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Epitopes on the Dengue 1 Virus Envelope Protein Recognized by Neutralizing IgM Monoclonal Antibodies

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Three of 41 IgM monoclonal antibodies derived from dengue 1 virus immunized mice neutralized dengue 1 infection *in vitro.* All three neutralizing monoclonal antibodies reacted with spatially related epitopes on the E protein of dengue 1 which were also recognized by antibodies in sera from dengue patients. Two neutralization-resistant populations of dengue 1 virus, D1-M10 and D1-M17, were selected by sequential passage of virus in C6/36 cells in the presence of neutralizing IgM monoclonal antibodies M10 and M17, respectively. Single nucleotide changes occurred in the E protein gene of each of these virus populations resulting in single amino acid substitutions at E279 (Phe–Ser) in D1-M10 and at E293 (Thr–Ile) in D1-M17. Both neutralization-resistant populations of virus were more sensitive to elevated temperature than was the wild-type dengue 1 virus and the infectivity and haemagglutinating ability of the neutralization-resistant populations decreased more slowly than that of wild-type virus when exposed to pH in the range 5.8 to 7.0. These are the first epitopes involved in neutralization to have been identified in dengue 1 virus and the first outside domain III of the E protein on any dengue virus.

Key Words: dengue virus; neutralizing epitopes; neutralization escape mutant.

INTRODUCTION

Dengue is perhaps the most significant mosquito-borne viral infection of humans in terms of the number of people at risk of infection and its economic impact (Gubler and Meltzer, 1999). The causative agent exists as four serotypes, each of which is able to cause disease varying in severity from a mild febrile illness to haemorrhagic fever and hypovolemic shock (World Health Organization, 1997). Although there are no dengue vaccines in use, the strategy for those in development has been to produce four separate vaccines, one against each virus serotype (Chambers *et al.,* 1997). An alternate strategy would be to make a chimeric vaccine composed of critical epitopes from all four serotypes. Such an approach, at this stage, would have to be empirical because of the limited information available concerning, for example, epitopes recognized by antibodies able to neutralize dengue virus infection.

The majority of the epitopes involved in neutralization are located on the envelope (E) protein of flaviviruses (Heinz and Roehrig, 1990) and Rey *et al.* (1995) have resolved the three-dimensional structure of a truncated flavivirus (tick-borne encephalitis [TBE] virus) E protein dimer. However, the serological topography of flavivirus E proteins is far from being mapped fully.

The identification of epitopes involved in antibody-mediated neutralization of flavivirus infection has been a priority because of its relevance to an understanding of disease processes and to vaccine development. Studies of the ability of mouse monoclonal antibodies (MAbs) to block binding of other MAbs to flavivirus virions have enabled maps of antigenic domains to be constructed, but most of these could not be linked directly to discrete regions of the E protein (Heinz *et al.,* 1983; Hall *et al.,* 1990; Cecilia *et al.,* 1988; Henchal *et al.,* 1985; Jianmin *et al.,* 1995; Simantini and Banerjee, 1995; Roehrig *et al.,* 1998). Furthermore, the resolution of these maps was influenced by the number and type of MAbs employed and by the competitive binding protocols used to construct them (Tsekhanovskaya *et al.,* 1993). Mandl *et al.* (1989) and Holzman *et al.* (1997) have prepared the most extensive map of serological epitopes in the E protein of any flavivirus involved in neutralization by sequencing the E protein gene of TBE virus mutants which were unable to be neutralized by MAbs. Because of the presumed structural similarities between flavivirus E proteins, efforts have been made to extrapolate these data to other flaviviruses.

Finer resolution of the antigenic map of dengue 2 E protein was achieved by measuring the ability of MAbs to combine with fragments of the E protein expressed in bacteria (Megret *et al.,* 1992) or with enzyme digests of the E protein (Roehrig *et al.,* 1998). Roehrig *et al.* (1998)

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TABLE 1

Characteristics of Selected Antidengue 1 IgM Monoclonal Antibodies

MAb	IFA[a]	Conformation-dependent epitope	HI titer	Neut. index	Ag capture[b]	Four-layer/indirect binding ratio[c]
D1-M02	D1	Y	<20	<1.0	≥1000	2.9
D1-M11	D1	N	<20	<1.0	<10	n.t.
D1-M17	D1,2	Y	160	2.7	≥1000	3.4
D1-M04	D1,3	Y	<20	<1.0	≥1000	3.0
D1-M10	D1,3	Y	80	3.7	≥1000	3.4
D1-M18	D1,3	N	<20	<1.0	<10	3.1
D1-M06	D1,4	N	<20	<1.0	<10	n.t.
D1-M12	D1,4	N	<20	<1.0	<10	n.t.
D1-M09	D1,2,3	Y	<20	<1.0	<10	0.7
D1-M20	D1,2,3	Y	<20	<1.0	<10	0.7
D1-M40	D1,2,3	Y	<20	3.0	≥1000	3.2
D1-M05	D1,2,4	N	<20	<1.0	<10	0.1
D1-M25	D1,3,4	N	<20	<1.0	<10	n.t.
D1-M15	D1,2,3,4	N	<20	<1.0	<10	1.9
D1-M19	D1,2,3,4	N	<20	<1.0	<10	2.1
D1-M26	D1,2,3,4	Y	<20	<1.0	<10	0.1
D1-M28	D1,2,3,4	N	<20	<1.0	<10	0.0
D1-M29	D1,2,3,4	Y	<20	<1.0	<10	n.t.

Note. n.t., not tested.

[a] Dengue serotype recognized in indirect immunofluorescence assays employing dengue 1, 2, 3, or 4 infected C6/36 cells.

[b] Titer of MAb yielding $A_{490/650}$ greater than 0.25 in capture ELISA with dengue 1 antigen.

[c] $A_{490/650}$ in four-layer ELISA/$A_{490/650}$ in indirect ELISA; value of 1.0 would indicate equal absorbance in both assays.

also were able to localize the binding site of one mouse neutralizing MAb to a 20 amino acid region (E333–351) of domain III of the dengue 2 E protein using oligopeptides.

