, the membrane was then washed and processed by the chemoiluminescence detection system (Upstate).

Analysis of the interaction between DV2E and Ubc9

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 1) were used to introduce mutations to pBTM-D2E to form pBTM-D2E-K51R; primers 3 and 4 (Table 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 [45]. The yeast cells were grown at 30 °C overnight until the A_{600} reached 1.3. The pellets were lysed with zymolase and incubated at 37 °C for 2 h before centrifugation, then the activity of β-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.

Table 1. Primers used for mutagenesis.

Primer name	Sequence
1	5′ AACAGAAGCCAGACAATCTGCC GCTCTAAG 3′
2	5′ CTTAGAGCGGCAGATTGTCTGGC TTCTGTT 3′
3	5′ TTGGTCACTTTCAAGAATCCCCAT GCGAAG 3′
4	5′ CTTCGCATGGGGATTCTTGAAAG TGACCAA 3′

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 h, cells were fixed in 4% paraformadehyde in PBS for 15 min at room temperature, and then incubated in 0.5% Triton X-100 in 1× PBS for 5 min. After washed three times with 1× PBS for 5 min each, the cells were blocked with 1% BSA in 1× PBS for 30 min. Then the cells were incubated with Anti-FLAG M2 monoclonal antibody (Sigma) overnight. After washed three times (5 min each) with 1× PBS, the cells were incubated with anti-mouse IgG-TRITC (Sigma) for 1 h at room temperature. Nuclei were visualized by DAPI (4′, 6′-diamidino-2-phenylindole) staining for an additional 1 h. 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 2×10^5 cells/ml in 6-well plates and incubated at 37 °C with 5% CO_2 for 24 h 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 h 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% CO_2 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

Ubc9 Interacting with DV2E in the yeast-two-hybrid analysis

To identify possible host cellular proteins interacting with the DV2E, we have constructed the N-terminal 402 amino acids of the E protein as the "bait" to screen on the "Functional Yeast Array" (Level Biotechnology Inc.). It has 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, we have co-transformed the L40 cells with different sets of plasmid and determined their growth on different media. As expected, all transformants were able to grow on -Trp/-Ura/-Leu/-Lys (-TULK) medium (Figure 2A). The DAXX protein, known to interact with Ubc9 and SUMO-1 [46, 47], was used as the positive control whereas the MST3 protein was the negative as described previously [46]. 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 (Figure 2B). 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, we have generated an anti-DV2E antibody to perform the pull down assay. To obtain the

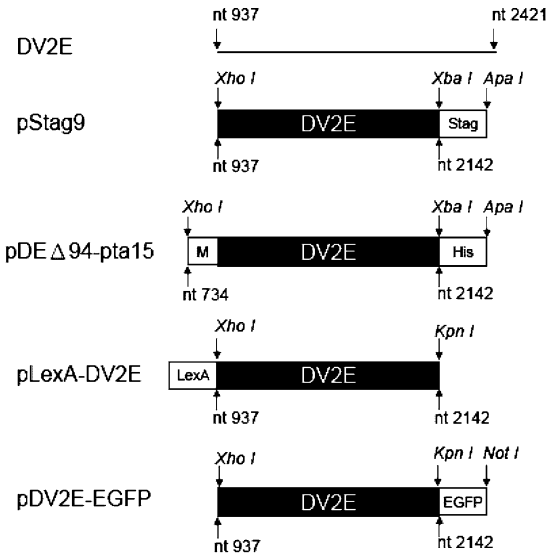

Figure 1. Schematic presentation of DV2E-related constructs in this study. The nucleotide sequence is denoted according to the DV2 genome sequence of NGC strain. DV2E: the whole ORF of DV2 E gene. The N-terminal DV2 E gene sequence is cloned into different vectors and fused in frame with in-vivo expression tags. pStag9 and pDEΔ94-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.

