

Biophysical Characterization and Vector-Specific Antagonist Activity of Domain III of the Tick-Borne Flavivirus Envelope Protein

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The molecular determinants responsible for flavivirus host cell binding and tissue tropism are largely unknown, although domain III of the envelope protein has been implicated in these functions. We examined the solution properties and antagonist activity of Langat virus domain III. Our results suggest that domain III adopts a stably folded structure that can mediate binding of tick-borne flaviviruses but not mosquito-borne flaviviruses to their target cells. Three clusters of phylogenetically conserved residues are identified that may be responsible for the vector-specific antagonist activity of domain III.

Flaviviruses (family *Flaviviridae*, genus *Flavivirus*) are organized into distinct vector-specific classes and serocomplex-specific subgroups (6), although little is known of the molecular determinants that dictate vector-specific pathogenesis, host cell specificity, and tissue tropism. Both mosquito-borne and tick-borne flaviviruses are responsible for epidemics throughout the developing world and pose serious public health threats in developed countries (7, 9, 10). Vaccines are only available to help control infection by yellow fever, Japanese encephalitis, Central European tick-borne encephalitis (TBE), and louping ill flaviviruses (1, 2, 16, 17). No antiviral therapy is available to treat any flavivirus infection.

Flavivirus infection requires attachment and entry into a target cell, mediated by binding of the viral envelope (E) proteins to cell surface receptors. The host cell receptor (or receptors) and the region (or regions) of the E protein responsible for flavivirus attachment are unknown, although heparan sulfate has been suggested as one factor mediating the interaction between dengue 2 virus and its target cells (3). It is unknown whether different cell surface receptors and/or envelope-receptor interactions are responsible for flavivirus pathogenesis, host range, and tissue tropism.

The structure of the E protein ectodomain from TBE virus has been determined (13). This ectodomain forms a homodimer, with each dimer subunit organized into three domains, designated I, II, and III. Comparisons of E proteins from wild-type viruses with those of attenuated or escape mutant viruses have identified a number of residues in domain III that may be responsible for receptor recognition (4, 8, 12–14). We examined the solution properties of domain III of the E protein from Langat virus (a tick-borne flavivirus) and the ability of this domain to function as an antagonist for virus

infectivity. We demonstrate that recombinant domain III adopts a highly stable folded structure in solution and shows reduced infectivity in tick-borne flaviviruses but not mosquito-borne flaviviruses. This suggests that interactions between the envelope protein and its host cell receptor may be vector specific, although it is likely that other factors also influence fla-

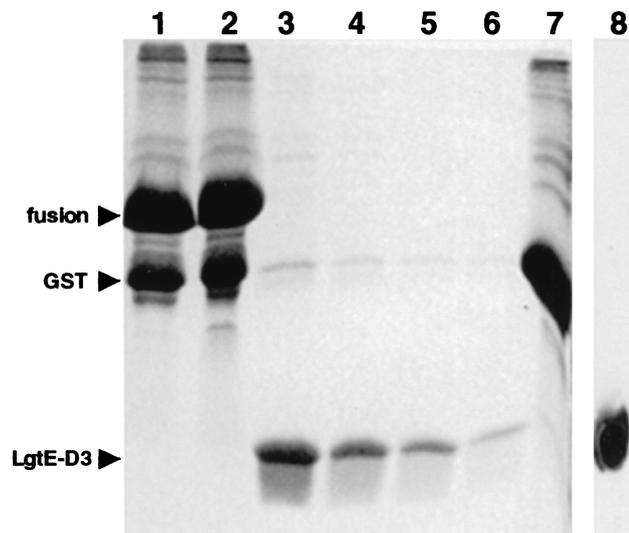


FIG. 1. Coomassie blue-stained composite sodium dodecyl sulfate-polyacrylamide gel showing expression and purification of recombinant LgtE-D3. Lane 1, GST-LgtE-D3 fusion protein bound to glutathione-conjugated agarose beads. Lane 2, total protein at the initiation of thrombin cleavage of bound GST-LgtE-D3. Lane 3, soluble protein after thrombin cleavage of bound GST-LgtE-D3. Lanes 4 to 6, soluble protein recovered after sequential washes of glutathione-agarose beads with low-salt buffer. Lane 7, protein bound to glutathione-agarose beads after thrombin cleavage. Lane 8, Western immunoblot of purified LgtE-D3 probed with rabbit polyclonal antibody generated against recombinant LgtE-D3 (Alpha Diagnostics, Inc.). The GST-LgtE-D3 fusion protein migrates as a single band at ~37 kDa. Recombinant LgtE-D3 migrates as a single band at ~10.7 kDa.

