Genetic interaction between NS4A and NS4B for replication of Japanese encephalitis virus

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Flavivirus NS4A and NS4B are important membrane proteins for viral replication that are assumed to serve as the scaffold for the formation of replication complexes. We previously demonstrated that a single Lys-to-Arg mutation at position 79 in NS4A (NS4A-K79R) significantly impaired Japanese encephalitis virus (JEV) replication. In this study, the mutant virus was subject to genetic selection to search for the potential interaction between NS4A and other viral components. Sequencing of the recovered viruses revealed that, in addition to an A97E change in NS4A itself, a Y3N compensatory mutation located in NS4B had emerged from independent selections. Mutagenesis analysis, using a genome-length RNA and a replicon of JEV, demonstrated that both adaptive mutations greatly restored the replication defect caused by NS4A-K79R. Our results, for the first time to our knowledge, clearly showed the genetic interaction between NS4A and NS4B, although the mechanism underlying their interaction is unknown.

INTRODUCTION

Japanese encephalitis virus (JEV) is the most common cause of viral encephalitis in eastern and southern Asia (Endy & Nisalak, 2002), and vaccination is the only effective way to prevent JEV infection. Development of effective antiviral therapeutics is still an urgent task. JEV belongs to the genus Flavivirus of the family Flaviviridae, which contains many other important human pathogens, such as West Nile virus (WNV), Dengue virus (DENV), Yellow fever virus (YFV) and Tick-borne encephalitis virus (TBEV). The flavivirus genome is a positive ssRNA of ~11 000 nt encoding a single ORF flanked by two UTRs at the 5′ and 3′ ends. Both the 5′ and 3′ UTRs contain conserved sequences and form complex secondary structures essential for viral gene expression and the onset of RNA replication (Brinton & Dispoto, 1988; Brinton et al., 1986; Shi et al., 1996; Zhang et al., 2008). The ORF encodes a large polyprotein, which is cleaved by host and viral proteases into three structural proteins (capsid, precursor membrane/membrane and envelope) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). The structural proteins form the virus particle, whilst the non-structural proteins play key roles in viral RNA replication, virus assembly and evasion of innate immune responses (Chang et al., 2006; Lin et al., 2004; Muñoz-Jordan et al., 2005).

During viral replication, most of the non-structural proteins associate to form a membrane-bound replication complex, which provides a platform for viral genome replication and virus assembly (Murray et al., 2008; Shi, 2014; Yi et al., 2012). NS5 has RNA-dependent RNA polymerase activity (Guyatt et al., 2001) and N-terminal methyltransferase activity involved in the capping of the progeny viral RNA genome (Dong et al., 2008). NS3 acts as a serine protease together with its cofactor NS2B (NS2B-3′pro) (Chambers et al., 1991; Falgout et al., 1991). In addition, NS3 also has RNA helicase/NTPase activities at the C terminus (Takegami et al., 1995). The glycoprotein NS1 plays a role in the early steps of viral replication (Lindenbach & Rice, 1997). In contrast, little is known about the functions of the small hydrophobic proteins NS2A, NS4A and NS4B. It has been suggested that these membrane proteins may be involved in anchoring the viral replicase components to intracellular membranes (Chambers et al., 1989).
NS4A is regarded as a central ‘organizer’ of the replication complex of flaviviruses (Lindenbach & Rice, 1999; Nemesio et al., 2012; Shiryaev et al., 2009), and is composed of a hydrophilic N-terminal portion residing in the cytoplasm, three internal hydrophobic regions associated with endoplasmic reticulum (ER) membranes (pTMS1–pTMS3) and a C-terminal fragment called 2K that acts as the signal sequence for translocation of the NS4B protein into the ER lumen (Miller et al., 2007). After translocation, the 2K fragment is cleaved off the N terminus of NS4B by the host signalase in the ER lumen. Signallase cleavage at the 2K-4B site requires a prior NS2B-3pro-mediated cleavage at the 4A-2K site just upstream of the N-terminal of the 2K fragment (Lin et al., 1993). Individual expression of NS4A protein (Kaufusi et al., 2014; Miller et al., 2007) or NS4A protein retaining the C-terminal 2K sequence (Roosendaal et al., 2006) was able to induce ER membrane remodelling that was similar to that observed in infected cells. Such events were proposed to anchor the viral replication complex to the ER membranes (Miller et al., 2007). Its N-terminal portion has also been reported to have multiple functions, such as facilitating NS4A homo-oligomerization (Stern et al., 2013) and interacting with cellular vimentin to regulate viral replication during DENV infection (Teo & Chu, 2014). The hydrophobic regions are proposed to be responsible for inducing membrane rearrangements, similar to those observed in infected cells, in order to anchor the viral replication complex to the ER membranes (Miller et al., 2007). NS4B is the largest hydrophobic non-structural protein of the flaviviruses (~27 kDa) and contains two hydrophobic segments (pTMD1 and pTMD2) that are probably associated with the ER lumen side of the membrane and three C-terminal transmembrane segments (pTMD3–pTMD5) (Miller et al., 2006). In addition to playing a similar role to NS4A in the formation of the viral replication complex (Miller et al., 2006; Yi et al., 2012), NS4B was demonstrated to be involved in IFN signalling suppression (Muñoz-Jordan et al., 2005). A recent study identified that NS4B existed as dimers either when the protein was expressed alone in cells or in cells infected with DENV (Zou et al., 2014). Additionally, both NS4A and NS4B proteins have been demonstrated to be able to interact genetically or physically with other viral proteins, including NS3 localized in the cytoplasm (Shiryaev et al., 2009; Umareddy et al., 2006) and NS1 positioned in the ER lumen (Lindenbach & Rice, 1999; Youn et al., 2012) to modulate viral replication. For example, the hydrophilic NS4A sequence functions as a cofactor of NS3 helicase and regulates the ATPase activity of NS3 helicase (Shiryaev et al., 2009). NS4B can dissociate NS3 from ssRNA and consequently enhance the helicase activity of NS3 through a physical interaction with the helicase domain of NS3 (Umareddy et al., 2006). The luminal NS1 could genetically or/and physically interact with NS4A and NS4B to transmit key signals into RNA replication occurring in the cytoplasm (Lindenbach & Rice, 1999; Youn et al., 2012).

