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The Murray Valley Encephalitis Virus prM Protein Confers Acid Resistance to Virus Particles and Alters the Expression of Epitopes within the R2 Domain of E Glycoprotein

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To study the role of the precursor to the membrane protein (prM) in flavivirus maturation, we inhibited the proteolytic processing of the Murray Valley encephalitis (MVE) virus prM to membrane protein in infected cells by adding the acidotropic agent ammonium chloride late in the virus replication cycle. Viruses purified from supernatants of ammonium chloride-treated cells contained prM protein and were unable to fuse C6/36 mosquito cells from without. When ammonium chloride was removed from the cells, both the processing of prM and the fusion activity of the purified viruses were partially restored. By using monoclonal antibodies (MAbs) specific for the envelope (E) glycoprotein of MVE virus, we found that at least three epitopes were less accessible to their corresponding antibodies in the prM-containing MVE virus particles. Amino-terminal sequencing of proteolytic fragments of the E protein which were reactive with sequence-specific peptide antisera or MAb enabled us to estimate the site of the E protein interacting with the prM to be within amino acids 200 to 327. Since prM-containing viruses were up to 400-fold more resistant to a low pH environment, we conclude that the E-prM interaction might be necessary to protect the E protein from irreversible conformational changes caused by maturation into the acidic vesicles of the exocytic pathway. © 1992 Academic Press, Inc.

INTRODUCTION

For enveloped viruses to enter into their target cells, virus-cell membrane fusion must take place to release the nucleocapsid into the cytoplasm. This occurs either directly at the plasma membrane, as with paramyxoviruses and herpesviruses, or in the endosomal vesicles after the viruses are taken up by receptor-mediated endocytosis, as with togaviruses and orthomyxoviruses (for review see Marsh and Helenius, 1989). Generally the fusion-mediating envelope proteins are activated by proteolytic cleavage of the spike precursor proteins by a host protease late during virus assembly. Cleavage activation of the glycoproteins involved in fusion has been demonstrated with the gPE2 of murine coronaviruses (Sturman and Holmes, 1984; Sturman *et al.*, 1985), the HA of influenza viruses (Wiley and Skehel, 1987), the gP160 of retroviruses (McCune *et al.*, 1988; Willey *et al.*, 1991), and the PE2 of alphaviruses (Lobigs and Garoff, 1990; Lobigs *et al.*, 1990; White *et al.*, 1983). The cleaved glycoprotein (e.g., HA) undergoes an irreversible conformational change in the acidic pH milieu of the endocytic vesicles which exposes a hydrophobic fusion site that mediates the virus-cell membrane fusion. This cleavage of the precursor protein is essential for virus infectivity but not particle formation. A Semliki Forest virus (SFV) mutant defective in PE2 cleavage expressed PE2 at the cell

surface but did not function in low pH-triggered cell-to-cell fusion (Lobigs and Garoff, 1990; Lobigs *et al.*, 1990).

Murray Valley encephalitis (MVE) virus is a member of the Flaviviridae, a family of small, positive-strand RNA viruses. The mature flavivirions contain three structural proteins: capsid (C), membrane (M), and envelope (E), with molecular masses of 14-15, 7-8, and 50-60 kDa, respectively. The E protein is the only structural protein responsible for the induction of neutralizing, hemagglutination-inhibiting, and protective antibodies, it is the receptor-binding protein and is also involved in acid-catalyzed membrane fusion (Heinz and Roehrig, 1990; Guirakhoo *et al.*, 1991). Little is known about the function of the M protein. The M protein is cleaved from its precursor protein (prM) after the consensus sequence R-X-R/K-R shortly before or after virus release (Rice *et al.*, 1986; Westaway, 1987). This cleavage is associated with a rearrangement of the oligomeric structures on the surface of the virion (Wengler and Wengler, 1989). By using monoclonal antibodies (MAbs) and protease maps, we previously demonstrated that the E glycoprotein of tick-borne encephalitis (TBE) virus undergoes an irreversible conformational change, predominantly in the epitopes of domain A, at mildly acidic pH (Guirakhoo *et al.*, 1989). Consistent with this, purified TBE virus demonstrated fusion from without (FFWO) of the mosquito cell line C6/36 only after exposure of cell-bound virus to low pH. The FFWO was abolished if the processing of prM

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to M was interrupted using the acidotropic agent ammonium chloride (Guirakhoo *et al.*, 1991).

In this paper we identify those epitopes on the E protein which are covered or dissociated by the presence of prM, demonstrate that prM-containing viruses are more resistant to low pH, localize the site on the E protein which is involved in the E-prM interaction, and finally hypothesize that the association of E-prM is necessary for the authentic expression of the E protein.

MATERIALS AND METHODS

Production and analysis of the immature virus particles

Aedes albopictus cells (clone C6/36) were grown on microcarriers (Cytodex 2, Pharmacia Fine Chemicals, Upsala, Sweden)² in Dulbecco's minimal essential medium (D-MEM) buffered with 25 mM HEPES and supplemented with 10% fetal calf serum (FCS, Hyclone Laboratories, Inc., Logan, UT). Cells were infected with MVE virus (strain Ord River) at a multiplicity of infection (m.o.i.) of 10 in D-MEM with 2% FCS. Ammonium chloride (25 mM) was added at 48 hr postinfection (p.i.) and the viruses were harvested on Day 6. At this time, ammonium chloride-containing medium was replaced with D-MEM, 2% FCS, without ammonium chloride, and the viruses were reharvested on Day 9 p.i. Viruses were precipitated with 8% polyethylene glycol 8000 (Fisher Scientific, Fair Lawn, NJ), purified by two cycles of glycerol-tartrate gradients (Obijeski *et al.*, 1976), and resuspended in 0.3 ml of D-MEM. Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). Untreated MVE viruses (without ammonium chloride) were propagated and purified as described above and were used as positive controls in all experiments. For the acid resistance experiments (see below) MVE viruses were also grown in SW13 mammalian cells in the presence or absence of ammonium chloride. To quantify the amount of viral structural proteins in each propagation, the purified viruses were subjected to SDS-PAGE on 12.5% acrylamide gels (Laemmli, 1970) and proteins were stained using a rapid silver stain kit (ICN, Cleveland, OH) according to the manufacturer's protocols. Gels were scanned by a microprocessor-controlled spectrophotometer (Gilford System 2600) and the peaks corresponding to each viral protein were identified and integrated to determine areas under the peaks, and the area ratios of prM to E were calculated.

² Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

Fusion from without assay

Purified viruses were diluted geometrically (from 200 to 6.25 $\mu\text{g/ml}$) in D-MEM containing 1% BSA. Thirty microliters of each dilution was added to prechilled C6/36 cells and the fusion assay was performed at pH 5.0. The number of nuclei and the number of cells in five microscopic fields (magnification 100-fold) were counted and the fusion index [$1 - (\text{number of cells}/\text{number of nuclei})$] was calculated.

Capture enzyme-linked immunosorbent assay (ELISA)

Microtiter plates were coated overnight at 4° with a 1:500 dilution of rabbit anti-MVE hyperimmune serum. This dilution of capture antibody was determined by box-titration against M- and prM-containing virus seeds, maximizing and standardizing the concentration of captured antigens. Wells were blocked with 3% goat serum in PBS, a 1:5 dilution of tissue cultures containing prM- or M-MVE virus was added, and plates were incubated for 1 hr at 37°. Ascites of the various MAbs were then titrated on both viruses for 1 hr at 37° starting at 1:100 or 1:1000 dilution depending on antibody potency. Bound MAbs were detected by goat anti-mouse alkaline phosphatase conjugate. Quantitation of bound conjugate was determined by adding Sigma 104 substrate and reading absorbance at 405 nm in a Titertek MC spectrophotometer after 30 min. The specificity and the biological activity of all MAbs except 6B4A-10 have been published elsewhere (Hawkes *et al.*, 1988). MAb 6B4A-10 was prepared against Japanese encephalitis virus, strain Nakayama, using standard hybridoma techniques. This MAb reacted with all members of the MVE virus serocomplex, blocked MVE virus-mediated hemagglutination, and neutralized MVE virus infectivity *in vitro*. The E glycoprotein epitope defined by 6B4A-10 has been designated E-8.

Analysis of the antigenic reactivities of MAbs after exposure of MVE virus to low pH

The acid sensitivity of the MVE virus epitopes was determined using a modification of the standard ELISA protocol. Two hundred and fifty microliters of purified MVE virus (100 μg total protein) in 0.2 M Tris-HCl, pH 8.0, was adjusted to pH 6.0 using 120 μl of 0.2 M HCl and incubated at 25° for 30 min. Both acid-treated virus and untreated virus (pH 8.0) samples were diluted to 5 ml with carbonate buffer, pH 9.5. Immulon II microtiter plates were coated with 100 μl virus per well and incubated overnight at 4°. The plates were blocked for 1 hr at 37° with 3% BSA in PBS. Protein

A-Sepharose-purified MAbs were diluted geometrically (from 10 μg /well total protein) and added to each well. After 2 hr at 25°, the plates were washed and further incubated with a 1:500 dilution of goat anti-mouse alkaline phosphatase conjugate. Bound antibody was detected by addition of Sigma 104 substrate, and absorbance was measured at 405 nm after 5 min. Although each antibody was tested in a dilution series, only the results with 1.25 μg antibody are reported.

Analysis of the MAb reactivities with reduced and nonreduced MVE virus

PAGE sample buffer (Laemmli, 1970) with or without the reducing agent, 2-mercaptoethanol, was added to 10 μg of purified MVE virus. Samples were boiled for 3 min at 100° and separated on 12.5% SDS gels. Proteins were then transferred to nitrocellulose sheet (Towbin *et al.*, 1979) and blocked with 3% goat serum. The reactivity of MAbs defining the epitopes E-4b, E-6, or E-8 with reduced or nonreduced forms of MVE virus E glycoprotein was assessed using goat anti-mouse alkaline phosphatase conjugate (Jackson Immuno Research, West Grove, PA).

Identification of MAb binding site

Proteolytic digestion of the native MVE virus was performed in D-MEM, pH 7.4, using predetermined concentrations of either trypsin or V8 protease (Sigma Chemical Co.) and the fragments reacting with MAb E-8 were identified by using goat anti-mouse alkaline phosphatase conjugate (see below). For the proteolytic digestion of the predenatured virus, 60 μg of purified MVE virus in D-MEM was precipitated in 60% TCA (end concentration 12%) on an ice bath for 30 min, washed with cold acetone, and resuspended in 50 μl 0.125 M Tris, pH 6.8, containing 0.5% SDS and 10% glycerol. After 24 hr incubation at 37° samples were boiled for 3 min and subjected to proteolytic digestion using either chymotrypsin or V-8 proteases (Sigma Chemical Co.) at the protein to enzyme ratios of 4:1 and 30:1, respectively, at 37° for 30 min. After addition of SDS and 2-mercaptoethanol (final concentrations 4%), the samples were boiled and separated on 12.5% SDS gels. Fragments were transferred to nitrocellulose membrane by electroblotting (Towbin *et al.*, 1979). Nitrocellulose was then blocked by 3% goat serum in PBS, pH 7.4, for 1 hr. Strips were cut and incubated with either MAb (defining epitope E-8) or mouse sera which were immunized with E-specific synthetic peptides as follows: MVE 02, amino acids 35–50; MVE 04, amino acids 122–141; MVE 06, amino acids 230–251; and MVE 17, amino acids 356–376. The sequence and characteristics of these antipeptides have been

published previously (Roehrig *et al.*, 1989). After 1 hr at 37°, goat anti-mouse alkaline phosphatase conjugate (Jackson Immuno Research) was added and the immune fragments were visualized by adding BCIP/NBT substrate (KPI, Inc., Gaithersburg, MD). For the amino-terminal sequencing, fragments were prepared as described above except that electroblotting was performed on polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, MA) using CAPS transfer buffer (10 mM CAPS, 0.5 mM dithiothreitol, DTT, and 10% methanol adjusted to pH 11.0 with NaOH). Fragments were stained with 0.1% Coomassie blue (Baker G-250) in 50% methanol and 10% acetic acid. The amino-terminal sequences were determined either on an Applied Biosystems 473A (Foster City, CA) or on a Porton Instrument 2090 (Tarzana, CA) protein sequencer using standard Edman Chemistry.

