

Mapping to completeness and transplantation of a group-specific, discontinuous, neutralizing epitope in the envelope protein of dengue virus

Olesia Lisova, Florence Hardy,† Vincent Petit‡ and Hugues Bedouelle

Correspondence

Hugues Bedouelle
hbedouel@pasteur.fr

Unit of Molecular Prevention and Therapy of Human Diseases (CNRS-URA3012), Institut Pasteur, 28 rue Docteur Roux, F-75724 Paris Cedex 15, France

Dengue is caused by a taxonomic group of four viruses, dengue virus types 1–4 (DENV1–DENV4). A molecular understanding of the antibody-mediated protection against this disease is critical to design safe vaccines and therapeutics. Here, the energetic epitope of antibody mAb4E11, which neutralizes the four serotypes of DENV but no other flavivirus, and binds domain 3 (ED3) of their envelope glycoprotein, was characterized. Alanine-scanning mutagenesis of the ED3 domain from serotype DENV1 was performed and the affinities between the mutant domains and the Fab fragment of mAb4E11 were measured. The epitope residues (307–312, 387, 389 and 391) were at the edges of two distinct β -sheets. Four residues constituted hot spots of binding energy. They were aliphatic and contributed to form a hydrophobic pocket (Leu308, Leu389), or were positively charged (Lys307, Lys310). They may bind the diversity residues of mAb4E11, H-Trp96-Glu97. Remarkably, cyclic residues occupy and block the hydrophobic pocket in all unrelated flaviviruses. Transplanting the epitope from the ED3 domain of DENV into those of other flaviviruses restored affinity. The epitope straddles residues of ED3 that are involved in virulence, e.g. Asn/Asp390. These results define the epitope of mAb4E11 as an antigenic signature of the DENV group and suggest mechanisms for its neutralization potency.

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INTRODUCTION

Dengue is a re-emerging viral disease. It is caused by four types of virus, dengue virus types 1–4 (DENV1–DENV4), which belong to the species *Dengue virus* in the genus *Flavivirus* (Heinz *et al.*, 2000). They are transmitted by *Aedes* mosquitoes and infect between 50 million and 100 million persons each year. The disease generally takes a mild form, dengue fever, but severe forms, dengue haemorrhagic fever and dengue shock syndrome, have recently become epidemic (Gubler, 2002).

The immune response against an infection by DENV involves both a humoral component, in the form of neutralizing antibodies, and a cell-mediated component (Guzman & Kouri, 2002). The preferential reactivation of the memory B cells that correspond to a primary infection, and an antibody-dependent enhancement of infection, might constitute triggering mechanisms of the severe forms

during a secondary infection by a different viral serotype (Halstead, 2003; Mongkolsapaya *et al.*, 2003). A molecular understanding of the events that lead to antibody neutralization, enhancement or escape is critical to the development of efficient and secure vaccines and therapeutics.

DENV1–DENV4 are enveloped RNA viruses, like all flaviviruses. The structures of the whole virus and of the envelope glycoprotein E (gpE) have been solved by electron cryomicroscopy and X-ray crystallography (Modis *et al.*, 2003, 2005; Zhang *et al.*, 2003). Ninety dimers of gpE cover the surface of the virus. Each monomer comprises three ectodomains, ED1–ED3, and a transmembrane segment. ED2 includes the dimerization interface, a glycosylation site and the peptide of fusion with the cellular membrane. ED3 is continuous and comprises residues 296–400 of gpE. Its fold is compact, immunoglobulin-like and stabilized by a disulfide bond between residues Cys302 and Cys333.

Several types of cellular receptor have been identified for DENV: highly sulfated heparan sulfates (hsHS) (Chen *et al.*, 1997), the lectins DC-SIGN and L-SIGN (Navarro-Sanchez *et al.*, 2003; Tassaneetrithep *et al.*, 2003) and the laminin-binding protein (Thepparit & Smith, 2004; Tio *et al.*, 2005). The structure of the complex between DENV and the carbohydrate-recognition domain of DC-SIGN has

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†Present address: Department of Biology, University of Cergy-Pontoise, F-95302 Cergy-Pontoise Cedex, France.

‡Present address: Unit of Molecular Retrovirology (CNRS-URA3015), Institut Pasteur, F-75724 Paris Cedex 15, France.

been determined by electron cryomicroscopy. This structure shows that DC-SIGN interacts with the glycosylation site of the ED2 domain, that the interaction involves two adjacent dimers of gpE and that ED3 remains available for interactions with other cellular receptors (Pokidysheva *et al.*, 2006). Numerous data indicate that ED3 plays an important role in the infectious process (Hung *et al.*, 2004; Se-Thoe *et al.*, 2000; Thullier *et al.*, 2001). In particular, many antibodies that are specific for ED3 are neutralizing (Crill & Roehrig, 2001; Roehrig, 2003). Of note, their epitopes are generally sensitive to elimination of the disulfide bond (Roehrig *et al.*, 2004).

Monoclonal antibody mAb4E11 was raised against DENV1. Immunofluorescence experiments on cells of the mosquito *Aedes albopictus*, infected with various flaviviruses, and Western blot experiments on their total extracts have shown that mAb4E11 recognizes exclusively the viruses of the dengue group, and not other flaviviruses (Megret *et al.*, 1992). Plaque-reduction assays, performed on monkey Vero cells, have shown that mAb4E11 neutralizes the four serotypes of DENV with varying efficacies (Thullier *et al.*, 1999). ELISAs using truncated recombinant gpE proteins as antigens have shown that the epitope of mAb4E11 is included within the ED3 domain (Megret *et al.*, 1992; Thullier *et al.*, 1999). Selection of peptides from a random library by the method of phage display has shown that residues 306–314 of gpE from the DENV1 serotype (gpE.DEN1) constitute a mimotope of the mAb4E11 epitope (Thullier *et al.*, 2001). Its paratope has been partially characterized by alanine-scanning mutagenesis of its third hypervariable loops (Bedouelle *et al.*, 2006; Renard *et al.*, 2003). This mutagenesis study of the Fab fragment of mAb4E11 has shown that the two residues that come from its diversity segment, H-Trp96 and H-Glu97 (Kabat's numbering), together provide >85% of the free energy of interaction with the ED3.DEN1 antigen.