Although these two membrane proteins exhibit a cooperative function in many cases, it is still unknown how, or even whether, NS4A and NS4B could interact with each other to facilitate viral replication. We previously demonstrated that a single Lys-to-Arg mutation at position 79 (NS4A-K79R) dramatically impaired JEV replication (Li et al., 2014a). In this study, we continuously passaged NS4A-K79R mutant viruses on Vero cells and identified two separate second-site mutations (NS4A-A97E and NS4B-Y3N) from several independent selections. Genetic analysis using the infectious clone and a subgenomic replicon system confirmed that either the NS4A-A97E or NS4B-Y3N mutation could efficiently rescue the replication defect caused by NS4A-K79R. These results demonstrated a genetic interaction between NS4A and NS4B during viral replication. We further investigated the effects of NS4A-K79R and its two adaptive mutations on the membrane rearrangement and process efficiency of NS4A–NS4B polypeptide, and no detectable difference relative to the WT was observed. This study established, for the first time to our knowledge, the genetic interactions between the two membrane proteins NS4A and NS4B, although how the interaction contributes to viral replication needs to be explored further.

**RESULTS**

**NS4A-K79R mutation dramatically decreases viral replication**

In our previous study, we found that a single Lys-to-Arg mutation at position 79 in the NS4A protein (NS4A-K79R) dramatically decreased JEV replication (Li et al., 2014a). To further analyse the role of NS4A in viral replication, and any possible genetic interaction between NS4A and other viral proteins, we continued to culture the recombinant virus with the NS4A-K79R mutation on Vero cells to select recovered virus. We found that the NS4A-K79R mutant containing a single nucleotide change (AGG) was prone to revert back to WT (AAG; data not shown). To reduce the possibility of WT reversion, we constructed a new full-length infectious clone bearing the NS4A-K79R mutation with three nucleotide changes (GGC) for the new round of selection. The phenotype of the new NS4A-K79R mutant was initially characterized, including viral protein expression, the specific infectivity value (SIV, i.e. the number of infectious virus after transfection of 1 μg RNA), plaque morphology and virus production. As shown in Fig. 1(a), immunofluorescence assay (IFA) analysis (monitoring viral envelope protein expression) showed that only WT RNA yielded IFA-positive cells as early as 24 h post-transfection (p.t.), whereas NS4A-K79R mutant RNA only yielded a few scattered positive cells at 72 h p.t. A SIV of only 40 p.f.u. (μg RNA)−1 was estimated for the NS4A-K79R (GGC) mutant, much lower than 1.3 × 104 p.f.u. (μg RNA)−1 of the WT RNA. Plaque assay revealed that the NS4A-K79R mutant generated smaller plaques than the WT virus.
(Fig. 1b). Virus production at 24, 48 and 72 h p.t. was examined by plaque assay. Consistent with the IFA and plaque morphology results, the NS4A-K79R (CGC) mutant yielded much lower viruses than the WT (Fig. 1c). These results were in agreement with our previous results observed in the NS4A-K79R mutant with an AGG mutation (Li et al., 2014a), which also demonstrated that it was the amino acid substitution that led to the replication defect of NS4A-K79R rather than the RNA sequence alteration.

