Association of dengue virus NS1 protein with lipid rafts

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Received 26 November 2007
Accepted 18 June 2008

During the replication of dengue virus, a viral non-structural glycoprotein, NS1, associates with the membrane on the cell surface and in the RNA replication complex. NS1 lacks a transmembrane domain, and the mechanism by which it associates with the membrane remains unclear. This study aimed to investigate whether membrane-bound NS1 is present in lipid rafts in dengue virus-infected cells. Double immunofluorescence staining of infected HEK-293T cells revealed that NS1 localized with raft-associated molecules, ganglioside GM1 and CD55, on the cell surface. In a flotation gradient centrifugation assay, a small proportion of NS1 in Triton X-100 cell lysate consistently co-fractionated with raft markers. Association of NS1 with lipid rafts was detected for all four dengue serotypes, as well as for Japanese encephalitis virus. Analysis of recombinant NS1 forms showed that glycosylated NS1 dimers stably expressed in HEK-293T cells without an additional C-terminal sequence, or with a heterologous transmembrane domain, failed to associate with lipid rafts. In contrast, glycosylphosphatidylinositol-linked recombinant NS1 exhibited a predilection for lipid rafts. These results indicate an association of a minor subpopulation of NS1 with lipid rafts during dengue virus infection and suggest that modification of NS1, possibly lipidation, is required for raft association.

INTRODUCTION

Dengue virus is an important mosquito-borne human pathogen causing illnesses ranging from mild febrile illness and dengue fever to life-threatening dengue haemorrhagic fever and dengue shock syndrome (Halstead, 1997). It consists of four serotypes and belongs to the genus Flavivirus of the family Flaviviridae, which includes viruses such as yellow fever virus, Japanese encephalitis virus (JEV), tick-borne encephalitis virus and West Nile virus (Lindenbach & Rice, 2001). The genome of flaviviruses is a single-stranded RNA that encodes at least 10 known proteins (C, prM, E, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). NS1, a relatively conserved glycoprotein, exists in multiple forms in different compartments of virus-infected cells (Flamand et al., 1992; Jacobs et al., 2000; Leblois & Young, 1995; Mason, 1989). Following proteolytic cleavage of the viral polyprotein, NS1 is present in the luminal side of the endoplasmic reticulum, mainly as homodimeric molecules (Falgout & Markoff, 1995). Intracellular dimeric NS1 associates with the cellular membrane, but the structural basis for membrane association is not known. NS1 lacks a transmembrane domain and there is no evidence for post-translational protein modification that can explain its affinity for the membrane. The possibility of glycosylphosphatidylinositol (GPI) linkage as suggested by in vitro transfection and HeLa cell infection studies (Jacobs et al.,...
2000) remains to be confirmed in cells of haematopoietic lineage. Ultrastructural localization studies have revealed that intracellular NS1 is associated with the virus replication complex in the perinuclear region, where it participates in viral RNA replication (Lindenbach & Rice, 1997, 1999; Mackenzie et al., 1996; Muylaert et al., 1996, 1997). NS1 is also present on the cell surface and is released into the extracellular compartment. In the latter, NS1 is found in the form of hexamers (Flamand et al., 1999). NS1 on the cell surface is implicated in signal transduction (Jacobs et al., 2000) and complement activation (Avirutnan et al., 2006; Brandt et al., 1970; Schlesinger et al., 1990).

Several proteins on the plasma membrane localize in lipid rafts to exert different functions. Lipid rafts are dynamic assemblies of liquid-ordered phase microdomains, highly enriched with cholesterol and sphingolipids, in the exoplasmic leaflet of the plasma membrane (Ikonen, 2001; Simons & Ikonen, 1997; Simons & Toomre, 2000). They selectively incorporate or exclude proteins on the cell membrane for particular cellular events, including signal transduction, endocytosis, transcytosis across endothelial cells and cholesterol homeostasis (Ikonen, 2001; Simons & Ikonen, 1997; Simons & Toomre, 2000). Lipid rafts are known to be preferred sites for the interaction between viruses and host cells. Simian virus 40 (Paragon & Lindsay, 1999), human immunodeficiency virus (HIV) (HIVmmartche et al., 1999; Mañes et al., 2000) and Epstein–Barr virus (Coffin et al., 2003; Dykstra et al., 2001; Higuchi et al., 2001) exploit raft-associated molecules on the target cell membrane for viral entry and evasion from host immune responses (van der Goot & Harder, 2001). Association of certain viral components with lipid rafts also facilitates the assembly and egress of viruses, as in the cases of influenza virus (Scheiffele et al., 1999), measles virus (Mani et al., 2000), HIV (Holm et al., 2003; Nguyen & Hildreth, 2000; Ono & Freed, 2003), filoviruses (Bavari et al., 2002) and herpesviruses (Lee et al., 2003).

