Mutagenesis of the RGD Motif in the Yellow Fever Virus 17D Envelope Protein

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The envelope protein of yellow fever virus 17D (YFV-17D) contains a solvent-exposed RGD motif, which has led to the suggestion that integrins may function as cellular receptors for YFV-17D. We found that mutating the RGD motif to RGE had no effect on viral titers, whereas changing RGD to TGD, TGE, TAD, TAE, or RGS led to reduced titers. Substitution of RGD by RAD or RAE yielded RNA genomes that replicated in mammalian cells but could not spread to neighboring cells at 37°C. These mutants did spread through the cell monolayer at 30°C (both in mosquito cells and in SW13 cells) and viruses grown at this temperature were capable of infecting mammalian cells at 37°C. These results strongly suggest that RGD-mediated integrin binding does not play a major role in YFV-17D entry, since the RGD to RAD mutation, as well as many or all of the other mutations studied, should disrupt all RGD-dependent integrin binding. However, the RGD to RAD or RAE mutations (as well as TAD and TAE) severely destabilized the envelope protein at 37°C, providing an explanation for the observed phenotype. Implications of these findings are discussed in light of the fact that mutations that alter tropism or virulence in different flaviviruses are often found within the loop containing the RGD motif.   © 1999 Academic Press

INTRODUCTION

Flaviviruses are a group of arthropod-borne, enveloped RNA viruses that enter their host cells by receptor-mediated endocytosis (for a review see Rice, 1996). Flavivirus particles contain three proteins, the nucleocapsid protein, the membrane protein (M), and the envelope protein (E). The M protein is synthesized as a precursor protein, prM, which is cleaved during or shortly before virus assembly to produce M and an N-terminal pr fragment, which is secreted from infected cells. The E protein is the viral moiety most likely to interact with a cellular receptor and to induce membrane fusion. The structure of the E protein of one flavivirus, tick-borne encephalitis virus (TBE), has been determined by X-ray crystallography (Rey *et al.,* 1995) and possesses a three-domain structure. Because mutations that affect virulence often map in the C-terminal domain III (which has an immunoglobulin-like fold), it was suggested that this domain is involved in cell attachment.

The mosquito-borne flaviviruses contain a 4-amino-acid, solvent-exposed loop in domain III (the FG loop) that consists of only a single glycine residue in TBE and other tick-borne flaviviruses (Rey *et al.,* 1995). In most mosquito-borne flaviviruses, the FG loop contains an Arg-Gly-Asp (RGD) motif or a very similar sequence. RGD motifs play critical roles in integrin–ligand interactions
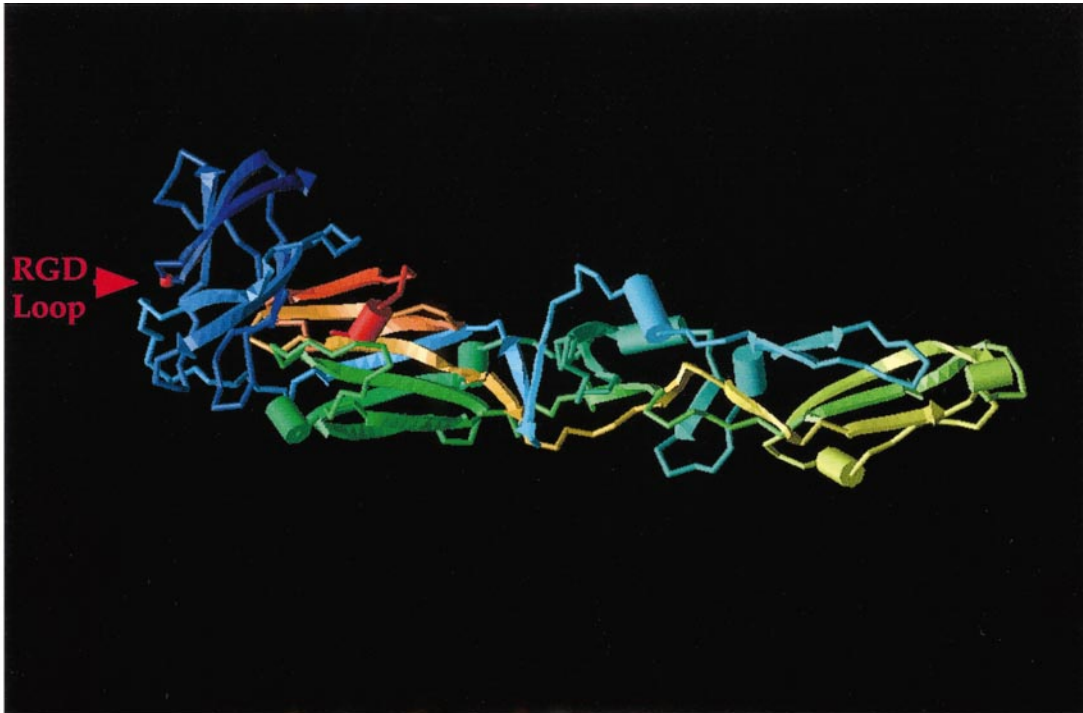
(Ruoslahti, 1997), and several viruses, e.g., foot-and-mouth disease virus (FMDV) (Mason *et al.,* 1994), cox-sackievirus (Roivanen *et al.,* 1991), and adenovirus (Bai *et al.,* 1993; Belin and Boulanger, 1993), enter cells by binding to integrins in an RGD-dependent fashion. In addition, the Tat protein of human immunodeficiency virus contains an RGD motif that facilitates integrin binding (Brake *et al.,* 1990) and this interaction of Tat with integrins appears to play a role in the generation of Kaposi sarcoma (Barillari *et al.,* 1993; Brake *et al.,* 1990). The presence of RGD motifs in the E proteins of Japanese encephalitis virus, Murray valley encephalitis virus (MVE), and yellow fever virus 17D (YFV-17D) has therefore fueled speculation that cell attachment of these viruses might also involve integrins (Kuhn and Rossmann, 1995; Lobigs *et al.,* 1990; Post *et al.,* 1992; Putnak *et al.,* 1997). Consistent with this, in both MVE and YFV-17D, several mutations that affect tropism map to the RGD motif. In the case of YFV, passaging of the pathogenic Asibi strain in chicken embryo fibroblasts (which yielded the attenuated 17D strain) led to 32 amino acid substitutions, including one that changed TGD in the Asibi strain to RGD in 17D (Hahn *et al.,* 1987). Passaging of MVE in human SW13 cells selected for mutations that changed the RGD sequence to RGG, RGH, or RGA (Lobigs *et al.,* 1990). These viruses were attenuated in mice.

With the exception of dengue virus, no cellular receptor for a flavivirus has been identified. For dengue virus, it has been shown that heparan sulfate plays a major role in cell attachment (Chen *et al.,* 1997). It is unknown whether dengue virus entry also requires additional, high-affinity receptors. The complexity of dengue virus

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FIG. 1. (a) Structure of the monomeric TBE E protein (Rey *et al.,* 1995). The color scheme goes from blue at the N-terminus through the colors of the rainbow to red at the C-terminus. The location of the FG-loop and the site of the RGD sequence in the YFV-17D E protein are indicated by the red ball. (b) The FG loop and its flanking sequences for YFV and several other flaviviruses. The RGD motif or the corresponding sequence in other flaviviruses is indicated in boldface type. Note that the FG loop in TBE lacks three amino acids. WNV, West Nile virus; KUN, Kunjin virus; SLE, St. Louis encephalitis virus; DEN, Dengue virus.

binding to cells is illustrated by binding assays in mosquito cells: dengue-2 virus was found to bind to 80- and 67-kDa proteins (Munoz *et al.,* 1998) and dengue-4 virus bound to 40- and 45-kDa proteins (Salas-Benito and Del Angel, 1997). Using a similar assay, Japanese encepha-

litis virus was shown to bind to a 74-kDa protein in Vero cells (Kimura *et al.,* 1994).

