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Prediction of Three-Dimensional Structure and Mapping of Conformational Epitopes of Envelope Glycoprotein of Japanese Encephalitis Virus

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Japanese encephalitis virus (JEV), a mosquito-borne flavivirus, is an important human pathogen. The envelope glycoprotein (Egp), a major structural antigen, is responsible for viral haemagglutination and eliciting neutralising antibodies. The three-dimensional structure of the Egp of JEV was predicted using the knowledge-based homology modeling approach and X-ray structure data of the Egp of tick-borne encephalitis virus as a template (Rey *et al.,* 1995). In the initial stages of optimisation, a distance-dependent dielectric constant of $4r_{ij}$ was used to simulate the solvent effect. The predicted structure was refined by solvating the protein in a 10-Å layer of water by explicitly considering 4867 water molecules. Four independent structure evaluation methods report this structure to be acceptable stereochemically and geometrically. The Egp of JEV has an extended structure with seven β-sheets, two α-helices, and three domains. The water-solvated structure was used to delineate conformational and sequential epitopes. These results document the importance of tertiary structure in understanding the antigenic properties of flaviviruses in general and JEV in particular. The conformational epitope prediction method could be used to identify conformational epitopes on any protein antigen with known three-dimensional structure. This is one of the largest proteins whose three-dimensional structure has been predicted using an homology modeling approach and water as a solvent.

Key Words: Japanese encephalitis virus; three-dimensional structure; envelope glycoprotein; homology modeling; conformational epitopes; sequential epitopes, antigenic determinant.

INTRODUCTION

Japanese encephalitis virus (JEV) is an RNA virus and is a member of the Flaviviridae family. It is endemic in Southeast Asia, including India (Banerjee, 1996; Monath and Heinz, 1996; Chambers *et al.,* 1997). Children between 5 and 15 years of age are maximally affected by JEV. It is also known that adults in the age group of 40–45 years are affected by JEV. Epidemics of JEV are distributed geographically among all of Southeast Asia (Umenai *et al.,* 1985; Monath and Heinz, 1996); thus there is an urgent need for effective JEV vaccine. The efficacy of the killed JEV vaccine, currently used, is not high. Furthermore, the protective immunity is induced only after the administration of two or three doses (Hoke *et al.,* 1988; Mohan Rao *et al.,* 1993), and the vaccine requires cold storage. In tropical countries, where JEV is endemic, the cost of immunisation becomes high (Tsai, 1994; Chambers *et al.,* 1997), so there is a need to develop cost-effective vaccines against JEV.

New approaches for vaccine development such as recombinant DNA vaccines or peptide vaccines require prior knowledge of antigenic structure and detailed delineation of conformational epitopes of proteins that are involved in protective immunity (Van Regenmortel, 1996, 1998a, 1998b). In flaviviruses, the envelope glycoprotein (Egp), being the major component of the virion surface, is the primary target for neutralization (Heinz, 1986; Heinz and Roehrig, 1990; Roehrig *et al.,* 1989). Antigenic structures of the Egp of flaviviruses have been studied extensively in past two decades, and three antigenic domains (A, B, and C) were suggested (Mandl *et al.,* 1989; Monath and Heinz, 1996; McMinn, 1997). However, these studies did not reveal the amino acid residues that are part of conformational epitopes in the Egp of JEV. Conformational epitopes are noncontiguous antigenic determinants whose specificity depends on the spatial folding or the conformation of the individual antigenic determinants. To our knowledge, there is no method to predict the conformational epitopes until now because of the subjectivity of the criteria that define conformational epitopes and the prerequisite knowledge of the three-dimensional (3-D) structure of an antigen.

In short, it would not be possible to discuss and define the conformational epitopes without knowledge of the detailed 3-D structure and the correlation of the 3-D structure with experimentally available antigenic data. At the present, high-resolution X-ray diffraction data of the Egp of only one flavivirus, Tick-borne encephalitis virus (TBEV), are available (Rey *et al.,* 1995). In the absence of experimental data, the 3-D structure of the Egp of JEV is

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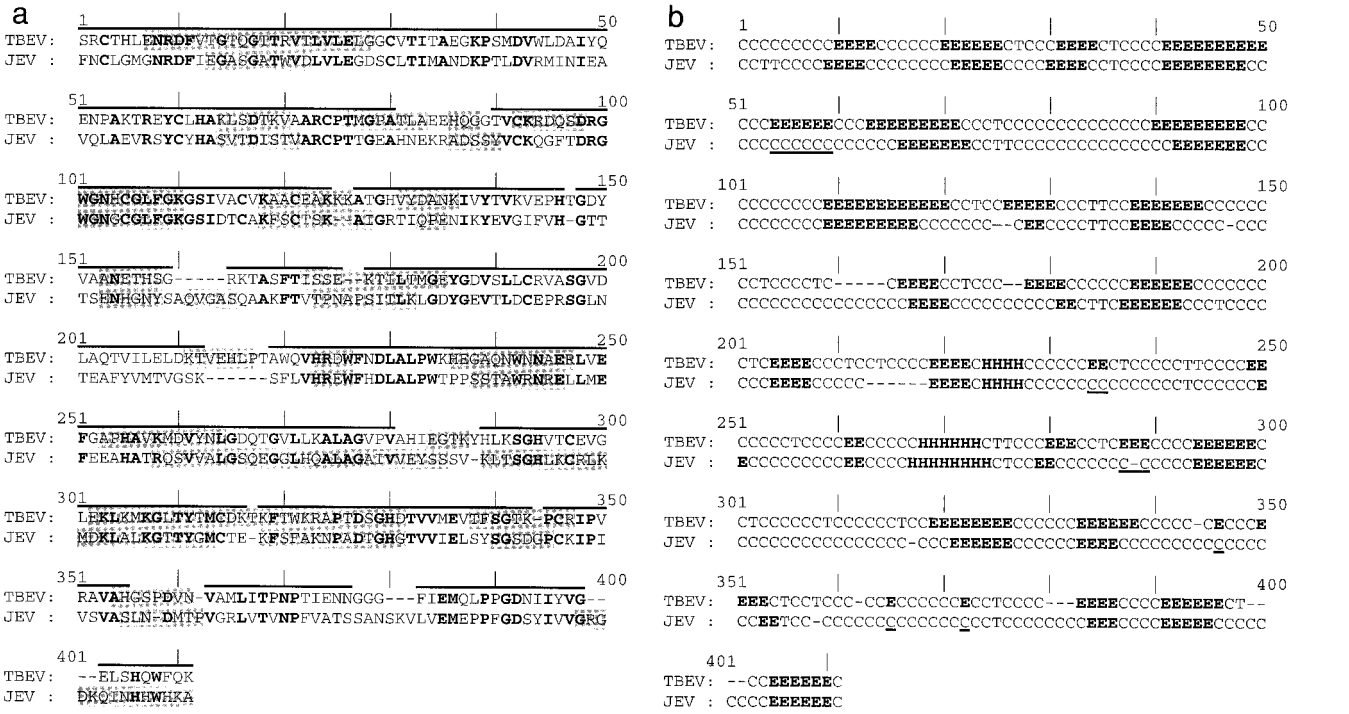