The involvement of lipid rafts in flavivirus replication is poorly understood. This study investigated a possible association of dengue virus NS1 with lipid rafts during virus infection. Infected HEK-293T cells were utilized in a double immunofluorescence staining assay to examine localization and co-fractionation of NS1 with known lipid raft-associated molecules. Stable transfectants expressing recombinant NS1 with or without a C-terminal additional sequence were then employed to explore the association of different recombinant NS1 molecules with lipid rafts.

**METHODS**

**Cells, viruses and antibodies.** Human embryonic kidney epithelial cells (HEK-293T) were cultured in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone), 37 μg penicillin ml⁻¹, 60 μg streptomycin ml⁻¹ and 2 mM L-glutamine at 37 °C in a 5% CO₂ humidified atmosphere. Stable HEK-293T cells expressing the recombinant forms rNS1s, rNS1tm or rNS1v1 were established and maintained as described previously (Noisakran et al., 2007). Four serotypes of dengue virus (serotype 1, strain Hawaii; serotype 2, strain 16681; serotype 3, strain H87; serotype 4, strain H241) and JEV (strain Nakayama) were propagated in C6/36 mosquito cells. For infection, a monolayer of HEK-293T cells was incubated with virus at 37 °C for 3 h. The culture medium was then replaced with RPMI 1640 plus 10% FBS and incubated for 3 days. Mouse monoclonal antibodies (mAbs) recognizing linear epitopes (NS1-1F.1 or 2E11, and NS1-3F.1 or 1F11; Putthikunt et al., 2003) or a conformational epitope (1A4; Sittisiri, 1994) on dengue virus NS1 were employed in the form of culture medium or purified proteins. Clone 2E11 reacted with NS1 of all serotypes of dengue virus, as well as with JEV. Clone 1F11 was dengue serocomplex-specific, whereas clone 1A4 reacted only with NS1 from serotype 2. Antibodies specific for human CD55 (clone IA10, IgG2a), CD59 (clone SH8, IgG1) and CD147 (clone M6-E9, IgG2a) were prepared as described previously (Kasinrerk et al., 1999; Kinoshita et al., 1985; Sugita et al., 1994). An anti-human CD71 mAb (clone 3B8 2A1, IgG1) was purchased from Santa Cruz Biotechnology.

**Double immunofluorescence staining.** To assess co-localization of NS1, CD55 or CD71 with ganglioside GM1 on the cell surface, dengue virus-infected cells or NS1 transfectants (5 × 10⁶) were harvested on day 3 after viral infection or subculture, washed twice with washing buffer (PBS containing 1% BSA and 10 mM NaN₃) and incubated with 10% human AB serum in washing buffer on ice for 30 min to minimize non-specific binding. After washing twice, cells were incubated with murine mAbs against NS1 (clone 1A4, IgG2a), human CD55 and human CD71 molecules, or isotype-matched (IgG1) control antibody on ice for 1 h. Cells were then washed twice and incubated with Cy3-conjugated goat anti-mouse IgG antibody (Fc fragment specific, 6 μg ml⁻¹; Jackson ImmunoResearch) and Alexa Fluor 488-conjugated cholera toxin B (5 μg ml⁻¹; Molecular Probes) in washing buffer for 30 min on ice, followed by cap induction at 37 °C for 30 min. Stained cells were fixed with 4% paraformaldehyde at room temperature for 5 min and cold methanol at −20 °C for 5 min, centrifuged at 200 g for 5 min and resuspended in 20 μl fluorescence mounting medium (Dako). Aliquots of cell suspension were placed onto a glass slide and observed under a laser-scanning confocal microscope (LSM510 META; Carl Zeiss) using the ×63 objective lens. Co-localization of NS1 with CD55 or CD71 was determined as described above by using a mixture of anti-NS1 antibody (2E11, IgM) and anti-CD55 antibody (IgG1) or anti-CD71 (IgG1) antibody, and a mixture of Cy3-conjugated goat anti-mouse IgG antibody (Fc fragment specific) and Alexa Fluor 488-conjugated goat anti-mouse IgM antibody (λ-chain specific; Molecular Probes) in the first and second staining steps, respectively.

