Dengue virus NS4B interacts with NS3 and dissociates it from single-stranded RNA

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INTRODUCTION

Dengue fever and its more severe form, dengue haemorrhagic fever, are mosquito-borne viral diseases that are caused by one of the four antigenically distinct serotypes of Dengue virus, DENV-1–DENV-4. Dengue fever affects 50–100 million people in the tropical and subtropical regions annually (Gubler, 1998, 2002). Contemporary demographic and lifestyle trends, such as population explosion and urbanization, have led to the spread of this disease to non-endemic regions. The pathogenesis of dengue fever remains poorly characterized and there are no antivirals or vaccines available to counter this emerging disease.

Dengue virus belongs to the family Flaviviridae that consists of enveloped, positive-sense, single-stranded RNA (ssRNA) viruses, such as those that cause yellow fever, Japanese encephalitis, West Nile fever and hepatitis C. Its RNA genome is encapsulated in an icosahedral nucleocapsid (30 nm) that is enveloped in a lipid bilayer (10 nm) (Kuhn et al., 2002) consisting of the membrane and envelope proteins. The 11 kb, capped RNA genome encodes a single polyprotein that is processed co- and post-translationally by host signalases, as well as the virus-encoded serine protease, into the three structural and seven non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. Except for enzymic activities contained within NS3 and NS5, the roles of the other proteins in virus replication and pathogenesis are not well defined. In this study, a physical interaction between NS4B and the helicase domain of NS3 was identified by using a yeast two-hybrid assay. This interaction was further confirmed by biochemical pull-down and immunoprecipitation assays, both with purified proteins and with dengue virus-infected cell lysates. NS4B co-localized with NS3 in the perinuclear region of infected human cells. Furthermore, NS4B dissociated NS3 from single-stranded RNA and consequently enhanced the helicase activity of NS3 in an in vitro unwinding assay. These results suggest that NS4B modulates dengue virus replication via its interaction with NS3.

The polymerase, helicase and protease enzymic activities encoded by the dengue virus genome ensure virus replication and polyprotein processing. NS3 (618 aa) is a multifunctional protein with protease, helicase, NTPase and 5’-terminal RNA triphosphatase activities (Arias et al., 1993; Benarroch et al., 2004; Falgout et al., 1991; Li et al., 1999; Zhang et al., 1992), whilst NS5 (900 aa) has RNA-dependent RNA polymerase and methyltransferase activities (Ackermann & Padmanabhan, 2001; Chu & Westaway, 1987; Egloff et al., 2002; Kapoor et al., 1995; Tan et al., 1996). These two proteins form a functional complex that is vital for flavivirus replication (Brooks et al., 2002; Johansson et al., 2001; Yon et al., 2005). The role of other non-structural proteins is not clear, except for NS2B, which is a cofactor for the protease activity of NS3 (Clum et al., 1997; Falgout et al., 1993). Interestingly, dengue virus NS4B has been reported to interfere with the interferon response in host cells by blocking the activation and nuclear translocation of Stat-1 (Muñoz-Jordán et al., 2003, 2005).

NS4B of members of the Flaviviridae is a small (248 aa), hydrophobic protein. NS4B proteins of dengue virus serotypes share 78–85 % amino acid sequence identity, whereas those of Yellow fever virus, West Nile virus and Dengue virus share 35 % identity. Hepatitis C virus (HCV) NS4B bears a negligible resemblance. Despite this divergence, the topology of NS4B, containing several endoplasmic reticular (ER) and cytoplasmic domains separated by transmembrane regions (Miller et al., 2006), is strikingly similar among members of the Flaviviridae, suggesting a conserved function of NS4B in the viral life cycle (Lundin et al., 2003).

Deletion of NS4B, as well as insertions in its sequence, inhibit replication of both Bovine viral diarrhea virus (BVDV) and Kunjin viruses (Balint et al., 2005; Grassmann et al., 2002; Chiu et al., 2003).
In this study, an interaction between the dengue virus non-structural proteins NS4B and NS3 was identified by using a yeast two-hybrid assay and validated in pull-down and immunoprecipitation studies. Furthermore, recombinant NS4B dissociated ssRNA from NS3 and consequently enhanced the overall helicase activity of NS3 in in vitro assays. Our results suggest a novel role for NS4B in dengue virus replication.

