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Carboxy-Terminally Truncated Dengue Virus Envelope Glycoproteins Expressed on the Cell Surface and Secreted Extracellularly Exhibit Increased Immunogenicity in Mice

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Recombinant vaccinia viruses expressing C-terminally truncated E's that ranged in length from 9 to 99% of the N-terminal sequence were constructed. The overall antigenicity of the E products was analyzed by radioimmunoprecipitation, using dengue virus hyperimmune mouse ascitic fluid (HMAF) or an anti-E peptide serum. Truncated E that was 79% or less in length did not bind HMAF efficiently, whereas E constructs greater than 79% were able to bind HMAF with high efficiency. The first 392 amino acids of the dengue type 4 virus E sequence, including the Arg-392 following the 79%E C terminus, appeared to be critical for proper antigenic structure required for efficient binding by HMAF. Truncated E's ranging from 59 to 81% in length were secreted extracellularly, whereas smaller or larger E's were retained intracellularly. Secreted E's contained carbohydrate side chains that were resistant to endoglycosidase H digestion, suggesting that the transport of E occurs via a pathway from the rough endoplasmic reticulum through the Golgi complex. 79%E-RKG (which possessed the three additional amino acids immediately downstream of 79%E) was expressed at a high concentration on the surface of recombinant virus-infected cells presumably being inserted into the plasma membrane by a hydrophobic C-terminal membrane anchor. Evaluation in mice of the protective efficacy of the various vaccinia virus E recombinants indicated that only truncated E's that were recognized efficiently by HMAF induced a high level of resistance to dengue virus encephalitis. 79%E-RKG which is expressed at a high concentration on the surface of infected cells was highly immunogenic when tested for induction of an E antibody response. This suggests that cell surface expression of 79%E-RKG was responsible for its enhanced immunogenicity. Finally, passive immunization studies indicated that serum antibodies to E played a major role in the complete or nearly complete resistance to dengue virus challenge induced by certain vaccinia virus-truncated E recombinants.

Disease caused by one or more of the four types of dengue virus is a major public health problem in many tropical or subtropical areas. Overall, the dengue viruses are responsible for a higher morbidity than any other members of the flavivirus family. Similar to other flaviviruses, dengue virus contains only three structural proteins, i.e., capsid protein (C; molecular mass, 12 to 14 kDa) which binds to the positive-strand genomic RNA forming the nucleocapsid and two lipid-associated membrane proteins termed the small membrane protein (M; molecular mass, 7 to 8 kDa) and the large membrane protein which is also called envelope glycoprotein (E; molecular mass, 55 to 60 kDa) (27). The envelope glycoprotein is the major virion antigen responsible for virus neutralization by specific antibodies and for several important antigenic properties such as binding to flavivirus-, dengue virus complex-, and type-specific antibodies (5, 29). Dengue virus and other flavivirus E's also exhibit a hemagglutinating activity that is presumably associated with virus attachment to the cell surface and subsequent virus uncoating (28). Results of epitope mapping with a library of monoclonal antibodies indicate that the antigenic structure of dengue virus E is similar to that of other flavivirus E's that contain several distinct antigenic sites as defined by serological specificity, functional activity, and competitive binding assay (12–14). More recently, complete or nearly complete sequences of the genomes of several dengue viruses and other major flaviviruses have been determined and their polyprotein sequences have been deduced (3, 6, 7, 10, 11, 15, 19, 21, 23–26, 30, 31). Comparison of amino acid sequences showed that there is significant sequence homology among the E glycoproteins of different flaviviruses.

Despite four decades of research effort, a safe and effective vaccine against dengue virus is still not available. However, several new strategies for vaccine development based upon the use of cloned dengue virus cDNA for synthesis of dengue virus protective antigens have yielded encouraging results. Infection of mice with a vaccinia virus recombinant expressing full-length E and NS1 glycoproteins [v(C-M-E-NS1-NS2A)], 93% of the N-terminal E sequence [v(93%E)], or only NS1 [v(NS1-NS2A)] induced complete or almost complete resistance to fatal encephalitis resulting from intracerebral inoculation of dengue type 4 virus (8, 32). Immunization of mice with baculovirus recombinant-expressed E and NS1 also induced a similar level of resistance (33). However, v(C-M-E-NS1-NS2A), v(93%E), and the baculovirus recombinant-infected cell lysate containing expressed E consistently failed to induce detectable antibodies to E or induced only a very low level of such antibodies (2, 33). A more recent study showed that monkeys immunized with baculovirus-expressed E also failed to develop antibodies to E as detected by radioimmunoprecipitation, and presumably only partial resistance to intravenous dengue virus challenge was induced for this reason (17). Thus, the low immunogenicity of E constitutes a major obstacle to the development of an effective dengue virus vaccine produced

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by recombinant DNA technology. For this reason, the sequence of E was analyzed by systematic C-terminal deletion and expression of the resulting truncated E product in a vaccinia virus recombinant in an attempt to delineate E sequences responsible for inducing resistance in mice and to improve the immunogenicity of recombinant DNA-expressed E. Unlike full-length E, several of the C-terminally truncated E products were secreted extracellularly or accumulated on the cell surface and most importantly, the latter property was associated with increased immunogenicity.

