Functional analysis of dengue virus (DENV) type 2 envelope protein domain 3 type-specific and DENV complex-reactive critical epitope residues

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The dengue virus (DENV) envelope protein domain 3 (ED3) is the target of potent virus neutralizing antibodies. The DENV-2 ED3 contains adjacent type-specific and DENV complex-reactive antigenic sites that are composed of a small number of residues that were previously demonstrated to be critical for antibody binding. Site-directed mutagenesis of a DENV-2 16681 infectious clone was used to mutate critical residues in the DENV-2 type-specific (K305A and P384A) and DENV complex-reactive (K310A) antigenic sites. The K305A mutant virus multiplied like the parent virus in mosquito and mammalian cells, as did the P384A mutant virus, which required a compensatory mutation (G330D) for viability. However, the K310A mutant virus could not be recovered. The DENV-2 type-specific critical residue mutations K305A and P384A+G330D reduced the ability of DENV-2 type-specific, but not DENV complex-reactive, mAbs to neutralize virus infectivity and this was directly correlated with mAb binding affinity to the rED3 mutants.

The disease dengue (DEN) is caused by four serologically and genetically related dengue viruses (DENVs) termed DENV-1, -2, -3 and -4. The DENV envelope (E) protein is composed of three domains: E protein domain I (ED1) is the central domain, ED2 is the dimerization domain and contains the conserved fusion loop, and ED3 is the putative receptor-binding domain (Modis et al., 2003). The E protein occupies the majority of the viral surface and is the primary antigen targeted by the humoral immune response (Kuhn et al., 2002; Pierson et al., 2008). Extensive antibody mapping studies using mouse mAbs have identified at least 12 different epitopes on the surface of the E protein, with those located on ED3 elicitng some of the most potent neutralizing antibodies (Pierson & Diamond, 2008; Roehrig et al., 1998). Furthermore, two major overlapping antigenic sites have been identified on ED3 and include the DENV type-specific and DEN complex-reactive antigenic sites (Gromowski & Barrett, 2007; Gromowski et al., 2008; Roehrig et al., 1998; Sukupolvi-Petty et al., 2007). Epitope mapping studies have demonstrated that these sites are composed of a small number of critically important residues (i.e. critical residues) that significantly reduce antibody binding to recombinant ED3 when mutated (Gromowski & Barrett, 2007; Gromowski et al., 2008; Hiramatsu et al., 1996; Sukupolvi-Petty et al., 2007). Critical residues composing the DENV-2 type-specific antigenic site are lysine at position 305 and proline at position 384 (Figs 1a and S1, available in the online Supplementary Material), whereas, the DEN complex-reactive antigenic site is composed of one critical residue, lysine at 310 (Fig. S1). It should be noted that in comparison to the studies with mouse mAbs where ED3 stimulates the most potent neutralizing antibodies, studies to date with human mAbs indicate the human antibody repertoire is different, and anti-ED3 antibodies do not appear to play an important role in induction of protective immunity (Smith et al., 2013; Fibriansah et al., 2014).

To maintain its transmission cycle, DENV must infect and replicate in both mosquito and human cells. Thus, DENV requires the conservation of important functional residues that mediate this interaction. Interestingly, the critical antibody-binding residues in ED3 are conserved
throughout the six DENV-2 genotypes and reside in regions hypothesized to play roles in the entry of DENV into both mammalian and mosquito cells (Chen et al., 1997; Erb et al., 2010; Hung et al., 2004; Pitcher et al., 2012). Specifically, the role of the ED3 F–G loop (containing the critical residue P384) for entry into C6/36 cells (mosquito larva) was demonstrated by an F–G loop peptide that blocked viral infection of C6/36 cells (Hung et al., 2004). However, substitution or deletion mutations of the F–G loop were able to infect C6/36 cells (Erb et al., 2010). Overall, the functional importance of the critical epitope residues K305, K310 and P384 for other events in the viral life cycle and for antibody-mediated neutralization has not been readily studied.

In order to examine the role of critical epitope residues composing the DENV-2 type-specific and DEN complex-reactive antigenic sites on viral biology, single-site alanine substitutions were engineered by site-directed mutagenesis into the DENV-2 strain 16681 infectious clone (IC) (Kinney et al., 1997). This approach was used successfully with a recombinant ED3 and effectively removed the amino acid side chains, while retaining the native ED3 conformation (Gromowski et al., 2010). The WT and three mutant ICs (K305A, K310A and P384A) were transcribed in vitro and electroporated into Vero cells (African green monkey kidney). The transfections yielded infectious virus from the WT, K305A and P384A ICs but not from the K310A IC, as determined by focus-forming assay and reverse transcriptase-PCR (RT-PCR) of cell culture supernatant up to 7 days post-transfection.