METHODS

Cloning. Non-structural protein sequences were amplified from cDNA of the dengue virus strain TSV01 and cloned into respective plasmids. The P104L mutation in NS4B was generated by site-directed mutagenesis of C to T at nucleotide position 7136 of tive plasmids. The P104L mutation in NS4B was generated by inducing morphological changes in the ER membrane (Yoshida et al., 2002; Grettton et al., 2005; Piccininni et al., 2002). Whilst these studies indicate that NS4B is a component of the replication complex of members of the Flaviviridae, co-immunoprecipitations of cell lysates using antibodies to double-stranded RNA (dsRNA) failed to reveal the presence of NS4B in the Kunjin virus replication complex (Chu & Westaway, 1992; Westaway et al., 2003).

Preparation of dsRNA/ssRNA substrate. Plasmid pGEM4Z was linearized by digestion with XbaI and was in vitro transcribed in the presence of [α-32P]GTP by using a Riboprobe kit (Promega). After incubation for 1 h at 37 °C, the reaction mixtures were treated with DNase I and extracted with phenol/CHCl₃. Unincorporated NTPs were separated by a Chromaspin-10 spin column (BD Clontech) and RNA was precipitated with ethanol. Radio-labeled in vitro transcription product of pGEM4Z was used as ssRNA substrate for electrophoretic mobility-shift assays (EMSSA). dsRNA substrate preparation has been described elsewhere (Xu et al., 2005).

dsRNA-unwinding assay. The dsRNA-unwinding assay was performed as described previously (Xu et al., 2005). Briefly, the reaction mixture for this assay contained 25 mM HEPES (pH 7.5), 1 mM ATP, 3 mM MnCl₂, 2 mM dithiothreitol (DTT), 100 μg BSA, 5 U RNasin, 0.25 pmol RNA substrate and 3 μg NS3 in a final volume of 20 μl. The mixture was incubated for 30 min at 37 °C and the reaction was terminated by adding 2.5 μl termination mix [100 mM Tris/HC1 (pH 7.5), 50 mM EDTA, 0.1% Triton X-100, 0.5% SDS, 50% glycerol, 0.1% bromophenol blue]. The helicase assay mixtures were resolved on a 1% native polyacrylamide gel and assayed with a Typhoon phosphorimager (Amersham Biosciences). For each value, the background from the negative control was subtracted and the fold variation of ssRNA release from each lane was calculated against ssRNA release by NS3-FL and plotted on a graph (Fig. 6b).

EMSA. The reaction mixture for this assay (20 μl) contained 20 mM HEPES (pH 7.5), 50 mM KCl, 1 mM EDTA, 5% glycerol, 1 mM DTT and 200 μg BSA ml⁻¹, along with [α-32P]-labeled ssRNA substrate and 1–3 μM NS3-FL or CF NS3 and 1–6 μM GST or GST–NS4B proteins. The mixtures were incubated for 5 min at 37 °C, 5 μl loading buffer (20% glycerol) was added and the mixtures were resolved on an 8% native polyacrylamide gel at 4 °C. Bands were identified by a Typhoon phosphorimager (Amersham Biosciences).

Cell culture, transfection and immunofluorescence. BHK-21, C6/36 [maintained in RPMI medium containing 10% fetal bovine serum (FBS)] and A549 (maintained in Dulbecco's modified Eagle's medium containing 10% FBS) cell lines were purchased from the ATCC. Medium components were purchased from Gibco/Invitrogen Corporation. Monolayers of A549 cells were cultured on coverslips in 24-well plates and co-transfected with 1 μg each of pXJ-NS4B and pXJ-NS3-FL plasmids by using Lipofectamine 2000 (Invitrogen). The cells were fixed in cold methanol 24 h post-transfection. For virus infections, A549 cells were seeded 24 h before infection with 5 m.o.i. TSV01 and fixed in cold methanol 3 days post-infection. Anti-NS3 and anti-NS4B antisera, generated in house, were used as primary antibodies. Texas red-conjugated anti-rabbit and fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary antibodies were used (Jackson Laboratories). Co-transfection images were captured by using a Leica fluorescence microscope, whereas the images of infected cells that were morphologically slightly different from uninfected cells were captured by using a confocal microscope (Olympus).