MATERIALS AND METHODS

Construction of dengue virus DNAs coding for full-length and C-terminally truncated E's. The dengue virus cDNA fragment that codes for the putative 15 amino acid N-terminal signal and the entire E sequence except the last 39 amino acids at the C terminus was obtained earlier (2). An extended DNA fragment coding for the N-terminal signal, the entire E plus the first 30 amino acids of the downstream NS1 nonstructural protein, was constructed from the previously derived DNA fragment terminating at the *Sst*I site of the dengue type 4 virus sequence and the *Sst*I-*Sau*3A DNA fragment (nucleotides 1931 to 2592), using the shared *Sst*I site for joining. This extended E DNA construct was inserted into the *Bgl*II site of intermediate vaccinia virus vector plasmid pSC11 (4, 9). At the downstream *Bgl*II site regenerated in the recombinant DNA construct, a linker sequence was inserted consisting of oligonucleotide (oligo) 2196 (GATCCTAGCTAGCTAGGTACC) and oligo 2197 (GATCGGTACCTAGCTAGCTAG) that contained stop codons in all three reading frames followed by a *Kpn*I cleavage site. The insertion of this linker sequence destroyed the joining *Bgl*II site, leaving the unique upstream *Bgl*II site in the recombinant plasmid pSC11.

In order to obtain a library of DNA fragments specifying full-length E and a series of C-terminally truncated E's, the extended DNA sequences cleaved from the plasmid by *Bgl*II and *Kpn*I were digested with BAL 31 and the progressively shortened DNA fragments were joined to a second *Kpn*I linker sequence consisting of oligo 2246 (TGAATGAAT GAGATCTGGTAC) and oligo 2247 (CAGATCTCATTCAT TCA) that contained stop codons in all three reading frames. After digestion with *Bst*EII at nucleotide 1438 of the dengue virus sequence, DNA fragments of various lengths were separated on an agarose gel and isolated so that the extended DNA region between the *Bst*EII and *Kpn*I sites in pSC11 E DNA could be replaced. This procedure allowed construction of DNA inserts coding for 40 to 100% of the E sequence. Recombinant 100*%E contained full-length E plus 4 N-terminal amino acids of the downstream NS1 nonstructural protein, while recombinant 100**% E contained full-length E plus the N-terminal 22 amino acids of the NS1 sequence. A similar scheme was employed to construct other shorter E DNAs. In these instances, *Bst*EII-linearized DNA was the starting material for digestion by BAL 31. The precise deletion junctions of each of these DNA constructs were determined by using oligo 1852 (CGTTTGCCATACGCT CACAG) located downstream of the DNA insert and within the pSC11 sequence as a negative-strand primer in a dideoxynucleotide sequencing reaction (Sequencing system purchased from Promega Corporation).

DNA coding for a truncated E terminating at a specific amino acid residue such as 79%E plus -R, -RK, -RKG, -RKGS, or -RKGSS, was constructed, using the polymerase chain reaction (PCR). The sequences used for the negative-strand primers in the PCR were as follows:

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79%E-RKGSS, oligo 2552 (AGATCTGGTACCTAGGAACTCCCTTTCCTGAA);
79%E-RKGS, oligo 2553  (AGATCTGGTACCTAACTCCCTTTCCTGAACCA);
79%E-RKG, oligo 2554   (AGATCTGGTACCTACCCTTTCCTGAACCAATG);
79%E-RK, oligo 2647    (AGATCTGGTACCTATTTCCTGAACCAATGGAG);
79%E-R, oligo 2648     (AGATCTGGTACCTACCTGAACCAATGGAGTGT).
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Oligo 1426 (GATCTATGACTGTCTTCTTTGTCCTAA) was used as the positive-strand primer for all of these PCR products. The PCR products were cleaved with *Bst*EII and *Kpn*I prior to their insertion into pSC11.

Construction of recombinant vaccinia viruses. CV-1 and human TK$^-$143 cells were grown and propagated in minimum essential medium plus 10% fetal calf serum (MEM10). Wild-type vaccinia virus strain WR was used, and recombinant vaccinia viruses were constructed by the procedure described earlier (4, 31). Recombinant vaccinia virus vSC8 that contained a *lacZ* gene insert was used as a control.

Labeling infected cells and protein analysis. Infection of CV-1 cells with recombinant vaccinia viruses was essentially the same as detailed earlier (2, 31). At 16 to 20 h after infection, the medium in a 6-well plate was replaced with methionine-free MEM2, and after 1 h of incubation, this medium was replaced with 0.75 ml of methionine-free MEM2 containing [^{35}S]methionine (100 μCi; specific activity, >800 Ci/mmol; Amersham Corp.). After a 2-h labeling period, cells were lysed in RIPA buffer and the lysate was centrifuged in a microfuge to remove particulate cell debris (9). The supernatant of the labeled lysate was used for analysis of viral proteins by immunoprecipitation. Intracellular and secreted forms of the various E products were also analyzed by using recombinant virus-infected cells that were labeled for 6 h. Fluid medium of infected-cell cultures was collected after the labeling period and analyzed directly by immunoprecipitation. Infected cells were disrupted in a volume of RIPA buffer equivalent to the volume of the fluid medium. Dengue virus hyperimmune mouse ascitic fluid (HMAF) prepared against dengue type 4 virus strain 814669 was used at a dilution of 1:5 or 1:20 for immunoprecipitation of the labeled lysates. A rabbit antiserum raised against a dengue type 4 virus E peptide (peptide 73, amino acids 259 to 272 of the E sequence) was also used at a dilution of 1:5 or 1:20 for immunoprecipitation. The immunoprecipitates collected on Pansorbin (Calbiochem-Boehringer) were subjected to further digestion with endoglycosidase F (endo F) or endoglycosidase H (endo H) (Boehringer-Mannheim Biochemicals) or analyzed directly by electrophoretic separation on a sodium dodecyl sulfate (SDS)–12% polyacrylamide gel (acrylamide/bis ratio = 60:1.6). Labeled protein bands were visualized by fluorography.

