mutations into rDENV-1(02–20)/pMW119 by inverse PCR (IPCR)-based site-directed mutagenesis [24]. For the construction of the replacement mutant NS4A(11–50^{JEV}), JEV sequences were amplified from recombinant clone rJEV (Mie/41/2002)/pMW119 [25] using primers as described in Table S1, and the fragments were ligated to the IPCR fragment. For construction of NS4A(11–34^{JEV}) and NS4A(35–50^{JEV}), two pairs of single-stranded JEV oligonucleotides from the respective regions were synthesized (M41.4A.nc.f and M41.4A.nc.r for the 11–34^{JEV} mutant and M41.4A.c.f and M41.4A.c.r for the 35–50^{JEV} mutant) and then annealed, and the double-stranded fragments were ligated to the IPCR fragment. Each rDENV-1 clone was digested at the 3′ end of the viral genome with a restriction enzyme, Sac II, and the linearized DNA was transcribed using the mMESSAGE mMACHINE T3 kit (Invitrogen) as described previously [24]. Recombinant viruses were recovered by transfection of Vero cells with in vitro-transcribed RNA, followed by a few passages of the culture supernatant.

Analysis of viral growth and plaque size

Vero cells (6×10^5) were plated in 6-well tissue culture plates and infected with wild-type (02–20) and mutant rDENV-1 at a multiplicity of infection (MOI) of 0.01 plaque-forming units (PFU)/cell. Small aliquots of media were recovered periodically, and the aliquots were titered by a plaque assay using Vero cells grown in 6- or 12-well culture plates. To measure plaque size, Vero cells were fixed with 3.7% (V/V) formaldehyde solution in phosphate-buffered saline for 1 h, the methylcellulose overlay was removed, and the cells were stained with methylene blue solution for 2 h.

Introduction of random mutations into the DENV-1 genome

A full-length rDENV-1(02–20) genome containing the 5′-proximal T3 promoter sequence in the rDENV-1(02–20)/pMW119 clone was amplified by PCR (35 cycles) using the moderately error-prone Taq polymerase Blend Taq (Toyobo, Japan) and primers pMW119F1 (5′-AGGCGATTAAGTTGGGTAACG-3′) and pMW119R1 (5′-GCTTCCGGCTCGTATGTTGTG-3′). The amplified fragment was digested with Sac II and then used for production of infectious virus as described above.

Results

To assess the importance of the N-terminal portion of DENV-1 NS4A and its functional compatibility with the corresponding portion of JEV, we constructed two recombinant DENV-1 clones in which a part of the N-terminal portion (from residues 11–50; 11–50 portion) was deleted (NS4A(d11–50)) and replaced by the corresponding sequences of JEV (Mie/41/2002 strain, GenBank accession number AB241119) (NS4A(11–50^{JEV})) from an infectious clone, rDENV-1 (02–20) [24] (Fig. 1a). To minimize the effect of the mutations on the processing efficiency of the NS3–NS4A junction, residues 1–10 of the N-terminal portion were not deleted or replaced in this study. Recombinant viral RNA was transcribed in vitro from the clones and then used to transfect Vero cells as described previously [24]. We used Vero cells for the production of recombinant viruses because these cells are defective in the production of type 1 IFN and therefore do not affect the inhibitory activity of NS4A on IFN signaling. The DENV-1 (02–20) strain and JEV Mie/41/2002 strain replicate efficiently in Vero cells [24, 25]. After two passages of the culture supernatant, the virus titer of the supernatant was measured using Vero cells (Table 1, experiments 1 and 2). No infectious viruses were found after transfection with RNA from NS4A(d11–50) or NS4A(11–50^{JEV}), indicating that the N-terminal 11–50 portion of DENV-1 NS4A is indispensable for replication of DENV-1 and that the 11–50 portion cannot be substituted by the corresponding portion of JEV NS4A.

