Identification and characterization of a linearized B-cell epitope on the pr protein of dengue virus

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The four serotypes of dengue virus (DENV) represent one of the major mosquito-borne pathogens globally; so far no vaccine or specific antiviral is available. During virion maturation, the pr protein is cleaved from its precursor form the prM protein on the surface of immature DENV by host protease. Recent findings have demonstrated that the pr protein not only played critical roles in virion assembly and maturation, but was also involved in antibody-dependent enhancement of DENV infection. However, the B-cell epitopes on the pr protein of DENV have not been well characterized. In this study, a set of 11 partially overlapping peptides spanning the entire pr protein of DENV-2 were fused with glutathione S-transferase and expressed in Escherichia coli. ELISA screening with murine hyperimmune antiserum against immature DENV identified the P8 peptide (57 KQNEPEDIDCWCNST 71) in the pr protein as the major immunodominant epitope. Fine mapping by truncated protein assays confirmed the 8-e peptide 57 KQNEPEDI 64 was the smallest unit capable of antibody binding. Importantly, the 8-e epitope reacted with sera from dengue fever patients. Site-directed mutagenesis revealed the asparagine residue at position 59 was important for epitope recognition. The 8-e epitope coincided well with the B-cell epitopes predicted by Immune Epitope Database analysis, and 3D structural modelling mapped the 8-e peptide on the surface of prM-E heterodimers. Overall, our findings characterized a linearized B-cell epitope on the pr protein of DENV, which will help to understand the life cycle of DENV and pathogenesis of dengue infections in human.

INTRODUCTION

Dengue virus (DENV) is a mosquito-borne flavivirus of global public health concern as almost 40% of the world’s population now lives at risk of contracting it (Farrar et al., 2007). Infection with any of the four serotypes of DENV results in a large range of disease manifestations, including dengue fever (DF), dengue haemorrhagic fever and a more severe form, life threatening dengue shock syndrome (Gubler, 2002; WHO, 2009). Despite several candidate vaccines being tested in clinical trials, no licensed vaccines against DENV are currently available (Whitehead et al., 2007).

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One supplementary table is available with the online version of this paper.

DENV contains a 10.6 kb positive-sense ssRNA genome, which has a single ORF with untranslated regions at both terminals. The ORF encodes a polyprotein precursor that is co-translationally processed by viral and cellular proteases generating three structural proteins and seven non-structural (NS) proteins. The three structural proteins: capsid (C), precursor membrane (prM) and envelope (E) protein constitute DENV virion together with the RNA genome (Kuhn et al., 2002). DENV virions are assembled on the membrane of the endoplasmic reticulum (ER) and the virus buds into the lumen of the ER as immature virions. Subsequently, the particles mature by passing through the Golgi and trans-Golgi network where the virion undergoes a conformational change and the cellular endoprotease furin cleaves prM into M and pr proteins. (Kuhn et al., 2002; Rodenhuis-Zybert et al., 2010a; Stadler et al., 1997). Upon release, the pr peptide dissociates from
the virion, resulting in the formation of mature progeny virions. The pr protein is a small (approx. 13 kDa) proteolytic fragment of its precursor form prM.

The functional roles of the prM protein are not yet fully understood. Laboratory and epidemiological studies have demonstrated that antibodies against prM protein distinguish between previous infection with DENV and Japanese encephalitis virus (JEV) (Cardosa et al., 2002; Hua et al., 2010). Recent findings demonstrated anti-prM antibodies are highly cross-reactive but possess limited neutralizing activity (Dejnirattisai et al., 2010; Kaufman et al., 1989). The prM-specific antibodies are known to play critical roles in human immune responses to DENV in both primary and secondary infections in addition to E-specific anti-DENV antibodies against prM protein (Dejnirattisai et al., 2010; Lai et al., 2008). Both mouse and human antibodies specific for the prM protein were shown to render normally non-infectious immature DENV (imDENV) highly infectious, and nearly as infectious as wt virus (Dejnirattisai et al., 2010; Rodenhuis-Zybert et al., 2010b). Since large numbers of prM-containing particles (imDENV) were present in DENV-infected mosquito and mammalian cells (Zybert et al., 2008), prM-specific antibodies were induced during natural DENV infections. The anti-prM antibody-mediated infection enhancement has been well demonstrated in vitro (Beltramello et al., 2010; Randolph et al., 1990) and in vivo (Zellweger et al., 2010), even for West Nile virus (WNV) (Colpitts et al., 2011). Further, the neutralization ability of anti-E antibodies is influenced by prM-containing immature virions (Nelson et al., 2008). Thus, anti-prM antibodies play critical roles in controlling DENV infections and pathogenesis.