Linear serological epitopes (6–8 amino acids) in the dengue 2 E protein have been identified using overlapping oligopeptides and polyclonal sera from dengue patients and dengue immune rabbits (Aaskov *et al.,* 1989; Innis *et al.,* 1989). While it was not possible to assign a function to any of the immunodominant epitopes identified, subsequent studies by Roehrig *et al.* (1990) demonstrated that a peptide (E35–50) containing part of two immunodominant linear epitopes was able to elicit neutralizing antibody in mice, as was a second peptide (E352–368), which contained none of the immunodominant epitopes identified in the two earlier studies.

Many epitopes recognized by neutralizing antibodies are conformational or discontinuous and so cannot be identified using oligopeptide antigens. Only two epitopes in dengue virus involved in neutralization, both in dengue 2, have been localized precisely (at E307, Lin *et al.,* 1994, and at E383–385, Hiramatsu *et al.,* 1996) and there is no evidence yet that either is recognized by antibodies from dengue patients.

As a prelude to attempting to prepare dengue vaccines containing chimeric E proteins, this study attempted to identify epitopes in the E protein of dengue 1 virus involved in neutralization and to identify phenotypic changes in the virus associated with amino acid changes at these sites.

RESULTS

Monoclonal antibody production and characterization

Forty-one clones of hybridomas produced IgM antibodies which reacted with dengue 1 virus in indirect ELISA and immunofluorescence assays (IFA), and which recognized dengue 1 virus E protein in Western blots. Eight of the 41 antibodies reacted with only dengue 1 virus-infected C6/36 cells in indirect IFA, 12 with two serotypes, nine with three serotypes, and 12 with all four serotypes (Table 1 contains representative results).

Only three of the 41 antibodies, D1-M10, D1-M17, and D1-M40, were able to neutralize more than 1 $\log_{10}$ of dengue 1 virus in BHK cells (Table 1). These three antibodies also neutralized 3 $\log_{10}$ of dengue 1 virus in C6/36 mosquito cells. No serotype cross-reactive neutralizing activity was detected. Two of the neutralizing IgM antibodies, D1-M10 and D1-M17, also inhibited haemagglutination by dengue 1 virus, but not by other dengue virus serotypes. None of the other IgM antibodies caused detectable haemagglutination inhibition.

The binding of neutralizing antibodies D1-M10, D1-M17, and D1-M40 to dengue 1 E protein in Western blots was abolished following reduction of viral antigen with 2-mercaptoethanol, indicating that these antibodies recognized conformation-dependent epitopes. Six of 24 nonneutralizing antibodies tested also failed to react with reduced E protein in Western blots (representative results are shown in Fig. 1).

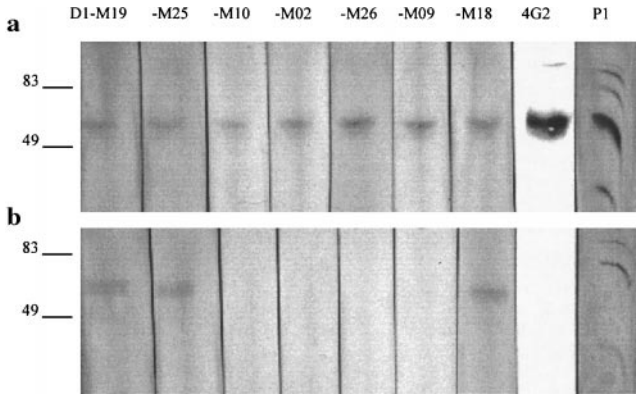

FIG. 1. Reactions of selected neutralizing (D1-M10) and nonneutralizing (remainder) antidengue 1 IgM MAbs, flavivirus cross-reactive MAb 4G2, and dengue-immune human serum P1 in Western blots with (a) native and (b) reduced dengue 1 virus-infected C6/36 cell lysates. Molecular weights (kDa) are indicated.

Competitive capture ELISA

These assays were performed to identify MAbs that bound the same, or spatially related, epitopes.

Preincubation of dengue 1 virus with MAbs, corresponding to those coated to ELISA plates to "capture" virus, resulted in significant (≥80%, $p < 0.001$) reductions in the amount of virus captured if the "blocking" antibody was diluted from 10^{-1} to 10^{-3} prior to incubation with virus. Higher dilutions of the blocking antibodies enabled more virus to be captured and there was no significant inhibition of capture of virus if the homologous blocking antibodies were diluted more than 10^{-5}. Blocking MAbs were diluted 1/100 in all competitive ELISAs described below (see Materials and Methods).

However, only five anti-dengue 1 MAbs, D1-M02, D1-M04, D1-M10, D1-M17, and D1-M40, and the flavivirus cross-reactive MAb 4G2 were able to capture dengue 1 virus after being coated to wells of an ELISA plate.

As the detection of captured virus was dependent on the binding of horseradish peroxidase (HRP) -labeled 4G2 antibody to the captured virions, blocking experiments were performed to determine if any of the antidengue 1 MAbs competed with this antibody for epitopes on the dengue 1 virion. MAb 4G2 and dengue-immune human serum P1 inhibited capture of dengue 1 virions by 4G2 antibody by more than 80% ($p < 0.001$) and MAbs D1-M02 and D1-M04 inhibited capture of dengue 1 by this antibody by approximately 55% ($p \leq 0.05$). No significant ($p \geq 0.05$) inhibition of capture of dengue 1 by 4G2 was observed with any of the other IgM antibodies tested.

MAbs D1-M10, -M17, -M40, -M02, and -M04 all inhibited capture of dengue virus by each other (Table 2). Within this field of competition, MAbs D1-M10, -M17, and -M40 (neutralizing) and D1-M02 and -M04 (nonneutralizing) competed more strongly with each other (≥80% blocking, $p < 0.001$). Some MAbs (D1-M08, -M19, and -M26) enhanced the capture of dengue virions by other MAbs.

Comparative binding of antibodies in indirect and "four-layer" ELISA

In light of the failure of many IgM MAbs coated to ELISA plates to capture dengue 1 virus antigen, the binding of capture (including neutralizing antibodies D1-M10, -M17, and -M40) and several "noncapture" antibodies to dengue 1 virus in an indirect and in a four-layer ELISA was compared (Table 1). Equal quantities of dengue 1 virus antigen (8 HA units) were coated to wells of ELISA plates, for indirect assays, or added to wells coated with dengue-immune human serum P1 (see Materials and Methods), for four-layer assays. More D1-M02, -M04, -M10, -M17, and -M40 antibody bound to virus captured by polyclonal human serum than to virions coated directly on to ELISA plates (two- to threefold increase in absorbance). In contrast, several noncapture antibodies (D1-M05, -M26, and -M28) which combined with dengue 1 virus in an indirect ELISA bound poorly to virus in a four-layer ELISA.

