Multiple regions in dengue virus capsid protein contribute to nuclear localization during virus infection

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During infection, the capsid (C) protein of many flaviviruses localizes to the nuclei and nucleoli of several infected cell lines; the underlying basis and significance of C protein nuclear localization remain poorly understood. In this study, double alanine-substitution mutations were introduced into three previously proposed nuclear-localization signals (at positions 6–9, 73–76 and 85–100) of dengue virus C protein, and four viable mutants, c(K6A,K7A), c(K73A,K74A), c(R85A,K86A) and c(R97A,R98A), were generated in a mosquito cell line in which C protein nuclear localization was rarely observed. Indirect immunofluorescence analysis revealed that, whilst C protein was present in the nuclei of PS and Vero cells throughout infection with a dengue serotype 2 parent virus, the substitution mutations in c(K73A,K74A) and c(R85A,K86A) resulted in an elimination of nuclear localization in PS cells and marked reduction in Vero cells. Mutants c(K6A,K7A) and c(R97A,R98A) exhibited reduced nuclear localization at the late period of infection in PS cells only. All four mutants displayed reduced replication in PS, Vero and C6/36 cells, but there was a lack of correlation between nuclear localization and viral growth properties. Distinct dibasic residues within dengue virus C protein, many of which were located on the solvent-exposed side of the C protein homodimer, contribute to its ability to localize to nuclei during virus infection.

INTRODUCTION

The positive-sense, single-stranded, approximately 11 kb RNA genome of dengue virus consists of an open reading frame from which a polypeptide of approximately 3400 aa is generated. The polyprotein is processed proteolytically by viral and host proteases into three structural proteins, capsid (C), pre-membrane (prM) and envelope (E), and seven non-structural (NS) proteins (Lindenbach & Rice, 2001). Following sequential cleavages of the polyprotein at the C–prM junction region by the viral protease, NS2B/NS3, and host signalase, mature C protein is released into the cytoplasm (Lobigs, 1993; Amberg et al., 1994), but a proportion of C protein may associate with the endoplasmic reticulum (ER) membrane via its hydrophobic segment at residues 45–65 (Markoff et al., 1997). The homodimeric form of the C protein is believed to interact with viral genomic RNA in the cytoplasm. The resulting nucleocapsid then buds into the ER lumen to form viral particles with the prM and E proteins (Lindenbach & Rice, 2001; Ma et al., 2004; Wang et al., 2004).

During dengue virus serotype 2 and 4 infection, C protein is also found in the nuclei and nucleoli of many infected cell lines (Tadano et al., 1989; Bulich & Aaskov, 1992;
Wang et al., 2002). This feature appears to be conserved among flaviviruses, as other investigators have detected C proteins of Kunjin virus, West Nile virus and Japanese encephalitis virus (JEV) in both the cytoplasm and nuclei of infected mammalian cells (Westaway et al., 1997; Mori et al., 2005; Oh et al., 2006). Three stretches of Arg/Lys-rich sequence in the dengue virus C protein have been proposed to serve as nuclear-localization signals (NLSs): KKAR, located at aa 6–9; KSK, located at aa 73–76; and the bipartite sequence RKEI<sup>GRML</sup>NILRRRRR, located at aa 85–100 (Bulich & Aaskov, 1992). Subsequently, Wang et al. (2002) showed, using recombinant dengue virus C protein expressed in HeLa cells, that dengue virus C protein nuclear localization is predominantly due to the bipartite sequence at aa 85–100. Whether the requirement for this NLS sequence in the nuclear localization of dengue virus C protein holds true in the context of virus infection remains unknown. This study aimed to assess the contribution of each of the proposed NLS sequences to the nuclear localization of C protein in infected cells and to examine the influence of the introduced mutations on dengue virus replication.