Subsequent efforts to recover the substitution K310A IC included transcription and transfection into C6/36 cells. RT-PCR indicated the presence of viral RNA in cell culture supernatants from day 2 onwards, but the RNA was not further quantified. Ultimately no infectious virus was detected in this or in subsequent C6/36 passages. These results imply an important structural role for residue K310, which forms a latch on a hydrophobic pocket containing the fusion loop and is also involved in inter-dimer interactions (Cockburn et al., 2012). The decreased mass and lack of charge of alanine may expose the hydrophobic pocket and also prevent the inter-dimer interactions required for infectious virus (Fig. 1c–e). Interestingly, a triple-substitution DENV-2 mutant IC containing the substitutions K305E, K307E and K310E was recently recovered in C6/36 cells, which suggests the added stability of glutamic acid in the IC stems from a comparable size to lysine and not from opposite chemical polarity (Fig. 1d) (Roehrig et al., 2013; Wahala et al., 2012).

Genetic stability of the recovered mutant ICs was evaluated by serial passage in Vero cells. The nucleotide sequence of

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**Fig. 1.** Pymol structural renderings of ED3 (PDB 1OAN). (a) ED3 epitopes for type-specific mAbs 3H5 and GTX77558, which differ by a single peripheral residue, aspartic acid at 329 (dashed circle). (b) Ribbon structure shows the location of critical residues and mutations in ED3, shown as sticks, with the A-strand shown in pink, B–C loop in green, and the F–G loop in red. (c–e) Ribbon structure of E protein, with ED1 shown in red, ED2 in yellow, ED3 in blue, the fusion loop in green (W101 shown), and the residue at position 310 in black. (c) WT ED3 with K310, (d) K310E substitution mutant; and (e) K310A substitution mutant.
the E protein gene of WT and K305A ICs remained stable throughout three passages. In contrast, the P384A IC developed a compensatory G330D substitution in ED3 following one passage, but additional passages did not result in further substitutions. Sequence data indicated that G330D was not in the P384A IC cDNA, arose following transfection of Vero cells, and was subsequently stable. Furthermore, G330D was encoded by the substitution of GGC to GAC, suggesting that selection for aspartic acid was not due to nucleotide drift. Interestingly the residues 330 and 384 are not contiguous in ED3, residing in different loops: B–C and F–G, respectively (Fig. 1b). The G330D substitution occurs naturally and is characteristic of the DENV-2 sylvatic genotype (Chambers et al., 1990; Pitcher et al., 2012; Sukupolvi-Petty et al., 2010).

The P384A mutant IC was retransfected into Vero cells to recover the single mutant independently of G330D, but no infectious virus was recovered, as determined by focus-forming assay, indicating that the substitution of P384A reduced the fitness of the virus. Interestingly, another DENV-2 16681 IC mutant, G304K, also resulted in the same compensatory G330D mutation after a single Vero cell passage (Roehrig et al., 2013). Moreover, F–G loop substitutions have the potential to disturb virus infectivity in Vero cell culture, a result recently observed with the DENV-2 ICs containing F–G loop deletions (VEPGA) or F–G loop transplantations from other flaviviruses (e.g. yellow fever and Japanese encephalitis viruses) (Erb et al., 2010).

The impact of the two type-specific critical epitope residue substitutions (K305A and P384A + G330D) on viral multiplication following an m.o.i. of 0.1 was compared with the WT IC in Vero and C6/36 cells. Both mutant ICs exhibited the same replication kinetics as the WT IC in both cell types (Fig. S2). Interestingly, all three ICs required 120 h to reach peak infectivity titres in C6/36 cells but only 48 h in Vero cells. Thus, the P384A + G330D IC-derived virus would suggest that residue P384A, but not residue K305A, of the DENV-2 type-specific antigenic site is critical for multiplication in mammalian or mosquito cell lines. This would be consistent with the mutant viruses generated by Erb and colleagues who demonstrated that removal of the ED3 F–G loop significantly reduced the infectivity of these viruses in mammalian cells and mosquito midguts, but not in C6/36 cells, and secondary mutations were necessary for replication of their F–G loop mutants (Erb et al., 2010).

