Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 309 (2003) 672–678
BBRC
www.elsevier.com/locate/ybbrc

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

Mei-Wui Chiu and Yun-Liang Yang*

Department of Biological Science and Technology, National Chiao Tung University, Hsinchu, Taiwan, ROC

Received 5 August 2003

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 this study, we 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.

Keywords: Dengue virus; Envelope (E) protein expression; Functional assay; In vivo expression tag

Dengue viruses (DVs) are human pathogens that have reemerged as an increasingly important public health threat. They belong to the family of Flaviviridae and they can be divided into four serotypes, 1–4 [7]. Other family members include yellow fever virus, Japanese encephalitis virus, West Nile virus, and Murray Valley encephalitis virus [11]. Dengue Viruses are transmitted by *Aedes* mosquitoes and cause infections mostly in tropical and subtropical regions worldwide [7]. Patients infected by DVs show various clinical symptoms ranging from no significant illness, mild fever to life-threatening dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS) [6]. Currently, there is only supportive treatment for those patients. Other treatments such as immunization are being developed in the hope of providing a realistic approach for controlling dengue infection [8]. Unfortunately, there is still no effective commercial DV vaccine available in spite of years of effort to develop live attenuated, inactivated whole virion vaccines.

Flaviviruses have a single-stranded positive-sense RNA genome. The 11-kb genome encodes only one open-reading-frame translated into a polyprotein. After being processed by proteases of DV and/or host enzymes, the polyprotein then produces three structural proteins (capsid, membrane protein, and envelope (E) protein) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [3].

Among the structural proteins, the E protein is the envelope glycosylated protein and 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 [10]. 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 [2,14]. It is also the primary antigen that induces protective immunity and

* Corresponding author. Fax: +886-3-572-9288.
E-mail address: yyang@cc.nctu.edu.tw (Y.-L. Yang).

0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2003.08.053

hence the major antigen for virus neutralization. Therefore, the protein directly affects the host range, cellular tropism, and in part, the virulence of DVs [14].

Several approaches, based on *Escherichia coli*, vaccinia virus, *Pichia* yeast, and baculovirus expression systems [5,17,18,22], 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 [5,17,18,22]. In order to study the structure and function of the E protein and to provide a potentially economic antigen source for subunit vaccine development, we 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, we report the functional expression of a recombinant E protein of DV-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.

Materials and methods

Cell lines and viruses. The DV-2 strain PL046 is a gift from Dr. Wen Chang (IMB, Academia Sinica, Taipei). BHK-21 cells were cultured at 37 °C with 5% CO_2 in MEM (Gibco) supplemented with 0.22% of sodium bicarbonate and 10% of fetal bovine serum (FBS) (Gibco). C6/36 cells were grown at 28 °C in MEM (Gibco) supplemented with 0.11% of sodium bicarbonate and 10% of FBS.

Cloning of the truncated DV-2 E gene. The cDNA fragment containing the DV-2 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