Plaque assay of prM- and M-containing MVE viruses after exposure to different pH buffers

Equal amounts of plaque forming units, PFUs (6×10^8 /ml for viruses grown in C6/36 cells and 4.5×10^5 for viruses grown in SW13 cells), from the supernatants of ammonium chloride-treated and untreated MVE virus-infected cells were incubated in different pH buffers, ranging from 7.5 to 4.5, using 1 M HEPES or 1 M MES containing D-MEM. After a 15 min-incubation at room temperature, the inoculum was exponentially diluted in BA-1 diluent (Hunt *et al.*, 1991) and examined for infectivity in Vero cells using the standard plaque assay.

RESULTS

The relationship between prM processing and the fusion activity

To understand the role of prM protein in virus maturation and its interaction with the E glycoprotein, we investigated the effect that ammonium chloride had on MVE viruses grown in C6/36 mosquito cells. Ammonium chloride has been shown to accumulate in the post-Golgi acidic vesicles and interrupt the processing of prM to M protein (Randolph *et al.*, 1990). The unprocessed prM protein was incorporated into the released viruses grown in the presence of ammonium chloride at 6 days p.i. (Fig. 1A, lane 2). When ammonium chloride was removed, the cells partially recovered, and the normal processing of the prM to M resumed (Fig. 1A, lane 3). Since some unprocessed prM proteins were also found in MVE-infected cells in the absence of ammonium chloride (Fig. 1A, lane 1), we attempted to quantify the amount of prM in each sample. This was

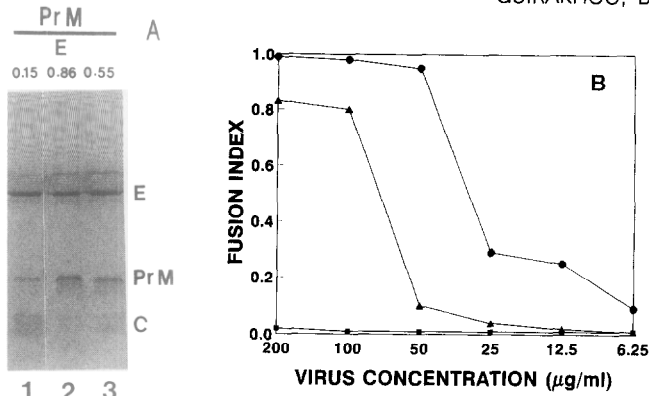


FIG. 1. The relationship between prM processing and fusion activity. (A) Ten micrograms of purified viruses was separated by PAGE using a 12.5% SDS gel. Viral proteins were silver stained and subjected to scanning, and the ratio of prM to E was calculated for each sample and is shown on the top of each lane. Lane 1, purified MVE viruses obtained from C6/36 cells in the absence of ammonium chloride. Lane 2, purified MVE viruses obtained from C6/36 cells in the presence of ammonium chloride. Lane 3, purified MVE viruses obtained from C6/36 after removal of ammonium chloride. (B) Fusion activities of the viruses shown in (A) were determined at pH 5.0 using the C6/36 cell lines as described under Materials and Methods. Symbols: ●, MVE virus shown in lane 1 of (A); ■, MVE virus shown in lane 2 of (A); ▲, MVE virus shown in lane 3 of (A).

achieved by scanning the stained viral proteins following separation by SDS-PAGE. By using nonradioactive viruses it was not always possible to clearly demonstrate the presence of the small M protein in minigels. The fact that the ratios of the viral structural proteins in the absence of ammonium chloride remain constant enabled us to evaluate the amount of prM processing indirectly by calculating its ratio to other structural proteins (E or C) which are more abundant in virions. The ratio of the area of each peak corresponding to C or prM proteins to E as well as prM to C protein was calculated. The ratio of C to E protein remained constant in the presence or absence of ammonium chloride (data not shown) whereas the ratio of prM to E protein was significantly increased in the presence of ammonium chloride (from 0.15 to 0.86) and reduced to 0.55 following removal of ammonium chloride (Fig. 1A). Similar results were obtained when the ratio of prM to C was calculated (data not shown). These data confirm that partial recovery of prM processing has occurred once the ammonium chloride has been removed.

To determine the fusion activity of the viruses obtained from the supernatants of virus-infected ammonium chloride-treated cells, the FFWO assay was performed using monolayers of C6/36 cells as described previously (Guirakhoo *et al.*, 1991). No polykaryocyte formation was observed (up to 200 µg/ml of purified virus) using viruses harvested in the presence of ammonium chloride at 6 days p.i.; however, when ammonium chloride was removed, the fusion activity of puri-

fied viruses obtained on Day 9 was significantly restored (Fig. 1B).

These results are in agreement with our previous finding with TBE virus which demonstrated a direct relationship between processing of prM and fusion activity of E glycoprotein (Guirakhoo *et al.*, 1991) and further manifest that both phenomena are reversible.

