Original Article

Single Amino Acid Mutation in Dengue Virus NS4B Protein Has Opposing Effects on Viral Proliferation in Mammalian and Mosquito Cells

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SUMMARY: Dengue virus (DENV) has a considerable impact on the global health and is known to cause morbidity and mortality every year. By passaging DENV2 in baby hamster kidney (BHK)-21 cells, we isolated a mutant clone of DENV2 that shows rapid cytopathic effects in BHK-21 cells as compared with that showed by the parent strain. To investigate the relationship between amino acid mutations and proliferation activity of the isolated DENV2 clone, we performed full genome sequencing and identified 3 amino acid mutations in the coding region, the envelope T120K, NS4A M85T, and NS4B G124A. Genetically modified recombinant DENV2 (rDENV2) carrying the NS4A M85T and NS4B G124A mutations produced higher titers of progeny virus in BHK-21, Vero, and Huh-7 cells than in the wild-type (WT) rDENV2. rDENV2 with mutations at NS4A M85T and NS4B G124A failed to produce any plaques in C6/36 mosquito cell lines. Furthermore, rDENV2 possessing only the NS4B G124A mutation showed no plaque production in C6/36 cells but had higher viral titers in Vero and Huh-7 cells than the WT rDENV2 had. Our results clearly showed that the DENV2 NS4B G124A mutation has opposing effects on the virus proliferation in mosquito and certain mammalian cell lines.

INTRODUCTION

Dengue and severe dengue are arthropod-borne viral diseases caused by 4 serotypes of dengue viruses (DENV) 1–4 belonging to the family Flaviviridae and genus Flavivirus. As DENV is mainly transmitted to humans and primates through the bites of DENV-infected Aedes mosquitoes, dengue and severe dengue are most prevalent in regions with tropical and sub-tropical climates that correspond with the distribution of these mosquitoes (1,2). According to the World Health Organization, 67–136 million dengue cases occur annually and approximately 12,500 deaths are reported (3,4).

Dengue virus has a single-stranded positive-sense RNA genome encoding 3 structural proteins, namely, the capsid (C), premembrane (prM), and envelope (Env) protein, and 7 nonstructural proteins, including NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. Structural and nonstructural proteins are translated as a single polyprotein, which is subsequently processed by signal peptidase, furin, and NS2B-NS3 protease (5–8).

Structural proteins involved in the formation of the viral particle interact with the host cell surface receptors, and the basic amino acids on Env bind to glycosaminoglycans (GAGs) such as heparan-sulfate (9–11). It has been previously reported that the cell-adapted mutant clones that have non-synonymous mutations in Env are produced by the serial passage of viruses in cell cultures (12–14). The passaging of the tick-borne encephalitis virus (TBEV) in baby hamster kidney (BHK)-21 cells results in the substitution of the positively charged amino acids in Env. As a consequence, the virus shows higher binding affinity for GAGs (15,16). Furthermore, amino acid substitutions, including T120K, N124D, and D249N, in the Env domain II of DENV2 have been previously reported that the cell-adapted mutant clones that have non-synonymous mutations in Env are produced by the serial passage of viruses in cell cultures (12–14). The passaging of the tick-borne encephalitis virus (TBEV) in baby hamster kidney (BHK)-21 cells results in the substitution of the positively charged amino acids in Env. As a consequence, the virus shows higher binding affinity for GAGs (15,16). Furthermore, amino acid substitutions, including T120K, N124D, and D249N, in the Env domain II of DENV2 have been observed in BHK-21 cells (17). In addition, non-synonymous mutations in flavivirus nonstructural proteins produced by serial passages of viruses in cell cultures have been previously reported (18,19). Flavivirus nonstructural proteins are important in virus production, particularly during viral genome replication (20), virus assembly, (21) and inhibition of immune responses through the blocking of type I interferon signaling in host cells (22). However, the role of non-synonymous mutations in nonstructural proteins of cell-adapted DENV mutant is incompletely understood.