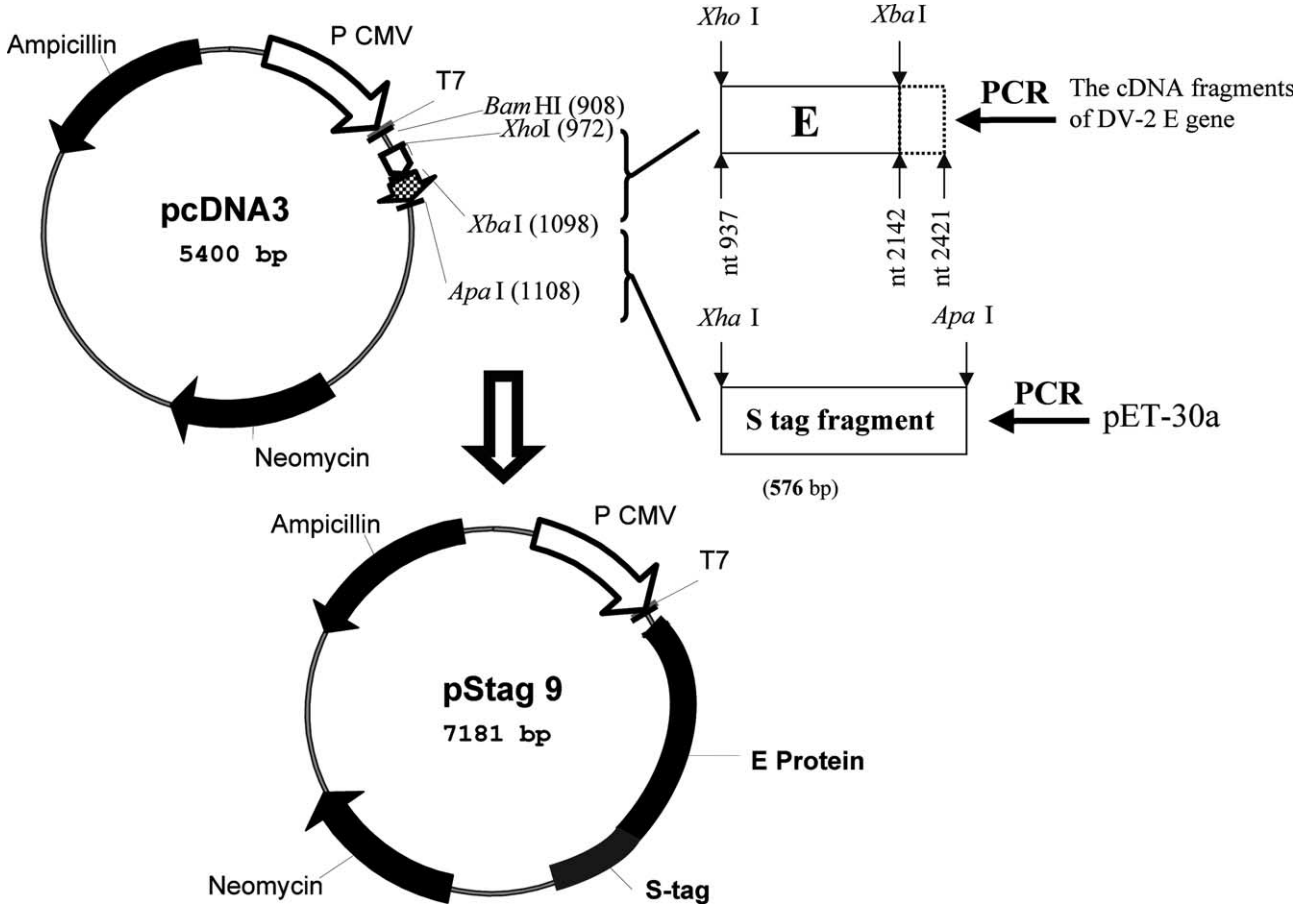

Fig. 1. Construction of pSatg9 plasmid: the DV-2 E gene sequence from nucleotide 937 to 2142 (full-length E gene is from 937 to 2421) was amplified by PCR and inserted to pcDNA3 expression vector with the addition of an *Xho*I site and ATG at the 5′ end and an *Xba*I site at the 3′ end. This truncated E sequence was then fused at the 3′, an S tag fragment amplified from the pET-30a and cloned into the *Xba*I and *Apa*I sites introduced by PCR onto the S tag fragment.

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′ AAATCTAGACTCAAGC ATTTGGCCGATAGA 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′ TTTGGGCCCACTACGTGAA CCATCACC 3′) contained *Apa*I site and the 5′ primer (5′ TTTTCTA GACTGGTGCCACGCGGTTCT 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 DV-2 E protein (nucleotides 937–2124 of DV-2 genome) with an in-frame S tag fused at C-terminus as shown in Fig. 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 °C until OD_{600} reached 0.4. Then, 0.4 mM of IPTG was added to induce the gene expression. After 3 h, the cells were harvested and centrifuged for 10 min at 5000 rpm. The pellets were resuspended with 1 mM Tris–HCl (pH 8.0) containing 1 mM PMSF and sonicated. The centrifuged cell lysates were re-suspended with inclusion-body solubilization buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl, 8 M urea, and 1 mM PMSF) before incubation for 1 h on ice. Removal of the urea was achieved by dialysis overnight in dialysis buffer (20 mM Tris–HCl, pH 7.0, 150 mM 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 (20 mM Tris–HCl, pH 7.0, 150 mM NaCl, 0.1% Triton X-100, and 3 M 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 analysis. 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, 10 mM Tris, pH 8.0 and 150 mM 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-DV-2 E domain III polyclonal antibody (provided by Dr. Wen Chang IMB, Academia Sinica, Taipei) or anti-S tag antibodies conjugated with HRP. The reaction was then incubated overnight at 4 °C. The membranes were then washed three times in TBST buffer (Tris-buffered saline, 10 mM Tris, pH 8.0, 150 mM 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 h. 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 4×10^5 cells per well in 6-well plates and incubated at 37 °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 5′ and 3′ 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. 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 DV-2 E gene of strain PL046 was subjected to sequence analysis. There are nine variations on the cDNA sequence in comparison to the DV-2 strain New Guinea-C (Accession No. M29095). As shown in Table 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 [13,15], 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. 2A) or by Western blot (Figs. 2B–D) using antibody against the S tag sequence or against domain III of E proteins (provided by Dr.