Epitope analysis of the E-proteins of prM- and M-containing viruses

A capture ELISA was performed to analyze the effect that blocking the prM cleavage had on the expression of E glycoprotein epitopes in native virions. The reactivities of MAbs defining nine distinct epitopes on the MVE E glycoprotein were compared on M- and prM-containing viruses using supernatants of ammonium chloride-treated or untreated virus-infected C6/36 cells. The antigenicity of the prM- and M-containing virus supernatants was equivalent in end-point ELISA antigen titration using a 1:500 dilution of polyclonal rabbit anti-MVE virus capture sera and anti-MVE virus mouse hyperimmune ascitic fluid (HIAF) as detector. Therefore, a 1:5 dilution of each supernatant was used in subsequent epitope mapping with MAbs. The equivalent antigenicity was confirmed by end-point ELISA titration of the MVE HIAF in the standardized capture assay (Fig. 2B). Even though these virus supernatants had equal antigenicity, the prM-containing virus supernatant was 10-fold less infectious than the M-containing virus supernatant (3.9×10^8 vs 2.3×10^9 PFU/ml). The reactivities of three E glycoprotein epitopes were significantly reduced in prM-containing viruses (Fig. 2C). Two of these epitopes were subcomplex- and complex-reactive (E-6 and E-8) and one was group-reactive (E-4b), being conserved in all flaviviruses (Hawkes *et al.*, 1988). The reactivities of other MVE virus-specific MAbs were identical with both viruses (Fig. 2A).

Structural properties of the epitopes corresponding to these MAbs were assessed by comparing the reactivities of MAbs after incubation of the purified viruses at low pH to those of untreated viruses (Fig. 3). As can be seen, the antigenic reactivities of all MAbs and the HIAF were significantly reduced upon low pH treatment of the virus.

Identification of the E-8 MAb binding site on the E glycoprotein

Figure 4A demonstrates the reactivities of the three MAbs defining the epitopes E-4b, E-6, and E-8, with the MVE virus E glycoprotein under reducing and nonreducing conditions. As can be seen neither the E-4b nor E-6 MAbs recognized the E protein in its re-

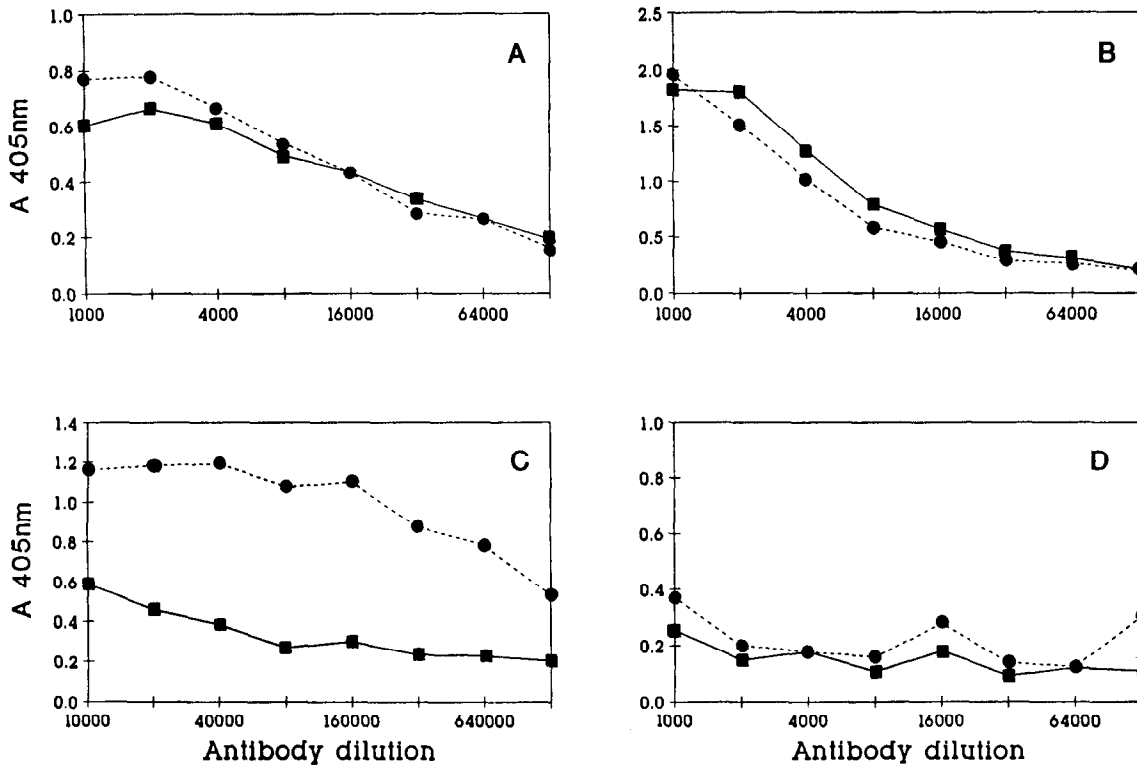


Fig. 2. Reactivities of the MVE virus E glycoprotein-specific MABs with prM- or M-containing MVE viruses in a capture ELISA. PrM- or M-containing MVE viruses were captured by rabbit anti-MVE immune serum-coated microtiter plates. Serial dilutions of the MVE virus E-specific MABs were added to the plates and the bound antibodies were detected by anti-mouse conjugate as described under Materials and Methods. (A) Representative curve (E-3) for the reactivity of MABs defining the epitopes E-1a, E-1c, E-1d, E-3, E-5, and E-7. (C) Representative curve (E-4b) for the reactivity of MABs defining the epitopes E-4b, E-6, and E-8 (for more details for epitope characteristics see Hawkes *et al.*, 1988). (B) Positive control MVE virus HIAF. (D) Negative control SLE virus-specific MAB 6B5A-2. Symbols: ●, M-containing viruses; ■, prM-containing viruses.