**Compensatory mutations accumulate in NS4A-K79R-derived viruses**

For the new selection, we performed seven independent selections of mutant viruses by continuing passage on Vero cells. We designated the supernatant harvested from NS4A-K79R mutant RNA-transfected BHK-21 cells as P0. The supernatant passaged on Vero cells for three rounds was designated P3 (Fig. 2a). The independent selections were designated Selection I–VII. To investigate the virus propagation properties during passaging, viral titres of each virus passage were detected by plaque assay. As shown in Fig. 2b, within three rounds of passaging on Vero cells, the titres of the seven independent selections increased rapidly and reached >10^6 p.f.u. ml^{-1} at P3 (Fig. 2b). As the P3 viruses produced high levels of viral titre and a marked cytopathic effect, plaque morphologies were compared between P0 and different selections of P3 viruses (Fig. 2c). The plaque size of P0 viruses was much smaller than that of the WT virus. Interestingly, the different selections of P3 viruses showed variant plaque morphologies. P3 viruses of Selections IV and V displayed a minimum size of plaque even smaller than the NS4A-K79R mutant, whilst the other five selections displayed a similar plaque size to that of the WT virus. The changes in plaque morphology indicated that some adaptive mutations, possibly accumulated in the passaged viruses, and the variable shapes and sizes of the plaques, may be due to different additional mutations. Complete genome sequencing of P3 viruses of all selections identified three groups of adaptive mutation: (i) revertant mutations (Selections I and II), (ii) a Tyr-to-Asn mutation...
at position 3 in the NS4B protein (NS4B-Y3N) (Selections IV and V) and (iii) an A97E change in the NS4A protein (NS4A-A97E) (Selections III, VI and VII) (Fig. 2d).

**Recovered mutations in NS4A and NS4B could rescue the viral replication defect of the NS4A-K79R mutation**

The recovered mutations NS4A-A97E and NS4B-Y3N were individually engineered into WT or NS4A-K79R mutant infectious cDNA clones to determine their biological functions in viral replication. Similar methods were used to measure the replication efficiencies of the mutant RNAs as described above. In contrast to NS4A-K79R, both NS4A-A97E + NS4A-K79R and NS4B-Y3N + NS4A-K79R transcribed genome-length RNAs produced increasing numbers of IFA-positive cells from 24 to 72 h p.t. At the same time, both NS4A-A97E and NS4B-Y3N mutant RNAs produced nearly the same amounts of IFA-positive cells as the WT at each time point (Fig. 3a), which indicated that

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**Fig. 2.** Plaque morphologies and genome sequencing of JEV-NS4A-K79R recovered virus. (a) Flowchart of the NS4A-K79R mutant (AAG→CGC) virus blind passage. p.i., Post-infection. (b) Viral titres of each virus passage of the seven independent selections. (c) Comparison of plaque sizes and virus titres of JEV-NS4A-K79R P0 virus and P3 recovered viruses. (d) Genome sequencing and adaptive mutation identification of the P3 NS4A-K79R recovered viruses.