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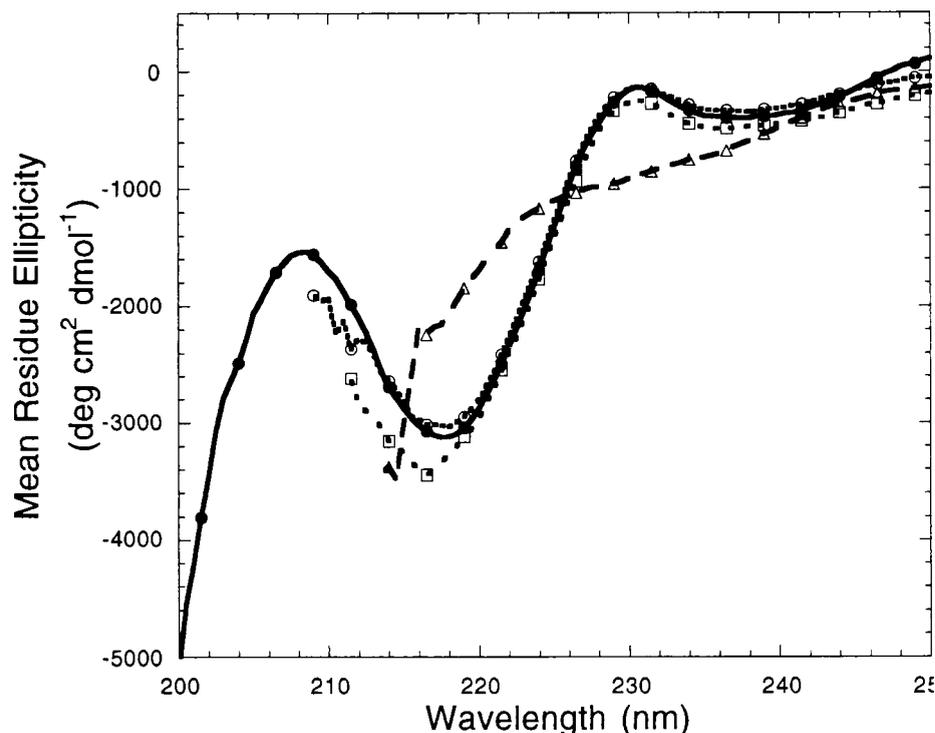


FIG. 2. Far-UV CD spectra of recombinant LgtE-D3 incubated with increasing amounts of denaturant. Spectra from LgtE-D3 in 0 M urea (solid curve, solid circles), 2 M urea (dotted curve, open circles), 4 M urea (dotted curve, open squares), and 8 M urea (dashed curve, open triangles) are shown. The LgtE-D3 concentration was 0.3 mg/ml in Tris buffer (pH 7.4). Spectra were collected on an Aviv 62 DS circular dichromer operating with a 0.5-nm step increment and a 1-s interval. Cylindrical quartz cuvettes with a 0.1-cm path length were used for all measurements. All sample spectra were recorded five times, averaged, and corrected for buffer contributions. Measurements were considered unreliable when the instrument dynode voltage exceeded 410 V and were not included in subsequent analyses.

vivirus vector preference. Sequence and structure analyses identified a small number of residues that may be responsible for vector-specific receptor binding.

