Identification of the homotypic interaction domain of the core protein of dengue virus type 2

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Dengue virus causes dengue haemorrhagic fever or dengue shock syndrome with a high mortality rate. The genome of dengue virus is a positive-sense, single-stranded RNA encoding three structural and seven non-structural proteins. The core protein is one of the three structural proteins and is the building block of the nucleocapsid of dengue virus. The core protein of dengue virus type 2 (DEN2) is composed of 100 aa with four $\alpha$-helix domains. An internal hydrophobic domain located at aa 44–60 was identified. The DEN2 core protein was shown to form homodimers. Deletion of aa 1–36 or 73–100 decreased but did not completely abolish the core-to-core homotypic interaction, whereas deletion of a portion (aa 44–60) within aa 37–72 completely abolished the ability of the DEN2 core proteins to interact with each other. A recombinant DEN2 core protein corresponding to aa 37–72 was able to undergo homotypic interaction and bound to a native DEN2 core protein. The results of this study indicated that the homotypic interaction domain of the DEN2 core protein is located at aa 37–72 and that the internal hydrophobic domain located at aa 44–60 plays a pivotal role in core-to-core homotypic interaction.

INTRODUCTION

Dengue virus is a mosquito-borne flavivirus and is present in more than 85 tropical and subtropical countries (Lam, 1993). It has been estimated to cause approximately 100 million new cases of benign dengue fever and 250 000 cases of life-threatening dengue haemorrhagic fever (DHF) or dengue shock syndrome (DSS) annually, with a mortality rate of around 5% (Gubler & Clark, 1995). Globally, approximately 2.5 billion people are at risk of dengue virus infection (Barrett, 1997; WHO, 2000). Although DHF was recognized in the 1950s (Hammon et al., 1960), the molecular biology of dengue virus and the pathogenesis of DHF are still not well understood and no effective treatment is available for DHF or DSS (Kurane & Ennis, 1992; Lei et al., 2001; Rothman & Ennis, 1999). A multivalent vaccine against all four known serotypes of dengue virus is being developed (Bhamarapravati & Sutee, 2000; Kanesathasan et al., 2001; Robertson et al., 2001).

The dengue virion is approximately 50 nm in diameter and is composed of a single-stranded, positive-sense RNA genome and three structural proteins: the core (capsid, C), membrane (M) and envelope (E) proteins. The dengue viral genome is approximately 11 000 nt in length and encodes a large polypeptide, which is processed to form three structural and seven non-structural (NS) proteins. The order of genes encoding these proteins is C-prM-EN1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 (Chambers et al., 1990; Westaway et al., 1985). The core protein has a molecular mass of 16 kDa and is the building block of the dengue nucleocapsid (Wang et al., 2002).

Although the core proteins of various flaviviruses share very little sequence homology, they all are rich in basic amino acid residues (approx. 25% Lys and Arg), which may be responsible for binding the core protein to the RNA genome (Chambers et al., 1990; Rice et al., 1985). Such interaction has been demonstrated in Kunjin virus and hepatitis C virus (HCV). The core proteins of these two viruses of the Flaviviridae family have been shown to bind both 5′- and 3′-untranslated regions of their respective RNA genomes in vitro (Fan et al., 1999; Khromykh & Westaway, 1996; Shimoike et al., 1999; Tanaka et al., 2000). The RNA-binding ability of the dengue virus core protein has not been demonstrated.

The core protein of dengue virus type 2 (DEN2) has exactly 100 aa and has recently been shown by far-UV circular dichroism and NMR analyses to be a helical protein containing four $\alpha$-helices located at aa 26–31, 45–55, 63–69 and 74–96, respectively (Jones et al., 2003). By analytical ultracentrifugation, NMR relaxation measurement and cross-linking with the lysine-specific reagent disuccinimidyl...
strain pl046 in eukaryotic cells (Wang et al., 2002). DNA fragments containing different portions of the DEN2 core gene were generated by PCR using pFC1-100 as the template and the primer pairs listed in Table 1. The PCR products were cloned into pFLAG-CMV-2 (Sigma-Aldrich) between the EcoRI and EcoRV sites, fusig different portions of the DEN2 core protein to the FLAG tag (Table 2 and Fig. 1a). Two of these DNA fragments encoding the entire core protein and aa 37–72 of the core protein, respectively, were also cloned between the EcoRI and Xhol sites of pCMV-DS (Chuang et al., 2001), which contains a DNA fragment encoding an epitope (11 aa) of the delta hepatitis virus. This delta epitope is recognized by the mAb HP6A1 (Hsu et al., 2000). Cloning of these DNA fragments into pCMV-DS fused different portions of the core protein to the delta epitope (Table 2 and Fig. 1b).

Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′→3′)*</th>
<th>Core amino acids</th>
</tr>
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<tbody>
<tr>
<td>P15</td>
<td>atgtctgaaacgcgagagaa</td>
<td>15–21</td>
</tr>
<tr>
<td>P37</td>
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<td>P1-72</td>
<td>TTAAatggtctcccctctctccag</td>
<td>72–66</td>
</tr>
<tr>
<td>P1-84</td>
<td>TTAAgacctcctaaaacataat</td>
<td>84–78</td>
</tr>
<tr>
<td>CoreTerm</td>
<td>TTATctgctccttcctgtaatag</td>
<td>100–94</td>
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<td>CoreX</td>
<td>ccgtctgacgTTCATctgcttcctgtaatag</td>
<td>100–94</td>
</tr>
<tr>
<td>P44A60for</td>
<td>ggacgagaccaacagcagaggtatcctgtaatag</td>
<td>40–61/61–66</td>
</tr>
<tr>
<td>P44A60rev</td>
<td>tcgtgctgtgtgtctccttcctgtaatag</td>
<td>63–61/43–37</td>
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<td>T7</td>
<td>taatacagatctatagga</td>
<td></td>
</tr>
<tr>
<td>CMV</td>
<td>cgttagctgtgtaggtgct</td>
<td></td>
</tr>
</tbody>
</table>

*The underlined sequence is a restriction site built into the primer. The sequences in upper-case are build-in stop codons.

The DEN2 core protein has been shown to form homodimers in solution without the involvement of other viral components (Jones et al., 2003). This result suggested that the DEN2 core protein has a domain responsible for the homotypic interaction similar to that of HCV (Matsumoto et al., 1996; Nolandt et al., 1997; Yan et al., 1998) and tick-borne encephalitis (TBE) virus (Kolfer et al., 2002). In this study, we have identified the homotypic interaction domain of the DEN2 core protein and found that this domain is located at aa 37–72, which overlaps the internal hydrophobic region of the DEN2 core protein.

**METHODS**

**Plasmid construction.** All recombinant plasmids used in this study were derived from pFC1-100, which was constructed to express an N-terminally FLAG-tagged, full-length core protein of DEN2 suberate, the DEN2 core protein. In a separate PCR, primer P44A60for was coupled with primer CoreTerm to amplify a fragment from pFC1-100 encoding aa 61–100 of the DEN2 core protein. Since the sequence of primer P44A60rev corresponded to aa 37–43 plus an additional sequence corresponding to aa 61–63 and that of primer P44A60for corresponded to aa 61–66 plus an additional sequence corresponding to aa 40–43, the 3′ end of the first PCR product and the 5′ end of the second PCR product overlapped by 21 bp (7 aa). These two PCR products were mixed, denatured, renatured and used as the template to amplify a DNA fragment containing the core gene with aa 44–60 deleted using primers CMV and CoreTerm. The amplified fragment was digested with EcoRI and inserted into pFLAG-CMV-2 between the EcoRI and EcoRV sites producing pFCA44-60 (Fig. 1a). pFCA44-60 encoded a FLAG-tagged DEN2 core protein without the helix II region between Leu-44 and Pro-60.

**Co-immunoprecipitation.** To demonstrate homotypic interaction of the core protein, both FLAG-tagged and delta-tagged core proteins were expressed in the same cells. If the core proteins bind to each other, immunoprecipitation of the FLAG-tagged core protein will also precipitate the delta-tagged core protein and vice versa. Eight micrograms (in 8 μl water) each of the plasmid encoding a delta-tagged core gene (e.g. pDC1-100) and another plasmid encoding a FLAG-tagged core gene (e.g. pFC1-100) were mixed and incubated with 30 μl lipofectamine 2000 (Invitrogen Life Technologies) at room temperature for 20 min. This liposome/DNA mixture was added slowly to the medium of a HeLa cell culture in a 100 mm well plate and cultured for 18 h at room temperature. The cells were washed once with PBS followed by fixation with formaldehyde (37%, Sigma-Aldrich) for 15 min at room temperature. After washing with PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. The cells were then blocked with 5% goat serum in PBS for 30 min at room temperature. After washing with PBS, the cells were incubated with a 1:200 dilution of HP6A1 (Hsu et al., 2000) for 1 h at room temperature. The cells were then washed with PBS and incubated with a 1:200 dilution of rabbit anti-human IgG (H+L) (Southern Biotech) conjugated with fluorescein isothiocyanate for 1 h at room temperature. The cells were then washed with PBS and mounted with 50% glycerol in PBS containing 4′,6-diamidino-2-phenylindole (1 mg/ml) (Invitrogen Life Technologies). The results were analyzed using a Zeiss Axioplan fluorescence microscope.