As a first step toward the identification of a receptor for YFV-17D, we have studied the role of the RGD motif in viral entry. To test this, we have mutated the RGD se-
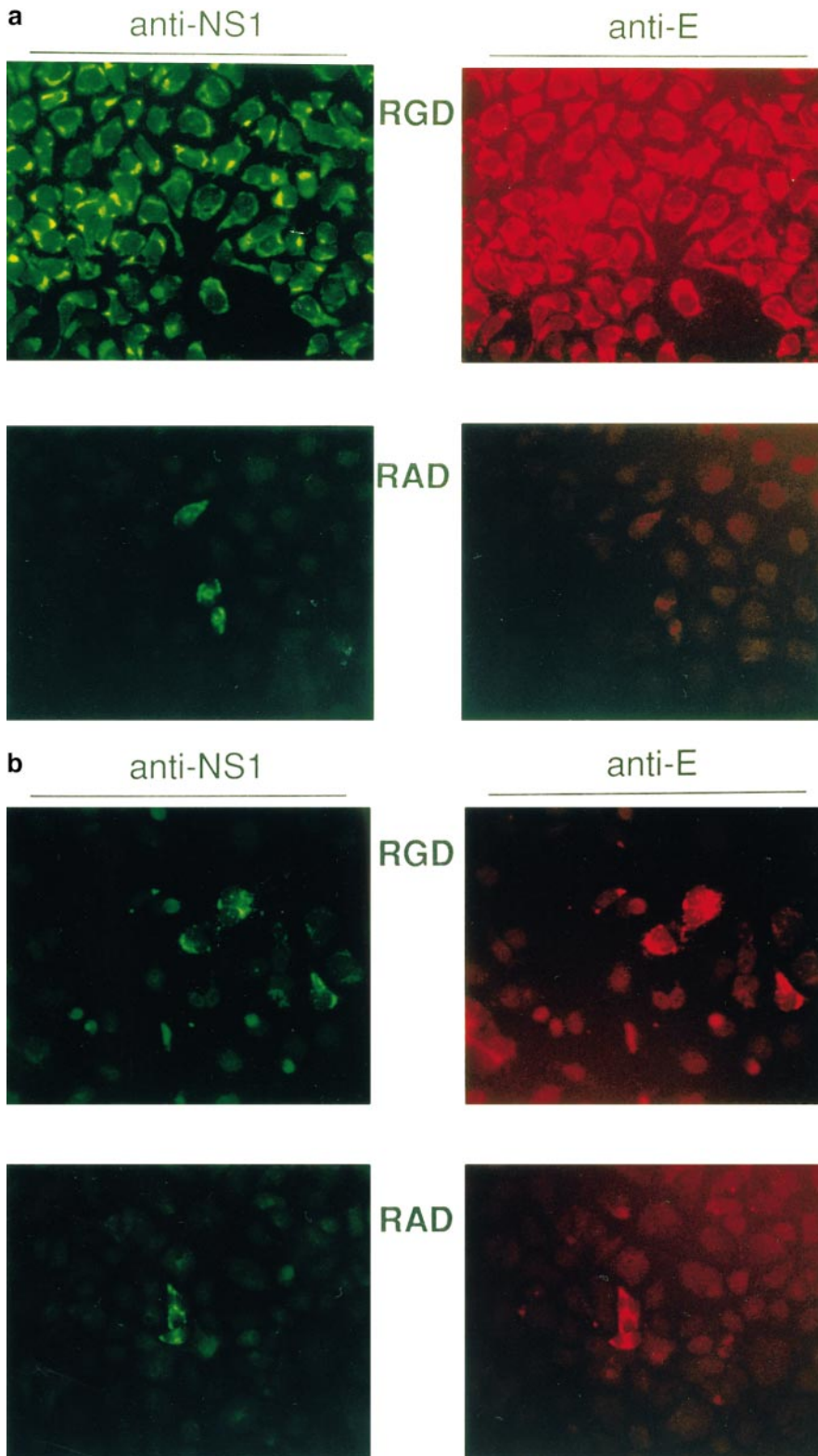
FIG. 5. SW13 cells were transfected with wild-type YFV-17D RNA (RGD) or with full-length RNA carrying the RAD mutation. Transfected cells were stained with mouse anti-E serum and with rabbit anti-NS1 serum at 48 h p.i. Transfected cells were incubated at 37°C (a) or at 30°C (b). Magnification, ×200.

quence in YFV-17D and analyzed the phenotypes of the resulting mutant viruses. We found that changing the RGD motif to RAD or RAE (which should disrupt integrin binding) yielded viruses that were no longer capable of spreading through a monolayer. However, these RAD- and RAE-containing viruses could infect cells. This ob-

Mutant	PFU/μg RNA[a]	Titer at 37°C (72 h)[b]	Titer at 30°C (120 h)[b]
RGD	1.0×10^4	1.1×10^8	6.7×10^6
RGE	1.2×10^4	8.3×10^7	2.0×10^6
TGD	1.2×10^4	7.4×10^6	8.8×10^5
TGE	1.1×10^4	4.8×10^6	8.6×10^5
RGS	1.7×10^4	1.9×10^5	1.0×10^5
TAE	1.3×10^4	1.2×10^5	3.0×10^4
TAD	1.8×10^4	1.2×10^4	1.0×10^4
RAE	0	ND	ND
RAD	0	13	<4

[a] Specific infectivities were determined by incubating SW13 cells transfected with 1 ng RNA for 96 h at 37°C under agarose and counting the plaques.

[b] Titers of virus in the culture fluid after incubation in liquid medium for the indicated time at the indicated temperature. Plaque assays were performed at 37°C. ND, not determined.

servation excludes binding to integrins in an RGD-dependent fashion as a major mechanism for YFV-17D entry. Instead, we found that the E proteins carrying the RAD and RAE mutations were far less stable than the wild-type E protein. The instability of the mutant E proteins most likely explains the dramatic phenotype of the RAD and RAE mutations and suggests a possible mechanism by which changes in the RGD sequences of mosquito-borne flaviviruses affect virulence.

RESULTS

Mutagenesis of the yellow fever virus RGD motif

Mosquito-borne flaviviruses possess a four-amino-acid, solvent-exposed loop (the FG loop; Rey *et al.,* 1995) in the C-terminal domain of the E protein (Fig. 1). The four residue FG loop is found only in mosquito-borne flaviviruses. In tick-borne flaviviruses, such as TBE, the FG loop contains only a single Gly residue (Fig. 1). The presence of RGD or RGD-like motifs in the solvent-exposed FG loops of mosquito-borne flaviviruses has led to the suggestion that these viruses enter their host cells by binding to integrins in an RGD-dependent fashion (Kuhn and Rossmann, 1995; Lobigs *et al.,* 1990; Post *et al.,* 1992; Putnak *et al.,* 1997). To test the idea that integrins function as receptors for YFV-17D, we have mutated the RGD motif in the YFV E protein and analyzed the phenotypes of the resulting mutant viruses (Table 1). The mutations were in part patterned on the RGD-like sequences found in other flaviviruses (Fig. 1). In addition, the Gly to Ala mutations were made because changing RGD into RAD has been shown to disrupt integrin–ligand interactions (Plow *et al.,* 1985). The mutations were introduced into cDNA clones of YFV-17D from which infectious RNA can be produced (Rice *et al.,* 1989).