The effects of critical epitope residue substitutions on mAb apparent affinity were examined using a panel composed of two DENV-2 type-specific mAbs [3H5 (Millipore) and GTX77558 (GeneTex)], and two DEN complex-reactive mAbs [GTX29202 (GeneTex) and MD-05-0104 (Ray Biotech)]. The reactivities of these antibodies were analysed with rED3s engineered with single site substitutions K305A, K310A, G330D and P384A and were compared with the WT rED3 of DENV-2 strain 16681 using an ELISA, as described previously (Pitcher et al., 2012). The apparent affinities of the DENV-2 type-specific mAbs 3H5 and GTX77558 for WT rED3 were 1.2 ± 0.1 nM and 0.5 ± 0.1 nM. The apparent affinities of these mAbs for rED3 containing K305A and P384A decreased significantly. Substitution of type-specific critical residues K305A or P384A eliminated the binding of mAb GTX77558 and resulted in a 17–36-fold reduction in mAb 3H5 affinity as compared with the WT rED3 (Table 1). Substitutions K310A and G330D did not significantly (P value > 0.05 by Student’s t-test) affect the apparent affinity of the DENV-2 type-specific mAbs, which only changed 0.7–2.2-fold for K310A and 0.8–1.1-fold for G330D. Both of the DEN complex-reactive mAbs GTX29202 and MD-05-0104 bound to WT rED3 with similar apparent affinities, 0.2 ± 0.02 nM and 0.5 ± 0.03 nM, respectively, but as expected the substitution K310A eliminated the binding of these DEN complex-reactive mAbs to rED3 (Table 1). Substitution of P384A weakly affected binding of the DEN complex-reactive mAbs, changing the affinities by 2.1–5.1-fold. Unlike K310A or P384A, the substitutions of K305A or G330D did not affect the binding of either DEN complex-reactive mAb (0.5–1.9-fold for K305A, and 0.5–1.3-fold for G330D; Table 1). Taken together, these results demonstrated that the DENV-2 type-specific and DEN complex-reactive antigenic sites are distinct (i.e. substitutions in one site do not affect the binding of mAbs to the other). These data also showed that the compensatory substitution G330D did not affect the binding of either class of mAb to rED3.

To evaluate the impact of the DENV-2 type-specific critical residues on antibody-mediated neutralization, the same panel of four mAbs was used in focus reduction neutralization tests (FRNT50). Additionally, a flavivirus group-reactive mAb that recognizes an epitope outside of ED3 (mAb 4G2; Millipore) was used as a control because it binds to the conserved fusion loop in ED2 (Huang et al., 2010) and should neutralize all viruses regardless of ED3 substitutions. As predicted from the rED3 ELISA physical binding studies, mAbs 3H5 and GTX77558 effectively neutralized the WT IC, but the FRNT50 concentrations differed significantly when assayed with viruses containing either K305A or P384A + G330D substitutions (Fig. 2a). Thus, the markedly reduced binding of mAb 3H5 to the K305A and P384A mutant rED3s detected via ELISA was corroborated in the neutralization assays. With mAb 3H5, substitution of K305A decreased neutralization efficacy by 14-fold compared with WT virus; 1.1 ± 0.3 nM for WT IC versus 16.3 ± 1.4 nM for the K305A IC (Table 1). Compared with the WT IC, neutralization by mAb 3H5 was further reduced with the P384A + G330D IC from 1.1 ± 0.3 nM to approximately 80 nM, a 70-fold reduction (Table 1). Neutralization by mAb GTX77558 was affected to a greater extent by the substitutions K305A and P384A + G330D because both substitutions eliminated detectable neutralization (Table 1). These results were consistent with the ELISA data that demonstrated that
K305A and P384A abrogated antibody binding. Overall, these data demonstrated the importance of critical epitope residues for neutralization and mAb affinity to ED3 was correlated with neutralization.

Amino acid residues K305 and P384 are not critical for the binding of DEN complex-reactive mAbs to rED3, but their role in neutralization by the DEN complex-reactive antibodies had not been examined. Both DEN complex-reactive mAbs GTX29202 and MD-05-0104 neutralized the WT and P384A+G330D ICs with similar FRNT_{50} concentrations (Fig. 2b, Table 1), but the K305A substitution weakly affected neutralization by 2–4-fold. Thus, a modest reduction in neutralization efficacy was observed that was not correlated with reduced affinity for the corresponding rED3 mutants. Lastly, as expected, there were no differences in neutralization efficacy of the WT, K305A and P384A+G330D ICs by the flavivirus group-reactive mAb 4G2 (Table 1). Overall the results with the DEN complex-reactive mAbs and the flavivirus group-reactive mAb 4G2 demonstrate that substitutions in the DENV-2 type-specific antigenic site do not have global effects on the E protein structure and only eliminate the neutralizing efficacy of type-specific mAbs.