The objective of our study was to map the energetic epitope of mAb4E11 as completely and precisely as possible, by alanine-scanning mutagenesis of the ED3 domain. A comparison of the mapped epitope with the available sequence, structure and functional data has enabled us to propose structural mechanisms for the cross-recognition of the four DENV serotypes by mAb4E11, the exclusion of the other flaviviruses and its neutralization potency.

METHODS

Strains, plasmids, buffers and chemicals. The *Escherichia coli* strains JM109, BL21(DE3) (Sambrook & Russell, 2001) and HB2151 (Carter *et al.*, 1985), and plasmids pPE1 (Bedouelle *et al.*, 2006), pET20b+ (Novagen) and those coding for the ED3 domain (Catteau *et al.*, 2003), have been described previously. The viral strains used are listed in Table 1. pPE1 codes for a hybrid, Fab4E11-H6, between the Fab fragment of mAb4E11 and a hexahistidine tag. SB medium was described by Sambrook & Russell (2001). Ampicillin was added at 200 µg ml⁻¹. Buffer A contained 20 mM Tris/HCl, 500 mM NaCl; buffer B contained 50 mM Tris/HCl, 150 mM NaCl. The pH of buffers A and B, 7.0 or 7.9, was chosen according to the protein and was distant by ≥1 unit from its pI. PBS was purchased from Sigma-Aldrich.

Genetic constructs. Plasmids coding for hybrids ED3-H6, between the ED3 domain and a hexahistidine tag (H6), were constructed through the following steps (Table 1). The DNA segment of the *E* gene that encodes ED3 was amplified by PCR from plasmid clones with oligonucleotide primers that introduced *Nco*I and *Xho*I restriction sites at its 5' and 3' ends, respectively. The PCR product was then inserted between the corresponding sites of pET20b+. For yellow fever virus (YFV), the *Nco*I site that pre-existed in the *E* gene was removed through a silent mutation before the PCR by changing codon GGC314 into GGT. For West Nile virus (WNV) and Japanese encephalitis virus (JEV), codons 400–406 or 399–405, respectively, of the *E* gene were absent from the parental plasmid clone. They were optimized for *E. coli* and reintroduced through one of the PCR primers. Mutations were constructed by site-directed mutagenesis with synthetic oligonucleotides and using previously published methods (Kunkel *et al.*, 1987; Urban *et al.*, 1997). All genetic constructs were verified by DNA sequencing.

Production and purification of proteins. The Fab4E11-H6 fragment was produced in *E. coli* strain HB2151 and purified as described previously (Bedouelle *et al.*, 2006). The ED3-H6 domains were produced in *E. coli* strain BL21(DE3) from recombinant plasmids (Table 1). The bacteria were grown at 24 °C in SB medium with ampicillin until *A*₆₀₀ reached 1.5–2.0 and then induced for 2.5 h with 1.0 mM IPTG to obtain expression of the recombinant gene. The following steps were performed at 4 °C in buffer A. The bacteria were harvested, resuspended in 1 mg polymyxin B sulfate (Sigma-Aldrich) ml⁻¹, 5 mM imidazole (25 ml for 1 l culture) with stirring for 1 h, then centrifuged at 15 000 g for 30 min. The supernatant (periplasmic fluid) was sonicated, filtered through a 0.22 µm pore filter and loaded onto a column of NiNTA resin [Qiagen; 1 ml (1 culture)⁻¹]. The bound protein was washed with 20 mM imidazole (20 vols resin), then eluted by a step gradient of imidazole. The protein fractions were analysed by SDS-PAGE under reducing conditions. The fractions that were homogeneous at >95% were pooled, dialysed against buffer B and stored at -80 °C.

Table 1. Parental plasmids and viral strains

Column 2 gives the segment of gpE residues that is encoded by the plasmid of column 1. Column 3 gives the positions of the Cys residues involved in a disulfide bond.

Plasmid	gpE residues	S-S bond	Virus	Strain	GenBank accession no.
pLB11	296–400	302–333	DENV1	FGA/89	AF226687
pVP4	294–398	300–330	YFV	17D	X03700
pVP5	299–406	305–336	WNV	IS98-ST1	AY033389
pVP6	298–405	304–335	JEV	SA14	U14163

Protein characterization. The concentration of the ED3-H6 domain was measured by spectrometry. The absorption coefficients were calculated as described by Pace *et al.* (1995). The concentration of the purified Fab4E11-H6 fragment was measured with a BioRad Protein Assay kit, using BSA (Roche) as a standard. The mass of ED3.DEN1-H6 was measured with an API 365 triple quadrupole mass spectrometer (Sciex) as follows. A purified preparation was dialysed extensively against 50 mM ammonium carbonate (pH 9.5) and then lyophilized. The sample was dissolved in a mixture of water, methanol and formic acid, and the measurement of its mass was performed exactly as described previously (Renard *et al.*, 2002).

Fluorescence experiments were performed with 1 μM protein at 20 °C in buffer B. The wavelength of the excitation light was 278 nm. The wavelength at which the intensity of the emitted light was maximal (λ_{max}) was obtained from the fluorescence spectra exactly as described previously (Monsellier & Bedouelle, 2005).

Ellman's test was performed as described by Creighton (1989). Each reaction contained $>3 \mu\text{M}$ protein. The amino acid Cys was used as a standard to establish a calibration curve, and BSA, which contains one free residue of Cys, was used as a positive control. The formation of nitrobenzoate from 5,5'-dithionitrobenzoic acid (Sigma-Aldrich) was measured from A_{412} . The lower limit of detection of the spectrophotometer was equal to an A_{412} of 0.005 and corresponded to 0.44 μM free Cys.