**METHODS**

**Cells, viruses and antibodies.** C6/36, a cell line derived from *Aedes albopictus* (Igarashi, 1978), and PS clone D, a small-cell clone of a porcine kidney epithelial cell line (Westaway, 1966; de Madrid & Porterfield, 1969), were grown at 29 and 37 °C, respectively, in Leibovitz’s L-15 medium supplemented with 10 % fetal bovine serum (FBS), 0.26 g % tryptose phosphate broth and glutamine/penicillin/streptomycin solution (Sriburi et al., 2001). Vero cells (ATCC) were grown at 37 °C in minimum essential medium supplemented with 10 % FBS in 5 % CO<sub>2</sub> in humidified air. A dengue serotype 2 virus, strain 16681, and its plasmid-derived counterpart, strain 16681<sup>Pst</sup> (–) (Sriburi et al., 2001), were propagated in C6/36 cells and stored at −65 °C. Monoclonal antibodies (mAbs) specific for dengue virus C protein [clone 6F3, which recognizes the epitope 9-RNTFPNFLKRE-19 on the C protein (Bulich & Aaskov, 1992)], flavivirus E protein [clone 4G2 (Henchal et al., 1982)] and dengue virus NS1 protein [clone NS1-1F (Putthikhunt et al., 2003)], or a placmazyma protein [MOPC21 (Sigma)], were employed in indirect immunofluorescence assays in the form of culture supernatant or ascitic fluid.

**Generation of C protein NLS mutant viruses.** Construction of mutant full-length cDNA clones was performed by using a full-length cDNA clone of strain 16681 (Sriburi et al., 2001). Mutations were introduced into the C coding region by using a PCR-based site-directed mutagenesis scheme (QuikChange; Stratagene) with a plasmid subclone, pBK(S15P6-1547)<sup>A402Pst</sup>, as the template, and oligonucleotide primers (see Supplementary Table S1, available in JGV Online). The DNA fragment containing the mutated sequence was removed from the plasmid by BglII digestion and used to substitute for the C gene in the 5′ half-genome. The full-length cDNA clone was generated by ligating a 6.2 kb *KpnI* fragment of the 3′ half-genome, corresponding to nt 4497–10723, to the unique *KpnI* site of the mutant 5′ half-genome and transforming the ligated products into *Escherichia coli* strain DH5αF<sup>(Invitrogen)</sup>. Synthesis of capped *in vitro* RNA transcripts and generation of mutant viruses from the RNA transcripts in C6/36 cells were carried out as described previously (Sriburi et al., 2001). Mutant viruses were amplified by one or two passages in C6/36 cells. Introduced mutations were confirmed by sequencing of the C coding region of the full-length cDNA clones and the corresponding amplified viruses as described previously (Sriburi et al., 2001). Quantification of infectious viruses was performed by using a focus immunosassay titration employing PS cells, as described previously (Keelapang et al., 2004).

**Indirect immunofluorescence analysis (IFA).** PS, Vero or C6/36 monolayers grown on glass coverslips in 35 mm dishes were incubated with virus suspension in a final volume of 1 ml for 2 h. The virus suspension was removed and 3 ml maintenance medium containing FBS at 3 % (PS cells), 2 % (Vero cells) or 1.5 % (C6/36 cells) was added, followed by incubation at appropriate temperatures. At designated time points, coverslips were removed manually, washed with PBS and fixed with 3.7 % formaldehyde for 10 min, followed by 2 % Triton X-100 permeabilization for 10 min at room temperature (Keelapang et al., 2004). Cells were then reacted with the mAbs, followed by Cy3-conjugated goat anti-mouse IgG antibody (6F3, 4G2 and MOPC21) or Alexa 488-conjugated goat anti-mouse IgM antibody (NS1-1F) (Jackson ImmunoResearch) and visualized under a fluorescence microscope (Provis AX; Olympus). Confirmation of the location of nucleoli was done by further staining of infected PS and Vero cells with a rabbit antibody specific for human nucleolin (Santa Cruz), followed by Alexa 488-conjugated goat anti-rabbit IgG antibody (Molecular Probes). Images were recorded without selection, each field under both transmitted light and fluorophore-specific excitation wavelength, for subsequent examination by visual inspection. Typically, five fields at fixed positions containing about 100–200 cells were analysed for each coverslip. Infected cells were identified as those with fluorescent signal in the cytoplasm and/or nucleus. The percentage of cells with nucleolar C protein localization was determined as the proportion of infected cells in which staining for the C protein was visible in the nucleoli. The nucleolus was identified as a prominent, well-circumscribed structure in the nucleus when observed under a light microscope using differential interference contrast (DIC). Positive fluorescence staining was considered to be nucleolar only when it occupied the same position, had the same shape and was in focus when viewed in the same plane as the nucleolar structure that was observed with DIC. Statistical analysis was performed by using a t-test.

