Yellow fever 17D virus: pseudo-revertant suppression of defective virus penetration and spread by mutations in domains II and III of the E protein

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Abstract

A yellow fever (YFV) 17D virus variant, which causes persistent infection of mouse neuroblastoma cells associated with defective cell penetration and small plaque size, yielded plaque-revertant viruses from cells transfected with viral transcripts encoding the adaptive mutation (Gly360 in the E protein). Reconstruction of a plaque-purified revertant which contained Gly360 and additional substitutions (Asn for Lys303 and Val for Ala261) yielded a virus whose infectious center size, growth efficiency, and cell penetration rate similar to the parental YF5.2iv virus, whereas viruses with Asn303 or Val261 alone with Gly360 yielded either a small-plaque virus or a parental revertant. These data indicate that the YFV E protein is subject to suppression of mutations in domain III that are deleterious for viral entry and spread by a second-site mutation in domain II. Position 261 lies within the hydrophobic ligand-binding pocket at the domain I–II interface, a site believed to be involved in the hinge-like conformational change of domain II during activation of membrane fusion-activity. Results of this study provide genetic data consistent with findings on flavivirus structure and implicate domain III in functions beyond simply cell surface attachment.

Introduction

Flaviviruses are small, enveloped positive-sense RNA viruses containing a single genomic segment of approximately 11 kb, which encodes a long open reading frame that produces three structural proteins C (capsid), prM (precursor to membrane protein), and E (envelope), and at least seven nonstructural proteins (Rice, 1996). Flavivirus infections involve initial attachment of virus to host cells through receptors that have yet to be definitively identified, and in some cases proteoglycans are involved in early virus–cell interactions (Anderson, 2003). The viral envelope protein governs the attachment of flaviviruses to specific receptors which then participate in receptor-mediated endocytosis and delivery of the virus to an endosomal compartment. Low pH-induced conformational changes render the E protein competent for fusion with host membranes resulting in release of the RNA genome into the cytoplasm (Heinz and Allison, 2000). Based on the structure of the soluble fragment of the tick-borne encephalitis (TBE) virus E protein (Rey et al., 1995), domain III has been implicated in binding to cellular receptors (Bhardwaj et al., 2001; Crill and Roehrig, 2001; Mandl et al., 2000). Consequently, some of the virulence determinants that affect the pathogenesis of flaviviruses in animal models have been mapped to the lateral edge of this domain (Rey et al., 1995), and these act presumably through effects on virus attachment, post-receptor-binding events associated with virus entry, or both.

Yellow fever (YFV) virus is the prototype member of the genus Flavivirus within the Flaviviridae (Rice, 1996). In a previous study, we characterized the properties of a YF 17D variant associated with persistent infection of a mouse neuroblastoma cell line (Vlaycheva and Chambers, 2002). This variant (NB15a) exhibited a lower efficiency of infection, impaired growth kinetics, reduced cell penetration, and poor cell-to-cell spread in different cell lines compared to parental virus (YF5.2iv, produced from YF17D infectious clone). A single nucleotide substitution encoding a glycine residue instead of aspartic acid at position 360 in domain III of the E protein was responsible for the cell culture properties...
of this variant. Although the NB15a virus exhibited a stable plaque phenotype, YF5.2iv virus engineered to encode this neuroblastoma-adaptive mutation (Gly360) yielded frequent plaque-revertant viruses after RNA transfection of Vero cells. To determine if this reversion phenomenon involved novel mutations in the structural protein region that might give insight into functionally important interactions among the domains of the E protein and its association with prM, we isolated and characterized a set of plaque-revertant viruses which were found to harbor second-site mutations. The mutations were evaluated for effects on plaque size and other cell culture properties which differentiate the neuroblastoma-adapted NB15a virus from the parental virus.