Mutagenesis of RGD to RAD or RAE prevents viral spread at 37°C

Full-length YFV-17D RNAs transcribed from the plasmids carrying the RGD mutations were first transfected into SW13 cells. Specific infectivities of these RNAs (defined as the number of plaques per microgram of RNA) were determined by incubating transfected SW13 cells under agarose overlays and counting the number of plaques. As shown in Table 1, all RNAs had similar specific infectivities, with the exception of viruses carrying the RAD and RAE motifs.

To study the phenotypes of the YFV-17D mutants, transfected cells were incubated under liquid medium for 3 days at 37°C. The culture fluids were then harvested for titration and the transfected cells were examined by immunofluorescence microscopy. Analysis of the viral titers revealed that the phenotypic effects of the mutations roughly fell into four categories (Table 1). Changing the Asp residue to Glu (RGD to RGE) had little effect on the virus titers obtained. Mutating the RGD sequence to TGD or TGE led to an approximately 20-fold reduction in virus titers, whereas the TAD, TAE, and RGS mutations resulted in 600- to 10,000-fold lower virus titers and a small plaque phenotype. Finally, substitution of the Gly residue by Ala (in RAD and RAE) reduced the viral titers $>10^6$-fold. Immunofluorescence analysis of transfected SW13 cells revealed that wild-type (RGD) and RGE-containing viruses spread throughout the entire monolayer (Fig. 2). In contrast, YF viruses carrying the RAD and RAE mutations replicated in transfected cells but failed to infect neighboring cells (Fig. 2). The other mutants were capable of limited spreading, consistent with the reduced titers and small plaque phenotype (data not shown). Similar results were obtained after transfection of BHK-21 cells or primary chicken embryo fibroblasts (data not shown). Thus, these data clearly show that the integrity of the RGD motif is important for production of infectious virus and that the Gly residue in particular is of crucial importance. Interestingly, the impact of the Gly to Ala substitution is much greater in the RGD/RGE context ($>10^6$-fold) than in the TGD/TGE context (50- to 500-fold).

To evaluate the potential temperature sensitivity of the mutant viruses, SW13 cells transfected with the various YFV RNA transcripts were incubated for 5 days at 30°C. Transfected cells were analyzed by immunofluorescence microscopy and progeny virus was titrated. The immunofluorescence experiment revealed that all mutant viruses, including RAD- and RAE-containing viruses, could infect neighboring cells at 30°C (Fig. 3; and data not shown). This result is in clear contrast to the situation at 37°C and indicates that the RAD- and RAE-containing viruses are temperature sensitive, with 30°C being a (semi)permissive temperature. As shown in Table 1, released virus was detected for the viruses carrying RGD, RGE, TGD, TGE, TAD, TAE, and RGS sequences, but not for RAD. Note that all
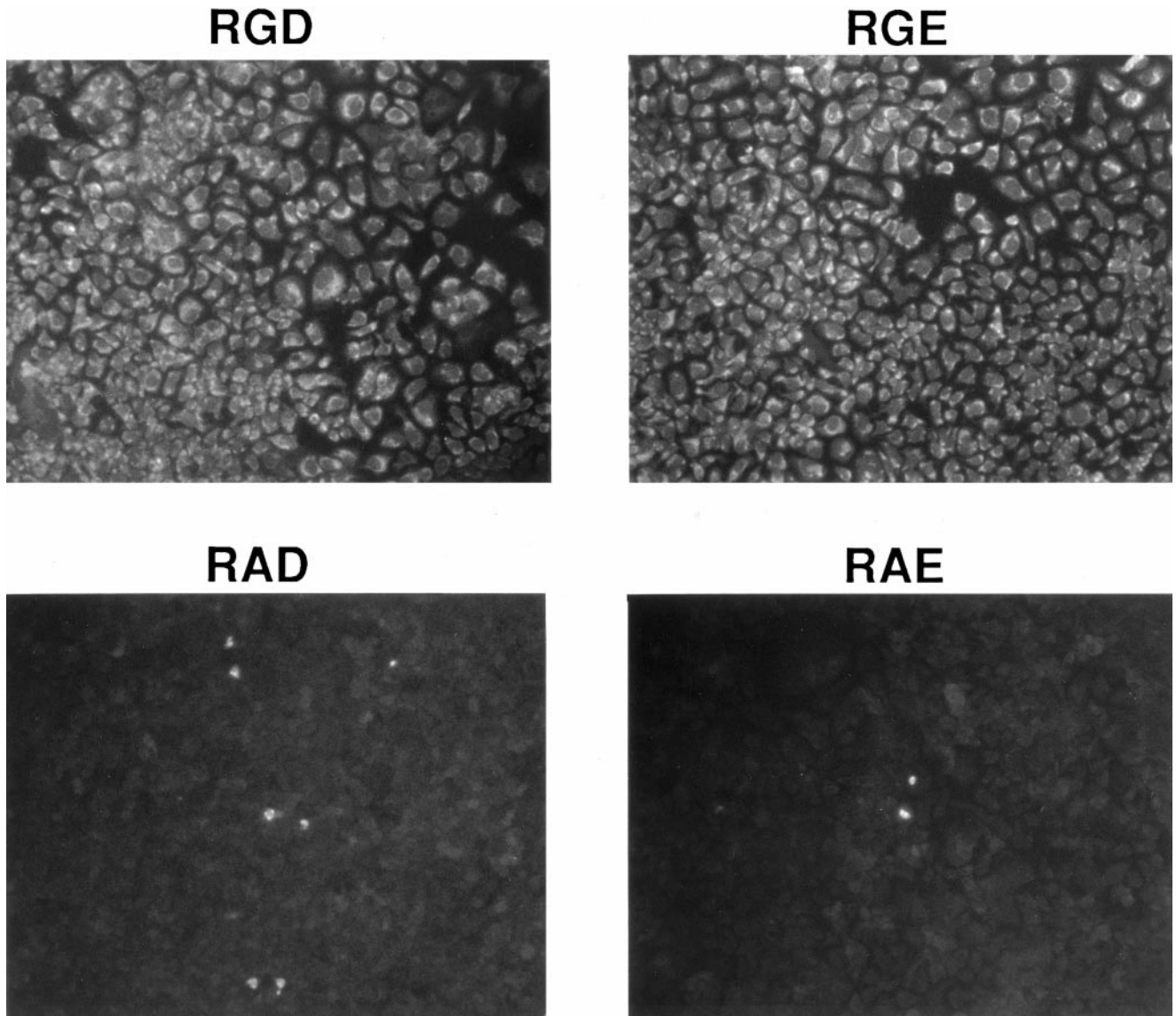
RGD

RGE

RAD

RAE

FIG. 2. SW13 cells were transfected with infectious YFV-17D RNA carrying the RGE, RAD, and RAE mutations and with wild-type RNA and incubated at 37°C. Cells were stained with anti-YFV serum at 72 h p.i. Wild-type (RGD) and mutant (RGE, RAD, and RAE) viruses are indicated by the sequence in their FG loop. Magnification, ×100.

plaque assays were done at 37°C and that RAD-containing virus does not grow at this temperature. Attempts to determine the titer of RAD virus by plaque assay at 30°C were unsuccessful since the virus did not grow well enough to produce visible plaques. Thus the spread of this virus at 30°C can be readily detected in immunofluorescence assays but not by plaque assay.