**Kinetics of virus replication.** Confluent PS, Vero or C6/36 cell monolayers in a T-25 flask were incubated with 2 ml virus suspension, using an m.o.i. of 0.01 for PS cells and of 0.001 for C6/36 and Vero cells, respectively. Following 2–4 h incubation at appropriate temperatures to allow virus adsorption, virus suspensions were removed. Cells were washed three times prior to the addition of maintenance medium with reduced FBS. Culture supernatants were collected on days 0, 1, 2, 3, 4, 5 and 7 for the quantification of infectious virus.

**RESULTS**

**Kinetics of C protein localization to nuclei and nucleoli of dengue virus-infected cells**

Several investigators have reported different patterns of dengue virus and other flavivirus C protein nuclear localization, or lack thereof, in many cell lines, which may reflect cellular differences and methodological variations (Tadano et al., 1989; Bulich & Aaskov, 1992; Westaway et al., 1997; Ng et al., 2001; Wang et al., 2002; Mori et al., 2005; Oh et al., 2006). To affirm the phenomenon in this study, subcellular localization of dengue virus C protein was assessed during infection of
staining of infected PS cells with mAb 6F3, an anti-C antibody, following formaldehyde and Triton X-100 treatment revealed that the C protein was found in the cytoplasm and the nuclei, particularly the nucleoli (Fig. 1a). Diffuse staining of the C protein in the nucleoplasm, which generally accompanied nucleolar staining, was less intense than nucleolar staining; it became apparent when cytoplasmic staining was weak or not detectable (Fig. 1b). In contrast, only positive cytoplasmic staining was observed with anti-E and anti-NS1 antibodies. An irrelevant antibody did not result in visible fluorescence (Fig. 1a).

Staining of the C protein in the nuclei was detected as frequently in strain 16681-infected Vero cells as in PS cells. Like in PS cells, staining of the C protein in the nucleoplasm of Vero cells was not as strong as nucleolar staining and was clearly visible when cytoplasmic staining was weak (Fig. 1b). Nucleolar localization of the C protein in dengue virus-infected PS and Vero cells was confirmed by double staining with mAb 6F3 and a rabbit antibody against human nucleolin (Fig. 1c). This anti-human nucleolin antibody failed to react with C6/36 cells (data not shown). Nucleolar staining also appeared to occur in infected C6/36 cells, although it was rarely observed. Fig. 1(d) shows examples of cytoplasmic only and cytoplasmic plus nuclear staining in all three cell types tested. Other fixation and permeabilization methods, including cold acetone fixation (Bulich & Aaskov, 1992), methanol fixation (Tadano et al., 1989) and formaldehyde fixation followed by methanol treatment (Westaway et al.,

two mammalian cell lines (PS and Vero) and a mosquito cell line (C6/36) with a dengue serotype 2 virus, strain 16681, by using indirect IFA. At 48 h after infection, infection and permeabilization, cells were reacted with 6F3, an anti-C mAb; 4G2, an anti-E mAb; MOPC21, an isotype-matched control antibody; or NS1-1F, an anti-NS1 mAb. This was followed by either Cy3-conjugated goat anti-mouse IgG antibody for the first three antibodies, or Alexa 488-conjugated goat anti-mouse IgM antibody in the case of NS1-1F. (b) Double staining of dengue virus C protein and nuclear DNA in infected PS and Vero cells. At 24 h (PS) or 48 h (Vero) after infection, cells were fixed, permeabilized and reacted with mAbs 6F3 and NS1-1F, followed by Cy3-conjugated goat anti-mouse IgG antibody, Alexa 488-conjugated goat anti-mouse IgM antibody and DAPI (4,6-diamidino-2-phenylindole). Results of anti-NS1 staining are not shown. (c) Double staining of C and nucleolin proteins in infected PS and Vero cells. At 24 h after infection, cells were fixed, permeabilized and reacted with mAbs 6F3 and 23 (H-250), an anti-nucleolin rabbit antibody, followed by Cy3-conjugated goat anti-mouse IgG antibody and Alexa 488-conjugated goat anti-rabbit IgG antibody. To the left of each fluorescence image are the same cells viewed under transmitted light illumination. Nc(−) and Nc(+) represent negative and positive nucleolar localization, respectively.