Results

Isolation and characterization of revertant viruses

Transfection of Vero cells with RNA transcripts from YF5.2iv virus containing the substitution of glycine for aspartic acid at amino acid residue 360 of the E protein yields a very small-plaque virus, similar to the original NB15a variant (Vlaycheva and Chambers, 2002). However, these transfections also yield virus progeny with heterogeneous plaque sizes ranging from small to parental (data not shown). To determine if any suppressor mutations were responsible for this plaque-revertant phenomenon, a total of four intermediate- and large-plaque revertants were plaque-purified and their prM-E regions sequenced (Table 1). A total of nine PCR clones of the two large-plaque viruses (four for L6 and five for L7), and six PCR clones of the two intermediate-plaque viruses (two for M2, and four for M8) were analyzed. None of the plaque revertants contained a reversion of glycine to aspartic acid at position 360. The large-plaque (L) revertants consistently contained substitutions at position 303 in the E protein (seven of nine clones), where a lysine residue was replaced with glutamic acid (three clones) or asparagine (four clones). Two clones of large-plaque revertants (L6.1 and L6.2) also showed an additional substitution of valine for alanine at position 261 of the E protein. Two additional large plaque clones (L6.3 and L6.4) contained no second-site mutations in the structural region. Among the intermediate-size (M) revertants, a substitution at residue 44 in the prM region appeared in two of six clones (M2.1 and M2.2). The other clones from a second revertant did not contain any mutations in the prM-E region. Some of these large- and medium-size plaque-revertant viruses may have contained additional mutations outside of the prM-E region that are involved in the revertant phenotype; however, we did not attempt to identify any such mutations in the present study.

Engineering of revertant viruses

To evaluate the role of the amino acid substitutions identified in Table 1 in the plaque reversion process of their respective viruses, a series of viruses containing these various substitutions either together with the original G360 mutation or with the parental YF5.2iv sequence were constructed and their viability and plaque sizes determined (Table 2). Viruses engineered to contain either asparagine or glutamic acid at position 303 with the parental aspartic acid at position 360 exhibited small plaque sizes. Viruses engineered to contain either asparagine or glutamic acid at position 303 together with the glycine substitution at position 303 also exhibited small-plaque sizes, similar to that of the NB15a virus. However, virus engineered to contain the substitutions of asparagine or glutamic acid at position 303 as well as the substitution of valine for alanine at position 261, together with the original G360 mutation, exhibited partial restoration of the parental plaque size (Fig. 1). The transfection harvest of this virus was very heterogeneous; however, plaque purification of one of the largest plaque isolates in this population yielded a virus whose plaque size was very nearly the same as parental YF5.2iv (not shown). This isolate (V261N303G360) was used for further characterization.

Because the presence of valine at position 261 was associated with reversion of plaque size, the YFV molecular clone was also engineered to encode this substitution either alone or together with glycine at position 360 to determine if valine was necessary and sufficient for the reversion. In two experiments with these two constructs, no viruses were recovered after transfection. Transfection harvests from a third experiment for each of these constructs yielded viruses with parental plaque sizes; however, nucleotide sequencing revealed reversions to the parental YF5.2iv sequence at positions 261 and 360 in both cases. A virus was also recovered in which valine at 261 was engineered together with asparagine at 303; however, this virus exhibited a small plaque size. These data indicate

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Table 1

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that a valine substitution alone is intrinsically a lethal mutation for the YF5.2iv virus either by itself, or in the context of either a glycine or aspartic acid residue at position 360. In contrast, valine at position 261 can be tolerated provided that a substitution of asparagine for lysine occurs at residue 303, suggesting that the latter mutation compensates in some way for the deleterious effects of the valine residue.