TABLE 2

Ability of Antidengue 1 Monoclonal Antibodies to Inhibit Capture of Dengue 1 Virions by Homologous and Heterologous Antibodies

	Blocking/enhancement of dengue 1 virus binding to capture MAb (%)[a]									
	Blocking MAb									
Capture MAb	D1-M10	D1-M17	D1-M40	D1-M02	D1-M04	D1-M08	D1-M19	D1-M15	D1-M26	D1-M01
D1-M10	83	89	88	57	57	−2	−4	−4	−6	1
D1-M17	93	98	97	48	49	−5	−7	−3	−4	−6
D1-M40	92	98	98	47	43	−17	−11	0	−22	0
D1-M02	65	54	39	94	89	−8	−8	2	0	8
D1-M04	44	38	22	79	93	−2	−2	−2	−4	8

[a] See Materials and Methods for the calculation of blocking (positive values)/enhancement (negative values). Shading indicates significant blocking/enhancement ($p < 0.05$). Boxed areas indicate "domains" of strongest competition ($p < 0.001$).

TABLE 3

Inhibition of Binding of Selected Antidengue 1 IgM MAbs and Control MAb 4G2 to Dengue 1 Virus by Serum from Dengue Patients P1 and P2 and from a Normal Flavivirus-Seronegative Donor, in an Indirect ELISA

	Blocking (%)[a]		
MAb	Serum P1	Serum P2	N.H.S.[b]
D1-M10	59[c]	31[c]	2
D1-M17	60[c]	26[c]	1
D1-M40	55[c]	25[c]	4
D1-M02	78[c]	1	4
D1-M04	84[c]	11[c]	−5
D1-M15	8	0	3
D1-M26	0	−3	−4
D1-M09	−16[c]	7	9
D1-M22	−7	n.t.	1
4G2	75[c]	74[c]	5

Note. n.t., not tested.

[a] Blocking calculated as a percentage using the formula: Blocking = 100 − ($A_{490/650}$ with blocking serum × 100/$A_{490/650}$ without blocking serum).

[b] Normal (flavivirus nonimmune) human serum.

[c] Significant blocking (positive values) or enhancement (negative values) of binding of MAbs to virus ($p < 0.05$; two-tailed Student *t*-test).

Competition with dengue-immune human sera

Binding of neutralizing antibodies D1-M10, -M17, and -M40 to dengue 1 virions in a competitive indirect ELISA was inhibited by sera from two dengue-immune human donors (P1 and P2; Table 3). Similarly, the binding of nonneutralizing antibodies D1-M02 and -M04 was inhibited by dengue-immune human serum, although binding of D1-M02 was not inhibited by serum P2, despite strong competition with serum P1. Binding of several other nonneutralizing antibodies to dengue 1 was not inhibited significantly by either human serum (Table 3). The binding of control MAb 4G2 was strongly inhibited by both serum P1 and P2. No significant ($p \geq 0.1$) inhibition of binding of MAbs was observed in experiments utilizing flavivirus nonimmune human serum.

Selection and nucleotide sequencing of neutralization escape mutant virus populations

Neutralization-resistant populations of dengue 1 virus were selected with monoclonal antibodies D1-M10 and D1-M17 following three and four passages, respectively, in C6/36 cells in the presence of these antibodies. No neutralization-resistant virus was recovered following two attempts using antibody D1-M40.

Despite the inability of antibodies D1-M10 and D1-M17 to neutralize infection of C6/36 cells by the mutant virus populations, they reacted with cells infected with the corresponding neutralization escape mutant virus population in indirect IFA.

Comparison of the nucleotide sequences of the prM and E protein genes of the wild-type dengue 1 (Jarrett) virus (Genbank Accession No. AF187271) with those of the mutant viruses, M10 and M17, revealed single nucleotide changes in the E protein gene at position 836 (TTT → TCT) and 878 (ACT → ATT), respectively. These mutations coded for single amino acid substitutions, at E279 (Phe → Ser) and E293 (Thr → Ile). Sequencing of the corresponding region of "passage control" viruses, which had been passaged in C6/36 cells in the absence of selecting antibody, revealed no changes from the nucleotide sequence of the wild-type virus.

Phenotypic characterization of mutant viruses

Wild-type and mutant viruses displayed similar HA profiles, with peak HA between pH 6.0 and 6.2 (data not shown). However, pretreatment of these virus populations at pH from 5.8 to 7.0 influenced their subsequent ability to agglutinate gander cells. The HA activity of wild-type virus was abolished by pretreatment at pH 6.2 or lower but that of the mutant viruses was not lost until the pH of the pretreatment reached 5.8 (Fig. 2a).

Infectivity of both mutant viruses was more susceptible to low pH or elevated temperature than that of wild-type virus (Figs. 2b, 2c). Infectivity of wild-type virus was unchanged following pretreatment at pH 7.0 or 6.7 but declined following exposure to more acidic pH, with the maximum reduction (3 logs) occurring at pH 6.4 or lower. In contrast, the infectivity of mutant viruses decreased following pretreatment at pH 6.7, with maximum loss (3 or 4 logs) at pH 6.2 or below (Fig. 2b).

Exposure to 45°C for 30 min caused a two to three log greater reduction in infectivity of mutant viruses than was observed for wild-type virus (Fig. 2c). Complete loss of mutant virus infectivity occurred within 90 min at 45°C, while infectivity of wild-type virus was only abolished after 90 to 180 min at this temperature.

Wild-type and neutralization escape mutant viruses grew at similar rates in C6/36 cells (Fig. 2d).