Indirect ELISA. ELISA experiments were performed in PBS and microtitre plates as described by Harlow & Lane (1988), except that the wells of the plates were washed three times with 0.05 % (v/v) Tween in PBS and three times with PBS alone between each step. The wells were coated with 1 μg ED3-H6 domain ml^{-1} and blocked with 3 % BSA (w/v). Measurements were performed at 25 °C in 1 % BSA. The immobilized domains were incubated with 0.4 nM Fab4E11-H6 for 1 h, except for the blank wells. The captured molecules of Fab4E11-H6 were revealed successively with an antibody specific for the Fab fragments of mouse IgG and conjugated with biotin, a conjugate between streptavidin and alkaline phosphatase, and *p*-nitrophenyl phosphate as a substrate (all from Sigma-Aldrich). A_{405} was measured and corrected by subtraction of the blank.

Determination of the equilibrium constants by competition

ELISA. The dissociation constants at equilibrium in solution, K_D , between the Fab4E11-H6 fragment and the ED3-H6 antigens or their mutant derivatives were measured by a competition ELISA (Friguet *et al.*, 1985). The assay was performed at 25 °C in PBS containing 1 % BSA. Fab4E11-H6 at a constant concentration (0.2 nM) and the ED3-H6 derivative at 12 different concentrations were first incubated together in solution for 20 h, to reach equilibrium. The concentration of free Fab4E11-H6 was then measured by an indirect ELISA in a microtitre plate whose wells had been coated with a 0.3 $\mu\text{g ml}^{-1}$ solution of ED3.DEN1-H6. The bound molecules of Fab4E11-H6 were revealed with a goat antibody specific for the Fab fragment of mouse IgG and conjugated with either alkaline phosphatase or biotin. In the latter case, the bound antibody was revealed with a conjugate between streptavidin and alkaline phosphatase (all from Sigma-Aldrich). The raw ELISA data were processed as follows. Let us consider the equilibrium of association and dissociation between an antigen L and an antibody P to give the complex L:P. If $[L]_0$ is the total concentration of L in the reaction, $[P]_0$ the total concentration of P, $[P]$ the concentration of the free molecules of P and K_D the dissociation constant of the complex L:P, then the laws of mass action and conservation imply that $[P]$ is the solution of the following equation (Rondard *et al.*, 1997):

$$[P]^2 + (K_D + [L]_0 - [P]_0)[P] - K_D[P]_0 = 0 \text{ (equation 1)}$$

The indirect ELISA for measuring $[P]$ was performed in conditions such that:

$$A = A_\infty + (A_0 - A_\infty)[P]/[P]_0 \text{ (equation 2)}$$

where A was the absorbance at 405 nm. We fitted equation 2 to the experimental values of A , with the solution of equation 1 as the expression of $[P]$, 2×10^{-10} M for $[P]_0$, 12 different and fixed concentrations for $[L]_0$, and K_D , A_0 and A_∞ as floating parameters.

The free energy of dissociation between L and P is then equal to $\Delta G = -RT \ln(K_D)$, and the variation of free energy on mutation of L is equal to $\Delta \Delta G = \Delta G(\text{wt}) - \Delta G(\text{mut})$, where wt and mut refer to wild-type and mutant molecules of L. The standard error (SEM) of $\Delta \Delta G$ was calculated from the SEM values of ΔG by using the formula:

$$[\text{SEM}(\Delta \Delta G)]^2 = [\text{SEM}\{\Delta G(\text{wt})\}]^2 + [\text{SEM}\{\Delta G(\text{mut})\}]^2 \text{ (equation 3)}$$

Structural analyses. The crystallographic structures of the gpE homodimer (PDB accession no. 1oan) and homotrimer (PDB accession no. 1ok8) from DENV2 (Modis *et al.*, 2003, 2004), and that of the gpE homodimer from WNV (PDB accession no. 1ztx) (Kanai *et al.*, 2006), were analysed with the WHAT IF program (Vriend, 1990). Computations were performed with the KALEIDAGRAPH software (Synergy Inc.).

RESULTS

Production of the ED3 domains from four flaviviruses

In this study, the residues of the ED3 domains are numbered according to their positions in the full-length envelope glycoprotein gpE. We constructed recombinant plasmids to obtain expression of the ED3 domains from DENV1, JEV, WNV and YFV in the periplasmic space of *E. coli*, a cellular environment where the disulfide bond between the two Cys residues could form (Table 1). These plasmids coded for hybrids ED3-H6 between ED3 and a hexahistidine tag under control of the phage T7 promoter and *pelB* signal sequence. The ED3-H6 domains and their mutant derivatives (see below) were purified from periplasmic extracts by affinity chromatography on a nickel-ion column. Analysis by SDS-PAGE under reducing conditions showed that their apparent and theoretical molecular masses (between 12 017 and 12 891 Da) were in agreement.

Structural integrity of the parental ED3 domains

The SignalP program (Bendtsen *et al.*, 2004) predicted that the products of the recombinant genes carried by plasmids pLB11, pVP4, pVP5 and pVP6 would be cleaved between the signal peptide and the first residue of the ED3 domain (Table 1). An analysis of the purified ED3.DEN1-H6 protein by mass spectrometry gave molecular masses equal to $12\,472.69 \pm 0.63$ and $12\,603.69 \pm 0.50$ Da. These experimental masses were equal to the theoretical masses for ED3.DEN1-H6 domains whose disulfide bond was formed correctly and whose N-terminal residue was either Gly296 or Met-Gly296 (12 472.20 and 12 603.40 Da, respectively). They did not correspond to the theoretical mass of an ED3.DEN1-H6 domain with reduced Cys residues. We confirmed the absence of free Cys residues in our purified preparations of

the ED3-H6 domains from DENV1, WNV, JEV and YFV by Ellman's test. The proportion of reactive Cys residues was below the lower limit of detection under our experimental conditions and corresponded to >94% of the ED3-H6 molecules with a disulfide bond in all cases (see Methods).