A recent investigation of acute B-cell response in dengue patients has confirmed that DENV infection causes significant B-cell activation 4–7 days after onset of fever (Balakrishnan et al., 2011). The humoral response is generally directed against viral structural proteins prM, E and non-structural protein NS1 (Beltramello et al., 2010; Cardosa et al., 2002). Identification of B-cell epitopes is important for development of vaccines and design of diagnostic reagents (Wu et al., 2003). Multiple potential B-cell epitopes in the E (Apt et al., 2006; Brien et al., 2010; Gromowski et al., 2008; Lin et al., 2012; Sukupolvi-Petty et al., 2007), NS1 (Steidel et al., 2012; Wu et al., 2003), NS4a and NS5 proteins for DENV have been well characterized. Prediction and analyses of B-cell epitopes of other flaviviruses have also focused on E, NS1 and NS5 proteins (Chiu et al., 2012; Hall et al., 2009; Sun et al., 2012). Although the importance of prM antibodies has been well demonstrated, only a few linear immunogenic determinants in DENV prM protein have ever been identified (Dejnirattisai et al., 2010; Vázquez et al., 2002). In this study, the potential B-cell epitopes in the pr protein of DENV were predicted with bioinformatics software, and peptide scanning and truncation assays mapped a linear B-cell epitope on DENV-2 pr protein.

**RESULTS**

**Identification of the antigenic determinants on the pr protein**

To determine the potential antigenic epitopes on the pr protein, 11 partially overlapping peptides (P1–P11) spanning the entire DENV-2 pr protein (Fig. 1a) were expressed as glutathione S-transferase (GST)-fused proteins in *Escherichia coli*. All the fusion proteins were successfully expressed under the induction of IPTG and purified on a glutathione-Sepharose 4B affinity column. The entire pr protein was expressed in the same system as a positive control. Western blot analysis with mouse anti-GST IgG antibodies showed all the 11 fusion peptides and entire pr proteins were successfully expressed with the expected molecular mass (Fig. 1b). Then, all the 11 truncated pr peptides were scanned by indirect ELISA with mouse hyperimmune antisera against imDENV-2. The results show that only the P8 peptide could react with the murine hyperimmune antisera against imDENV-2, together with the entire pr fusion protein (Fig. 1c). All the other 10 peptides and the GST control failed to bind with the antisera. Thus, the P8 peptide, 57KQNEPEDIDCWCNST71, represented the potential B-cell epitope in DENV pr protein responsible for antibody binding.

**Characterization and fine mapping of the P8 peptide**

To locate the core sequence of the P8 epitope on DENV pr protein, a series of truncated peptides were designed and expressed in the same GST fusion system. One or two amino acid residues were removed from either the N-terminal or the C-terminal of the P8 peptide (Fig. 2). All the truncated peptides were then fused with GST and expressed as described above (data not shown). All the truncated peptides were then analysed by ELISA using the mouse hyperimmune antisera against imDENV-2 described above. The results showed that removal of any amino acid residue from the N-terminal end significantly decreased the reactivity, while the removal of at least seven amino acid residues from the C-terminal end did not affect the reactivity of P8 peptide with the antisera (Fig. 2). The GST control showed no reactivity with the antisera as expected. Together, these results demonstrated that peptide 8-e (57KQNEPEDIDCWCNST71) was the minimal requirement for the linearized epitope.

Then, to confirm the reactivity of the identified 8-e epitope with human sera, ELISA screening was performed with human sera from both DF patients and healthy adults. As shown in Fig. 3, the 8-e peptide was recognized by convalescent sera from all four DF patients (nos 12-12, 12-27, 12-45 and 12-60) infected with DENV-2; however sera from healthy adults (nos NC1 and NC2) failed to bind with the 8-e peptide together with the GST control. These results strongly demonstrated specific antibody response against the 8-e epitope was induced during natural DENV infection.

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Site-directed mutagenesis was then carried out to determine the critical residues responsible for antibody binding with the 8-e epitope. Each amino acid residue of the 8-e peptide was mutated individually into alanine (A, encoded by GCA), respectively. All the mutated peptides were fused with GST and expressed as described above (data not shown). ELISA analysis using mouse anti-imDENV-2 hyperimmune antisera revealed that only the N59A mutation significantly decreased the reactivity of 8-e peptide with the antisera against imDENV-2 (Fig. 4).

Collectively, these results demonstrated that the 8-e peptide 57KQNEPEDI64 represented as a linearized B-cell epitope in the DENV pr protein and the N59 residue was critical for antibody recognition.