Table 2. Plasmid constructs for DEN2 core protein expression

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Region (aa)</th>
<th>Primer pair</th>
<th>Template</th>
<th>Vector</th>
<th>Cloning sites</th>
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</thead>
<tbody>
<tr>
<td>pFC1-84</td>
<td>1–84</td>
<td>CMV/P1-84</td>
<td>pFC1-100*</td>
<td>pFlag-CMV-2</td>
<td>EcoRI and EcoRV</td>
</tr>
<tr>
<td>pFC1-72</td>
<td>1–72</td>
<td>CMV/P1-84</td>
<td>pFC1-100*</td>
<td>pFlag-CMV-2</td>
<td>EcoRI and EcoRV</td>
</tr>
<tr>
<td>pFC15-100</td>
<td>15–100</td>
<td>P15/T7</td>
<td>pFC1-100*</td>
<td>pFlag-CMV-2</td>
<td>EcoRV and BamHI</td>
</tr>
<tr>
<td>pFC37-100</td>
<td>37–100</td>
<td>P37/T7</td>
<td>pFC1-100*</td>
<td>pFlag-CMV-2</td>
<td>EcoRV and BamHI</td>
</tr>
<tr>
<td>pFC47-100</td>
<td>47–100</td>
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<td>pFC1-100*</td>
<td>pFlag-CMV-2</td>
<td>EcoRV and BamHI</td>
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<tr>
<td>pFC37-84</td>
<td>37–84</td>
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<td>pFC1-100*</td>
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<td>EcoRV and EcoRV</td>
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<td>37–72</td>
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<td>EcoRV and EcoRV</td>
</tr>
<tr>
<td>pFCA44-60</td>
<td>1–43/61–100</td>
<td>CMV/P44A60rev</td>
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<td>EcoRV and EcoRV</td>
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<tr>
<td>pDC1-100</td>
<td>1–100</td>
<td>CMV/CoreX</td>
<td>pFC1-100*</td>
<td>pCMV-DS*</td>
<td>EcoRI and Xhol</td>
</tr>
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<td>pDC37-72</td>
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<td>pDC1-100*</td>
<td>pCMV-DS*</td>
<td>SmaI</td>
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*Plasmids pCMV-DS and pFC1-100 were generated previously (Chuang et al., 2001; Wang et al., 2002).*
cell debris was removed by centrifugation at 15,000 g for 1 min. The Protein A–agarose immune complex pellet was washed twice with cold PBS at 20 °C for 5 min.

Nucleus translocation assay. The nucleus translocation assay was performed as described previously (Wang et al., 2002). Plasmids encoding a FLAG-tagged and a delta-tagged core protein were co-transfected into HeLa cells as described above. The transfected cells were washed twice with cold PBS at 20 h post-transfection and fixed with methanol:acetone (1:1) mixture at −20 °C for 5 min. Nuclear localization of the core protein was determined by an immunofluorescence assay using either the ANTI-FLAG mAb M2 or the anti-delta epitope HP6A1 mAb as the primary antibody and FITC-conjugated anti-mouse antibody as the secondary antibody. The cells on slides were examined by confocal microscopy to determine the subcellular localization of the core protein.