Fluorescence of Trp and Tyr residues varies with the folding state of proteins. The ED3.DEN1-H6 domain comprises a unique and conserved residue of Trp, in position 391, and three residues of Tyr. The fluorescence intensity of ED3.DEN1-H6 decreases by 2.5-fold between 0 and 8 M urea upon excitation at 278 nm and its λ_{\max} shifts from 340 to 350 nm. These variations of the fluorescence intensity and λ_{\max} are consistent with the unfolding of ED3.DEN1-H6 by urea, the partial burial of Trp391 in the interior of the folded domain, its full exposure to the solvent in the unfolded domain and the solvent-accessible surface area of its side chain (20.7%) in the crystal structure of gpE.DEN2 (Modis *et al.*, 2003; Monsellier & Bedouelle, 2005). Thus, the native ED3.DEN1-H6 domain had the expected molecular mass, disulfide bond and partial burial of Trp391 in the interior of a folded structure, according to the above biochemical and biophysical criteria.

Recognition between Fab4E11 and four flaviviruses

We used a recombinant Fab fragment of mAb4E11 in our recognition experiments to avoid the problems associated with the bivalency of a whole antibody. We measured the constant of dissociation, K_D , at 25 °C in solution between the Fab4E11-H6 fragment and the wild-type ED3.DEN1-H6(wt) domain by a competition ELISA in solution, in which the concentration of the antigen varied (Fig. 1; see Methods). The value of K_D was equal to 0.12 ± 0.01 nM and was fully consistent with the value that we obtained previously with another recombinant form of the antigen (Bedouelle *et al.*, 2006).

To confirm the specificity of mAb4E11 for the flaviviral group of DENV, we performed indirect ELISAs of the interaction between the Fab4E11-H6 fragment and various ED3-H6 domains, i.e. those from DENV1, JEV, WNV and YFV (Table 1). The wells of a microtitre plate were coated with a high concentration of ED3-H6 ($1 \mu\text{g ml}^{-1}$). At the end of a reaction period that was prolonged intentionally (3 h at 20 °C and then 20 h at 4 °C), the ELISA signal was equal to 3.4 ± 0.4 (mean \pm SEM) for ED3.DEN1-H6, 0.025 ± 0.004 for ED3.JE-H6 and lower for ED3.WN-H6 and ED3.YF-H6. Therefore, the signals for JEV, WNV and YFV were <1% of the signal for DENV1. These results showed that Fab4E11-H6 did not recognize the ED3 domains of the three non-cognate flaviviruses.

Energetic contributions of gpE residues 306–314 to the epitope

A synthetic nonapeptide, corresponding to residues 306–314 of gpE.DEN1, constitutes a mimotope of the mAb4E11

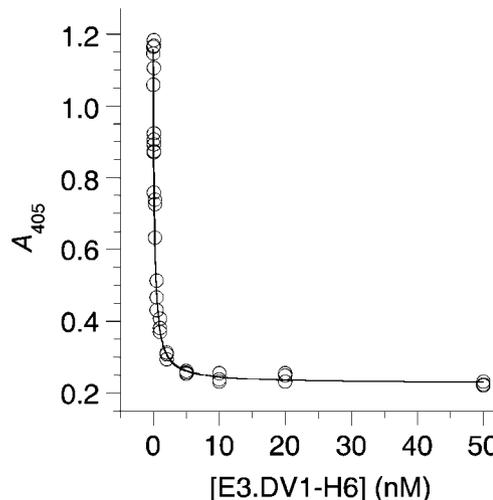


Fig. 1. Determination of the dissociation constant between Fab4E11-H6 and ED3.DEN1-H6 by competition ELISA in solution. The total concentration of ED3.DEN1-H6 in the binding reaction is given along the x-axis; the A_{405} signal, which is proportional to the concentration of free Fab4E11-H6 in the binding reaction, is given along the y-axis. The curve was obtained by fitting the equation of the equilibrium to the experimental data (see Methods). Twelve concentrations were used and each data point was done in triplicate.

epitope (see Introduction). To analyse the energetic contributions of these nine residues to the binding of mAb4E11 in the environment of the ED3.DEN1-H6 domain, we changed them individually into Ala by site-directed mutagenesis, except for Ala313, which was changed into Gly. We produced and purified the mutant ED3.DEN1-H6 domains with yields similar to that for the wild type, except for the F306A mutant, which we were unable to produce. We determined the K_D values for the interaction between the mutant ED3.DEN1-H6 domains and Fab4E11-H6 as for the wild type (Table 2). The values of K_D and the corresponding free energies of interaction, ΔG , showed that residues 307–312 of gpE.DEN1 belonged to the energetic epitope of Fab4E11 [$0.5 < \Delta\Delta G < 5.0 \text{ kcal mol}^{-1}$ ($1 \text{ kcal mol}^{-1} = 4.184 \text{ kJ mol}^{-1}$)], contrary to residues 313 and 314. Three residues, Lys307, Leu308 and Lys310, constituted hot spots of binding energy ($\Delta\Delta G > 1.5 \text{ kcal mol}^{-1}$). The sum of the $\Delta\Delta G$ values for the mutations of residues 307–312 was equal to $13.5 \text{ kcal mol}^{-1}$ and was therefore close to the free energy of interaction between the wild-type ED3.DEN1-H6 domain and Fab4E11-H6, $\Delta G = 13.6 \text{ kcal mol}^{-1}$ (Table 2). This comparison showed that residues 307–312 constituted a major part of the mAb4E11 epitope.

In search of the whole epitope

Several pieces of data suggested to us that the epitope of mAb4E11 extended beyond residues 307–312 of gpE. First,

Table 2. Equilibrium constants and associated free energies for the dissociation between Fab4E11-H6 and wild-type or mutant ED3.DEN1-H6 domains

K_D was measured at 25 °C in solution by a competition ELISA. The mean and associated SEM values of K_D , ΔG and $\Delta\Delta G$ in three independent experiments are given. In addition, each ELISA measurement was done in triplicate. WT, Wild type. The SEM value on $\Delta\Delta G$ was calculated as described in Methods.