Bioinformatics prediction and structural modelling of the 8-e epitope

The pr protein sequences of DENV strains and other flavivirus strains (JEV and WNV) were retrieved from GenBank for alignment. The results revealed that the pr protein sequence was highly conserved among DENV strains, with amino acid identity of over 70%, but was less than 50% in JEV and WNV. Notably, the amino acid residue glycine in position 59 was absent in the DENV pr protein but not in that of WNV and JEV (Fig. 5). The potential B-cell epitopes on DENV-2 pr protein were then predicted by using the Immune Epitope Database (IEDB) online prediction server. Meanwhile, the hydrophilicity, accessibility, charge distribution, flexibility and secondary structure of the pr protein of DENV-2 were evaluated (Table S1, available in JGV Online). Four potential B-cell immunodominant epitopes (Pre-Ep1–Ep4) were selected for further evaluation (Fig. 5). Among those peptides, Pre-Ep3 (57KQNEPEDI65) had the highest calculated score of 8.587. The results supported the peptide scanning results, and Pre-Ep3 well matched the identified 8-e peptide.

Finally, the 8-e peptide was mapped on the 3D crystal structure of prM-E heterodimer at neutral pH [Protein Data Bank (PDB) 3C6E] by using the PyMOL 1.1 program. The results showed that the 8-e peptide was well exposed on the surface of the heterodimer, and the critical amino
acid residue N59 was located at the prM/EDII interface (Fig. 6). This finding supports the importance of this single amino acid residue during antibody recognition.

**DISCUSSION**

In this study, by using bioinformatics analysis and peptide scanning, we characterized a potential linear B-cell epitope in the pr protein of DENV-2. IEDB analysis with multiple methods identified Pre-Ep3, 57KQNEPEDID65, to have the highest score, and protein scanning and truncation assays confirmed the 8-e epitope 57KQNEPEDID64 was the minimal requirement unit for the linearized epitope. In our experiments, mouse hyperimmune antisera against imDENV-2 showed strong reactivity with the identified 8-e epitope (Fig. 2). Most importantly, human convalescent sera from DF patients with DENV-2 also specifically recognized the 8-e epitope (Fig. 3). These results showed specific antibodies against the 8-e epitope were induced in both DENV-infected mice and human, indicating the importance of the 8-e epitope during natural infection.

Previously, five synthetic peptides in the pr protein were shown to induce robust humoral response in mice, and two of the peptides B20-2 (position 45–67) and B19-5 (position 57–92) well covered the 57KQNEPEDID64 epitope identified in this study (Vázquez et al., 2002). Huang et al. also demonstrated that a mouse mAb against the pr protein of DENV was mapped to amino acids 53–67 (Huang et al., 2006). Very recently, a conformational epitope containing partial segments of pr protein was identified by screening of a human Fab-phage library with a DENV prM-specific antibody, and L56, Q58 and N59 in the pr protein were identified as critical residues responsible for antibody binding (Chan et al., 2012). These results fit well with our findings and support the importance of the 8-e epitope characterized in this study.

IEDB analysis predicted four potential B-cell epitopes; however, ELISA scanning showed that Pre-Ep1, Pre-Ep2 and Pre-Ep4 contain peptides that had no reactivity in the ELISA experiments. Only Pre-Ep3 (57KQNEPEDID65), contained the identified antigenic determinants (8-e epitope) was reactive with the hyperimmune antisera
against imDENV-2. Site-directed mutagenesis of the 8-e peptide indicated that the N^{59} residue in the pr protein was the principle residue involved in antibody binding. Sequence alignment of the pr protein of flaviviruses indicated that position 59 is absent in the DENV pr protein compared with that of WNV and JEV (Fig. 5). Previously, studies with mAbs also indicated that the N^{59} residue is involved in the interaction between the antibody and the pr protein (Chan et al., 2012). Structural modelling indicated that N^{59} is located in the interface between the prM and E proteins (Fig. 6), indicating its potential role in antibody recognition and conformational changes. The amino acid residue D^{65} following the 8-e peptide is highly conserved among flaviviruses (position in pr: DENV 65; tick-borne encephalitis virus 67; JEV/WNV 66) and has been identified with critical functions in virion assembly and secretion (Yoshii et al., 2012).