Immunization of mice. Induction of resistance to experimental dengue virus encephalitis was evaluated by infecting mice with a recombinant vaccinia virus and subsequently challenging these animals intracerebrally with 100 50% lethal doses of dengue type 4 virus strain H241 as described earlier (2). Passive immunization was also evaluated, using pooled sera from groups of mice immunized with recombinant vaccinia virus v(59%E), v(79%E-RKG), v(81%E), or v(100*%E). Female BALB/c mice (5 to 6 weeks old) were each inoculated with 0.6 ml of pooled sera by the intraperitoneal route. The next day the inoculated mice were challenged intracerebrally with 100 50% lethal doses of dengue type 4 virus strain H241 and observed for symptoms of encephalitis and mortality as described previously (8).

Seroanalysis by radioimmunoprecipitation. E-specific antibodies in individual or pooled sera of immunized mice were

TABLE 1. Predicted amino acids of C-terminally truncated dengue virus envelope glycoproteins expressed by recombinant vaccinia viruses[a]

Recombinant vaccinia virus	Predicted amino acids
v(9%E)	M_1–D_{42}
v(19%E)	M_1–C_{92}LNE
v(27%E)	M_1–L_{135}DE
v(37%E)	M_1–T_{182}LNE
v(50%E)	M_1–A_{245}
v(59%E)	M_1–E_{290}NE
v(66%E)	M_1–E_{327}GE
v(70%E)	M_1–K_{344}DE
v(79%E)	M_1–F_{391}E
v(81%E)	M_1–K_{399}MNE
v(88%E)	M_1–H_{436}E
v(94%E)	M_1–W_{464}MNE
v(99%E)	M_1–G_{489}
v(100*%E)	M_1–A_{494}DMGC
v(100**%E)	M_1–$A_{494}D_1$---V_{22}

[a] Dengue type 4 virus envelope glycoprotein contains 494 amino acids. The first and the last amino acids in the truncated E's are indicated by amino acid numbers in the dengue type 4 virus E sequence. Additional amino acids translated from the vector sequence at the C terminus are underlined. The percent E expressed is shown in parentheses in vaccinia virus recombinants. v(100*%E) contained the entire E sequence plus the first 4 amino acids of NS1, and v(100**%E) contained the full-length E plus 22 amino acids of NS1.

analyzed by immunoprecipitation of a ^{35}S-labeled lysate of dengue virus-infected $LLCMK_2$ cells as described previously (33).

Indirect immunofluorescence assay. Confluent CV-1 cells in a chamber slide were infected with recombinant virus at a low multiplicity (less than 10 PFU per well). At 16 to 24 h after infection, cells were rinsed with MEM and fixed with cold acetone (fixed cells). Alternatively, cells were rinsed with MEM but not treated with acetone (live cells) and were examined for cell surface expression of E. HMAF was used at 1:100 dilution as the first antibody and fluorescein-conjugated rabbit anti-mouse immunoglobulins (immunoglobulin G and immunoglobulin M) were used as the second antibody in an indirect immunofluorescence test. Stained cells were observed in a fluorescence microscope.

RESULTS

Synthesis of C-terminally truncated dengue virus E glycoproteins. Initially, cDNAs coding for C-terminally truncated E of various lengths were constructed, and the expressed products were analyzed for their ability to bind dengue type 4 virus HMAF. Because the polyclonal antibodies present in HMAF were raised against full-length intact E, it was expected that these antibodies should react with a wide variety of linear and conformational antigenic determinants that are normally present on E. Recombinant DNAs coding for the full-length E (100%), or 99, 94, 88, 81, 79, 70, 66, 59, 50, 37, 27, 19, or 9% of the N-terminal sequence of E were constructed and sequenced to determine the truncation site. The predicted C-terminal amino acid sequences are shown in Table 1. Recombinant vaccinia viruses containing this series of E DNAs were then constructed and used for infection of CV-1 cells. [^{35}S]methionine-labeled lysates were prepared from infected cells and precipitated with HMAF, and the precipitates were analyzed by polyacrylamide gel electrophoresis. Each of the eight recombinants that contained 59% or more of the coding sequence produced a dengue virus

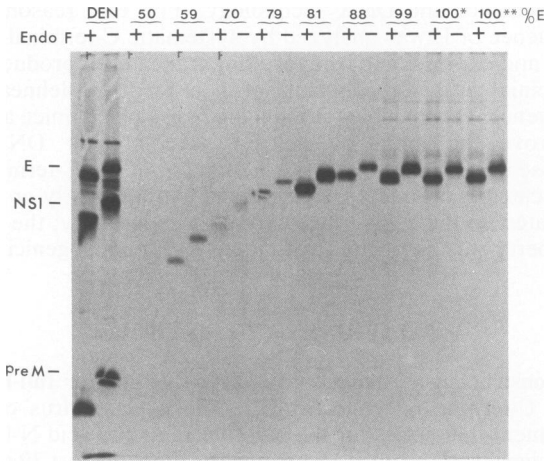

FIG. 1. Analysis of dengue virus C-terminally truncated and full-length envelope glycoproteins expressed by recombinant vaccinia viruses. [^{35}S]methionine-labeled lysates of CV-1 cells infected with various recombinant virus constructs were prepared and immunoprecipitated with a HMAF. Each immunoprecipitate was divided into two aliquots: one was treated with endo F (+), the other was not treated (−). Samples were subsequently analyzed on a SDS-12% polyacrylamide gel. The predicted length of the N-terminal E sequence expressed by each recombinant is indicated. Two recombinants, one expressing the full-length E plus 4 amino acids of NS1 and the other expressing full-length E plus 22 amino acids of NS1, were designated 100*% and 100**%, respectively. The HMAF precipitate of [^{35}S]methionine-labeled lysate of dengue virus-infected cells was used as dengue virus protein size markers (DEN).