Mutation	K_D (nM)	ΔG (kcal mol ⁻¹)	$\Delta\Delta G$ (kcal mol ⁻¹)
WT	0.117 ± 0.009	13.64 ± 0.05	0.0 ± 0.1
K307A	78.0 ± 10.0	9.71 ± 0.08	3.9 ± 0.1
L308A	4.10 ± 0.20	11.44 ± 0.02	2.2 ± 0.1
E309A	0.70 ± 0.20	12.54 ± 0.14	1.1 ± 0.1
K310A	541 ± 210	8.55 ± 0.23	5.1 ± 0.2
E311A	0.32 ± 0.07	12.99 ± 0.11	0.6 ± 0.1
V312A	1.30 ± 0.60	12.34 ± 0.20	1.3 ± 0.2
A313G	0.09 ± 0.02	13.74 ± 0.14	-0.1 ± 0.1
E314A	0.17 ± 0.06	13.44 ± 0.20	0.2 ± 0.2
A386G	0.18 ± 0.07	13.41 ± 0.19	0.2 ± 0.2
L387A	0.35 ± 0.05	12.91 ± 0.09	0.7 ± 0.1
K388A	0.16 ± 0.06	13.44 ± 0.21	0.2 ± 0.2
L389A	2.10 ± 0.40	11.88 ± 0.15	1.8 ± 0.2
S390A	0.14 ± 0.02	13.47 ± 0.09	0.2 ± 0.1
W391A	0.79 ± 0.09	12.43 ± 0.07	1.2 ± 0.1

the value of K_D for the synthetic peptide that corresponds to residues 306–314 of gpE.DEN1 is equal to 3 μ M and is thus 37 000-fold higher than the value for the whole ED3.DEN1-H6 domain (Thullier *et al.*, 2001). Second, we have shown in a previous study that the diversity segment of mAb4E11 comprises two residues, H-Trp96 and H-Glu97; these two residues are major contributors to the interaction with ED3.DEN1 ($\Delta\Delta G \geq 6$ kcal mol⁻¹ for

each mutation into Ala), and they are highly exposed to the solvent in a three-dimensional model of the Fab4E11 fragment (Bedouelle *et al.*, 2006). Thus, the epitope of mAb4E11 should contain both positively charged residues to interact with the negatively charged H-Glu97, and a hydrophobic pocket to bind H-Trp96.

Our alanine-scanning mutagenesis of residues 306–314 of the ED3.DEN1-H6 domain showed that the positively charged residues Lys307 and Lys310 and the hydrophobic residue Leu308 of gpE.DEN1 were hot spots of binding energy ($\Delta\Delta G = 3.9$, 5.0 and 2.1 kcal mol⁻¹, respectively). We therefore analysed the structural environment of residue 308 in the crystal structure of gpE.DEN2 (Modis *et al.*, 2003). We observed that it belongs to a major hydrophobic cluster at the surface of gpE.DEN2, including Ile308 and Ile312 on one β -strand and Leu387, Leu389 and Trp391 on an adjacent and parallel β -strand (Fig. 2).

To test whether residues 386–391 of the ED3.DEN1-H6 domain belonged to the epitope of mAb4E11, we changed them individually into Ala, except for Ala386, which was changed into Gly. The binding properties of the mutant ED3.DEN1-H6 domains were characterized as described above (Table 2). We found that three hydrophobic residues, Leu387, Leu389 and Trp391, contributed significantly to the energy of interaction with Fab4E11-H6 ($\Delta\Delta G > 0.5$ kcal mol⁻¹) and that Leu389 constituted a hot spot of binding energy ($\Delta\Delta G = 1.8 \pm 0.2$ kcal mol⁻¹). These results showed that the epitope of mAb4E11 was discontinuous.

Epitope transplantation

The proportion of identical amino acid residues between gpE of DENV and those of other flaviviruses is about 40 %. As expected at this level of identity, the structures of the

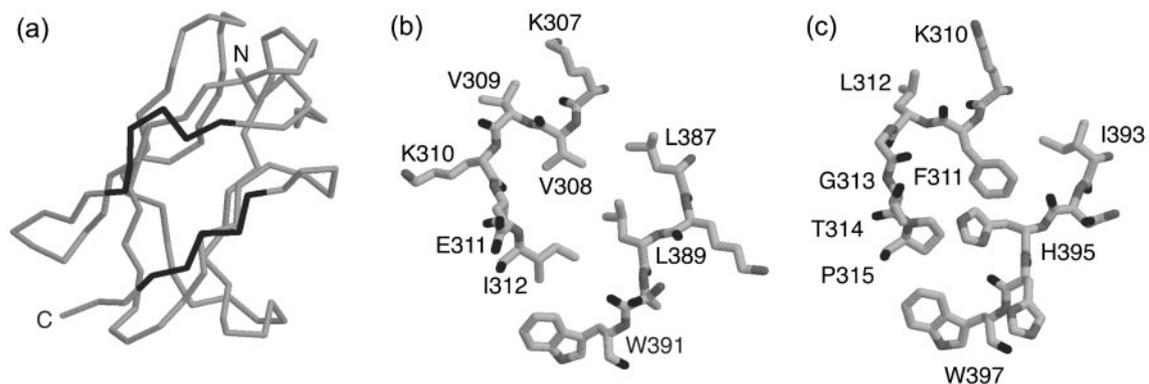


Fig. 2. Position of the mAb4E11 epitope in the crystal structure of gpE from DENV2 and comparison with the structure from WNV. (a) C_α trace of the ED3.DEN2 domain. Residues 307–312 and 387–391 are represented in black. (b) Epitope in the structure of gpE.DEN2. The nine residues of the epitope are labelled. The interface between residues 307–312 and 387–391 forms a hydrophobic pocket. Note that several residues of the epitope differ between DENV1 and DENV2 (Fig. 3). (c) Equivalent residues in the crystal structure of gpE.WN. The interface between residues 310–315 and 393–397 is occupied by bulky cyclic residues Phe311, Pro315 and His395.