FIG. 1. (a) Pairwise optimal alignment of sequences of the Egp of TBEV and JEV obtained using the Homology module of Insight II of MSI and the Conformational Similarity Weight Matrix (CSW) (Kolaskar and Kulkarni-Kale, 1992). The identical amino acids are shown in bold, the thick lines mark the SCRs, and the highlighted regions correspond to antigenic determinants predicted using the algorithm described. (b) The secondary structures of the Egp of TBEV and JEV, as calculated by the DSSP program (Kabsch and Sander, 1983), are mapped on the optimal alignment of sequences of the Egp of TBEV and JEV. The secondary structure of each amino acid residue is described in terms of E-extended, H-helix, T-turn, and C-coil. Note that the residues 54–59, 234–235, 287–289, 345, 365, and 372 are not in extended conformation in the Egp of JEV and are underlined. There are slides/insertions/deletions at the level of secondary structure of both of the proteins, which have similar tertiary structures.

predicted using the knowledge-based homology modeling approach. Knowledge-based homology modeling has proved to be the best approach for the prediction of protein structure if the query and reference protein or proteins have similar function as well as sequence similarity of >40% (Johnson *et al.,* 1994; Sali, 1995; Sanchez and Sali, 1997; Moult *et al.,* 1997; Martin *et al.,* 1997; Davis *et al.,* 1998).

In the next sections, we discuss the knowledge-based homology modeling approach used to predict the 3-D structure of the Egp of JEV. The predicted structure is evaluated using four independent evaluation methods to gain confidence about the correctness and accuracy of the predicted structure. The predicted structure of the Egp of JEV was found to be acceptable according to the structure evaluation methods and was used to predict conformational and sequential epitopes. The validity of the prediction method was tested using the Egp of TBEV and coat proteins VP1, VP2, VP3, and VP4 of human Rhinovirus 14 because experimental data on 3-D structure and antigenicity are available for these protein antigens (Rey *et al.,* 1995; Mandl *et al.,* 1989; Holzmann, 1995; Rossmann *et al.,* 1985; Sherry *et al.,* 1986).

RESULTS AND DISCUSSION

Model building and refinement

In knowledge-based homology modeling, an optimal alignment leads to a more accurate model. The optimal alignment of the Egp of TBEV (template) and the Egp of JEV (query), as shown in the Fig. 1a, points out 40% identity at amino acid level. Furthermore, all 12 cysteines and glycosylation site (NYS) are conserved (Fig. 1a). The structurally conserved regions (SCRs) are marked using the Homology module of MSI and are also shown in Fig. 1a. Care was taken not to insert breaks in the regions that were part of the regular secondary structural motifs. As can be seen from Fig. 1a, 44 residues are in the loop regions and the remaining residues are part of 13 SCRs.

Prediction of the structure of the loop regions is the most important task in modeling proteins. As discussed, a hybrid approach of energy minimisation and molecular dynamics was used to perform the conformational search for every loop and to obtain the conformation of loop regions. Molecular dynamics were carried out at the room temperature for 500 ps to avoid the bias of the starting conformation and to reach the global minimum conformation for the residues in the loop regions. The

number of cycles of dynamics and minimisation were carried out particularly for loop regions because conformations of these regions are sensitive and give rise to inaccuracies (Johnson *et al.,* 1994). The inaccuracies in the prediction of the loop regions are due to the fact that loops are often variable in length, sequence, and conformation, even among the proteins of the same family. Because they are situated at the molecular surface and not in the hydrophobic core region, loops are also found to undergo large movements about the mean position (Moult *et al.,* 1997).