Antibody production. Anti-NS4B antiserum was prepared in mice against NS4B expressed as a GST fusion protein and eluted from polyacrylamide gels. This was purified by using an Esherichia coli lysate column to remove any non-specific antibodies and was tested on virus-infected C6/36, BHK-21 and A549 cell lines, as well as

2001; Khromykh et al., 2000; Li & McNally, 2001). BVDV NS4B interacts with NS3 and NS5A (Qu et al., 2001) and HCV NS4B plays a role in viral RNA replication, possibly by inducing morphological changes in the ER membrane (Egger et al., 2002; Grettton et al., 2005; Piccininni et al., 2002).
as transient transfections of NS4B in 293T, HeLa and A549 cells by Western blotting, immunoprecipitation and immunofluorescence. Polyclonal rabbit anti-DENV-2 NS3 was generated by injecting purified NS3 into rabbits. The serum was collected after 4 weeks and tested as described above.

**Virus infection and immunoprecipitation assays.** C6/36 and A549 cell lines were seeded in T75 cell-culture flasks 24 h prior to infection and, when about 80% confluent, infected with 10 m.o.i. TSV01. Infected and mock-infected cells were lysed in 2 ml cold m-RIPA buffer [50 mM Tris/HCL (pH 7.5), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 × protease inhibitor cocktail (Sigma)] 72 h post-infection. Lysates were pre-cleared with Protein A–agarose beads and normalized for protein concentration. About 10 μl NS4B antibody (roughly 1 μg) was added to 500 μg extract and incubated overnight at 4°C with gentle agitation. The complexes were captured by incubation for 1 h with 50 μl Protein A–agarose beads. Beads were washed three times each with m-RIPA buffer and PBS, boiled in 20 μl loading buffer and Western blotting was performed with anti-NS3 antibody.

**Protein purification.** NS3-FL protein purification has been described elsewhere (Xu et al., 2005). Briefly, BL21-RIL E. coli cells expressing NS3-FL and CF NS3 were induced for 16 h at 16°C with 10 μM IPTG and lysed in 50 mM HEPES (pH 7.5), 300 mM NaCl, 5% glycerol in a cell disrupter. The supernatant was loaded onto a GST column (5 ml; Centricon-75; Amersham Biosciences) pre-equilibrated with 50 mM Tris (pH 8.0). Proteins were eluted with 10 mM reduced glutathione. Peak fractions were pooled and concentrated by ultrafiltration at 3000 g (300 kDa) using Tris buffer to obtain 30%; cut-off, 30 kDa) and passed through a gel-filtration column (Sephadex-75; Amersham Biosciences) using Tris buffer and PBS, boiled in 20 μl loading buffer and Western blotting was performed with anti-NS3 antibody.

**RESULTS**

Identification of an interaction between NS4B and NS3 using the yeast two-hybrid system

The yeast two-hybrid interaction method is a powerful tool to detect molecular interactions (Fields & Song, 1989). To elucidate the role of NS4B in virus replication, we carried out yeast two-hybrid screening to find interacting partners of NS4B among the other non-structural proteins of Dengue virus. Full-length NS4B (NS4B), N-terminal aa 1–135 (NS4B N) and C-terminal aa 136–248 of NS4B (NS4B C) were engineered into the yeast bait vector (Table 1). A single point mutation of a conserved residue in NS4B (P104L) of DENV-4 has been reported to enhance virus replication in mosquito cells while decreasing its replication in mammalian cells (Hanley et al., 2003). We therefore also included this NS4B mutant (NS4B-M) in the screen. Full-length NS1, NS2A, NS2B, NS4A and NS4B

**Table 1. Yeast two-hybrid results**

Screening was done with NS4B, NS4BM, NS4B N and NS4B C as bait (represented in rows) and non-structural proteins as prey (represented in columns). The grid shows strong (+ + +), weak (+) or no (−) interaction in the yeast two-hybrid screen. NS4B shows a strong homo-association and the region of this interaction was narrowed down to aa 91–136 of its N-terminal domain (data not shown). NS4B interacts strongly with NS3_{303–618} and very weakly with NS3_{1–303}. NS4B N, NS4B C and NS4BM do not interact with NS3_{303–618}, suggesting that this interaction is conformation-dependent. ND, Not determined.
and different domains pertaining to NS31–303, NS3303–618 and NS51–405, NS5405–900 were cloned into the prey vector and tested for expression. Co-transformants were then grown either on low-stringency plates (lacking leucine, tryptophan and adenine) to identify weak interactions or on high-stringency plates (X-a-gal plates lacking leucine, tryptophan, adenosine and histidine) to allow identification of strong interactions.

