Inhibition of West Nile virus entry by using a recombinant domain III from the envelope glycoprotein

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The envelope glycoprotein located at the outermost surface of the flavivirus particle mediates entry of virus into host cells. In this study, the involvement of domain III of West Nile virus (WNV-DIII) envelope protein in binding to host cell surface was investigated. WNV-DIII was first expressed as a recombinant protein and purified after a solubilization and refolding procedure. The refolded WNV-DIII protein displays a content of β-sheets consistent with known homologous structures of other flavivirus envelope DIII, shown by using circular dichroism analysis. Purified recombinant WNV-DIII protein was able to inhibit WNV entry into Vero cells and C6/36 mosquito cells. Recombinant WNV-DIII only partially blocked the entry of dengue-2 (Den 2) virus into Vero cells. However, entry of Den 2 virus into C6/36 was blocked effectively by recombinant WNV-DIII. Murine polyclonal serum produced against recombinant WNV-DIII protein inhibited infection with WNV and to a much lesser extent with Den 2 virus, as demonstrated by plaque neutralization assays. Together these results provided strong evidence that immunoglobulin-like DIII of WNV envelope protein is responsible for binding to receptor on the surface of host cells. The data also suggest that similar attachment molecule(s) or receptor(s) were used by WNV and Den 2 virus for entry into C6/36 mosquito cells.

INTRODUCTION

West Nile virus (WNV), a single-stranded positive sense RNA envelope virus, was first isolated and identified in the West Nile region of Uganda in 1937 from a febrile female adult (Smithburn et al., 1954). It has been classified as a member of the family Flaviviridae using cross-neutralization tests with polyclonal antisera (Boctor et al., 1989). Being neuroinvasive (George et al., 1984), severe human meningoencephalitis might occur as seen clearly in the outbreaks in North America (CDC, 1999, 2002). During 1999–2002, the virus extended its range throughout much of the eastern parts of the USA, and its range within the western hemisphere is expected to continue to expand. Birds are the natural reservoir hosts, and WNV is maintained in nature in a mosquito-bird-mosquito transmission cycle primarily involving Culex species mosquitoes.

The WNV, like all flavivirus, is made up of three structural proteins, the large envelope protein (E), a single nucleocapsid protein (C) and a lipid membrane protein (M).

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et al., 2003). Each E protein monomer folds into three structural domains predominantly composed of β-strands. Domain I is centrally located in the structure and carries the N-glycosylation site. Structural domain II of the E protein promotes dimerization and bears the fusion loop that inserts into the target host membrane during the pH-dependent fusion of the virus (Modis et al., 2004; Bressanelli et al., 2004). Located at the C-terminal end of the molecule, domain III, which was named domain B in earlier antigenic mapping studies, carries several epitopes able to elicit virus-neutralizing antibodies (Roehrig, 2003). In addition, studies with several flaviviruses including TBE (Mandl et al., 2001) have also suggested that domain III, which has a fold typical of an immunoglobulin constant domain, could also mediate flavivirus attachment to host cells (Anderson, 2003).

Since the C-terminal domain can fold independently into a stable conformation, it is therefore an attractive target to block infectivity either through the design of molecules that would compete with the whole virus to enter cells or by eliciting neutralizing antibodies. In addition, it should also be useful to help identify receptors and co-receptors located at the cell surface. In the present study, the putative receptor binding WNV-DIII was cloned, expressed, refolded and purified. The recombinant protein was subjected to circular dichroism (CD) spectroscopy to estimate its secondary structure, and the antigenic and antagonist properties were studied.

**METHODS**

**Cells and viruses.** Vero cells (ATCC) were maintained in Medium 199 (M199; Gibco) containing 10% inactivated fetal calf serum (FCS) while C6/36 cells (ATCC) were grown in L15 (Gibco) containing 10% FCS. Flaviviruses, West Nile virus [Sarafend, WNV(S)] and Den 2 (New Guinea) (gifts from E. G. Westaway, Sir Albert Sakzewski Virus Research Laboratory, Queensland, Australia) were propagated in Vero cells throughout this study.

