Highly Conserved Residues in the Helical Domain of Dengue Virus Type 1 Precursor Membrane Protein Are Involved in Assembly, Precursor Membrane (prM) Protein Cleavage, and Entry

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Background: The role of α-helical domain (MH) of prM protein in DENV replication remains unknown.

Results: Alanine substitutions of nine highly conserved MH domain residues affected assembly of replicon particles and entry, which correlated with impairment in prM cleavage.

Conclusion: MH domain residues modulate assembly, maturation, and entry.

Significance: These highly conserved residues are potential targets of antiviral strategy.

The envelope and precursor membrane (prM) proteins of dengue virus (DENV) are present on the surface of immature virions. During maturation, prM protein is cleaved by furin protease into pr peptide and membrane (M) protein. Although previous studies mainly focusing on the pr region have identified several residues important for DENV replication, the functional role of M protein, particularly the α-helical domain (MH), which is predicted to undergo a large conformational change during maturation, remains largely unknown. In this study, we investigated the role of nine highly conserved MH domain residues in the replication cycle of DENV by site-directed mutagenesis in a DENV1 prME expression construct and found that alanine substitutions introduced to four highly conserved residues at the C terminus and one at the N terminus of the MH domain greatly affect the production of both virus-like particles and replicon particles. Eight of the nine alanine mutants affected the entry of replicon particles, which correlated with the impairment in prM cleavage. Moreover, seven mutants were found to have reduced prM-E interaction at low pH, which may inhibit the formation of smooth immature particles and exposure of prM cleavage site during maturation, thus contributing to inefficient prM cleavage. Taken together, these results are the first report showing that highly conserved MH domain residues, located at 20–38 amino acids downstream from the prM cleavage site, can modulate the prM cleavage, maturation of particles, and virus entry. The highly conserved nature of these residues suggests potential targets of antiviral strategy.

The four serotypes of dengue virus (DENV1, DENV2, DENV3, and DENV4) belonging to the genus Flavivirus in the family Flaviviridae are the leading cause of mosquito-borne diseases in humans in the tropical and subtropical regions (1, 2). Although most DENV infections are asymptomatic or cause a self-limited illness, known as dengue fever, some individuals may develop severe and potentially life-threatening diseases, known as dengue hemorrhagic fever or dengue shock syndrome (3). DENV is a positive-sense, single-stranded RNA virus containing a genome of ~10.6 kb in length. Between the 5′- and 3′-nontranslated regions, there is a single open reading frame encoding a polyprotein precursor, which is cleaved by cellular and viral proteases into three structural proteins, capsid, precursor membrane (prM) and envelope (E), and seven nonstructural proteins (4).

DENV enters the cell through receptor mediated endocytosis (4–8). After uncoating, translation, and genome replication, assembly of viral particles takes place in the membrane of rough endoplasmic reticulum (ER), where the immature virions, containing prM and E proteins on surface, bud into the lumen of ER and transport through the secretory pathway (4, 8–10). In the trans-Golgi, the prM protein on immature virions is cleaved by furin or furin-like protease to produce mature virions, although the cleavage was inefficient for DENV (11–15). A common feature of flaviviral replication is the formation of small and slowly sedimenting subviral particles (4, 16). Co-expression of prM and E proteins can generate recombinant virus-like particles (VLPs), which are similar to the infectious virions in the biological and antigenic features (17–19). Several studies have employed VLPs as a model system to study the function of prM/E proteins, assembly of particles (19, 20), infectious serodiagnostic antigens, and potential vaccine candidates (21–

2 The abbreviations used are: DENV, dengue virus; E, envelope; [E], concentration of E protein; ER, endoplasmic reticulum; M, membrane; MH, α-helical domain of M protein; MT, transmembrane domain of M protein; prM, precursor membrane; VLP, virus-like particles.

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In addition, flaviviral replicon particles, in which a flaviviral replicon containing reporter gene is trans-supplied with structural proteins (CprME or prME) to generate replicon particles capable of completing one-round of infection, have been shown as convenient and useful tools to study flavivirus replication, inhibitors, and neutralization (26–32).