RESULTS

Demonstration of homotypic interaction of the DEN2 core protein by co-immunoprecipitation

Co-immunoprecipitation was performed to demonstrate homotypic interactions of the core protein. Two different recombinant core proteins were expressed: one was tagged with the FLAG epitope and the other was tagged with the delta epitope. If the core protein binds to itself in cells, immunoprecipitation with antibody against either the delta or the FLAG epitope will precipitate both FLAG- and delta-tagged core proteins. The FLAG-tagged core protein was encoded by the recombinant plasmid pFC1-100 (Fig. 1a) and the delta-tagged core protein was encoded by pDC1-100 (Fig. 1b). These two plasmids were co-transfected into HeLa cells. To ensure that both the FLAG- and delta-tagged core proteins were expressed in transfected cells, a Western blot assay was first performed on the lysate of transfected cells using the anti-delta mAb HP6A1 (Fig. 2, lanes 1–3) or the ANTI-FLAG mAb M2 (Fig. 2, lanes 10–12). Cell lysates from three different groups of cells were used: (i) cells co-transfected with pFC1-100 and pCMV-DS, which was the vector used to construct pDC1-100; (ii) cells co-transfected with pFC1-100 and pDC1-100; and (iii) cells co-transfected with pDC1-100 and pFLAG-CMV-2, which was the vector used to construct pFC1-100. As shown in Fig. 2, the core protein was detected with either the anti-delta mAb HP6A1 (Fig. 2, lanes 2 and 3) or the ANTI-FLAG mAb M2 (Fig. 2, lanes 10 and 11) in cells transfected with pDC1-100 (groups 2 and 3) or pFC1-100 (groups 1 and 2), respectively, indicating that both the FLAG- and delta-tagged core proteins were expressed in transfected cells. The observation that mAb HP6A1 did not react with the group 1 cell lysate (Fig. 2, lane 1) indicated that this antibody did not cross-react with FLAG-tagged proteins. Similarly, the fact that mAb M2 did not react with the group 3 cell lysate (Fig. 2, lane 12) indicated that this antibody did not cross-react with delta-tagged proteins.

Co-immunoprecipitation was then performed on these cell lysates using either anti-delta mAb HP6A1 or ANTI-FLAG mAb M2. The three groups of cell lysates were first reacted with mAb M2, which was conjugated to Protein A–agarose. The antigen–antibody complexes thus formed were pelleted and electrophoresed on polyacrylamide gels in duplicate. The Western blot of one set of gels was reacted
with anti-delta mAb HP6A1 and that of the other set was reacted with ANTI-FLAG mAb M2, followed with a peroxidase-conjugated anti-mouse IgG. The gel reacted with the ANTI-FLAG antibody showed the expected FLAG-tagged core protein (Fig. 2, lanes 13 and 14). The gel reacted with the anti-delta antibody showed that the delta-tagged core protein DC1-100 was precipitated with the ANTI-FLAG antibody only in the presence of the FLAG-tagged core protein FC1-100 (Fig. 2, lane 5). No core protein bands were seen in the sample from cells transfected with pFLAG-CMV-2 and pDC1-100, indicating that no delta-tagged core protein was immunoprecipitated with the ANTI-FLAG antibody in the absence of the FLAG-tagged core protein FC1-100 (Fig. 2, lane 6). ANTI-FLAG mAb-precipitated proteins were also reacted with ANTI-FLAG mAb to ensure successful precipitation of theFLAG-tagged core protein (lanes 7–9). ANTI-FLAG mAb-precipitated proteins were also reacted with anti-delta-epitope mAb to ensure successful precipitation of the delta-tagged core protein (lanes 8–9). Likewise, anti-delta-epitope mAb-precipitated proteins were also reacted with anti-delta-epitope mAb to ensure successful precipitation of the delta-tagged core protein (lanes 10–12). Open arrows indicate delta-epitope-tagged core proteins (DC) and solid arrows indicate FLAG-tagged core proteins (FC). ‘L’ indicates the immunoglobulin light chain of the primary antibody that reacted with the secondary antibody; ‘ns’ denotes a protein that reacted non-specifically with the antibody.

