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# Recombinant Subviral Particles from Tick-Borne Encephalitis Virus Are Fusogenic and Provide a Model System for Studying Flavivirus Envelope Glycoprotein Functions

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**Recombinant subviral particles (RSPs) obtained by coexpression of the envelope (E) and premembrane (prM) proteins of tick-borne encephalitis virus in COS cells (S. L. Allison, K. Stadler, C. W. Mandl, C. Kunz, and F. X. Heinz, J. Virol. 69:5816–5820, 1995) were extensively characterized and shown to be ordered structures containing envelope glycoproteins with structural and functional properties very similar to those in the virion envelope. The particles were spherical, with a diameter of about 30 nm and a buoyant density of 1.14 g/cm$^3$ in sucrose gradients. They contained mature E proteins with endoglycosidase H-resistant glycans as well as fully cleaved mature M proteins. Cleavage of prM, which requires an acidic pH in exocytic compartments, could be inhibited by treatment of transfected cells with ammonium chloride, implying a common maturation pathway for RSPs and virions. RSPs incorporated [$^{14}$C]choline but not [$^3$H]uridine, demonstrating that they contain lipid but probably lack nucleic acid. The envelope proteins of RSPs exhibited a native antigenic and oligomeric structure compared with virions, and incubation at an acidic pH (pH <6.5) induced identical conformational changes and structural rearrangements, including an irreversible quantitative conversion of dimers to trimers. The RSPs were also shown to be functionally active, inducing membrane fusion in a low-pH-dependent manner and demonstrating the same specific hemagglutination activity as whole virions. Tick-borne encephalitis virus RSPs thus represent an excellent model system for investigating the structural basis of viral envelope glycoprotein functions.**

For many years, the hemagglutinin of influenza virus was the only fusion-active protein and the only receptor-binding protein from an enveloped virus whose structure was known at atomic resolution (40). Recently, however, the solution of the X-ray crystal structure of the major envelope protein of a flavivirus, tick-borne encephalitis (TBE) virus (35), has provided new opportunities to carry out structure-based studies in a completely different virus system. Such investigations, however, require suitable experimental models that allow detailed analysis of structure-function relationships.

It is a common characteristic of flavivirus infections that, in addition to infectious virions, noninfectious subviral particles which contain the viral envelope proteins but lack the nucleocapsid are released (36). It was demonstrated by Mason and coworkers (29) that similar particles can be obtained in secreted form when flavivirus envelope proteins are coexpressed in the absence of the capsid protein. It has been suggested that these represent capsidless empty viral envelopes (29).

Flavivirus subviral particles have since been reported in a number of other studies in which cloned envelope protein genes were expressed by using poxvirus vectors (6, 21–24, 32, 37, 42) or a Sindbis virus replicon system (33). Although these studies have demonstrated the potential importance of subviral particles for the development of recombinant flavivirus vaccines, they have not been extensively characterized with respect to their other functional properties, and their utility as an experimental model for studying viral functions has not yet been evaluated.

TBE virus, like the related flaviviruses yellow fever virus, Japanese encephalitis virus (JEV), and the dengue viruses, is an important human pathogen (30). The mature virions are composed of a nucleocapsid consisting of the viral RNA and a single capsid protein (C), surrounded by a lipid membrane containing the major envelope protein E and the small membrane protein M.

Protein E is responsible for mediating viral entry functions and inducing a protective immune response (reviewed in reference 15). In contrast to the influenza virus hemagglutinin, the E protein of TBE virus has been shown to be a head-to-tail homodimer which apparently lies parallel to the viral membrane, rather than forming protruding spikes (35). Biochemical and structural evidence has suggested that these dimers are organized in a network-like structure involving lateral contacts between dimers (2, 12, 35).

Exposure of virions to mildly acidic conditions corresponding to the pH of membrane fusion induces a rearrangement of the envelope structure in which the protein E dimers are quantitatively converted to trimers, apparently because of conformational changes which simultaneously weaken dimeric contacts and strengthen lateral interactions between adjoining monomers from different dimers (2). The two oligomeric states of the TBE virus E protein reflect the bifunctional nature of this protein and its role in both receptor binding and acid-induced fusion (35). The native dimeric configuration is probably required for recognition of a still-unidentified cellular receptor, whereas conversion to the trimeric form at low pH appears to be necessary for membrane fusion (2, 10, 16, 38).

The small membrane protein M, whose function in mature

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virions is unknown, is initially synthesized as a larger precursor, designated prM (4). Proteins prM and E form heterodimers (3, 39), and these are incorporated into immature virions which are assembled in the endoplasmic reticulum (reviewed in reference 31). The presence of prM in these immature particles apparently renders them unable to undergo low-pH-induced rearrangements (2, 8, 16) and fusion (8, 10), suggesting that one of its principal functions is to protect the virion against premature fusion activation during intracellular transport through acidic compartments. A proteolytic cleavage of prM by furin or a similar cellular protease (reviewed in reference 19) shortly before release from the cell apparently serves to activate the fusion potential of the virus.

Recently, it was shown that recombinant subviral particles (RSPs) can be produced in a virus-free system by transfection of COS-1 cells with a recombinant plasmid encoding the prM and E proteins of TBE virus (3). We have now characterized these particles in detail, and in this report we provide evidence that RSPs are ordered structures in which the envelope proteins are organized and function in a way similar to those in the virion envelope. They resemble whole virions in their ability to undergo low-pH-induced rearrangements and to induce membrane fusion, making them an excellent model system for investigating the structural basis for viral entry functions.