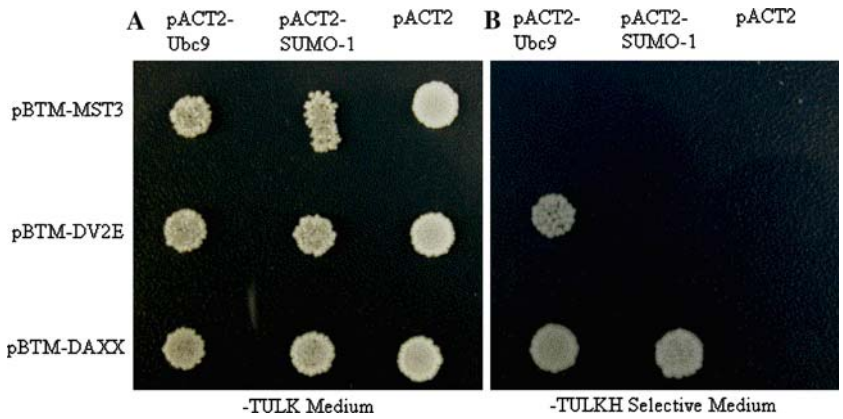

Figure 2. Yeast two-hybrid analysis to assess the protein-protein interaction between Ubc9 and DV2E. *Saccharomyces cerevisiae L40* was co-transformed with a pBTM-based plasmid encoding the protein as ‚bait'' and a pACT2-based plasmid encoding the protein as ''prey''. The co-transformation were grown on -TULK plate (A) and selected on -TULKH plate (B). The gene sequences carried are indicated as in the names of the plasmids.

anti-DV2E antibody, we have transformed the plasmid Stag9 into *E. coli* BL21 cells and purified the EStag9 protein as described in the section of material and method, and used it 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 (Figure 3A, lane 3) and the purified EStag9 at about 53 kDa (Figure 3A, lane 4). In the lysate of cells containing no Estag9-expressing plasmid, there was no detectable band at the corresponding position (Figure 3A, lane 1–2). The polyclonal antibody also recognize an additional band at about 47 kDa (Figure 3A, lanes 3–4, indicated by a star), and it was very likely resulted from the degradation or different precessing of EStag9 protein [48]. 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 65 kDa (Figure 2B, indicated by arrow), which was correspond to the expected molecular mass of the dengue virus E protein after it was processed and glycosylated [48].

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

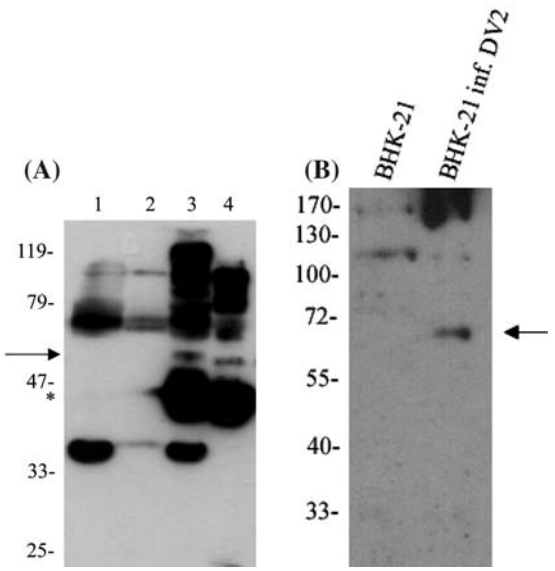

Figure 3. Detection of DV2E protein with anti-EStag9 polyclonal antibody. (A). Western blotting on EStag9 protein. Lane 1, *E. coli* lysates from cells transformed with pcDNA3 vector; Lane 2, the supernatant of *E. coli* lysates from cells transformed with pStag9; Lane 3, *E. coli* lysates from cells transformed with pStag9; and lane 4, proteins purified from *E. coli* cells transformed with pStag9. The arrow indicates the expected Estag9; * indicates the position of a band detected by the antibody. (B) Western blotting on DV2 PL046 strain. 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.

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 (Figure 4A, indicated by an arrow), whereas DV2E-EGFP was not precipitated by GST (Figure 4A, 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 Figure 4B, 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[49]. 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 Figure 4C, The Flag-Ubc9 could be precipitated down by DEΔ94-pta15 and detected by anti-Flag antibody (Figure 4C, indicated by an arrow), while the Flag-Ubc9 was not precipitated by the control