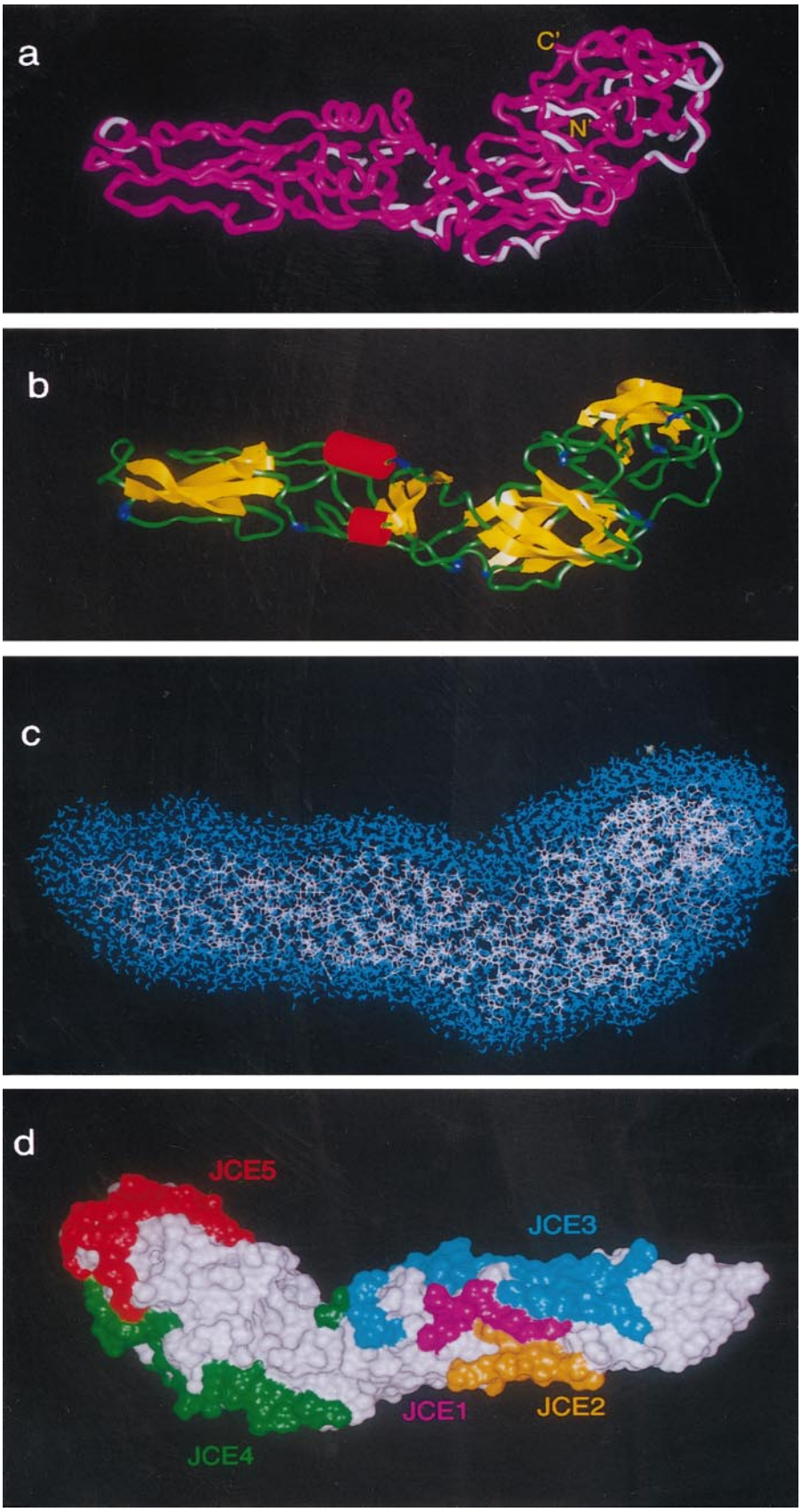
The predicted 3-D structure of the Egp of JEV is shown in Fig. 2. The use of distance-dependent dielectric constant (ϵ) has helped to simulate the solvent effect even in the early stages of optimisation. The solvation of the protein molecule, the Egp of JEV, in the 10-Å layer of water showed little changes in the conformation of the protein (Fig. 2c). On solvation, the conformation of the protein was stabilised by the formation of 962 hydrogen bonds between the protein and water molecules. Intramolecular potential energy had also decreased by ~5.0%, indicating that the readjustments in the orientation of the group of atoms have made the molecule more stable, and the strains, particularly in the chain reversal regions, were reduced after solvation. The RMS deviation in the conformations of the solvated and unsolvated but optimised protein molecule was found to be 1.13 Å for all atoms including side chains and 0.87 Å when only main chain atoms were compared. Further analysis of solvated and unsolvated models of the Egp of JEV showed that the conformation of side chains of 15 residues changed appreciably as seen from χ values. In all 15 residues, only one χ angle has changed, except for Asn^{82}, where both χ_1 and χ_2 have changed by >60 degrees. Main chain conformation of 41 residues on the surface has also changed. The residues involved in the glycosylation were among these 41 residues that were found to be reoriented after solvation. However, the secondary structure and overall 3-D structure of solvated and unsolvated models of the Egp of JEV were found to be similar. Thus 3-D structure prediction with explicit water helps to relieve the strains in the model and enables further optimisation so the predicted structure would be biologically meaningful.

Evaluation of the model

The water-solvated predicted structure, as mentioned, was evaluated using four independent approaches. The evaluation of the model using more than one method is essential in this particular case mainly because the predicted 3-D structure, as shown in Fig. 2, is highly extended and open, compared with most globular protein structures, which are highly compact. The model was evaluated in terms of stereochemical and geometric parameters such as bond lengths, bond angles, torsion angles, and packing environment and was found to satisfy all stereochemical criteria. As can be seen from Fig. 3, (ϕ, ψ) values calculated for each amino acid residue of the model structure lie in the allowed region (extreme limit included) of the Ramachandran plot (Ramachandran and Sasisekharan, 1968). The rotations around peptide bond were restricted by applying a 20-kcal barrier of rotation, so for all amino acids, $\omega = 180 \pm 10$ degrees except for the residues Lys^{118}, Lys^{289}, and Met^{301}; for these residues, $\omega = 180 \pm 15$ degrees. The PROCHECK G factors for dihedral angles is −0.28, and that for main chain covalent forces is 0.31. The overall average G factor is 0.0; the G factor is essentially a log-odds score based on the observed distributions of various stereochemical parameters and indicates that the overall structure is stereochemically correct (Laskowski *et al.,* 1993). The WHAT-CHECK score of merit is −1.5 and thus confirms that it is a well-refined model with "unusual structure" (Vriend, 1990). A further objective check on the model's quality was obtained using the program Prosall (Sippl, 1993). The energy graphs drawn using Prosall display the energetic architecture of protein folds as a function of amino acid sequence position. The energies are represented as the ratio of *E*/*kT* values. The combined (pair plus surface) energy graphs of the Egp of JEV (thick line) and the Egp of TBEV (thin line) are shown in Fig. 4. The graphs are smoothed by a window size of 60 residues. As can be seen from Fig. 4, not only is the molecule threaded properly but also its overall structure is satisfactory. None of the amino acid residues have a positive *E*/*kT* value for combined, pair and surface energy. In addition, we calculated surface as well as pair energies using both C_α–C_α and C_β–C_β interactions (data not shown), and the results show that the structure is acceptable. Figure 4 points out that the structure of the Egp of JEV in the region around 200 residues (±20) is energetically more favourable than the corresponding region in the experimental structure of the Egp of TBEV. The Prosall *Z*-score for the Egp of JEV gave a *Z*-score of −10.27. This *Z*-score falls in the range typical for native folds. Thus all four independent structure evaluation methods have pointed out that the predicted structure of the Egp of JEV is acceptable.