In the present study, we performed serial passages of DENV2 in BHK-21 cells to investigate adaptive mutations in the nonstructural proteins and identify mutant DENV2. We synthesized genetically modified recombinant DENV2s (rDENV2s) with amino acid mutation(s) to clarify whether mutations in the nonstructural proteins are related to the proliferation of DENV2 in mammalian cells and mosquito-derived cells.
MATERIALS AND METHODS

Cells and viruses: BHK-21, Vero (African green monkey kidney), and Huh-7 (human hepatocellular carcinoma) cells were cultured in Dulbecco’s modified Eagle’s medium with high glucose (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin, and 0.5 mg/ml of streptomycin at 37°C in a humid 5% CO₂ incubator. C6/36 (Aedes albopictus) cells were cultured in Eagle’s minimum essential medium (EMEM; Nissui, Tokyo, Japan) supplemented with 10% FBS, 100 units/ml of penicillin, 0.5 mg/ml of streptomycin, 200 nM of L-glutamine (Sigma), 1 mM sodium pyruvate solution (Sigma), and MEM non-essential amino acids (Life Technologies, Carlsbad, CA, USA) at 28°C in a 5% CO₂ incubator.

The DENV-2-hu/INDIA/09-74 strain clinically isolated in Japan was kindly provided by Dr. Tomohiko Takasaki, and the genomic RNA sequence of the DENV-2-hu/INDIA/09-74 strain was submitted to the DDBJ/EMBL/GenBank databases under the accession number LC367234. The virus was initially amplified in BHK-21 cells after its inoculation at a multiplicity of infection (MOI) of 0.1 until cytopathic effects (CPE) were confirmed, as described by Wada et al. (23). Thereafter, supernatants from DENV2-infected cells were harvested and virus titers were measured by the plaque assay. Supernatants containing DENV-2-hu/INDIA/09-74 were stored at −80°C until use.

Titration of DENV2 by plaque assay: Sub-confluent BHK-21 cells in 24-well plates were inoculated in duplicates with 5-fold dilutions of DENV2 and the cells were incubated at 37°C for 1 h. After washing with phosphate-buffered saline (PBS), the cells were overlaid with EMEM containing 2% FBS, 200 nM of L-glutamine, 1 mM sodium pyruvate, and 1% methylcellulose. The culture plates were further incubated for 4 days. After fixation of the cells with buffered formalin for 30 min, the cells were stained with 1% crystal violet. The titer of DENV2 was calculated as the number of plaques and represented as plaque-forming unit per milliliter (pfu/ml).

Isolation of a mutant clone of DENV2: DENV-2-hu/INDIA/09-74 was amplified in BHK-21 cells cultured in 150 cm² flasks after the viral inoculation at an MOI of 0.1. After 2-day incubation, DENV2-infected cells were seeded in 12-well plates and cultured until CPE were observed. The supernatants from the DENV2-infected cells were harvested and transferred to fresh BHK-21 cells cultured in 12-well plates. Cell passage was performed every 2–3 days. Viruses harvested after 28 days of passage were used for plaque cloning.

Plaque cloning: Sub-confluent BHK-21 cells grown in 12-well plates were inoculated with DENV2s and incubated at 37°C for 1 h. After washing with PBS, the cells were overlaid with RPMI-1640 (Sigma) containing 4% FBS and 0.9% SeaPlaque agarose (Lonza, Walkersville, MD, USA). The culture plates were further incubated for 5 days, followed by the treatment of the cells with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide at 37°C for 4 h. Single colonies were collected with a pipette tip and subsequently seeded into plates carrying BHK-21 cells. Cloned viruses were obtained after a single round of plaque cloning.

Genome sequence analysis: Full genome sequence analysis was performed using the Ion PGM system (Life Technologies), as previously described by Sasaki et al. (24). In brief, the viral RNA was extracted with the PureLink Viral RNA/DNA mini kit (Thermo Fisher Scientific, San Jose, CA, USA) from the supernatants of the infected BHK-21 cells and reverse transcribed into double-stranded complementary DNA (ds-cDNA) by a sequence-tagged random hexamer (5’-CGCTTTCCGATCTNNNNNN-3’) using the PrimeScript double strand cDNA synthesis kit (Takara Bio, Shiga, Japan). Purified ds-cDNA was amplified by the KOD-plus-Neo (Toyobo, Tokyo, Japan) using a tag sequence primer (5’-CGCTTTCCGATCT-3’). Polymerase chain reaction (PCR) products were fragment amplified using the Covaris S2 Focused-ultrasonicator (Covaris, Woburn, MA, USA) and used to prepare a 400-base-read library using the Ion Plus Fragment Library kit (Life Technologies) and E-Gel SizeSelect 2% agarose gels (Life Technologies). Emulsion PCR was performed with the Ion PGM Template Hy-Q OT2 400 kit (Life Technologies). Sequencing was carried out with an Ion PGM sequencer with Ion PGM 400 kit and Ion 318 Chip V2 (Life Technologies). Data analysis was performed with the CLC Genomic Workbench 7.5.1 (CLC bio Japan, Tokyo, Japan).