Comparison of the amino acid sequences of the 11–50 portion of DENV-1 and JEV NS4A showed that this portion could be divided into three subportions based on the degree of sequence identity: 11–23, 27–34, and 35–50 (Fig. 1b). To determine the subportion in the 11–50 portion of DENV-1 that is important for DENV-1 replication, four replacement mutants of the recombinant DENV-1 clone, NS4A (11–34^{JEV}), NS4A(35–50^{JEV}), NS4A(11–23^{JEV}), and NS4A (27–34^{JEV}), were constructed (Fig. 1a), and the viral RNAs of the mutants were synthesized and used for the production of virus particles (Table 1, experiments 3 and 4). Infectious particles were recovered when Vero cells were transfected with the NS4A(35–50^{JEV}) and NS4A(11–23^{JEV}) RNAs, whereas no infectious viruses were found when the NS4A(11–34^{JEV}) and NS4A(27–34^{JEV}) RNAs were used for transfection. DENV-1 RNA was also detected by RT-PCR in the supernatants of NS4A(35–50^{JEV}) and NS4A(11–23^{JEV}) RNA-transfected cells, but not in the supernatants of NS4A(11–34^{JEV}) and NS4A(27–34^{JEV}) RNA-transfected cells (data not shown). Complete nucleotide sequences of the NS4A(35–50^{JEV}) and NS4A (11–23^{JEV}) mutants were determined, and we confirmed that no additional non-synonymous mutations were present in these genomes (Table 1). These results suggest that the corresponding sequence of JEV NS4A does not substitute for the 27–34 subportion of DENV-1 NS4A in DENV-1 replication, and therefore, the 27–34 subportion of DENV-1

Table 1 Summary of the yield and sequence analysis of the recombinant DENV-1

Experiment no.	Virus (NS4A)	Titer (PFU/ml)	Additional nucleotide change	Amino acid change	Region
1*	d11–50	<20	–	–	–
	11–50^{JEV}	<20	–	–	–
2*	d11–50	<20	–	–	–
	11–50^{JEV}	<20	–	–	–
3*	11–34^{JEV}	<10	–	–	–
	35–50^{JEV}	2.9×10^5	NF	–	–
	11–23^{JEV}	5.2×10^6	ND	–	–
	27–34^{JEV}	<10	–	–	–
4*	11–34^{JEV}	<10	–	–	–
	35–50^{JEV}	2.0×10^6	A6967C/A¶	(silent)	–
	11–23^{JEV}	3.0×10^6	NF	–	–
	27–34^{JEV}	<10	–	–	–
5†	27–34^{JEV}-A3225U	6.5×10^5	G7165U	L2357F	NS4B
	27–34^{JEV}-A5628C	ND	C7152U/C¶	T2353I/T	NS4B
			A8997U	Y2968P	NS5
	27–34^{JEV}-A7714U	ND	C7152U	T2353I	NS4B
6†	27–34^{JEV}-A3225U	ND	C7140U	P2349L	NS4B
	27–34^{JEV}-A5628C	ND	C7152U/C¶	T2353I/T	NS4B
	27–34^{JEV}-A7714U	ND	C7152U/C¶	T2353I/T	NS4B
7†	11–34^{JEV}	<10	–	–	–
	27–34^{JEV}	2.6×10^6	G7184A/G¶	A2364T/A	NS4B
8†	11–34^{JEV}	<10	–	–	–
	27–34^{JEV}	6.1×10^6	C7152U/C¶	T2353I/T	NS4B
9*	27–34^{JEV}-G7165U	2.8×10^7	NF	–	–

NF not found; *ND* not determined

* Vero cells were transfected with recombinant viral RNA and the first supernatant was then transferred to Vero cells. The amplification step was repeated once more and then the third supernatant was recovered and used for measuring virus titer and determining the nucloetide sequence of viruses

† The amplification step was repeated three times, and the fourth supernatant was then recovered and used for the assays

¶ Mixed population

NS4A acts more specifically for DENV-1 replication compared to the other subportions.