duced form (treated with 2-mercaptoethanol), whereas E-8 Mab was still reactive after protein reduction. This reactivity was further sustained after the reduction and alkylation of the cysteine residues of the E glycoprotein using DTT/SDS followed by iodoacetamide treatment (Johnson *et al.*, 1990) (data not shown). Since the reactivity of the E-8 Mab was reduced with prM-containing viruses and the fact that it could recognize the E protein in immunoblots in its linear conformation, we attempted to obtain more information about the site involved in prM-E interaction by identifying the E-8 binding site. The proteolytic digestion of the native E protein with trypsin (which cleaves after K or R) or V8 endoproteinase (which cleaves after E or D) did not produce fragments smaller than 30 kDa that were reactive with E-8 Mab (Fig. 4B). In order to produce smaller fragment reactive with the E-8 Mab, the MVE virus was denatured by SDS prior to protease digestion. Using chymotrypsin (which cleaves after F, T, or Y) or V8 protease, 20- and 22-kDa fragments, Fr 20 and Fr 22, were identified, respectively. These two fragments, which reacted with E-8 Mab, were also recog-

nized by mouse antiserum immunized with synthetic peptide MVE 06 (Figs. 4C and 4D). A 23-kDa fragment, Fr 23, and a 19-kDa fragment, Fr 19, were produced using V8 or chymotrypsin, respectively, which were recognized by MVE 04 antibodies but not by MVE 06 antibodies or E-8 Mab. Antisera prepared against MVE 17 also recognized a V8 fragment of 16 kDa Fr 16 (Fig. 4D). Fr 23, 22, 16, and 14 were sequenced at their amino-terminal ends and the results are shown in Table 1. Fr 23 (reactive with MVE 02 and MVE 04 antibodies but not with MVE 06 and MVE 17 antibodies or E-8 Mab) starts at the amino-terminus of the E glycoprotein and contains approximately 199 amino acids with a calculated MW of 21,210. Fr 22 (reactive with MVE 06 and E-8 Mab but not with MVE 02, MVE 04, or MVE 17 antisera) (Fig. 4D and Table 1) is derived by cleavage between E-138 and V-139, ends approximately at position 326, and has a calculated MW of 20,560. Fr 16 (reactive only with MVE 17 antisera) is about 152 amino acids long, starts at L-327, ends approximately at D-478, and has a calculated MW of 16,010. Fr 14 (reactive with MVE 06 antibody and E-8 Mab but not

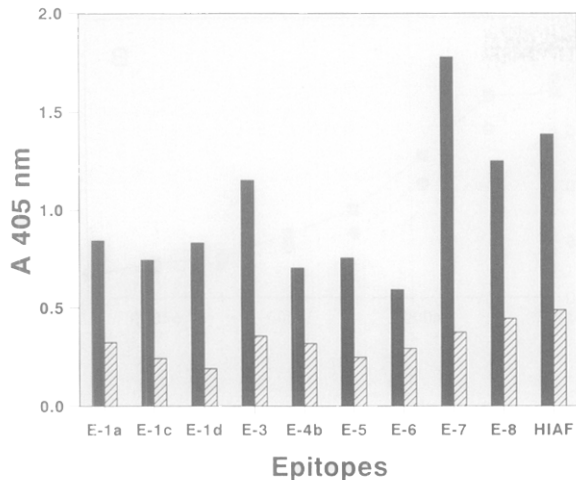


Fig. 3. Epitope sensitivity of the MVE virus E glycoprotein to low pH. Purified MVE viruses were treated at pH 6.0 and used to coat microtiter plates. The reactivity of each MAb with the low pH-treated virus was compared with that of the untreated virus (pH 8.0). For reporting, only the results with 1.25 μ g/well antibodies have been shown. Solid bars: the reactivity of the MVE virus E-specific MAbs with untreated virus (pH 8.0). Cross-hatched bars: the reactivity of the MVE virus E-specific MAbs with treated virus (pH 6.0).

with MVE 02, MVE 04, or MVE P17 antisera) starts with A-200, is 127 amino acids long, ends apparently at the carboxy-terminal of E-326, and has a calculated MW of 14,207. Since the published sequences for MVE virus (Dalgarno *et al.*, 1986) predict only one potential N-linked glycosylation site on the E protein at position N-154 and the fact that this site might contain carbohydrate of the complex type (endo H resistant) (Winkler

et al., 1987), we added an extra 2 kDa for the fragments comprising position 154 (e.g., Fr 23 and Fr 22). Because the E-8 MAb reacted with Fr 22 (starting at position 139) as well as with Fr 14 (starting at position 200), but not with Fr 23 (ending approximately at position 199) or Fr 16 (starting at position 327), we predict its binding site to be between amino acids 200 and 327 on the E glycoprotein (Fig. 5).

Low pH sensitivity of prM- and M-containing MVE viruses

Because it has been shown with West Nile virus that the E and prM glycoproteins remain as heterodimers during transport and maturation through acidic compartments of the exocytic route (Wengler and Wengler, 1989), we compared the infectivity of the prM-containing MVE virus with that of the M-containing MVE virus following virus exposure to acidic conditions. Equal PFUs of the prM- and M-virus containing supernatants from C6/36 or SW13 cells were subjected to environments of different pH values and their resultant infectivity in a plaque assay was examined (Fig. 6). No significant difference in infectivity of either virus preparation was observed after the incubation of viruses at pH values between 7.5 and 6.5. Upon incubation at pH 6.0, the infectivity of both M-containing viruses (grown in C6/36 or SW13 cells) was drastically abolished; however, this loss of infectivity was less dramatic for prM-containing viruses than for M-containing viruses (150-fold loss versus 1360-fold loss for viruses grown in C6/36 cells, Fig. 6A; and 17-fold loss versus 7000-fold loss