E-specific protein of the predicted size (Fig. 1). Digestion with endo F reduced the molecular size of each product to the value estimated for the unglycosylated protein. The products of recombinants v(100*%E) and v(100**%E), each containing the full-length E plus additional NS1 sequences, were similar in size to the product of v(99%E), suggesting that the additional NS1 sequences were proteolytically cleaved from the full-length E product. The gel mobility of 88%E was similar to that of the full-length E. Additional posttranslation modification such as glycosylation could explain the gel migration of this E or E's larger than 88%, including the 93%E described in an earlier study (2). The most interesting observation was that dengue virus E that was 81% or longer was detected with similar high efficiency, and this level was significantly higher than that detected for 79%E or other smaller E products. 50%E was very weakly immunoprecipitated by HMAF, as were other smaller E's not shown in Fig. 1. The reduced detection of these E's was not due to incomplete immunoprecipitation, since a threefold increase of HMAF failed to precipitate more labeled E product.

E sequence critical for binding HMAF. It was possible that the reduced level of detection of 79%E or other small E species was because these shortened E's were unstable following synthesis and therefore accumulated to a lower concentration within infected cells. This does not appear to be the case for 79%E, 70%E, 66%E, or 59%E, as indicated by an experiment using an antiserum prepared in rabbits against a 14-amino-acid peptide of E (peptide 73, amino acids 259 to 272 of E) for immunoprecipitation of the radiolabeled E products (20). The sequence of this peptide is located 55% from the N terminus of E. HMAF detected 81%E at a high level but 79%E at a much lower level. On the other hand,

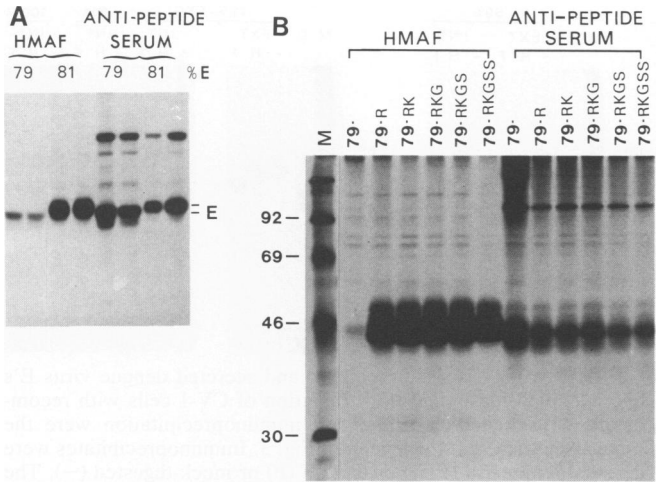

FIG. 2. Differential HMAF binding affinities of truncated dengue virus E's and amino acid sequence involved in binding transition. (A) The labeled lysates of cells infected with recombinants v(79%E) and v(81%E) were the same as described in the legend to Fig. 1. Each lysate was divided into four equal aliquots: two were precipitated with HMAF in one- and threefold concentrations. The other two precipitated with a rabbit anti-peptide serum also in one- and threefold concentrations. E-specific protein bands are indicated. Other bands labeled present in the high-molecular-size region in the precipitates with the rabbit serum were apparently not related to dengue virus E. (B) [^{35}S]methionine-labeled lysates were prepared from CV-1 cells infected with recombinant vaccinia viruses that expressed 79%E, 79%E-R, 79%E-RK, 79%E-RKG, 79%E-RKGS, and 79%E-RKGSS. Equal aliquots of each lysate were precipitated separately with HMAF and with rabbit anti-peptide 73 serum followed by analysis on a SDS-polyacrylamide gel. M shows sizes of protein markers in kilodaltons.

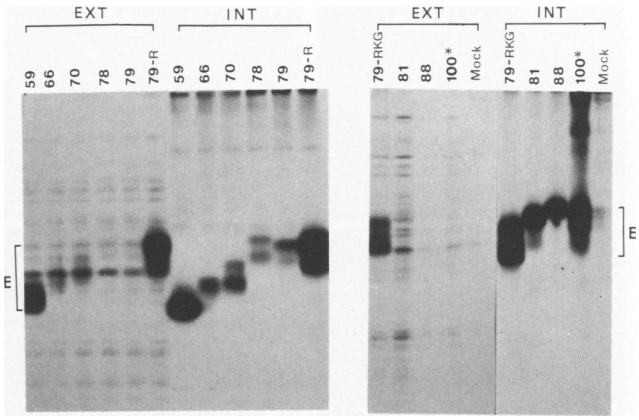

FIG. 3. Analysis of dengue virus E's in intracellular and extracellular fractions. CV-1 cells were infected with various recombinants expressing C-terminally truncated dengue virus E's of the sizes indicated or not infected (Mock). At 18 h after infection, cells were labeled with [^{35}S]methionine for 6 h. The intracellular fraction (INT) and the extracellular (medium) fraction (EXT) were prepared and immunoprecipitated with HMAF for separation on a SDS-polyacrylamide gel. Dengue virus E's in the gel lanes are indicated. The recombinant construct 100%E contained dengue virus DNA coding for the full-length dengue virus E plus 4 amino acids of NS1.

peptide 73 antiserum precipitated both E's to the same extent, suggesting that 79%E was as stable as 81%E but was not precipitated efficiently by HMAF (Fig. 2A). Similarly, an increased level of detection by peptide 73 antiserum was observed for 70%E, 66%E, or 59% E (data not shown). The finding that the binding of HMAF was similar for E's ranging from 81% to the full-length E but significantly reduced by an additional 2% C-terminal deletion of E (i.e., 79%E) suggested that the sequence 2% upstream of the C terminus of 81%E acts to maintain the configuration of E required for major conformational epitopes of E recognized by HMAF.

