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Analysis of self-association of West Nile virus capsid protein and the crucial role played by Trp 69 in homodimerization

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Abstract

The understanding of capsid (C) protein interactions with itself would provide important data on how the core is organized in flaviviruses during assembly. In this study, West Nile (WN) virus C protein was shown to form homodimers using yeast two-hybrid analysis in conjunction with mammalian two-hybrid and in vivo co-immunoprecipitation assays. To delineate the region on the C protein which mediates C–C dimerization, truncation studies were carried out. The results obtained clearly showed that the internal hydrophobic segment flanked by helix I and helix III of WN virus C protein is essential for the self-association of C protein. The crucial role played by Trp 69 in stabilizing the self-association of C protein was also demonstrated by mutating Trp to Gly/Arg/Phe. Substitution of the Trp residue with Gly/Arg abolished the dimerization, whereas substitution with Phe decreased the self-association significantly. The results of this study pinpoint a critical residue in the C protein that potentially plays a role in stabilizing the homotypic interaction.

Keywords: Capsid; Tryptophan; Homotypic interaction; Yeast two-hybrid; Mammalian two-hybrid; Co-immunoprecipitation

The family *Flaviviridae* comprises of more than 70 closely related RNA viruses and is one of the most medically important groups of emerging arthropod-borne viruses that includes West Nile (WN), dengue (DEN), yellow fever (YF), and tick-borne encephalitis (TBE) viruses [1–3]. Flaviviruses are small, enveloped animal viruses containing a single positive-strand genomic RNA [4]. The positive-stranded RNA genome, enclosed within the nucleocapsid, is approximately 11 kb in length. The genome encodes a single large polyprotein, which is processed by viral and host proteases to three structural proteins; the capsid (C), membrane (M), and envelope (E) as well as seven non-structural (NS) proteins. The order of these proteins on the polyprotein is 5′ C-prM-E-NS1-NS2a-NS2b-NS3-NS4a-NS4b-NS5 3′ [4,5].

The C protein is distinctly made up of four α helices and comprises of an internal hydrophobic domain that is flanked by two stretches of positively charged amino acids. Such arrangement is highly conserved among the flaviviruses, which is suggestive that the hydrophobic and positively charged clusters may play important roles in virus capsid assembly and RNA recruitment. In DEN-4 virus, the conserved internal hydrophobic domain was shown to assist in the anchoring of the capsid to the cellular ER membrane [6]. Although large deletions in the internal hydrophobic domain of TBE impaired the formation of infectious virus particles, it was still capable of secreting significant amounts of subviral particles [7]. Impact of the removal of the entire hydrophobic domain can also be limited by mutating the remaining amino acids to increase the overall

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hydrophobicity, which demonstrated the importance of the internal hydrophobic cluster [8].

Secondary structures in C proteins of YF and DEN viruses were recently characterized by nuclear magnetic resonance spectroscopy (NMR) [9,10] to reveal the dimeric nature of flavivirus C protein. Crystal structures of Kunjin virus (subtype of WN virus) C proteins in both dimeric and tetrameric forms were also determined [11] but the study on the functional domains on the WN virus C protein is limited. The mechanism involved in the nucleocapsid formation and encapsulation is still poorly understood. Thus, the molecular and biochemical characterization of the C protein and its interaction with itself and other viral proteins could be very helpful in suggesting how the core is organized in this important group of viruses.

In this study, the yeast two-hybrid in conjunction with mammalian two-hybrid and in vivo co-immunoprecipitation techniques were employed to delineate the region on the C protein, which mediates C–C dimerization. We have also shown the importance of Trp 69 in mediating/stabilizing the dimerization of C protein.

Materials and methods

Cells and viruses. Vero cells (African green monkey kidney) used throughout all experiments were grown in medium 199 (M199) containing 10% heat-inactivated foetal calf serum (FCS). All cell cultures were carried out in a humidified 5% CO_2 incubator at 37 °C. WN [(Sarafend) WN(S)] virus, a kind gift from E.G. Westaway (Sir Albert Sakzewski Virus Research Laboratory, Queensland, Australia), was used for the extraction of the viral RNA.

Plasmid construction. The cDNA coding sequences of WN(S) virus C were amplified by PCR using Advantage 2 polymerase mix (Clontech). The primers used for amplification and cloning of WN(S) virus genes are listed in Tables 1A and B. The PCR products were digested with *Eco*RI and *Bam*HI or *Eco*RI and *Xho*I, gel purified by Qiagen kit, and ligated with T4 ligase (Promega) into the yeast expression vectors pGBKT7 and pGADT7 or the mammalian expression vectors pCMV-Myc and pCMV-HA (Clontech), respectively. These vectors had previously been digested with *Eco*RI and *Bam*HI or *Eco*RI and *Xho*I. The ligation mixture was transformed into *E. coli* DH5α cells, which were subsequently grown under either kanamycin or ampicillin selection. Plasmid DNA was extracted from selected transformants and screened using restriction digestion and sequencing.

To mutate the amino acid Trp 69 of the C protein, primer based site-directed mutagenesis was carried out using the primers listed in Table 1C and ligated into pCMV-HA vector. We chose Gly to check whether the smaller size of the residue has an effect on the self-association of the C protein. Trp to Arg mutation was performed to illustrate whether hydrophilicity or positive charges affect the dimerization. Trp to Phe substitution was carried out to mark the importance of hydrophobicity at the dimer interface.

Yeast two-hybrid analysis. Yeast two-hybrid experiments were performed as described in the Clontech Manual for the MATCHMAKER GAL4 Two-Hybrid System and in the Clontech Yeast Protocols Handbook (Clontech, USA). The yeast strain *S. cerevisiae* AH109 (Clontech) was transformed by using the lithium acetate method with pGBKT7 (*TRP1*) and pGADT7 (*LEU2*) constructs carrying C proteins, in every possible pairwise combination. Control plasmids used were pGBKT7-Lamin C, pGBKT7-53, pGADT7-T, and empty vectors [(pGBKT7 and pGADT7) (Clontech)]. This resulted in 7 pairwise transformations that were plated onto SD-Trp/-Leu/-His medium (Triple dropout = TDO). About 18 colonies from each transformation were subsequently plated onto SD-Trp/-Leu/-His/-Ade medium (Quadruple dropout = QDO, high stringency plates) to assess the transcriptional activation of the *ADE2* reporter gene. All the experiments were performed in triplicate. In order to eliminate false positives, colonies from QDO plates were assayed for *lacZ* reporter gene activity on nitrocellulose filters using X-gal substrate in a β-galactosidase (β-gal) assay. The pCL1 was used as a positive control in β-gal assay. Each enzyme activity assay was performed with at least 12 independent colonies. Protein expression was confirmed using Western immunoblot analysis probed with either anti-HA or anti-c-Myc antibodies (Clontech).