Cloning and expression of domain III of Langat virus E protein. cDNA encoding domain III of the Langat virus E protein (E protein residues 300 to 395; LgtE-D3) was amplified by PCR. Template DNA for the PCR was plasmid pUC18 containing cDNA encoding Langat virus structural proteins. The PCR product was subcloned into pGEX-2T expression vector (Pharmacia), and the fidelity of the cloned sequence was confirmed by DNA sequencing (T. Woods, unpublished data). *Escherichia coli* DH5 α cells were transformed with pGEX-2T

expressing LgtE-D3 and grown at 37°C in 2xYT medium. The cultures were chilled to 16°C, and expression of a glutathione *S*-transferase (GST)-LgtE-D3 fusion protein was induced by the addition of 1 mM isopropyl-1- β -D-galactopyranoside. Cultures were maintained at 16°C for 6 to 12 h, pelleted, and frozen. Pellets were resuspended in ice-cold PBST buffer (10 mM sodium phosphate, 2 mM potassium phosphate [pH 7.4], 140 mM NaCl, 3 mM KCl, 0.1% [vol/vol] Tween 20) and lysed with mild sonication. Glutathione-agarose beads were added to the supernatant and mixed gently at 4°C for 2 to 4 h. The beads were washed with PBST buffer and high-salt PBST

TABLE 1. Qualitative examination of CPE in Vero cells^a

Flavivirus	Vector	CPE of LgtE-D3 ^b
Dengue virus type 4	Mosquito	+
Japanese encephalitis virus	Mosquito	+
Yellow fever virus	Mosquito	+
Langat virus	Tick	-
Powassan virus	Tick	-

^a Cells were grown until 60 to 70% confluent, washed with cold PBS, and incubated on ice with 20 μ M LgtE-D3. Cells were challenged with virus at a multiplicity of infection of 0.5 and incubated again for 30 min on ice. The cells were then supplemented with 2 ml of Dulbecco's modified Eagle's medium containing 2% (vol/vol) fetal bovine serum (viral maintenance medium) and incubated at 37°C in 5% carbon dioxide.

^b Presence (+) or absence (-) of CPE compared to control cells.

TABLE 2. Decreased tick-borne flavivirus production in protected Vero cells^a

Challenge virus	Virus titer (log PFU)		Protection factor ^b
	Without LgtE-D3	With LgtE-D3	
Langat virus	6.6	5.7	7.9
Powassan virus	6.0	5.1	7.9

^a Cells were challenged with virus as described in the text. Aliquots of the supernatant were harvested at selected time points and frozen. Virus titers were determined by plaque assay using confluent Vero cells overlaid with 5 ml of 1% agar overlay (2% agar with an equal volume of viral maintenance medium). Plates were incubated at 37°C in 5% CO₂ for 5 to 10 days and plaques were visualized with 1% agar overlay supplemented with 2% (vol/vol) neutral red (Sigma).

^b Protection factor = (PFU without LgtE-D3)/(PFU with LgtE-D3).