The prM protein of DENV contains 166 amino acids; it is cleaved at position 91 by furin or furin-like protease to produce the pr peptide and M protein, which consists of an N-terminal loop (first 20 residues), an α-helical domain (MH), and two transmembrane domains (MT1 and MT2) (33) (see Fig. 1). Based on a cryo-EM study of mature DENV2 virion at high resolution, the first part (N terminus) of the MH domain is outside the head group region of membrane, and the second part (C terminus) is buried in the head group region of membrane (33, 34). After synthesis in the rough ER, prM protein forms a heterodimer with E protein and has been reported to function as a chaperone for proper folding of E protein (35, 36) and prevent premature fusion of E protein within acidic compartments along the secretory pathway (18, 37, 38).

Previously, several residues of prM protein, mainly in the pr peptide, have been shown to play important roles in the replication of different flaviviruses. A tyrosine residue at position 78 of prM protein was reported to be involved in the assembly of West Nile virus (39); mutations introduced to threonine at position 20 and lysine at position 31 were found to affect the production of West Nile virus VLPs and/or virions (40). In addition, a highly conserved proline at position 63 of tick-borne encephalitis virus prM protein was reported to affect the assembly of VLPs (41). Loss of the N-linked glycosylation site at position 15 of Japanese encephalitis virus prM protein was found to affect the production of virion (42). A histidine residue at position 99 of Japanese encephalitis virus prM protein was reported to involve in the prM–E interaction (43). However, little is known about the role of M protein including the MH domain (positions 112–131) in the replication cycle of DENV. Previously, Pryor et al. (44) reported that a histidine residue at position 130 is important for assembly of DENV2 virions; we reported that proline substitutions of residues in the MH domain (positions 120, 123, and 127) of DENV prM protein greatly affect the entry and assembly of VLPs and virions, suggesting the importance of the C terminus of MH domain (45).

Sequence analysis of the MH domain revealed nine residues highly conserved among different strains of four DENV serotypes (see Fig. 1).

In this study, we investigated the roles of these nine highly conserved MH domain residues in the replication cycles of DENV by site-directed mutagenesis in a DENV1 prME expression construct and found that alanine substitutions introduced to four highly conserved residues at the C terminus and one at the N terminus of the MH domain greatly affect the production of both replicon particles and VLPs. In the entry assay, eight of the nine alanine mutants were found to affect the entry of replicon particles, which correlated with the impairment in the cleavage of prM protein. Reduced prM–E interaction at low pH was found in seven mutants, which may affect the formation of smooth immature particles and exposure of prM cleavage site during maturation and contribute to the inefficient prM protein cleavage. Together, our findings suggest that highly conserved residues in the MH domain of prM protein modulate the cleavage of prM protein, maturation of particles, and virus entry.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The prME expression construct of DENV1 (Hawaii strain), pCB-D1, was described previously (45). To generate mutants at the MH domain of prM protein, a two-step PCR mutagenesis was performed using pCB-D1 as template and primers. After the second round PCR, the 977-bp products containing the MH mutations were digested with KpnI and DraIII and cloned into pCB-D1. All constructs were confirmed by sequencing the entire inserts to rule out second site mutations. The sequences of all primers for mutagenesis will be provided upon request.