Mammalian two-hybrid assay. Mammalian two-hybrid (M2H) assays were performed using Invitrogen's Mammalian Two-Hybrid kit with TOPO tools technology. The bait genes were amplified by PCR primers (Tables 1D and E) according to TOPO tools protocol which was subsequently joined to the pSV40-GAL4 5′ element and SV40 pA 3′ element to create a linear expression construct for the bait protein of interest. Similarly, a linear expression construct for the prey proteins was constructed using the pSV40-VP16 5′ element and the SV40 pA 3′ element. Co-transfection was performed using the DNA linear constructs generated from above along with the reporter plasmid; pGAL/*lacZ* using the Lipofectamine reagent (Invitrogen). Appropriate controls were included in this assay (Fig. 1A). The experiments were performed at least eight to twelve times for each transfection. The pSV40-GAL4 5′ element carries a Gal4 DNA binding domain (BD), while the pSV40-VP16 5′ element carries a VP16 DNA activation domain (AD). In the case of an interaction between the two proteins, the BD and AD domains form a transcriptional activation complex and activate the *lacZ* reporter gene on the reporter plasmid pGAL/*lacZ*. Reporter activity was measured by β-gal assay.

β-Gal assay (M2H). The β-gal assay was carried out using β-Gal Assay Kit (Invitrogen) following manufacturer's protocol. In brief, 48 h post-transfection, cell monolayers were harvested by scraping cells into phosphate-buffered saline (PBS) and centrifuged at 250*g* for 7 min. After discarding the supernatant, the pellet was resuspended in lysis buffer. The sample was then freeze–thawed for four times. The supernatant (cell lysate) was separated from the insoluble material by centrifugation at 15,000*g* for 5 min. Cleavage buffer with β-mercaptoethanol and ortho-nitrophenyl-β-D-galactopyranoside (ONPG) was added to the cell lysate and mixed. Stop buffer was added after incubating the sample at 37 °C for 5–15 min. Absorbance at 420 nm against a blank containing ONPG and cleavage buffer without the lysate was measured using a spectrophotometer. The specific activity of β-gal activity was calculated as described in the kit.

In vivo co-immunoprecipitation and Western blotting. To reaffirm the results observed from yeast two-hybrid and mammalian two-hybrid assays, another independent assay, co-immunoprecipitation was carried out. Vero cells were co-transfected with the plasmids expressing epitope (Myc/HA) tagged C/truncated C proteins using the Iipofectamine reagent (Invitrogen). At 48 h post-transfection, cells were washed with PBS and lysed using cold mild lysis buffer. Cell lysate was then mixed with either anti-HA or anti-c-Myc magnetic microbeads for 30 min on ice and purified using a μMACs separator column (Miltenyi Biotec, Germany). The column was further rinsed thrice with wash buffer and eluted with pre-heated elution buffer. For Western blotting, samples obtained from co-immunoprecipitation assays or co-transfected cell lysates were electrophoresed in a 10% sodium dodecyl sulphate–polyacrylamide gels (SDS–PAGE) and transferred onto polyvinylidene difluoride membranes. The membrane was then incubated with anti-HA or anti-c-Myc antibodies (3:10,000; Clontech) followed by goat anti-rabbit or goat anti-mouse IgG conjugated with alkaline phosphatase (1:1000; Chemicon) and the immunoreactive bands were visualized by development with 5-bromo-4-chloro-3-indolylphosphate (BCIP) and *p*-nitroblue tetrazolium (NBT) substrates (Chemicon).