Because 79%E and 81%E exhibited a marked difference in HMAF binding affinity, the extended C-terminal 8 amino acids, RKGSSIGK, of dengue virus sequence present in 81%E was examined in an attempt to identify the precise amino acid at which there was an abrupt transition of HMAF binding efficiency. Additional recombinants that extended the C terminus of 79%E to include R, RK, RKG, RKGS, or RKGSS were constructed. HMAF efficiently precipitated 79%E-R and also the other longer E species, but not 79%E. On the other hand, peptide 73 antiserum detected 79%E, 79%E-R, and other larger E products with similar efficiency (Fig. 2B). Thus, inclusion of the Arg residue immediately downstream of the C terminus of 79%E is required for the formation and maintenance of the conformational E epitopes recognized by HMAF, presumably through a charged amino acid interaction between this Arg and the other regions of the molecule.

Detection of extracellularly secreted E. We considered the possibility that the poor immunogenicity of the full-length E expressed by v(C-M-E-NS1-NS2A) stemmed from the intracellular targeting of the E product. Earlier, we constructed DNA sequences that specified 93%E lacking the entire C-terminal hydrophobic sequence in an attempt to produce an "anchor-minus" E so that the product would be secreted extracellularly and possibly exhibit increased immunogenicity. However, 93%E was not detected in the medium, and it also failed to induce detectable antibodies in immunized mice (2). It was possible that sequences other than those present at the C-terminal hydrophobic region might be involved in the intracellular targeting of E. The series of E's that had sustained additional truncation at the C terminus allowed us to determine whether such specific targeting sequences had been removed, allowing the resulting E's to be secreted extracellularly. CV-1 cells were infected with a recombinant expressing 59%E, 70%E, 79%E-R, 79%E-RKG, 81%E, 88%E, or 100%E. HMAF detected the synthesis of each of the E's in the cell lysate fraction, although 79%E-R, 79%E-RKG, and the three other larger E species were more efficiently precipitated than 79%E and other smaller E's, as observed earlier (Fig. 3). 59%E, 66%E, 70%E, 79%E-R, and 79%E-RKG were detected in significant amounts in the fluid medium ranging from 10 to 50% of the total labeled product. Similarly, 30 to 50% of label in 79%E-RK, 79%E-RKGS, or 79%E-RKGSS was also detected in the medium fraction during the 6-h labeling period (data not shown). Notably, 81%E that contains three amino acids, IGK, downstream of the C terminus of 79%E-RKGSS was not secreted efficiently. Other E's larger than 81%E were detected only in the cell fraction.

Detection of truncated E's on the cell surface. An indirect immunofluorescence assay was employed to determine whether any of the truncated E's accumulated on the surface of recombinant virus-infected cells. Figure 4 shows the result of this assay for several representative recombinants including v(59%E) and v(79%E-RKG), which expressed extracellular E's, and v(81%E) and v(100*%E), whose E product was not secreted extracellularly. Live-cell immuno-

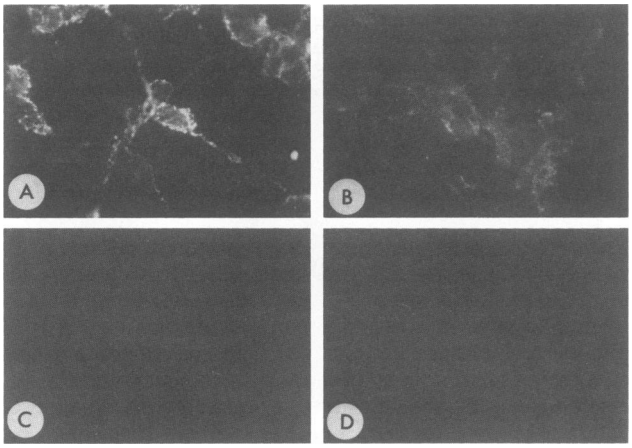

FIG. 4. Detection of truncated E's of dengue virus on the surface of recombinant vaccinia virus-infected cells. An immunofluorescence assay on live cells was performed as described in Materials and Methods. Cells infected with v(79%E-RKG) (A), v(81%E) (B), v(59%E) (C), or v(100%E) (D).