Our data demonstrate that the N-terminal portion of NS4A is essential for viral replication, but the function and molecules that are associated with this portion of NS4A remain poorly understood. To gain insight into the role of DENV-1 NS4A subportion 27–34, we attempted to isolate a suppressor mutant of the NS4A(27–34^{JEV}) virus using PCR-based random mutagenesis. The full-length replacement mutant NS4A(27–34^{JEV}) cDNA was amplified using a moderately error-prone Taq polymerase to introduce random mutations into the NS4A(27–34^{JEV}) genome, and this amplicon was used as a template for the synthesis of recombinant viruses. Two suppressor mutant pools of the NS4A(27–34^{JEV}) mutant were obtained from the two experiments (RM1 and RM2 in Table 2). Sequence analysis revealed that there were 11 nucleotide changes accompanying amino acid changes in C, PrM, E, NS1, NS3, NS4B, and NS5. NS3 and NS5 have enzymatic activities that are critical for viral replication, and a previous report has demonstrated a genetic interaction between NS1 and NS4A [11]. Therefore, we next introduced single mutations into NS1 (A3225U), NS3 (A5628C), and NS5 (A7714U) of the NS4A(27–34^{JEV}) clone to produce additional mutant viruses (Table 1, experiments 5 and 6). Although recombinant viruses were produced, these viruses had additional non-synonymous mutations in NS4B. Moreover, some of the NS4B mutations were identical to the mutations observed in random mutants of NS4A(27–34^{JEV}). We attempted again to isolate suppressor mutants of NS4A(11–34^{JEV}) and NS4A(27–34^{JEV}) by increasing the number of passages of the culture supernatant (Table 1, experiments 7 and 8). NS4A(27–34^{JEV}) viruses, but not NS4A(11–34^{JEV}) viruses, were amplified by repeating three passages. Nucleotide sequencing analysis indicated that the NS4A(27–34^{JEV}) viruses also had mutations in NS4B.

Table 2 Non-synonymous mutations observed in the genomes of NS4A(27-34^{JEV}) random mutants

Experiment no.	Nucleotide change	Amino acid change	Region
RM1	A362U/A	N90Y/N	C
	U1683C/U	V530A/V	E
	A3225U/A	H1044L/H	NS1
	A5628C/A	N1845T/N	NS3
	C7140U/C	P2349L/P	NS4B
	G7172A/G	V2360 M/V	NS4B
RM2	A470G/A	V126 M/V	PrM
	A6983G/A	M2297 V/M	NS4B
	C7152U/C	T2353I/T	NS4B
	U7173C/U	V2360A/V	NS4B
	A7714U/A	R2540S/R	NS5

Vero cells were transfected with recombinant viral RNA, and the first supernatant was then transferred to Vero cells. The amplification step was repeated once more, and the third supernatant was then recovered and used for determining the nucleotides sequence of the viruses

Furthermore, a cytosine residue at position 7,152 of the NS4A(27–34^{JEV}) viruses in experiment 8 was completely replaced with a uracil residue (NS4A(27–34^{JEV})-C7152U) after one additional passage of the viruses. These results raise the possibility that these mutations in NS4B play a crucial role in rescuing the NS4A(27–34^{JEV}) mutant virus. To test this possibility, we constructed a new mutant clone, NS4A(27–34^{JEV})-G7165U, which had an additional mutation in NS4B (G7165U) of the NS4A(27–34^{JEV}) clone (Table 1, experiment 9). Infectious NS4A(27–34^{JEV})-G7165U virus was obtained easily by fewer passages compared with the other NS4A(27–34^{JEV}) mutants and had no additional mutations except for the mutations introduced, indicating that the mutations in NS4B are required for the replication of the NS4A(27–34^{JEV}) mutant.

Our findings lead to two alternative hypotheses: (1) the mutations in NS4B enhance the replication of DENV-1, or (2) the interaction between the 27–34^{JEV} mutant of NS4A and NS4B is restored by the mutation of NS4B. To evaluate the effect of the non-synonymous mutations in NS4B, two recombinant DENV-1 clones, C7152U and G7165U, which have a mutation at nucleotide position 7,152 or 7,165 of the NS4B-coding region of the wild-type DENV-1(02–20) clone, were constructed, and infectious viruses were produced. The mutant viruses could be recovered easily (after only one passage) compared with the other mutants (data not shown). Analysis of growth kinetics showed that the C7152U and G7165U mutants grew faster in Vero cells than wild-type DENV-1, while NS4A (27–34^{JEV})-C7152U and NS4A(27–34^{JEV}-G7165U grew more slowly than wild-type (Fig. 2a). In Vero cells, the plaque sizes of the C7152U and G7165U mutants were larger than those of wild-type, NS4A(27–34^{JEV})-C7152U,