ED3 domains from DENV2, DENV3, DENV4, JEV and WNV, solved by nuclear magnetic resonance or X-ray crystallography, are similar (Kanai *et al.*, 2006; Modis *et al.*, 2003, 2005; Nybakken *et al.*, 2005; Volk *et al.*, 2004, 2007; Wu *et al.*, 2003). Among the nine residues that belonged to the energetic epitope of mAb4E11 in ED3.DEN1-H6, six are different in YFV and seven in JEV and WNV (Table 3). To confirm that these nine residues belong to the epitope of mAb4E11, to test whether they constitute its whole epitope and to check whether they are responsible for its specificity of recognition, we introduced them at the homologous positions of the ED3.JE-H6, ED3.WN-H6 and ED3.YF-H6 domains by site-directed mutagenesis. We measured the K_D values between the Fab4E11-H6 fragment and the chimeric domains by competition ELISA in solution (Table 3). These values (4.0, 5.9 and 204 nM, respectively) showed that it was possible to introduce the discontinuous epitope of mAb4E11 into different viral backgrounds and yet maintain its recognition by Fab4E11-H6 with a low-nanomolar affinity. This showed the quasi-completeness of the mapped epitope.

Conversely, we changed the three aliphatic residues at positions 308, 312 and 389 of ED3.DEN1-H6 simultaneously into the cyclic residues that are present in the homologous positions of WNV (column 2 of Table 3). We found that the signal of interaction between the Fab4E11-H6 fragment and the purified mutant domain ED3.DEN1-H6(L308F, V312P, L389H) was <1% of the signal for the wild-type domain ED3.DEN1-H6(wt) in an indirect ELISA (see Methods). This result showed that the introduction of bulky cyclic residues at these three positions of the mAb4E11 epitope abolished the interaction between its Fab fragment and ED3.DEN1-H6.

Structural integrity of the ED3.DEN1-H6 derivatives

We could not produce the F306A mutant of the ED3.DEN1-H6 domain in *E. coli*. The side chain of residue Phe306 is fully buried in the crystal structure of the gpE.DEN2 protein and interacts with the side chain of

Cys302. Therefore, mutation F306A could prevent the correct folding of ED3.DEN1-H6 and hence its production. More generally, some mutations might induce a misfolding of ED3.DEN1-H6 and thus affect the interaction with Fab4E11-H6 indirectly. To test this assumption, we performed Ellman's test on all mutant ED3.DEN1-H6 domains with $\Delta\Delta G > 0.5$ kcal mol⁻¹ and recorded their fluorescence spectra. We found results in Ellman's test and the fluorescence spectra that were identical for the wild-type and mutant ED3.DEN1-H6 domains, except for the spectrum of the W391A mutant, which did not contain any Trp residue. Therefore, the mutations that we introduced, including W391A, did not grossly modify the folding of ED3.DEN1-H6, and the measured variations of the K_D and ΔG values resulted from local effects of the side-chain change.

DISCUSSION

Structure of the mAb4E11 epitope

The 15 mutations to Ala that we constructed enabled us to identify nine residues of the ED3.DEN1 domain that contributed to the energetic epitope of mAb4E11 ($\Delta\Delta G > 0.5$ kcal mol⁻¹): residues 307–312, 387, 389 and 391. Residues Lys307, Leu308, Lys310 and Leu389 constituted hot spots of binding energy ($\Delta\Delta G > 1.5$ kcal mol⁻¹). In the crystal structure of gpE.DEN2, the polypeptide backbone of residues 306–314 associates with and is antiparallel to that of residues 320–326, at the edge of a first β -sheet. Similarly, the backbone of residues 387–393 is antiparallel to that of residues 374–380 at the edge of a second β -sheet. Consequently, the epitope of mAb4E11 straddles the edges of two β -sheets that associate through tertiary interactions (Fig. 2a). It is a discontinuous and conformational epitope. The structure shows that the complete epitope is formed only if ED3.DEN1 is folded properly, and we verified that none of the mutations that we constructed, except F306A, affected this folding.

Residues Lys307 (restricted to the aliphatic portion of its side chain), Leu308 and Val312 are engaged in tertiary interactions with residues Leu387, Leu389 and Trp391 (Fig. 2b). These six residues form a hydrophobic pocket at the surface of the ED3 domain. Therefore, the epitope of mAb4E11 includes two basic residues, Lys307 and Lys310, and a hydrophobic pocket that could interact with the diversity residues, H-Trp96-Glu97, of mAb4E11. These two residues constitute the main hot spots of binding energy with the ED3.DEN1 antigen (Bedouelle *et al.*, 2006).

Completeness of the epitope

The variations of the free energy of interaction between ED3.DEN1 and Fab4E11 for the nine mutations that had significant effects ($\Delta\Delta G > 0.5$ kcal mol⁻¹) had a sum equal to 17.1 kcal mol⁻¹. This sum comprised 13.5 kcal mol⁻¹ coming from residues 307–312 and 3.6 kcal mol⁻¹ from

Table 3. Equilibrium constants for the dissociation between Fab4E11-H6 and the wild-type (wt) or chimeric ED3-H6 domains

See the legend to Table 2 for details. NA, Not applicable.