Structural description of the model

The predicted 3-D structure of the Egp of JEV is shown in Fig. 2. As can be seen, the Egp of JEV is highly extended and consists of three domains. Domain I, which is referred to as the central domain, consists of 128 residues from 1–51, 137–196, and 293–311. There are two disulphide bonds in this domain (Cys^3–Cys^{30} and Cys^{193}–Cys^{297}). This domain contains the glycosylation site in addition to the epitopes with serological or biological activities. The second domain, domain II, consists of 171 residues (52–136 and 197–292) and three disul-


a
C'
N'
b
c
d
JCE5
JCE3
JCE1
JCE2
JCE4

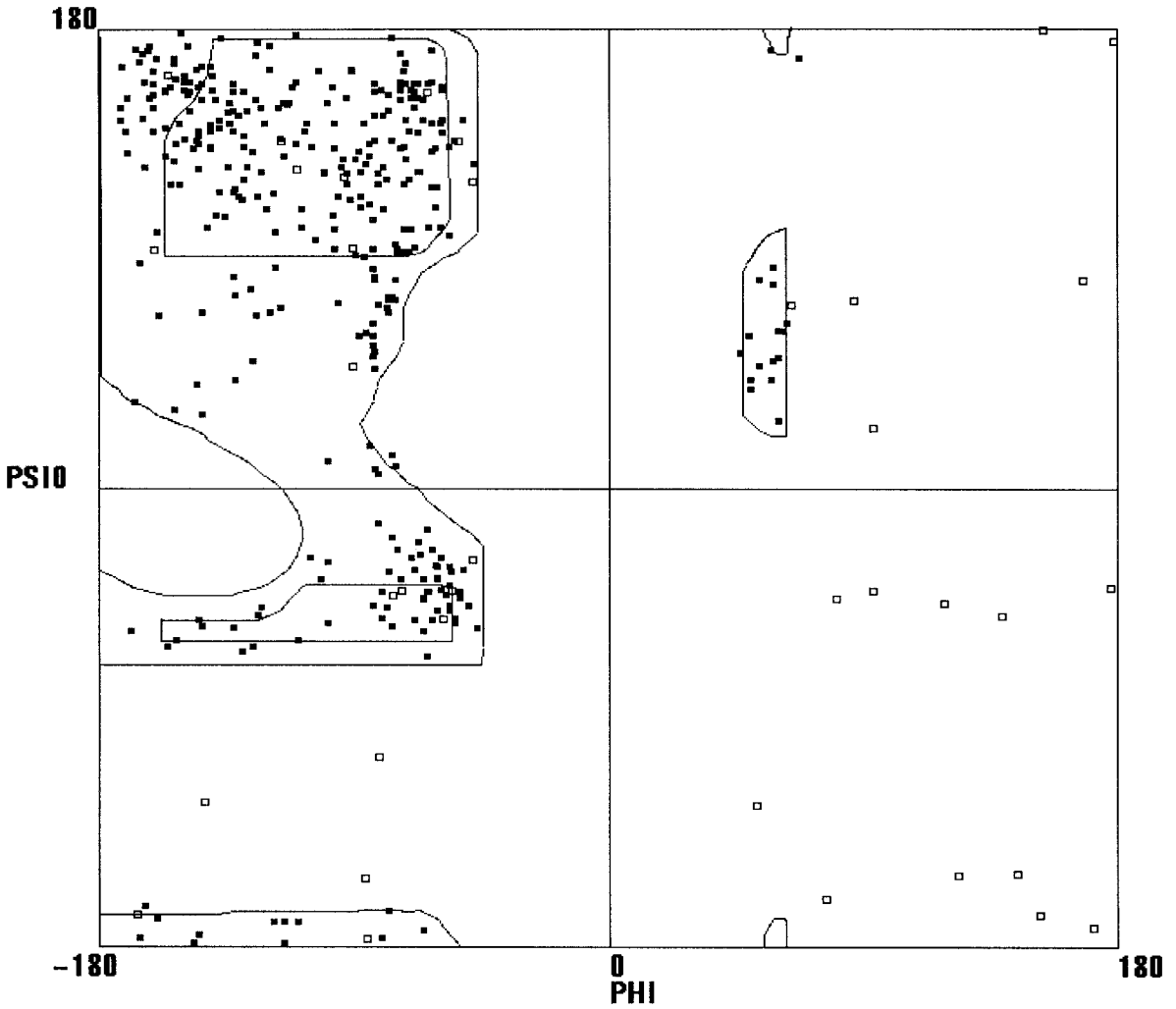


FIG. 3. The main chain dihedral angles (ϕ, ψ) are plotted for the predicted 3-D structure of the Egp of JEV. The (ϕ, ψ) values of every residue lie within the allowed regions of the Ramachandran plot. ■, Non-Gly amino acids. □, Gly residues.

phide bonds (Cys^{60}–Cys^{121}, Cys^{74}–Cys^{105}, and Cys^{92}-Cys^{116}). This domain is called the dimerisation domain. Domain II has a hydrophilic region and contains epitopes involved in neutralisation and hemagglutination. The region 98–111 is a highly conserved region among flaviviruses, has a β-hairpin motif, and has been suggested as the one involved in the fusion activities (Roehrig *et al.,* 1990). Domain III is a contiguous stretch of 100 residues (310–411) with only one disulphide bridge (Cys^{314}–Cys^{346}). Site-directed mutagenesis studies of this domain proved that it is an important domain not only in JEV but also in other viruses such as Murray Valley encephalitis virus, TBEV, and Looping ill virus (Lobigs *et al.,* 1990; Cecilia and Gould, 1991; Holzmann *et al.,* 1990; Jiang *et al.,* 1993). Domain III has also been suggested to be involved in the receptor binding activities. The 399-RGD-401 sequence motif, present in domain III, is unique to the mosquito-borne flaviviruses and was proposed to form part of the receptor binding site (Lobigs *et al.,* 1990). The three domains mentioned are related to previously defined antigenic domains for the Egp of JEV. Domain I corresponds to antigenic domain C, and domains II and III