Fig. 1. Indirect immunofluorescence staining of C protein in dengue virus strain 16681-infected cells. (a) PS cells grown on coverslips were infected (m.o.i. = 0.5) for 48 h. After fixation and permeabilization, cells were reacted with 6F3, an anti-C mAb; 4G2, an anti-E mAb; MOPC21, an isotype-matched control antibody; or NS1-1F, an anti-NS1 mAb. This was followed by either Cy3-conjugated goat anti-mouse IgG antibody for the first three antibodies, or Alexa 488-conjugated goat anti-mouse IgM antibody in the case of NS1-1F. (b) Double staining of dengue virus C protein and nuclear DNA in infected PS and Vero cells. At 24 h (PS) or 48 h (Vero) after infection, cells were fixed, permeabilized and reacted with mAbs 6F3 and NS1-1F, followed by Cy3-conjugated goat anti-mouse IgG antibody, Alexa 488-conjugated goat anti-mouse IgM antibody and DAPI (4,6-diamidino-2-phenylindole). Results of anti-NS1 staining are not shown. (c) Double staining of C and nucleolin proteins in infected PS and Vero cells. At 24 h after infection, cells were fixed, permeabilized and reacted with mAbs 6F3 and 23 (H-250), an anti-nucleolin rabbit antibody, followed by Cy3-conjugated goat anti-mouse IgG antibody and Alexa 488-conjugated goat anti-rabbit IgG antibody. To the left of each fluorescence image are the same cells viewed under transmitted light illumination. Nc(−) and Nc(+) represent negative and positive nucleolar localization, respectively.

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1997), were attempted with PS and C6/36 cells. All gave similar staining patterns, but with less clarity (data not shown).

Due to the distinctive shape of nucleoli and their strong signal intensity following staining with anti-C mAb, the kinetics of C protein nuclear localization during virus infection of PS and Vero cells were next assessed by counting cells with nucleolar staining among infected cells. Confluent monolayers were infected with dengue virus strain 16681 at an m.o.i. of 6 and cells were harvested at regular intervals post-infection. In PS cells, nucleolar staining was visible in virtually all infected cells at 6 h after infection and remained at high levels throughout the course of infection (Fig. 2a). Similarly high proportions of infected cells with C protein nucleolar localization were observed when a lower m.o.i. of 0.6 was employed (data not shown). In Vero cells, nucleolar staining with mAb 6F3 was detected in some infected cells at 6 h and then in the vast majority of infected cells from 12 to 72 h post-infection (Fig. 2b). However, infected C6/36 cells with localization of C protein in the nucleoli were rarely observed in the cell population chosen for the analysis at any time point (Fig. 2c). In all three cell lines, the proportion of infected cells, identified as those stained by mAb 6F3 in any part of the cell, increased steadily and reached 100% by 72 h post-infection. It was remarkable that, whilst dengue virus was able to replicate efficiently in the three cell lines employed, nuclear localization of C protein was not uniformly present in these cell lines. Our observations of C protein localization to the nucleolus in the two infected mammalian cell lines were similar to those of Tadano et al. (1989) and Wang et al. (2002), and also concurred with the findings of Bulich & Aaskov (1992) that C protein nucleolar localization in C6/36 cells is a rare event.

A study of the influenza virus nucleoprotein suggested that cell density could influence the nuclear localization of this viral protein (Bui et al., 2002). To investigate the effect of
initial cell density on nucleolar localization of dengue virus C protein, a range of concentrations of PS cells were seeded in 35 mm dishes to obtain very low (<50 % confluence), moderate, medium and high (100 % confluence) cell densities. At 24 h after seeding, cells were infected with strain 16681 at an m.o.i. of at least 4, then collected and processed for the detection of intracellular C protein. As shown in Fig. 2(d), the percentage of infected cells with C protein nucleolar localization was 65 % or higher at the three time points in all initial cell inputs. Under these conditions, the initial cell density did not affect the proportion of PS cells with C nucleolar localization.