**Flotation gradient centrifugation assay.** Virus-infected cells or NS1 transfectants (1 × 10⁶) were removed by scraping and centrifuged at 200 g at 4 °C for 10 min. Cell pellets were washed twice with ice-cold PBS, resuspended in 1 ml lysis buffer [10 mM Tris/HCl (pH 7.5), 75 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100 and protease inhibitor cocktail] and incubated on ice for 30 min. Cell lysate was passed through a 26-gauge needle 15 times and then centrifuged at 1000 g at 4 °C for 10 min to remove cellular debris and nuclei. Clarified lysate was mixed with 1 ml 80% sucrose (w/v) in Tris buffer, placed at the bottom of an ultracentrifuge tube and overlaid with 8 ml 35% sucrose (w/v) in Tris buffer and 2.5 ml 5% sucrose (w/v) in Tris buffer. The lysate was centrifuged at 4 °C in an SW40 rotor (Beckman) for 20 h at 250,000 g. Thereafter, 1 ml fractions were collected from the top and subjected to immunoblot analysis for the detection of NS1, CD55, CD59, CD71 and CD147.

**Immunoblot analysis.** Individual sucrose gradient fractions (15–30 μl) were mixed with loading buffer [50 mM Tris/HCl (pH 6.8), 2% SDS, 0.1% bromophenol blue and 10% glycerol] in the presence

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or absence of 5 % β-mercaptoethanol, boiled (95 °C, 5 min) or left unheated, and subjected to electrophoresis by 10% SDS-PAGE. Proteins were transferred to PVDF membrane (Millipore) by using a semi-dry electroblotter. Non-specific binding sites on the membrane were blocked with 5 or 10 % skimmed milk in PBS for 1 h and the membrane was incubated with a murine mAb specific for NS1 (clone 1F11), CD55, CD59, CD71 or CD147 at 4 °C overnight. Following three washes with PBS, rabbit anti-mouse Ig antibody conjugated with horseradish peroxidase (Dako) at a dilution of 1:1000 was applied for 1 h at room temperature. The membrane was again washed three times and immunoreactive proteins were visualized using an enhanced chemiluminescence detection kit (Western Lightning Chemiluminescence Reagent Plus; Perkin Elmer). The size of protein bands was determined by comparison with a pre-stained protein ladder (Fermentas).

RESULTS

Localization of NS1 with lipid raft markers on the cell surface

An association of NS1 with lipid rafts on the surface of dengue virus-infected cells was examined by double immunofluorescence staining. HEK-293T cells were infected with strain 16681 at an m.o.i. of 1 for 3 days and then reacted with mAbs specific for NS1 (clone 1A4), CD55 and CD71, followed by Cy3-conjugated anti-mouse IgG antibody and Alexa Fluor 488-conjugated cholera toxin B. In this double staining, CD55, a GPI-anchored raft resident protein (Hatano et al., 2007; Stuart et al., 2002), clearly localized in the same areas as ganglioside GM1 on the cell surface, whereas CD71, a transmembrane protein known to be excluded from lipid rafts (Bavari et al., 2002; Pickl et al., 2001), was observed in distinct locations from ganglioside GM1 (Fig. 1a, middle and lower panels). Similar to CD55, NS1 showed a significant overlap in the locations of fluorescent signal with ganglioside GM1 on the cell surface (Fig. 1a, top panel). Co-localization of NS1 with raft-associated molecules was further assessed by simultaneous staining of NS1 and CD55, or NS1 and CD71. In the second set of immunofluorescence stainings, a different antibody of the IgM class (clone 2E11) and Cy3-conjugated anti-mouse IgM antibody were employed for the detection of NS1. Again, NS1 localized in the same patches as CD55, but not CD71, on the surface of virus-infected cells (Fig. 1b). These results indicated that NS1 on the surface of strain 16681-infected cells associates with lipid rafts.