Table 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)

Nucleotide position	Nucleotide changes		Amino acid changes (M29095→PL046)
	M29095	PL046	
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

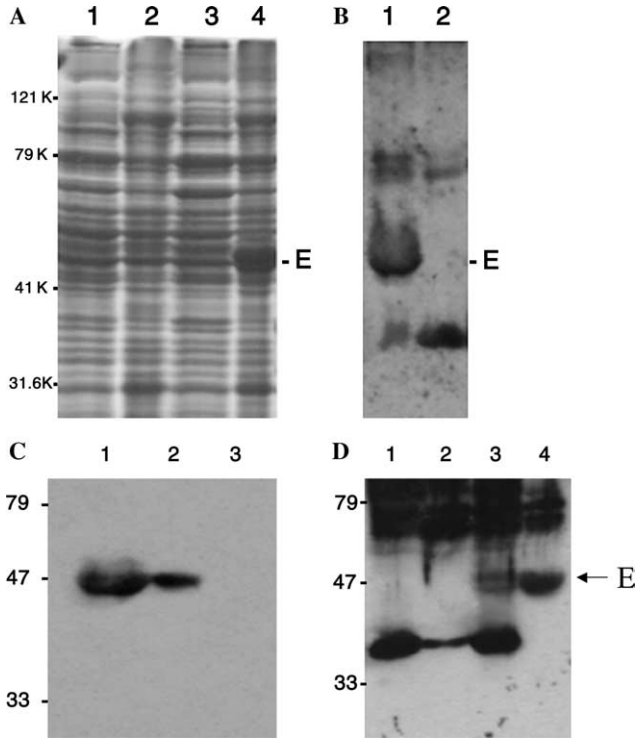

Fig. 2. Detection of recombinant EStag9 protein expression. *E. coli* cells were sonicated and followed by centrifugation before resolution by 12% SDS–PAGE. The detection of protein expression was by (A) SDS–PAGE and with Coomassie blue staining. Total proteins from supernatant (lanes 1 and 3) and pellet (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-DV-2 domain III antibody. 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-DV-2 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.

Wen Chang, IMB, Academia Sinica, Taipei). As shown in Fig. 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). 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 8 M 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 every 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 DV-2 PL046 strain. If the purified EStag9 can still bind host cells, it will compete with the DV-2 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. 3 and 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. 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 DV-2 virions for the infection of host cells. As shown in Fig. 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 g of EStag9 protein reduced the PFU down to less than 10% of that of wild-type, while the addition

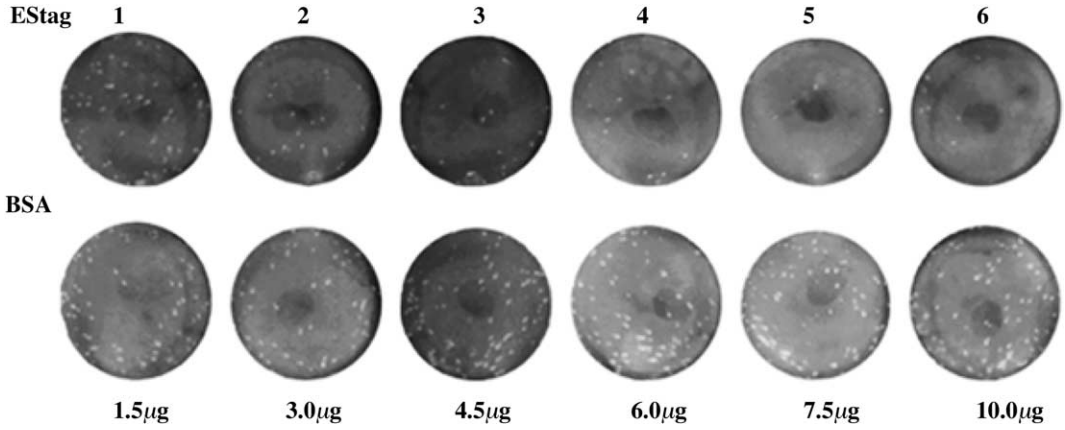

Fig. 3. Competitive blocking assay. Purified EStag9 proteins and BSA were serial diluted with medium without FBS and mixed with DV-2 virions before the addition to BHK-21 cells growing in 6-well plates. After absorption for 1 h at 37 °C with 5% CO_2, the media were aspired and the cells overlaid with agarose. After incubation for 5 days at 37 °C, 5% CO_2, cells were fixed and stained with crystal violet before the plaque numbers were scored.