DISCUSSION

Epitopes associated with neutralization by mouse MAbs have been identified in all three structural domains of the TBE virus E protein (Mandl *et al.,* 1989; Holzmann *et al.,* 1989, 1997; Rey *et al.,* 1995). Serological maps of similar detail have not been prepared for any other flavivirus. In the case of dengue viruses, the locations of only three epitopes associated with neutralization have been identified, all in dengue 2 and all in structural domain III of the E protein (E307, Lin *et al.,* 1994; E383–385, Hiramatsu *et al.,* 1996; E335–351, Roehrig *et al.,* 1998). However, binding of neutralizing antibodies to large fragments representing the other structural domains of the dengue 2 E protein has been reported (Megret *et al.,* 1992). This study is the first to localize epitopes associated with neutralization of dengue 1 virus

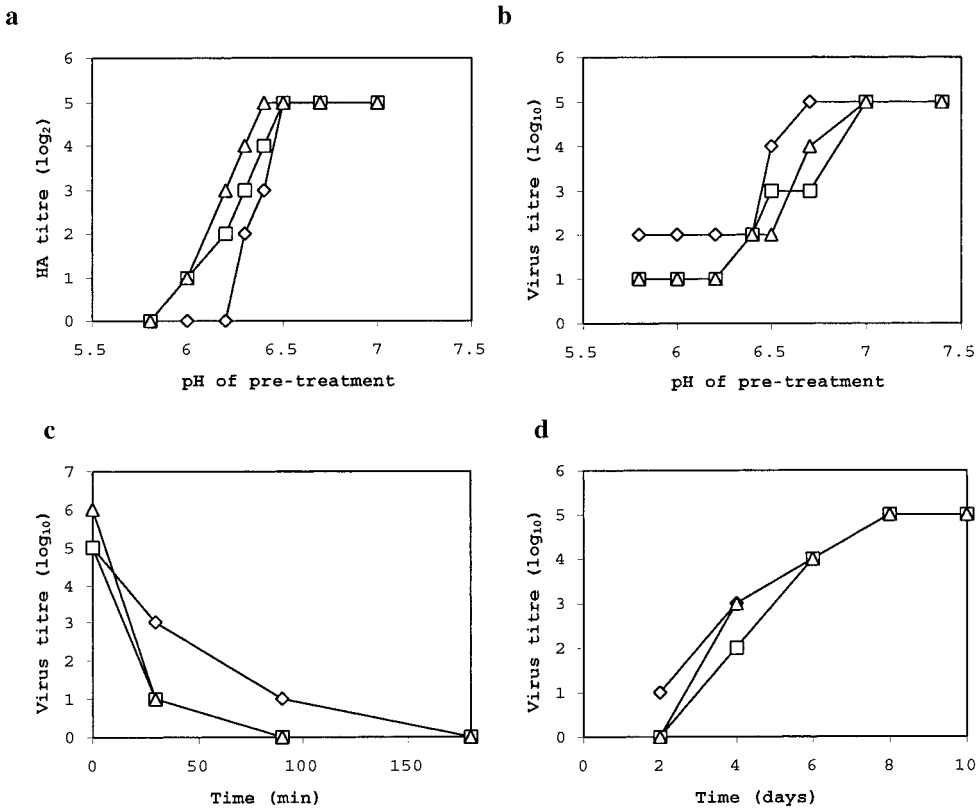

FIG. 2. Comparison of the phenotypic properties of wild-type (w.t.) and neutralization escape mutant dengue 1 viruses (M10 and M17). (a) Effect of low pH pretreatment on haemagglutination (HA) at pH 6.2. (b) Effect of low pH pretreatment on infectivity in C6/36 cells. (c) Effect of exposure to elevated temperature (45°C) on infectivity in C6/36 cells. (d) Growth rates in C6/36 cells. (◇) w.t.; (□) M10; (△) M17.

and the first to identify such epitopes outside domain III of the dengue E protein. Furthermore, the results of competitive binding experiments suggested that these epitopes at E279 and E293 were the same as, or spatially related to, epitopes recognized by antibodies from dengue patients.

Comparison of the amino acid sequences of all dengue E proteins lodged in the SWISS-PROT database indicated that Phe at E279 was conserved in almost all wild-type dengue viruses, although surrounding residues varied. Lee *et al.* (1997) reported a similar Phe–Ser substitution in the E protein of dengue 3 virus following four or more passages in Swiss white mice, and this mutation was associated with altered pH sensitivity of that virus. The Thr–Ile substitution at E293 has been reported for two other dengue 1 viruses; clones 45AZ5 and 45AZ5-PDK27 derived from the WestPac dengue 1 isolate by mutagenesis and *in vitro* cell passage (Puri *et al.,* 1997). Both clones were sensitive to elevated temperatures as was the D1-M17 neutralization escape mutant with a similar amino acid change at this site. Neither Lee *et al.* (1997) nor Puri *et al.* (1997) investigated the serological consequences of the changes they observed.

The failure to recover a neutralization-resistant population of dengue 1 viruses using MAb D1-M40 may have been due to the absence of any subpopulations of virus with changes in the epitope recognized by this antibody or amino acid changes at this site may have been lethal for the virus. Coincidentally, MAb D1-M40 was the only one of the three antidengue 1 neutralizing antibodies not to inhibit haemagglutination by dengue 1 virus.

Given the more precise localization of the serological epitope in dengue 2 virus E protein recognized by MAb 3H5 which was achieved by selective nucleotide substitutions in the E protein gene of a dengue 2 infectious clone (Hiramatsu *et al.,* 1996), there may be value in attempting a similar approach with dengue 1 virus at the sites of nucleotide changes in the neutralization escape mutant viruses selected in this study.

The substitution of Ser for Phe at E279 of the dengue 1 neutralization-resistant virus population was a nonconservative change that would have increased the hydrophilicity of this region of the protein (mean hydrophobicity scores for the region encompassing residues E277–281 were 1.60 for the wild-type virus and 0.88 for mutant M10, when calculated using the values assigned by Kyte and Doolittle, 1982). Also, in structural models of the dengue 1 E protein predicted using the Swiss-Model server (Guex and Peitsch, 1997) this change resulted in a displacement of the β-sheet in which it was located (Fig. 3c). The Thr–Ile substitution at E293 was more conservative, would have increased the hydrophobicity of the