Co-fractionation of NS1 in cell lysate with CD55

A number of proteins associated with lipid rafts can be identified based on the insolubility of lipid rafts in non-ionic detergent and low buoyant density in equilibrium flotation centrifugation at low temperature (Simons & Toomre, 2000). A flotation gradient centrifugation assay was thus employed to verify an association of NS1 with lipid rafts. Clear lysates of virus-infected HEK-293T cells were prepared in ice-cold lysis buffer containing 1 % Triton X-100, adjusted to 40 % sucrose and subjected to centrifugation in a discontinuous sucrose gradient. Fractions of 1 ml were collected from the top and assessed for the presence of NS1, CD55 and CD71 by immunoblot analysis. Following centrifugation, CD55 was detected mainly in fractions 2–4, with a peak in fraction 3, indicating the migration of lipid rafts and raft-associated molecules to these upper fractions during centrifugation (Fig. 2a). In contrast, CD71, a non-raft resident protein, was observed in fraction 10 and more prominently in fractions 11 and 12 at the bottom of the gradient (Fig. 2b).
CD59, a known raft resident (Pickl et al., 2001), and CD147, a known non-raft resident (Staffler et al., 2003), also exhibited similar fractionation profiles to those of CD55 and CD71, respectively (Fig. 2c and Fig. 2d). CD55 and CD71 were utilized throughout the study as controls for raft- and non-raft-associated molecules. Under the same conditions, NS1 in strain 16681-infected cell lysate exhibited a bimodal distribution in raft fractions 2–4 and non-raft fractions 10–12 (Fig. 2e). The intensity of NS1 signal was quite different in these two sets of fractions, with the majority residing in the lower non-raft fractions. A titration experiment revealed that NS1 in fraction 3 constituted about 1/20 of that present in fraction 11 (Fig. 2f), indicating that only a small proportion of NS1 in virus-infected cells is raft-associated. This minor subpopulation of NS1 was consistently detected in four subsequent Triton X-100 lysis and flotation gradient centrifugation experiments (Fig. 2g).

Further investigations were carried out to determine whether NS1 raft association is unique to strain 16681 or represents a common phenotype that is shared with related flaviviruses. HEK-293T cells were infected with virus strains belonging to dengue serotypes 1, 3 and 4, as well as with JEV. Infected cells were lysed in Triton X-100 and cell lysates were subjected to flotation gradient centrifugation separation. The fractionation profiles of CD55 and CD71 observed in lysates of cells infected with these four viruses were similar to those of strain 16681-infected cells, although with some variation in the locations of the fraction with peak CD55 signal (Fig. 3). In these cases, a small proportion of NS1 partitioned into raft fractions 2–4 overlapping with CD55, whilst the remainder was found in fractions 10–12 (Figs 2 and 3). NS1 and CD55). The disparity in signal intensity of NS1 between the two sets of fractions was comparable among the viruses tested. These results suggested that the association of a minor
subpopulation of NS1 with lipid rafts is a general property of dengue virus and possibly other flaviviruses as well.

**Lipid raft association of GPI-linked recombinant NS1**

Localization of proteins in lipid rafts can be mediated by several mechanisms, including a transmembrane association, a GPI linkage or other post-translational modifications such as palmitoylation (Pike, 2004). NS1 lacks a transmembrane hydrophobic domain and the evidence for a GPI anchor and other lipid modifications of NS1 during virus infection remain unclear. In an attempt to determine whether glycosylated NS1 is inherently able to associate with lipid rafts or whether additional modification is required for raft association, HEK-293T cell lines expressing distinct recombinant forms of NS1 were utilized (Noisakran et al., 2007). These transfectants expressed glycosylated NS1 dimers without C-terminal additional sequence (rNS1s form), with a C-terminal transmembrane domain (rNS1tm form) or with a short NS2A sequence that allowed GPI-linkage of NS1 (rNS1v1 form) (Noisakran et al., 2007). Among the three recombinant NS1 forms, only rNS1tm and rNS1v1 associated with cellular membrane and were detectable on the cell surface. The rNS1s form was absent from the cell surface as it was mainly secreted into the culture medium (Noisakran et al., 2007).

As observed with virus-infected cells, flotation gradient centrifugation analysis of transfected cell lysates revealed that CD55 was present largely in fractions 2–4, whereas CD71 was detected in fractions 11 and 12 (Fig. 4a–c). Under the same conditions, rNS1s and rNS1tm partitioned into the lower non-raft fractions (Fig. 4b and c), indicating an inability of NS1 without C-terminal addition or with a C-terminal transmembrane domain to associate with lipid rafts. In contrast, rNS1v1 was detected predominantly in the raft fractions with a minimal amount in the non-raft fractions (Fig. 4a). The proportions of NS1 in the raft versus the non-raft fractions obtained from the rNS1v1 transfectants were markedly different from those obtained from virus-infected cells. These findings suggested that recombinant GPI-linked NS1 molecules have a predilection for lipid rafts.