**Cloning and expression of recombinant WNV-DIII protein.** The sequence corresponding to aa 299–401 of the C-terminal domain III of the WNV(S) envelope protein was amplified by PCR using Advantage II polymerase enzyme (Clontech) and the following set of primers: forward primer, 5'-CATATGGTGTATGCTC-3' and reverse primer, 5'-ACCAGGATCC-TTACCCAGATTTGTGCCAGTG G-3'. The XhoI and BamHI restriction sites are underlined. The PCR product of 440 bp was digested with the restriction enzymes XhoI and BamHI and ligated into the BamHII/XhoI sites of the pET16b vector (Novagen). The nucleotide sequence was confirmed by using DNA sequence analysis. *E. coli* cells [strain BL21(DE3)] with the pET16b vector containing the inserted fragment were grown until OD600 of the culture reached 0-6, and protein expression was induced by adding IPTG to a final concentration of 0.5 mM at 30°C for 4-6 h. Cells were harvested by centrifugation at 5000 g for 20 min at 4°C and washed twice with a buffer containing 1% NP40, 20 μg ml⁻¹ DNase and 1 mM PMSF. Cell lysis was performed in a French pressure cell at 1200 p.s.i. (8280 kPa), followed by centrifugation at 48000 g for 30 min at 4°C (Beckman). The recombinant protein was expressed in the insoluble fraction as inclusion bodies as shown by SDS-PAGE.

**Purification of recombinant WNV-DIII protein.** Inclusion bodies were solubilized in 8 M urea and passed through a Ni-NTA agarose column (Qiagen) for affinity purification. The protein was eluted at pH 2-4. The pH was then adjusted to 7-9 and the protein diluted to a concentration of 5–10 μg ml⁻¹. Refolding was carried out through extensive dialysis at 4°C against a buffer containing 50 mM Tris/HCl, 1 mM EDTA, 20% glycerol, 100 mM NaCl and 3 mM DTT at pH 8-0. After concentration by ultracentrifugation using a molecular mass cut-off of 5 kDa (Amicon), the protein was purified by size exclusion chromatography (Superdex 75; Amersham) in a buffer containing 12 mM Tris/HCl, 200 mM NaCl and 5 mM DTT at pH 8-0. Western blot analysis was performed using an anti-penta-histidine antibody (Qiagen). In order to confirm the identity of the protein cleavage of the His tag was carried out using Factor Xa (Qiagen) following the manufacturer’s recommendations, and sequencing of the first 10 aa from the cleaved product were obtained using Edman degradation on an Applied Biosystems sequencer.

**Analysis by CD.** The CD spectra were recorded on a Jasco J810 spectro-polarimeter by using three accumulations of data at 0-1 nm intervals and were smoothed using the noise reduction routines provided with the instrument, including solvent background subtraction. The buffer used was 12 mM Tris/HCl, 10 mM sodium phosphate, 2 mM DTT at pH 8-0 and the protein concentration was 0.1 mg ml⁻¹. Deconvolution of the CD far UV-spectrum was carried out with the CDNN and CONTIN software (Sreearama & Woody, 2000). Mass spectrometry analysis using a matrix-assisted laser desorption ionization time of flight mass spectrometer (MALDI-TOF) was used to determine the molecular mass of the sample.

**Generation of murine polyclonal antibodies against soluble WNV-DIII protein.** Soluble WNV-DIII protein was incubated with ImmunEasy mouse adjuvant (Qiagen) at a concentration recommended by the manufacturer. The antigen–adjuvant mixture was used to immunize BALB/c mice five times subcutaneously at 14-day intervals. Mouse sera were collected 12 days after the last booster. Mouse sera were purified using Econo-Pac serum IgG purification kits (Bio-Rad) and dialysed overnight with PBS. The purified immunoglobulins were stored at −20°C. Sera were tested by Western blot detection for the presence and specificity of antibodies against the WNV-DIII protein as described previously in Chu & Ng (2002).

**Inhibition of WNV infection by soluble WNV-DIII protein.** Soluble WNV-DIII protein or BSA (in the concentration range of 5–100 μg ml⁻¹) was incubated with either Vero or C6/36 cells (1 × 10⁶ cells) in cell culture medium at 4°C for 1 h. Unbound protein molecules were removed by washing the cells three times with PBS. This was followed by incubating with 1 × 10⁶ p.f.u. ml⁻¹ of 35S-methionine labelled WNV or Den 2 virus for 1 h at 37°C. The ratio of WNV-DIII molecules to WNV/Den 2 virus particles is 5:1. After the incubation period, excess or unbound virus were inactivated with acid citrate buffer, pH 2-8 (Chu & Ng, 2003) and removed by washing three times with PBS. The cells were then lysed with 1% SDS and the specific radioactivity was determined. Three independent experiments were carried out.