Characterization of the Val261Asn303Gly360 virus

The Val261Asn303Gly360 virus recovered from RNA transfection as shown in Table 2 was plaque-purified and subjected to nucleotide sequence analysis, and the presence of the three substitutions in the E protein was verified. The virus was then characterized with regard to several cell culture properties which differentiate the neuroblastoma-adapted NB15a virus from the parental YF5.2iv virus. To confirm that the increased plaque size of the Val261Asn303Gly360 virus reflected efficient cell-to-cell spread, infected Vero cell monolayers were examined using an immunofluorescent focus assay (Fig. 2). Cells infected at low multiplicity were serially examined for the size of infectious centers in comparison to the size generated by the parental YF5.2iv virus. In this type of assay, viruses with different plaque sizes can be readily distinguished as early as 2 or 3 days postinfection. In particular, the Gly360 virus exhibits a markedly restricted infectious focus size that does not enlarge much over a period of 8 days postinfection (Vlaycheva and Chambers, 2002). The V261N303G360 virus formed fluorescent foci at day 3 postinfection that were similar in size to those of YF5.2iv, consistent with the results of the plaque assay. However, it displayed a somewhat distinct pattern of spread, as the edges of the foci were less well defined relative to those formed by YF5.2iv, which were rounder with sharper edges.

Cell culture growth

To determine if the plaque-revertant phenotype of the V261N303G360 virus was associated with growth efficiency that resembled the parental virus, the kinetics of virus growth were examined in Vero cells after low multiplicity of infection. In previous studies, we had observed that the small-plaque neuroblastoma cell-adapted NB15a virus exhibited reduced growth efficiency in various cell lines of different host origin in comparison to parental YF5.2iv virus (Vlaycheva and Chambers, 2002). The growth profile of the engineered Val261Asn303Gly360 virus was very similar to that of the YF5.2iv virus (Fig. 3), with peak virus titers of approximately 6.5 logs PFU/ml attained after 3 days of incubation. This suggests that full reversion of this property had taken place. Furthermore, a homogenous plaque size of the Val261Asn303Gly360 virus was maintained in all time points tested in this experiment, suggesting that additional mutations, which might increase growth efficiency, had not accumulated. However, this possibility was not formally investigated by nucleotide sequencing of viruses from the later time points. Although these data suggest that there is no difference between Val261Asn303Gly360 virus and YF5.2iv virus in growth properties, we also cannot exclude the possibility that host-restricted differences that might exist in other cell lines.

Cell penetration

The ability of the Val261Asn303Gly360 virus to penetrate cells was examined using a penetration assay, based on the acquisition of low pH-resistance by cell-associated virus as a function of time (Fig. 4). In this assay, which uses a very low multiplicity of infection, virus adsorption to the monolayer is essentially complete within the initial 10 min of infection (Vlaycheva and Chambers, 2002), thereby minimizing virus binding efficiency as a factor affecting penetration rate and efficiency. Using this assay, substantial impairment of penetration efficiency of YF virus for different cell lines has been demonstrated by substitution of glycine for aspartic acid at position 360 of the E protein and with various other amino acid substitutions at this site (Vlaycheva and Chambers, 2002, and unpublished data). In particular, both the NB15a and Gly360 viruses exhibit a

![Fig. 1. Plaque morphology of parental YF5.2iv virus, the G360 (NB15a) virus, and the engineered pseudo-revertant G360V261N303. Plaque assays were stained with crystal violet after incubation of the infected monolayers for 8 days. For the G360 virus, a plaque-purified stock was used, and for G360V261N303, a harvest from cells 5 days post-transfection was used.](image-url)
penetration efficiency of only 10–15% on Vero cells after 60 min, compared to 70–90% for the parental YF5.2iv virus (Vlaycheva and Chambers, 2002). In the current study, YF5.2iv virus exhibited approximately 30% penetration of Vero cells at 15 min, 55% at 30 min, and 90% at 60 min (Fig. 4). The Val261Asn303Gly360 virus exhibited slightly greater extents of penetration at the same time points; however, the differences were not significant. These data suggest that the introduction of the substitutions of valine at 261 and asparagine at 303 suppresses the defect in cell penetration associated with a glycine residue at position 360, as seen in the NB15a and G360 viruses.

Discussion

In this study, we characterized plaque-revertant viruses that emerged from cell culture of a small-plaque, neuroblas-

Fig. 2. Fluorescent focus assay. Vero cells were infected with parental or plaque-purified G360V261N360 virus at low M.O.I. Samples were processed for indirect immunofluorescence at day 3 after infection, as described in Materials and methods. Images were taken at 100 × and 40 × .