RGD mutagenesis does not prevent spreading in mosquito cells

We next analyzed the phenotypes of the various mutants in C6/36 mosquito cells that were maintained at 30°C. Titration of supernatants from transfected C6/36 cells at 3 days after transfection revealed three phenotypic groups (Table 2). RGD and RGE viruses grew to similar levels, titers

(determined at 37°C) from RAD and RAE viruses were extremely low or below the detection limit, and the other mutants had titers that were approximately 100-fold lower than the wild-type virus. Immunofluorescence assays of transfected mosquito cells revealed that the RAD and RAE viruses did replicate and spread in mosquito cells, as the infection clearly spread to neighboring cells (Fig. 4). The fact that the RAD- and RAE-containing viruses grow in mosquito cells at 30°C is consistent with the temperature-sensitive phenotype of these viruses.

RAD- and RAE-containing viruses can infect cells at 37°C

The observation that RAD- and RAE-containing viruses replicated in transfected cells but did not spread at 37°C
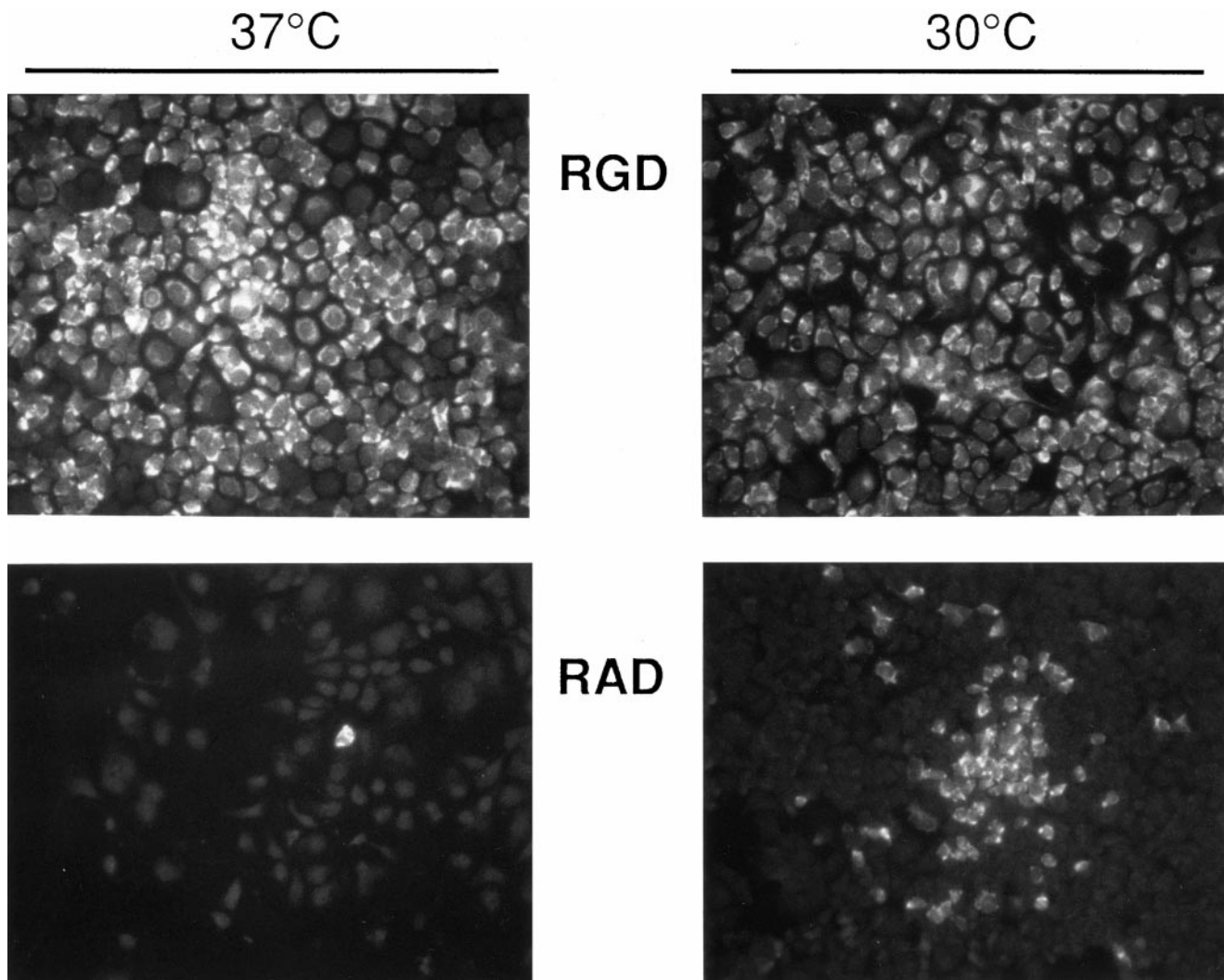
FIG. 3. SW13 cells were transfected with wild-type YFV-17D RNA or with RNA from the RAD mutant and incubated at 37 or 30°C. Cells were stained with anti-YFV serum at 72 h p.i. Incubation temperatures are indicated. Wild-type (RGD) and mutant (RAD) viruses are indicated. Magnification, ×100.

and that most of the other mutants grew to much lower titers could be consistent with a role of the RGD motif in virus–receptor interaction. However, there are other possible explanations for these results. The mutations could destabilize the E protein, leading to reduced quantities or complete absence of intracellular E, or the substitutions could interfere with virus particle formation. To address this issue, we first asked whether RAD/RAE viruses are capable of infecting cells at the nonpermissive temperature (37°C). Virus grown in C6/36 cells or in SW13 cells at 30°C was tested for the ability to infect mammalian cells at 37°C. We reasoned that if the Gly to Ala substitution interferes with virus entry, the virus produced at 30°C would not be able to infect cells at 37°C. If, however, the mutation affects other steps in the viral life cycle, we expected that RAD/RAE-containing viruses would infect mammalian cells at 37°C but would not be able to spread to neighboring cells.

We performed two experiments to test this. First, SW13

TABLE 2

RGD Mutants and Their Phenotypes in C6/36 Cells

Mutant	PFU/μg RNA[a]	Titer (72 h)[b]
RGD	70	8.0×10^3
RGE	80	1.0×10^4
TGD	60	2.0×10^2
TGE	80	1.2×10^2
RGS	140	4.0×10^2
TAE	30	1.2×10^2
TAD	60	8.0×10^2
RAD	50	<5
RAE	50	<5

[a] Specific infectivities were determined by incubating C6/36 cells transfected with 100 ng RNA for 72 h at 30°C under agarose, staining the cells with anti-YFV serum, and counting the fluorescent centers.