Cell Lysates, VLPs, and Western Blot Analysis—293T cells prepared in a 10-cm culture dish at 5 × 10^5 cells/dish 1 day before were transfected with 10 μg of plasmid DNA by the calcium phosphate method. At 48 h post-transfection, culture supernatants were collected (see below), and cells were washed with PBS and treated with 1% Nonidet P-40 lysis buffer (100 mM Tris, pH 8.0, 150 mM NaCl, 20 mM EDTA, 1% Nonidet P-40, 0.5 mM sodium deoxycholate, and protease inhibitors; Roche Diagnostics), followed by centrifugation at 20,000 × g at 4 °C for 30 min to obtain cell lysates. Culture supernatants were clarified by centrifugation at 1,250 × g for 20 min, filtered through a 0.22-μm pore-sized membrane (Millipore), layered over a 20% sucrose buffer, and ultracentrifuged at 65,000 × g at 4 °C for 5 h to obtain pellets, which were resuspended in 60 μl of TNE buffer (45). For Western blot analysis, cell lysates or pellets were added to nonreducing buffer (2% SDS, 0.5 M Tris, pH 6.8, 20% glycerol, 0.001% bromphenol blue; final concentrations) and subjected to 12% PAGE, followed by transfer to nitrocellulose membrane, blocking, and incubation with primary antibody including human dengue-immune serum, mouse anti-calnexin mAb E-10 (Santa Cruz), mouse anti-NS1 mAb (DB29-1), or rabbit anti-M serum (against a M peptide, positions 92–130 of DENV2 M protein), which were added to nonreducing buffer (2% SDS, 0.5 M Tris, pH 6.8, 20% glycerol, 0.001% bromphenol blue; final concentrations) and subjected to 12% PAGE, followed by transfer to nitrocellulose membrane, blocking, and incubation with primary antibody including human dengue-immune serum, mouse anti-calnexin mAb E-10 (Santa Cruz), mouse anti-NS1 mAb (DB29-1), or rabbit anti-M serum (against a M peptide, positions 92–130 of DENV2 prM protein), and secondary antibody (45). After final washing, the signals were detected by enhanced chemiluminescence reagents (PerkinElmer Life Sciences) (45). The intensities of prM and M bands recognized by anti-M serum were analyzed by ImageQuant (GE Healthcare) (46, 47), and the relative prM cleavage of a mutant equals (the intensity of mutant M band/the intensity of WT M band)(the intensity of WT prM band)/(the intensity of WT M band).

Quantitative Capture ELISA—A flat-bottomed 96-well plate was coated with serum from a confirmed dengue case at 4 °C overnight, followed by blocking with 1% BSA in PBS for 1 h and adding cell lysates, pellets, or recombinant DENV1 E protein. After adding mouse anti-DENV1 E mAbs (DA6–7) and anti-mouse IgG conjugated with HRP (Jackson) each at 37 °C for 1 h, TMB substrate, and stop solution, the absorbance at wavelength of 450 nm (A_{450}) with reference wavelength of 650 nm was read (46, 47). The concentration of E protein, [E], was determined by interpolating the A value to the standard curve generated by known concentrations of recombinant E protein (in serial dilution) (GraphPad Prism5, GraphPad Software Inc.).
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**Immunoprecipitation**—For immunoprecipitation at neutral pH, protein A-Sepharose beads (GE Healthcare) were incubated with mouse anti-DENV1 E mAb (FL0251; Chance Biotechnology, Taiwan) at 4 °C for 4 h and then incubated with cell lysates in 1% Nonidet P-40 (pH 8.0) at 4 °C overnight. For immunoprecipitation at low pH, protein A-Sepharose beads containing FL0251 were incubated with cell lysates in 1% Nonidet P-40 (pH 6.0) at 4 °C overnight. After washing with 1% Nonidet P-40 buffer four times, the beads were mixed with 20 μl of 2× sample buffer (500 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 0.4% bromphenol blue), heated at 95 °C for 5 min, and centrifuged at 20,000 × g for 1 min. The solubilized fraction was subjected to 12% PAGE and Western blot analysis using human centrifuged at 20,000 × g. The six mAbs included two human (DVB18.5 and anti-prM mAbs recognizing pr (Ref. 48 and data not shown). The recognition index of an anti-prM mAb to a mutant prM protein equals (intensity of mutant prM dot/intensity of WT prM dot)

**Dot Blot Assay**—Aliquots of cell lysates or pellets (containing VLPs) derived from the above transfectants were diluted in bromphenol blue-containing PBS and dot blotted using a 96-dot formatted dot blotter (Biotzero, Germany) to nitrocellulose membrane (Hybond-C; Amersham Biosciences). After blocking of the membrane with 4% milk in wash buffer, incubation with primary (mixed mAbs consisting a pool of four mouse anti-prM mAbs or each anti-prM mAb) and secondary (horse-radish peroxidase-conjugated anti-mouse or anti-human IgG) antibodies (Pierce), and final washing, the signals were detected by enhanced chemiluminescence reagents (PerkinElmer Life Sciences). The intensities of E and prM bands of WT pCB-D1 and mutants were analyzed by ImageQuant (GE Healthcare) (47), and the prM/E index of a mutant equals (the intensity of mutant prM band/mutant E band)/(the intensity of WT prM band/WT E band).