Altered nuclear localization of mutant C proteins

Of the three regions of basic amino acids proposed as possible NLSs within the dengue virus C protein (Bulich & Aaskov, 1992), only residues 85–100 were previously demonstrated to facilitate nuclear localization (Wang et al., 2002). To test the role of the proposed NLS in the context of dengue virus infection directly, five full-length cDNA clones containing double alanine-substitution mutations at various positions of the C coding region were constructed (Table 1). C6/36 cells were chosen for the generation of virus mutants, as the rare occurrence of C nucleolar localization in C6/36 suggested that the phenotype loss would not affect viral replication severely in this cell line. The mutant cDNA clones generated four viable viruses, c(K6A,K7A), c(K73A,K74A), c(R85A,K86A) and c(R97A,R98A), from this cell line (Table 1). A mutant with quadruple alanine substitutions on both sides of the bipartite sequence at positions 85–86 and 97–98 was not viable; however, whether the failure to recover infectious particles was due to the intended quadruple mutation in the C coding region or to spurious mutation in other locations of the genome was not tested rigorously.

Nuclear localization of the mutated C protein was assessed in PS cells by infecting with the parent strain, 16681Pst(−), and the four mutants at an m.o.i. of 0.6, followed by indirect IFA using the anti-C mAb 6F3. This mAb recognizes a linear epitope encompassing residues 9–19 of the C protein; the introduced mutations in the C protein were unlikely to interfere with its binding. Whilst the nucleoli were positively stained with the anti-C mAb in >80 % of 16681Pst(−)-infected cells at 24, 48 and 72 h post-infection, staining of the nucleoli in c(K73A,K74A)- and c(R85A,K86A)-infected cells was reduced significantly at all three time points compared with the parent strain (Figs 3 and 4a). The reduction in nucleolar staining was not accompanied by an increase in nucleoplasmic staining, indicating that these mutations affected nuclear localization of the C protein, but did not result in its redistribution between the nucleoli and nucleoplasm. Intriguingly, in PS cells infected with c(K6A,K7A) and c(R97A,R98A), the initially high levels of nucleolar staining at 24 and 48 h were followed by significant reductions at 72 h (Figs 3 and 4a). The latter sets of mutations of the putative NLSs appeared to affect nuclear localization in a time-dependent manner in this cell line.

IFA of intracellular localization of the C protein generated by the parent and mutant dengue virus strains in infected Vero cells revealed similar outcomes. At 24, 48 and 72 h after infection, C proteins generated by the parent strain, mutants c(K6A,K7A) and c(R97A,R98A) were found in the nucleoli in the majority of infected cells, indicating that the two mutations did not affect the nuclear-localization phenotype in Vero cells (Figs 3 and 4b). As observed in PS cells, C proteins generated by c(K73A,K74A) and c(R85A,K86A) were found significantly less frequently in the nucleoli of Vero cells than that of the parent virus at all three time points (Figs 3 and 4b). Thus, only mutations at positions 73–74 and 85–86 affected subcellular localization of the C protein in Vero cells.

In summary, our results suggest that at least two of the predicted NLSs of the dengue virus C protein are required for nuclear localization of C in the two mammalian cell lines tested, although the IFA technique used does not reveal the mechanism by which this phenomenon occurs. Previous observations in which the C protein without its putative NLSs appeared to affect nuclear localization in a time-dependent manner in this cell line.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Amino acid residues*</th>
<th>Titre (f.f.u. ml⁻¹)</th>
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<tbody>
<tr>
<td>16681</td>
<td>KKA R</td>
<td>KKS K</td>
</tr>
<tr>
<td>16681Pst(−)</td>
<td>KKA R</td>
<td>KKS K</td>
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<tr>
<td>c(K6A,K7A)</td>
<td>AAR A</td>
<td>KKS K</td>
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<tr>
<td>c(K73A,K74A)</td>
<td>KKA A</td>
<td>ASK K</td>
</tr>
<tr>
<td>c(R85A,K86A)</td>
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<td>KKS K</td>
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<tr>
<td>c(R97A,R98A)</td>
<td>KKA K</td>
<td>KKS K</td>
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<tr>
<td>c(R85A,K86A,R97A,R98A)</td>
<td>KKA K</td>
<td>KKS K</td>
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Table 1. Dengue virus strains employed in the study

* Amino acids predicted to be responsible for C protein nuclear localization are indicated in bold. Mutated amino acid residues are underlined. Numbers indicate position of the amino acid residues.
et al., 2002) may indicate that C protein nucleolar localization is an active process; however, passive nuclear localization of the C protein cannot be ruled out, as the protein is small enough for this to occur.