Table 1
Primers used for cloning of WN(S) virus genes

Primer	Nucleotide sequence (5′–3′)	Polypeptide
(A) Yeast two-hybrid analysis		
Y2HCF (F)	CGTT**GAATTC**ATGTCTAAGAAACCAGGA	FC, C3′ △18, C3′ △39, C3′ △54, and C3′ △63
Y2HC5′ △15 (F)	CGTT**GAATTC**ATGCTAAAACGCGGTATGC	C5′ △15
Y2HC5′ △20 (F)	CGTT**GAATTC**ATGCCCCGCGGATTGTCCT	C5′ △20
Y2HC5′ △25 (F)	CGTT**GAATTC**TCCTTGATTGGACTGAAGA	C5′ △25
Y2HC5′ △30 (F)	CGTT**GAATTC**AAGAGGGCTATGCTGAGTCTG	C5′ △30
Y2HC5′ △45 (F)	CGTT**GAATTC**TTCGTGTTGGCTCTTCTGG	C5′ △45
Y2HC3′ △18 (R)	CG**GGATCC**TTATCTTTTCTTTTGTTTTGTGC	C3′ △18
Y2HC3′ △39 (R)	CG**GGATCC**TTAGAAACTCAAGAGATGCTTC	C3′ △39
Y2HC3′ △54 (R)	CG**GGATCC**TTACCATCTGTCCAGCACCGCACG	C3′ △54
Y2HC3′ △63 (R)	CG**GGATCC**TTAAGCAATTGCAGTGAATCT	C3′ △63
YTCREV (R)	CG**GGATCC**TTAAGCTCCAGCACAGGCGA	FC, C5′ △15, C5′ △20, C5′ △25, C5′ △30, C5′ △45
(B) In vivo co-immunoprecipitation		
HACF (F)	CCG**GAATTC**GGATGTCTAAGAAACCAGGA	HA-C, Myc-C, HA-C3′ △18, HA-C3′ △39, HA-C3′ △54, HA-C3′ △63
HAC5′ △15 (F)	CCG**GAATTC**GGATGCTAAAACGCGGTATGC	HA-C5′ △15
HAC5′ △20 (F)	CCG**GAATTC**GGATGCCCCGCGGATTGTCCT	HA-C5′ △20
HAC5′ △25 (F)	CCG**GAATTC**GGTCCTTGATTGGACTGAAGA	HA-C5′ △25
HAC5′ △30 (F)	CCG**GAATTC**GGAAGAGGGCTATGCTGAGT	HA-C5′ △30
HAC5′ △40 (F	CCG**GAATTC**AAGGGCCCAATACGTTTC	HA-C5′ △40
HAC5′ △45 (F)	CCG**GAATTC**GGTTCGTGTTGGCTCTTCTG	HA-C5′ △45
HAC3′ △18 (R)	CCG**CTCGAG**TTATCTTTTCTTTTGTTTTGTGC	HA-C3′ △18
HAC3′ △39 (R)	CCG**CTCGAG**TTAGAAACTCAAGAGATGCTTC	HA-C3′ △39
HAC3′ △54 (R)	CCG**CTCGAG**TTACCATCTGTCCAGCACCGCACG	HA-C3′ △54, HA-C40–69
HAC3′ △63 (R)	CCG**CTCGAG**TTAAGCAATTGCAGTGAATCT	HA-C3′ △63
HACREV (R)	CCG**CTCGAG**TTAAGCTCCAGCACAGGCGAT	HA-C, Myc-C, HA-C5′ △ 15, HA-C5′ △20, HA-C5′ △25, HA-C5′ △30, HA-C5′ △45
(C) In vivo co-immunoprecipitation-mutagenesis primers		
CO-IPCF (F)	CCG**GAATTC**GGTCCTTGATTGGACTGAAGAGG	HAC26–69, Myc-C26–69, HAC26–68, HACphe, HACgly, HACarg
CO-IPCR69 (R)	CCG**CTCGAG**TTACCATCTGTCCAGCACCGCA	HAC26–69
CO-IPCR68 (R)	CCG**CTCGAG**TTATCTGTCCAGCACCGCACG	HAC26–68
CO-IPCRGly (R)	CCG**CTCGAG**TTA<u>CCC</u>TCTGTCCAGCACCGCA	HACgly
CO-IPCRArg (R)	CCG**CTCGAG**TTA<u>CCG</u>TCTGTCCAGCACCGCA	HACarg
CO-IPCRPhe (R)	CCG**CTCGAG**TTA<u>AAA</u>TCTGTCCAGCACCGCA	HACphe
(D) Mammalian two-hybrid analysis		
M2HCF (F)	CGGAAC**AAGGG**ATGTCTAAGAAACCAGGA	FC, C3′ △18, C3′ △39, C3′ △54, and C3′ △63
M2HC5′ △15 (F)	CGGAAC**AAGGG**ATGCTAAAACGCGGTATGC	C5′ △15
M2HC5′ △20 (F)	CGGAAC**AAGGG**ATGCCCCGCGGATTGTCCT	C5′ △20
M2HC5′ △25 (F)	CGGAAC**AAGGG**TCCTTGATTGGACTGAAGA	C5′ △25
M2HC5′ △30 (F)	CGGAAC**AAGGG**AAGAGGGCTATGCTGAGTCTG	C5′ △30
M2HC5′ △45 (F)	CGGAAC**AAGGG**TTCGTGTTGGCTCTTCTGG	C5′ △45
M2HC3′ △18 (R)	TGAGTC**AAGGG**TCTTTTCTTTTGTTTTGTGC	C3′ △18
M2HC3′ △39 (R)	TGAGTC**AAGGG**GAAACTCAAGAGATGCTTC	C3′ △39
M2HC3′ △54 (R)	TGAGTC**AAGGG**CCATCTGTCCAGCACCGCACG	C3′ △54
M2HC3′ △63 (R)	TGAGTC**AAGGG**GCAATTGCAGTGAATCT	C3′ △63
M2HCR (R)	TGAGTC**AAGGG**GCTCCAGCACAGGCGA	FC, C5′ △15, C5′ △20, C5′ △25, C5′ △30, C5′ △45
(E) Mammalian two-hybrid analysis-mutagenesis primers		
M2HCF26 (F)	CGGAAC**AAGGG**TCCTTGATTGGACTGAAGA	C26–69, C26–68, Cphe, Cgly, Carg
M2HCR69 (R)	TGAGTC**AAGGG**CCATCTGTCCAGCACCGCA	C26–69
M2HCR68 (R)	TGAGTC**AAGGG**TCTGTCCAGCACCGCACG	C26–68
M2HCRGly (R)	TGAGTC**AAGGG**<u>CCC</u>TCTGTCCAGCACCGCA	Cgly
M2HCRArg (R)	TGAGTC**AAGGG**<u>CCG</u>TCTGTCCAGCACCGCA	Carg
M2HCRPhe (R)	TGAGTC**AAGGG**<u>AAA</u>TCTGTCCAGCACCGCA	Cphe

(F) denotes forward primer and (R) denotes reverse primer. Restriction site in each primer used for yeast two-hybrid and co-immunoprecipitation studies is in bold. The sequence complementary to the topoisomerase I recognition site is shown bold in the primers used in the mammalian two-hybrid analysis. Mutations were introduced in the primers which are underlined.

Results

Interactions between WN virus capsid proteins

Yeast two-hybrid analysis

In our first approach, the C protein of WN virus was cloned into the yeast two-hybrid vectors resulting in an N-terminal in-frame fusion of the GAL4-DNA binding domain and the GAL4 activation domain to the C protein. Capsid-encoding pGADT7 was co-transformed into *Saccharomyces cerevisiae* AH109 strain with pGBKT7 plasmids expressing C proteins. AH109 strain contains four reporter genes; *HIS3*, *ADE2*, *LacZ*, and *MEL1*, and each comes under different distinct GAL4-responsive promoter elements. Co-transformants that grew on TDO plates were scored as potential positive interactors, confirmed by restreaking on QDO plates, and tested for β-gal production (first row in Table 2). To eliminate autoactivation, all the pGBKT7 fusion plasmids were co-transformed with empty pGADT7 vector. Likewise, specificity of interaction was tested by co-transforming C-encoding pGADT7 with pGBKT7 containing human lamin C or empty pGBKT7 vector. No intrinsic or non-specific activation for all the fusions was observed (first row in Table 2).