**Paque neutralization assay.** Purified murine polyclonal antibodies generated against soluble WNV-DIII protein or pre-immune sera (control) were evaluated using plaque reduction assay to determine the neutralization ability of these antibodies on WNV or Den 2 virus infectivity. Fifty microlitres of antibodies (500 μg ml⁻¹) in twofold serial dilutions (from 1:2 to 1:8192) were prepared in microcentrifuge tubes. The WNV or Den 2 virus was adjusted to 500 p.f.u. in 50 μl of virus diluent (10% concentrated Hanks’ balanced salt solution, 0.1% BSA; pH 7.2–7.4) and added to the tube containing serial diluted antibodies. The antibody and virus was mixed, pulse centrifuged and then incubated at 37°C for 1 h.
24-well plate with confluent monolayer of Vero cells was used for virus infection. Before inoculation of the antibody–virus mixture, the cell monolayer was rinsed once with virus diluent, after which 100 µl of the antibody–virus mixture was added to the appropriate wells. The plates were left at 37°C for 1 h, and rocked at 15 min intervals. After incubation, the inocula were removed and the cell monolayer was rinsed once with virus diluent. Overlay medium (2% carboxymethyl cellulose in M199 containing 2% FCS) was added and incubated further at 37°C for 4 days, and virus plaques were stained with 0.5% crystal violet. Three independent experiments were carried out.

RESULTS

Purification and characterization of the solubilized recombinant WNV-DIII protein

The products from the various purification steps were analysed in a 15% SDS-PAGE (Fig. 1). The total protein profile of E. coli BL21 was shown in lane 1. Lane 2 shows the total protein profile after induction of protein expression with IPTG (the arrow shows the expressed DIII protein). The supernatant from the inclusion bodies (lane 3) after solubilization showed the presence of the WNV-DIII protein (arrow). The protein sample was refolded (lane 4, arrow) and purified (lane 5, arrow).

The homogeneity of WNV-DIII protein was assessed using MALDI-TOF and showed the presence of a single peak with the expected molecular mass of 13.7 kDa (data not shown). The protein could be concentrated up to about 15 mg ml⁻¹ in Tris/HCl buffer (pH 7.4). At higher concentration, the protein started to form aggregates, which are consistent with the presence of several additional exposed hydrophobic residues. Purification by size-exclusion chromatography demonstrated that the protein eluted as a monomer.

The amino acid sequences of the recombinant WNV-DIII protein [WNV(S) and WNV (NY)] were aligned with the sequences of other flaviviruses namely: Yellow fever virus [(vaccine strain 17D) (17D YF)], TBE virus, Den 2 virus, Japanese encephalitis virus (JE), Kunjin (Kun) virus, Murray Valley encephalitis (MVE) virus and Langat virus (Lgt) (Fig. 2). Secondary structure elements (ß-strands 1–7) are represented above the sequences as reported by Rey et al. (1995). The single conserved disulphide bond is indicated by a dotted line. The RGD/RGD integrin-binding motif present in YF, JE and MVE viruses is located in the loop between ß6 and ß7 strands of DIII. The known antigenic regions 307, 330–332 and 390 are indicated in bold characters.

The CD spectrum of the recombinant protein revealed a secondary structure predominantly composed of anti-parallel ß-sheets as shown by the presence of a large positive maximum at approximately 200 nm and a shallow negative peak at 217 nm (Fig. 3). This pattern which is found in immunoglobulin structures (Tetin et al., 2003) allows for an accurate determination of their secondary structure content. Deconvolution of the CD far UV-spectrum using several publicly available programs (Sreerama & Woody, 2000) reveals the presence of about 45% of ß-sheets in the protein. This result is consistent with a content of 45.3% of residues in a ß-strand conformation as observed in the crystallographic 3D structure of the C-terminal domain of TBE virus (residues 301–395) (Rey et al., 1995; PDB code 1svb). This is slightly higher than the 35% (residues 301–401) reported for the closely related recombinant domain III protein of JE virus determined by NMR (Wu et al., 2003; PDB code 1pwj). The effect of increasing concentrations of urea on the CD spectrum of the recombinant WNV-DIII protein showed that the protein is completely devoid of regular secondary structures at a concentration of 2 M urea.