Association of recombinant NS1 with lipid rafts was next assessed by double staining of NS1 and ganglioside GM1 on the cell surface. Only transfectants expressing rNS1v1 and rNS1tm were used, as the rNS1s form was not detected on the cell surface (Noisakran et al., 2007). On the surface of rNS1v1 and rNS1tm transfectants, CD55 resided in the same overlapping areas as ganglioside GM1 (Fig. 5a, b), whilst a distinct pattern of surface localization was observed with CD71 (Fig. 5a, b). As expected, rNS1v1 localized with ganglioside GM1 on the cell surface (Fig. 5a). The majority of rNS1tm, however, was detected in areas different from the ganglioside GM1 (Fig. 5b). A lack of rNS1tm co-localization with ganglioside GM1 on the cell surface agreed well with the finding that rNS1tm did not associate with lipid rafts in the flotation gradient centrifugation analysis. Taken together, analysis of recombinant NS1-expressing transfectants indicated that NS1 molecules without any modification at the C terminus, or with a C-terminal transmembrane hydrophobic domain, are unable to associate with lipid rafts. GPI linkage of NS1, as in the rNS1v1 form, allowed NS1–raft association, which was able to occur independently of other viral proteins.

**DISCUSSION**

Lipid rafts are specialized microdomains on the plasma membrane that assist in the lateral movement of associated molecules in the lipid bilayer and function as a platform for...
membrane traffic, protein sorting and cell signalling (Ikonen, 2001; Verkade & Simons, 1997). Cellular proteins that localize in lipid rafts include GPI-linked proteins, certain transmembrane proteins, heterotrimeric G proteins, immune receptors and doubly acylated or cholesterol-linked proteins (Simons & Toomre, 2000). In this study, dengue virus NS1 was found to associate with lipid rafts during infection with all four serotypes of dengue virus, as well as with JEV. Evidence in support of NS1–raft association was generated from flotation gradient centrifugation analysis of infected-cell lysates and double immunofluorescence staining of cell-surface NS1 with known raft-associated molecules. The flotation gradient centrifugation analysis reveals that a minority of total NS1 in infected cells was associated lipid rafts. Co-localization of NS1 with ganglioside GM1 and CD55 on the cell surface identified the plasma membrane as a location where NS1–raft association can occur. In addition, some of the raft-associated NS1 might be found inside virus-infected cells, as NS1 comes into contact with lipid rafts in the Golgi apparatus, where lipid rafts are formed (Ikonen, 2001; Nichols et al., 2001; van Blitterswijk et al., 2003), during the transport of NS1 to the cell surface. Whether NS1 can be found in association with lipid rafts in other specific intracellular locations is an important issue that needs to be addressed.

Dengue virus NS1 lacks a hydrophobic transmembrane domain and the mechanism by which it associates with the membrane remains unclear. An increase in the overall hydrophobicity following NS1 homodimerization has been proposed to explain its affinity for the cellular membrane (Winkler et al., 1989). However, our previous finding that recombinant NS1 expressed in stably transfected cells without C-terminal modification was absent from the cell surface (Noisakran et al., 2007) suggests that dimerization alone is insufficient for NS1 association with the plasma membrane. Analysis of the transfectants in this study revealed that both the 26-residue N-terminal region of NS2A, which serves as the signal sequence for GPI anchoring of NS1 (Jacobs et al., 2000; Noisakran et al., 2007), and the transmembrane region derived from platelet-derived growth factor receptor conferred the ability to associate with plasma membrane, but only the former allowed lipid raft association. The finding that the GPI-linked rNS1v1 form associated with lipid rafts in stable transfectants is suggestive of a possibility that, during dengue virus infection, GPI-linked or otherwise lipidated NS1 molecules, if there are any, can associate with lipid rafts. This result by itself does not constitute proof for the existence of GPI-linked NS1 and an association of such molecules with lipid rafts in dengue virus-infected cells. Although GPI linkage is a common mechanism for proteins in the secretory pathway to associate with lipid rafts, this is unlikely to be the only mechanism. S-Palmitoylated Wnt-1 and cholesterol-linked and N-palmitoylated hedgehog proteins are examples of non-GPI-linked, luminal proteins that associate with lipid rafts (Rietveld et al., 1999; Taipale et al., 2000; Zhai et al., 2004). It is also evident that, whilst the minority of native NS1 from infected HEK-293T cells was associated with lipid rafts, the opposite situation was observed in rNS1v1-transfected cells. The disparity hints at a greater complexity of post-translational modifications and protein interactions that take place in virus-infected cells compared with single-gene transfectants. A short hydrophobic sequence at