Fig. 3. Panel A Comparison of growth in cell culture of the G360V261N360 virus (VNG) with the parental YF5.2iv virus. Monolayers of Vero cells were infected at a multiplicity of 0.002 in triplicates. Virus was harvested at different time points after infection, and yields were determined by plaque assay. Values represent mean ± SD, as shown by error bars.

Fig. 4. Comparison of cell penetration of parental YF5.2iv and G360V261N360 (VNG) viruses. Penetration assay was performed using the plaque-purified engineered virus on Vero cells as described in Materials and methods. The percentage of penetrated virus was determined as the ratio of PFUs of acid-resistant virus to that of control virus (×100) for each virus as a function of time. Data represent combined results of two independent experiments where samples for each virus at each time point were done in duplicate. Error bars represent standard deviations of composite results from the two experiments.
toma cell-adapted YF 17D virus variant (NB15a). Their plaque sizes ranged from intermediate to parental size, suggesting that genetic heterogeneity in the E protein region and perhaps elsewhere in the viral genome was involved in the reversion process. This was confirmed by nucleotide sequencing, which revealed mutations at several positions in the prM-E region of viruses chosen to represent the variation in plaque size. All plaque revertants had the original substitution of glycine for aspartic acid at residue 360, which by itself confers the properties of the neuroblastoma cell-adapted NB15a virus. Thus, second-site mutations in the E protein conferred the revertant phenotype. Genetic engineering of the YF5.2iv molecular clone was used to identify the mutations responsible for this phenomenon. The majority of mutations tested yielded small-plaque viruses. However, one virus, Val261Asn303Gly360, had cell culture properties that resembled the parental YF5.2iv virus, indicating that its mutations compensated for the deleterious effect of glycine at position 360. Although substitutions at both positions 261 and 303 were required to enable the pseudo-reversion, position 261 is the critical determinant, since asparagine at position 303 with glycine at 360 yielded only a small-plaque virus. Rather, asparagine at 303 appears to counteract the otherwise lethal effects of the valine substitution.

Second-site substitutions involving either lysine or asparagine at residue 303 were frequent among plaque revertants, suggesting a selection for these substitutions in viruses harboring glycine at residue 360. Position 303 lies within a loop connecting the Ax and Aβ strands of the Ax-Cx-Dx β-sheet located on the upper lateral solvent-exposed surface of domain III of the E protein (Rey et al., 1995) (Fig. 5A).

**Fig. 5.** Representation of the YF17D E protein based on the TBE virus structure and localization of the mutations in the pseudo-revertant V261N303G360 virus. Panel A shows the YFV E protein domain III, with the region containing residue 303 depicted. Only side chains for residues 303 and 380 are shown for clarity. Potential hydrogen bond interactions between residues 303 and 380, through side-chain nitrogens and through side-chain nitrogen of 380 and backbone oxygen of 303, are shown by dotted lines. Panel B shows predicted effects of substitution with asparagine at position 303. The arginine at 380 maintains similar hydrogen bond interactions, and hydrogen bond between the carbonyl oxygen of asparagine and arginine at 380 replaces that contributed by lysine 303.
Lysine at position 303 is predicted to interact with arginine at position 380 to form a charge cluster that might be involved in cell-surface binding or conformational changes in domain III that occur during membrane fusion (Modis et al., 2003, 2004). This region is conserved in tick-borne and most of the mosquito-borne flaviviruses, and several studies have shown that substitutions at position 303 alter their phenotypic properties. For example, a lysine to asparagine substitution in the YF 17D E protein was associated with fatal vaccine-associated encephalitis (Jennings et al., 1994). Residue 303 is adjacent to an antibody epitope which distinguishes wild type from vaccine strains of YFV and is associated with neutralization-escape mutations (phenylalanine 305 to valine or serine) (Ryman et al., 1998) that modulate neurovirulence properties conferred by a determinant in a distal region of domain II (residue 240). In addition, mutagenesis studies of the corresponding region of the TBE virus E protein demonstrated that lysine 311 (equivalent to YFV lysine 303 in the E protein alignment) modulates the effects of adjacent substitutions on virus growth efficiency, plaque size, and mouse neuroinvasiveness (Mandl et al., 2000). This was proposed to result from disruption of a potential cellular receptor-binding determinant. However, the mechanisms have not been defined, and recent findings suggest that other functions of domain III beyond cell attachment may also be involved (see below).