This confirmed that the positive results obtained by yeast two-hybrid assay are specific for WN virus capsid proteins. Positive interaction control (PIC) used was a co-transformation of pGBKT7 with murine p53 sequences and pGADT7 containing SV40 large T-antigen. The PIC transformants showed obvious growth on TDO or QDO within 3 or 4 days of incubation and generated strong β-galactosidase signals. Strong homotypic interaction for C protein was detected within 3 days (first row in Table 2).

Mammalian two-hybrid assay

As some post-translational processing may be absent in yeast, mammalian two-hybrid system was used to corroborate the findings in yeast two-hybrid assay. The C protein constructs were generated as described under Materials and methods. Both linear constructs (bait and prey) and the reporter plasmid, pGAL, which en-

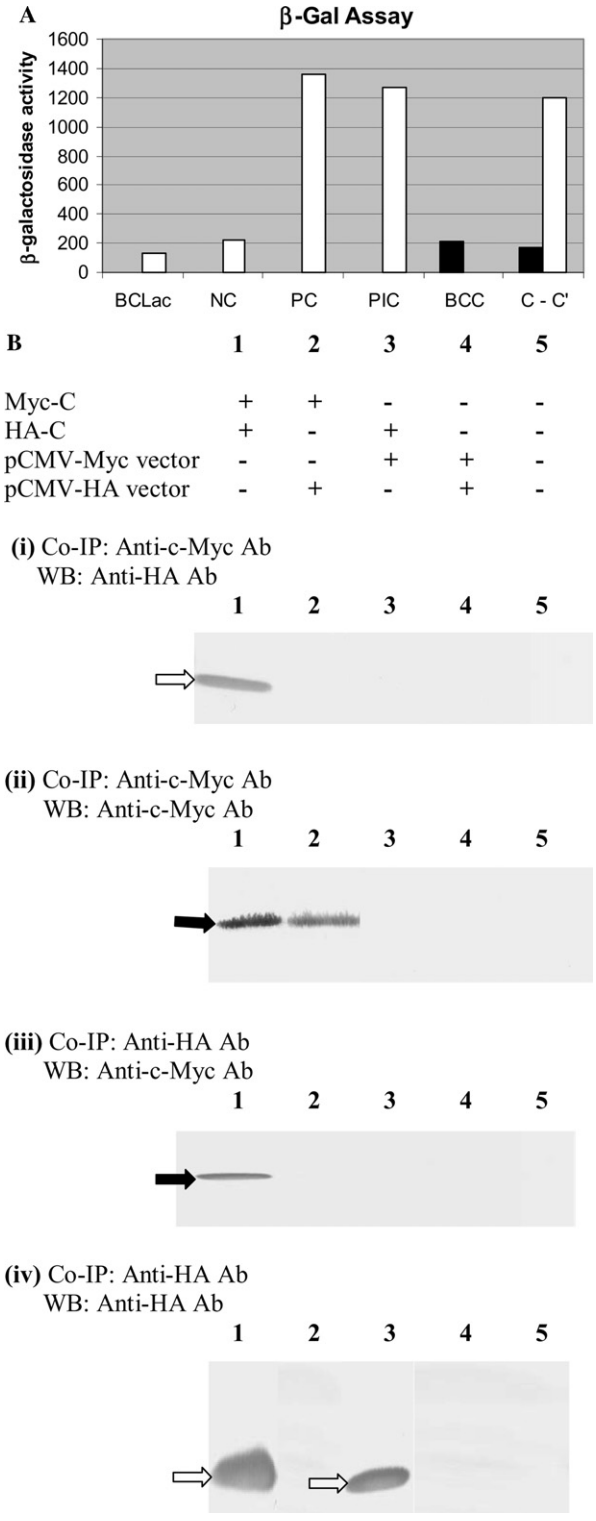

Fig. 1. Interaction between the C proteins of WN virus. (A) β-Gal assay (M2H). BC_{Lac} and NC are negative controls. PC and PIC are positive controls. BC_C represents the background control for the C protein (bait). The black bar represents the background controls to check if the C protein functions as a transcriptional activator in the absence of the interacting partner. C–C′ proteins represent the interacting partners. Strong interaction signal was observed for C–C′ pair. (B) In vivo co-immunoprecipitation. Vero cells were co-transfected with (i) Myc-C and HA-C; (ii) Myc-C and pCMV-HA vector; (iii) pCMV-c-Myc vector and HA-C; (iv) pCMV-c-Myc vector and pCMV-HA vector; and (v) mock-transfected cells. Homotypic association of C protein was detected by immunoprecipitation with anti-c-Myc Ab followed by Western blotting of the precipitated immune complexes using anti-HA Ab (i). Anti-c-Myc Ab precipitated proteins were also reacted with anti-c-Myc Ab (ii) to ensure successful precipitation of the Myc-C. Similarly, immunoprecipitation was carried out with anti-HA Ab followed by probing the blot with anti-c-Myc Ab (iii). Anti-HA Ab precipitated proteins were also reacted with anti-HA Ab (iv) to ensure successful precipitation of the HA-tagged C protein. The results showed the C–C protein self-association. Open arrows denote HA-C (i and iv) and solid arrows indicate Myc-C proteins (ii and iii).