Fig. 4. Flotation gradient analysis of recombinant NS1. HEK-293T cells stably expressing rNS1v1 (a), rNS1s (b) or rNS1tm (c) were harvested on day 3 after subculture and lysed with ice-cold lysis buffer containing 1% Triton X-100. Clear lysates were adjusted to 40% sucrose and then subjected to a discontinuous sucrose density-gradient centrifugation. Fractions of 1 ml were collected from the top (fraction 1) to the bottom (fraction 12) of the centrifuge tube and assessed for the presence of NS1, CD55 and CD71 by immunoblot analysis using specific mAbs. High-molecular-mass NS1 bands have been observed previously in lysate of rNS1s stable transfectants, possibly due to aggregation (Noisakran et al., 2007).
the C terminus of the rNS1v1 form may allow efficient GPI linkage to occur in the transfectants but not in virus-infected cells, as this NS2A-derived segment would be connected to the rest of the NS2A sequence in infected cells. During virus infection, the interactions between intracellular NS1 and other viral non-structural proteins may affect the quantity and rate of transport of NS1 to the Golgi apparatus, where the GPI linkage occurs; such interactions are absent in the transfectants. Also, other viral proteins may influence the profiles and levels of host protein expression, viral and host protein interactions and host cellular responses that occur during virus infection. Whether the raft-associated NS1 molecules in dengue virus-infected cells are actually GPI linked or otherwise modified is currently under investigation.

In addition to serving as a trigger of complement activation (Avirutnan et al., 2006; Brandt et al., 1970), NS1 on the surface of infected cells has been implicated in signal transduction (Jacobs et al., 2000). An ability of NS1 to associate with lipid rafts appears to be conserved among dengue viruses. An explanation for this conservation is the possibility that raft association represents an essential feature of NS1 that is required for its participation in signal transduction events. Several proteins of viral pathogens that are involved in signal transduction, such as measles virus glycoprotein complexes (Avota et al., 2004), the Tip of herpesvirus saimiri (Cho et al., 2006), the latent membrane proteins 1 and 2A of Epstein–Barr virus (Coffin et al., 2003; Dykstra et al., 2001) and the Nef protein of HIV-1 (Krautkrämer et al., 2004), are raft-associated. As in these examples, association of cell-surface NS1 with lipid rafts may play a crucial role in assisting the activation of intracellular signalling cascades in response to dengue virus infection. Cross-linking of membrane-associated NS1 with specific antibodies has been shown to stimulate tyrosine phosphorylation of cellular proteins in dengue virus-infected HeLa cells and NS1-expressing transfected cells (Jacobs et al., 2000). The relevance of this observation in the pathogenesis of dengue is not known. Nevertheless, this experimental approach may serve as an in vitro model for assessing the requirement for lipid raft association in the signal transduction and downstream cellular activation processes mediated by NS1 on the cell surface.

Comparison of the intracellular location of NS1 in dengue virus-infected HEK-293T cells and transfectants by indirect immunofluorescence staining revealed that native NS1 localizes in discrete intracellular regions within infected cells, whereas recombinant NS1 forms, irrespective of their association with membrane, are scattered throughout the cytoplasm (Noisakran et al., 2007). The differences probably reflect the recruitment of NS1 to restricted areas within infected cells through an interaction with other viral proteins or virus-induced cellular components. In ultrastructural studies of dengue virus and other flaviviruses, intracellular NS1 co-localizes with double-stranded viral RNA as well as other non-structural proteins, including NS2A, NS3, NS4A and NS5 (Mackenzie et al., 1996, 1998; Westaway et al., 1997), in the RNA replication complex. A recent analysis indicated that the viral replication complex is located inside non-ionic detergent-resistant membrane structures originating from virus-induced membranes of
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Acknowledgements

We thank Dr Sa-Nga Pattanakitsakul for assistance with ultracentrifugation, and Ms Panthip Rinkaew and Ms Suchada Sengsai for help in immunofluorescence studies and immunoblotting analysis. This work was supported by the Thailand Research Fund (BRG4980003 to N.S.), Thailand-Tropical Diseases Research (T2) Program (01-1-DEN-02-006 to S.N.) and National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Thailand (BT-B-02-MG-B4-4801 to S.N.).

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