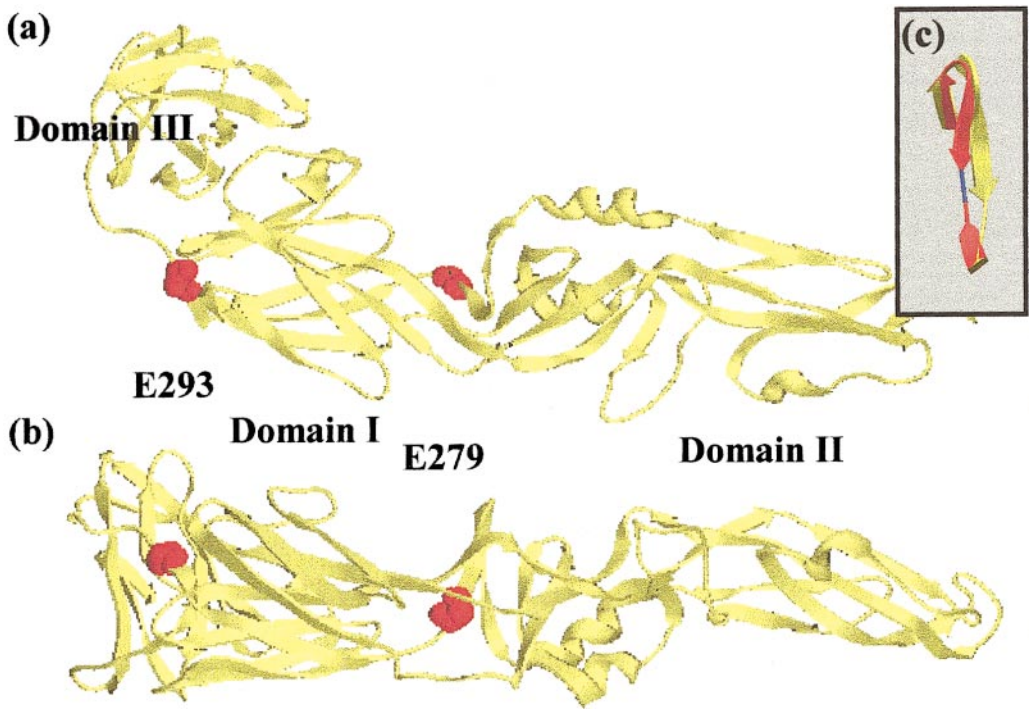

FIG. 3. Location of amino acid substitutions at E279 and E293, associated with escape from neutralization, on a model of the dengue 1 virus E protein derived from that for tick-borne encephalitis virus. (a) Superior view. (b) Lateral view. (c) Predicted displacement of beta-sheet by the Phe–Ser substitution at E279 (shown in blue on red ribbon).

region in which it was located (mean hydrophobicity for E291–295 was −0.18 for the wild-type virus and 0.86 for mutant M17), but caused no change in the predicted structural model.

Attempts to map epitopes involved in neutralization of dengue viruses have been hampered by the variable plaquing efficiency of these viruses, particularly following repeated passage in cell culture, and, in the case of dengue 1 and 3, the small proportion of monoclonal antibodies which neutralized infection *in vitro* (Simantini and Banerjee, 1995; Serafin and Aaskov, 1996). In this study, the problem of plaquing was overcome by using C6/36 mosquito cells and assessing infectivity rather than plaque formation. The antidengue 1 MAbs used to select neutralization escape mutant virus populations neutralized dengue 1 virus infection of C6/36 and BHK cells to similar levels (2.7–3.0 $\log_{10}$). The issue of whether dengue 1 virus has epitopes involved in neutralization spread throughout its E protein, as the TBE virus data might suggest (Mandl *et al.,* 1989; Holzmann *et al.,* 1997), remains unresolved.

We have no explanation as to why such a small proportion of the antidengue 1 MAbs neutralized infection *in vitro,* but these data are in broad agreement with those of Simantini and Banerjee (1995), who reported that only 2 of a panel of 10 antidengue 1 MAbs were able to neutralize dengue 1 virus and suggest that very large panels of MAbs may be required to provide for dengue 1 as extensive a neutralization epitope map as that which has been prepared for TBE virus. If the disparity in the proportion of neutralizing to nonneutralizing MAbs produced in mice infected with dengue 1 (this study; Simantini and Banerjee, 1995) and dengue 3 (Serafin and Aaskov, 1996) compared to dengue 2 virus (Gentry *et al.,* 1982; Henchal *et al.,* 1985; Jianmin *et al.,* 1995; Roehrig *et al.,* 1998) also occurs in humans, it may have significant implications for vaccination strategies. However, Roehrig *et al.* (1990) have suggested that the restricted MHC background of inbred mice might influence immune responses of these animals to dengue virus antigens. Further experiments are in progress to determine what role the genetic background of the mice used for MAb production has in determining the ratio of neutralizing to nonneutralizing antidengue antibodies.

Several antibodies produced in this study could not capture dengue 1 virus if they were first coated to ELISA plates and failed to react with virus in a four-layer ELISA despite binding to virus in indirect ELISA and IFA. Holzmann *et al.* (1993) described a number of monoclonal antibodies, specific for the E protein of TBE virus, that bound to viral antigens in indirect ELISAs but did not bind in a procedure similar to the four-layer ELISA used in this study. The coating of protein antigens to plastic surfaces has been reported to disrupt their native structure (Jemmerson, 1987), and Holzmann *et al.* (1993) suggested that their antibodies may have recognized epitopes whose conformation was altered in the ELISA plate-bound virus, or which were not surface exposed in

the native virion. It is possible that antidengue 1 antibodies which did not bind native virions in capture or four-layer ELISAs may have recognized such cryptic epitopes.

Three of the five anti-dengue 1 antibodies which reacted strongly with native virions in capture and four-layer ELISA procedures were able to neutralize dengue 1 virus infection *in vitro,* suggesting that antibodies recognizing epitopes on the surface of virions are more likely to inhibit virus infectivity than antibodies reacting with cryptic epitopes. In the future, screening of hybridomas using a four-layer ELISA protocol should allow easier and more reliable selection of surface-reactive (and, hence, potentially neutralizing) antidengue 1 antibodies than the indirect ELISA/IFA protocols which were used in this study.

There are a number of reports of neutralization escape mutant virus populations with amino acid changes occurring at sites distant from the antibody binding site (Blondel *et al.,* 1986; McCahon *et al.,* 1989; Wilson *et al.,* 1990; Cecilia and Gould, 1991; Holzmann *et al.,* 1997). Dimmock (1993) proposed that neutralization could occur when a conformational change in a viral protein was triggered by antibody binding and that a mutation outside the antibody binding site allowed the virus to overcome this effect without direct modification to the antibody binding site. Although the selecting MAbs D1-M10 and D1-M17 reacted with C6/36 cells infected with neutralization escape mutant virus in indirect IFA, this may not necessarily indicate that the amino changes observed at E279 and E293 were distinct from the antibody binding site.