## MATERIALS AND METHODS

**DNA transfections.** RSPs were generated by transfection of COS-1 cells (ATCC CRL 1650) with the recombinant plasmid SV-PEwt (1), which contains the TBE virus prM and E genes under the control of the simian virus 40 early promoter, and has been shown previously to lead to secretion of RSPs when expressed in COS cells (3). In some experiments, 50 mM ammonium chloride was added to the medium 20 to 22 h posttransfection to inhibit cleavage of prM, and after 1 h the supernatants were discarded and replaced by fresh ammonium chloride-containing medium. Incubation was then continued for another 24 h until supernatants were harvested.

**Purification of RSPs and virions.** Cell supernatants containing RSPs were cleared by centrifugation at 10,000 rpm (16,000 × *g*) for 30 min at 4°C in a Sorvall F16/250 rotor. The particles were then pelleted by centrifugation at 44,000 rpm for 120 min at 4°C in a Beckman 45 Ti rotor and resuspended in TAN buffer (0.05 M triethanolamine [pH 8.0], 0.1 M NaCl). The RSPs were purified first by rate zonal centrifugation in a 5 to 20% sucrose gradient at 38,000 rpm at 4°C for 90 min, followed by an overnight equilibrium density centrifugation in a 20 to 50% sucrose gradient at 35,000 rpm and 4°C. All gradients were made with TAN buffer and were centrifuged in a Beckman SW-40 rotor. Fractions of 0.6 ml were collected by upward displacement and assayed for hemagglutination (HA) activity at pH 6.4 by the method of Clarke and Casals (5), with goose erythrocytes. For experiments in which highly purified material was not required, RSPs were pelleted from clarified supernatants, resuspended in TAN buffer, and used directly for analysis. In some experiments 0.1% bovine serum albumin was included in the buffer as a stabilizer.

TBE virus strain Neudoerfl (28) grown in primary chick embryo cells and purified as described previously (14) was used as the virus control in all experiments. Protein E in virus and RSP preparations was quantitated by four-layer enzyme-linked immunosorbent assay (ELISA) as described previously (17).

**Buoyant density determination.** Equilibrium density gradients for analysis of RSPs and virions were as described in the purification procedure. The sucrose density in gradient fractions was determined both by precision weighing at 4°C and by refractometry at 21°C with an Abbe refractometer (Atago), with corrections for temperature by using standard tables (ISCO).

**Electron microscopy.** Purified virus or RSP samples were adsorbed for 5 min onto glow-discharged Formvar/carbon-coated cupron grids (Agar Scientific, Stansted Essex, England) and stained for 4 min with 1% uranyl acetate (pH 4.5). The preparations were visualized using a Zeiss EM 10 electron microscope.

**Gel electrophoresis, immunoblotting, cross-linking, and endoglycosidase treatment.** The proteins of purified virions and RSPs were analyzed by precipitation with trichloroacetic acid, separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (25), and staining with PhastGel Blue R (Pharmacia).

For immunodetection, proteins were blotted from gels onto polyvinylidene difluoride membranes (Bio-Rad) with a Bio-Rad Trans-blot semidry transfer cell. After incubation at 4°C in phosphate-buffered saline (pH 7.4) containing 1% BSA and 0.1% Tween 20 to block nonspecific binding, membranes were incubated for 60 min at room temperature with a polyclonal rabbit serum obtained by immunization with whole purified TBE virus. Membranes were then washed three times, and specifically bound immunoglobulin was visualized with peroxidase-labeled donkey anti-rabbit immunoglobulin together with 3,3′-diaminobenzidine-$H_2O_2$ and $NiCl_2$ as described by the manufacturer (Bio-Rad).

Cross-linking analysis of solubilized protein E oligomers with dimethylsuberimidate (DMS; Pierce Chemical Co.) in the presence of 0.5% Triton X-100 was done as described previously (2), with the exception that viral proteins were detected by immunoblotting rather than Coomassie blue staining.

*N*-Glycosidase F (PNGase F) and endoglycosidase H (endo H) were purchased from Boehringer Mannheim and used according to the manufacturer's instructions. Glycosidase-treated protein E samples were analyzed by SDS-PAGE and immunoblotting as described above.

**Metabolic labeling.** For the analysis of incorporation of lipids in RSPs, 250 μCi of [methyl-$^{14}$C] choline chloride (Amersham) in 40 ml of culture medium was added immediately after transfection of COS cells. After a labeling period of 22 h the medium was discarded, the cells were washed, and incubation was continued 26 h in the absence of radioactive lipid. RSPs were harvested, partially purified by rate zonal sucrose density centrifugation, and analyzed by equilibrium density centrifugation as described above. The radioactivity in gradient fractions was determined by liquid scintillation counting.

For the analysis of RNA incorporation, transfected COS cells or BHK-21 cells infected with TBE virus at a multiplicity of infection of approximately 1 were labeled for 48 h with 0.5 mCi of [5,6-$^3$H]uridine (Amersham) in 40 ml of culture medium. Virus and RSPs were then harvested, partially purified, and analyzed as described above.

**Low-pH incubation.** RSP or virion preparations at a concentration of 5 to 15 μg/ml in TAN buffer plus 0.1% BSA were acidified by the addition of a buffer (0.1 M MES [morpholineethanesulfonic acid] or Bis-Tris, 0.05 M NaCl, 0.1% BSA) that had been pretitrated to yield the desired final pH. After a 10-min incubation at 37°C, the pH was adjusted back to 8.0 with a predetermined amount of alkaline buffer (0.1 M triethanolamine, 0.1 M NaCl, 1% BSA).