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Fig. 3. Analysis of adaptive mutations in full-length genomic cDNA clone. (a) IFA of BHK-21 cells transfected with genome-length RNAs containing various mutations. (b) SIV and plaque morphology in BHK-21 cells transfected with genome-length RNAs containing various mutations. The SIV is the mean value from three independent measurements. (c) Virus production of the supernatant of adaptive mutants RNAs transfected cells at the indicated time points following transfection. The data represent the mean ± SD from three independent transfections.
the NS4A-A97E or NS4B-Y3N mutation alone does not substantially affect viral replication. Similar results were also found with regard to the SIV (Fig. 3b). NS4A-K79R produced a much lower SIV (75 p.f.u. µg⁻¹ RNA), whilst the addition of individual NS4A-A97E and NS4B-Y3N to the NS4A-K79R mutation increased SIV to 2.7 × 10⁴ and 2.2 × 10⁵ p.f.u. (µg RNA)⁻¹, respectively. Plaque morphologies were compared amongst these recombinant viruses. Both recombinant viruses of NS4A-A97E + NS4A-K79R and NS4B-Y3N + NS4A-K79R produced similar plaque sizes (Fig. 3b) to their original recovered viruses (Fig. 2c). Finally, the supernatants from NS4A-K79R and the adaptive mutant RNA-transfected cells were harvested to detect virus production by plaque assay. Compared with NS4A-K79R, the additional NS4A-A97E or NS4B-Y3N mutation could rescue viral replication efficiently (Fig. 3c). Overall, the results indicated that either the NS4A-A97E or NS4B-Y3N compensatory mutation could rescue the replication defect of NS4A-K79R RNA.

Analysis of the compensatory mutations in the JEV replicon system

To directly detect the effects of these mutations on viral RNA replication, a transient replicon system of JEV was used. In the Renilla luciferase replicon (JEV-Rluc-Rep; Fig. 4a), a large fragment of the structural protein regions in-frame were deleted and the Rluc gene was inserted to the corresponding position, as described in our previous study (Li et al., 2014a). The luciferase activity at 2 h p.t. represented the input RNA translation, and the increasing luciferase activity after 12 h p.t. indicated RNA synthesis, which was used for investigation of RNA replication (Shi et al., 2002). BHK-21 cells were transfected with equal amounts of WT and mutant replicon RNAs, and assayed for luciferase activities at indicated time points after transfection. All replicons produced roughly the same levels of luciferase signals at 2 h p.t. (Fig. 4b, c), indicating that none of the mutations affected the input RNA translation and that the transfection efficiency was similar for the various replicon RNAs. However, luciferase signals showed differences amongst various replicons at ≥ 24 h p.t. Consistent with our previous study (Li et al., 2014a), the luciferase activity of the WT replicon continued to increase from 24 to 72 h p.t., whilst for the NS4A-K79R replicon, luciferase signals kept decreasing with extended time. In contrast, the addition of either the NS4A-A97E or NS4B-Y3N mutation to the NS4A-K79R replicon greatly improved viral RNA replication. At 72 h p.t., the NS4B-Y3N + NS4A-K79R and NS4A-A97E + NS4A-K79R replicons produced ~1000 times higher luciferase activities than the NS4A-K79R replicon. Notably, in the context of the WT replicon, neither NS4A-A97E nor NS4B-Y3N substitution conferred a replication advantage to the WT, exhibiting similar or even lower levels of luciferase activity relative to the WT (Fig. 4b, c). Taken together, these results further demonstrated that the compensatory mutations NA4A-A97E and NS4B-Y3N could restore the replication defect of the NS4A-K79R mutation.

No visible alterations of membrane rearrangement induced by NS4A/NS4B mutants are observed

It has been suggested that both NS4A and NS4B play important roles in triggering membrane rearrangement during flavivirus replication, and exhibit a dot-like staining pattern in the cytoplasm (Kaufusi et al., 2014; Miller et al., 2006, 2007). Thus, we tried to address whether the differential replicative capabilities of NS4A-K79R and its
adaptive mutations were attributed to their differential abilities to induce membrane rearrangement. To this end, we constructed a series of plasmids expressing WT and mutant NS4A-EGFP and NS4B-mCherry fusion proteins, and analysed NS4A and NS4B localization in transfected cells using the polyclonal antibodies against GFP and NS4B, respectively. As it was reported that the 2K fragment is essential for WNV-KUN NS4A-induced membrane rearrangement (Roosendaal et al., 2006), but is not required for DENV NS4A (Miller et al., 2007) and WNV-NY99 NS4A (Kaufusi et al., 2014), we first determined the role of 2K peptide in JEV NS4A-induced membrane rearrangement by comparing the localization of the full-length WT NS4A-2K-EGFP and the JEV NS4A-EGFP lacking the 2K peptides. As shown in Fig. 5a, GFP alone was distributed throughout the cells. Cells transfected with the construct of WT NS4A-EGFP lacking the 2K fragment displayed a dot-like structure in the cytoplasm; however, WT NS4A-2K-EGFP had a diffused distribution (Fig. 5a), which suggested that the 2K sequence was not required for JEV NS4A-induced membrane remodelling. Therefore, NS4A-EGFP without 2K was used as the backbone in subsequent studies. A similar dot-like staining pattern was also observed in cells transfected with the WT NS4B-mCherry expression plasmid, whilst mCherry alone was distributed throughout the cells. In addition, we did not observe significant alterations of the NS4A/NS4B staining pattern in cells transfected with the NS4A-K79R or NS4B-Y3N mutant plasmid relative to the WT (Fig. 5a). However, such similarities in the staining patterns between NS4A and NS4B prompted us to further determine whether NS4A and NS4B co-localize with each other. A distinct co-localization of NS4A and NS4B at cytoplasmic dot-like structures was observed when NS4A and NS4B were co-expressed. Additionally, the NS4A-K79R and NS4B-Y3N mutations had little effect on their co-localization. Taken together, these results demonstrated that: (i) the 2K fragment was not required for JEV NS4A-induced membrane rearrangement, (ii) JEV NS4A and NS4B could induce foci formation and co-localize with each other at the foci structure, and (iii) the NS4A-K79R mutation (dramatic replication defect) and the NS4B-Y3N adaptive mutation (slight replication defect) had little effect on the membrane alteration and co-localization of NS4A and NS4B.