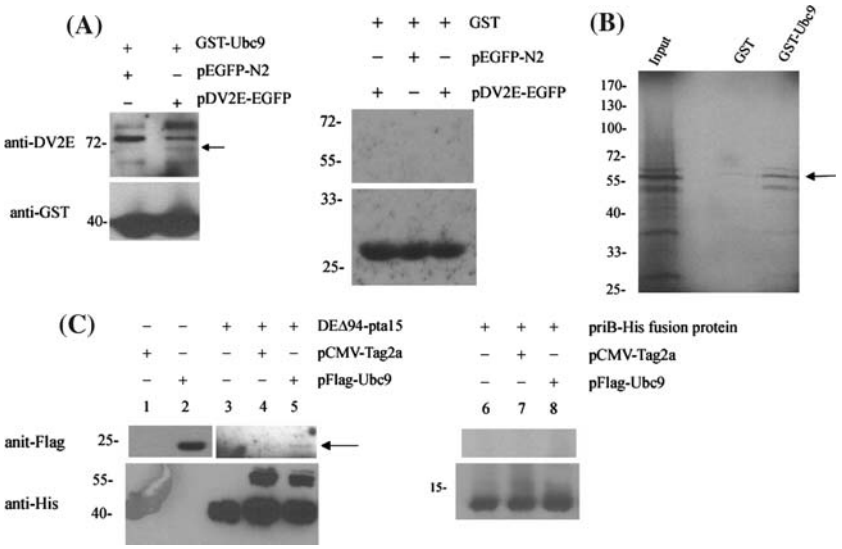

Figure 4. Ubc9 interacting with DV2E *in vitro*. (A). GST pull down assay. GST-Ubc9 were expressed in *E. coli BL21*(*DE3*) and purified by GST affinity. The purified proteins were immobilized on GST-Sepharose 4B and incubated with the cell lysate of 293 expressing pDV2E-EGFP or pEGFP-N2 vector. Bound 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; priB-His, the purified control His-tag fusion protein; pCMV-Tag2a, the lysate from cells transfected with the 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.

His fusion protein (Figure 4C, 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, we analyzed the sumoylation consensus motif of DV2E 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 2) among seven potential candidates. Next, according to the X-ray crystallography [50] 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 [50, 51]. 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 2.

To determine the effects of the mutated residues on DV2E, we 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 5, 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, we 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 6. 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 (Figure 6), which is consistent with the quantitative β-gal activity assay (Figure 5). 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 (Figure 6). 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, we transiently expressed the DV2E-EGFP and Flag-Ubc9 together in BHK-21

Table 2. The candidates of interaction sites between Ubc9 and DV2E.

Name of constructs	Mutation position (Amino acid)	Sumoylation consensus motif (ΨK*X*E)
pBTM-D2E- K51R	K51	LIETEA<u>K</u>QPATLRK
pBTM-D2E- K241R	K241	ETLVTF<u>K</u>NPHAKKQ
pBTM-K51R-K241R	K51 and K241	LIETEA<u>K</u>QPATLRK LKLNWF<u>K</u>KGSSIGQ

The mutation sites are indicates by underline.

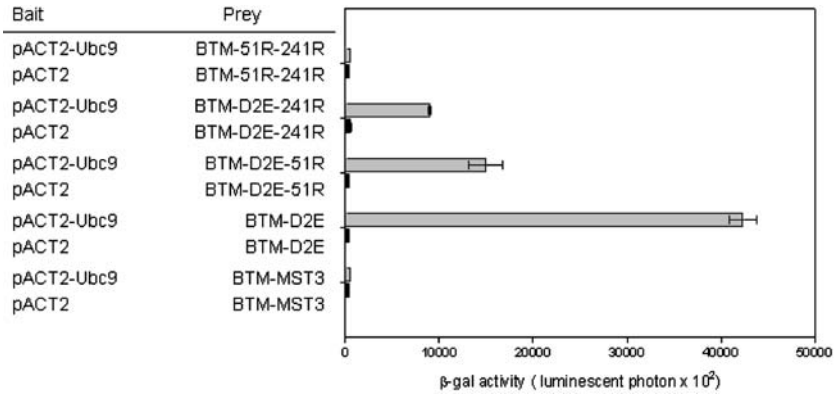

Figure 5. β-gal activity assay to quantify the interaction between DV2E and Ubc9 in yeast cells. *Saccharomyces cerevisiae L40* was cotransformed with constructs pBTM-D2E-K51R, pBTM-D2E-K241R, pBTM-K51R-K241R, pBTM-MST3 (negative control) separately with either pACT2-Ubc9 or pACT2 (control prey vector). The interactions between the encoded proteins are indicated by the level of β-gal activity.

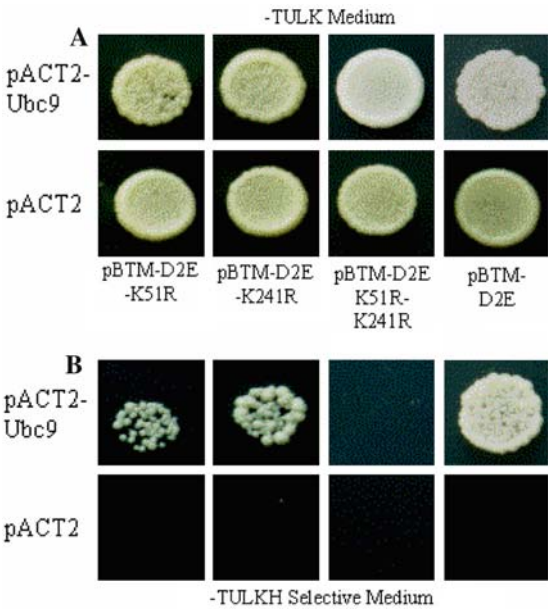

Figure 6. Double mutations on DV2E abolishing the interaction between the DV2E and Ubc9. *Saccharomyces cerevisiae L40* cells were co-transformed with the pBTM-based plasmids encoding the mutated or wild-type DV2E proteins as the "bait" and the pACT2-based plasmid encoding the Ubc9 protein as the "prey". 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.