**Sedimentation analysis.** For determination of the oligomeric state of protein E based on sedimentation properties (2), samples were treated for 1 h at room temperature with 0.5% Triton X-100 and analyzed by centrifugation (38,000 rpm, 20 h, 15°C) in 7 to 20% sucrose gradients (in TAN buffer [pH 8.0]) containing 0.1% Triton X-100 to prevent aggregation. Fractions (0.6 ml) were collected by upward displacement, and protein E was quantitated by four-layer ELISA (17).

**Antigenic structure analysis.** To assess the conformational state of protein E in virions and RSPs, the binding of 18 different protein E-specific monoclonal antibodies (MAbs) (9, 18) was determined in a four-layer ELISA as described previously (17). All samples were tested at a protein E concentration of 1 μg/ml with a single MAb dilution which was adjusted to an absorbance value between 0.5 and 1.8.

**Cell fusion assay.** To determine fusion activity, a fusion-from-without (FFWO) assay with C6/36 mosquito cells was used as described previously (10). Cells grown in 96-well Primaria tissue culture plates (Falcon) were precooled, incubated for 90 min on ice with RSPs that had been collected by centrifugation from cell supernatants, and resuspended at a final protein E concentration of approximately 500 μg/ml. As a control, an equivalent amount of purified TBE virus was used. After the mixture was washed to remove unbound material, cells were incubated for 2 min at 40°C in culture medium buffered with either 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 7.6]) or 20 mM MES (pH 5.5). The medium was then replaced by growth medium, and incubation at 40°C was continued for 2 h. Cells were fixed with a 1-to-1 mixture of methanol and acetone and stained with Giemsa's solution (Fluka).

## RESULTS

**Physical properties of RSPs.** RSPs secreted from COS cells expressing the TBE virus prM and E proteins (3) were purified by rate zonal and equilibrium density gradient centrifugation in order to investigate their structural and functional properties.

Figure 1A shows the equilibrium banding profiles of RSPs and virions in sucrose density gradients. The RSPs banded at a sucrose density of 1.14 g/cm$^3$, compared with 1.19 g/cm$^3$ for whole virions. The latter value is consistent with earlier published measurements with purified TBE virus (11). The difference in buoyant density between RSPs and whole virions can be attributed to the lack of a nucleocapsid in the RSP (see below).

Electron microscopy of purified RSPs stained with uranyl acetate (Fig. 1B) revealed spherical particles with a diameter of about 30 nm, which is about two-thirds of that of whole virions (Fig. 1B).

**Analysis of glycoproteins.** Comparison of proteins from purified RSPs and virions by SDS-PAGE demonstrated the presence of mature M and E proteins with identical electrophoretic

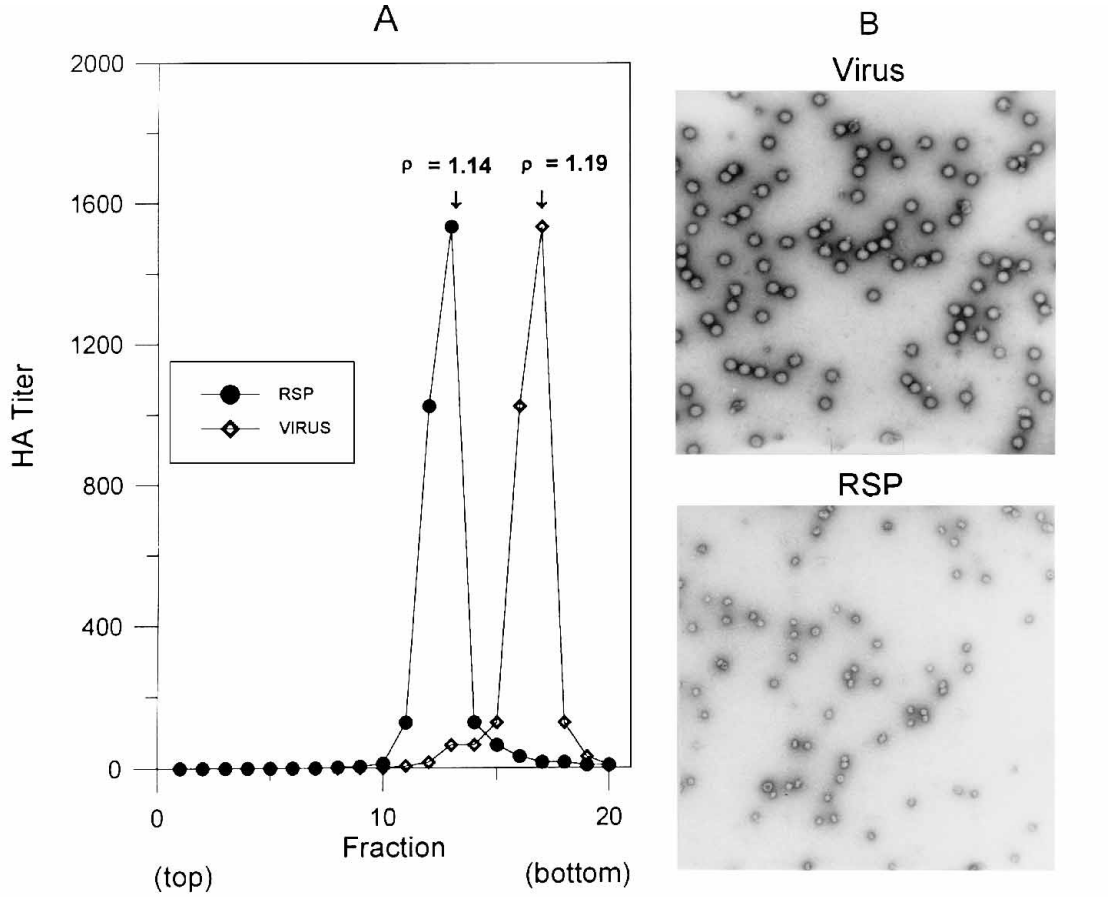


FIG. 1. Comparison of physical properties of RSPs and virions. (A) Equilibrium banding profiles of RSPs and virions in parallel sucrose density gradients. Equivalent amounts of virus and RSPs were loaded onto each gradient to yield equal HA titers. The buoyant density values for RSPs and virions are indicated above the peaks. (B) Electron micrographs at 50,000-fold magnification of virions (upper panel) and RSPs (lower panel) stained with uranyl acetate.

mobilities, but the RSPs, as expected, lacked the capsid protein (Fig. 2A).