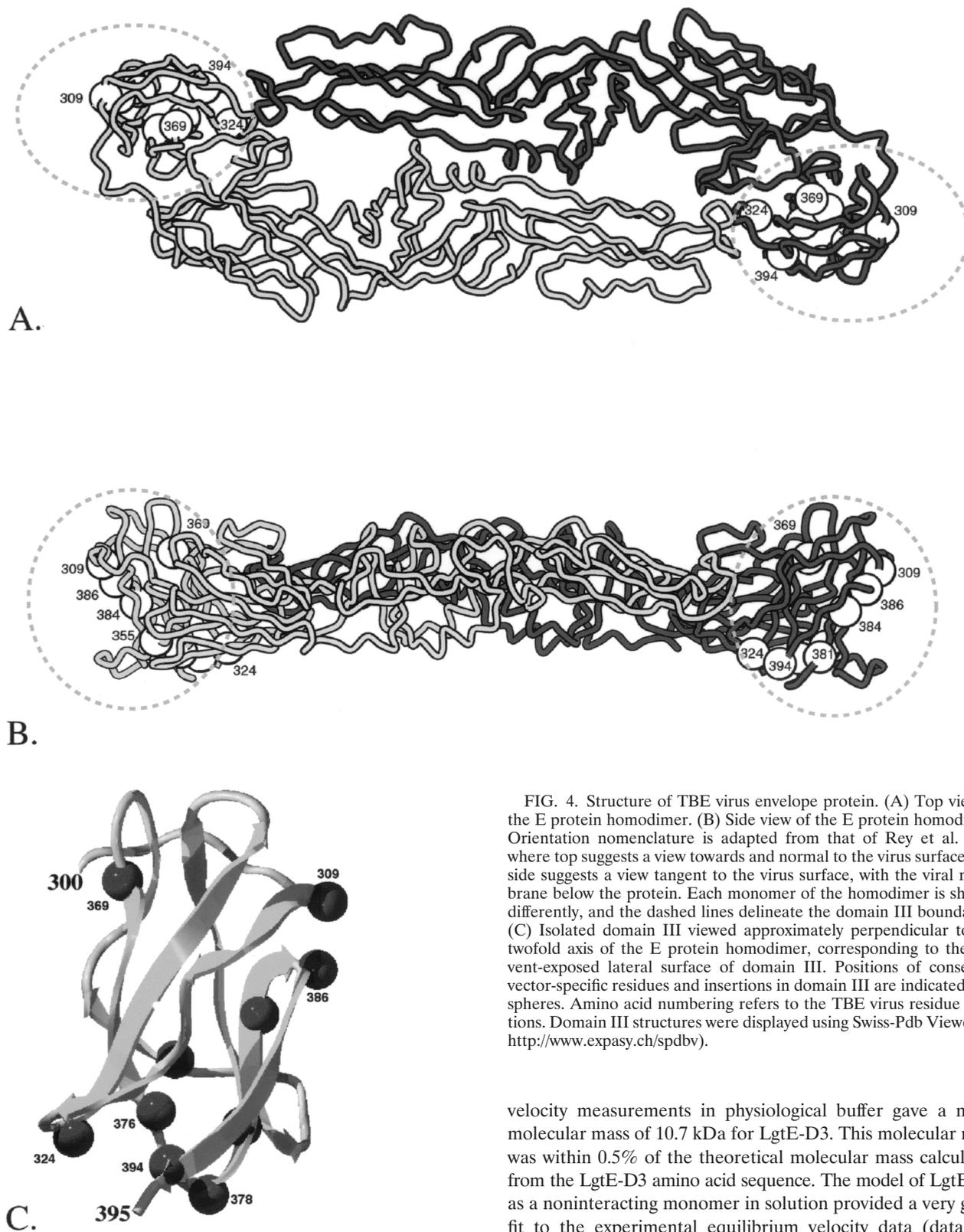


FIG. 4. Structure of TBE virus envelope protein. (A) Top view of the E protein homodimer. (B) Side view of the E protein homodimer. Orientation nomenclature is adapted from that of Rey et al. (13), where top suggests a view towards and normal to the virus surface, and side suggests a view tangent to the virus surface, with the viral membrane below the protein. Each monomer of the homodimer is shaded differently, and the dashed lines delineate the domain III boundaries. (C) Isolated domain III viewed approximately perpendicular to the twofold axis of the E protein homodimer, corresponding to the solvent-exposed lateral surface of domain III. Positions of conserved vector-specific residues and insertions in domain III are indicated with spheres. Amino acid numbering refers to the TBE virus residue positions. Domain III structures were displayed using Swiss-Pdb Viewer (5; <http://www.expasy.ch/spdbv>).

velocity measurements in physiological buffer gave a mean molecular mass of 10.7 kDa for LgtE-D3. This molecular mass was within 0.5% of the theoretical molecular mass calculated from the LgtE-D3 amino acid sequence. The model of LgtE-D3 as a noninteracting monomer in solution provided a very good fit to the experimental equilibrium velocity data (data not shown).

Antagonist activity of LgtE-D3. The ability of LgtE-D3 to protect cells from infection with tick-borne and mosquito-borne flaviviruses was tested. LgtE-D3 significantly reduced the amount of observed cytopathic effect (CPE) in Vero cells

a Beckman Optima XL-A analytical ultracentrifuge showed that LgtE-D3 was a single low-molecular-weight species with a sedimentation coefficient (15) of 1.3S in physiological buffer at room temperature (data not shown). Analysis of equilibrium