**Localization of the homotypic interaction region of the DEN2 core protein by co-immunoprecipitation**

To localize the homotypic interaction domain of the core protein, we constructed several recombinant plasmids (pFC1-84, pFC1-72, pFC15-100, pFC37-100 and pFC47-100) to express N- or C-terminal-truncated core proteins. pFC1-84 and pFC1-72 encoded FLAG-tagged C-terminal-truncated core proteins lacking aa 85–100 and 73–100, respectively. These two constructs were used to investigate the role of aa 85–100 and 73–100 in core-to-core interactions. The FLAG-tagged core proteins encoded by pFC15-100, pFC37-100 and pFC47-100 were missing aa 1–14, 1–36 and 1–46, respectively, and were used to determine whether these regions are involved in homotypic interactions of the core protein. Each of these plasmids was co-transfected with pDC1-100 into HeLa cells to co-transfected with pDC1-100 and pCMV-DS, pFC1-100 and pDC1-100, or pFLAG-CMV-2 and pDC1-100. Cell lysates of transfected cells were examined directly by Western blotting (WB) using anti-delta-epitope mAb (αD HP6A1) (lanes 1–3) or ANTI-FLAG mAb (αF M2) (lanes 10–12) to detect the expressed core proteins. Core-to-core homotypic interaction was detected by immunoprecipitation with ANTI-FLAG mAb followed by Western blotting of the precipitated antigen–antibody complexes using anti-delta-epitope mAb (lanes 4–6) or by immunoprecipitation with anti-delta-epitope mAb followed by Western blotting of the precipitated antigen–antibody complexes using ANTI-FLAG mAb (lanes 7–9). ANTI-FLAG mAb-precipitated proteins were also reacted with ANTI-FLAG mAb to ensure successful precipitation of the FLAG-tagged core protein (lanes 13–15). Likewise, anti-delta-epitope mAb-precipitated proteins were also reacted with anti-delta-epitope mAb to ensure successful precipitation of the delta-tagged core protein (lanes 16–18).

Fig. 2. Core-to-core homotypic interaction. HeLa cells were co-transfected with pFC1-100 and pCMV-DS, pFC1-100 and pDC1-100, or pFLAG-CMV-2 and pDC1-100. Cell lysates of transfected cells were examined directly by Western blotting (WB) using anti-delta-epitope mAb (αD HP6A1) (lanes 1–3) or ANTI-FLAG mAb (αF M2) (lanes 10–12) to detect the expressed core proteins. Core-to-core homotypic interaction was detected by immunoprecipitation with ANTI-FLAG mAb followed by Western blotting of the precipitated antigen–antibody complexes using anti-delta-epitope mAb (lanes 4–6) or by immunoprecipitation with anti-delta-epitope mAb followed by Western blotting of the precipitated antigen–antibody complexes using ANTI-FLAG mAb (lanes 7–9). ANTI-FLAG mAb-precipitated proteins were also reacted with ANTI-FLAG mAb to ensure successful precipitation of the FLAG-tagged core protein (lanes 13–15). Likewise, anti-delta-epitope mAb-precipitated proteins were also reacted with anti-delta-epitope mAb to ensure successful precipitation of the delta-tagged core protein (lanes 16–18). Open arrows indicate delta-epitope-tagged core proteins (DC) and solid arrows indicate FLAG-tagged core proteins (FC). ‘L’ indicates the immunoglobulin light chain of the primary antibody that reacted with the secondary antibody; ‘ns’ denotes a protein that reacted non-specifically with the antibody.
cells. The cell lysate of the transfected cells was then assayed for production of the FLAG-tagged truncated core protein and the ability of this core protein to bind the full-length delta-tagged core protein (the ‘bait’).

The Western blot of the cell lysate reacted with the anti-delta mAb HP6A1 revealed that the delta-tagged core protein DC1-100 was successfully expressed in the transfected cells (Fig. 3a, lanes 17–23). Immunoprecipitation of the cell lysates was then performed using the Protein A–agarose-conjugated ANTI-FLAG mAb M2 to precipitate the FLAG-tagged core protein and the delta-tagged core protein DC1-100 that bound to it. The immune complexes were electrophoresed, blotted and reacted with ANTI-FLAG mAb M2 or anti-delta mAb HP6A1 as described above. The Western blot reacted with ANTI-FLAG mAb M2 revealed that all transfected cells expressed the FLAG-tagged truncated core proteins of expected sizes (Fig. 3a, lanes 9–14). No core protein band that reacted with ANTI-FLAG antibody, various amounts of the delta-tagged core protein DC1-100 were detected in different samples (Fig. 3a, lanes 1–6). Cells co-transfected with pFC15-100 and pDC1-100 were found to have the same amounts of delta-tagged core protein DC1-100 as those transfected with pFC1-100 and pDC1-100, indicating that aa 1–14 of the core protein were not essential for core-to-core homotypic interaction (Fig. 3a, lanes 1 and 4). Cells co-transfected with pDC1-100 and pFC1-84, pFC1-72 or pFC37-100 were found to have greatly reduced amounts of protein DC1-100 that bound to these FLAG-tagged truncated core proteins (Fig. 3a, lanes 2, 3 and 5), suggesting that aa 85–100, 73–100 and 15–36 play some roles in the homotypic interaction. Cells co-transfected with pDC1-100 and pFC47-100 only had trace amounts of delta-tagged core protein DC1-100 that bound to the FLAG-tagged truncated core protein FC47-100 (Fig. 3a, lane 6). These results suggested that the domain most critical for homotypic interaction of the core protein is located at aa 37–72.