There are several indirect lines of evidence that suggest the observed amino acid changes in the neutralization escape mutants were at or near the site on the dengue 1 E protein where the MAb bound. The selecting MAbs were more cross-reactive in indirect IFA assays employing dengue virus-infected C6/36 cells than in either HI or neutralization tests (Table 1). This may indicate that there are nonfunctional mimotopes of the epitopes at E279 and E293 to which the MAbs also bound and these were responsible for the reactions detected in indirect IFA assays performed with cells infected with mutant viruses. Such mimotopes have recently been described for MAb 4G2 in dengue 2 virus at E349–359 and E274–283 (Falconar, 1999). In addition, the amino acid sequences surrounding residues E279 and E293 were sufficiently different from the sequences in other dengue viruses to account for the serotype specificity of the selecting MAbs. Also, the epitopes are close enough for antibodies binding to them to interfere stearically with each other (Fig. 3), as was observed in competitive binding studies with MAbs D1-M10 and -M17. Both antibodies also failed to interfere with the binding of antibody 4G2 in competitive binding experiments. This is compatible with the location of the 4G2 neutralizing epitope being on a separate structural domain (domain III; Megret *et al.,* 1992; Falconer, 1999) from that on which the M10 and M17 domains were located. Furthermore, both the D1-M10 and -M17 antibodies inhibited haemagglutination by dengue 1 virus. Previous studies (Aaskov, 1989) suggested regions E30–42 and E127–134 of the dengue 2 E protein might be involved in HA. The epitope surrounding E293 is immediately adjacent to the region E30–42 in the flavivirus E protein and E279 is adjacent to region E127–134 such that antibodies reacting with an epitope at E279 or E293 might be able to stearically hinder any interaction between these putative HA determinants and the surface of an erythrocyte.

Other flaviviruses also have serological epitopes at or near sites corresponding to these two neutralizing epitopes in the dengue 1 E protein. The conformational epitope at E293 of dengue 1 corresponds to an immunodominant, linear epitope of unknown function in the dengue 2 E protein (E291–298, Jianmin *et al.,* 1995) and would be adjacent to an epitope at E307, in the dengue 2 protein, involved in neutralization (Lin *et al.,* 1994). The conformational epitope at E279 of dengue 1 also corresponded to a linear, immunodominant epitope of unknown function in dengue 2 virus recognized by sera from dengue patients (Innis *et al.,* 1989). MVE virus has a discontinuous epitope in this region of its E protein (E274–277), which is recognized by neutralizing MAbs (McMinn *et al.,* 1995).

Rey *et al.* (1995) suggested that the region between domains I and II of the flavivirus E protein might act as a hinge for low pH-induced conformational changes accompanying the E protein dimer–trimer transition which occurs prior to its fusion with host cell membranes. Stuart and Gouet (1995) have suggested the region between domains I and III as a possible hinge for these changes because of its simpler structure. Mutant dengue 1 virus M10 had a change in the middle of the putative domain I–II hinge and the M17 mutant had a change at the edge of the putative domain I–III hinge (Fig. 3). The infectivity of both mutant dengue 1 virus populations was more sensitive to decreases in pH than that of wild-type virus suggesting that the E protein dimer–trimer transition may be accompanied by conformational changes in both these regions. This is consistent with the results of Heinz *et al.* (1994) who reported that epitopes located in both domains I and II are disrupted by low pH treatment of TBE virus.

The precise localization of epitopes recognized by neutralizing antibody in two domains of the dengue virus E protein (domain III of dengue 2 [Lin *et al.,* 1994; Hiramatsu *et al.,* 1996; Roehrig *et al.,* 1998] and domain I of dengue 1 [this study]) suggests that the production of dengue virus vaccines containing chimeric E proteins, capable of stimulating neutralizing antibody responses against the E protein of two or more dengue virus serotypes, may be possible.

MATERIALS AND METHODS

Virus

Suckling mouse brain (smb) preparations of dengue viruses were obtained from the Yale Arbovirus Research Unit (dengue 1 Hawaii, dengue 2 NGC, dengue 4 H241), the Centers for Disease Control (dengue 3 PRS225489), or the QUT/Queensland Health WHO Arbovirus Reference Centre (dengue 1 Jarrett). Working stocks of each virus were prepared by infecting cultures of C6/36 *Aedes albopictus* cells (Igarashi, 1978).

Polyclonal and monoclonal antisera

Flavivirus cross-reactive monoclonal antibody 4G2 (Gentry *et al.,* 1982) was prepared as tissue culture supernatant (tcs) or ascitic fluid from hybridomas obtained from the Walter Reed Army Institute of Research (WRAIR).

Serum samples from dengue-immune patients and flavivirus nonimmune donors were obtained from the collection maintained by the WHO Arbovirus Reference Centre, Brisbane, Australia. Dengue-immune serum "P1" was collected from a patient with primary dengue 3 infection who had prior Yellow fever and Japanese encephalitis virus vaccinations. Serum "P2" was from a patient with primary dengue 1 infection. Both sera had antidengue 1 IgG antibody titers $>$ 6400 measured in an indirect ELISA.

Production of antidengue 1 IgM monoclonal antibodies

Female BALB/c mice (Animal Resources Centre, Perth, Australia) were immunized intravenously (iv) with approximately 10^3 plaque forming units (PFU) of a dengue 1 Hawaii smb preparation diluted in sterile phosphate buffered saline (PBS). Mice were boosted with the same dose of virus approximately 1 week later and sacrificed 3 to 5 days after the second immunization and their spleens harvested. Spleen cells were eluted into RPMI 1640 tissue culture medium (Gibco, USA) and fused with SP2/0 myeloma cells using standard protocols (Zola, 1987). Production of antidengue 1 IgM antibody by hybridomas was detected by indirect ELISA (see below), and individual hybridomas from cultures producing antibody of interest were transferred to single wells in a 96-well tissue culture plate to replicate. These hybridoma clones were retested by indirect ELISA, and positive reactions confirmed in indirect IFA (Jianmin *et al.,* 1995) against dengue 1 infected and uninfected baby hamster kidney (BHK) cells. Hybridomas were injected into BALB/c mice, which had been pretreated by intraperitoneal (ip) injection of Pristane (Sigma, USA) to produce ascitic fluid.