Substitutions of DENV-2 type-specific critical epitope residues in rED3 significantly decreased the apparent

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**Table 1.** Comparison of mAb binding (apparent affinity) and neutralization titre in Vero cells

<table>
<thead>
<tr>
<th>Relative K_{D} compared with WT rED3</th>
<th>DENV-2 type-specific</th>
<th>DEN complex-reactive</th>
<th>Flavi-reactive</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>3H5</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT 16681</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>K305A</td>
<td>17.7 ± 2.1 nM</td>
<td>0.5 ± 0.1 nM</td>
<td>0.6 ± 0.1 nM</td>
</tr>
<tr>
<td>P384A</td>
<td>35.8 ± 7.1 nM</td>
<td>5.2 ± 2.1 nM</td>
<td>16.3 ± 1.4 nM</td>
</tr>
<tr>
<td>K305A</td>
<td>11.1 ± 1.4 nM</td>
<td>80 ± 10 nM</td>
<td><strong>ND</strong></td>
</tr>
<tr>
<td>G330D</td>
<td>0.8 ± 0.5 nM</td>
<td>1.3 ± 0.5 nM</td>
<td><strong>ND</strong></td>
</tr>
<tr>
<td><strong>MD-05-0104</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT 16681</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>K305A</td>
<td>17.7 ± 2.1 nM</td>
<td>0.5 ± 0.1 nM</td>
<td>0.6 ± 0.1 nM</td>
</tr>
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<td><strong>ND</strong></td>
</tr>
<tr>
<td>G330D</td>
<td>0.8 ± 0.5 nM</td>
<td>1.3 ± 0.5 nM</td>
<td><strong>ND</strong></td>
</tr>
</tbody>
</table>

*ND, Neutralization not detected at up to 80 nM for DENV-2 type-specific mAbs.
*Assay could not be performed.

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**Fig. 2.** FRNT_{50} curves of mAb 3H5 (a) and mAb MD-05-0104 (b) neutralization assays with WT 16681 IC (blue), 16681 IC K305A (green) and 16681 IC P384A+G330D IC (purple). Each point represents the mean ± SEM of two separate experiments, each performed in triplicate. Type-specific and DEN complex-reactive mAb neutralization titres of ICs were determined by non-linear regression of FRNT_{50} curves.
affinity of two DENV-2 type-specific mAbs for rED3 (approx. 20–40-fold for mAb 3H5 and loss of binding for GTX77558). ICs engineered with the K305A and P384A+G330D substitutions were used to determine how major changes in mAb affinity affected the biological process of neutralization. The efficacy of mAb 3H5-mediated neutralization of mutant ICs decreased (15–73-fold reduction from WT IC) when evaluated with viruses containing either DENV-2 type-specific critical residue, thereby demonstrating the correlation between reductions in apparent affinity and neutralizing efficacy for mAb 3H5. Similarly, these substitutions also abrogated mAb GTX77558 binding and neutralization. The differences between the two type-specific mAbs may be due to the different residues that constitute the epitopes recognized by the mAbs (Gromowski & Barrett, 2007) (Fig. 1a). In contrast to the results attained with the DENV-2 type-specific mAbs, the neutralizing ability of the DEN complex-reactive mAbs was relatively unaffected by substitution of critical type-specific epitope residues.

The goal of this study was to determine the functional role of DENV-2 type-specific and DEN complex-reactive critical epitope residues on viral biology and antibody-mediated neutralization. Substitutions of these residues in the DENV-2 IC and recovery of mutant viruses demonstrated that the substitutions K305A and P384A with a compensatory G330D mutation were tolerated, but K310A was not. The multiplicity kinetics of the K305A and P384A+G330D showed that these specific amino acid side chains were dispensable for viral multiplication in mammalian and mosquito cells, but the G330D compensatory mutation was necessary for viability of the P384A mutant and both critical type-specific residues were required for potent antibody-mediated neutralization by the DENV-2 type-specific mAbs. In addition, substitution of critical residues in the DENV-2 ED3 type-specific antigenic site did not have major effects on the binding of mAbs to the adjacent DEN ED3 complex-reactive or distant ED2 fusion loop antigenic sites. Overall, these data demonstrate a strong correlation between the apparent affinity of antibodies for their epitopes and neutralization potency. DENV neutralizing antibodies with critical epitope residues that are functionally important for the virus life cycle are attractive targets for vaccines and antivirals because it would be more difficult for the virus to acquire resistance mutations.

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**References**