To demonstrate further the homotypic interaction ability of aa 37–72, two recombinant plasmids encoding the FLAG-tagged FC37-72 and the delta-tagged DC37-72 were constructed. These two plasmids were co-transfected into cells to determine whether FC37-72 and DC37-72 could
bind to each other using the methods described above. In this experiment, DC37-72, instead of DC1-100, was used as the bait for binding. pFC37-84 encoding the FLAG-tagged C37-84 core protein and pFLAG-CMV-2, the vector used to construct FLAG-tagged core proteins, were also co-transfected with pDC37-72 to serve as controls. As with the experiment using DC1-100 described above, a Western blot of the lysate of transfected cells was first performed using the ANTI-FLAG antibody. The results indicated that FC37-84 and FC37-72 were successfully expressed in transfected cells (Fig. 3b, lanes 7 and 8). Immunoprecipitation was then performed using Protein A–agarose-conjugated anti-delta mAb HP6A1 and the Western blots of the immune complexes were reacted with peroxidase-conjugated ANTI-FLAG or anti-delta antibody. Reaction with the anti-delta antibody revealed a 6.5 kDa protein band (Fig. 3b, lanes 4–6), indicating that the delta-tagged core protein DC37-72 was precipitated with the antibody. Reaction with the ANTI-FLAG antibody detected a protein with a molecular mass of 7 kDa in the sample derived from cells transfected with pDC37-72 and pFC37-84 (lane 1) and a protein of 6 kDa in the sample derived from cells transfected with pDC37-72 and pFC37-72 (lane 2), indicating that these FLAG-tagged core proteins were bound to the bait (the delta-tagged core protein DC37-72) and were co-precipitated with the anti-delta antibody. No core protein band was detected in the control cells that were co-transfected with pFLAG-CMV-2 and pDC37-72 (Fig. 3b, lane 3). These results indicated that the region spanning aa 37–72 of the core protein was indeed responsible for the core-to-core homotypic interaction.

The region spanning aa 37–72 overlaps the hydrophobic domain of the DEN2 core protein located at aa 44–60 (Jones et al., 2003). To determine whether this hydrophobic domain is involved in core-to-core homotypic interaction, pFCA44-60 was constructed to express a FLAG-tagged core protein FCA44-60 lacking aa 44 (leucine) to 60 (proline). Although FCA44-60 was successfully expressed in cells transfected with pFCA44-60 (Fig. 4, lane 5), it was unable to bind the bait FC1-100 in the co-immunoprecipitation experiment (Fig. 4, lane 2). This result indicated that this hydrophobic domain is indeed involved in core-to-core homotypic interaction.

Detection of the homotypic interaction domain of the DEN2 core protein by the nucleus translocation assay

The experiments described above showed that aa 37–72 of the DEN2 core protein can mediate core-to-core homotypic interaction in vitro. To determine whether this interaction also occurs in vivo, a nucleus translocation assay was performed. The core protein has been shown to be able to enter the nucleus and nucleolus, and the sequence (RKeigrunlnR) responsible for this nuclear localization is located at aa 85–100 (Wang et al., 2002). If the FLAG-tagged core protein FC37-72 binds to another core protein, such as DC1-100, it will be transported into the nucleus. To perform the experiment, cells were transfected with pDC1-100 or pFC37-72 alone or co-transfected with pDC1-100 and pFC37-72. The transfected cells were then stained with FITC-conjugated anti-delta or ANTI-FLAG antibody and examined by confocal microscopy as described previously (Wang et al., 2002). The results showed that the delta-tagged core protein DC1-100 in cells transfected with pDC1-100 alone was mainly found in the nucleolus (Fig. 5a). In comparison, the FLAG-tagged core protein FC37-72 was found only in the cytoplasm in cells transfected with pFC37-72 alone (Fig. 5b). Interestingly, the FLAG-tagged core protein FC37-72 was detected in both the nucleus and cytoplasm in cells co-transfected with
The homotypic interaction domain that we have identified encompasses the core protein for transport into the nucleus.