Table 2
Interactions between the full length C and C/truncated C proteins of WN virus in the yeast two-hybrid system, assayed for β-galactosidase activity and HIS autotrophy

AD fusion	BD fusion			
	C	Lamin-C	No insert	53
C	**+++** (+++)	**−** (−)	**−** (−)	**ND** (ND)
C5′ △15	**+++** (+++)	**−** (−)	**−** (−)	**ND** (ND)
C5′ △20	**++** (+)	**−** (−)	**−** (−)	**ND**(ND)
C5′ △25	**++** (++)	**−** (−)	**−** (−)	**ND** (ND)
C5′ △30	**−** (−)	**−** (−)	**−** (−)	**ND** (ND)
C5′ △45	**−** (−)	**−** (−)	**−** (−)	**ND** (ND)
C3′ △18	**+++** (++)	**−** (−)	**−** (−)	**ND** (ND)
C3′ △39	**++** (++)	**−** (−)	**−** (−)	**ND** (ND)
C3′ △54	**++** (+)	**−** (−)	**−** (−)	**ND** (ND)
C3′ △63	**−** (−)	**−** (−)	**−** (−)	**ND** (ND)
No insert	**−** (−)	**−** (−)	**−** (−)	**ND** (ND)
SV40 T	**ND** (ND)	**ND** (ND)	**ND** (ND)	**+++** (+++)
Positive control (pCL1)		**+++**		

The results obtained from the β-galactosidase assay are shown in bold. The relative strength of the interaction was judged by the intensity of the blue phenotype after 8 h when the positive control showed deep-blue colonies. +++, deep-blue colonies (strong interaction); ++, light-blue colonies (weak interaction); −, white colonies (no interaction); and ND, not determined. All results shown are representative of at least 12 independent transformants. The results obtained from the HIS autotrophy assay are shown in brackets. Growth was recorded after 2 days when the positive control showed clear growth. +++, clear growth (strong interaction); ++, moderate growth (moderate interaction); +, slow growth (weak interaction), −, no growth (no interaction); and ND, not determined. All results shown are representative of at least 12 independent transformants.

coded for *LacZ* gene, were co-transfected into Vero cells. To ensure that neither the bait nor prey constructs functioned as transcriptional activators, they were individually co-transfected with only the reporter plasmid to serve as background controls. Cells transfected with only the reporter plasmid served as background control for *LacZ* activity (BC_{Lac}) in Vero cell line. To verify induction of the reporter gene, positive control plasmid (PC), pCR 2.1/Gal4 containing VP16, was co-transfected with the reporter gene. The pCR 2.1/LgT Prey control and pCR 2.1/p53 Bait control plasmids served as positive interaction control (PIC) while pCR 2.1/VP16-CP plasmid and pCR 2.1/p53 bait control served as the negative control (NC). As depicted in Fig. 1A, high level of β-gal activity was detected only for full-length C–C′ transfection.

In vivo co-immunoprecipitation

To ensure that the interaction of the structural proteins was not limited by the requirement of nuclear translocation in two-hybrid systems, the C genes were cloned in mammalian expression vectors pCMV-Myc and pCMV-HA. Vero cells were then co-transfected with plasmids expressing Myc-tagged C (Myc-C) and HA-tagged C (HA-C) proteins. Cell lysates from the co-transfected cells were first immunoprecipitated with anti-c-Myc monoclonal antibody (mAb) followed by Western blotting using anti-HA polyclonal Ab. Lane 1 in Fig. 1B(i) was loaded with a sample co-transfected with Myc-C and HA-C and confirmed that the HA-C protein was precipitated with the anti-c-Myc Ab. Co-transfection with empty vector (pCMV-Myc or pCMV-HA) and each of the plasmids (Myc-C and HA-C) was carried out to check for non-specific interaction brought about by the epitope tags (lanes 2 and 3). Lysates from mock-transfected cells as well as from cells transfected with both vectors (pCMV-Myc and pCMV-HA) served as negative controls (lanes 4 and 5). To ensure the successful precipitation of the Myc-C proteins, the anti-c-Myc Ab precipitated immune complexes were also probed with anti-c-Myc antibody [Fig. 1B(ii)]. To verify the specificity of the co-immunoprecipitation performed using anti-c-Myc antibodies, reciprocal co-immunoprecipitations were performed using the anti-HA antibodies followed by Western blotting using either anti-c-Myc Ab [Fig. 1B(iii)] or anti-HA Ab [Fig. 1B(iv)]. These results demonstrated that the interacting proteins were specifically co-immunoprecipitated with both the anti-HA and anti-c-Myc antibodies, and confirmed that the C proteins do interact with each other.

Truncation studies to map the homotypic association domain of WN virus C protein

Yeast two-hybrid analysis

To further the understanding on how the capsid monomers self-interact, the constructs of C protein mutants containing varying lengths of deletions from the amino (N) and carboxy (C) termini were constructed. Three truncated mutants from the N terminus, which carried terminal 15, 30, and 45 amino acid deletions, were generated in conjunction with 2 mutants carrying 18 and 39 amino acid deletion from the C terminus as depicted in Fig. 2. All the truncated mutants were subcloned into a pGADT7 vector and the fusion plasmids were co-transformed with pGBKT7 encoding full-length

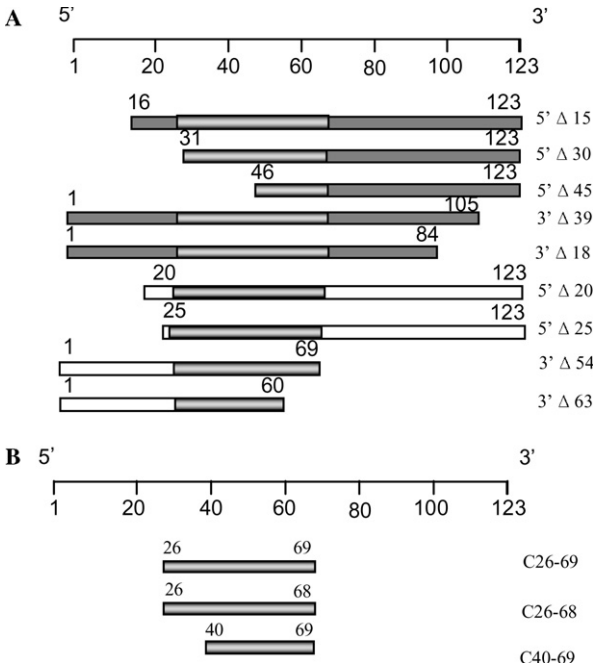

Fig. 2. Deletion mapping of the regions in C protein responsible for homologous interaction. (A) Deletion mapping of the regions in WN virus C protein responsible for homologous interaction. The highlighted region represents the homotypic interaction domain of C protein. (B) Truncated mutants containing the homotypic interaction domain alone (C26–69) and Trp 69 (C26–68) and helix I (C40–69) deleted mutants.