As indicated in Table 1 and Fig. 1, full-length NS4B interacted strongly with the C-terminal region of NS3 (NS3303–618), which encompasses its helicase motif. The interaction is specific, because NS4B interacted very weakly with N-terminal NS3 (NS31–303), which contains the protease and NTPase motifs, and with NS2A, whilst no interaction was detected with NS1, NS2B, NS4A or NS5. Neither the NS4B N nor NS4B C terminus interacted with NS3303–618, suggesting that protein conformation is important for this interaction, whilst NS4BM did not interact with NS3 (Fig. 1). We identified a strong, homotypic interaction of NS4B through its N-terminal domain, indicating that NS4B may exist as an oligomer. The significance of this interaction was not investigated in this study. Also, there was no strong interaction of the small non-structural proteins NS2A, NS2B and NS4A with NS4B in our yeast two-hybrid assay. However, we cannot exclude the possibility that, being highly hydrophobic, these proteins could not be transported into the yeast nucleus.

**Verification of interaction by pull-down and immunoprecipitation assays**

In order to corroborate our yeast two-hybrid results, pull-down experiments were performed. Equal amounts of 35S-labelled, in vitro-translated, myc-tagged, full-length NS4B were incubated with or without recombinant His-tagged NS3 (NS3303–618), which encompasses its helicase motif. The interaction is specific, because NS4B interacted very weakly with N-terminal NS3 (NS31–303), which contains the protease and NTPase motifs, and with NS2A, whilst no interaction was detected with NS1, NS2B, NS4A or NS5. Neither the NS4B N nor NS4B C terminus interacted with NS3303–618, suggesting that protein conformation is important for this interaction, whilst NS4BM did not interact with NS3 (Fig. 1). We identified a strong, homotypic interaction of NS4B through its N-terminal domain, indicating that NS4B may exist as an oligomer. The significance of this interaction was not investigated in this study. Also, there was no strong interaction of the small non-structural proteins NS2A, NS2B and NS4A with NS4B in our yeast two-hybrid assay. However, we cannot exclude the possibility that, being highly hydrophobic, these proteins could not be transported into the yeast nucleus.

![Fig. 2. Pull-down analyses of the NS4B and NS3 interaction.](image)

(a) Pull-down of NS4B by NS3. Ni–NTA agarose beads were incubated with 35S-labelled, in vitro-translated NS4B with or without recombinant His-tagged NS3303–618 protein. Lane 1 depicts in vitro-translated NS4B protein as a positive control. Ni–NTA beads do not pull down NS4B in the absence of NS3303–618 protein (lane 2) and NS3303–618 pulls down NS4B specifically (lane 3). One microlitre of in vitro-translated NS4B (from a total reaction volume of 50 μl) was loaded in lane 1 and one-third of the material from the pull-downs was loaded in lanes 2 and 3. (b) Pull-down of NS3 by NS4B. Ten microlitres each of glutathione–Sepharose beads that were pre-incubated with crude cell lysates of bacteria expressing GST, GST–NS4B or GST–NS4BM was incubated with or without 7 μg recombinant CF NS3 protein, run on an SDS-PAGE gel and Coomassie-stained for detection of proteins. Lane 1, 7 μl CF NS3; lanes 2 and 3, 10 μl GST beads without and with CF NS3, respectively; lanes 4 and 5, 10 μl GST–NS4B beads without and with CF NS3, respectively; lanes 6 and 7, 10 μl GST–NS4BM beads without and with CF NS3 respectively. Note that GST–NS4B, but not GST–NS4BM, pulls down CF NS3.