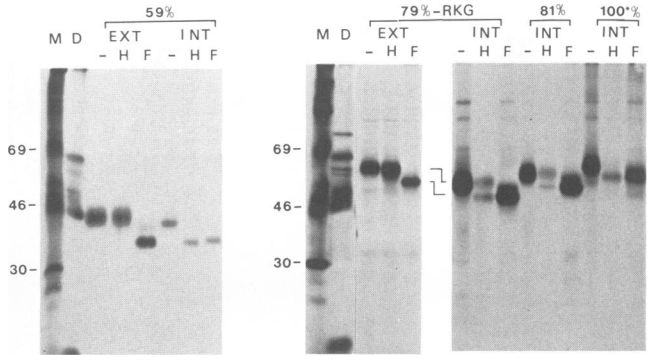

FIG. 5. Analysis of intracellular and secreted dengue virus E's by endoglycosidase digestion. Infection of CV-1 cells with recombinant viruses, radiolabeling, and immunoprecipitation were the same as described in the legend to Fig. 3. Immunoprecipitates were digested with endo H (H) or endo F (F) or mock-digested (−). The intracellular fraction (INT) or the extracellular fraction (EXT) of the recombinant E products was prepared for this analysis. M shows the molecular sizes of marker proteins in kilodaltons. D is the immunoprecipitate of dengue virus proteins for size comparison.

fluorescence tests indicated that the E product of v(79%E-RKG) was present at a high concentration on the cell surface, whereas v(81%E)-infected cells exhibited considerably less cell surface antigen. 79%E-R, 79%E-RK, 79%E-RKGS, and 79%E-RKGSS were also assayed and shown to express a high concentration of E at the surface of infected cells (data not shown). Cells infected with v(59%E) or v(100*%E) were negative by this assay. These observations indicate that the addition of Arg-392 at the C terminus of 79%E alters this molecule so that it becomes stably associated with the cell membrane and accumulates at a high concentration at this site. As shown earlier, Arg-392 is also critical for the formation and maintenance of the native antigenic structure of E and for efficient secretion from recombinant virus-infected cells. Although both 59%E and 79%E-RKG were secreted extracellularly, only 79%E-RKG was detected on the cell surface. On the other hand, 81%E accumulated at a low concentration on the cell surface but was not secreted. Finally, 100%E was detected only inside recombinant virus-infected cells. Of particular interest was the finding that a high level of 79%E-RKG was detected extracellularly and on the cell surface.

Endoglycosidase analysis of intracellular and extracellular E's. In order to determine whether extracellular E's were secreted by a protein export pathway or were released during cell lysis at a late stage of virus infection, both extracellular and intracellular E's were subjected to endoglycosidase digestion. The intracellular fraction of 59%E was sensitive to digestion by endo H or endo F, indicating that its carbohydrate moiety was of the mannose-rich type that is added during the first stage of glycosylation during protein synthesis (Fig. 5). On the other hand, extracellular 59%E was very resistant to endo H digestion, indicating that it entered the secretory pathway through the Golgi apparatus where endo H-resistant carbohydrates were added prior to secretion into the medium. Extracellular 79%E-RKG, similar to extracellular 59%E, was resistant to endo H digestion, whereas the intracellular form of 79%E-RKG was partially sensitive to endo H digestion. In contrast, intracellular full-length E was completely sensitive to endo H digestion. Thus, it appears that both C-terminally truncated 59%E and 79%E-RKG enter into the export pathway but this pathway is not taken by full-length E. The intracellular form of 81%E contained both endo H-sensitive and endo H-resistant carbohydrate moieties similar to those found for intracellular 79%E-RKG, indicating that 81%E was also directed in part into the export/secretory pathway which is consistent with the observation that a small fraction of this protein was detected on the cell surface. Despite the similarity of glycosylation pattern of 79%E-RKG and 81%E, the latter accumulated to a lesser extent on the cell surface and failed to be secreted extracellularly.

Protective immunity induced by C-terminally truncated E's. The protective efficacy of 15 recombinant vaccinia viruses that expressed full-length E or C-terminally truncated dengue virus E ranging from 9 to 99% of the colinear N-terminal sequence was evaluated in four separate studies by challenging immunized mice with dengue type 4 virus intracerebrally. Mice immunized with v(79%E-RKG) or with a recombinant expressing 81%E, 88%E, 94%E, 99%E, or 100%E were completely protected or almost completely protected against dengue virus challenge, i.e., the overall protection rate was 94 to 100% (Fig. 6). On the other hand, mice immunized with v(79%E), or a recombinant expressing a smaller product (i.e., 70%E, 66%E, 59%E, 50%E, 37%E, 27%E, or 19%E) were partially protected (40 to 78% overall protection rate) with the exception of v(59%E) which consistently exhibited a high protection rate (9 of 10, 9 of 10, and 6 of 7 in separate experiments) against dengue virus challenge. Significant resistance induced by 19%E indicates that at least one protective antigenic site is located within the first 92 amino acids of E. v(9%E) had the lowest protection rate, 17%, which was similar to the value observed for the control recombinant vSC8. An interesting correlation became apparent when the protection rate and HMAF binding affinity were compared. Recombinant E's that induced the highest level of resistance to dengue virus encephalitis also exhibited high HMAF binding affinity, suggesting that mature conformational structure of the dengue virus E glycoprotein is an important factor in inducing effective protective immunity.

Antibody response of mice immunized with recombinant vaccinia viruses expressing E of various lengths. The level of E-specific antibodies in sera of immunized mice was analyzed by radioimmunoprecipitation to determine whether