**Reductions of mutant virus replication in PS, Vero and C6/36 cells**

To assess how well the mutant viruses could replicate in comparison with the parent strain, all four mutants and the parent strain were subjected to multi-step kinetic studies in PS, Vero and C6/36 cells at an m.o.i. that resulted in gradual accumulation of infectious virus over a period of several days. The four C protein mutants replicated in PS cells to plateau levels approximately 5–50 times lower than that for the parent strain (Fig. 5a). In Vero cells, whilst the replication of c(K6A,K7A), c(K73A,K74A) and c(R85A,K86A) was markedly lower than that of the parent virus, the replication of c(R97A,R98A) was undetectable (Fig. 5b). An inability of c(R97A,R98A) to replicate in Vero cells was unexpected, as this C protein mutant was not defective in nuclear localization (Figs 3 and 4). It is possible that c(R97A,R98A) was able to initiate infection of Vero cells, but that one or more subsequent step(s) in the

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**Fig. 4.** Kinetics of parent and mutant C protein nucleolar localization in PS and Vero cells. (a) PS and (b) Vero cells were infected with strain 16681Pst(−) and the four mutant strains. At 24, 48 and 72 h after infection, nucleolar localization of C protein was analysed by indirect immunofluorescence staining. Data represent means and SEM from three or five separate experiments. *C protein nucleolar localization was reduced significantly compared with the parent strain at the corresponding time points (P<0.005).
generation and release of infectious virus particles was faulty. All four mutants replicated to similar titres in C6/36 cells, but these were lower than the titre of the parent strain in this cell line (Fig. 5c). Thus, all four C protein mutants exhibited reduced replication in these cell lines, regardless of their C protein nucleolar-localization ability.

In an attempt to examine whether the observed kinetics of virus replication reflected growth characteristics of original mutant viruses or revertants that might have emerged during replication in mammalian cells, mutant viruses collected after infection of PS cells were subjected to RNA extraction followed by RT-PCR of the C gene and sequencing analysis. The sequence data revealed that, on day 7, two mutants, c(R85A,K86A) and c(R97A,R98A), retained their introduced mutations with no additional mutation in the C gene. Whilst c(K6A,K7A) had gained a very low level of an additional Ala-to-Val substitution at position 6 by day 7, c(K73A,K74A) contained an equal mixture of the intended mutations and an additional point mutation (C317A), resulting in an Ala-to-Asp substitution at position 74, on day 7 and, albeit at lower levels, days 3 and 5 after infection. Following infection of Vero cells, additional mutations in c(K73A,K74A), generating additional Ala-to-Pro substitutions at positions 73 and 74, were observed on days 5 and 7. As these additional mutations in c(K73A,K74A) did not involve basic residues and therefore were unlikely to revert functionally as NLSs, it appeared that there was no selective pressure for c(K73A,K74A) to regain its nucleolar-localization ability. These data suggest further that C protein nucleolar localization is not needed for the replication of dengue virus.

**DISCUSSION**

In this study, we have investigated the nuclear-localization property of dengue virus C protein containing double alanine substitutions of the three putative NLSs in the context of virus infection in two mammalian cell lines. Our results concurred with previous data based on observations of recombinant dengue virus C protein expressed in HeLa cells, which implicated R85–K86 in C protein nuclear localization (Wang et al., 2002). Contrary to previous observations, K73–K74 were also found to contribute to C protein nuclear localization. Our observations that mutation of either K73–K74 or R85–K86 led to significant reductions of C protein nuclear localization in both mammalian cell lines suggest that K73–K74 and R85–K86 may function in a concerted manner, perhaps through cooperative binding to the same carrier molecule during transport into the nucleus. Two other stretches of basic amino acids, K6–K7 and R97–R98, appeared to facilitate C protein nuclear localization in a time-dependent manner in PS cells only.