To confirm the interaction between endogenously expressed NS4B and NS3, a co-immunoprecipitation assay was carried out using dengue virus-infected mosquito and mammalian cell lysates. C6/36 and A549 cells were infected with DENV-2 (TSV01 strain) at an m.o.i. of 10 and harvested 48 and 60 h post-infection, respectively. The presence of both NS3 and NS4B was detected in the infected cells by immunoblotting with the respective antibodies, which did not cross-react (see Supplementary Fig. S2, available in JGV Online). NS4B was immunoprecipitated with a mouse polyclonal anti-NS4B antibody raised in house. The immunoprecipitated material was separated by SDS-PAGE and immunoblotted with anti-NS3 antibody. NS3 could be co-immunoprecipitated by anti-NS4B antibody from infected C6/36 (Fig. 3b) and A549 (Fig. 3c) cell lysates, but not from uninfected lysates. Together, the in vivo co-immunoprecipitation and the in vitro pull-down results provide evidence that a specific interaction exists between NS4B and NS3.
NS3 and NS4B share similar subcellular-localization patterns

Kunjin virus NS4B localizes to the nucleus in the early stages of infection (Westaway et al., 1997), whilst HCV NS4B is an integral ER membrane protein (Hügle et al., 2001). Interestingly, dengue virus NS4B does not translocate into the nucleus even in later stages of infection, but resides primarily in cytoplasmic foci of ER origin (Miller et al., 2006). NS4B expressed along with an N-terminal 2K fragment shows a reticular staining and co-localizes with ER markers (Muñoz-Jordán et al., 2005).

We examined the localization of transiently expressed NS4B and NS3 in A549 cells by immunofluorescence. Co-transfected cells were double-labelled with rabbit anti-NS3 and mouse anti-NS4B antibodies and observed under a fluorescent microscope. Forty-eight hours after transfection, both NS3 and NS4B showed a reticular staining pattern that surrounded the nucleus and extended through the cytoplasm, typical of the ER localization, and they co-localized with each other when the two labelings were merged (Fig. 4). Further confirming the co-localization of the two molecules in vivo, A549 cells infected with DENV-2 showed a similar co-localization pattern 48 h post-infection (Fig. 4). Cells infected with Dengue virus showed marked morphological changes in the ER compartment, similar to those seen in HCV infection (Egger et al., 2002; Gretton et al., 2005). Our results are in agreement with another report that NS4B co-localizes with NS3 and dsRNA, arguing that NS4B is part of the membrane-bound virus replication complex (Miller et al., 2006).

NS4B dissociates NS3 from ssRNA

The C-terminal domain of flaviviral NS3 has been proposed to function in RNA and protein recognition (Xu et al., 2005; Yon et al., 2005). In order to test whether its interaction with NS4B would affect the RNA-binding property of NS3, we carried out EMSA. A radiolabelled ssRNA probe was generated by incorporation of $^{32}$P during in vitro transcription and EMSA was performed as described in Methods. Briefly, equal amounts of the ssRNA were incubated with proteins at 37°C for 5 min to allow binding and the protein–RNA complexes were resolved on an 8% native polyacrylamide gel under non-denaturing conditions.

Increasing amounts of GST–NS4B decreased the binding of NS3-FL (bacterially expressed full-length NS3) to ssRNA, whilst GST and GST–NS4B did not bind to ssRNA. Interestingly, this dissociation of NS3 from ssRNA occurred only when the stoichiometric ratio of NS4B to NS3 was at least 2:1 (Fig. 5a). Also, CF NS3, but not NS3-FL, formed higher-order complexes, possibly protein–RNA concatamers [denoted by * in Figs 5(a, b)], that disappeared when the concentration of CF NS3 was reduced to accommodate the stoichiometry of GST/GST–NS4B versus CF NS3 (lanes 12 and 13). This suggests that the formation of these concatamers is dependent on concentration of the protein, as well as the presence of its cofactor. However, dissociation of NS3 from ssRNA in the presence of NS4B was independent of the NS2B cofactor, as shown in Fig. 5(b), where NS4B abolished ssRNA binding of both NS3 (NS3-FL) and NS3 with cofactor (CF NS3). In a control experiment, GST–NS4BM did not show any effect on ssRNA binding of NS3 (Fig. 5c).
NS4B modulates the dsRNA-unwinding activity of NS3