Since the E protein of the neuroblastoma cell-adapted YF NB15a virus loses a negative charge through substitution of glycine for aspartic acid at residue 360, the additional substitution of glutamic acid or asparagine for lysine at position 303 might affect the properties of this virus in part by restoration of the net charge of domain III. For instance, loss of lysine at residue 303 might also contribute to a small-plaque phenotype by reducing interaction of the E protein with cell surface glycosaminoglycans (GAGs), as these substances affect the infectivity of YF and dengue viruses (Chen et al., 1997; Germi et al., 2002), and loss of GAG binding correlates directly with reduced plaque size (Lee and Lobigs, 2000; Mandl et al., 2001). Similar to glutamic acid at position 303, asparagine is predicted to interact with arginine at 380 through hydrogen bond formation, without any obvious change to the local structure surrounding this residue (Fig. 5B). Although both asparagine and glutamic acid were tolerated at position 303, neither substitution resulted in plaque reversion, indicating that charge restoration alone is insufficient to restore the properties of the parental virus.

Since substitutions at position 303 did not revert the plaque size of virus containing glycine at position 360, substitution of valine at residue 261 appears to be a primary factor in the pseudo-revertant phenomenon. Introduction of valine at 261 was either lethal or resulted in reversion to the parental alanine residue, suggesting that valine at this position causes a functional alteration in the E protein. Studies on the structure of dengue-2 virus have shown that the corresponding residue is in the hinge region of domain II, within a conserved hydrophobic pocket (residues 268–280), which includes the “k–l loop”. This loop is implicated in a conformational change of domain II that is required for the dimer-to-trimer conversion of the E protein (Modis et al., 2003), and studies have shown that mutations in the hinge alter the pH threshold for membrane fusion and affect flavivirus virulence in animal models (Beasley and Aaskov, 2001; Cecilia and Gould, 1991; Hasegawa et al., 1992; Hurrelbrink and McMinn, 2001; Lee et al., 1997; Monath et al., 2002). In particular, substitutions with hydrophobic amino acids having smaller side chains are proposed to lower the pH threshold by allowing tighter closure of the hinge (Modis et al., 2003). Residue 261 in the YFV E protein (corresponding to residue 267 of the dengue-2 E protein) lies at the edge of the k–l loop (Fig. 6). Modeling of valine at 261 suggests that it occupies a hydrophobic pocket associated with residues tryptophan at 203, leucine at 258, methionine at 262, and valine at 264, which form the base of the loop. It is conceivable that replacement of alanine with the larger valine residue destabilizes closure of the k–l loop, resulting in deleterious effects on the function of the E protein. Interestingly, a mutation at position 260 of the FNV E protein was observed to strongly attenuate mouse neurovirulence in association with reduced binding of virus to brain membrane receptor preparations (Ni et al., 2000). Regardless of the individual effects of substitutions at positions 261 and 303, it remains unclear how they compensate for the defect caused by a glycine at position 360, and why the substitution at 303 is required. A possible explanation is that the valine substitution at 261 promotes fusion by lowering the pH threshold and facilitating the hinge-like conformational change of domain II, but this process may have lethal effects in the absence of an additional mutation at position 303. In this regard, the recent report of the post-fusion structure of the dengue-2 virus E protein describes a major conformational change in domain III, wherein it folds back over and contacts the adjacent domain I, as the carboxy terminus moves towards the fusion loop (Bressanelli et al., 2004; Modis et al., 2004). Substitution with glutamic acid or asparagine at residue 303 might restrict this movement of domain III during the conformational change, and counteract a lethal effect of the valine residue at position 261. Additional studies which examine the effects of mutations at residues 261 and 303 on cell entry and membrane fusion are needed to determine the mechanism underlying the pseudo-reversion and provide insight into the molecular basis for the function of the E protein during virus entry.