Serology

Indirect ELISAs were performed using plates coated with dengue 1 Hawaii C6/36 tcs preparations diluted in borate saline pH 9.0. After coating, plates were "blocked" using 5% v/v "milk diluent blocking solution concentrate" (milk diluent; Kirkegaard and Perry Laboratories, USA) in PBS, then washed with PBS containing 5% v/v Tween-20 (PBS/Tween). Hybridoma culture supernatants (undiluted) or ascitic fluids (diluted 1/100 in PBS/Tween) were added, plates were incubated at room temperature for 45 min, and then washed with PBS/Tween. Antibody binding was detected by addition of specific horseradish peroxidase-labeled secondary antibodies, anti-mouse immunoglobulin (Dako, Denmark) or anti-mouse IgM (Southern Biotechnology Associates, USA), and hydrogen peroxide/*o*-phenylenediamine dihydrochloride (H_2O_2/OPD) substrate/chromagen solution. Color reactions were stopped by addition of 3 M HCl and absorbances were read at 490 nm with a reference wavelength of 650 nm in an automated ELISA plate reader (Dynatech MR 5000).

For four-layer ELISAs, 96-well plates were coated with flavivirus-immune human serum (P1) diluted 1/1000 in borate saline, blocked with 5% v/v milk diluent and dengue 1 antigen (eight haemagglutinating units diluted in PBS/Tween) dispensed in each well, and incubated at room temperature for 45 min. After washing with PBS/Tween, MAbs (hybridoma tissue culture supernatant) were added, and their binding to captured virus was detected by addition of HRP-labeled anti-mouse immunoglobulin antisera and H_2O_2/OPD substrate/chromagen. The absorbance of the reactions was read on an automated ELISA plate reader as described above.

Haemagglutination inhibition assays were performed using methods described by Clarke and Casals (1958) and Sever (1962). Eight haemagglutinating units of virus, derived from C6/36 cell cultures, were used in each reaction.

Neutralization assays and virus titrations were performed using suspensions of BHK cells and protocols based on those of Morens *et al.* (1985). Neutralization titers were calculated as a "neutralization index" (Jianmin *et al.,* 1995). Dengue 1 virus (Jarrett) was used in neutralization assays as it produced plaques more consistently than the prototype strain of dengue 1 and grew to higher titers in tissue culture.

Western blotting was performed using lysates from dengue 1 virus-infected C6/36 cell cultures disrupted in 10% w/v SDS with or without 1 M 2-mercaptoethanol. Proteins were separated on a discontinuous (4% stacking/15% separating) 30:0.5 acrylamide:bisacrylamide gel (Laemmli, 1970) and transferred to 0.2 μm nitrocellulose according to the method of Towbin *et al.* (1979). Nitrocellulose sheets were blocked overnight at 4°C in a solution of 3% w/v skim milk in Tris-buffered saline (TBS), after which monoclonal antibody (ascitic fluids) diluted 1/20 or

1/50 in TBS containing 0.2% v/v Tween-20 (TBS/Tween) were added. The flavivirus cross-reactive, E protein specific antibody 4G2 and a dengue-immune human serum sample (P1) were included as a control. Nitrocellulose was washed three times in TBS/Tween and bound antibody detected by addition of HRP-labeled anti-mouse immunoglobulin or HRP-labeled anti-human immunoglobulin antisera (Dako, Denmark) and hydrogen peroxide/chloronapthol substrate/chromagen solution.

Competitive binding assays

Competitive capture ELISAs with monoclonal antibodies were performed as described by Jianmin *et al.* (1995). Briefly, 96-well ELISA plates were coated overnight at 4°C with monoclonal antibody ascitic fluid (the "capture antibody") diluted 1/100 in borate saline. Plates were then blocked with 5% v/v milk diluent and washed with PBS/Tween. Dengue 1 virus was mixed with blocking monoclonal antibody (ascitic fluid diluted 1/100 in PBS/Tween) and incubated at room temperature for 45 min, and these virus/antibody mixtures were added to quadruplicate wells of the capture MAb-coated plate and incubated at room temperature for 45 min. Virus incubated with normal mouse serum (nms) served as a "no blocking" control. The dilution of blocking antibody used was that which caused most inhibition of virus binding in homologous competition experiments (i.e., where the same antibody was used as capture and blocking antibody). After washing in PBS/Tween, captured virus was detected by addition of HRP-labeled 4G2 antibody followed by H_2O_2/OPD substrate/chromagen as described previously. Mean absorbances for each blocking reaction were determined and significant changes in absorbance identified using a two-tailed Student *t*-test. The degree to which the blocking antibody inhibited capture of virus by the capture antibody was calculated as a percentage relative to absorbance in wells containing normal mouse serum as follows:

$$\text{Blocking} = 100 - [(\text{Abs. blocking MAb} + \text{virus}) / (\text{Abs. nms} + \text{virus})].$$

Competitive binding experiments utilizing dengue-immune human serum samples were performed using a competitive indirect ELISA. Dengue 1 antigen (8–16 HA units) diluted in borate saline was coated in 96-well plates (4°C overnight). Plates were blocked as described previously, and 10-fold dilutions of human serum (either dengue-immune or flavivirus nonimmune) in the range 10^{-1} to 10^{-3} were added. After incubation (room temperature/45 min), plates were washed and monoclonal antibodies (either undiluted hybridoma tcs or ascitic fluid diluted 1/100 in PBS/Tween) were added. After further incubation and washing, binding of monoclonal antibodies was detected by addition of HRP anti-mouse immunoglobulin antisera and substrate/chromagen as described previously. Absorbances were read at 490/650 nm and statistically significant changes in absorbance identified using a two-tailed Student *t*-test.

Selection of dengue 1 virus neutralization escape mutants in C6/36 cells

Dengue 1 virus (Jarrett), diluted in RPMI 1640 (10^{-1} to 10^{-6}), was mixed with neutralizing antibody (1/20) or RPMI 1640 and incubated at 37°C for 60 min. These virus/antibody mixtures were then transferred to monolayers of C6/36 cells in 24-well plates (Nunc, Denmark) and incubated at 28°C for 3 h to allow uptake of virus. The cell monolayers were then rinsed with serum-free RPMI 1640 and either the selecting antibody diluted in RPMI 1640 or RPMI 1640 alone was added to each well.