Flaviviruses are initially assembled as immature virions containing the M precursor protein, prM, and it has been shown that proteolytic cleavage of prM, which occurs during exocytosis, can be blocked by treatment with ammonium chloride or other agents that raise the pH of acidic exocytic compartments (8, 10, 16, 34). To determine if the assembly and maturation of RSPs occur in a similar manner, RSPs from cells treated with 50 mM ammonium chloride were compared with those from untreated cells by immunoblotting. As shown in Fig. 2B, RSPs from the $NH_4Cl$-treated cells appeared to contain almost exclusively uncleaved prM, migrating at the same position as prM from immature virions, whereas the untreated controls contained mature M.

The glycosylation state of protein E in RSPs was examined with protein PNGase F, which cleaves both high-mannose and complex-type oligosaccharides, and endo H, which is specific for high-mannose-type oligosaccharides. Comparison of glycosidase-treated RSPs and virions by SDS-PAGE and immunoblotting showed that like their viral counterparts, the E proteins in the recombinant particles contained complex glycans which were susceptible to cleavage by PNGase F but resistant to endo H (Fig. 2C), indicating that both virus and RSP pass through the Golgi during cellular transport. However, while the RSPs contained almost exclusively endo H-resistant glycans, the virion preparations, consistent with earlier observations (41), contained a mixture of endo H-resistant and -sensitive forms. Incomplete glycan processing in virion E proteins was observed consistently regardless of whether the virus was grown in chick embryo cells (Fig. 2C) or in COS cells (data not shown).

**Evidence that RSPs contain a lipid membrane.** Flavivirus subviral particles are sensitive to treatment with Triton X-100 (3, 23), a property which is consistent with, but does not prove, the presence of a lipid membrane. To investigate whether RSPs actually contain membrane lipids, transfected cells were treated with [$^{14}$C]choline, and the secreted particles were partially purified and analyzed on sucrose density gradients. As shown in Fig. 3, radioactive material was found almost exclusively in the hemagglutinating fractions containing the RSPs. To confirm that the radioactivity of the particles was actually due to incorporation of lipid, the RSPs were subsequently solubilized in 0.5% Triton X-100 and then subjected to rate zonal centrifugation to separate the lipid and protein components. After solubilization, essentially all of the radioactivity previously associated with the particle was found in the lipid fraction, separate from the proteins (data not shown). These data provide further evidence that RSPs contain a lipid membrane.

**Evidence that RSPs lack nucleic acid.** Since RSPs do not possess a capsid protein and do not have full-length viral RNA available for packaging, it was not clear whether nucleic acid would represent a significant component of these particles. To

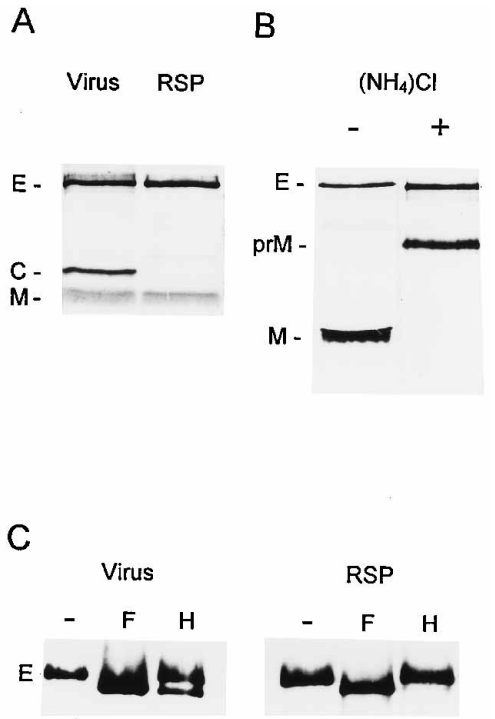


FIG. 2. Analysis of RSP proteins. (A) Coomassie blue-stained SDS-polyacrylamide gel of proteins from RSPs and virions purified on rate zonal and equilibrium density sucrose gradients. The positions of the E, C, and M protein bands are indicated. (B) Inhibition of cleavage of prM by treatment of cells with 50 mM ammonium chloride. Proteins from RSPs purified from supernatants of untreated (left lane) or ammonium chloride-treated cells (right lane) were analyzed by SDS-PAGE and immunoblotting with a polyclonal rabbit antiserum that recognizes the E, prM, and M proteins, whose positions are indicated. (C) Endoglycosidase treatment of E protein in virions and RSPs. Samples were treated with PNGase F (lanes 2 and 5) or Endo H (lanes 3 and 6) and compared with untreated controls (lanes 1 and 4) by SDS-PAGE and immunoblotting. Lanes: 1 to 3, virions; 4 to 6, RSPs.