cells for 24 and 48 h, and then examined the cells using confocal microscopy. As shown in Figure 7, the expression of DV2E-EGFP was present predominantly within cytoplasm (7-A, 7D; green fluorescence) 24 h and 48 h after the transfection. The Flag-Ubc9 distributed predominantly in the cytoplasm after 24 h (7-B; red fluorescence) has moved predominantly into nucleus after 48 h (7(A–E). Comparing Figure 7C and F shows that in the presence of pDV2E-EGFP, some Flag-Ubc9 proteins remained in the cytoplasm and co-localized around the nuclear membrane with the DV2E-EGFP protein 48 h after the transfection (the yellowish patches indicated by arrows in 7 F). 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 (Figure 7G–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 DV-2 virus, the pFlag-Ubc9 was transfected into BHK-21 cells cultured in 6-well plates. After incubation for 48 h, the transfected cells were infected with DV-2 virus strain PL046. Additional 48 h later, one well of the transfected cells were harvested to determine the expression of proteins (Figure 8B). As shown in Figure 8A, there is about 60% reduction in plaque formation

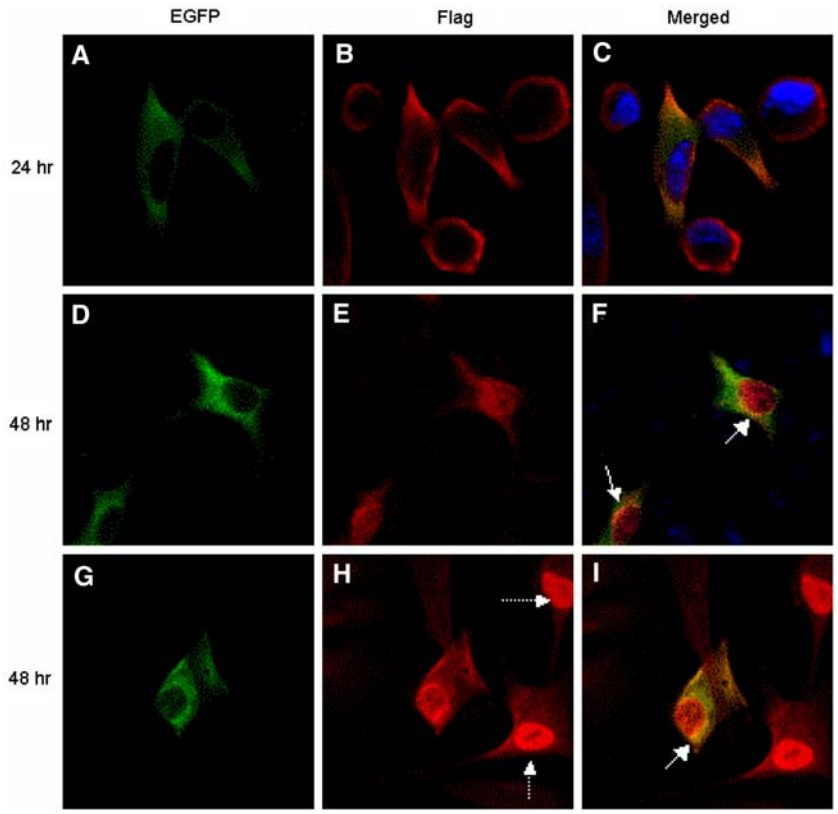

Figure 7. Subcellular localization of DV2E and Ubc9 by immunofluorescence. BHK-21 cells were co-transfected with pDV2E-EGFP and pFlag-Ubc9, and then were fixed at 24 and 48 h post-transfection. Flag-Ubc9 proteins were detected with primary antibody anti-FLAG M2, and labeled with secondary antibody anti-mouse 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 dotted arrows indicate the presence of nuclear foci (H).