[b] Titers of virus in the culture fluid after a 72-h incubation in liquid medium. The plaque assays were performed in BHK cells at 37°C.
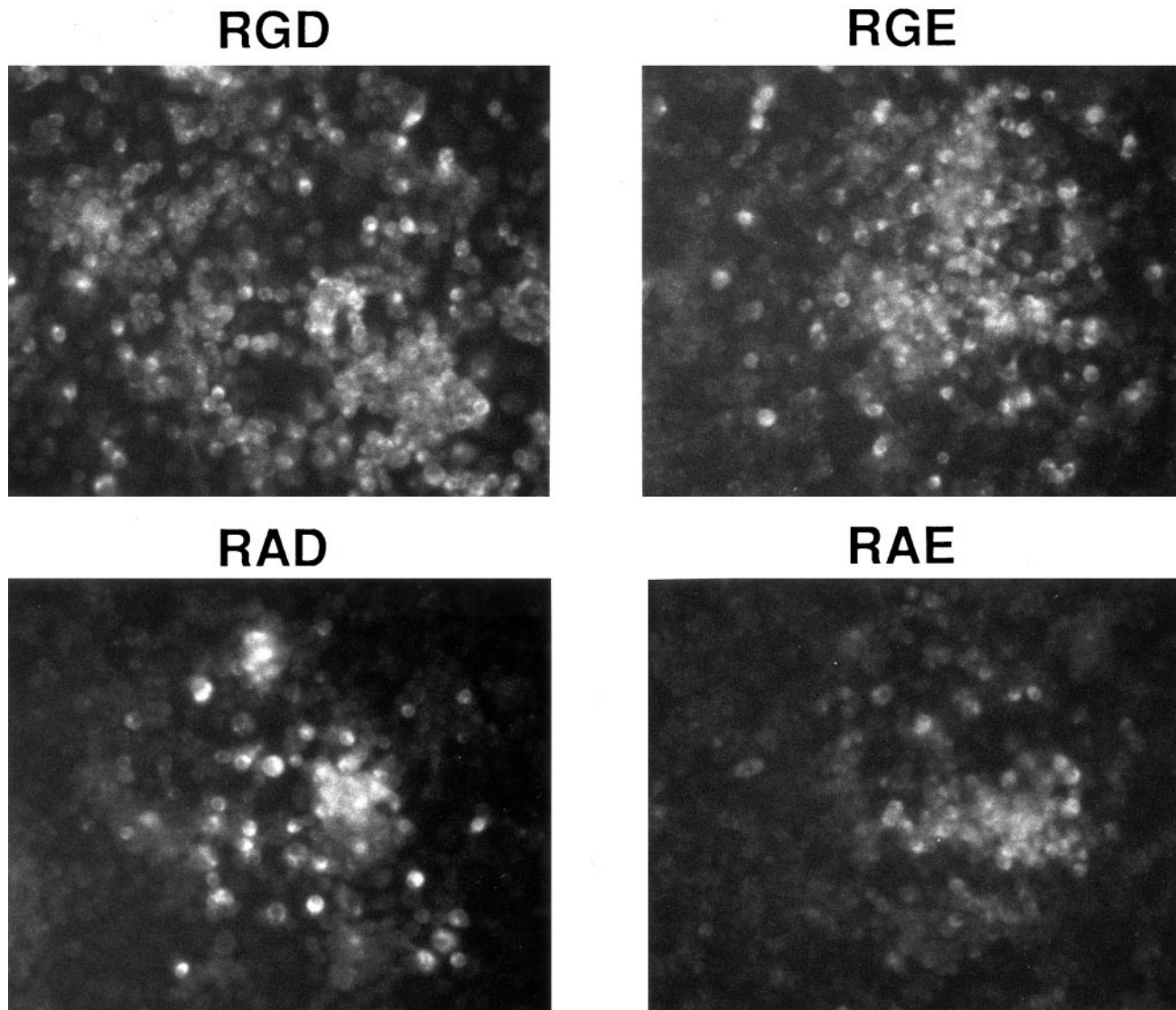
FIG. 4. C6/36 cells were transfected with infectious YFV-17D RNA carrying the RGE, RAD, and RAE mutations and with wild-type RNA. Cells were stained with anti-YFV serum at 72 h p.i. Wild-type (RGD) and mutant (RGE, RAD, and RAE) viruses are indicated. Magnification, ×100.

cells were transfected with wild-type RNA or with RNA from the RAD mutant and grown at 30°C. Supernatants were harvested at 72 h after transfection and immediately used to infect fresh SW13 cells at 30 or at 37°C. As shown by immunofluorescence microscopy, infection of SW13 cells was evident for wild-type YFV-17D and for the RAD mutant under both inoculation conditions (data not shown). As observed in previous experiments, RAD-containing viruses did not, after the initial infection, spread to any neighboring cells at 37°C.

In a second experiment, we transfected C6/36 cells at 30°C with wild-type RNA or with RNAs from the RGE, RAE, and RAD mutants and used the supernatants to infect BHK-21 cells. The BHK-21 cells were either infected at room temperature or at 37°C and were then incubated under agarose for 48 h at 37°C. To quantify infectivities, we counted the number of fluorescent centers under each condition (which equals the number of transfected cells for the RAE and RAD mutants). Confirming the results from the experiment with the SW13 cells, we found that RAD and RAE viruses grown at 30°C in C6/36 cells could infect BHK-21 cells under both conditions with about the same efficiency (Table 3), but no spread to neighboring cells occurred during incubation at 37°C (data not shown). As shown in Table 3, the virus titers determined by counting fluorescent centers were very similar for room temperature or 37°C inoculations. Thus, mutating the RGD motif to RAD or RAE does not prevent the resulting mutant viruses (grown at 30°C) from entering mammalian cells at either room temperature or 37°C. This indicates that the phenotype of the RAD mutant cannot be explained by a defect in binding to a putative integrin receptor.

Mutant	RT inoculation	37°C inoculation
RAD	12	8
RAE	49	53

[a] BHK-21 cells were inoculated with RAD or RAE virus stocks at the indicated temperature and incubated for 48 h at 37°C under agarose. Fluorescent cells were counted after the monolayers were stained with anti-YFV serum.

RAD- or RAE-containing E proteins are unstable at 37°C

Since RAD/RAE-containing viruses can infect mammalian cells at 37°C, it appears that the Gly to Ala substitution affects the biosynthesis or stability of E or the formation of virus particles. To assess whether E is present in cells infected or transfected with RAD or RAE viruses at 37°C, we visualized E directly by immunofluorescence microscopy. Fixed cells were stained with an E-specific antiserum and with an antiserum directed against NS1. The double staining served two purposes. First, it allowed us to identify productively transfected cells in the monolayer (and, since the NS1 ORF is downstream of the E ORF in the YFV polyprotein sequence, it also proves that E was translated). Second, it provides us with an internal standard on intracellular protein levels because E and NS1 are synthesized in equimolar amounts.

SW13 cells were transfected with transcripts from RGD, RGE, RAD, and RAE cDNAs and subjected to E and NS1 staining at 48 h after transfection. At 37°C, both proteins were readily visualized for wild-type YFV-17D and the RGE virus (Fig. 5a; and data not shown). For the RAD and RAE mutants, E-specific staining was extremely weak at this temperature, whereas a fluorescent signal for NS1 was readily detected (Fig. 5a; and data not shown). At 30°C, E could clearly be detected in cells transfected with RAD- and RAE-containing viruses (Fig. 5b; and data not shown), confirming the temperature-sensitive phenotype of these mutants. These data indicate that the intracellular levels of the RAD and RAE E proteins are very low in cells incubated at 37°C, which may reflect a stability problem of the mutant proteins.