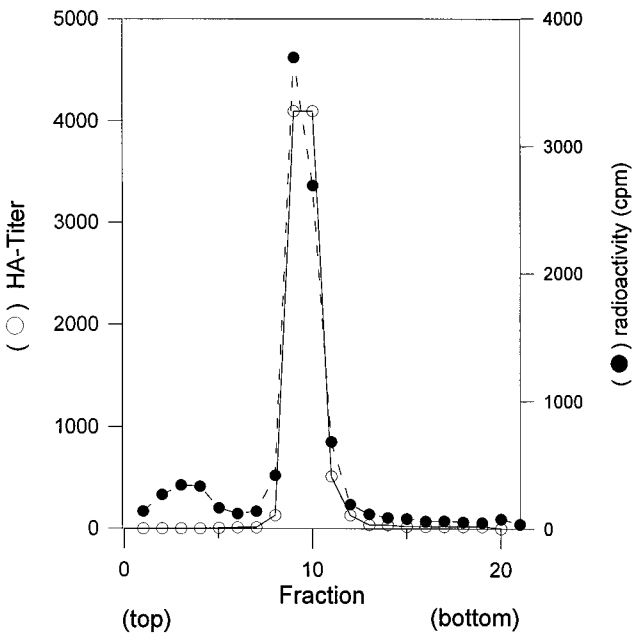


FIG. 3. Sucrose density gradient centrifugation of partially purified RSPs from COS cells labeled with [$^{14}$C]choline. The HA activity (open circles) and $^{14}$C radioactivity (closed circles) of the gradient fractions are shown.

address this question, RSPs were isolated from the supernatant of transfected COS cells that had been labeled with [$^{3}$H]uridine, and equilibrium centrifugation in a sucrose density gradient was used to assess whether radioactive nucleic acid had been incorporated into the particles. As a control, virus-infected BHK cells were treated in an identical manner, and the resulting virions were analyzed in a parallel sucrose gradient after dilution to the same HA titer as the RSP preparation. As shown in Fig. 4A, the virion particles clearly cobanded with a peak of $^{3}$H radioactivity due to incorporation of labeled uridine into the viral genomic RNA. In contrast, the RSPs did not appear to have incorporated nucleic acid (Fig. 4B). This preparation contained two broad peaks of radioactive material which did not coincide with the hemagglutinating peak and probably represent impurities in the sample. However, since one of these peaks tailed slightly into the RSP peak, the possibility of some low-level incorporation cannot be rigorously excluded.

Previous attempts to detect JEV-specific RNA in JEV-derived subviral particles by reverse transcription and PCR (23) have yielded negative results. The data shown here not only support those earlier conclusions but also argue against the presence of any nucleic acid, whether of viral or cellular origin, as a significant structural component of RSPs.

**Conformation, oligomeric state, and low-pH-induced rearrangements of protein E.** To assess the state of the envelope proteins on the surface of RSPs, the quaternary and tertiary structures of protein E on native and low-pH-treated RSPs and virions were compared, and their potential to undergo irreversible structural rearrangements at low pH were investigated.

Native virions and RSPs were pretreated for 10 min at pH 8.0 or pH 6.0, neutralized to pH 8.0, solubilized in 0.5% Triton X-100, and analyzed by rate zonal centrifugation on sucrose gradients under conditions allowing protein E monomers, dimers, and trimers to be distinguished (2). Consistent with earlier observations (2, 13), Triton X-100-solubilized virions at pH 8.0 yielded a defined protein E peak in sucrose gradients at the position predicted for the E dimer (Fig. 5A). The dimeric nature of this material (Fig. 5A, inset) was further confirmed by dimethyl sulfoxide DMS cross-linking as shown previously (2, 12). Cross-linking of oligomeric proteins with such reagents is typically incomplete (7) because of saturation of reactive sites before intersubunit cross-links can be formed. When RSPs at pH 8.0 were solubilized and analyzed in the same manner, they also yielded dimeric E proteins, whose sedimentation behavior (Fig. 5B) and cross-linking pattern (Fig. 5B, inset) were identical to those of the virion-derived E dimers (Fig. 5A).

A 10-min exposure of virions to pH 6.0, as expected, caused a conversion of protein E dimers to trimers (2) as demonstrated by a shift in the sedimentation profile as well as the appearance of a trimeric protein E band after cross-linking (Fig. 5A). An identical change in sedimentation velocity and cross-linking pattern was also observed with RSPs (Fig. 5B), indicating that a similar quantitative irreversible rearrangement had occurred on exposure of RSPs to acidic pH.

As a means of evaluating the E protein tertiary structure, native virions and RSPs were tested for reactivity with a panel of 18 MAbs by a four-layer ELISA (17). This method allows the detection of irreversible changes only, since the samples are first back-neutralized, and subsequent incubation steps are carried out at pH 7.4. These MAbs have been shown to rec-