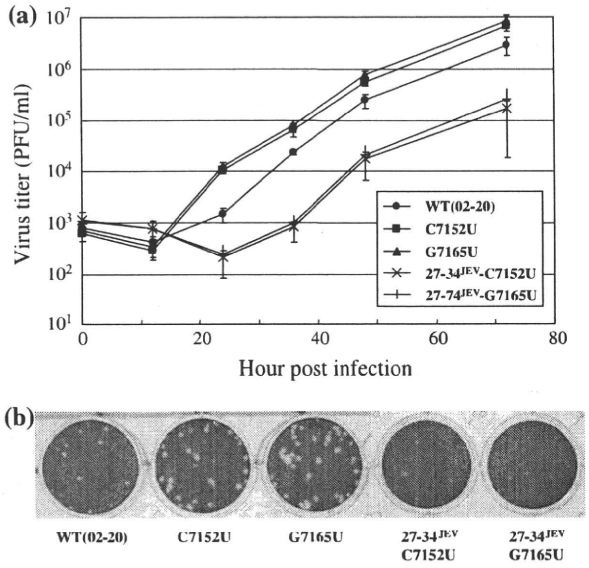

Fig. 2 Comparison of the growth properties of the wild-type (parent 02–20 strain) and mutants of DENV-1. **a** Growth curves of the wild-type DENV-1 and the C7152U, G7165U, NS4A(27-34^{JEV})-C7152U, and NS4A(27-34^{JEV})-G7165U mutants in Vero cells. Cells were infected with these viruses at a multiplicity of infection (MOI) of 0.01 plaque-forming units (PFU) per cell. Values represent the means and SD of three independent tests. **b** Plaque morphology of the wild-type DENV-1 and the C7152U, G7165U, NS4A(27–34^{JEV})-C7152U, and NS4A(27–34^{JEV})-G7165U mutants in Vero cells

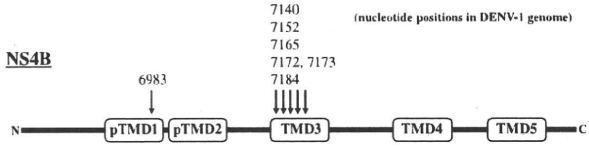

Fig. 3 Positions of the DENV-1 NS4B mutations observed in this study. Mutation sites are described as the nucleotide positions in the DENV-1(02–20) genome. (p)TMD, (predicted) transmembrane domain [16]

and NS4A(27–34^{JEV})-G7165U (Fig. 2b). These results suggest that, irrespective of the 27–34^{JEV} mutation, the mutations in NS4B enhance the rate of replication of DENV-1, and therefore, the very weak replication ability of NS4A(27–34^{JEV}) could be partially overcome by mutations in NS4B.

The mutations in NS4B found in this study were mapped, and interestingly, most of the mutations were concentrated in transmembrane domain 3 (TMD3) (Fig. 3).

Discussion

In this study, we demonstrated that the N-terminal cytoplasmic portion of DENV-1 NS4A plays an important role in viral replication. The corresponding portion of JEV

NS4A could not substitute for the 11–50 portion of DENV-1, even though this portion is homologous among flaviviruses [23]. Furthermore, analysis using replacement mutants of subportions revealed that the subportion comprising amino acid residues 27–34 could not be substituted by the corresponding subportion of JEV. Amino acid sequence comparison between DENV-1 and JEV indicates that the 27–34 subportion shows no identity compared to other subportions 11–23 and 35–50 of NS4A (Fig. 1b), and our experimental data are consistent with this result. In contrast, the amino acid sequence of the 27–34 subportion is highly conserved among DENV-1 isolates; there is only one isolate with amino acid differences in 386 randomly selected DENV-1 isolates, and moreover, this isolate differs by only one amino acid. These findings provide evidence that the 27–34 subportion is the most virus-specific subportion in the N-terminal portion of DENV-1 NS4A. NS4A(11–23^{JEV}) and NS4A(35–50^{JEV}) viruses could be recovered without any additional mutation, but the mutants showed lower infection titers, as shown in Table 1, and smaller and weaker plaques (data not shown) when compared with those of the parent DENV-1(02–20). These observations indicate that the 11–23 and 35–50 subportions are not completely replaced by the corresponding subportions in JEV. Previous findings and our results also support the idea that this portion of NS4A interacts with other viral proteins and functions in viral replication [11, 15, 27]. A recent report demonstrated that this portion regulates the ATPase activity of the NS3 helicase [23], raising the possibility that the DENV-1 NS4A mutant with the N-terminal portion of JEV could not act as a cofactor of DENV-1 NS3, and consequently NS3 function was less than optimal. Identification of a viral protein that binds directly to the N-terminal portion is needed to elucidate the role of this portion.