Neither the NS4A-K79R mutation nor its adaptive mutation NS4B-Y3N has an effect on proteolytic processing of the NS4A–NS4B polyprotein

As the adaptive mutation NS4B-Y3N is located adjacent to the host protease cleavage site of the 2K–NS4B junction, we speculated whether NS4A-K79R alters the release efficiency of individual NS4A and NS4B proteins, and whether this function is restored partly by the addition of the NS4B-Y3N compensatory mutation. To test this possibility, we performed an in vitro cleavage assay using a NS4A–NS4B polyprotein as the substrate of NS2B-3pro. Plasmids encoding the full-length WT or mutant NS4A–NS4B polyprotein with a FLAG-tag at the C-terminus were constructed. The NS2B-3pro expression plasmid constructed previously (Li et al., 2014a) was used here. Equal amounts of WT and mutant NS4A–NS4B plasmids were transfected or co-transfected with NS2B-3pro expression plasmid into 293T cells, and cell lysates were analysed by Western blot assay using the anti-FLAG antibody. As shown in Fig. 6, a distinct 44 kDa band corresponding to the full-length NS4A–NS4B-FLAG polyprotein was observed in the cells transfected with NS4A–NS4B-FLAG plasmid alone and a 28 kDa NS4B-FLAG cleavage product was produced following coexpression with NS2B-3pro, which suggested that all the NS4A–NS4B mutants could be successfully cleaved by NS2B-3pro protease. Overall, neither the individual NS4A-K79R mutation nor the NS4A-K79R + NS4B-Y3N combination had any effect on NS4A–NS4B expression and processing, NS4B-Y3N rescued viral replication through an alternative mechanism other than regulating efficiencies in the processing of NS4A–NS4B polyprotein.

DISCUSSION

In this study, we demonstrated that a single Y3N adaptive mutation in JEV NS4B could rescue the replication defect of the NS4A-K79R mutant virus. Tajima et al. (2011) previously used a PCR-based random mutagenesis method and identified a set of NS4B mutations clustered within the region of pTMD3 (aa 93–146) that could restore the replication defect of mutant DENV-1 bearing N-terminal mutations (aa 27–34) of NS4A. However, their findings were different from ours. The NS4B mutations identified in their study were demonstrated to be able to enhance the growth of WT DENV-1, irrespective of the sequence of the aa 27–34 subportion NS4A, and therefore the very weak replication ability of mutant DENV-1 with NS4A mutations could be partially overcome by mutations in NS4B. In contrast, in our study the NS4B-Y3N mutation was generated by traditional viral genetic selection. The addition of the NS4B-Y3N mutation greatly improved the viral replication of the NS4A-K79R mutant virus, whilst it did not confer a growth advantage for the WT virus. Thus, the current study, for the first time to our knowledge, clearly establishes the genetic interaction between NS4A and NS4B that is important for JEV replication.