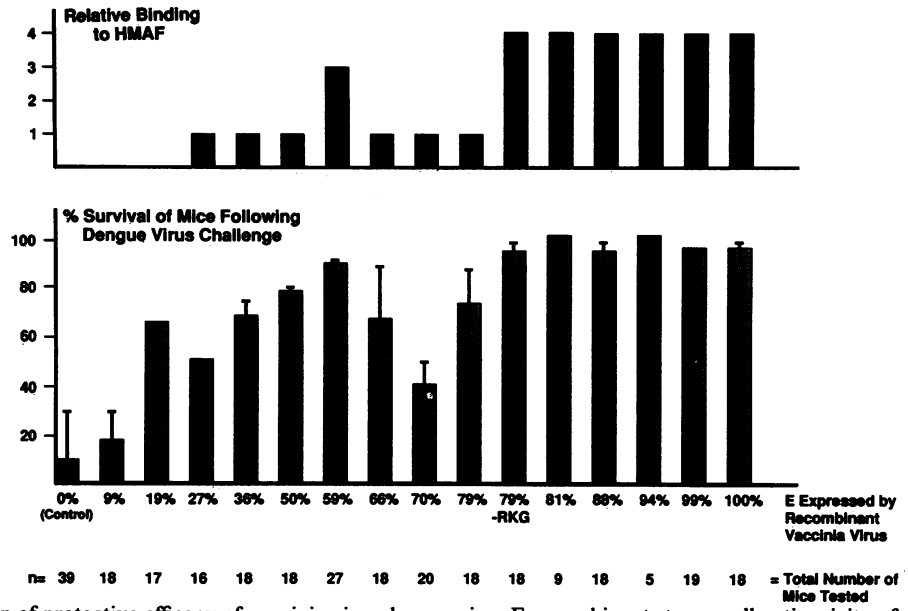

FIG. 6. Relationship of protective efficacy of vaccinia virus dengue virus E recombinants to overall antigenicity of expressed E products. The top panel shows relative binding of the expressed E products to HMAF as detected by radioimmunoprecipitation. On a scale of 1 to 4, the highest number was assigned to the E products which exhibit high HMAF-binding affinity, and the lower numbers (1 and 3) were assigned to E's which bind less efficiently to HMAF. The lower panel shows the cumulative protection rates as expressed by percent survival following dengue virus challenge of mice immunized with various vaccinia virus recombinants expressing 9 to 100% dengue virus E's. The results were derived from four separate mouse protection studies. The total number of mice tested for each recombinant is indicated.

C-terminal truncation of E increased its immunogenicity. Groups of mice that were partially protected in the challenge studies failed to develop detectable E antibodies in response to immunization. Also, not all groups of mice that exhibited complete or almost complete resistance to dengue virus challenge developed an E antibody response to immunization. For example, mice immunized with 81%E developed a low level of E antibodies, while mice immunized with 100%E failed to develop detectable E antibodies. Among the various truncated and full-length E constructs tested, 79%E-RKG was most immunogenic. The level of E antibodies in individual sera of mice immunized with v(79%E-RKG) was equivalent to that present in a 1:80 or 1:160 dilution of HMAF as measured by immunoprecipitation (data not shown). Pooled sera from mice immunized with v(100%E), v(81%E), v(79%E-RKG), and the vSC8 control were also tested for dengue virus-neutralizing activities by the plaque-reduction neutralization test (1). Sera from mice immunized with 79%E-RKG or 81%E consistently inhibited plaque formation compared with sera from mice immunized with the vSC8 control recombinant, but inhibition at a 1:10 serum dilution did not exceed 50%. Passive transfer of sera to mice followed by dengue virus challenge was also performed to determine whether serum antibodies induced by the various forms of E played a role in the resistance induced by immunization. Sera from mice immunized with v(79%E-RKG) or v(81%E) conferred on recipient mice solid protection against intracerebral dengue virus challenge (Table 2). Sera from groups of mice immunized with v(100*%E) or with v(59%E) provided a somewhat lower passive protective effect (i.e., 80 or 50% survival, respectively). In addition, morbidity was observed in these animals following dengue virus challenge, whereas this was not the case in mice passively protected by serum from mice immunized with 79%E-RKG or 81%E. It appears that E antibodies induced in mice following immunization with v(79%E-RKG), or v(81%E) played a major role in the resistance to lethal dengue virus challenge exhibited by these vaccinated animals.

DISCUSSION

The full-length E of dengue type 4 virus contains 494 amino acids including 12 Cys residues, all of which are conserved in some 20 flavivirus E's that have been sequenced. The most C-terminal Cys in dengue type 4 virus E is at position 333 of the E sequence. Thus, 67% or more of the N-terminal E should contain all 12 Cys residues. In flavivirus West Nile E, each of the 12 Cys residues appeared to be involved in disulfide bond formation (22). In dengue type 4 virus E, two potential N-linked glycosylation sites are located at positions 67 and 153 and a third N-glycosylation site that is probably not used is present at position 471 within the C-terminal hydrophobic region. N-terminal 31%E contains both potential glycosylation sites. However, the E glycoproteins of two flaviviruses, West Nile virus and Kunjin virus, lack glycosylation sites, suggesting that N glyco-

TABLE 2. Protection of mice against dengue virus challenge following passive transfer of sera from donor mice immunized with recombinant vaccinia virus expressing the full-length and C-terminally truncated E's

Recombinant vaccinia virus	Response to dengue virus challenge[a]	Protection rate (%)
v(100*%E)	8/10	80
v(81%E)	10/10	100
v(79%E-RKG)	10/10	100
v(59%E)	5/10	50
vSC8	1/10	10

[a] Number of mice that survived/number of mice that received sera.

sylation is not essential to the antigenic, structural, and functional integrity of the flavivirus envelope glycoprotein (6, 30). Antigenic analysis of our series of C-terminally truncated dengue virus E's using polyclonal HMAF or an antipeptide serum suggested that inclusion of Arg at position 392 is required for the formation and maintenance of the native conformation of E. This position in the linear E sequence appears to represent a transition between the two classes of E's that are distinguishable by their binding affinities to HMAF (Fig. 2B). Since this Arg is located C terminal of the last Cys residue, it is likely that this charged amino acid influences the folding of the E molecule during nascent synthesis so that Cys residues can be brought into close proximity for disulfide bond formation, which is a prerequisite for maintenance of a stable configuration.