**Replicon, CprME Constructs, and in Vitro Transcription**—The DENV1 replicon construct (DENV-1-RLuc2A-Rep) was described previously (32). To generate CprME construct, a PCR fragment containing the Kozak sequence at the 5′-end, C gene, and partial prM gene was digested with KpnI and PstI and cloned into pCB-D1 (designated as CprME). To generate MH mutations in the context of CprME, MH mutants in pCB-D1 were digested with KpnI and XcmI and cloned into CprME. All constructs were confirmed by sequencing the entire inserts to rule out second site mutations. The sequences of primers for mutagenesis and cloning will be provided upon request. Replicon RNA was transcribed in vitro using DENV-1-RLuc2A-Rep that was linearized with SacI as template and an AmpliScribe™ T7 High Yield transcription kit according to the manufacturer’s instructions (Epicenter) (45).

**Replicon Particle Assay**—Replicon particles of DENV1 were prepared by trans-supplying CprME proteins to replicon RNA. BHK-21 cells (4 × 10⁶ cells) were electroporated with 10 μg of DENV1 replicon RNA according to the manufacturer’s instructions (Neon transfection system; Invitrogen). The transfected cells were resuspended in DMEM with 10% FBS, incubated at 37 °C for 24 h, and electroporated again with 10 μg of DNA containing WT or mutant CprME. After incubation at 30 °C for 48 h, culture supernatants were centrifuged to remove cellular debris, followed by ultracentrifugation at 65,000 × g at 4 °C for 5 h to obtain pellets containing replicon particles, which were resuspended in medium and stored at –80 °C (45). To determine the amount of RNA in replicon particles, replicon RNA was extracted and dissolved in 50 μl of RNase free water, of which 10 μl was quantified by a real time RT-PCR assay using primers targeting the DENV1 3′-nontranslated region (5′-ACACCAAGGGGAAGCTGTACCCCTGGA-3′ and 5′-CATTCC-CATTTTCTGGGCCTTCT-3′) (49), the iScript™ one-step RT-PCR kit with SYBR Green (Bio-Rad) and serial dilutions of a control plasmid D1–3′-nontranslated region with known copy numbers as a standard. The amount of RNA was expressed as genome equivalent (GE) copy number as described previously (50). For replicon particle infection assay, Vero cells (1 × 10⁵ cells/well) in a 24-well plate were infected with equal amounts of WT or mutant replicon particles (normalized by RNA GE copy number). At 72 h postinfection, cells were washed once with PBS, lysed, and assayed for luciferase activity using a Renilla luciferase assay kit (Promega) and a luminescence microplate reader (PerkinElmer Life Sciences).

**RESULTS**

**Highly Conserved Residues at the MH Domain Are Involved in the Production of DENV1 VLPs**—To investigate the roles of nine highly conserved MH domain residues (Fig. 1) on the assembly of VLPs, site-directed mutagenesis was carried out to replace each of these residues with an alanine in a DENV1 prME expression construct, pCB-D1 (45). Substitutions with alanine are commonly employed to study the effect of removing side chain with the helical structure preserved (51, 52). As shown in Fig. 2A, prM/E proteins of mutants in cell lysates were readily detectable compared with those of WT pCB-D1, suggesting that alanine substitutions do not greatly affect the expression of prM and E proteins. In contrast, greatly reduced prM and E protein bands were found in pellets (containing VLPs) derived from ultracentrifugation of culture supernatants for mutants E114A, E124A, W126A, L128A, and R129A, when compared with those of WT (Fig. 2A). We further performed a quantitative capture ELISA to measure the concentration of E protein, [E], in pellets and cell lysates. Consistent with the result of Western blot analysis, the ratios of [E] in pellets to [E] in cell lysates were greatly reduced for mutants E114A, E124A, W126A, L128A, and R129A (Fig. 2B), suggesting that mutations introduced to four highly conserved residues at the C terminus and one at the N terminus of MH domain greatly affect the production of VLPs.