According to the membrane topology of NS4A (Miller et al., 2007) and NS4B (Miller et al., 2006), all the mutated sites, including NS4A-K79R as well as its two adaptive mutations NS4A-A97E and NS4B-Y3N, reside in hydrophobic segments of proteins (pTMS2 for NS4A and pTMD1 for NS4B). It has been reported that NS4A and NS4B may serve as a scaffold for replication complex formation through inducing membrane rearrangement (Lindenbach & Rice, 1999; Nemésio et al., 2012; Shiryaev et al., 2009; Tajima et al., 2011). Therefore, we further studied the effects of the mutations on membrane structure through microscopic characterization of the localization of
NS4A-EGFP and NS4B-mCherry fusion proteins. As reported previously, WT NS4A and NS4B induced cytoplasmic dot-like structures (Kaufusi et al., 2014; Miller et al., 2006, 2007). In comparison with WT proteins, no visible alterations in membrane remodelling were observed in cells expressing mutant proteins. Notably, the characteristic dot-like structures induced by NS4A occurred only when the cells were transfected with the plasmid of NS4A-EGFP lacking the 2K fragment, which indicated that the 2K fragment in JEV was not required for NS4A function in the regulation of membrane rearrangement. The result is consistent with those for DENV (Miller et al., 2007) and WNV-NY99 (Kaufusi et al., 2014), and is contradicted by the observation in WNV-KUN (Roosendaal et al., 2006). In addition, coexpression of NS4A-EGFP and NS4B-mCherry resulted in a clear colocalization between the two proteins, suggesting that a physical interaction might also exist between JEV NS4A and NS4B proteins. The bimolecular fluorescence complementation results further indicated that JEV NS4A might physically interact with NS4B (Fig. S1, available in the

**Fig. 5.** Subcellular localization of expressed NS4A and NS4B observed by IFA. (a) Localization of JEV NS4A and NS4A-K79R, NS4B and NS4B-Y3N in Vero cells transfected with the NS4A-EGFP or NS4B-mCherry expression plasmids. (b) Effect of the NS4A-K79R mutation and the NS4B-Y3N adaptive mutation on NS4A and NS4B co-localization in Vero cells co-transfected with NS4A-EGFP and NS4B-mCherry expression plasmids. Nuclear DNA was stained with DAPI.
online Supplementary Material). Our results are contrary to the recent study by Yu et al. (2013) which did not find any co-localization or physical interaction between WNV (strain B956, the low-pathogenic lineage II strain) NS4A (with the 2K fragment) and NS4B using confocal microscopy and fluorescence resonance energy transfer. As it is uncertain whether the 2K fragment is engaged in NS4A-induced membrane remodelling during WNV-B956 replication, additional studies need to be carried out to clarify this issue.

Previous studies demonstrated that flavivirus RNA replication is regulated by differential cleavage of the polyprotein precursor and that mutations affecting polyprotein processing are detrimental to viral replication (Herod et al., 2014; Nestorowicz et al., 1994). We investigated the cleavage efficiency of the NS4A-2K-NS4B polyprotein with different mutations using the same method as described previously (Li et al., 2014a). The NS4A-2K-NS4B polyprotein is initially cleaved by NS2B-3pro to release mature NS4A, followed by the subsequent cleavage by cellular signalase between 2K and NS4B (Lin et al., 1993). In comparison with the WT group, NS4A-K79R and NS4B-Y3N substitutions had no significant effect on the cleavage of NS4A-2K-NS4B polyprotein. These data suggested that the compensatory mutation Y3N in NS4B may rescue viral replication of the NS4A-K79R mutant through an alternative mechanism.