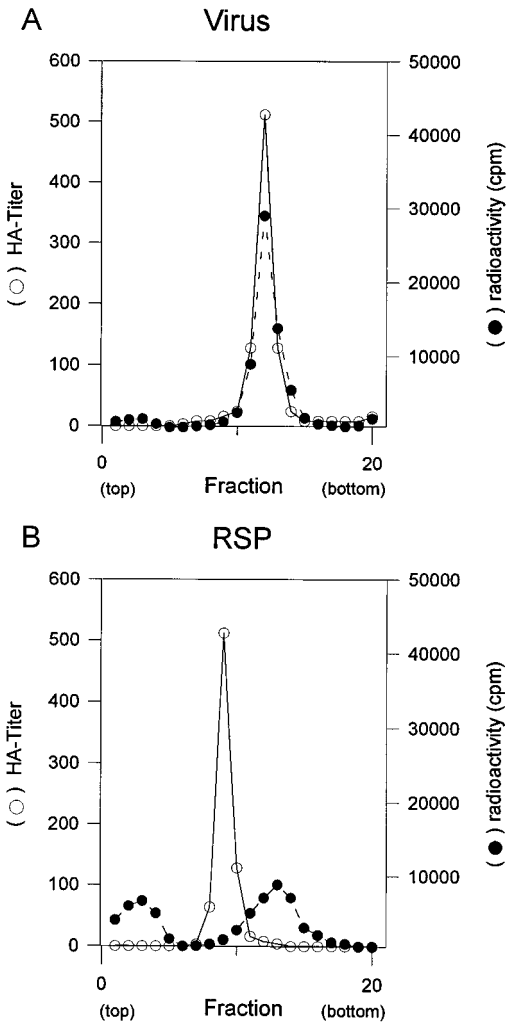


FIG. 4. Sucrose density gradient centrifugation of partially purified virions (A) and RSPs (B) labeled with [$^3$H]uridine. The HA activity (open circles) and $^3$H radioactivity (closed circles) are shown.

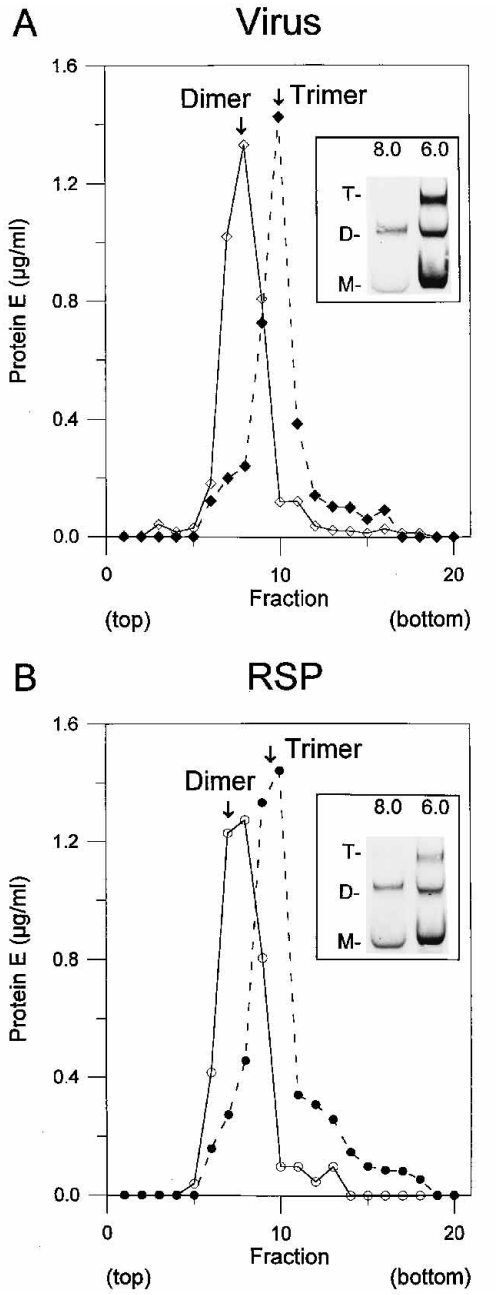


FIG. 5. Oligomeric rearrangement of protein E after low-pH treatment. Preparations of virus (A) and RSPs (B) were incubated either at pH 8.0 (solid lines) or at pH 6.0 followed by neutralization (broken lines), solubilized with 0.5% Triton X-100, and analyzed by sedimentation in 7 to 20% sucrose gradients containing 0.1% Triton X-100. The amount of protein E in the fractions was determined by four-layer ELISA. (Insets) Immunoblots of peak fractions cross-linked with DMS and separated by SDS-PAGE. The positions of protein E monomers (M), dimers (D), and trimers (T) are indicated.

ognize distinct epitopes within the three structural domains of protein E (9, 18, 27), and this finding has been confirmed by X-ray crystallography (35). Most of these MAbs recognize discontinuous conformation-sensitive epitopes and have been shown to be useful for assessing the conformational state of the protein (9, 18, 27) and conformational changes occurring at low pH (16, 18).

When MAb reactivities of the native (pH 8.0) forms were compared (Fig. 6A), virions and RSPs yielded nearly identical reactivity profiles. After treatment at low pH, the same dramatic change was observed in the MAb reactivity profiles of both forms, indicating that in each case a similar irreversible conformational change had occurred. It therefore appears that both forms contain structurally native E proteins and that these proteins undergo a low-pH-induced rearrangement in essentially the same manner, yielding protein E trimers with very similar overall structures.

For a closer examination of the pH dependence of these changes, RSPs and virions were treated for 10 min at different pH values and back-neutralized. Trimer formation and reactivity with MAb i2, which exhibits a significantly reduced reactivity with the low-pH-treated forms, were then monitored in a four-layer ELISA as described above. In agreement with earlier observations (2), formation of E trimers and loss of reactivity with MAb i2 under these conditions began to occur in virions at a threshold of approximately pH 6.5 and was complete by pH 6.0 (Fig. 7). When virions were compared with RSPs treated in the same manner, no significant differences in pH dependence were observed, and the pH threshold was approximately 6.5 in each case.