Variant	Epitope sequence	K_D (nM)
DEN1(wt)	306 FKLEKEVAE ... 387 LKLSW	0.12 ± 0.01
JE(wt)	308 F SFAKNPAD ... 392 INHHW	NA
JE/DEN1	308 FKLEKEVVD ... 392 LNLHW	4.0 ± 0.6
WN(wt)	309 FKFLGTPAD ... 393 INHHW	NA
WN/DEN1	309 FKLEKEVAD ... 393 LNLHW	5.9 ± 1.5
YF(wt)	305 MFFVKNP TD ... 385 LTYQW	NA
YF/DEN1	304 MKLEKEVTD ... 385 LTLQW	204 ± 26

Leu387, Leu389 and Trp391. It was higher than the free energy of interaction, $\Delta G=13.6 \text{ kcal mol}^{-1}$, between ED3.DEN1 and Fab4E11. This comparison suggested that the nine identified residues constituted the entirety of the epitope and indicated that the effects of some mutations were not fully independent. We showed the total absence of recognition between Fab4E11 and the ED3 domains from JEV, WNV and YFV in indirect ELISA experiments. We introduced the nine residues of the mAb4E11 epitope from ED3.DEN1 into the equivalent positions of ED3.JE, ED3.WN and ED3.YF. As a result of these transplantations, the K_D values for the interaction between Fab4E11 and either the chimeric ED3.JE/DEN1 domain or the chimeric ED3.WN/DEN1 domain were in the low-nanomolar range. These experiments confirmed the quasi-completeness of the mAb4E11 epitope, as characterized here. The values of K_D were higher for the chimeric domains than for the wild-type ED3.DEN1 domain, probably because the epitope residues were in different structural contexts. Consistently, the polypeptide backbones of ED3.DEN2 and ED3.WN are not exactly superimposable in the corresponding crystal structures (Kanai *et al.*, 2006; Modis *et al.*, 2003).

The interaction between mAb4E11 and the synthetic peptide that corresponds to residues 306–314 of gpE.DEN1 has a K_D value equal to $3 \mu\text{M}$ and an associated ΔG value equal to $7.5 \text{ kcal mol}^{-1}$ (Thullier *et al.*, 2001). Therefore, the sum of the $\Delta\Delta G$ values for residues 307–312 of the ED3.DEN1 domain was higher than the value of ΔG for the linear mimotope by 6 kcal mol^{-1} . This comparison indicated that the synthetic peptide mimics residues 307–312 of ED3.DEN1 only partially and that the structural context of these residues is important for their recognition by mAb4E11. Consistently, residues 309–311 form a bulge outside β -strand 306–314 and the surface of gpE (Fig. 2).

Specificity for the DENV group

We analysed the sequences and structures of ED3 domains from representative flaviviruses in the region of the epitope to understand the specificity of mAb4E11 towards DENV and its discrimination against the other flaviviruses (Figs 2 and 3). Residues 307 and 309 of DENV1 are variable among DENV; therefore, they cannot be involved in the cross-reactivity of mAb4E11 towards the four serotypes of DENV. Residues 310, 387 and 391 of DENV1 are conserved among the flaviviruses and take the values Lys/Arg, Leu/Ile and Trp, respectively. Therefore, they cannot be involved in the specificity of mAb4E11 for the flaviviral group of DENV. In contrast, the triad of aliphatic residues Leu/Ile308, Val/Ile/Met312 and Leu/Ile389, which participate in the formation of a hydrophobic pocket at the surface of the DENV virion, is replaced by bulky cyclic residues, Trp/Phe, Pro and His/Tyr, respectively, in the other flaviviruses. These bulky residues could prevent the binding of a hydrophobic residue, e.g. the diversity residue H-Trp96 of mAb4E11. Consistently, we found that the triple mutation (L308F, V312P, L389H) of the ED3.DEN1 domain

<i>Mosquito-borne</i>			
	*****		* * *
DEN1:	306 FKLEKEVAE ...	382	AGEKALKLSW
DEN2:	306 FKVVKEIAE ...	382	VEPGQLKLDW
DEN3:	304 FVLKKEVSE ...	380	IGDKALKINW
DEN4:	306 FSIDKEMAE ...	382	VGNSALTLHW
JE :	308 FSFAKNPAD ...	387	RGDKQINHHW
MVE :	309 FTFSKNPAD ...	388	RGDKQINHHW
KUN :	309 FRFLGTPAD ...	388	RGEQQINHHW
WN :	309 FKFLGTPAD ...	388	RGEQQINHHW
SLE :	309 FTFSKNPTG ...	388	RGTTQINYHW
YF :	304 MFFVKNPTD ...	380	RGDSRLTYQW
<i>Tick-borne</i>			
TBE :	312 FTWKRAPTD ...	387	----ELSYQW
LI :	312 FAWKRTPTD ...	387	----ELSHQW
LGT :	312 FTWKRAPTD ...	387	----DLNHQW
POW :	312 FKWKRVPTD ...	388	----DLSQQW
<i>Neither</i>			
APOI:	304 FEWIKKPVV ...	381	----DIDVQW
RB :	303 FVWHKRPVA ...	380	----GAVFQW

Fig. 3. Sequences of representative flaviviruses in the region of the mAb4E11 epitope. The virus acronyms (Buchen-Osmond, 2003) and vector classes are indicated. Adapted from Bhardwaj *et al.* (2001) with the following modifications: DENV1 (GenBank accession no. AF226687), DENV2 (M19197; the PDB file 1OAN has Asn390), JEV (U14163), WNV (AY033389).

abolished its recognition by the Fab4E11 fragment. Thus, the identities of the residues in positions 308, 312 and 389 could explain both the cross-reactivity of mAb4E11 towards the four serotypes of DENV and the rejection of the other flaviviruses.

Potential mechanism of neutralization

Residue 390 of gpE.DEN2 has been implicated repeatedly in the biology of the virus. Two different genetic types of DENV2 circulate. The American genotype is associated with dengue fever only, whereas the South-East Asian genotype has the potential to cause dengue haemorrhagic fever and dengue shock syndrome. Residue Asp390 is present in gpE of American viral strains, whereas Asn390 is present in South-East Asian strains (Leitmeyer *et al.*, 1999). Studies of natural and engineered variants of DENV2 have shown that residue 390 of gpE controls the replicative efficiency of the virus in cultured cells and its virulence in the mouse (Pryor *et al.*, 2001; Sanchez & Ruiz, 1996). Antibody mAb4E11, which interacts with the neighbouring residues in positions 389 and 391, could prevent essential interactions between the virus and some cellular components by steric hindrance and thus be neutralizing. Interestingly, the epitope of mAb4E11 remains exposed to the solvent in the trimeric post-fusion structure of gpE (Modis *et al.*, 2004).