With the exception of K6 and K7, the other basic residues, i.e. K73, K74, R85, K86, R97 and R98, are distributed on the solvent-exposed side of the α4–α4’ helices and adjacent loops of the C protein homodimeric molecule, which are enriched with positive charges and have been proposed to be involved in RNA binding (Ma et al., 2004) (Fig. 6a, b). Although the positions of K6 and K7 are unknown, due to a lack of structural information, they might also be located on the same side of the protein as the other positively charged residues. It is possible that all of these basic residues serve to bind viral RNA or nuclear-translocation mediators through charge–charge interactions. In JEV, residues involved in nuclear localization of the C protein
were mapped to G42 and P43 (Mori et al., 2005). These two residues are located in a relatively hydrophobic bend between the α1 and α2 helices on the hydrophobic side of the C dimer and may contribute to nuclear localization of the JEV C protein through a different mechanism.

The mechanism by which the dengue virus C protein localizes to the nucleus is currently unknown. The C protein may be transported into the nucleus via an active process involving one or more carrier proteins within the importin α/β superfamily (reviewed by Görlich & Kutay, 1999; Pemberton & Paschal, 2005). This scenario is plausible as the three regions of basic amino acids at residues 6–9, 73–76 and 85–100 of dengue virus C protein have the appearance of classical NLSs (Dingwall & Laskey, 1991). Furthermore, recombinant C protein lacking aa 73–100 or 85–100 did not localize to the nucleus of transfected HeLa cells and residues 85–100 were able to mediate nuclear localization of multiple copies of green fluorescent protein (Wang et al., 2002). Alternatively, the C protein may interact directly with components of the nuclear-pore complex, as observed with Stat1 (signal transducer and activator of transcription factor 1; Marg et al., 2004), or ‘piggyback’ on other cellular proteins that are transported actively to the nucleus, as in the cases of the mouse DNA primase p46 subunit (Mizuno et al., 1996) and the splicing factor SIPP1 (Llorian et al., 2005). Intriguingly, a number of other viral proteins, such as dengue virus NS5 (Brooks et al., 2002), adeno-associated virus capsid (Grieger et al., 2006) and influenza A virus NS1 proteins (Melén et al., 2007), also possess more than one NLS, and some of these NLS sequences are utilized in other protein–protein or protein–DNA/RNA interactions. For example, one of the NLSs of dengue virus NS5 binds NS3 in the formation of RNA replicase complex (Brooks et al., 2002). Having multiple NLSs may, therefore, be useful in sustaining the nuclear-localization process when certain NLSs are being used for other purposes.

It is not yet feasible to exclude the possibility that dengue virus C protein diffuses passively through the nuclear-pore complex and that the basic amino acid stretches act as nuclear/nucleolar-retention sequences, as do those in the avian infectious bronchitis virus nucleocapsid protein (Reed et al., 2006), thus preventing the C protein from escaping from the nucleus back into the cytoplasm. Mutation affecting the retention function of these basic amino acid stretches should also result in a reduced accumulation of the C protein in the nucleus. The reduction of C protein nuclear localization observed only at the late time point after infection of PS cells with mutants c(K6A,K7A) and c(R97A,R98A) may also be due to the time course-dependent impairment of this function.

**Fig. 6.** (a) Structure of the dengue virus C protein homodimer with positions of the mutated residues. The structure is viewed from the charge-rich side of the protein. (b) As for (a), but rotated 90° about a horizontal axis. A ribbon–cylinder diagram and a space-filling model are shown in the left and right panels, respectively. Grey depicts hydrophilic amino acid residues; blue and red depict basic and acidic amino acid residues, respectively; yellow represents mutated residues. Mutated residues, except for K6 and K7, are labelled. The coordinates were obtained from the Protein Data Bank, accession number 1R6R (Ma et al., 2004), and the structure was generated by using the program Cn3D viewer, v. 4.1 (Wang et al., 2000).
The three cell lines used for the study of nuclear localization of dengue virus C protein in this study were chosen for their ability to support efficient dengue virus replication, with PS and Vero cells derived from mammalian sources and C6/36 cells from one of its natural mosquito vectors. Based on these cell lines, a striking contrast in the nuclear localization of wild-type C protein was observed when testing was performed in the mosquito cells compared with the mammalian cells. Whilst nuclear localization of dengue virus C protein has been detected previously in other mammalian cell lines (Tadano et al., 1989; Bulich & Aaskov, 1992; Wang et al., 2002), suggesting the conservation of this C protein function in host cells of mammalian origin, the paucity of C protein nuclear localization in C6/36 cells needs to be confirmed, most relevantly in infected mosquitoes. Moreover, our C protein mutant viruses differed in the nuclear-localization property of the C protein and their replication in the two mammalian cell lines employed. It is possible that the differences in nuclear localization of mutated C proteins in PS and Vero cells are due to the heterogeneity and availability of carrier proteins in these mammalian cell lines, whereas no carrier protein in C6/36 cells is able to perform this function for both the wild-type and mutated C proteins. Nuclear localization of cellular proteins is partly controlled by differential expression of carrier proteins in different tissues (Nachury et al., 1998; Kohler et al., 1999; Hogarth et al., 2006), and their expression may be influenced by various cellular stimulations (Mizrachy et al., 2004; Tao et al., 2004). A situation where different carrier proteins may interact with the same cargo to facilitate nuclear localization under different conditions (Jans et al., 2000) may underlie the differences that are observed when C protein mutant viruses are tested in cell lines with differences in host species of origin, tissue type and differentiation status.