Materials and methods

Cells and viruses

Vero cells, originally obtained from Jerry Jennings, USAMRIID, were grown at 37 °C in alpha minimal essential
medium supplemented with 10% fetal bovine serum (FBS) and antibiotics [penicillin/streptomycin (Gibco/BRL)]. NB41A3 cells were obtained from the American Type Culture Collection and grown as previously described (Vlaycheva and Chambers, 2002). YF5.2iv virus was derived from an infectious clone of the YF 17D virus (Rice et al., 1989). The NB15a virus was previously obtained by passaging YF5.2iv in mouse neuroblastoma cells (Vlaycheva and Chambers, 2002). The G360 virus, which contains a single amino acid substitution (glycine for aspartic acid at residue 360 of the E protein), was engineered using a full-length YF5.2iv infectious RNA template, as previously described (Vlaycheva and Chambers, 2002). Titers of all viruses were determined by plaque assays on Vero cells at 37°C.

Derivation of revertant viruses

Plaque revertants were isolated from Vero cell cultures transfected with infectious YFV RNA encoding the substitution of glycine at amino acid 360 of the E protein. Plaque assays of the transfection harvests revealed heterogeneous plaque sizes involving as much as 10–20% of the population of the progeny virus. Based on plaque assay of 8 days duration, sizes of plaques were assessed as follows: large: >5 mm diameter (parental YF5.2iv virus), medium: 2–3 mm, and small: <1 mm. Two of both the medium-size and large-size plaque revertants were plaque-purified on Vero cells and amplified on NB41A3 cells before further characterization.

Nucleotide sequencing

Total RNA was extracted from infected cells using TRIzol Reagent (Life Technologies) according to the manufacturer’s protocol. RNA was resuspended in 10 mM Tris–chloride, pH 7.5, containing 0.1 mM EDTA, aliquoted, and stored at −70°C. Nucleotide primers were designed to amplify the region of the YF 17D virus from nucleotides 424 to 2500 by reverse transcription and PCR (RT-PCR). First-strand cDNA was synthesized using Superscript II reverse transcriptase (Gibco/BRL) and approximately 1.5 µg of total RNA at 46°C for 1 h, followed by incubation at 70°C for 10 min. cDNA products were then subjected to PCR amplification using Deep Vent DNA polymerase (New England Biolabs) using the same 3’ primer and a 5’ primer to produce a PCR product of approximately 2.0 kb long. PCR was run at 95°C for 2 min, followed by 36 cycles of 1 min at 95°C, 2 min at 50°C, and 2 min at 72°C, and then 7 min at 72°C. PCR products were isolated from low-melting-temperature agarose gels (BioWhittaker) and purified using Wizard PCR Preps kits (Promega). Purified PCR products were cloned into pCR-Blunt II-TOPO using the Zero Blunt-TOPO PCR cloning kit (Invitrogen), and the ligation products were transformed into TOP10 E. coli cells. Selected colonies were grown in small-scale cultures in LB broth plus kanamycin. DNA was purified from bacterial cells using Wizard PCR Prep DNA Purification System (Promega). Plasmids were analyzed by restriction enzyme digestion for presence of YF 17D sequences, and subjected to nucleotide sequencing using the Beckman cycle sequencing protocol.
Construction of viruses