FIG. 2. Predicted structure of the Egp of JEV using the knowledge-based homology modeling approach. (a) Structural superimposition of the Egp of JEV (model) and the Egp of TBEV (template). Structurally unique regions in the Egp of JEV are shown in white. (b) Secondary rendering of the model of the Egp of JEV. The β-sheets form the major secondary structure, and only two short α-helices are present. (c) Model of the Egp of JEV soaked in the 10-Å water layer consisting of 4867 water molecules. (d) Predicted conformational epitopes on the Egp of JEV. An algorithm developed to predict conformational epitopes (JCE1, JCE2, JCE3, JCE4, and JCE5) is discussed in the text. Note that these epitopes are distributed in all three domains and that most of the residues are accessible for interaction with antibody. The solvent-accessible surface of the Egp of JEV is drawn using an implementation of the Connolly (1983) algorithm.

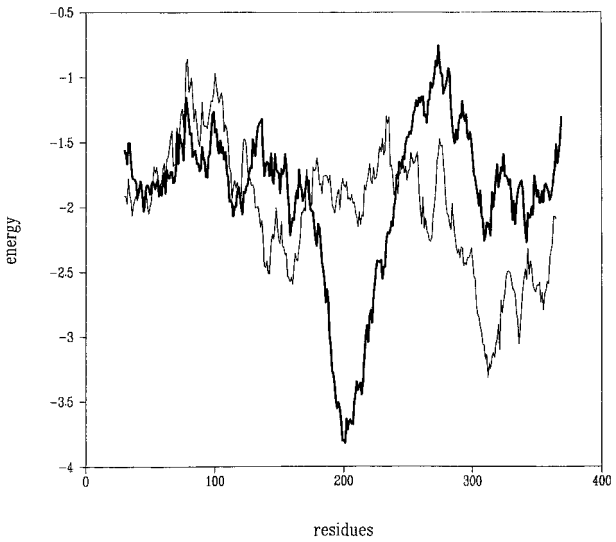


FIG. 4. The energy profile is shown of every residue for the Egp of JEV (thick line) and the Egp of TBEV (thin line) calculated using ProsaII. Both the graphs are smoothed using a window size of 60 residues. Note that energy for every residue in the predicted 3-D structure of the Egp of JEV is negative.

correspond to antigenic domains A and B, respectively (Mandl *et al.,* 1989; Monath and Heinz, 1996; McMinn, 1997).

The secondary structure of the final model was determined using the Dictionary of Protein Secondary Structure Patterns (DSSP) program (Kabsch and Sander, 1983). To compare the secondary structures of the Egp of JEV with that of TBEV, the secondary structure of the Egp of TBEV was determined using the atomic coordinate data (Rey *et al.,* 1995) and the DSSP program. These results are shown in Fig. 1b. It can be seen from Figs. 1b and 2b that the major secondary structure of the Egp of JEV is β-sheet as in the case of the Egp of TBEV. There are 25 strands with seven β-sheets in the Egp of JEV, which are distributed in three domains. There are only two short α-helices in the Egp of JEV (224–227 and 267–274). The domain I is a up-down β-barrel with nine strands. The elongated part of domain II consists of a sandwich of a three-stranded β-sheet and a β-hairpin. The base of this domain has an $\alpha + \beta$ structure with a β-sheet of three short strands and two short α-helices. Domain III has an immunoglobulin-like fold.

Superimposition of the predicted structure of the Egp of JEV on the template structure of the Egp of TBEV shows very high similarity at 3-D structure level among these two proteins, although sequence similarity is only 40% (Figs. 1b and 2a). The RMS deviations between the superimposed structure of the template and the model for the Cα trace (391 atoms) and the backbone (1173 atoms) were found to be 1.93 and 2.07 Å, respectively. Although these RMS deviation values are small, there are minor differences at the secondary structure level (Figs. 1b and 2a). The relative orientation of the helices in the Egp of JEV is different from that of the Egp of TBEV. A comparison of the β-strands of the Egp of JEV and the Egp of TBEV shows that the residues at positions 54–59, 234–235, 287–289, 345, 365, and 372 are not in the extended conformation in the Egp of JEV. In addition, slides/insertions/deletions were observed in a few β-strands. Thus Fig. 1b clearly points out the similarities and differences in the secondary structures of the Egp of JEV and the Egp of TBEV. At the tertiary structure level, as expected, the differences were observed among the residues on the surface. As can be seen from Fig. 2a, the residues 131–136, 152–165, 337–345, 377–383, and 397–403 have different conformations compared with the conformations of corresponding residues in the Egp of TBEV. It is interesting to note that the region 152–165 has glycosylation site, NYS, and the conformation of the glycosylation site is different in these two flaviviruses. The differences in the conformations of the residues 337–345, 377–382, and 397–403 in domain III may help us to understand the variations in antigenic and neutralising properties of flaviviruses. Most of the phenotypically variant properties, like serotypic differences, variants of vaccine strains, and neutralisation escape variants, map within domain III (Cecilia and Gould, 1991; Gritsun *et al.,* 1995).

Conformational epitope predictions: Assessment and application

Sequential antigenic determinants on the Egp of JEV were predicted according to Kolaskar and Tongaonkar (1990), Parker *et al.* (1986), and the distance criteria discussed in this paper. These antigenic determinants are listed in Table 1, which shows that the use of 3-D structure to predict antigenic determinants provides results that are in agreement with both of the earlier methods.