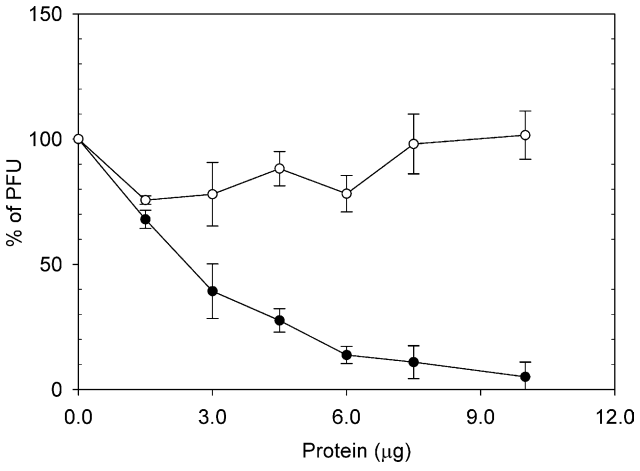

Fig. 4. Quantitative representation of dosage-dependent competitive blocking assay of viral infection by the purified recombinant EStag9 proteins (●) and BSA (○). Proteins were serial diluted with medium without FBS and mixture with DV-2 strain PL046.

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 DV-2 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 DV-2 E protein homodimer has been solved [13] and multiple lines of evidence indicated that the structure of E protein is conserved across the *Flaviviridae* [15]. 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 [15,16]. According to the model of Rey et al. [15], 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 [13]. 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 [1]. 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 [1]. 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 [21] and could strongly block virus adsorption [4]. This suggests that interactions between this region and target cells could mediate virus entry [20]. Mutations in this region may affect the entry and/or infection of the virus. Hence, it is not surprising that we found no variations in this area.

Previous reports have shown that DV E proteins expressed in *E. coli* form inclusion bodies [12,19], 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 [19]. This is consistent with our data that the expressed ES-tag9 proteins were presented in the precipitated fraction of cell lysate (Fig. 2). It is a possibility that over-expression of the E protein in *E. coli* facilitates the formation of inclusion body. Here, we have overcome this issue by denaturing the cell lysates containing protein inclusion bodies in 8 M urea and then we refolded the proteins by dialysis to remove urea.

We 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 [9,17,18]. By using competitive assay on viral infections, we directly tested the relevant biological function of the purified EStag9 proteins. We 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, we 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.

Acknowledgments

We are grateful to Dr. Y.-L. Lin for the gift of plasmid containing the DV-2 E gene fragment, Dr. W. Chang for the antibody against E protein domain III and DV-2 virus. This work is supported by National Genomic Medicine Project: grant number AS91IMB7PP.