Generation of rDENV2s: D2/hu/INDIA/09-74 genomic RNA was extracted and ds-cDNA was generated as described above. The viral genome was amplified with PrimSTAR GXL (Takara Bio) and the amplified fragment was purified and sub-cloned into pMW119 (Nippon Gene, Tokyo, Japan) under a T3 promoter using an In-Fusion HD cloning kit (Clontech, Palo Alto, CA, USA). Construct accuracy was confirmed by Sanger sequencing using the Big Dye Terminator V3.1 Cycle Sequencing Kit and the Applied Biosystems 3130 and 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Mutations of infectious cDNA clones were performed by site-directed mutagenesis. Constructed DENV2 infectious cDNA clones were transcribed into the viral genome using mMESSAGE mMACHINE T3 Transcription Kit (Life Technologies) and transfected into sub-confluent BHK-21 cells in 6-well plates using Lipofectamine Messenger MAX transfection reagent (Life Technologies). At 5–6 days post-transfection, the supernatants containing the synthesized rDENV2s were harvested and their titers were measured as described above.

Immunofluorescence assays (IFA): We inoculated rDENV2s in BHK-21, Huh-7, Vero, and C6/36 cells and incubated cells for different time points. After incubation, these cells were fixed with 100% methanol at −30°C for 30 min and blocked with a Block Ace solution (DS Pharma Biomedical, Osaka, Japan) at room temperature for 45 min. The cells were overnight incubated with a mouse monoclonal antibody to DENV (DENV1-4 D1-11 (3) [GeneTex, Irvine, CA, USA]) at 4°C. After washing with PBS, the cells were incubated with Alexa Fluor 488-labeled goat anti-mouse IgG (Molecular probes in Life Technologies) and 4′, 6-diamidino-2-phenylinde (DAPI) at room temperature for 30 min. After washing with PBS, fluorescent signals were observed using a IX70-FL/DIC microscope.
Quantitative reverse transcription polymerase chain reaction (qRT-PCR): Sub-confluent Vero and C6/36 cells cultured in 24-well plates were inoculated in duplicates with rDENV2 and incubated at 37°C for 1 h. After washing with PBS, the cells were cultured with EMEM supplemented with 2% FBS. At 24, 72, and 120 h post-infection, supernatants in the culture plates were removed and the cells were washed with PBS. Total RNA was isolated with TRIzol Reagent (Thermo Fisher Scientific) and Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA, USA). Extracted RNA was reverse transcribed and amplified using EXPRESS One-Step SuperScript qRT-PCR kit (Thermo Fisher Scientific) with DENV2 primers (forward: 5′-AGTG-GACACGGAACCCAGA-3′ and reverse: 5′-TTG-GCCGTGATTITTCATTAG-3′) and the probe (FAM-AAAAGAAGGCACGAAGAA-MGB). Genome copy numbers were normalized to the total extracted RNA concentration (ng/µl).

Statistical analysis: Statistical analysis was performed with Student’s t-test or Tukey-Kramer test based on one-way analysis of variance (ANOVA) from 3 independent experiments and significance was indicated by asterisks (*: p < 0.05, **: p < 0.01).

RESULTS

Sequence analysis of DENV2 mutant clones and proliferation activity of rDENV2s in BHK-21 cells: DENV-2-hu/INDIA/09-74 was serially passaged in BHK-21 cells for 28 days. In comparison with the parent strain, the passaged virus rapidly developed CPE in BHK-21 cells. We performed plaque cloning of these DENV2s. Full genome sequence analysis was carried out using genomic RNA extracts from 3 plaque-cloned DENV2s. As a result, all the 3 cloned viruses had 3 amino acid mutations in their coding regions, including Env T120K, NS4A M85T, and NS4B G124A (Table 1).

To clarify whether these mutations were related to the proliferation of DENV2, we synthesized genetically modified recombinant rDENV2s with 3 specific amino acid mutations.