The infectivity of DENV2 may depend on the binding of gpE to hsHS decorating the surface of some cell types (Chen *et al.*, 1997). Moreover, the ED3.DEN2 and ED3.DEN4 domains bind hsHS *in vitro* and four conserved

Lys residues have been implicated in this interaction, including Lys310 (Hung *et al.*, 2004; Pattnaik *et al.*, 2007). The binding of mAb4E11 to gpE could therefore sterically hinder the interaction between hsHS molecules and gpE.

Comparison with neutralizing epitopes in other flaviviruses

Several neutralizing epitopes have been mapped in the ED3 domain of flaviviruses through point mutations, synthetic peptides and structural studies of complexes between antibodies and antigens (Table 4). Here, we used alanine-scanning mutagenesis because it is the most sure and most well-documented method to map functional epitopes (Greenspan & Di Cera, 1999; Rondard & Bedouelle, 1998). We used isolated ED3 domains as antigens because their conformation is independent of concentration, which varied between 10 pM and 10 µM in our experiments, whereas the oligomerization state and conformation of the corresponding gpE ectodomains vary between flaviviruses and with concentration, which would preclude reliable measurements and comparisons of K_D values (Kanai *et al.*, 2006; Modis *et al.*, 2004). Moreover, we checked that the mutations did not affect the structure of the ED3.DEN1

domain by using two sensitive conformational probes that were located in proximity to the epitope, i.e. the formation of a disulfide bond between Cys302 and Cys333 and the fluorescence of Trp391. The available data on the neutralizing epitopes of other flaviviruses show that some antibodies interact with the same regions as mAb4E11 (italicized residues in Table 4). However, none of these antibodies makes functional interactions with the residues that are homologous to Leu387, Leu389 or Trp391 in DENV1, and none makes functional interactions simultaneously with the two polypeptide segments that together form the epitope of mAb4E11. Therefore, the mechanism of recognition between mAb4E11 and gpE seems fully novel in the flaviviruses.

Conclusion

We mapped the energetic epitope of mAb4E11 at the surface of the ED3 domain and showed that the mapped epitope is quasi-complete by transplantation of its nine residues into other viral contexts. We observed that the epitope of mAb4E11 straddles residues of gpE that have been implicated in the replicative efficiency and virulence of the virus. This observation suggested a potential

Table 4. Epitopes of neutralizing antibodies in the ED3 domain of flaviviruses

The epitopes were defined by point mutations, peptide mapping or structural data on the complex between antibody and antigen. The point mutations affected the binding of the neutralizing antibody, except where indicated. The italicized residues are homologous to residues of the mAb4E11 epitope.

Virus	Epitope residues	Reference(s)
Point mutations		
DENV1*	K307, L308, E309, K310, E311, V312, L387, L389, W391	This work
DENV2†	<i>K307, E311</i> , H317, E383, P384, G385	Goncalvez <i>et al.</i> (2004); Hiramatsu <i>et al.</i> (1996); Lin <i>et al.</i> (1994); Lok <i>et al.</i> (2001)
DENV3	K386	Serafin & Aaskov (2001)
JEV‡	E306, S331, D332, G333, I337, F360, M387	Cecilia & Gould (1991); Lin & Wu (2003); Wu <i>et al.</i> (1997, 2003)
WNV§	S306, K307, <i>K310, L312, P315</i> , T330, T332, D381, <i>N394</i>	Beasley & Barrett (2002); Chambers <i>et al.</i> (1998); Li <i>et al.</i> (2005); Oliphant <i>et al.</i> (2005); Volk <i>et al.</i> (2004)
YFV	K303, <i>F305</i> , S325	Jennings <i>et al.</i> (1994); Ryman <i>et al.</i> (1998); Schlesinger <i>et al.</i> (1996)
TBEV†	G368, Y384, S389	Holzmann <i>et al.</i> (1990); Mandl <i>et al.</i> (1989)
LIV	D308, S310, K311	Gao <i>et al.</i> (1994); Jiang <i>et al.</i> (1993, 1994)
Peptides		
DENV1*	306–314	Thullier <i>et al.</i> (2001)
DENV2	279–298, 333–351	Kanai <i>et al.</i> (2006); Roehrig <i>et al.</i> (1998)
JEV	327–333, 373–399	Seif <i>et al.</i> (1996); Wu & Lin (2001)
TBEV	306–339	Niedrig <i>et al.</i> (1994)
WNV	281–300	Kanai <i>et al.</i> (2006)
Structure of complex		
JEV‡	302–312, 322–339, 360–372, 385–392	Wu <i>et al.</i> (2003)
WNV	302, 306–309, 330–333, 365–368, 389–391	Nybakken <i>et al.</i> (2005)

*Energetic epitope of mAb4E11.

†H317Q in DENV2 and G368R in TBEV affect neutralization, but not binding.

‡Epitope of mAbE3.3, except for G333.

§S306, K307, T330 and T332 belong to the energetic epitope of mAbE16.

||Epitope of mAbE16.

mechanism of neutralization by occupancy of gpE sites that are involved in viral infection. The mutations that we introduced and characterized in ED3 suggest types of structural change that could lead to the escape of the virus from neutralization. A comparison of sequences and structures of flaviviral gpE has revealed a molecular mechanism that is potentially responsible for the specificity of mAb4E11 for the DENV group, allowing us to predict that mAb4E11 would recognize no other flavivirus and, thus, to define an antigenic signature of the DENV complex. Overall, our results should contribute to elucidation of the molecular basis for antibody-mediated protection against DENV. It could help to develop vaccines or therapeutic molecules specific for this important disease through a rational approach.

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