References

[1] S.L. Allison, J. Schalich, K. Stiasny, C.W. Mandl, F.X. Heinz, Mutational evidence for an internal fusion peptide in flavivirus envelope protein E, J. Virol. 75 (2001) 4268–4275.
[2] T.J. Chambers, C.S. Hahn, R. Galler, C.M. Rice, Flavivirus genome organization, expression, and replication, Annu. Rev. Microbiol. 44 (1990) 649–688.
[3] G.J. Chang, Molecular biology of dengue viruses, in: D.J. Gubler, G. Kuno (Eds.), Dengue and Dengue Hemorrhagic Fever, CAB International, New York, NY, 1997, pp. 175–198.
[4] W.D. Crill, J.T. Roehrig, Monoclonal antibodies that bind to domain III of dengue virus E glycoprotein are the most efficient blockers of virus adsorption to Vero cells, J. Virol. 75 (2001) 7769–7773.
[5] C. Delenda, I. Staropoli, M.P. Frenkiel, L. Cabanie, V. Deubel, Analysis of C-terminally truncated dengue 2 and dengue 3 virus envelope glycoproteins: processing in insect cells and immunogenic properties in mice, J. Gen. Virol. 75 (1994) 1569–1578.
[6] R. George, L.C.S. Lum, Clinical spectrum of dengue infection, in: D.J. Gubler, G. Kuno (Eds.), Dengue and Dengue Hemorrhagic Fever, CAB International, New York, NY, 1997, pp. 23–44.
[7] D.J. Gubler, Dengue and dengue hemorrhagic fever: its history and resurgence as a global public health problem, in: D.J. Gubler (Ed.), Dengue and Dengue Hemorrhagic Fever, CAB International, New York, NY, 1997, pp. 1–22.
[8] R.O. Jimenez, B.A.L. da Fonseca, Recombinant plasmid expressing a truncated dengue-2 virus E protein without co-expression of prM protein induces partial protection in mice, Vaccine 19 (2001) 648–654.
[9] E.P. Kelly, J.J. Greene, A.D. King, B.L. Innis, Purified dengue 2 virus envelope glycoprotein aggregates produced by baculovirus are immunogenic in mice, Vaccine 18 (2000) 2549–2559.
[10] E. Lee, M. Lobigs, Substitutions at the putative receptor-binding site of an encephalitic flavivirus alter virulence and host cell tropism and reveal a role for glycosaminoglycans in entry, J. Virol. 74 (2000) 8867–8875.
[11] P. Leyssen, E. De Clercq, J. Neyts, Perspectives for the treatment of infections with Flaviviridae, Clin. Microbiol. Rev. 13 (2000) 67–82, table.
[12] P.W. Mason, M.U. Zugel, A.R. Semproni, M.J. Fournier, T.L. Mason, The antigenic structure of dengue type 1 virus envelope and NS1 proteins expressed in *Escherichia coli*, J. Gen. Virol. 71 (1990) 2107–2114.
[13] Y. Modis, S. Ogata, D. Clements, S.C. Harrison, A ligand-binding pocket in the dengue virus envelope glycoprotein, Proc. Natl. Acad. Sci. USA 100 (2003) 6986–6991.
[14] T.P. Monath, F.X. Henize, Flaviviruses, in: B.N. Fields, D.M. Knipe, P.M. Howley (Eds.), Fields Virology, vol. 1, third ed., Lipponcott-Raven Publishers, Philadelphia, PA, 1996, pp. 961–1034.
[15] F.A. Rey, F.X. Heinz, C. Mandl, C. Kunz, S.C. Harrison, The envelope glycoprotein from tick-borne encephalitis virus at 2 Å resolution, Nature 375 (1995) 291–298.
[16] J.T. Roehrig, R.A. Bolin, R.G. Kelly, Monoclonal antibody mapping of the envelope glycoprotein of the dengue 2 virus, Jamaica, Virology 246 (1998) 317–328.

[17] I. Staropoli, J.M. Clement, M.P. Frenkiel, M. Hofnung, V. Deubel, Dengue virus envelope glycoprotein can be secreted from insect cells as a fusion with the maltose-binding protein, J. Virol. Methods 56 (1996) 179–189.

[18] I. Staropoli, M.P. Frenkiel, F. Megret, V. Deubel, Affinity-purified dengue-2 virus envelope glycoprotein induces neutralizing antibodies and protective immunity in mice, Vaccine 15 (1997) 1946–1954.

[19] R.J. Sugrue, T. Cui, Q. Xu, J. Fu, Y.C. Chan, The production of recombinant dengue virus E protein using *Escherichia coli* and *Pichia pastoris*, J. Virol. Methods 69 (1997) 159–169.

[20] P. Thullier, C. Demangel, H. Bedouelle, F. Megret, A. Jouan, V. Deubel, et al., Mapping of a dengue virus neutralizing epitope critical for the infectivity of all serotypes: insight into the neutralization mechanism, J. Gen. Virol. 82 (2001) 1885–1892.

[21] P. Thullier, P. Lafaye, F. Megret, V. Deubel, A. Jouan, J.C. Mazie, A recombinant Fab neutralizes dengue virus in vitro, J. Biotechnol. 69 (1999) 183–190.

[22] H.Y. Wei, L.F. Jiang, Y.H. Xue, D.Y. Fang, H.Y. Guo, Secreted expression of dengue virus type 2 full-length envelope glycoprotein in *Pichia pastoris*, J. Virol. Methods 109 (2003) 17–23.