The method developed based on distance criteria to predict the conformational epitopes was evaluated using high-resolution X-ray structure data for antigen–antibody complexes for which 3-D structure data are available in the PDB. Four complexes are used in this study [PDB: 1JHL (Chitarra *et al.,* 1993); 2JEL (Prasad *et al.,* 1993); 1GC1 (Kwong *et al.,* 1998), and 1A14 (Malby *et al.,* 1993)]. 1JHL is a complex between anti-hen egg lysozyme antibody D11.15 and pheasant egg lysozyme. 2JEL is a complex of Fab fragment of antibody jel42 and histidine-containing protein. 1GC1 is a complex of human immunodeficiency virus type 1 Gp120 core with CD4 and a neutralizing human antibody. 1A14 is a complex between Nc10, anti-influenza virus neuraminidase antibody, and influenza virus neuraminidase. The compilation of the antigen–antibody interacting residues, obtained using the P-P server (Jones and Thornton, 1996), and proximate residues, obtained using the 5 A° distance criteria discussed, is given in Table 2, which shows that 80% of the

TABLE 1

Prediction of Antigenic Determinants Using 3-D Structural Information of Egp of JEV

Number	Predicted antigenic determinants	Kolaskar and Tongaonkar 1990	Parker *et al.* (1986)
1	^{13}EGASGATWVD^{22}	18–33	
2		40–46	
3	^{64}SVTDISTV^{71}	48–75	
4	^{86}ADSSY^{90}	88–95	75–92
5	^{101}WGNGCGLFGK^{110}		
6	^{118}KFSCTSKAI^{128}	113–124	
7	^{133}QPE^{135}		
8		138–148	
9	^{153}ENHGNY^{158}		150–160
10	^{161}QVGAS^{165}	158–184	
11	^{173}TPNAPSITLK^{182}	158–184	
12		188–194	
13		202–209	
14		211–219	
15	^{223}HREW^{226}		
16	^{238}SSTAWRNRE^{246}		235–246
17	^{258}RQSVVALGSQEGGLHQALAGAIVVEYSS^{285}	271–277; 282–300	252–260
18	^{289}KLTSGHLKCRLKMDKLALKGTTYGM^{313}	282–300; 302–308	
19	^{318}KFSFAKNPADTGHG^{331}		320–330
20		331–340	
21	^{340}SGSDGP^{345}		339–346
22	^{355}SLNDMTP^{362}		
23		363–370	
24		379–387	375–381
25	^{398}GRGDKQINHHWWHKA^{411}	391–399	

Note. Determinants predicted by Kolaskar and Tongaonkar (1990) and Parker *et al.* (1986) are also listed for comparison. See text for algorithm. Note that the amino acid numbers are the alignment positions from Fig. 1a. Amino acid residues with <30% accessibility are underlined.

residues that interact with the antibody have an accessible surface area (ASA) of ≥30%. The remaining 20% residues interact with antibody even though their ASA is <30%. Thus the criteria of an ASA of ≥30% and a distance of ≤5 Å seem to be appropriate to identify residues involved in the conformational epitopes in that only 6 of 72 interacting residues obtained by P–P interaction server were missed with the use of our method (Table 2).

TABLE 2

List of The Antigen–Antibody Interacting Residues Using The P-P Server (Jones and Thornton, 1996) and Proximate Residues Using the Distance Criteria of 5 Å

			Interacting residues listed by P-P server			Proximate residues listed by distance criteria		
N	PDB ID	Antigen Length	Total IR	IR ≥30% ASA	IR <30% ASA	Total PR	PR ≥30% ASA	PR <30% ASA
1	1JHL	129	17	13	4 (Y_{23}: 26%; G_{104}: 15%; W_{111}: 7%;V_{120}: 13%)	15	12	4 (Y_{23}: 26%; G_{104}: 15%; W_{111}: 7%; V_{120}: 13%)
2	2JEL	85	19	17	2 (S_{64}: 21%; E_{70}: 22%)	17	15	2 (S_{64}:21%;E_{70}:22%)
3	1GC1	297	16	12	4 (C_{119}: 24%; C_{205}: 28%; I_{420}: 25%; Y_{435}: 21%)	15	12	4 (C_{119}: 24%; C_{205}: 28%; I_{420}: 25%; Y_{435}: 21%)
4	1A14	390	20	14	6 (P_{328}: 21%; P_{331}: 20%; Y_{341}: 21%; S_{367}: 25%: S_{370}: 22%; S_{372}: 12%)	21	15	6 (P_{328}: 21%; P_{331}: 20%; Y_{341}: 21%; S_{367}: 25%; S_{370}: 22%; S_{372}: 12%)

Note. Only six interacting residues reported by the P-P server are missed by the distance criteria. Both of the methods report identical residues with ASA <30% as IR and PR, respectively (headings IR <30% ASA and PR <30% ASA). The residue names, numbers (in subscript), and percent ASA are given. IR, interacting residue(s); PR, proximate residue(s).

As can be seen from Table 2, except for 6 residues, the interacting and proximate residues are identical. The distance of these six residues, which are absent in the proximate residue list, is >5 Å by a margin of 0.1–0.8 Å. Table 2 lists the residues that are not accessible (ASA <30%) and are interacting with the antibody. For example, ASA for Val^{120} from PDB: 1JHL is 13.3%, and it is reported to interact with the antibody and is part of the epitope 116–121 (Chitarra *et al.,* 1993). In this epitope, all residues except Val^{120} have an ASA of >30%, but all residues (including Val^{120}) interact with the antibody. Such observations based on crystal structure data of Ag–Ab complexes support the idea of an extension of antigenic determinants in both directions if accessible amino acid or acids are present after an inaccessible residue. This algorithm allows the identification of sequential epitopes in addition to conformational epitopes. The predicted conformational epitopes on the Egp of JEV are given in Table 3A and are shown in Fig. 2d. The presence of conformational epitopes on the the Egp of JEV and other flaviviruses has been demonstrated earlier by experimental studies involving competitive binding of monoclonal antibodies (Henchal *et al.,* 1985; Kaufman *et al.,* 1987; Hawkes *et al.,* 1988; Kimura-Kuroda and Yasui, 1983, 1986; Hirabayashi *et al.,* 1996). Although these results have demonstrated the existence of spatially separable conformational epitopes, they have not identified the amino acids in these epitopes. For the first time, the results given in Table 3A and Fig. 2d identify amino acid residues in the conformational epitopes. Similarly sequential epitopes that are not part of any conformational epitopes are also listed.