Competitive inhibition of WNV entry with soluble recombinant WNV-DIII protein

By using Western blotting, the expressed soluble recombinant WNV-DIII was detected by monoclonal antibodies against the WNV envelope protein as well as anti-His antibodies (not shown), hence indicating the antigenicity of the expressed protein. Since WNV-DIII has been proposed to be the receptor-binding domain, the ability of recombinant WNV-DIII binding to the cell and blocking the entry of WNV infection was investigated. Vero cells were first incubated with a range of concentrations (5–100 µg ml⁻¹) of soluble recombinant WNV-DIII protein or BSA (to rule out any possibility of steric hindrance that block virus binding) for 1 h at 4°C. Radiolabelled WNV or Den 2 virus was added to the pre-treated cells and quantified for

Fig. 1. Analyses of expressed and purified WNV-DIII using 15% SDS-PAGE. M, Molecular mass markers (New England Biolabs). Lanes: 1, total protein from E. coli BL 21; 2, total proteins after induction of protein expression with IPTG; 3, supernatant from inclusion bodies solubilized in urea; 4, sample after the refolding step and 5, purified fraction from the gel filtration column. The arrow indicates the WNV-DIII protein.
virus entry (Fig. 4). Fig. 4(a) shows that pre-treatment of Vero cells at a concentration of 100 \(\mu\)g ml\(^{-1}\) of soluble WNV-DIII protein resulted in more than 60% inhibition in WNV entry. The inhibition was dose-dependent, decreasing to 20% when the Vero cells were pre-treated with low concentration (5 \(\mu\)g ml\(^{-1}\)) of WNV-DIII protein. Although the inhibition of WNV entry was not complete, it was still very significant when compared to cells that were pre-treated with BSA, at high concentration of 100 \(\mu\)g ml\(^{-1}\).

There was a baseline of 5–10% non-specific inhibition of WNV entry. The solubilized WNV-DIII protein was only able to partially inhibit Den 2 virus entry (30% inhibition) into the pre-treated Vero cells. Again the inhibition was dose-dependent, decreasing to about 1% inhibition in cells that were pre-treated with WNV-DIII protein concentrations ranging from 5 to 10 \(\mu\)g ml\(^{-1}\).

Fig. 2. Alignment of the amino acid sequences of domain III of flaviviruses E protein. Amino acid numbering refers to WNV. Evolutionary conserved residues are shaded. The disulphide bond between Cys305 and Cys336 is indicated by a dotted line. Secondary structure elements are labelled from \(\beta\)1 to \(\beta\)7. The three known antigenic regions located around residues 307, 330–332 and 390, which are likely to be involved in receptor binding, are indicated in bold characters.

Fig. 3. CD spectrum of recombinant WNV-DIII protein. A large maximum at 200 nm and a shallow minimum at approximately 217 nm which are indicative of the presence of anti-parallel \(\beta\)-strands in the structure are visible.
Similar experimental procedures were carried out to determine if WNV-DIII protein was responsible for binding to the cell surface of mosquito cells, C6/36. Entry of WNV can be effectively blocked by the recombinant WNV-DIII (>70%, Fig. 4b) and, interestingly, the entry of Den 2 virus into C6/36 cells was also significantly inhibited (>60%, Fig. 4b). Again, BSA has minimal effect in blocking the entry of WNV and Den 2 virus. Studies are also currently being carried out to determine the ability of recombinant Den 2 virus E DIII protein in blocking the binding of WNV to vertebrate and mosquito cells. These will provide further information as to whether similar attachment or receptor molecules are utilized by WNV and Den 2 virus in different cell types.

To rule out the presence of other bacterial proteins that may co-purify with WNV-DIII protein and participate in blocking virus attachment, supernatant of bacterial cell lysate not expressing WNV-DIII (purified in the same manner as for recombinant WNV-DIII protein) was used to assess virus binding. The E. coli cell lysate inhibited WNV and Den 2 virus binding to Vero and C6/36 cells at a low level of not more than 2%. This was less than that observed using BSA as a competitor (approx. 5%) (data not shown). Therefore, these results confirm that WNV-DIII protein is responsible for binding to the cell surface of both vertebrate and invertebrate cells and the plausibility of WNV and Den 2 virus sharing the same attachment/receptor molecule(s) in C6/36 cells.