The other two domains are located at aa 123–134 and 147–162, respectively (Matsumoto et al., 1996; Nolandt et al., 1997; Yan et al., 1998). In this study, we demonstrated that the DEN2 core protein also has a homotypic interaction domain and that this domain is located at aa 37–72. This conclusion was based on the observation that deletion of the first N-terminal 36 aa or the last C-terminal 28 aa of the core protein did not completely abolish the homotypic interaction ability of the core protein (Fig. 3a, lanes 5 and 3, respectively). Furthermore, a truncated core protein containing aa 37–72 showed homotypic interaction activity (Fig. 3b, lane 2) and was able to bind a full-length core protein for transport into the nucleus.

The homotypic interaction domain that we have identified encompasses α-helices II (aa 45–55) and III (aa 63–69) of the DEN2 core protein (Jones et al., 2003). Using NMR, Ma et al. (2004) recently determined the three-dimensional structure of the DEN2 core protein and predicted that helices II (aa 45–55) and IV (aa 74–96) would form the majority of the dimer contact surface. Although we did not find helix IV to be critical for homotypic interaction of the DEN2 core protein, deletion of the region encoding helix IV did greatly decrease the homotypic interaction ability of the core protein, as shown by the reduced binding of FLAG-tagged truncated core protein FC1-72 or FC1-84 to the delta-tagged DC1-100 (Fig. 3a, lanes 2 and 3). We also found that truncation of helix I (aa 26–31) reduced core-to-core interactions (Fig. 3a, lane 5, FC37-100). This result is consistent with the prediction that the side chains of Val-26 and Leu-29 in helix I, Phe-47 and Val-51 in helix II and Ile-65, Arg-68 and Trp-69 in helix III form a hydrophobic interior that stabilizes the core homodimers (Ma et al., 2004).

The internal hydrophobic sequence (aa 44–60) is also located in the homotypic interaction domain that we have identified. The function of the DEN2 internal hydrophobic region has not been determined. It has been shown in vitro that the internal hydrophobic region of the DEN4 core protein is associated with the endoplasmic reticulum (ER) membrane, which may be the site for morphogenesis of DEN4 virus (Markoff et al., 1997). Based on the predicted three-dimensional structure of the DEN2 core protein, this internal hydrophobic region would create a large contiguous apolar patch that may interact with the ER membrane (Ma et al., 2004). It is possible that DEN2 core protein binds to the ER membrane through this hydrophobic domain, forming a base for capsid assembly. The capsid may then capture the viral genome to form the viral nucleocapsid. Interaction of the core protein of Kunjin virus with the viral genome has been shown to be mediated through the basic amino acid residues present in both N and C termini of the core protein (Khromykh & Westaway, 1996). Both the N- and C-terminal regions of the DEN2 core protein are also rich in basic amino acid residues, which may have a similar function in RNA binding to that of Kunjin virus. The HCV core protein has been shown to undergo heterotypic interactions with the HCV E1 protein (Lo et al., 1996), in addition to homotypic interaction with itself (Matsumoto et al., 1996; Nolandt et al., 1997; Yan et al., 1998). Heterotypic interactions with other structural proteins may influence viral budding and fusion processes (Lopez et al., 1994). Whether the DEN2 core protein also undergoes heterotypic interactions remains to be investigated.

The core protein of TBE virus was predicted to possess two leucine zipper–like domains, which may mediate homotypic interaction (Kofler et al., 2002). Although structural analysis...
Flavivirus capsid is a dimeric alpha-helical structure. The lack of a typical leucine zipper structure on the DEN2 core protein suggests that DEN2 core-to-core homotypic interactions are not as strong as those of TBE. This speculation is supported by our finding that the DEN2 core-to-core interaction was easily disrupted by washing the complex under more stringent conditions, such as washing with a triple-detergent lysis buffer (150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 100 µg PMSF ml⁻¹, 50 mM Tris/HCl, pH 8.0) (data not shown). In a yeast two-hybrid assay, the DEN2 core-to-core interaction was also found to be weak (data not shown). The identification of the DEN2 homotypic interaction domain in this study will enable a more detailed study of the assembly of the DEN2 nucleocapsid.

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REFERENCES