Development of an effective dengue vaccine is considered a high public health priority. However, dengue is unique and complex, and it is especially the antibody-dependent enhancement (ADE) issues that have critically restricted vaccine development. The first large-scale clinical trial of tetravalent dengue vaccine failed to consistently prevent DENV-2 infections in children (Sabchareon et al., 2012). It may be worthwhile to consider an alternative vaccine design approach, and strategies minimizing the cross-reacting antibody responses may contribute to the success of the next generation of dengue vaccines (Crill et al., 2012; Dejnirattisai et al., 2010). Identification and characterisation of the B-cell epitopes in the pr protein of DENV will help in the rational design of a novel vaccine candidate with reduced risks for ADE infection.

**METHODS**

**Viruses and antisera.** DENV-2 strain 43 was isolated from a DF patient in Guangxi, China, and the complete genome sequence was submitted to GenBank under accession no. AF204178.1 (Liu et al., 2010). Immature DENV-2 particles were prepared as previously described (Zybert et al., 2008). Murine hyperimmune antisera were prepared following immunization with three doses of imDENV-2 as previously described (Deng et al., 2011). Human convalescent sera from DF patients (nos 12-12, 12-27, 12-45 and 12-60) and healthy adults (NC) were from Guangzhou No. 8 People’s Hospital, Guangzhou, China. All serum samples were inactivated at 56 °C for 30 min.

**Fig. 5.** Sequence alignment and bioinformatics prediction of potential B-cell epitopes on the pr protein of DENV. The amino acid sequences of the pr protein from selected DENV strains and other flaviviruses are shown above. All sequences were retrieved from GenBank for alignment with MEGA software. The potential B-cell epitopes in the pr protein were predicted (Pre-Ep) using IEDB and basic information for these epitopes are denoted below.

**Fig. 6.** Localization of the 8-e peptide sequence on the 3D crystal structure of a prM-E heterodimer. The 8-e epitope was mapped against the 3D structure using PyMOL 1.1 with the crystal structures of the prM-E heterodimer at neutral pH (PDB accession code: 3C6E). (a) Side view; (b) top view. The prM protein is shown in pink, and the domains I, II and III of the envelope proteins are shown in red, yellow and blue, respectively. The 8-e epitope is shown in green, and the N^{59} residue in the pr protein is in black indicated by arrow.
30 min before assay. The use of human sera was approved by the ethical committee of Guangzhou No. 8 People’s Hospital.

Computer analysis. To analyse the conservation of the pr protein, a total of 2205 strains of all four serotypes of DENV, 82 strains of JEV and 462 strains of WNv were retrieved from GenBank for alignment analysis with MEGA software. The epitope prediction was performed using the IEDB prediction program (SMM; http://tools.immuneepitope.org/tools/bcell/iedb_input). The potential antigenic regions and evaluation methods used in this study were listed as follows: hydrophilicity (Kyte–Doolittle method), accessibility (Emini method), antigenicity (Jameson–Wolf method), flexibility (Karplus–Schulz method), charge distribution (Kolaskar–Tongkook method), secondary structure (Chou–Fasman and Gamier–Robson methods). The 8-e epitope was mapped against 3D structure using the PyMOL 1.1 with the crystal structures of prM-E heterodimer at neutral pH (PDB accession code: 3C6E).

Expression, purification and identification of bolting fusion peptides. All peptides were expressed in Escherichia coli Rosetta as a fusion protein with GST tags. For each short peptide, a pair of oligonucleotide strands was synthesized. The two strands were annealed, and the resultant dsDNA contained a BamHI and an XhoI cohesive terminus at the 5’ and 3’ ends, respectively. The annealed fragment was then cloned into the pGEX-4T-1. All the recombinant plasmids were confirmed by DNA sequencing and then transformed into E. coli Rosetta competence cells. The expression of the recombinant proteins was induced with 0.1 M IPTG at 37 °C for 4 h. The expressed recombinant fusion peptides were analysed with SDS-PAGE, and purified on glutathione-Sepharose 4B affinity column (GE Healthcare Life Sciences) according to the manufacturer’s instructions. Subsequently, the bound fusion protein was eluted with glutathione elution buffer for further analysis. Bolting fusion peptides were identified by Western blotting with mouse anti-GST antibody.

ELISA. To scan for specific epitopes that directly bind with the pr protein, ELISA was performed with mouse hyperimmune antisera against imDENV-2 or human sera. The ELISA plate was coated with protein, ELISA was performed with mouse hyperimmune antisera (P, D, and O) and goat-anti-human IgG, and each sample was assayed in triplicate.

Statistical analysis. All statistical analyses were performed by using an unpaired Student’s t-test with SPSS 13.0. Graphs were performed using the Prism software (GraphPadPrism5). The data were presented as means plus SD from three independent experiments. A P-value <0.005 (*** ) was considered statistically significant.

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