To evaluate the prediction method based on the distance criteria, the sequential and conformational epitopes were predicted on the Egp of TBEV and coat proteins VP1, VP2, VP3, and VP4 of human rhinovirus 14. For these proteins, both the high-resolution X-ray crystal structure data and the experimental data on antigenic residues are available (Rey *et al.,* 1995; Mandl *et al.,* 1989; Holzmann, 1995; Rossmann *et al.,* 1985; Sherry *et al.,* 1986). The predicted conformational and sequential epitopes on the Egp of TBEV are listed in Tables 3B and 3D, respectively; however, the prediction data on coat proteins of human rhinovirus 14 are not included. In both cases, the experimentally known antigenic residues are part of predicted sequential and conformational epitopes except for three residues in the case of human rhinovirus 14 and two residues of TBEV, which are excluded as being inaccessible. In the case of TBEV, an additional antigenic residue was missed with the use of our method because the criterion of three consecutive accessible residues was not met. The results of predicted antigenic determinants on the Egp of JEV and that of TBEV are mapped on their aligned sequences (see Fig. 1a). It can be seen from Fig. 1a and Table 3 that there are unique conformational and sequential epitopes on the Egp of JEV and TBEV. Even for the common epitopes, not all amino acids are identical. Therefore, as can also be seen from Fig. 1a and Table 3, the structure of TBEV and sequence alignment of the Egps of TBEV and JEV are not sufficient to predict the unique epitopes on the Egp of JEV. Furthermore, there could be false-positive predictions based on the unique epitopes on the Egp of TBEV. These results point out the usefulness of the algorithm for epitope prediction. Results of experimental studies under way on peptides JSE3 and JSE4 agree with the predictions (data not shown).

The known sizes of Fab footprints on protein antigens are in the range of 500–900 Å and involve 15–20 contact residues (Davies *et al.,* 1990; Smith *et al.,* 1993; Wang *et al.,* 1992). It also has been pointed out that the Ag–Ab interactions involve sequentially distant antigenic determinants and most of the epitopes are expected to be discontinuous (Van Regenmortel, 1986, 1998a,b). The results in Table 3 are consistent with the above observations that most of the epitopes are conformational epitopes and that only a selected few are sequential. The method described here and used to map conformational and sequential epitopes on the 3-D model of the Egp of JEV is general in nature and can be used to delineate conformational and sequential epitopes for any protein antigen with known 3-D structure.

Conclusions

The knowledge-based 3-D structure prediction method with suitable modifications has been used to predict the structure of one of the largest protein, the Egp of JEV. The final predicted structure was obtained after soaking the protein in a 10-Å layer of water. This is one of the first protein structures in which 4867 water molecules were explicitly considered and the whole system was optimised without any constraints. The predicted structure was evaluated by using four independent standard methods; each method gave the results that the predicted structure of the Egp of JEV is acceptable stereochemically and energetically although it is extended and unusual.

Like the Egp of TBEV (template structure), the Egp of JEV has three domains and the structure is extended. The observed differences could help in understanding the antigenic and serological properties of these viruses. The 3-D structural information has been used effectively to predict conformational and sequential epitopes on the Egp of JEV. A distance-based method has been developed to predict the sequential and conformational epitopes on protein antigens with known structure. The predicted epitopes, on well-characterised protein antigens, were found to be in agreement with the experimental data. This approach could be used to delineate conformational and sequential epitopes on any protein

TABLE 3

Predicted Epitopes on Egp of JEV and TBEV

A

Predicted Conformational Epitopes on Egp of JEV (Also Shown in Fig. 2d)

Number	Determinants that are part of conformational epitopes	Accessible residues that are within 5 Å from conformational epitopes
JCE1	^{64}SVTDISTV^{71} + ^{118}KFSCTSKAI^{128}	H^{81}, S^{260}
JCE2	^{86}ADSSY^{90} + ^{238}SSTAWRNRE^{246}	K^{118}
JCE3	^{258}RQSVVALGSQEGGLHQALAGAIVVEYSS^{285} + ^{289}KLTSGHLKCRLKMDKLALKGTTYGM^{313} + ^{64}SVTDISTV^{71}	V^{51}, S^{211}, K^{212}, F^{220}, W^{226}
JCE4	^{289}KLTSGHLKCRLKMDKLALKGTTYGM^{313} + ^{13}EGASGATWVD^{22} + ^{173}TPNAPSITLK^{182}	V^{24}, E^{26}, D^{28}, D^{37}, I^{46}, Q^{161}, D^{185}, E^{188}, E^{194}, R^{196}, S^{197}, N^{200}, P^{345}, S^{366}, R^{399}
JCE5	^{318}KFSFAKNPADTGHG^{331} + ^{340}SGSDGP^{345} + ^{398}GRGDKQINHHWHKA^{411}	N^{379}, F^{390}

B

Predicated Conformational Epitopes on Egp of TBEV

Number	Determinants that are part of conformational epitopes	Accessible residues that are within 5 Å from conformational epitopes
TCE1	^{7}ENRDFVTGTQGTTRVTLVLELG^{28} + ^{302}EKLKMKGLTYTMCDKTKFTWKRAPTDSGHD^{331}	E^{36}, D^{46}, K^{291}, S^{292}, H^{294}
TCE2	^{64}KLSDTKV^{70} + ^{118}KAACEAKKK^{126} + ^{253}APHAVKMDVYNL^{264}	Q^{87}, E^{250}
TCE3	^{76}TMGPATLA^{83} + ^{86}HQG^{88} + ^{92}CKRDQSD^{98} + ^{101}WGNHCGLFGK^{110} + ^{253}APHAVKMDVYNL^{264}	K^{69}, V^{70}, R^{73}, K^{118}, N^{241}
TCE4	^{118}KAACEAKKK^{126} + ^{211}KTVEHLP^{217} + ^{236}HEGAQNWNNAER^{247}	K^{55}, R^{57}, Q^{87}, A^{231}, E^{284}
TCE5	^{131}VYDANK^{136} + ^{172}ISSEKTILTMGE^{185} + ^{302}EKLKMKGLTYTMCDKTKFTWKRAPTDSGHD^{331}	R^{20}, A^{54}, K^{55}, K^{166}, T^{167}, S^{169}, D^{188}, R^{194}, A^{196}, H^{289}, L^{290}
TCE6	^{302}EKLKMKGLTYTMCDKTKFTWKRAPTDSGHD^{331} + ^{338}TFSGTKPCR^{347}	E^{36}, G^{37}, D^{188}, E^{403}, S^{405}, Q^{407}

C

Predicated Sequential Epitopes on Egp of JEV

Number	Sequential determinants
JSE1	^{101}WGNGCGLFGK^{110}
JSE2	^{133}QPE^{135}
JSE3	^{153}ENHGNY^{158}
JSE4	^{161}QVGAS^{165}
JSE5	^{223}HREW^{226}
JSE6	^{355}SLNDMTP^{362}

D

Predicated Sequential Epitopes on Egp of TBEV

Number	Sequential determinants
TSE1	^{153}ANETHS^{158}
TSE2	^{223}HRDW^{226}
TSE3	^{284}EGTK^{287}
TSE4	^{354}AHGSPDVN^{361}

Note. The amino acid numbers are the alignment positions from Fig. 1a.

antigen with known 3-D structure and could help in designing vaccine strategies.