To extend these results, we compared the stabilities of wild-type E and the E protein carrying the RAD mutation in a pulse–chase experiment. For this experiment, we used a full-length, infectious YFV-17D cDNA clone, in which the entire YFV-17D cDNA was cloned into a single-copy-number plasmid vector. Full-length YFV-17D RNAs transcribed from these plasmids were transfected into BHK-21 cells and were metabolically labeled at 16 h posttransfection for 30 min, followed by a 5-h chase period. As shown in Fig. 6, the E-RAD protein was de-

tected during the pulse labeling, but it was far less abundant than its wild-type counterpart. After a 5-h chase, the wild-type E protein was still abundant, whereas the mutant E protein was virtually undetectable (Fig. 6). These data clearly confirm the immunofluorescence data.

Expression of prM and E using Sindbis virus expression vectors

To study the intracellular levels of RAD-E in the absence of the other YFV proteins, the prM and E genes from YFV-RGD and YFV-RAD were expressed using a Sindbis virus expression vector (Bredenbeek et al., 1993; Frolov et al., 1997). Because it has previously been shown that Sindbis virus interferes with the processing and secretion of flavivirus proteins (Pugachev et al., 1995; and our unpublished results), we used a Sindbis virus expression vector that contains a single amino acid substitution in the nsP2 protein (Pro to Ser at position 726). Mutant Sindbis viruses carrying this substitution do not inhibit host cell protein synthesis as much as wild-type Sindbis virus does and are not cytopathic (Dryga et al., 1997). Importantly, noncytopathic Sindbis viruses that carry a different mutation at the same position (Pro to Leu at position 726) do not interfere with YFV-17D replication and polyprotein processing (Lindenbach and Rice, 1997).

Wild-type and mutant prME cassettes were placed downstream of the Sindbis virus capsid ORF in the ex-
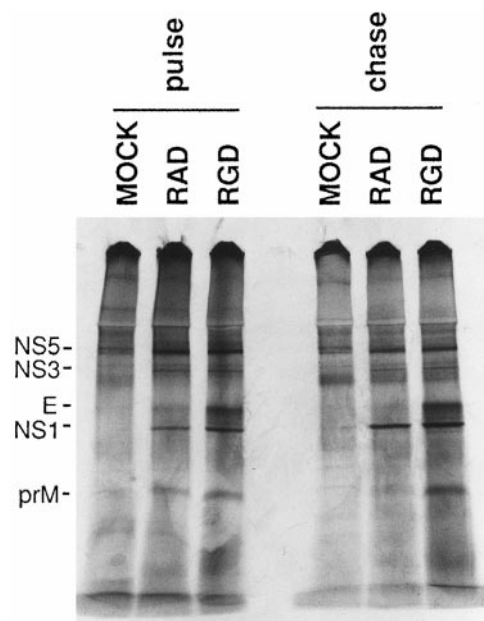

FIG. 6. BHK-21 cells were transfected with wild-type YFV-17D RNA (RGD) or with RNA from the RAD mutant (RAD). Cells were metabolically labeled at 16 h p.i. for 30 min and chased for 5 h. Cell lysates were subjected to radioimmunoprecipitation and analyzed on a 12% polyacrylamide gel.
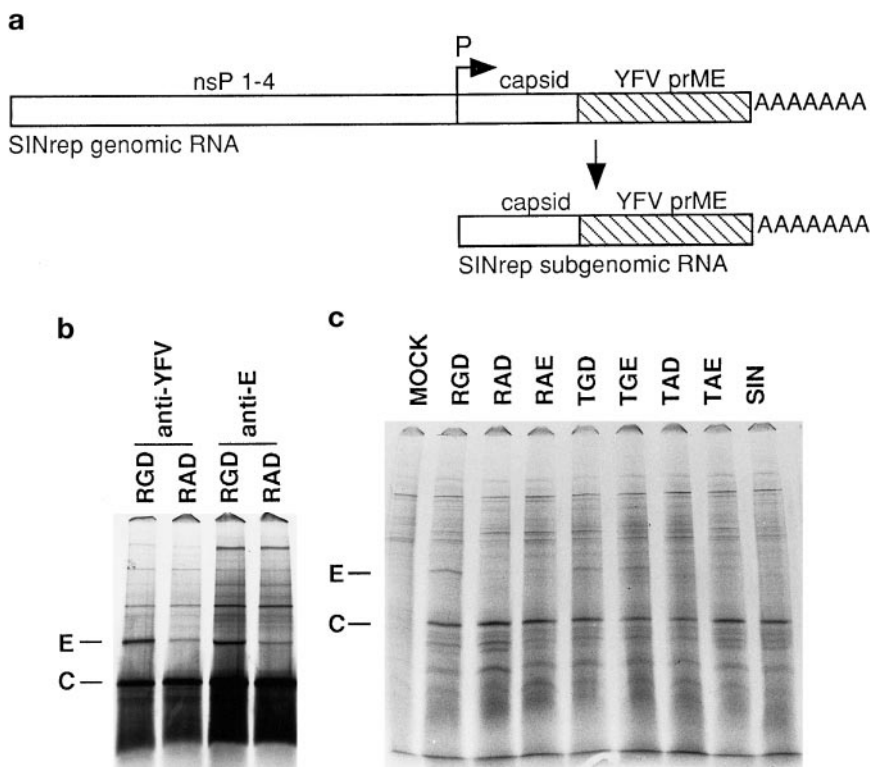
FIG. 7. Expression of prM and E. (a) Recombinant SINrep construct. YFV-17D prM and E sequences (prME) are indicated by shaded boxes. The Sindbis virus capsid protein (C), the subgenomic promoter (P) and the nonstructural proteins (nsP1–4) are indicated. (b) BHK-21 cells infected with recombinant Sindbis viruses were pulse-labeled and chased for 5 h. Cells lysates were immunoprecipitated with mouse anti-E serum or with anti-YFV serum as indicated. Protein samples were analyzed on a 12% polyacrylamide gel. (c) BHK-21 cells were infected and labeled as described above. Lysates were immunoprecipitated with anti-YFV serum and analyzed on a 12% polyacrylamide gel. The wild-type and mutant viruses are indicated by the RGD or mutant RGD sequences.

pression vector SINrep/capsid (Frolov et al., 1997) in which the Pro to Ser mutation had been introduced (Fig. 7a). The prM and E proteins are cleaved from the Sindbis virus capsid protein by an autoproteolytic activity in the capsid protein (Strauss and Strauss, 1990). The capsid protein, prM, and E are synthesized in equimolar amounts. To obtain SINrep/prME virus stocks, BHK-21 cells were transfected with transcripts from the recombinant SINrep/prME replicon plasmids and with a helper RNA providing the Sindbis virus structural proteins (tRNA-BB-CΔ3) (Frolov et al., 1997). Supernatants were harvested at 48 h after transfection and used to infect fresh monolayers of BHK-21 cells, which were metabolically labeled for 30 min at 16 h after infection and then chased for 5 h. Radioimmunoprecitation with either anti-E serum or anti-YFV serum revealed that the E-RAD protein was much less abundant than the wild-type E protein after the 5-h chase (Fig. 7b). Analysis of the supernatants yielded similar results, demonstrating that the difference was not the result of accelerated secretion (data not shown). Although the Sindbis virus capsid protein precipitates nonspecifically (Rice and Strauss, 1982) and is therefore not an ideal internal standard, the similar levels of Sindbis virus capsid protein for each anti-

serum in Fig. 7b suggest that similar amounts of RGD E and RAD E were synthesized.