We confirmed the relationship between Env T120K mutation and DENV proliferation activity, as previously reported (17). As shown in Fig. 1A, rDENV2 with single amino acid mutation of T120K in Env (EnvT120K) exhibited higher proliferation activity than the WT rDENV2 in BHK-21 cells, as evident from the plaque assay and IFA and consistent with the results of the previous report (17). In addition, rDENV2 with 3 amino acid mutations in Env, NS4A, and NS4B (EnvT120K/NS4AM85T/NS4BG124A) had significantly higher proliferation activity than EnvT120K (Fig. 1A). It was suggested that the amino acid mutations NS4A and NS4B were also related to DENV2 proliferation activity in BHK-21 cells.

We next examined whether NS4A M85T and NS4B G124A mutations would affect DENV2 proliferation in BHK-21 cells. rDENV2 with both NS4A M85T and NS4B G124A (NS4AM85T/NS4BG124A) mutations had significantly higher virus titers than the WT rDENV2 had (Fig. 1B).

Table 1. Genetic changes in mutant clones of DENV2

| Clones | Nucleotide change | Protein | Frequency (%) | Amino acid change |
|--------|------------------|---------|---------------|------------------|
| 1      | ACA→AAA          | Env     | 98.7          | T120K            |
|        | ATG→ACG          | NS4A    | 56.3          | M85T             |
|        | GGA→GCA          | NS4B    | 99.5          | G124A            |
| 2      | ACA→AAA          | Env     | 99.0          | T120K            |
|        | ATG→ACG          | NS4A    | 61.5          | M85T             |
|        | GGA→GCA          | NS4B    | 99.5          | G124A            |
| 3      | ACA→AAA          | Env     | 98.9          | T120K            |
|        | ATG→ACG          | NS4A    | 54.2          | M85T             |
|        | GGA→GCA          | NS4B    | 99.5          | G124A            |

Non-synchronous mutations in the DENV2 coding regions at least 50% frequency through the passages for 28 days in BHK-21 cells.
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in BHK-21 cells; however, viruses with a single mutation either in NS4A (NS4A^{M85T}) or NS4B (NS4B^{G124A}) had proliferation activity similar to that of the WT in BHK-21 cells (Fig. 1B).

Proliferation activity of rDENV2s with NS4B mutation in Vero, Huh-7, and C6/36 cells: We examined the growth of viruses carrying NS4A^{M85T}/NS4B^{G124A} mutations in different cell lines, including Vero, Huh-7, and C6/36 cells. Viruses carrying M85T and G124A mutations exhibited significantly higher growth rates than the WT rDENV2 in Vero and Huh-7 cells. However, the supernatants from C6/36 cells infected with NS4A^{M85T}/NS4B^{G124A} virus showed no plaque formation activity as compared to supernatants from the cells infected with the WT rDENV2 (Fig. 2A-C).

To clarify the critical amino acid residue involved in DENV2 proliferation in C6/36 cells, we examined the relationship between amino acid mutations of NS4A and NS4B and proliferation of DENV2 in C6/36 cells. In comparison with the WT, that carrying NS4A^{M85T} mutation showed significantly lower proliferation activity in C6/36 cells (Fig. 2C). Furthermore, the plaque assay performed using the supernatants from C6/36 cells infected with NS4B^{G124A} virus revealed no plaque formation (Fig. 2C). Thus, NS4B 124G plays a critical role in DENV2 proliferation in C6/36 cells. On the contrary, the infection of DENV2-susceptible mammalian cells, including Vero and Huh-7 cells, with NS4B^{G124A} mutant resulted in significantly higher viral proliferation activity than that observed following infection with WT strain (Fig. 2A-B).

Replication of rDENV2 with NS4B mutation in Vero and C6/36 cells: We investigated if G124A in NS4B affects viral genome replication in Vero and C6/36 cells. As shown in Fig. 3A-B, DENV2 copies increased in Vero cells infected with NS4A^{M85T}/NS4B^{G124A} and NS4B^{G124A} but decreased in C6/36 cells infected with NS4A^{M85T}/NS4B^{G124A} and NS4B^{G124A} as compared with the cells infected with WT strain at 24, 72, and 120 h.