MATERIALS AND METHODS

Model building

To build the initial 3-D structure of the Egp of JEV, the X-ray structure data of the Egp of TBEV at 2.0 Å resolution (Rey *et al.,* 1995) were used as the template structure (PDB entry: 1SVB). The Homology module of Insight II (MSI, version 95; Biosym) was used to identify the SCRs between these two proteins (Fig. 1a). In the case of nonidentical substitutions, the coordinates of the backbone were taken from the template protein and the coordinates of the side chains were assigned only after performing the conformational search procedure using the Rotamer search library for the favored side chain conformations (Benedetti *et al.,* 1983; Ponder and Richards, 1987). Short contacts, if any, were removed by manually rotating the side chains. Initial conformations of the loop regions were assigned using the procedure described by Hobohm and Sander (1994). The junction regions between SCRs and loops were adjusted by fixing the dihedral angle, $\omega = 180$ degrees, and standard bond length to peptide bonds.

Model refinement

Conformation of each loop region was refined using the protocol described below. The initial conformation of the loop region was perturbed by carrying out molecular dynamics at room temperature (300K) for 500 ps and equilibration of 100 ps. Energy of the loop was calculated using the AMBER all atom force field (Seibel *et al.,* 1990) and distance-dependent dielectric constant $4r_{ij}$. Minimisation was carried out using Steepest descents (200 cycles) followed by the Conjugate gradient method until the RMS derivative reached 0.001 kcal/mol/Å. The Discover module of MSI was used for these studies. The bond lengths, bond angles, and ω values were checked and corrected if they were outside the acceptable range. For bond length and bond angle, the acceptable range was fixed as (standard bond length/bond angle $\pm$ 3σ), and for ω, it was 180 $\pm$ 15 degrees. If any of the molecular geometry parameters were found to be unacceptable as per the criteria given above, then all of the steps described above were repeated.

In the next step, the SCRs adjacent to the loop were included in energy minimization studies. SCR_{n-1}, $loop_n$, and SCR_{n+1} were minimized using Steepest descents and the Conjugate gradient method to the same level of convergence as mentioned above. This procedure of energy minimization of loop with flanking SCRs was carried out for every loop.

There are three domains in the reference protein Egp of TBEV. A similar domain structure is assumed for the Egp of JEV. Each domain was minimized without any constraints, and minimum energy conformations of all domains were obtained. The whole molecule, which consists of 399 amino acids, was then minimized to reach the convergence limit of 0.001 kcal/mol/Å. The main chain dihedral angles and geometrical parameters were calculated to determine whether they are in the acceptable range. Those geometrical parameters, which were found to be deviating from the standard geometry, were assigned standard values. The non-Gly residues that were found to have (ϕ, Ψ) values in the disallowed region of the Ramachandran plot were also assigned their nearest (ϕ, Ψ) values in the allowed region (Ramachandran and Sasisekharan, 1968). The energy of the molecule was then minimized in an unconstrained fashion. This step was repeated until (ϕ, Ψ) values for every residue were in the allowed region of the Ramachandran plot.

Model for solvated protein

To obtain the conformation of the protein solvated in water, the following procedure was used. The Egp of JEV molecule, in its minimum energy conformation, was soaked in a water layer of 10 Å. The system size was increased to 20648 atoms from 6047 atoms of the Egp of JEV with the addition of 4867 water molecules. Initial relaxation of the water molecules was carried out using 200 iterations of Steepest descents and 2000 iterations of Conjugate gradients. The water molecules were allowed to thermalise for 75 ps at 400K while the conformation of the protein was fixed. A short-duration dynamics (25 ps) was carried out on the water molecules alone so they could orient themselves with respect to the protein and form hydrogen bonds among themselves as well as with the protein. The energy of the whole system consisting of the Egp of JEV and 4867 water molecules was then minimised to the convergence limit of 0.001 kcal/mol/Å using the procedure described above.

Model evaluation

The essential accuracy and correctness of the model were evaluated using four independent methods: PROSTAT (module in Homology), Prosall (Sippl, 1993), PROCHECK (Laskowski *et al.,* 1993), and WHAT-CHECK program from WHAT-IF suite (Vriend, 1990).

An algorithm to predict conformational epitopes

The percent ASA of each amino acid residue in the predicted structure of the Egp of JEV was calculated using an implementation of the Lee and Richards (1971) algorithm as modified by Shrake and Rupley (1973). The following procedure was developed to identify conformational and sequential epitopes.

- Residues with ≥30% ASA were termed as accessible residues.

• A contiguous stretch of more than three accessible residues was termed as an antigenic determinant.

• A determinant was extended to N- and C-terminals only if accessible amino acid or acids were present after an inaccessible amino acid residue.

• The distance between every atom of residues from the i^{th} determinant and every atom of residues from the j^{th} determinants was calculated.

• If the distance between any pair of atoms of the residues from these sequentially distinct determinants was found to be ≤5 Å, then j^{th} sequential determinant was considered part of a conformational epitope that consists of i^{th} and j^{th} determinants.

• Such distance calculations were carried out with every sequential determinant ($j = 1, n$ and $j \neq i$) with i^{th} determinant as a reference. The reference sequential determinant was then varied from $i = 1$ to n. The list of conformational epitopes, thus identified, is given in Table 3.

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