In an attempt to isolate the suppressor mutant of NS4A (27–34^{JEV}), we identified 11 non-synonymous mutations in 7 viral proteins (Table 2). Among these proteins, NS1, NS3, NS4B and NS5 have been suggested to interact with NS4A and form a replication complex [11, 15, 17, 27]. Unexpectedly, our analysis suggested that the mutations in NS4B, but not in NS1, NS3, and NS5, were involved in the recovery of the replication activity of the 27–34 mutant virus. Furthermore, the NS4B mutations C7152U and G7165U alone increased the growth of wild-type DENV-1. These results suggest that the NS4B mutations rescue the replication activity of the 27–34 mutant of recombinant DENV-1, but the mechanism may be attributed to the increased activity of NS4B rather than to the recovery of the interaction between the 27–34 subportion and NS4B. Previous studies have shown that single non-synonymous mutations in NS4B enhance the replication of DENV-4 in Vero cells, and most of the mutations are concentrated in TMD3 [1, 6]. Similar mutations in NS4B were reported in Vero-cell-adapted JEV [29]. These findings are consistent with our results and suggest that NS4B, particularly TMD3, is a key determinant in the adaptation of DENV for efficient replication [1]. The mechanism by which the mutations in TMD3 of NS4B increase the efficiency of virus replication remains unknown. TMD3 is located outside the region responsible for inhibition of interferon signaling [16, 19], and Vero cells do not produce type I interferon, indicating that the growth enhancement by the NS4B mutations is independent of the inhibitory activity of NS4B on interferon signaling. It has been shown that WNV NS4B also plays an important role in rearranging the cytoplasmic membranes that facilitate efficient replication of the viral genome [21], suggesting that the mutations in NS4B may enhance the replication of flaviviruses by altering the degree of rearrangement of the membranes in Vero cells. A recent study has also demonstrated that DENV NS4B interacts with NS3 and dissociates it from single-stranded viral RNA [26]. This dissociation may allow NS3 to interact with the next double-stranded viral RNA and thereby increase the overall unwinding activity of NS3 [26]. Therefore, it appears that the mutations in NS4B result in the increased ability of the protein to interact with NS3. However, Umareddy et al. have also shown that a P104L mutation, which corresponds to the C7140U mutation in DENV-1, disrupts the interaction between NS3 and NS4B in both yeast two-hybrid and pull-down assays without any effect on the RNA-binding or helicase activities of NS3 [26]. This observation implies that P104L and the other NS4B mutations detected in our study may cause a change in the structure or conformation of NS4B that results in altered replication in vivo, and therefore, two-hybrid and pull-down assays may not be suitable for evaluation of the activity of NS4B mutants. The C7129U mutation in DENV-4 NS4B, which corresponds to the C7140U mutation in DENV-1, enhances the replication activity of DENV-4 in Vero cells and decreases the ability of the virus to infect mosquito cells [6]. This result suggests that some of the mutations in NS4B may also be involved in the species tropism of DENV-1 and modulate the balance of efficient replication in mosquito and mammalian cells. Identification of the cellular factors that interact with NS4B may be required for understanding the opposing effects of NS4B mutations in mosquito and mammalian cells. The effects of the NS4B mutants on viral pathogenicity remain unknown. A recent report demonstrated that a mutation C102S mutation in WNV NS4B, a mutation is proximal to TMD3, attenuates viral growth in Vero cells and virulence in mice, raising the possibility that the NS4B mutations may influence the pathogenicity of DENV-1 [28].

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