Alignment analysis of amino acid sequences of NS4A showed that residue 79 is relatively preserved amongst flaviviruses as a positively charged Arg (YFV and TBEV) or Lys (JEV, DENV and WNV), implying the importance of these positively charged residues in NS4A function. The pTMS2 region where these residues localize is an interface helix lying in-plane in the luminal membrane leaflet and might act like a wedge resulting in NS4A-induced membrane curvature by insertion of the luminal leaflet of the ER membrane (Miller et al., 2007). Lys and Arg have long aliphatic side-chains with a positively charged amine or guanidinium group at the end. Their aliphatic hydrocarbon backbone is much more deeply inserted into the hydrophobic region of the bilayer, whereas the positively charged group prefers the more polar interface region. This is designated the ‘snorkelling effect’ (Segrest et al., 1990). Thus, it is very likely that these positively charged residues at position 79 might help the pTMS2 segment to penetrate inside the membrane and subsequently bind tightly to it. Amongst these, the effect of Lys is most prominent, with a mean snorkelling of the polar atom of 2.5 Å, compared with 1.6 Å for Arg (Granseth et al., 2005). We reasoned that substitution of K79R may influence the interaction of pTMS2 with ER membranes due to the shortened snorkelling distance, resulting in a disturbance of membrane-initiated signalling induced by NS4A, although no visible significant alterations in membrane rearrangement induced by NS4A-K79R were observed using an indirect IFA approach. Indeed, the occurrence of the compensatory mutations also supported the hypothesis. Two separate mutations, NS4A-A97E and NS4B-Y3N, were identified from independent selections of recovered viruses. For the A97E adaptive mutation in the same pTMS2 region, as the acidic residue Glu at the membrane/water interface is most often directed away from the membrane, substitution of Ala97 with Glu may be able to secure pTMS2 helix to the membrane, partly restoring NS4A function in JEV replication. In terms of an alternative adaptive mutation in NS4B, NS4B-Y3N, two lines of evidence indicate that it may contribute to the restoration of NS4A function. (i) The site of position 3 resides in the first hydrophobic segment at the face of the ER lumen. Such spatial localization makes it possible for the interaction between NS4B and NS4A. (ii) Tyr and Asn residues tend to anti-snorkel and snorkel, respectively, when residing in the interface region of membrane proteins (Liang et al., 2005). Changing the anti-snorkelling Tyr to the snorkelling Asn at NS4B position 3 could alter the ER membrane structure, which could compensate for the defects of NS4A-K79R-induced membrane curvature. Therefore, study of the effects of NS4A/NS4B mutant proteins on the fine structure of the ER membrane, using alternative approaches, is required in future work.

**METHODS**

**Cells and antibodies.** BHK-21 and Vero cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS, 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹ in 5% CO₂ at 37°C. The primary antibodies used in our study included mouse anti-St. Louis encephalitis mAb (Chemicon), rabbit polyclonal anti-EGFP antibody (provided by Z.-M.Y.), mouse polyclonal anti-JEV NS4B antibody (the antiserum from an immunized mouse with
purified NS4B-GST protein) and mouse monoclonal anti-FLAG antibody (Sigma). The secondary antibodies contained FITC-conjugated goat anti-rabbit IgG and Texas red-conjugated goat anti-mouse IgG (ProteinTech) used in the IFA, and HRP-coupled goat anti-mouse antibody used in Western blot analysis.

**Plasmid construction.** We used the full-length JEV strain JEV-SA14 infectious cDNA clone (pACYC-JEV-SA14) and a Renilla luciferase reporting replicon (JEV-Rluc-Rep) (Li et al., 2014a) as the backbones for the introduction of the mutations (NS4A-K79R, NS4A-A97E and NS4B-T32N) in the infectious cDNA clone and replicon, respectively. Overlap PCR was performed to produce the mutant DNA fragments which were then engineered into pACYC-JEV-SA14 and JEV-Rluc-Rep at BamHI and XhoI sites. For the construction of the plasmids encoding NS4A-EGFP fusion proteins, the mutant NS4A fragments were amplified by PCR using the corresponding infectious cDNA clones as the template and then cloned in-frame with EGFP into pEGFP-N1 via EcoRI and BamHI sites. For the construction of the plasmids encoding NS4B-mCherry fusion proteins, we first constructed a mCherry expression vector (designated Vector-1) by replacing the EGFP-coding sequence with the mCherry-coding sequence. The NS4B-mCherry expression plasmid was then produced by engineering the NS4B gene in-frame at HindIII and BamHI restriction sites of Vector-1. The plasmid encoding the full-length NS4A–NS4B polyprotein was constructed by inserting the amplified NS4A–NS4B-FLAG fragment from pACYC-JEV-SA14 into a pCAGGS vector at KpnI and XhoI sites. All clones were sequenced prior to subsequent experiments.

**RNA transcription and electroporation.** All infectious cDNA and replicon plasmids were linearized by XhoI. Genome-length RNA and replicon RNA were transcribed using a MEGAscript T7 kit (Ambion) according to the manufacturer’s protocols. For transfection, ~5 μg RNA was electroporated into 8 × 10^6 BHK-21 cells in 0.8 ml ice-cold PBS buffer (pH 7.5) in a 0.4 cm cuvette with a GenePulsor electroporation system (Bio-Rad) at 0.85 kV and 25 μF, pulsing three times at 3 s intervals. After 10 min recovery at room temperature, the transfected cells were mixed with 25 ml pre-warmed DMEM containing 10% FBS, plated out into six-well plates and incubated at 37 °C with 5% CO_2_. Supernatants of the RNA-transfected cells were harvested at different time points following transfection and stored at ~80 °C.