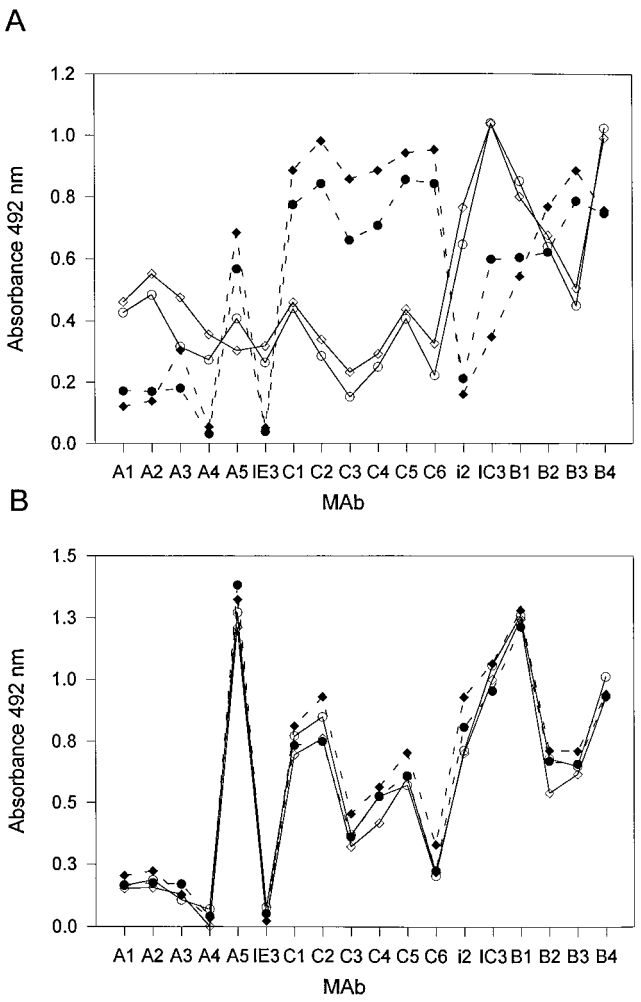


FIG. 6. Reactivity of 18 E-specific MAbs with mature (A) and immature (B) virions (diamonds) and RSPs (circles). Reactivities at neutral pH are indicated by open symbols, and those after incubation at pH 6.0 and neutralization are indicated by solid symbols.

**Function of prM in RSPs.** It has been shown previously that the low-pH-induced rearrangements described above do not occur in immature virions containing uncleaved prM proteins (2, 16). To see whether the presence of prM in RSPs would have a similar inhibitory effect, prM-containing RSPs obtained from ammonium chloride-treated cells (Fig. 2B) or immature virions (16) were treated at pH 8.0 and pH 6.0, and their MAb reactivity profiles were compared. As shown in Fig. 6B, consistent with earlier observations with virions (16), the presence of prM caused significant changes in several epitopes of the E protein (compare Fig. 6A), which were identical for prM-containing RSPs and immature virions. In both cases, the reactivity profile remained unchanged after low-pH treatment (Fig. 6B). Also consistent with earlier studies with immature virions (2), sedimentation analysis after solubilization of low-pH-treated prM-containing RSPs with Triton X-100 did not reveal any E trimer formation or other change in oligomeric state (data not shown). These results suggest that prM in RSPs interacts with E and functions in a manner identical to that in virions.

**Low-pH-dependent functions of membrane fusion and hemagglutination.** TBE virus-mediated membrane fusion requires an acidic pH (10, 38), and it is believed that this pH dependence is related to the ability to undergo the low-pH-induced structural rearrangements described above. To test whether RSPs exhibit fusion activity, a fusion from without (FFWO) assay with the C6/36 mosquito cell line was employed (10). As shown in Fig. 8, the addition of RSPs to the cells followed by a brief treatment at pH 5.5 resulted in extensive syncytium formation, similar to that observed with whole virions under the same conditions. In contrast, no fusion was observed when cells were incubated with virions or RSPs at pH 7.6. Thus, RSPs are functionally active and, like whole virions, are capable of inducing membrane fusion in a pH-dependent manner.

The specific HA activity of RSPs, determined according to the method of Clarke and Casals (5) at pH 6.4 with goose erythrocytes, was found to be identical to that of virions (titer of 128 at a E protein concentration of 1 μg/ml). Since HA activity of flaviviruses is a phenomenon occurring only at acidic

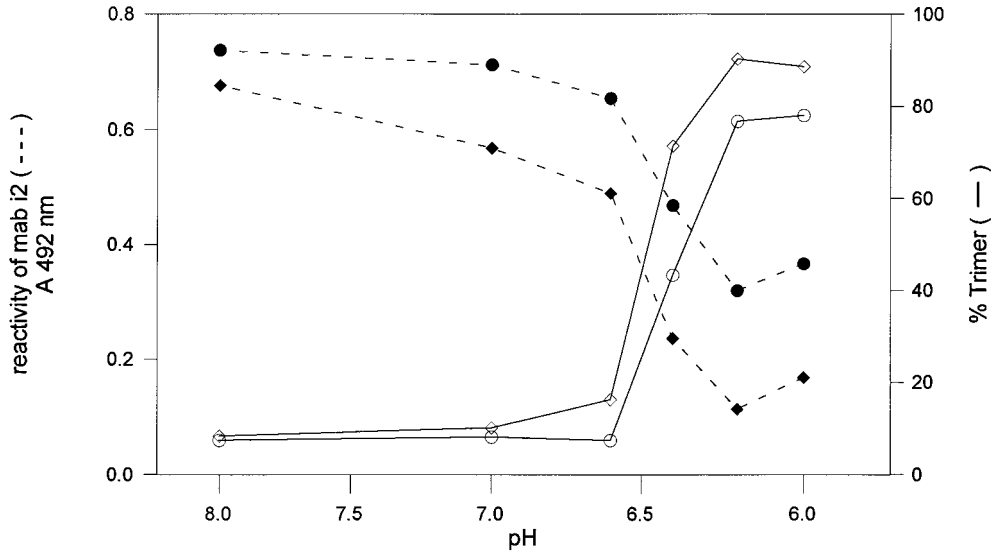


FIG. 7. pH dependence of the low-pH-induced conformational change and oligomeric rearrangement in virions (diamonds) and RSPs (circles). The closed symbols show the pH dependence of the reactivity of MAb i2, which binds preferentially to the neutral pH form of protein E. The open symbols indicate the percentage of protein E present in a trimeric state, which was estimated by solubilizing the sample with Triton X-100, separating the dimers and trimers on sucrose gradients as shown in Fig. 5, and measuring the amount of protein E in the peak fractions.