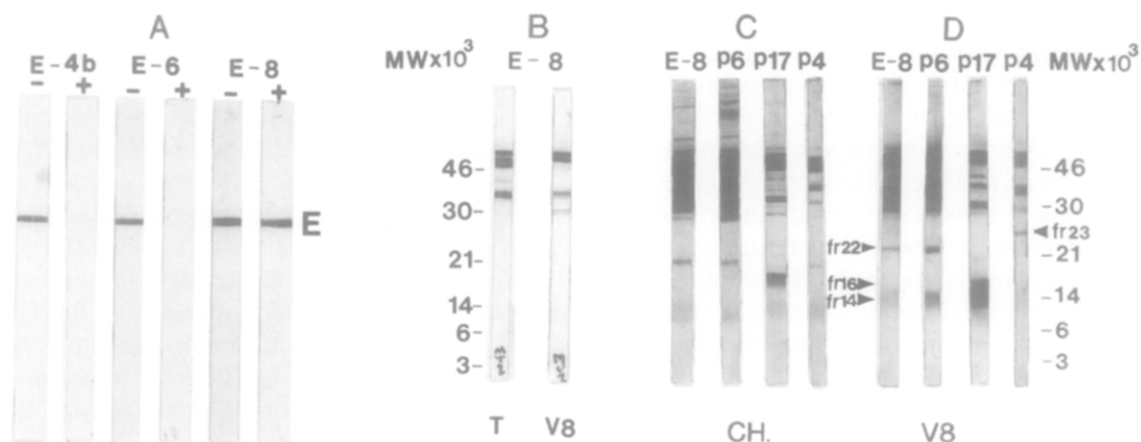


Fig. 4. Analysis of the immune fragments of the MVE virus E glycoprotein in Western blots. (A) Reactivity of the MAbs E-4b, E-6, and E-8 with SDS-denatured MVE virus (-) or SDS-denatured and reduced MVE virus (+). (B) Reactivity of the E-8 MAb with trypsin (T)- or V8-digested native MVE virus in Western blots. (C) and (D) Proteolytic digestion of MVE virus. MVE virus was denatured by SDS and subjected to limited digestion by either chymotrypsin or V8 protease. Proteins were separated by PAGE on 12.5% SDS gels and transferred to the nitrocellulose membrane by electroblotting. The reactivities of the synthetic peptide antisera P4 (MVE 04), P6 (MVE 06), P17 (MVE 17), or MAb E-8 with fragments generated by chymotrypsin (C) or V8 protease (D) have been shown. Those fragments which were subjected to the N-terminal sequencing (Table 1) have been marked by arrowheads.

TABLE 1
AMINO-TERMINAL SEQUENCES OF V-8 FRAGMENTS OF MURRAY VALLEY ENCEPHALITIS ENVELOPE PROTEIN

Fragments (kDa) ^a	Reactivities with peptide antisera and MAb ^b					Amino-terminal sequence
	P2	P4	P6	P17	E-8	
23	+	+	-	-	-	F-N-X-L-G-M
22	-	-	+	-	+	V-G-V-F-V-H-G
16	-	-	-	+	-	L-Q-Y-T-G-S-D-G
14	-	-	+	-	+	A-Y-Y-V-M-T-I-G

^a Fragments were generated by V-8 digestion of SDS-denatured purified MVE virus, separated on 12.5% SDS gels, and transferred to PVDF membranes by Western blotting. Membranes were stained with Coomassie blue and subjected to amino-terminal sequence analysis.

^b Fragments were generated as described above, transferred to nitrocellulose, and subjected to immune detection using MVE antipeptide antibodies (P2, P4, P6, P17; for sequence and length see Materials and Methods) or Mab E-8 as described under Materials and Methods.

for viruses grown in SW13 cells, Fig. 6B). Maximal loss of infectivity was achieved upon incubation at pH 4.5; nevertheless, the prM-containing viruses were still 10-fold (those grown in C6/36 cells) and 400-fold (those grown in SW13 cells) more resistant to the low pH environment than the M-containing viruses.

DISCUSSION

The proteolytic cleavage of prM occurs after a dibasic amino acid motif, presumably by a trypsin-like host enzyme similar to those responsible for normal processing of prohormones (Fischer and Scheller, 1988; Steiner *et al.*, 1984). Recently, an enzyme designated paired basic amino acid cleaving enzyme (PACE) has been cloned and purified from a human liver cell line (Wise *et al.*, 1990). A similar endoprotease was also isolated from the *trans*-Golgi membranes of rat liver cells, which was shown to cleave *in vitro* the F₀ protein of New Castle disease virus after a dibasic residue motif (Sakaguchi *et al.*, 1991). We believe that the late maturational processing of prM at the R-X-R/K-R cleavage motif is the mechanism used to activate the flavivirus fusion protein (E glycoprotein). The infectivity of prM-containing viruses (grown in C6/36 cells) for mammalian cells was 10-fold lower than that of the mature MVE (M-containing) viruses, although the amount of antigen detected by polyclonal antibodies was similar for both viruses. These results indicate that prM processing is required for full virus infectivity. A similar observation has been made with West Nile (WN) virus in which the ratio of physical particles to PFU was about 600 for the fully infectious extracellular (M-containing) viruses and was about 60-fold higher for the cell-associated (prM-containing) viruses (Wengler and Wengler, 1989).

Upon subsequent infection and endocytosis, the E glycoprotein probably undergoes a conformational

change in the acidic environment of endocytic vesicles, which results in membrane fusion and release of the nucleocapsid into the cytoplasm. Consistent with this hypothesis are our previous findings that the E glycoprotein of TBE virus undergoes irreversible conformational changes at pH less than 6.2. These changes, which predominantly occur in the A-domain epitopes and the i2 site (Guirakhoo *et al.*, 1989; Mandl *et al.*, 1989), are probably necessary to trigger the fusion activity of the E glycoprotein. It has been shown that the fusion activity of the TBE virus can be blocked effectively by three MAbs, two of which were specific for epitopes of the A-domain, and one recognized the i2 epitope (Guirakhoo *et al.*, 1991). The A-domain contains an amino acid sequence (G L F G) that is conserved in the fusion sequences of other enveloped viruses. We have also observed with dengue virus that the region containing the conservative fusion sequence is more accessible to antipeptide antibodies following low pH treatment (Roehrig *et al.*, 1990). By using MAbs specific for MVE virus E glycoprotein in a capture ELISA, we demonstrated that if the processing of prM was interrupted at least three of these epitopes on the E glycoprotein were less accessible on the virus particle. The observation that all of the epitopes were acid sensitive indicates that they are probably located in the A-domain. The sensitivity of the HIAF to low pH also suggests that these epitopes are the predominant epitopes recognized following immunization with intact virus. It may be significant that these three epitopes are associated with hemagglutination of red blood cells. At least one of these epitopes (E-8) also elicits virus-neutralizing antibodies. Since other functionally important epitopes which induce neutralizing, hemagglutination-inhibiting, and protective antibodies (Hawkes *et al.*, 1988) are still present in the prM-containing viruses (Fig. 2 A), and the fact that these viruses are still capable of binding to and infecting their host cells, we