C protein to test for their interactions. Interaction between the full-length capsid (FC) with C5′ △15, C3′ △18, and C3′ △39 was detected in high stringency QDO plates and showed significant β-gal activity (Table 2). No positive interaction was observed in co-transformation of full length C (FC) and C5′ △30 or C5′ △45. Initial truncation study indicated that the first 15 amino acids from the N-terminal of the C protein and the last 39 amino acids from the C-terminal were not required for C–C homodimerization. Further truncation of 30 amino acids and 45 amino acids from the N-terminal abolished C–C interaction. These results suggested the domain responsible for homomeric association of capsid lies between the amino acids 16 and 84.

In order to delineate the actual domain that is responsible for C–C homotypic interaction, four additional mutants were constructed. Two of the mutants carried 20 (C5′ △20) and 25 amino acid (C5′ △25) deletion from the N terminal and the other two mutants carried 54 (C3′ △54) or 63 amino acid (C3′ △63) deletion from its C terminal (Fig. 2). When these four constructs were co-transformed with FC, interactions were detected for all the pairings, except the combination of FC and C3′ △63. To ensure that there was no non-specific transactivation of the reporter genes, all the constructs were concurrently assayed for reporter gene activation by transforming singly or with the control pGBKT7-Lamin or empty pGBKT7 plasmids. As depicted in Table 2, no intrinsic or non-specific activation for all the fusions was observed. This narrowed the homointeracting domain for C down to the amino acids 26–69 of the capsid polypeptide.

Likewise, similar truncated C constructs (Fig. 2) were created for mammalian two-hybrid assay and in vivo co-immunoprecipitation. As shown in Fig. 3A (β-gal assay-M2H) and Fig. 3B (in vivo co-immunoprecipitation), the results obtained from these two assays corroborated with the results from yeast two-hybrid analysis. All these observations confirmed that the domain most critical for homotypic interaction of the C protein is located at amino acids 26–69.

Homotypic interaction ability of amino acids 26–69 of WN virus C protein and the role of helix I in stabilizing the self-association

To demonstrate further the homotypic interaction ability of amino acids 26–69 of the C protein, two recombinant plasmids encoding the HA-tagged HA-C26–69 and the Myc-tagged Myc-C26–69 were constructed (Fig. 2B). These two plasmids were co-transfected into Vero cells to determine whether HA-C26–69 and Myc-C26–69 could bind to each other using in vivo co-immunoprecipitation.

In order to elucidate the role of helix I in stabilizing the homotypic interaction between C proteins of WN virus, the recombinant plasmid encoding the HA-tagged HA-C40–69 was constructed (Fig. 2B). Together with Myc-C26–69, HA-C40–69 was co-transfected into Vero cells and immunoprecipitation was then performed using anti-c-Myc mAb and the precipitated antigen–antibody complexes were analysed by Western blotting using anti-c-Myc mAb (Fig. 4A) or anti-HA Ab (Fig. 4B). The immunoblot probed with anti-c-Myc mAb showed the bands, indicating that Myc-C26–69 was precipitated with the antibody (Fig. 4A, lanes 1–3). On the other hand, the blot probed with anti-HA Ab showed the immuno-reacting band corresponding to HA-C26–69 protein (Fig. 4B, lane 1). No band was seen in the control lanes that were co-transfected with Myc-C26–69 and HA-C40–69/pCMV-HA vector (Fig. 4B, lanes 2 and 3, respectively). The positive interaction between HA-C26–69 and Myc-C26–69 (Fig. 4B, lane 1) illustrated that the homotypic interaction domain lies between the amino acids 26 and 69 and is functional. The lack of interaction between HA-C40–69 and Myc-C26–69 (Fig. 4B, lane 2) showed the importance of helix I in the self-association of WN virus C proteins.

Role of Trp 69 in stabilizing the dimer interface

The amino acid tryptophan at position 69 is conserved among the flaviviruses. To elucidate the importance of