infected with the two tick-borne viruses but not with the mosquito-borne viruses (Table 1). In addition, there was a significant delay in the appearance of CPE in cells challenged with tick-borne viruses and protected with LgtE-D3 relative to unprotected cells (data not shown). The protection afforded Vero cells by LgtE-D3 was quantitated by examining virus titers produced in cells challenged with tick-borne viruses. Approximately 10-fold less tick-borne virus was produced from cells protected with LgtE-D3 than from unprotected cells (Table 2). These observations suggest that domain III functioned as a vector-specific antagonist to interfere with flavivirus binding to host cell receptors. This is the first direct experimental evidence that domain III is involved in host cell receptor binding. Recent immunofluorescence studies support the conclusion that LgtE-D3 binds to a membrane-associated host cell receptor (M. Holbrook, personal communication). The observed vector-specific differences could result from the tick-borne and mosquito-borne flaviviruses' interacting with an identical receptor binding site via different intermolecular contacts, with the mosquito-borne flaviviruses binding to this site with greater affinity than the tick-borne flaviviruses. Alternatively, vector specificity could result from flaviviruses' recognizing different cell surface receptors or different binding sites on the same cell surface receptor.

Molecular basis of vector-specific antagonist activity. Since LgtE-D3 displays vector-specific antagonist activity, an analysis of vector-based invariant residues was performed to identify domain III regions that mediate host cell binding (Fig. 3). Analogous sequence comparisons in the hemagglutinin receptor-binding site of influenza A viruses have identified phylogenetically conserved residues that determine receptor specificity (11, 18). Domain III amino acid sequences showed ~80 to 95% sequence identity among tick-borne flaviviruses, ~50 to 90% identity among mosquito-borne flaviviruses, and ~50% identity among two non-vector-borne flaviviruses. Twelve residues were invariant, and nine residues were highly conserved (>93%) in the sequences examined. Six amino acids, at positions 324, 355, 381, 382, 384, and 394 (TBE virus numbering), were completely conserved within a flavivirus vector class but differed between the tick-borne and mosquito-borne viruses. In addition, vector-specific insertions and deletions occurred at positions 309, 369, 376, 378, and 386, either at loops between β -sheets or in extended coils (Fig. 4).

The 11 vector-specific positions were clustered in three spatially distinct regions in domain III (Fig. 4), which may be responsible for the vector-specific antagonist activity of domain III. Region 1, formed by residues 309, 384, and 386, was located on the solvent-exposed lateral face of domain III. These residues were flanked by invariant residues, and at residue 386 three homologous residues were inserted in mosquito-borne flaviviruses relative to tick-borne flaviviruses. Thus, the structural and chemical properties of region 1 may be similar among vector-specific flaviviruses, allowing this region to form vector-specific interactions with cell surface receptors. Recent studies showed that mutations in region 1 were associated with virus attenuation (8, 13, 14). Region 2, formed by residues 324, 355, 376, 378, 381, 382, and 394, made extensive contacts between the β -sheet termini of domain III (Fig. 4). The majority of these residues were partially buried within the domain III structure and thus likely shielded from intermolecular interac-

tions. Region 3, containing residue 369, was part of a solvent-exposed loop (Fig. 4). Between residues 369 and 370 of the mosquito-borne flaviviruses, one additional residue was inserted in the dengue and yellow fever viruses and three additional residues were inserted in the non-dengue and non-yellow fever viruses relative to the tick-borne flaviviruses. Invariant residues were not adjacent to residue 369, implying that the tertiary structure and chemical properties of region 3 are different for different mosquito-borne flaviviruses.

Vector-specific residues in the non-vector-borne flaviviruses had similarities to both the tick- and mosquito-borne flaviviruses. The non-vector-borne and mosquito-borne flaviviruses had the same residues at positions 324, 355, and 381 and had a conserved deletion at position 309. The non-vector-borne and tick-borne flaviviruses had similar residues at positions 376 and 394 and conserved deletions at positions 378 and 386. The lysine residue at position 384 in the non-vector-borne flaviviruses was distinct from the conserved residue found in the mosquito- and tick-borne flaviviruses.

Domain III mutants can be used to delineate E protein receptor interaction surfaces and test if different E protein interaction surfaces are responsible for tissue tropism. Domain III and its derivatives may be useful first-generation vector-specific antagonists and as targets against which to generate antiviral agents that prevent binding of flavivirus to its host cell receptor. In addition, recombinant domain III may be valuable in the development of flavivirus vaccines and diagnostic reagents.

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