In a second experiment, most of the RGD mutations were introduced into the SINrep/prME expression vector, and the encoded proteins were analyzed in a pulse–chase experiment similar to that described above. As shown in Fig. 7c, the levels of the E proteins containing the different mutations correlated with the titers of the corresponding YFV variants. Wild-type E was easily detected, the levels of the E proteins containing TGE and TGD were somewhat lower, and RAD, RAE, TAD, and TAE E proteins were undetectable. Thus, these expression data are consistent with the immunofluorescence results and the YFV-17 expression studies described above and indicate that the Gly to Ala substitution strongly reduces the quantity of intracellular E. The most likely explanation is that the mutation destabilizes the E protein.

DISCUSSION

Integrins do not function as major YFV-17D receptors

In the present study we have investigated the role of the RGD motif in the C-terminal domain of the YFV-17D envelope protein. The RGD motif is located in a loop that

is exposed to the solvent (Fig. 1), and it has been suggested that this motif might be involved in YFV-17D receptor binding (Kuhn and Rossmann, 1995; Lobigs *et al.,* 1990; Post *et al.,* 1992; Putnak *et al.,* 1997). We have tested this hypothesis by mutating the RGD motif and studying the phenotypes of the resulting mutant YF viruses. Results obtained with FMDV serve to illustrate this approach: for FMDV, mutagenesis of the RGD motif to KGD, RGE, and KGE yielded viruses that were unable to bind to or enter cells (Mason *et al.,* 1994).

The experiments described here strongly suggest that integrins do not function as major YFV-17D receptors in an RGD-dependent fashion. Initially, the observation that viruses carrying a mutant RAD motif (which should disrupt integrin binding) replicate in transfected cells but fail to spread to neighboring cells suggested that this mutation might block viral entry. However, further experiments revealed that these mutant viruses are capable of infecting mammalian cells. The phenotype of the RAD-containing viruses can be explained by the low levels of intracellular E protein in infected cells at 37°C. Furthermore, viruses carrying the RGE sequence grew to the same levels as wild-type YFV-17D, although integrin binding should be severely affected by this mutation (Pytela *et al.,* 1985). In fact, RGE-containing peptides are often used as negative controls in integrin-binding experiments involving RGD peptides (Pytela *et al.,* 1985). Thus, if integrins were major YFV-17D receptors, any RGD-mediated integrin binding should be blocked by the RGD to RGE mutation and lead to reduced titers, which is clearly not the case. In addition, the observation that all the other mutants (including one in which all three amino acids are mutated, i.e., TAE) can still spread through the monolayer appears to be inconsistent with a role of the RGD motif in integrin binding. Our conclusion is further supported by experiments using synthetic RGD-containing peptides. It was shown that even at maximum concentrations these peptides did not inhibit infection of primary chicken embryo fibroblasts by YFV-17D (Preugschat and Strauss, unpublished results). These results are even more striking considering that the change from TGD (YFV Asibi) to RGD (17D) was selected for by passaging YFV on primary chicken cells. The TGD to RGD mutation was apparently not selected to allow the virus to use integrins as major receptors. It is of interest, however, that the TGD-containing YFV-17D produced less virus than the RGD-containing YFV-17D. Although other mutations also occurred during the selection of YFV-17D during passaging in chicken cells, the higher yield of 17D containing RGD under our conditions could explain in part the selection of the RGD mutation.

Since our data seem to exclude a role for RGD-mediated integrin binding in YFV-17D entry, the nature of the receptor remains unclear. For dengue virus, it has been found that heparan sulfate functions as a receptor (Chen *et al.,* 1997). The authors of that study predicted that these structures might also function as receptors for other flaviviruses (Chen *et al.,* 1997). However, we recently found that heparin did not affect the infectivity of YFV-17D, although binding of soluble E protein or virus particles to cells could be inhibited by heparin or treatment of the cells with heparinase (unpublished results). Thus, although YFV-17D appears to bind to sulfated glycoaminoglycans, these molecules do not function as receptors. We are currently trying to screen cDNA expression libraries with soluble E protein in order to identify a cellular receptor.

YFV E proteins carrying RAD or RAE sequences are unstable at 37°C

The more puzzling result of our study is the observation that the RGD to RAD or RAE substitutions have very dramatic effects on the stability of the E protein. These Gly to Ala substitutions clearly had the strongest impact on viral titers, but most of the other mutations in the RGD motif also reduced virus titers, indicating that the sequence or conformation of the FG loop is of critical importance. The immunofluorescence data and the YFV and Sindbis virus expression studies indicate that the intracellular levels of RAD/RAE-containing E proteins are considerably lower than their RGD/RGE counterparts at 37°C. Some of the other RGD mutations also appear to affect stability: for the TAD and TAE mutants in particular, intracellular E proteins levels were much lower than wild-type E at 37°C. The very low levels of intracellular E-RAD and E-RAE most likely account for the failure of the mutant viruses to spread. A stability problem of E-RAD and E-RAE would be the most obvious explanation, and this would also be consistent with the temperature-sensitive phenotype of the mutant viruses. An additional indication of the instability of E-RAD comes from our attempts to produce soluble E protein for direct cell-binding studies. Whereas the wild-type E protein could be obtained at levels of approximately 1 μg/ml culture medium using a baculovirus expression system, it proved to be very difficult to produce and purify the E-RAD protein.

It is difficult to envisage why a relatively minor substitution like Gly to Ala is capable of affecting protein levels so dramatically, given that this change appears to occur in a short loop of four amino acids. Possible explanations include a folding problem, failure of the mutant protein to dimerize or to interact with the prM protein, leading to instability, or failure of the mutant E to properly interact with putative chaperones. One possibility is that the more hydrophobic nature of the Ala residue (compared to the wild-type Gly) destabilizes the FG loop. A more thorough understanding of this question will require the elucidation of the YFV E structure by X-ray crystallography. Another way of obtaining a closer insight in the role of the FG loop in the E protein structure would be to

perform passage experiments of the RAD and RAE viruses at 30°C and analyze second-site revertants.

Our data make it clear, however, that the sequence of the RGD loop is critical for the conformation of the E protein. Minor changes in this area can have drastic effects on the stability of the protein. It is feasible that such changes in structure or stability can affect the spectrum of cells infected. For instance, the relatively small changes that are seen in the RGD motif after passaging MVE in SW13 cells (which yields viruses that are attenuated in mice) could induce significant changes in the structure of the protein, which in turn could affect the viral tropism.

Our finding that the conformation and stability of the E protein are very sensitive to the sequence of the FG loop could facilitate the design of attenuated flaviviruses for vaccine purposes. This loop is present in all mosquito-borne flaviviruses, and most of them carry a sequence that is very similar to the YFV-17D RGD motif. It is conceivable that mutations in this region in other flaviviruses will affect viral fitness as well.

MATERIALS AND METHODS

Cells and virus

BHK-21, SW13, and C6/36 cells were propagated in MEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, antibiotics, and nonessential amino acids (Gibco BRL). Virus titers were determined by plaque assay on BHK-21 cells at 37°C.