**Glutamic Acid of Residue 114 Is Involved in prM-E Interaction**—To investigate whether these mutations affect the prM-E interaction, lysates of 293T cells transfected with each of these mutants were subjected to an immunoprecipitation assay using an anti-E mAb (45). As shown in Fig. 2C, the amounts of prM protein relative to E protein of mutants M111A, S112A, G115A, and W117A were generally comparable with that of the
WT, suggesting that alanine substitutions introduced to these residues do not greatly affect the prM-E heterodimeric interaction. In contrast, the amounts of prM protein relative to E protein in mutant E114A were greatly reduced, suggesting that glutamic acid at residue 114 is critical for prM-E interaction. Notably, the amounts of prM protein relative to E protein in mutants E124A, W126A, L128A, and R129A were increased, suggesting stronger prM-E interaction by these mutations.

It was noted that the prM band of mutant E124A, although expressed well, migrated slower than WT prM band (Fig. 2A), suggesting that this mutation might affect certain conformation of prM protein. To rule out the possibility that other alanine mutations affect the conformation of prM protein, we employed a panel of mouse and human anti-prM mAbs in a dot blot binding assay in which cell lysates derived from transfection of each mutant were prepared in 1% Nonidet P-40 lysis buffer that contains a mild nonionic detergent without reducing agent such as H9252

FIGURE 1. Schematic drawing of prM protein and different domains of M protein of DENV1. The prM protein is cleaved at position 91 by furin or furin-like protease to generate the pr peptide and M protein, which contains an N-terminal loop (first 20 residues), an α-helical domain (MH), and two transmembrane domains (MT1 and MT2). Sequences of the MH domain of different strains of four DENV serotypes were obtained from GenBank; one or two strains from the same outbreak or group were included to ensure coverage of diverse strains from each serotype. There are nine residues (bold type) highly conserved by four DENV serotypes. Single-letter designations of amino acid are shown with the numbers above indicating amino acid positions and the numbers beneath indicating the number of strains containing such residue.

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MH Domain Residues Are Involved in the Assembly of Replicon Particles—To further investigate the effect of these mutations on the assembly of replicon particles and infectivity, we employed a previously described replicon particle packaging system, in which a DENV1 luciferase-reporting replicon was trans-supplied with DENV1 WT or mutant structural proteins (CprME) by sequential transfection (Fig. 3A) (32, 45). Nine CprME constructs, each containing an alanine substitution of these MH domain residues, were generated. Seventy-two hours after transfection, luciferase assay and Western blot analysis of cell lysates revealed that the luciferase activities and the amounts of NS1 (Fig. 3C) and E (Fig. 3D) proteins in the mutant-transfected cells were comparable with those in the WT-transfected cells, suggesting that these mutations do not affect the replication of replicon RNA and expression of non-structural or structural proteins in the packaging cells.

We next used quantitative capture ELISA to examine the amounts of E protein in the replicon particles at 72 h post-transfection. Compared with that of WT, the [E] was reduced...
for all four mutants at the C terminus (E124A, W126A, L128A, and R129A) and three at the N terminus (M111A, S112A, and E114A) of MH domain (Fig. 3E), suggesting that mutations introduced to the highly conserved residues of MH domain affect the assembly of replicon particles, a finding generally in agreement with that of VLPs (Fig. 2B).

We further examined the amounts of RNA in replicon particles at 72 h post-transfection by a quantitative RT-PCR assay. Compared with the WT, all four mutants at the C terminus (E124A, W126A, L128A, and R129A) and three at the N terminus (S112A, E114A, and W117A) showed more than one log reduction in the amounts of replicon RNA (Fig. 3F). This is generally consistent with the effect of these mutations on the assembly of replicon particles except that W117A had mild reduction of [E] in replicon particles. Notably, three mutants (E124A, L128A, and R129A) had more than 2 logs reduction in