DISCUSSION

It has been reported that NS4B 124G is located at the transmembrane domain (TMD) 3 (Fig. 4A) (25) and is related to the suppression of host RNAi, resulting in the promotion of DENV replication (26). Our results (Figs 2A and 3A) suggested that the G124A mutation in NS4B may affect the viral proliferation activity by promoting viral RNA replication through RNAi suppression in Vero cells; however, rDENV2 with NS4B G124A mutation was shown to exhibit a virus titer almost
similar to that of the WT strain in BHK-21 cells (27), consistent with our result (Fig. 1B). This observation suggests that NS4B G124A may interact with different host factors in Vero and BHK-21 cells. In addition, both the mutations of NS4A M85T and NS4B G124A were thought to be necessary for higher DENV2 proliferation activity in BHK-21 cells (Fig. 1B). DENV2 NS4A directly interacts with NS4B via amino acid residues.

**Fig. 3.** Viral genome replication of rDENV2s in Vero and C6/36 cells. DENV2 copies in rDENVs-inoculated (A) Vero (120 hpi, MOI of 0.1) and (B) C6/36 (120 hpi, MOI of 0.1) cells. Genome copy numbers were evaluated using total RNA extract from rDENVs-inoculated cells and normalized to total extracted RNA concentrations (ng/µl). Significance was analyzed by Tukey-Kramer test based on One-way ANOVA analysis and indicated by asterisks (*: p < 0.05, **: p < 0.01).

**Fig. 4.** Multiple alignment analysis of flaviviruses NS4A and NS4B. Amino acid sequences were downloaded from the National Center for Biotechnology Information (NCBI) database. The amino acid positions of NS4A and NS4B are numbered according to DENV2. (A) Schematic diagram of the membrane topology model of DENV2 NS4B. NS4B 124 G is located at TMD3. (B) Multiple alignment analysis of part of arthropod-borne flaviviruses NS4B. (C) Multiple alignment analysis of part of arthropod-borne flaviviruses NS4A. JEV, Japanese encephalitis virus; TBEV, Tick-borne encephalitis virus; WNV, West Nile virus; SLEV, Saint Louis encephalitis virus; YFV, Yellow fever virus; ZIKV, Zika virus.
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40–76 of NS4A and 84–146 of NS4B, the minimal determinants required for NS4A-NS4B interaction (28). Although NS4A M85T mutation is not located at this minimal determinant region of the NS4A-NS4B interaction, NS4B G124A mutation is located within this region. Further experiments are warranted to investigate whether the NS4A-NS4B interaction is related to high viral proliferation activity in BHK-21, Vero, and Huh-7 cells.

As NS4B G124A failed to demonstrate any plaque formation activity in C6/36 cells (Fig. 2C), we thought that NS4B G124A may be defective in replication in C6/36 cells. As shown in Fig. 3B, NS4B G124A showed decreased replication activity as compared with the WT strain but was not completely defective in replication. Modification in the endoplasmic reticulum (ER) membrane was shown to provide a platform for capsid formation in DENV assembly (29), and single amino acid mutation in Hepatitis C virus NS4B, the hydrophobic protein regulating ER membrane rearrangements similar to that with DENV NS4B, was known to affect the virion assembly (30). Thus, G124A mutation in NS4B may be involved in viral genome replication as well as in viral particle production.

Studies have shown that DENV4 NS4B P101L, which is homologous to DENV2 NS4B 104P (Fig. 4B), has trade-off effects on DENV4 proliferation in Vero and Huh-7 cells versus C6/36 cells (31); however, DENV4 NS4B 101P is not conserved in arthropod-borne flaviviruses such as JEV, WNV, and SLEV. In our study, although DENV2 NS4B G124A mutation had effects similar to those observed with DENV4 NS4B 101P in Vero and Huh-7 cells versus C6/36 cells, the plaque-forming activity was undetected using supernatants of NS4B G124A-infected C6/36 cells (Fig. 2C). This phenomenon may help in the development of a live-attenuated vaccine because the DENV2 vaccine candidate strain with G124A mutation may fail to produce infectious viral particles in mosquitoes. Although NS4A 85M is not conserved among DENVs (Fig. 4C), NS4B 124G is highly conserved among arthropod-borne flaviviruses, including DENVs (Fig. 4B). Therefore, further investigations on NS4B 124G and related amino acid residues are desirable to clarify the mechanisms underlying flavivirus proliferation in their host vectors.

Taken together, NS4B 124G in DENV2 plays an important role in the viral proliferation in C6/36 cells and certain mammals cells. This observation may be useful to study DENV proliferation mechanisms and develop live-attenuated vaccines with low transmission potential in mosquitoes.

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Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships. The authors HN and AS are employees of Shionogi & Co., Ltd., Japan.

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