In an earlier study, we were not successful in producing a secreted E by removing the C-terminal hydrophobic sequence nor were we able to anchor the protein on the cell surface using the N-terminal anchor sequence of respiratory syncytial virus G glycoprotein (2). Upon further truncation of the C terminus, efficient extracellular secretion was detected for 79%E-RKGSS, but not for 81%E that contained three additional amino acids, IGK, of dengue virus E plus three vector-derived amino acids, MNE, at its C terminus. Since other longer E's were also not secreted, it appears that the three dengue virus C-terminal amino acids of 81%E-IGK play a role in directing the cellular distribution of E by preventing it from being exported and secreted. Deletion of these amino acids resulted in secretion of the E product. In addition to being secreted extracellularly, the series of five E's ranging in size from 79%E-R to 79%E-RKGSS, as exemplified by 79%E-RKG, was expressed in high concentration on the surface of recombinant virus-infected cells. This suggests that 79%E-RKG is an integral membrane glycoprotein containing an anchor sequence for insertion into the cell membrane. Analysis of the C-terminal sequence suggested a mechanism for insertion of this protein into the plasma membrane and secretion of a fraction of the protein extracellularly. There are 17 hydrophobic or neutral amino acids in a 20-amino-acid stretch preceding the hydrophilic RKG sequence at the C terminus. This hydrophobic structure resembles the C-terminal anchor of many surface glycoproteins in that a hydrophobic transmembrane domain of length sufficient to span the lipid bilayer (usually 20 to 30 amino acids) is followed by a charged cytoplasmic domain of various lengths. The 3-amino-acid sequence in the C-terminal domain of 79%E-RKG (especially the one charged amino acid (R) in the C-terminal domain of 79%E-R) is shorter than the cytoplasmic domains of other viral integral membrane proteins and may affect its stable association with membranes, as a similar finding has been described previously (16). Perhaps for this reason, 79%E-RKG or 79%E-R failed to become firmly anchored on the membrane, and as a consequence, a fraction of the expressed protein was secreted. Consistent with this interpretation is the observation that 81%E containing three additional amino acids IGK at the C terminus of 79%E-RKGSS was not secreted.

Using the mouse encephalitis model, the current study revealed that 79%E-R or other larger E species induced complete or nearly complete protection, whereas 79%E or other shorter E's induced less protection against dengue virus challenge. Mouse protective efficacy appeared to correlate with the ability of an E construct to bind antibodies in dengue type 4 virus HMAF. Presumably assumption of the mature conformation of E is required for proper display of major protective sites on E. Consistent with this view was the observation that the protective efficacy and HMAF binding affinity of 59%E were higher than that of 50% or 70%E. In this regard, it appears that 59%E displays one or more major protective antigenic sites. Among the truncated E molecules that were able to bind antibodies in dengue virus HMAF efficiently, 79%E-RKG was highly immunogenic when tested for induction of E antibodies. This truncated E was unique in being expressed at a high concentration on the surface of infected cells. 79%E-RKG was also secreted extracellularly, but other truncated E constructs that were also secreted efficiently did not induce a detectable E antibody response. This suggests that cell surface expression of 79%E-RKG was responsible for its enhanced immunogenicity. The properties of 81%E were also consistent with the importance of cell surface expression of E in immunogenicity. Unlike longer E constructs that were not expressed on the cell surface, 81%E was expressed at a low level on the cell surface and this truncated E was also able to induce a detectable level of E antibodies. There is evidence from studies with the S antigen of *Plasmodium falciparum* that anchoring the secreted plasmodial antigen on the cell membrane increased immunogenicity severalfold (18). Although the protective efficacy of v(79%E-RKG) and v(81%E) compared with other recombinant vaccinia viruses expressing larger E species appeared to be similar as measured by survival rate, a notable difference in the morbidity following dengue virus challenge was observed. Mice immunized with v(79%E-RKG) or with v(81%E) showed few, if any, symptoms of encephalitis (ruffled fur, hunched backs) after dengue virus challenge compared with other groups of mice immunized with recombinant v(88%E), v(94%E), v(99%E), or v(100%E). Thus, in addition to being more immunogenic than longer E constructs, 79%E-RKG and 81%E induced a higher level of resistance to experimental dengue virus disease.

There is significant conservation of E sequence among flaviviruses. Amino acid homology among different dengue serotype viruses ranges from 62 to 70%, while homology among different flaviviruses ranges from 40 to 50%. Also, conservation of cysteine residues suggests conservation of the three-dimensional structure of E. These similarities suggest that a strategic truncation at the C terminus analogous to the 79%E-RKG of dengue type 4 virus may yield an E product that exhibits increased immunogenicity and protective efficacy. We have recently evaluated the applicability of this strategy to another dengue virus serotype by constructing dengue type 2 virus (PR159, S1 strain) truncated E similar to 79%E-RKG of dengue type 4 virus. Immunization with the recombinant vaccinia virus expressing this C-terminally truncated E of dengue type 2 virus induced solid resistance in mice to challenge with type 2 virus. In contrast, immunization with a recombinant expressing the full-length E of dengue type 2 virus induced only partial protection with a survival rate of less than 50%, whereas all unprotected animals died following dengue virus challenge (1a). This observation suggests that it may be possible to extend this strategy to construct highly immunogenic E glycoproteins of other flaviviruses, such as Japanese encephalitis virus and tick-borne encephalitis virus that constitute important public health problems.

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