Antisera

Anti-YFV ascites fluid was obtained from the ATCC (Rockville, MD) and polyclonal rabbit anti-NS1 serum was provided by Dr. Charles Rice (Washington University, St. Louis, MO). A polyclonal mouse anti-E serum was generated at the California Institute of Technology Monoclonal Antibody Facility by injecting two Swiss Webster mice with purified, recombinant E protein obtained from the supernatants of Hi-5 cells (Invitrogen) infected with a recombinant baculovirus. Mice were injected two times with 5 μg protein in RIBI adjuvant, followed by three booster immunizations with 10 μg protein in the absence of adjuvant (Ou *et al.,* 1993). Ascites fluid was induced after the immunizations, as described (Ou *et al.,* 1993).

Construction of plasmids

Site-directed mutagenesis was done by fusion PCR using the proofreading enzymes Vent polymerase (New England Biolabs) or *Pfu* polymerase (Stratagene), following a protocol previously described by Yao *et al.* (1996). The nucleotide sequences of PCR products were verified by automated sequencing. The sequences of the oligonucleotide primers that were used are shown in Table 4.

TABLE 4

Oligonucleotides Used in PCR Mutagenesis

Oligo-nucleotide	Sequence
RV7	TCGTTGGGA(C/G)AG(G/C)AGA(G/T)TCACGTCTCACTT
RV8	AGACGTGA(A/C)TCT(C/G)CT(G/C)TCCCAACGATAATG
RV9	ATCGTTGGGAGAGGATCGTCACGTCTCACTTAC
RV10	GTAAGTGAGACGTGACTCTCCTCTCCCAACGAT
RV26	GAATTGCCTTAACTCAGGAGGCACCC
RV27	GGGTGCCTCCTGAGTTAAGGCAATTC
RV28	AAGTCTGCCACTGCATGGTAG
RV29	CAGCTATTGTATCGACACTGC
GB19	GTGGAAGTGGCGGGGTGTG
GB38	CTACGCGTACGTTACGCCCCAACTCCTAGA
JC01	GGTGGTAGAAAGACGGGG
JC02	CCGGCTCGAGCGTACGAGTGGTTTTGTGTT
1	CTACGCGTTACCCCTGAAAGGCAGA
3	CTTCATGAGGTCCCATGATGTTCTGA
20	CGGGATCCCTCAAGGGGACATCC

Mutagenesis of the RGD motif. To mutate the RGD sequence of YFV-17D, we used two sets of degenerate overlapping fusion primers: RV7/RV8 and RV9/RV10. As templates we used the plasmids pYF5′3′IV and pYFM5.2 (Rice *et al.,* 1989), which together contain the entire YFV-17D cDNA sequence. Using pYF5′3′IV as a template, two 0.3-kb fragments were amplified with either primers 20 and RV8 or primers 20 and RV10 (fragments A and C, respectively). Two 0.2-kb fragments were amplified from pYFM5.2, with primers RV7 and 1 and primers RV9 and 1 (fragments B and D). The fusion DNAs were obtained by mixing either fragments A and B or fragments C and D, followed by amplification with primers 1 and 20. The 0.5-kb AB and CD fusion PCR products were digested with *Pst*I and *Eco*RI and inserted into pYF5′3′IV. Inserts from the resulting plasmids were sequenced. *Sse*8387I-*Bsm*BI fragments (163 nt) from these plasmids were inserted into pYFM5.2, yielding a set of pYFM5.2 derivatives carrying the different mutations.

Construction of pBAC/YFV. A plasmid containing the entire YFV-17D cDNA sequence was constructed by first ligating a blunt-ended *Not*I–*Ssp*I fragment from pYF5′3′IV into the *Not*I-digested and blunt-ended bacterial artificial chromosome (BAC) vector pBeloBAC11 (Shizuya *et al.,* 1992; Wang *et al.,* 1997). The resulting plasmid was cut with *Nsi*I and *Aat*II and an *Nsi*I–*Aat*II fragment from pYFM5.2 was inserted, yielding pBAC/YFV. The linearization site was changed from *Xho*I to *Bsi*WI by PCR mutagenesis using primers JC01 and JC02. In order to introduce the RGD to RAD mutation into this plasmid, a 1.0-kb *Sse*8387I-*Mlu*I fragment was excised from the pYFM5.2 plasmid carrying the RAD mutation and inserted into *Sse*8387I–*Mlu*I cut pBAC/YFV. All transformations involving BAC plasmids were done by electroporation using ultracompetent *Escherichia coli* cells (Research Genetics, Huntsville, AL).

Mutagenesis of Sindbis virus nsP2. To change the Pro residue at position 726 of the Sindbis virus nsP2 protein to Ser (Dryga *et al.,* 1997), we carried out two PCRs using the replicon plasmid SINrep/capsid (Frolov *et al.,* 1997) as a template. A 0.5-kb fragment amplified with primers RV26 and RV28 was mixed with a 1.2-kb fragment amplified with primers RV27 and RV29. A 1.7-kb fusion product was amplified with RV28 and RV29, cut with *Cla*I and *Avr*II, and inserted into *Cla*I–*Avr*II digested SINrep/capsid. The replicon plasmid carrying the Pro to Ser substitution was referred to as SINrep/capsid*.

Insertion of prME sequences into SINrep/capsid.* A 1.9-kb fragment encompassing the YFV-17D prM and E sequences was amplified from pYF5′3′IV (primers 1 and 3), digested with *Bsp*HI and *Mlu*I, and ligated into *Bsp*HI–*Mlu*I cut pSINrep/capsid*, yielding pSINrep/prME. The full-length 3′ termini containing the wild-type sequence or the different mutations were amplified from the appropriate pYFM5.2 derivatives using primers GB19 and GB38 and ligated into *Sse*8387I–*Mlu*I-digested SINrep/prME.

Transcription and transfection

In vitro ligation of the pYF5′3′IV and pYFM5.2 plasmids was done as described using *Nsi*I–*Aat*II (Roche Molecular Biochemicals) digested DNA fragments (Rice *et al.,* 1989). *In vitro* ligated YFV cDNAs, SINrep/capsid plasmids, and the Sindbis virus helper plasmid, tRNA-BB-CΔ3 (Frolov *et al.,* 1997), were linearized with *Xho*I and transcribed using SP6 RNA polymerase. pBAC/YFV and pBAC/YFV(RAD) were linearized with *Bsi*WI. RNA transcripts (approximately 100 ng) were transfected into SW13 or C6/36 cells using lipofectin (Gibco BRL), as described (Rice *et al.,* 1989). For transfection of BHK-21 cells lipofectamine was used (Gibco BRL).

Metabolic labeling and radioimmunoprecipitation

Cells were metabolically labeled using Express-^{35}S Protein Labeling Mix (NEN). Cell lysis and immunoprecipitations were done according to standard protocols. Protein samples were analyzed on 12% polyacrylamide gels (Bio-Rad).

Immunofluorescence

Cells were fixed in either 100% methanol (15 min, −20°C) or 2% paraformaldehyde/0.1% Triton X-100 (30 min at 4°C). Methanol fixation was used for the anti-YFV ascites fluid (ATCC), and paraformaldehyde/Triton fixation was used for the anti-E and anti-NS1 sera. Fixed cells were incubated for 40 min with primary antiserum in PBS (Gibco BRL) at room temperature (anti-YFV and anti-NS1 were diluted 1:100, and anti-E was diluted 1:50), washed three times with PBS, and incubated for 40 min at room temperature with the secondary antiserum. Goat anti-mouse R-phycoerythrin, sheep anti-mouse FITC, and goat anti-rabbit-FITC were purchased from Sigma (St. Louis, MO) and were used in a 1:100 dilution in PBS. Cells were examined using a Nikon fluorescence microscope.

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