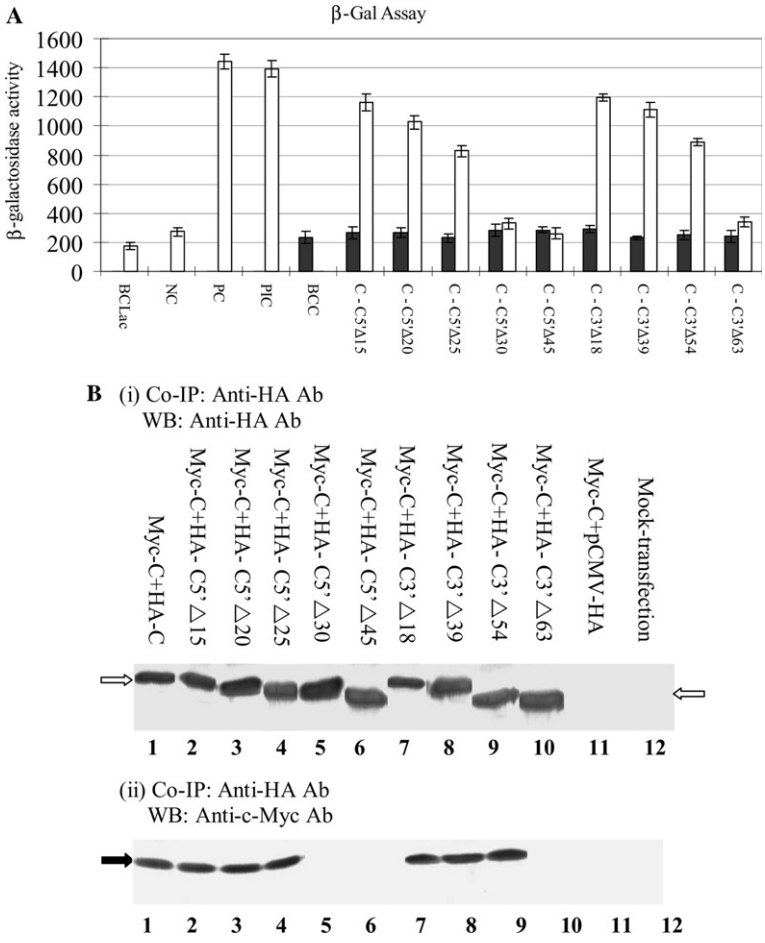

Fig. 3. Determination of C protein self-interacting domain. (A) β-Gal assay (M2H). BC_{Lac} and NC are negative controls. PC and PIC are positive controls. BC_C is the background control for the C protein. The black bar represents the background controls to check if one of the truncated C proteins functions as a transcriptional activator in the absence of the interacting partner. Strong interaction signal was observed for all the truncated proteins, except for the C–C5′ △30, C–C5′ △45, and C–C3′ △63 pairs. (B) In vivo co-immunoprecipitation. Vero cells were co-transfected with Myc-C and HA-C (lane 1)/HA-C5′ △15 (lane 2)/HA-C5′ △20 (lane 3)/HA-C5′ △25 (lane 4)/HA-C5′ △30 (lane 5)/HA-C5′ △45 (lane 6)/HA-C3′ △18 (lane 7)/HA-C3′ △39 (lane 8)/HA-C3′ △54 (lane 9)/HA-C3′ △63 (lane 10)/pCMV-HA vector (lane 11). Lane 12 is loaded with mock-transfected cell lysate. Immunoprecipitation was performed using anti-HA Ab and the precipitated complexes are analysed by Western blotting using anti-HA Ab (i) or anti-c-Myc Ab (ii). Open arrow denotes HA-tagged C protein or its truncated mutants and solid arrows indicate Myc-C proteins. These results demonstrated that amino acids from 26 to 69 are important for the homotypic interaction of C protein.

Trp 69 in the self-association of C protein, we either deleted this amino acid from the functional dimerization domain (amino acids 26–69) or mutated the Trp 69 to Gly or Arg or Phe. Mammalian two-hybrid analysis (Fig. 5A) in conjunction with co-immunoprecipitation studies (Fig. 5B) showed the significance of Trp 69 in stabilizing the dimer interface of the C protein. Trp to Gly or Arg substitution in the self-association domain completely abolished the ability of the C protein to form dimers whereas Trp to Phe substitution restored the property of homodimerization albeit partially ($p = 0.001$; Student's t test was performed using Minitab Release 14) as shown in Fig. 5A. This suggested that the indole ring of Trp 69 may play a crucial role in mediating self-association.

Based on these results, it is concluded that the homotypic interaction domain in WN virus lies between the amino acids 26 and 69 which include the internal hydrophobic domain and few positively charged amino acids and Trp 69 are crucial for the self-association of WN virus C protein.

Discussion

The mechanism of nucleocapsid assembly in flaviviruses is still poorly understood, despite the recent publications of various NMR and X-ray crystallography of capsid structures in DEN, YF, and Kunjin viruses [9–11]. In order to form the virus core, it is critical for C protein monomers to associate with one another prior to viral RNA recruitment and virus particle packaging. Although structural studies have postulated the ability of capsid monomers to intermingle, there are no

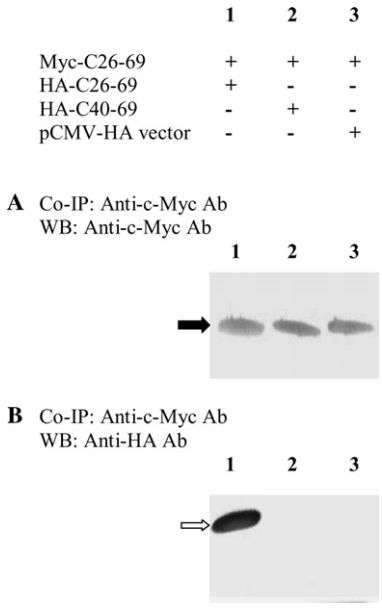

Fig. 4. Illustrating the role of helix I and the homotypic interaction ability of the amino acids 26–69. Vero cells were co-transfected with Myc-C26–69 and HA-C26–69 (lane 1)/HA-C40–69 (lane 2)/pCMV-HA vector (lane 3). Immunoprecipitation was performed using anti-c-Myc Ab followed by probing the blot with anti-c-Myc Ab (A) or anti-HA Ab (B). Solid arrows indicate the protein Myc-C26–69 and open arrow denotes HA-C26–69. The positive interaction between HA-C26–69 and Myc-C26–69 illustrated that the homotypic interaction domain lies between the amino acids 26 and 69, and is functional. The lack of interaction between HA-C40–69 and Myc-C26–69 showed the importance of helix I in the self-association of WN virus C proteins.

biochemical studies to confirm the domain on WN virus C protein that catalysed the formation of complete virus core. Furthermore, the process by which the nucleocapsid is packaged into complete infectious particle is yet to be elucidated. This prompted the embarkment of this study to investigate the homotypic interplay between the WN virus C proteins. The understanding of capsid interaction with itself or other viral components would provide important data for the formation of an infectious particle.

This is the first study demonstrating the C–C homodimerization in WN virus. The ability of C protein to form dimers is important as this triggers the cascade of oligomerization that leads to the formation of the viral nucleocapsid which protects the genome from extracellular agents. Dimerization of C proteins also suggested that the assembly of nucleocapsid core could occur in the absence of other viral proteins. Indeed, in many viruses, nucleocapsid proteins have been shown to dimerize and multimerize [13–22]. Kiermayr et al. [23] recently demonstrated that in vitro production of core-like particles was possible just by expressing only C protein of TBE virus. In addition, N terminus of Hepatitis C protein was also shown to contain all the necessary information for nucleocapsid formation in vitro and in vivo [24]. These observations strengthened the possibility of capsid proteins to catalyse self-assembly in WN virus.