A two-plasmid system for generation of infectious YF 17D virus (Rice et al., 1989) was used to reconstruct YF virus harboring second-site mutations found in the revertants. RT/PCR-derived clones harboring the mutations from the plaque-revertant viruses in pZeroBlunt-TOPO were used for these constructions. To construct pYFM5.2 derivatives containing the second-site mutations by themselves, NsiI/Sse8387I restriction fragments from the zero-blunt plasmids were exchanged for the corresponding fragments from YFM5.2. To construct pYFM5.2 derivatives containing the second-site mutations together with the G360 mutation, NsiI/Sse8387I restriction fragments from the zero-blunt plasmids were exchanged for the corresponding fragments from YFM5.2 containing this mutation. The transcription templates for the YF genomes were generated by in vitro ligation using T4 DNA ligase (NEB), of AatII and NsiI restriction fragments from two plasmids, pYFS' 3' IV and pYFM5.2. Transcription with SP6 RNA polymerase in the presence of 5' cap analog was done to produce full-length viral RNA for transfection into cells, essentially as previously described (Rice et al., 1989). Approximately 350 ng of RNAs were transfected into Vero cells in the presence of 20 μg of Lipofectin (Gibco/BRL), followed by incubation at 37 °C in alpha MEM plus 5% FBS. Viruses were harvested from the cell culture at time of onset of cytopathic effects (typically 5–8 days post-transfection) and the yields were determined by plaque assay on Vero cells.

Fluorescent focus assay

Infectious-center assay was performed using fluorescence as previously described (Vlaycheva and Chambers, 2002). Confluent monolayers of Vero cells were infected for 1 h at 37 °C with approximately 20 PFU of virus per well. Virus was removed, and monolayers were overlaid with 1% agarose (BMA) in alpha-MEM plus 5% FBS, and incubated at 37 °C. At 3 days postinfection, cells were processed for immunofluorescence by fixation in 4% paraformaldehyde and removal of agarose. Cells were permeabilized with 100% cold methanol, incubated with primary antiserum (anti-YF hyperimmune ascitic fluid (ATCC) diluted 1:500 in PBS plus 1% FBS), washed with PBS, and then incubated with secondary antibody [affinity-purified fluorescein-conjugated goat anti-rabbit IgG antibody (ICN), 1:50 in PBS plus 1% FBS]. Cells were examined using a Nikon TE-300 fluorescent microscope equipped with a standard FITC fluorescence cube and a SPOT camera (Diagnostic Instruments).

Cell penetration assay

Penetration rate was determined by a previously described method (Vlaycheva and Chambers, 2002). Mono-layers of Vero cells in 60-mm culture plates in quadruplicates were adsorbed at 37 °C with approximately 50 PFU of virus per plate. At various intervals up to 60 min, unbound virus was removed, and replicate samples were treated with acid glycerine buffer (pH 3.0) (0.1 M glycine and 0.1 M sodium chloride) for 3 min to inactivate uninternalized virus, whereas a third sample (nonacid-treated control) was washed with PBS. In previous studies, this treatment was found to inactivate the majority (>90%) of input viruses (Vlaycheva and Chambers, 2002). All samples were then washed once with PBS, overlaid with agarose, and incubated at 37 °C to allow plaque formation. Percentage of penetrated virus for each time point was calculated as the ratio of the number of plaques on the acid-treated cells to the number of plaques on the nonacid-treated cells for each time point × 100.

Molecular modeling

A homology-based model for the YF17D E protein was derived using the comparative protein modeling server (Swiss-Model) and the structure of the soluble ectodomain of the tick-borne encephalitis virus E protein (pdb code 1svb), as described previously (Nickells and Chambers, 2003). The model was used for analysis of the effects of substitutions at amino acid position 303 using various rotamer configurations to determine energy parameters and predicted hydrogen bond formations for the most favorable conformations of these residues. The region surrounding residue 261 was modeled using the “open” form of the dengue-2 virus E protein crystallized in the presence of n-octyl-β-D-glucoside (pdb code 1oke) as the template. Figures were drawn using the Swiss-Modeller graphics platform.

Acknowledgments

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References


Crill, W.D., Roehrig, J.T., 2001. Monoclonal antibodies that bind to domain III of dengue virus E glycoprotein are the most efficient blockers of virus adsorption to Vero cells. J. Virol. 75, 7769–7773.


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