As NS4B interfered with the RNA binding of NS3, we asked whether this affects the helicase activity of NS3 in a dsRNA-unwinding/helicase assay (Xu et al., 2005). Briefly, a radiolabelled dsRNA substrate was incubated with NS3-FL alone or with GST, GST–NS4B or GST–NS4BM at 37°C for 30 min; the mixture was then run on a gel to separate the ssRNA from the dsRNA. A 1:1 or 1:2 molar ratio of GST or GST–NS4B to NS3 was employed in the assay and ssRNA release was measured by autoradiography. As seen in Fig. 6(a), there was an approximately twofold increase in the helicase activity of NS3 upon addition of GST–NS4B, as opposed to the addition of GST. These experiments were repeated three times, the autoradiography signals were quantified and the helicase activity is represented in Fig. 6(b). Statistical relevance of these results was calculated by using a two-tailed t test (Fig. 6b). GST, GST–NS4B and GST–NS4BM did not exhibit any unwinding activity on their own. GST–NS4BM did not enhance the helicase activity of NS3 (data not shown). Taken together, these results suggest that NS4B enhanced the overall dsRNA-unwinding activity of NS3 by dissociating it from ssRNA and thereby enabling it to bind to a new duplex.

DISCUSSION

The roles of dengue virus NS3, NS5 and NS2B in replication have been fairly well characterized, but those of the other non-structural proteins, NS1, NS2A, NS4A and NS4B, have remained somewhat elusive. We sought to ascertain the role of NS4B in replication by searching for its interacting partner among the other non-structural proteins. The yeast two-hybrid interaction-trap method has been used successfully to characterize the interactions of HCV NS4B, which (similar to dengue NS4B) is a hydrophobic protein with several transmembrane domains (Dimitrova et al., 2003). Hence, we used this assay to search for interacting partners of dengue virus NS4B.

In this study, we identified an interaction between NS4B and the C-terminal part of NS3 (aa 303–618) that contains a helicase motif. Full-length NS4B, but neither the N- nor C-terminal truncations of NS4B, interacted with NS3 (aa 303–618) in our yeast two-hybrid assay, suggesting that this interaction is dependent on NS4B conformation. We validated this interaction by using biochemical pull-downs with recombinant proteins and co-immunoprecipitations of endogenously expressed proteins in infected cell lysates and have shown that they co-localize to similar subcellular compartments.

Structural analysis of NS3 helicase suggests that it binds to RNA as well as proteins through its C-terminal region (Wu et al., 2005; Xu et al., 2005). As NS4B interacted with the C-terminal region of NS3, it seemed likely that RNA binding of NS3 might be affected by this interaction. Our RNA-binding experiments have shown that wild-type NS4B, but not the mutant (NS4BM), dissociates NS3 from ssRNA. Interestingly, the dissociation is dependent on stoichiometry of the molecules. At least two molecules of NS4B per molecule of NS3 are needed to have a pronounced effect on ssRNA binding of NS3. These data are supported by our yeast two-hybrid results, wherein NS4B interacted with itself, suggesting that a functional NS4B molecule may be an oligomer. Dengue virus proteins are translated as a polyprotein, wherein a 1:1 stoichiometry of molecules seems logical. However, there are examples of other dengue virus proteins, such as NS1, which acts as a hexamer (Flamand et al., 1999; Winkler et al., 1988), and E protein, which forms a heterodimer with the prM protein (Zhang et al., 2003), that are known to form functional oligomers, suggesting the existence of such stoichiometry of molecules in vivo.

In the case of HCV, NS4A increases the ability of NS3 to bind to RNA and thereby enhances its helicase activity (Gallinari et al., 1999; Howe et al., 1999; Morgenstern et al., 1997; Pang et al., 2002). As dengue virus NS4B dissociates NS3 from RNA, we hypothesized that NS4B might act as a negative modulator of NS3. Surprisingly, NS4B
not decrease the dsRNA-unwinding activity of NS3, but enhanced it in an in vitro helicase assay. The helicase activity of dengue virus NS3 protein is coupled functionally to its NTPase activity (reviewed by Rocak & Linder, 2004). However, NS4B had no effect on the ATP hydrolysis activity of NS3-FL in a colorimetric assay described previously (Lanzetta et al., 1979; Silverman et al., 2003; Xu et al., 2005) (data not shown). This suggests that NS4B does not regulate the helicase activity of NS3 via its NTPase function. We hypothesize that NS3 is displaced from ssRNA in