**SIV assay.** About 5 × 10^5 BHK-21 cells were seeded in six-well plates 1 day prior to RNA electroporation. A series of 1:10 dilutions of RNA-transfected cell suspensions was made in a total volume of 2 ml and seeded to the six-well plates containing BHK-21 monolayer cells. After 6 h incubation under 5% CO_2 at 37 °C, culture medium was aspirated and cells were then covered with the first layer of agar. The second layer of agar containing neural red was plated at 72 h.p.t. By counting plaque number, the SIV was calculated as p.f.u. (μg RNA)^-1_.

**IFA and plaque assay.** Cells transfected with genome-length RNA were seeded on a Chamber Slide (Nalge Nunc). At 24, 48 and 72 h.p.t., the cells were fixed in cold (~20 °C) 5% acetic acid in methanol for 10 min at room temperature, washed three times with PBS and incubated with mouse anti-St. Louis encephalitis mAb (1:250 dilution with PBS) for 1 h. After washing with PBS three times, the cells were then incubated with goat anti-mouse IgG conjugated with Texas red at room temperature for a further 1 h. Following PBS washing for three times and 5 min incubation with DAPI (10 μg ml^-1_) to stain the nuclei, the slides were mounted with 95% glycerol and analysed under a fluorescence microscope at ×200 magnification. Virus titre and morphology were determined by single- and double-layer plaque assay with standard protocols as described previously (Li et al., 2014a, b).

**Luciferase assay.** Replicon RNA-transfected cells were seeded in 12-well plates in various amounts. Briefly, 1, 1, 0.5, 0.25 and 0.05 ml transfected cells were seeded, and cell lysates were reclaimed at 4, 24, 48, 60 and 72 h.p.t, respectively, and stored at ~80 °C for subsequent luciferase assay. Triplicate wells were seeded for each time point. Luciferase activity was measured in a Microplate Reader (Varioskan Flash; Thermo Fisher) by mixing 20 μl lystate with 50 μl substrate (Promega).

**DNA transfection of Vero cells and IFA.** NS4A-EGFP and NS4B-mCherry expression plasmids were transfected into Vero cells. For each experiment, Vero cells were seeded to 35-mm-diameter dishes or six-well plates and grown overnight to 60–70% confluent monolayer cells. The cells were washed once with PBS and fed with 2 ml complete medium per well. Plasmid DNA (2 μg) was transfected into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocols. After 12 h p.t, cells were fixed in cold (~20 °C) 5% acetic acid in methanol for 10 min at room temperature and washed with PBS three times. For the detection of NS4A protein, anti-EGFP rabbit antibody (1:200 dilution with PBS) and FITC-conjugated goat anti-rabbit IgG antibody (1:125 dilution with PBS) were used as the primary and secondary antibodies, respectively. For the detection of NS4B protein, anti-NM4B mouse antibody (1:200 dilution with PBS) and Texas red-conjugated goat anti-rabbit IgG antibody (1:125 dilution with PBS) were used as the primary and secondary antibodies, respectively. Following incubation with the secondary antibody, cells were subject to 5 min incubation with DAPI (10 μg ml^-1_) to stain the nuclei. The fluorescence images were then obtained with a fluorescence microscope.

**Western blot analysis.** Equal amounts of WT and mutant NS4A–NS4B-FLAG expression plasmids were mock-transfected or co-transfected with NS2B-3′ coding region expression plasmid (Li et al., 2014a) into 293T cells using Lipofectamine 2000 as described above. At 36 h.p.t., cells were lysed with 200 μl lysis buffer containing 20 mM Tris (pH 7.4), 100 mM NaCl, 0.5% n-dodecyl β-D-maltoside (Sigma) and EDTA-free protease inhibitor cocktail (Roche). Cell lysates were centrifuged at 17 000 g for 20 min, and the supernatants were collected and heated at 75 °C for 10 min. Samples were separated using 15% SDS-PAGE gels and transferred to PVDF membranes (Millipore), followed by blocking with 5% skim milk (Bio-Rad) in TBST buffer (50 mM Tris/HC1, pH 7.5, 150 mM NaCl and 0.1% Tween 20) at room temperature for 1 h. Following blocking, membranes were subject to sequential incubation with anti-FLAG mouse antibody (1:2000 dilution) and secondary anti-mouse IgG conjugated to HRP (Bio-Rad). After washing three times with TBST buffer, the signals were detected with a chemiluminescence system (ChemilDoc; Bio-Rad).

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