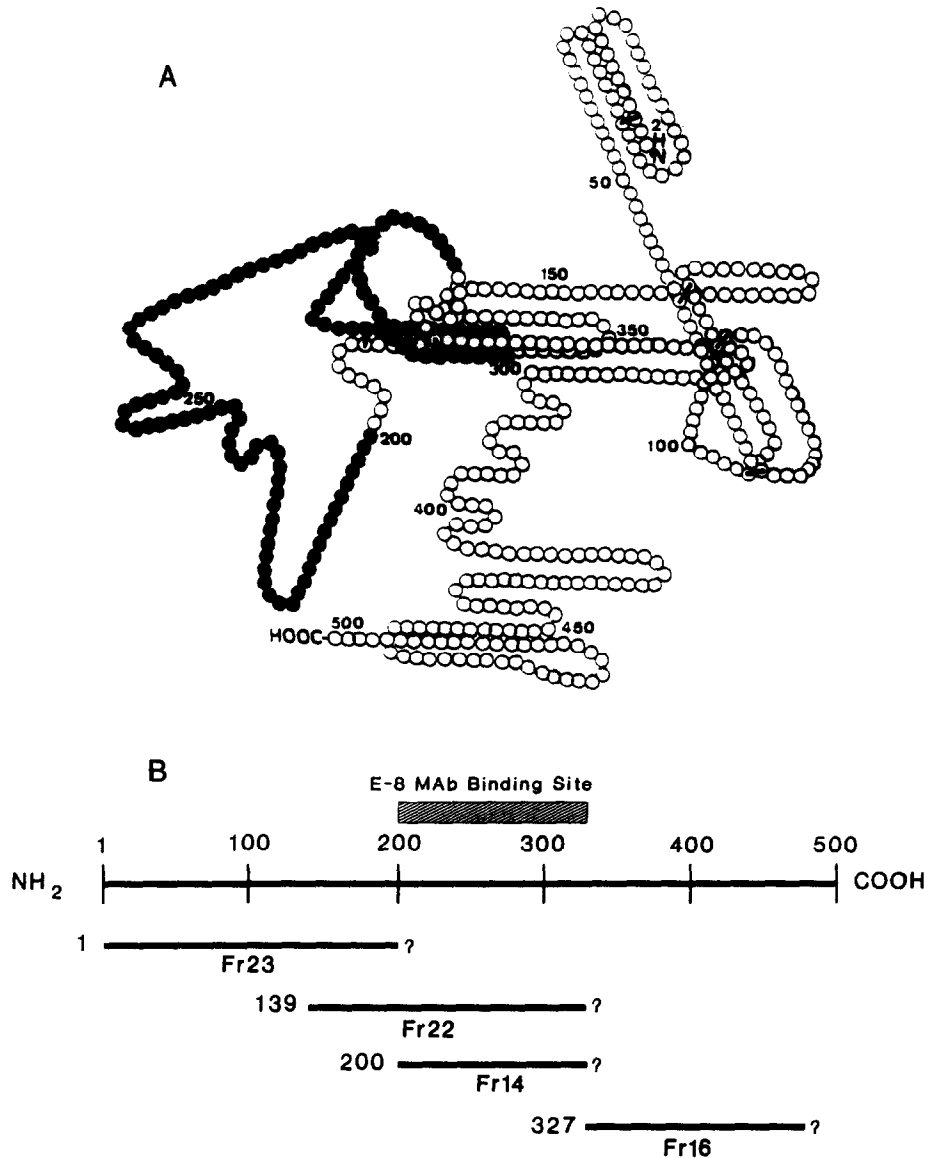


FIG. 5. Estimation of the area on the E protein which interacts with prM by identification of the E-8 binding site. (A) The site on the E glycoprotein which interacts with prM (darkened amino acid circles) was estimated by the identification of the segment containing the E-8 MAb binding site (cross-hatched area on B). The protein model has been adapted from Roehrig *et al.* (1989). (B) Fragments generated by V8 digestion of the MVE virus are shown on the linear sequence of the E protein. The carboxy-terminal end (?) of each fragment was approximated considering the size of fragments (for those fragments which included position 154, 2 kDa was added to the calculated MW for the carbohydrate moiety), the reactivity pattern with the antipeptide antibodies (some of which have been shown in Figs. 4C and 4D), and the specificity of the V8 protease for E or D amino acid residues.

did not expect that the fusion peptide or the receptor-binding sites would be directly involved in the prM-E interactions. This was confirmed when the immune-reactive fragments generated by V8 protease were sequenced. It was shown that predominantly three sites on the E glycoprotein were exposed to the proteolytic attack of the V8 enzyme. This cleavage, which occurred on positions 138, 199, and 326, generated four fragments reactive with sequence-specific antipeptide antibody or E-8 MAb. Similar segments on the E pro-

tein of WN virus, which are located in L1 and R3, have also been shown to be exposed to proteolytic attack (Wengler *et al.*, 1987). Combining results of the reactivity pattern of the antipeptide antibodies with proteolytic fragments and also information obtained from N-terminal sequence analysis of the immune fragments, we were able to map the binding site of the E-8 MAb which was covered or dissociated by the presence of prM. This site which reside between amino acids 200 and 327 on the E protein includes more than 80% of the R2