**Fig. 5.** NS4B abolishes ssRNA binding of NS3. (a) NS4B dissociates NS3 from ssRNA in a dose-dependent manner. Equal amounts of the $^{32}$P-labelled ssRNA substrate were incubated with proteins at 37 °C for 5 min to allow binding and the complexes were resolved on an 8% native polyacrylamide gel under non-denaturing conditions. Gels were scanned by using a Typhoon phosphorimager (Amersham Biosciences). Arrows represent free probe and shifted bands. Lanes 1 and 2, negative control and NS3 pro; lane 3, CF NS3; lanes 4 and 5, GST and GST–NS4B; lanes 6–13, increasing molar ratios of GST and GST–NS4B with CF NS3. (b) Dissociation of NS3 from ssRNA by NS4B is independent of the NS2B cofactor. Lane 1, negative control; lanes 2 and 3, 3 mM each of CF NS3 and NS3-FL, respectively; lanes 4 and 6, 6 mM GST with NS3-FL and CF NS3, respectively; lanes 5 and 7, 6 mM GST–NS4B with NS3-FL and CF NS3, respectively. (c) Mutant NS4B does not alter RNA binding of NS3. Lane 1, negative control; lanes 2 and 3, GST and GST–NS4BM, respectively; lane 4, NS3-FL; lane 5, GST–NS4BM with NS3-FL.
the presence of NS4B, enabling it to interact with the next duplex and thereby increasing the overall processivity of the enzyme in vitro. In this light, it will be interesting to see whether ssRNA and NS4B compete to bind to the same region of NS3.

A single amino acid mutation in NS4B (P104L), which has been reported previously to have pleiotropic effects on dengue virus replication in mosquito versus human cells (Hanley et al., 2003), disrupted the interaction between NS3 and NS4B in both yeast two-hybrid and pull-down assays. This NS4B mutant had no effect on the RNA-binding or helicase activities of NS3. Proline at amino acid position 104 of NS4B is conserved in DENV-1–DENV-4, but, interestingly, Japanese encephalitis virus, Kunjin virus and West Nile virus each possess a leucine at that position. Therefore, Hanley et al. (2003) proposed that the substitution of proline by leucine in position 104 of NS4B causes a change in its structure or conformation that results in altered replication in vivo. This hypothesis might explain why, in our yeast two-hybrid and in vitro studies, neither truncated NS4B nor the P104L mutant interacted with NS3.

An in vivo implication of the NS4B–NS3 interaction is in the formation of a functional complex that holds the two strands of the RNA apart. It has been proposed that flaviviral NS3 and NS5 act as a functional complex (Brooks et al., 2002; Yon et al., 2005). The physical interaction of NS3 and NS4B demonstrated in this study might imply that all three molecules (NS3, NS4B and NS5) form a complex that holds the separated strands apart as the helicase moves along the duplex. Further in vivo studies will be needed to verify this hypothesis and to determine the role of the non-structural protein complex in flavivirus replication.

Finally, it is evident that an understanding of the flavivirus replication cycle will require characterization of the physical and functional interactions of the proteins that form the replication complex, including unidentified host proteins. Whereas many studies have indicated how NS3 and NS5 might participate in the replication process, this is the first report of the role of flaviviral NS4B in virus replication. Further work on the finely balanced interactions between all of these components should ultimately provide a working model for the control of flavivirus replication.

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**Fig. 6.** Modulation of RNA-unwinding activity of NS3 by NS4B. (a) NS4B enhances unwinding activity of NS3. Lane 1, negative control; lane 2, heat-denatured duplex; lane 3, 3 μM NS3-FL; lanes 4 and 5, 6 μM each of GST and GST–NS4B; lanes 6 and 7, 3 μM GST and GST–NS4B, respectively; with 3 μM NS3-FL; lanes 8 and 9, 6 μM GST and GST–NS4B, respectively, with 3 μM NS3-FL. In all reactions, 0.25 pmol dsRNA substrate and 1 mM ATP were used, incubated at 30 °C for 30 min, terminated and resolved on a 10% native gel. (b) Quantification of autoradiography signals. Amount of ssRNA in each lane was quantified using ImageQuant software and represented as fold variation with respect to the ssRNA released by NS3-FL. The variation in the unwinding activity of NS3 in the presence of GST–NS4B is significant (P = 0.0025 and 0.0029) as opposed to that seen in the presence of GST (P = 0.33058 and 0.0637). The values in the graph represent means of three staggered experiments.
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