Infection of cells was detected seven days later by IFA using flavivirus-immune human serum and FITC-labeled anti-human immunoglobulin (Dako, Denmark). If virus was neutralized by antibody, this process was repeated using supernatant from the well containing the highest dilution of virus that infected cells in the presence of the neutralizing antibody. This process was repeated until the selecting antibody no longer neutralized infection (i.e., neutralization index < 1.0).

Supernatant from the culture infected with the highest dilution of neutralization-resistant virus was used to infect C6/36 cell monolayers in 25 cm^2 tissue culture flasks (Nunc, Denmark), in the presence of the selecting antibody, to prepare stocks of mutant virus.

A passage control virus population was derived from the sequential passage of virus in the absence of neutralizing antibody.

Effects of low-pH pretreatment on HA activity of dengue 1 viruses

Wild-type or mutant dengue 1 viruses were mixed with an equal volume of borate saline (pH 9.0), and two volumes of phosphate buffer (Clarke and Casals, 1958) were added to individual tubes to yield final pH values in the range 5.8 to 7.0. These mixtures were incubated at room temperature for 10 min, and the pH of each was then adjusted back to 7.4 by addition of 1 M NaOH. Standard HA assays were then performed with these virus preparations at pH 6.2.

Effects of low-pH treatment on infectivity of dengue 1 viruses

Aliquots of wild-type or mutant dengue 1 viruses (20 μL) were mixed with equal volumes of borate saline (pH 9.0), and 40 μL of sterile phosphate buffer was added to individual tubes to yield pH in the range 5.8 to 7.0. Forty microliters of RPMI 1640 was added to one tube as an "untreated" control (pH $\geq$ 7.4). Tubes were incubated at 37°C for 10 min, and the pH in each adjusted to 7.4 by addition of 1 M NaOH.

TABLE 4

Oligonucleotide Primers Used for Amplification and Sequencing of Dengue 1 Envelope Protein Gene Fragments

Primer	Sense	Sequence[a]	Location
D1-404	+	5′ GCTGCCCACAGCCTTG	C404–420
D1-925A	−	5′ CCTATTCCCACGCATCG	E4–20
D1-810U	+	5′ CCCGGGTTCGGTGATAGCCCTTTTTC	prM128–145
D1-1557L	−	5′ GTAGATCTCACGAGGTCCAAGGCAGTG	E666–650
D1-1391U	+	5′ CCCGGGTGCAACCATAACACCTCAA	E484–501
D1-2221L	−	5′ GTAGATCTCACGCAGTCCCAAAAATC	E1329–1314
D1-1058U	+	5′ CCCGGGTACAAACCCTGCCGTCCTG	E151–168
D1-1705U	+	5′ CCCGGGAGCGACAGAAATCCAAAC	E798–815
D1-1101L	−	5′ GTAGATCTCAGGTGGTGGTGTTTGATA	E210–194
D1-1830L	−	5′ GTAGATCTCAAGCCACTTCCTTCTCTA	E939–923

[a] Underlined regions are added restriction sites and/or stop codons.

Tenfold dilutions (10^{-1} to 10^{-6}) of each sample were prepared in RPMI 1640 and dispensed into wells of a 48-well tissue culture plate (Nunc, Denmark) containing monolayers of C6/36 cells. Plates were incubated as described previously and, seven days postinfection, the presence of virus in each well was detected by performing HA with culture supernatants at pH 6.2.

Temperature sensitivity assays

Aliquots of wild-type and mutant dengue 1 viruses were incubated at 45°C for 30, 90, and 180 min. At each time interval, an aliquot of virus was collected, 10-fold dilutions prepared in serum-free RPMI 1640, and monolayers of C6/36 cells in 48-well tissue culture plates infected as described previously. Seven days postinfection, culture supernatant from each well was tested for presence of virus by HA at pH 6.2. The titer of the infecting virus was the highest dilution that resulted in infection of the C6/36 cells.

Nucleotide sequencing

RNA was extracted from wild-type, passage control, and mutant virus populations using a guanidinium isothiocyanate lysis procedure based on that of Lanciotti *et al.* (1992), and cDNA template prepared using avian myeloblastosis virus (AMV) reverse transcriptase (Boehringer Mannheim, Germany) according to the method of Bielefeldt-Ohmann *et al.* (1994).

Oligonucleotide primers (Table 4) were designed to amplify three overlapping fragments of the dengue 1 polyprotein gene corresponding to the prM protein, and the structural domains and part of stem anchor region of the E protein (amino acids E1–420). Additional primers were prepared to allow complete forward and reverse sequencing of these products.

PCR amplification of dengue polyprotein gene fragments was carried out using "AmpliTaq" Taq polymerase (Perkin–Elmer, USA) according to the manufacturer's protocols and under the following cycling conditions: 94°C/4 min (1 cycle); 94°C/1 min, 54°C/1 min, 72°C/4 min (45 cycles); 72°C/10 min (1 cycle). Primer pairs D1-404/D1-925A, D1-810U/D1-1557L, and D1-1391U/D1-2221L were used to amplify the three gene fragments for sequencing. PCR products were purified from 2% agarose–Tris acetate EDTA gels using a Bresaclean kit (Bresatech, Australia) according to the manufacturer's instructions and sequenced on an ABI 373A DNA sequencer (Applied Biosystems, USA) using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin–Elmer) according to the manufacturer's protocols. Products from at least two RT-PCR reactions were sequenced in both forward and reverse directions.

Nucleotide sequences, and derived amino acid sequences, were compiled using programs from the WISCONSIN PACKAGE software suite and the EGCG extensions (version 8.1-Unix/version 8.1.0; Genetics Computer Group Inc., USA), and where ambiguity existed, fresh RT-PCR reactions were performed and the products sequenced.

Computer modeling of dengue 1 E protein structure

Structural models of the dengue 1 virus E protein were prepared from the published structure of the TBE virus E protein (Rey *et al.,* 1995; obtained from the Brookhaven Protein Data Bank) using the Swiss-Model structure prediction server (Guex and Peitsch, 1997).

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