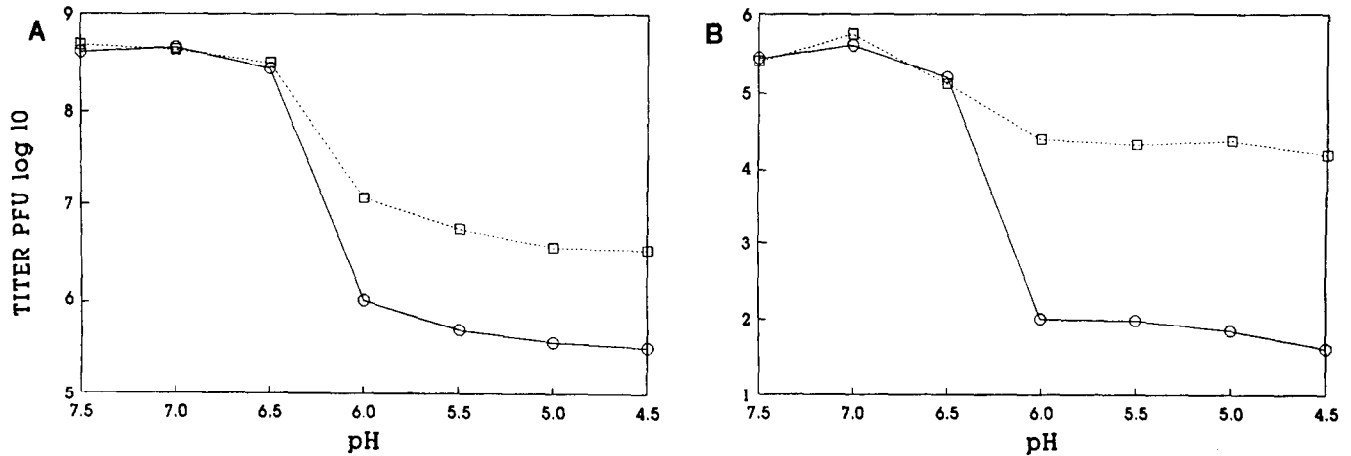


Fig. 6. Resistance of prM- and M-containing MVE viruses to various pHs. Equal amounts of PFU from the supernatants of ammonium chloride-treated and untreated MVE virus-infected cells were incubated at different pH values, ranging from 7.5 to 4.5, and examined for infectivity in Vero cells using the standard plaque assay. (A) The pH sensitivities of M- and prM-containing MVE viruses grown in C6/36 cells. (B) The pH sensitivities of M- and prM-containing MVE viruses grown in SW13 cells. Symbols: \circ , M-containing MVE viruses; \square , prM-containing MVE viruses.

domain proposed for WN (Nowak and Wengler, 1987) and MVE (Roehrig *et al.*, 1989) viruses. No actual biological function of the R2 domain had been previously identified. It is noteworthy that the synthetic peptide MVE 06, which consists of 21 amino acids within the R2 domain (230–251), was cross-reactive with other members of the flaviviruses (Roehrig *et al.*, 1989), indicating the biological importance of this region.

Several studies have indicated that when influenza A virus and Semliki Forest virus were incubated at low pH they became irreversibly inactivated (Doms *et al.*, 1985; Kielian and Helenius 1985; Stegman *et al.*, 1989; White *et al.*, 1982). This is probably due to exposure and self-aggregation of the hydrophobic fusion peptide which, therefore, can no longer interact with target membranes (Nir *et al.*, 1990). If the flavivirus E glycoprotein were not protected during transport to the cell surface, the acidic milieu of the post-Golgi vesicles could inactivate it. Because it has been shown with WN virus that the E and prM proteins remain as heterodimers during exocytosis (Wengler and Wengler, 1989), we reasoned that the function of the prM is to prevent conformational changes in the E glycoprotein at low pH, thus assuring safe transport of these proteins through post-Golgi acidic vesicles during maturation until the virions are released. A similar role has also been proposed for the PE2 of alphaviruses, which forms a stable dimer with the E1 protein (critical for infection and fusion) in the acidic compartment of the exocytic route (Lobigs and Garoff, 1990; Presley *et al.*, 1991; Wahlberg *et al.*, 1989). Different approaches might be utilized by other viruses to secure the authentic expression of the envelope proteins on the infected cells. It has been shown that the M2 protein of influ-

enza virus raises the pH of the post-Golgi vesicles by about 0.8 unit, thus preventing the expression of the low pH conformation of the HA molecules on the plasma membranes of the infected cells (Ciampor *et al.*, 1992). The high resistance of the prM-containing viruses to low pH might indicate that the prM could shield some segments of the E protein which are otherwise protonated in a low pH environment, leading to the exposure of the fusion peptide and consequent loss of infectivity. Concomitant with the low pH sensitivity of the M-containing viruses was a loss of epitope reactivity in ELISA following incubation at pH 6.0 or below. Because prM-containing viruses are still infectious, apparently the prM protein can be cleaved after the virus has undergone endocytosis. Using chymotrypsin, trypsin, and thermolysin it was demonstrated that the prM protein of the WN virus is highly sensitive to proteases (Wengler *et al.*, 1987). In our laboratory we also observed that by using a low concentration of trypsin, the prM protein of the MVE virus is cleaved prior to the E protein (data not shown).

Our results predict that the expression of authentic E glycoprotein requires the coexpression of prM. This would explain why flavivirus vaccine candidates produced from the expression of only E glycoprotein were partially protective against homotypic challenge, whereas those prepared from the expression of both prM and E glycoproteins gave a significant level of protection (Bray and Lai, 1991). Similar results were observed with Japanese encephalitis virus, where high neutralizing and hemagglutination-inhibiting antibodies which correlated with a high level of protection were found in mice inoculated with recombinant vaccine viruses expressing both prM and E but not E pro-

tein alone (Konishi *et al.*, 1991; Mason *et al.*, 1991; Yasuda *et al.*, 1990).

We are currently performing experiments to define further the role of prM in virus infectivity and the events occurring after prM-containing viruses enter their target cells. We are also attempting to determine if a similar area on the E protein of other flaviviruses is involved in the prM interaction.

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