Murine polyclonal antibodies to recombinant WNV-DIII protein neutralized WNV

To affirm further that WNV-DIII protein is indeed the receptor-binding domain, murine polyclonal antibodies against recombinant WNV-DIII were produced and used for plaque neutralization assay of WNV. The murine polyclonal was specific in detecting WNV-DIII protein in a Western blot (data not shown). Plaque neutralization assays were then carried out for both WNV and Den 2 virus with a dilution series of antibodies against WNV-DIII protein. At dilution of 1:64, there was at least 90% neutralization of the WNV (Fig. 5a). High neutralization (80%) of the WNV was maintained until the murine polyclonal antibodies were diluted to 1:256. At higher dilutions, there was an exponential decrease in the degree of neutralization reaching 0% when the dilution factor was 1:8192. When the WNV was reacted with the pre-immunized sera, no neutralization effect was observed. In contrast, Den 2 virus was only partially inhibited (50%) at low dilution (<1:64) of the antibodies and minimal neutralization effect was observed with higher dilution of the antibodies (>1:256) (Fig. 5b).

DISCUSSION

Among the three structural domains of the flavivirus major envelope glycoprotein E, DIII forms a continuous polypeptide segment that can fold independently (Bhardwaj et al.,
It was earlier identified as a stable tryptic fragment of about 10 kDa, which required formation of a native intramolecular disulfide bond to correctly present B-cell epitopes (Roehrig et al., 1998). Due to the absence of the interacting central domain I, the recombinant truncated protein is poorly soluble, presumably because of the exposure of additional hydrophobic residues which are buried in the context of the native homodimer. To overcome solubility problems, several investigators have expressed the DIII of flaviviruses as a recombinant protein fused either with thioredoxin as for JE virus (Wu et al., 2003) or with GST for Langat virus (Bhardwaj et al., 2001).

In the protocol used in this study, the truncated protein was obtained within inclusion bodies and a refolding procedure was performed. Despite the relative high yield and ease of purification, the procedure introduced an uncertainty about the exact proportion of protein molecules, which have the native fold. Indeed, some proteins associated as dimers or trimers via the formation of non-native inter-monomer disulfide bonds. Most of these misfolded proteins were eliminated during the size-exclusion chromatography step. Proper folding of the expressed recombinant WNV-DIII protein is of paramount importance since it has been shown that a reduced and denatured WNV E glycoprotein was unable to elicit neutralizing antibodies in mice (Wengler & Wengler, 1989). The secondary structure composition of the recombinant WNV-DIII protein derived from CD indicates an anti-parallel β-strands conformation consistent with the experimentally determined structures of other flaviviruses envelope protein ectodomains (Rey et al., 1995; Modis et al., 2003) or DIII fragments (Wu et al., 2003; Volk et al., 2004).

The sites of mutations that affect host range or virulence have been mapped on the major flavivirus envelope protein (Rey et al., 1995). They cluster at the hinge junction between domain I and III next to the fusion loop, which is buried in the dimer interface and in domain III. In the virus structure (Kuhn et al., 2002; Mukhopadhyay et al., 2003) the lateral face of domain III is largely exposed. In particular, residues located around positions 305–308, 330–333 and 384–386 are clustered on the upper surface of WNV-DIII, which is exposed to the solvent and hence accessible to antibodies. Residue 307 is a lysine in WNV-DIII protein and also in TBE virus but is acidic in other flaviviruses (JE virus and MVE virus). Position 307 is a hot spot for mutation enabling the generation of flavivirus escape mutants (Beasley & Barrett, 2002; Chambers et al., 1998).

Residues Ser331 and Asp332 [corresponding respectively to Lys332 and Asp333 in WNV(S)] belong to a neutralizing epitope on JE virus (Lin & Wu, 2003). Thus, a number of these residues which belong to virus-neutralizing epitopes (Beasley & Barrett, 2002; Lin & Wu, 2003; Wu et al., 2003; Volk et al., 2004) account for the antigenic fine structure of the flavivirus E protein, and are likely to be involved in determining the binding specificity for different cell types (Hung et al., 2004). In this study, a polyclonal anti-WNV-DIII protein serum was produced in mice. The serum was able to neutralize WNV (Fig. 5a). This suggested that the conformational epitopes on the purified refolded protein were correctly presented at the external surface and the WNV-DIII recombinant protein adopts a native fold.