FIGURE 2. Expression, production of VLPs, and prM-E interaction of nine alanine mutants in the MH domain. A, expression and production of VLPs. At 48 h post-transfection, 293T cell lysates and pellets derived from culture supernatants were subjected to Western blot analysis using a human dengue-immune serum. Anti-calnexin mAb was tested for cell lysates (lower left gel). The long exposure gel for prM bands in pellets was shown (lower right gel) (45). One representative experiment of three is shown. B, quantification of [E] in cell lysates and pellets by a quantitative capture ELISA, which was based on the standard curve generated by known concentrations of a recombinant DENV1 E protein. Shown are the [E] pellets/[E] cells of each mutant relative to that of the WT. The dashed line indicates relative [E] pellets/[E] cells of 0.25. C, prM-E interaction determined by immunoprecipitation of transfected 293T cell lysates using anti-E mAb FL0251 at pH 8.0, SDS-12% PAGE, and Western blot analysis using a human dengue-immune serum and mouse anti-prM mAb 14-4. The sizes of molecular mass markers are shown in kDa. The prM/E index of each mutant is shown (47); the dashed line indicates prM/E index of 0.5. One representative experiment of three is shown. The data are means with standard deviation from three experiments. *, p < 0.03 compared with WT, two-tailed Mann-Whitney test. D, recognition of mutant prM protein by six anti-prM mAbs. WT and mutant prM proteins prepared in 1% Nonidet P-40 lysis buffer were subjected to dot blot binding assay using each anti-prM mAb and mixed mAbs. Layout of the dot blot assay is shown at the left. Decreasing amounts of WT prM protein were included in the left row of each membrane to control for exposure of the signal; the relative intensities of such control dots were shown below each membrane (white bars) (46, 47).
the amounts of replicon RNA, suggesting that these mutations might affect the incorporation of RNA or nucleocapsid into replicon particles. This was further supported by the ratio of replicon RNA to [E], of which these three mutants showed severe reduction (Fig. 3).

Mutations Introduced to MH Domain Residues Affect Entry of Replicon Particles and Cleavage of prM Protein—To further investigate the effect of these mutations on virus entry, we inoculated WT and mutant replicon particles, after normalizing the amount of RNA in replicon particles, into Vero cells. Compared with WT, the luciferase activities in most (eight of nine) mutant-infected cells were greatly (more than 1 log) reduced at 48 h postinfection except mutant S112A (Fig. 4A). Because these mutations, present on the surface of replicon particles, did not affect the replication of replicon RNA (Fig. 3B) or expression of structural and nonstructural proteins (Fig. 3, C and D), the reduced luciferase activities in the mutant-infected cells suggest that alanine substitutions introduced to these eight residues affect the entry of replicon particles.

We next examined whether these mutations inhibit the cleavage of prM protein and thus produce more immature particles; immature DENV particles (prepared from furin-deficient cells) have been shown to be 10,000-fold less infectious compared with DENV particles prepared from normal cells.
Western blot analysis of the pellets containing VLPs revealed that in addition to E protein, prM protein can be readily detected by a human dengue-immune serum (Fig. 4B), suggesting that VLPs derived from 293T cells contain immature particles. This is in agreement with a previous report (54). We then used a rabbit anti-M serum (against M peptide) in the Western blot analysis and found a predominant prM band with some M band in the WT VLPs, suggesting that VLPs derived from 293T cells contain predominant immature or partially immature particles and small proportion of mature particles (54, 55). The amounts of M protein relative to prM protein in VLPs derived from most (eight of nine) mutants were lower than those of WT except mutant S112A (Fig. 4B), suggesting that these mutations affect the cleavage of prM protein. The extent of prM cleavage in WT and mutant VLPs (Fig. 4C) revealed a pattern generally in agreement with the luciferase activities in replicon particles-infected cells (Fig. 4A), suggesting that most (eight of nine) of these mutations affect the entry of replicon particles by inhibiting the cleavage of prM protein and thus generating predominant immature particles.

Mutations Introduced to MH Domain Residues Affect prM-E Interaction at Low pH—During the maturation of DENV in the trans-Golgi, spiky immature particles become smooth immature particles under low pH environment, and the prM cleavage site was repositioned to expose to furin protease (15, 57). Because the MH domain is close to E protein domain II in mature virions, it was proposed that the membrane-associated MH domain attracts E protein to membrane under low pH to facilitate the formation of smooth immature particles. This was supported by the stronger binding of MH peptide to E protein and to liposome at low pH compared with that at neutral pH (58). To test the possibility that these alanine mutants affect the interaction between MH domain and E protein at low pH, we examined prM-E interaction at pH 6.0 by immunoprecipitation assay. As shown in Fig. 5, the amounts of prM protein relative to E protein were reduced in seven mutants (the exceptions being
G115A and E124A), suggesting that reduced binding between the MH domain and the E protein at low pH and thus reduced formation of smooth immature particles and less exposure of prM cleavage site might contribute to the inefficient prM protein cleavage in most of these mutants.