The importance of nuclear localization of the C protein in virus replication has been documented in a number of studies. Mutants of JEV and porcine reproductive and respiratory syndrome virus, whose C proteins lack nuclear-localization ability, did not replicate to high titers, and reversions were associated with enhanced replication (Mori et al., 2005; Lee et al., 2006). In this study, the four dengue virus mutants did not show marked variations in replication in PS cells, despite a lack of C protein nuclear localization in c(K73A,K74A) and c(R85A,K86A). In Vero cells, c(K73A,K74A) replicated to a much higher level than c(R85A,K86A) and the other mutants with intact C protein nuclear-localization capability [c(K6A,K7A) and c(R97A,R98A)]. Furthermore, there appeared to be no selective pressure for c(K73A,K74A) and c(R85A,K86A) to regain their nuclear-localization ability during their replication in the mammalian cell lines employed. These results suggest that C protein nuclear localization may not be associated directly with efficient dengue virus replication in the cell lines tested. To investigate the significance of C protein nuclear localization further, the mutant viruses could be tested in cell types that may be more related to natural dengue virus infection, such as human dendritic cells, monocytes and macrophages. It cannot be ruled out that the reduced replication of these mutants may be attributed to disruption of other functions of the C protein, such as genomic RNA binding (Khromykh & Westaway, 1996; Ma et al., 2004) and, albeit less likely, being part of the substrate for C–prM cleavage by NS2B/NS3 protease in the case of c(R97A,R98A) (Amberg & Rice, 1999; Li et al., 2005; Niyomrattanakit et al., 2006).

Upon translocation into the nucleus, dengue virus C protein may perform a number of functions. A proportion of dengue virus genomic RNA is synthesized in the nuclei of infected cells (Uchil et al., 2006) and the C protein may facilitate trafficking of the RNA out of the nucleus. This, however, may not represent its major function, as the viral RNA-dependent RNA polymerase activity was detected at the nuclear periphery (Uchil et al., 2006), whereas previous and current studies did not reveal a preference of the C protein for this location (Tadano et al., 1989; Wang et al., 2002). On the other hand, it is remarkable that the C protein was observed in the nucleoli of infected cells as early as 6 h after infection, well before the formation of infectious viruses (Keelapang et al., 2004). Nucleolar accumulation of the C protein may result from its interaction with rRNA or certain nucleolar proteins, whose diverse functions include ribosome synthesis, mRNA processing and DNA replication, as well as regulation of the cell cycle and stress responses (Andersen et al., 2005; Boisvert et al., 2007). Localization of the C protein to the nucleolus early in infection may modulate the functions of these nucleolar proteins so as to maintain the infected cells at the stage that allows optimal virus replication (Helt & Harris, 2005; Hiscox, 2007). Also, the C protein may help to avert certain cellular stress responses to the infection, including apoptosis (Marianneau et al., 1997; Avirutnan et al., 1998), to minimize premature cell dysfunction and death. Comparison of the cell-cycle profiles and the activation of apoptotic pathways of cells infected with wild-type dengue virus or a mutant strain lacking C protein nuclear-localization ability may indicate whether intranuclear C protein is capable of affecting the cell cycle and apoptotic cell death. These studies, together with the identification of nuclear protein(s) that interact specifically with the C protein, should provide a better understanding of the functional consequences of C protein nuclear localization and how this process could be advantageous to dengue virus.

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