At the surface of the virion, DIII cluster around fivefold

![Fig. 5. Plaque neutralization of WNV with murine polyclonal antibodies against WNV-DIII protein. The polyclonal antibodies against WNV-DIII protein are diluted in a twofold series. Equal volume (50 μl) of anti-WNV-DIII antibodies and (a) WNV or (b) Den 2 virus (500 p.f.u.) were incubated for 1 h before this mixture is overlaid onto Vero cells monolayer. Plaques were stained with 0.5% crystal violet. Virus diluent was used as a negative control instead of anti-WNV-DIII antibodies. (a) Greater than 90% of the WNV is neutralized when the antiserum used is at a dilution of <1:64. The neutralization capability is maintained at 80% with the antiserum dilution up to 1:256. The neutralization percentages decrease exponentially after that to 0% at the antiserum dilution of 1:8192. (b) Partial neutralization of Den 2 virus with polyclonal anti-WNV-DIII is only observed at low dilutions. Up to 40% neutralization is observed with dilutions of 1:16. The neutralization capability is reduced to less than 10% with dilution of 1:256 or higher.](image)
icosahedral axis (Kuhn et al., 2002; Mukhopadhyay et al., 2003) and such pentameric clusters might be a more efficient way to elicit neutralizing antibodies that would possibly interfere with receptor binding. It is interesting to note that the Langat virus DIII protein crystallizes with five monomers per asymmetric unit thus possibly mimicking the arrangement found on the viral surface (White et al., 2003).

Domain III was initially postulated to form the receptor-binding site for the virus particles because of its exposed location at the surface of the virus (Rey et al., 1995; Kuhn et al., 2002; Mukhopadhyay et al., 2003). In addition, the presence of a tripeptide Arg-Gly-Asp motif in the loop connecting strands $\beta 6$ and $\beta 7$ of several mosquito-borne flaviviruses E protein (e.g. MVE virus and several strains of JE virus) (Fig. 2) suggested that this motif could bind to the integrin family of cell-surface matrix receptors. This hypothesis, however, was not supported by mutagenesis studies of the YF virus (Van der Most et al., 1999) and cellular receptors are still not unequivocally identified for flaviviruses. Indeed a range of different surface molecules could act as flavivirus receptors on different cell types (Anderson, 2003). The fact that the recombinant WNV-DIII protein was able to substantially inhibit infection with the Den 2 virus in C6/36 cells (Fig. 4b) indicated that the attachment/receptor molecules situated at the surface of the mosquito cells may be common for both WNV and Den 2 virus. Work is in progress to address this interesting issue.

However, the lack of high antagonistic effect of WNV-DIII on Den 2 virus infection (Fig. 4a) in Vero cells as compared to WNV, may suggest that the two viruses utilized a different set of attachment or receptor molecules for binding to Vero cells. This was consistent with the observation that the murine polyclonal antibodies against WNV-DIII also failed to exhibit high neutralization effect on Den 2 virus (Fig. 5b).

Glycosaminoglycans such as heparan sulfate and chondroitin sulfate (negatively charged carbohydrates) present on the surface of many vertebrate cells were proposed to be involved in the attachment and entry of Den 2 virus (Chen et al., 1997). However, treatment with heparinase on vertebrate cells (Chu & Ng, 2003) did not significantly inhibit infection with WNV. Instead, a 105 kDa protease-sensitive, N-linked glycoprotein has been implicated to mediate attachment and entry of WNV into permissive vertebrate cells (Chu & Ng, 2003). Other studies also suggested that heparan sulfate may not necessary serve as the universal flavivirus attachment or receptor molecule on cell surfaces (Bielefeldt-Ohmann et al., 2001; Kroschewski et al., 2003).

The entry process of WNV into both vertebrate and invertebrate is poorly understood and this process is important in explaining tissue tropism and pathogenesis of WNV infection. To our knowledge, this study has defined direct evidence, for the first time, that DIII of the WNV envelope protein is involved in binding to cell surface molecules for both Vero and C6/36 cells. However, we cannot exclude any possibility that other domains of the envelope protein may also be necessary for post-binding process of WNV entry into host cells. Future work is also necessary to elucidate the molecular mechanism of WNV entry mechanism. Nevertheless, the identification of the receptor-binding domain of WNV envelope protein can serve as a potential target for the design of anti-viral agent development to eradicate this emerging flavivirus infection.

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had died of encephalitis. 