It is worth noting that the prM protein of mutants G115A and E124A, which showed increased prM-E interaction at low pH (Fig. 5), cannot be recognized well by anti-M serum compared with WT (Fig. 4B), suggesting that these two mutations might affect the local conformation of M protein required for recognition by this anti-M serum; this serum was raised against a M peptide from positions 92–130. Such local conformational change of M protein on VLPs might also contribute to inefficient prM protein cleavage in these two mutants. To rule out the possibility that these mutations affect the overall conformation of prM protein on VLPs, pellets containing WT and mutant VLPs (prepared in 1 × PBS) were dotted on membrane in the same format as in Fig. 2D and probed with mouse and human anti-prM mAbs. Compared with the dot containing WT VLPs, the dots containing mutant VLPs were recognized well by mixed mAbs (Fig. 6A), suggesting that similar amounts of VLPs were dotted. For each of the six mAbs, the intensities of dots containing mutant prM proteins were comparable with those containing WT prM protein (Fig. 6, A and B), suggesting that these mutations do not affect the overall conformation of prM protein on VLPs.

**DISCUSSION**

In this study, we investigated the roles of nine highly conserved MH domain residues in the replication cycles of DENV by site-directed mutagenesis using a DENV1 prME expression construct and examined the production of VLPs, assembly and entry of replicon particles. We found that alanine substitutions introduced to four highly conserved residues at the C terminus and one at the N terminus of the MH domain greatly affected the production of both VLPs and replicon particles. In the entry assay, eight of the nine mutants severely affected the entry of replicon particles, which correlated with the impairment in the cleavage of prM protein, suggesting that these MH mutations affect the maturation of particles thus the entry step of infection. To our knowledge, this is the first study of complete characterization of MH domain and showing that MH domain residues, located at 20–38 amino acids downstream from the prM cleavage site, can affect the prM cleavage, maturation of particles, and infectivity.

The cleavage of cellular precursor proteins by furin, a member of the mammalian subtilisin/kex2p-like endoprotease family, generally requires a consensus sequence motif containing basic amino acids (Arg-Xaa-Arg/Lys-Arg) proximal to the cleavage site (59, 60). Previous studies of flaviviral prM protein demonstrated the importance of the Arg-Xaa-Arg/Lys-Arg motif (at positions P4 to P1) for the cleavage of prM protein (11); moreover, charged residues adjacent to the consensus motif such as those at positions P3, P5, and P6 have been shown to affect the efficiency of prM cleavage (61). Based on the crystal structure of furin, the active site cleft consisting of 18 clustered negatively charged residues and S1, S2, and S4 subsite pockets may explain the stringent requirement of arginine at P1 and P4 and lysine at P2 (62). Our findings that highly conserved prM residues at positions 111–29, 20–38 residues downstream from the prM cleavage site, affect the prM cleavage were surprising. A recent cryo-EM study of mature DENV2 virions at high resolution (3.5 Å) revealed that several residues at the N-terminal loop of M protein (prM protein positions 91–110) interact with domain II residues of E protein (34). These interactions were also shown by another cryo-EM study of mature and immature DENV1 virions at 4.5 and 6 Å resolution, respectively (63). Based on these observations and previous structural studies of prM/E proteins and E protein at pre- and postfusion states, a model of maturation was proposed (33, 34, 56, 64–66). After low pH-induced conformational change of prM and E proteins including the tightening of the unfolded pr and pulling down of E domain II by the folded head of pr in the trans-Golgi, spiky immature particles become smooth immature particles where the prM cleavage site was repositioned to expose to furin protease. After furin cleavage, the N-terminal loop of M protein passed through the hole between two E monomers and internalized below E dimer (34).