Truncation studies have clearly defined the localization of C protein dimerization domain as the segment that lies between the amino acids 26 and 69 of the virus C protein. Further inward deletion beyond these amino acid locations from either ends obliterated C protein association (Figs. 4 and 5). The identified homo-interaction domain included α helix I to III which was reflective of the structural stability prediction of the three helices in DEN virus C protein [10]. Although deletion of α helix I

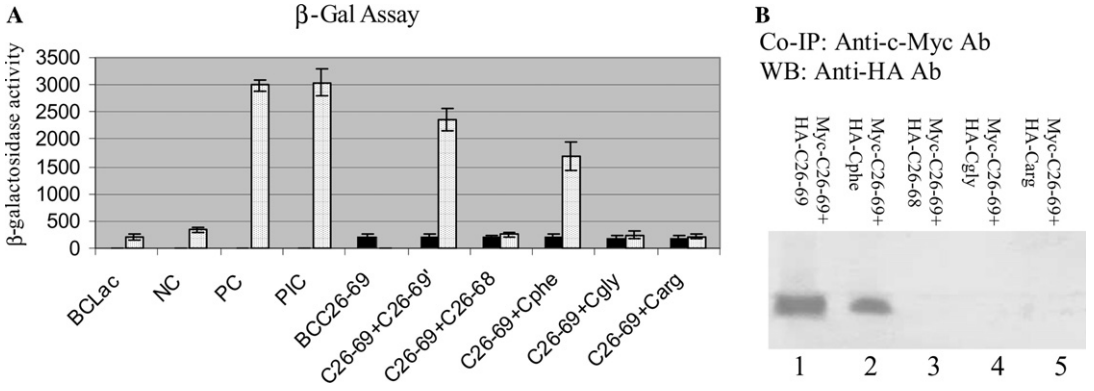

Fig. 5. Importance of Trp 69 in stabilizing the dimer interface. (A) β-Gal assay (M2H). BC_{Lac} and NC are negative controls. PC and PIC are positive controls. BCC26–69 represents the background control for the functional self-association domain (bait). The black bar represents the background controls to check if one of the mutant functions as a transcriptional activator in the absence of the interacting partner. Strong interaction signal was observed for C26–69 + C26–69′pair, whereas Trp to Phe substitution (C26–69 + Cphe pair) restored the property of homodimerization but significantly reduced ($p = 0.001$; Student's t test was performed using Minitab Release 14. The data shown here are representative of 10 independent experiments). On the other hand, removal or mutation of Trp 69 to Gly (Cgly) or Arg (Carg) completely abolished the ability to form the dimers which explains the crucial role played by Trp 69. (B) In vivo co-immunoprecipitation. Vero cells were co-transfected with Myc-C26–69 and HA-C26–69 (lane 1)/HA-Cphe (lane 2)/HA-C26–68 (lane 3)/HA-Cgly (lane 4)/HA-Carg (lane 5). Immunoprecipitation was performed using anti-c-Myc Ab followed by probing the blot with anti-HA Ab. The positive interaction between Myc-C26–69 and HA-C26–69 (lane 1)/HA-Cphe (lane 2) explained the importance of hydrophobicity at position 69. Twice the amount of Myc-C26–69 + HA-Cphe was loaded to visualize the band clearly on lane 2 which highlights the importance of indole ring.

reduced C and C protein interaction in DEN-2 virus [12], it totally abolished homotypic C interaction in WN virus. This indicated the importance of flanking positive residues/helix I in mediating the stability of the C protein for WN virus (Fig. 4B). It is of some interest to note that deletion of helix IV did not affect dimerization, even though the structural evidence shows that helix IV is involved in dimer formation [10,11]. However, we did observe that deletion of helix IV (C3′ △54) slightly weakened the self-association of the C proteins (Fig. 3A and Table 2). The amino acids participating in stabilizing the interaction in WN virus C protein were relatively similar to the predicted amino acids in DEN virus C protein by structural studies [10]. The possibility of false positive was highly minuscule as stringent controls were employed in all three assays and results that were obtained, correlated consistently from all three techniques.

The amino acid tryptophan at position 69 is conserved among the flaviviruses. Mutagenesis studies clearly showed that the removal of Trp 69 from the functional homotypic interaction domain completely abolished the process of homodimerization, which supported the importance of Trp 69 in capsid protein. Several experiments demonstrated that the change of hydrophobicity to hydrophilicity of amino acid residues exposed to the solvent at the surface of proteins is an efficient strategy to stabilize proteins [24–28]. Strub et al. [29] also showed that the stability of the proteins might be increased by mutating the exposed hydrophobic amino acids to arginine. However, both the mutations Trp 69 to Arg 69 and Trp 69 to Gly 69 disrupted the functional ability of the homotypic interaction domain.

This suggested that Trp 69 at the dimer interface has some stabilization effects or mutation to Arg or Gly disrupted the hydrophobic interior of the 3-helix core. When Trp 69 was replaced by another hydrophobic residue Phe, the self-association of WN virus C protein was significantly decreased ($p = 0.001$) as shown in Fig. 5A. This might be due to the significant alteration of the backbone structure, changes in solvent accessible surface area, solvent transfer free energy or the conformational changes induced by the substitution [30]. When Trp 69 was replaced with Phe, dimerization was retained, demonstrating the importance of a hydrophobic environment at this position. The significant reduction in dimerization process after the substitution of Phe indicated that the indole ring of Trp 69 might play a critical role in stabilizing the interaction. Structural studies [10] also suggested that Trp 69 might play a role in the structural stabilization of the dimer interface. This explains the loss of dimerization after mutation or removal of Trp 69 from the functional domain in this study.

In summary, this study using three independent protein interaction assays has demonstrated the homotypic association of capsid proteins in WN virus and delineated the internal segment from the amino acids 26 to 69 as the functional domain that mediates C–C dimerization. We also showed the importance of helix I and Trp 69 in mediating and stabilizing the self-association of WN virus C protein. Mutagenesis studies clearly revealed the importance of a hydrophobic environment at the position of 69th amino acid (Trp 69). The significant reduction in dimerization process after the substitution of Phe indicated that the indole ring of Trp 69 might play a critical role in stabilizing the interaction. These findings helped to expand the current understanding of nucleocapsid assembly and pinpoint the internal hydrophobic domain flanked by few positively charged amino acids (helix I, helix II, and helix III) of WN virus capsid as potential target of future antiviral agents.

Acknowledgments

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