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 [17]. In this study we have showed 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 mutation on E protein at the K51 and K241 separately could reduce the interaction with Ubc9 about 50∼80% (Figure 8), but when both mutations were introduced together, the interaction between DV2E and Ubc9 was lost completely (Figure 8). 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 [8, 29] or the stability of the proteins [52], as well as DNA replication and repair [53, 54]. The Ubc9 is known to mediate

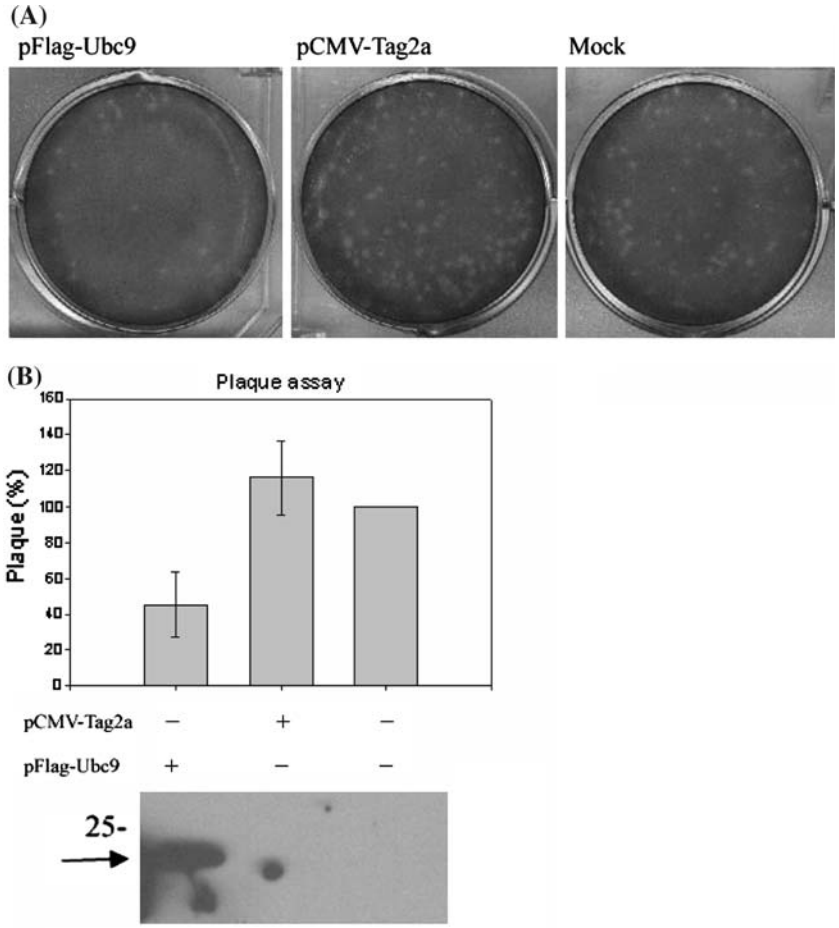

Figure 8. Determination of the Effect of Ubc9 over-expression on the propagation of dengue virus type 2 by plaque formation assay. BHK-21 cells were grown in 6-well plates and transfected with pFlag-Ubc9 or pCMV-Tag2a before the addition of dengue virus type 2 PL046. After absorption for 1 h at 37 °C, the media were aspired and the cells overlaid with growth medium/methyl cellulose. Then, the cultures were incubated at 37 °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.

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 [55–57]. Here we 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 (Figure 2). This is consistent with several reports revealing that Ubc9 can recognize the substrate protein directly [6, 18–21]. Ubc9 binding may play an important role in substrate recognition as well as in substrate modification [22]. There are both direct and indirect evidences that the presence of viruses can alter the sumoylation status of host cellular proteins [24, 26, 27, 29, 32–35, 58]. 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 mecha-

nisms or for promoting an intracellular state that is supportive of viral reproduction [14]. 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 [17]. On the other hand, it is also a possibility that modification of viral proteins by the sumoylation is part of a cellular antiviral mechanism [17], which is consistent with our data that expression of Ubc9 reduces the plaque formation (Figure 8). 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 [32], to enhance transcriptional activity [59, 60], 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 [23, 61, 62].

In mammalian cells, Ubc9 has been found in the nucleus but it is particularly prominent on the cytoplasmic side of the nuclear pore fibrils [13, 32, 63–65]. Therefore, it is thought that Ubc9 controls intracellular targeting of substrate proteins either directly through protein interactions or indirectly via sumoylation [13, 66]. 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 [35, 37, 38]. 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 [38]. Second, Weldon et al. [37] 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 [37]. 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 (Figure 7), and DV2E-EGFP also effected the distribution of pFlag-Ubc9 from predominantly in nucleus to near the nuclear membrane (Figure 7) at 48 h after transfection. Furthermore, the plaque assay has showed that over-expression of Ubc9 would interfere with the number of the plaque formed (Figure 8). 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, we 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.

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