Although the extended part of pr and M protein including the prM cleavage site are predicted to undergo a large conformational change during the maturation process and be repositioned for furin protease, there is no definite structural information to support the cleavage intermediate because of the unclear electron density of the extended part of pr and M protein (positions 82–112) in the crystal structure of recombinant prM-E proteins (65). The close proximity of (∼8 Å) of E protein domain II and MH domain after maturation led to the hypothesis that the membrane-associated MH domain might attract the E protein to membrane under low pH in trans-Golgi to facilitate the formation of smooth immature particles. This was supported by the stronger binding of MH peptide to E protein and to liposome at low pH compared with neutral pH (58). In this regard, our findings of reduced prM-E interaction at low pH in MH domain mutants suggest that reduced formation of smooth immature particles and thus less exposure of prM cleavage site might contribute to the inefficient prM protein cleavage in most of these mutants (Fig. 7). Notably, two prM mutants (G115A and E124A), which showed no reduced prM-E interaction at low pH, might interfere prM protein cleavage by affecting the local conformation of M protein, because both mutant proteins can be recognized well by six anti-prM mAbs tested (Fig. 6) but not by an anti-M serum (Fig. 4B). Interestingly, quantification of the ratio of the intensity of mutant prM band to E band in cell lysates revealed that mutant E124A had increased prM/E ratio, whereas other mutants had comparable or mild decreased (less than 2-fold) prM/E ratios compared with that of the WT (data not shown). Mutant E124A might affect the local conformation of prM protein and enhance the recognition by a human dengue-immune serum in the Western blot analysis (Fig. 2A). The phenotypes of these nine mutants were summarized in Fig. 7B. It is worth noting that four mutants at the C terminus (E124A, W126A, L128A, and R129A) showed increased prM-E interaction at pH 8; this may affect the release of E protein to form trimer during the process of fusion with endosomal membrane in the new target cells and
further contribute to the impairment in entry of these four mutants.

Previously, we reported that proline substitutions introduced to residues in the C terminus of MH domain (positions 120, 123, and 127) greatly affected the assembly of VLPs and virions; these observations and analysis of adaptive mutations suggest the importance of the C-terminal helical structure of MH domain for assembly of particles (45). In this study, we focused on alanine substitutions of nine highly conserved MH domain residues and found four at the C terminus and one at the N terminus of the MH domain that greatly affected the production of both VLPs and replicon particles, suggesting the importance of these highly conserved residues. In addition, proline substitutions intro-

**FIGURE 6.** Recognition of prM proteins of WT and MH domain mutants on VLPs by different anti-prM mAbs based on dot blot binding assay. A, at 48 h, pellets (containing VLPs) derived from ultracentrifugation of culture supernatants of 293T cells transfected with WT or mutant constructs (in 1 × PBS) were dotted on nitrocellulose membrane and subjected to dot blot binding assay using six anti-prM mAbs and mixed mAbs. The layout and controls of the assay are presented as in Fig. 2D. B, the recognition index (R.I.) of each mAb to mutant prM protein as described under “Experimental Procedures” (46, 47) is shown in gray bars. The data are means with standard deviation from two experiments.
duced to these nine highly conserved residues revealed that all four mutants at the C terminus and two mutants at the N terminus greatly affected the production of VLPs (data not shown). Because the N terminus of MH domain is close to but outside the membrane and the C terminus is buried in the head group region of membrane (33, 34), it is conceivable that the mutations introduced to most MH domain residues at the C terminus and some at the N terminus affect the curving and bending of the membrane during assembly in ER. In summary, we showed that highly conserved residues in the MH domain of prM protein are involved in multiple steps of DENV replication cycle, including assembly, cleavage of prM protein, maturation of particles, and virus entry. The highly conserved nature of these residues suggests that they are potential targets of future antiviral strategy against DENV. It should be noted that of the nine residues, three at the C terminus (positions 124, 126, and 129) are also conserved by other flaviviruses including Japanese encephalitis virus, YFV, West Nile virus, and tick-borne encephalitis virus (Fig. 1). This suggests that the C terminus of MH domain is probably also involved in the assembly, prM protein cleavage, maturation, and entry of other flaviviruses.
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