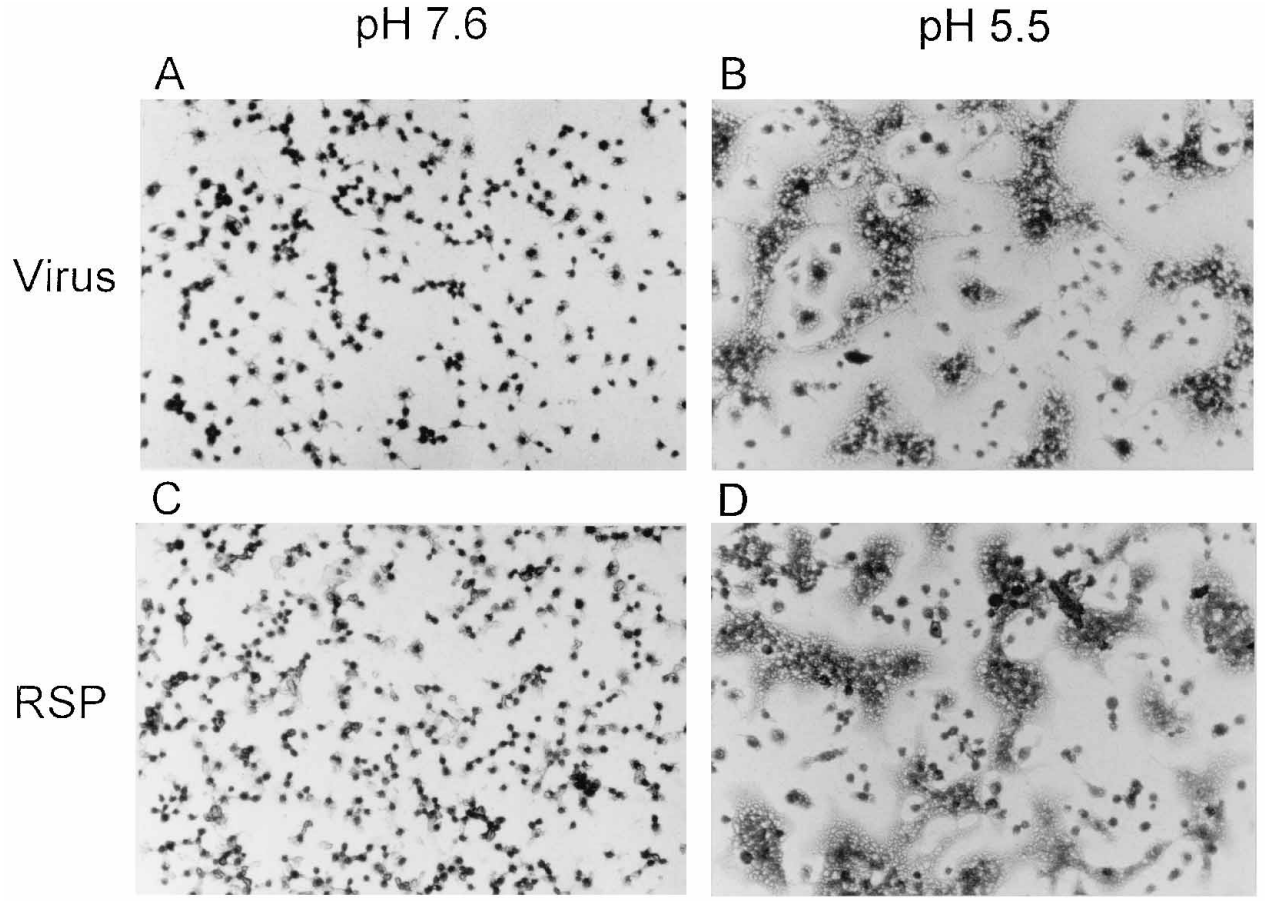


FIG. 8. Low-pH-induced fusion of C6/36 mosquito cells by virus and RSP. Giemsa-stained cells that had been incubated with virus (A and B) or RSP (C and D) and treated at pH 7.6 (A and C) or pH 5.5 (B and D) as described in Materials and Methods are shown.

pH (5) it, like fusion activity, appears to be dependent on low-pH-induced structural changes. Consistent with this notion, prM-containing immature RSPs and virions, which did not rearrange at low pH (Fig. 6B), also lacked HA activity.

## DISCUSSION

The results of this study indicate that RSPs derived from TBE virus are ordered structures with specific properties that resemble the virion envelope in many respects including (i) protein composition, (ii) maturation pathway, (iii) the presence of a lipid membrane, (iv) antigenic structure, (v) oligomeric structure, (vi) low-pH-induced rearrangements, (vii) low-pH-dependent HA activity, and (viii) the ability to fuse membranes. These properties are summarized in Table 1.

It was shown previously (3, 6, 20–24, 26, 29, 32, 33, 37, 42) that coexpression of flavivirus prM and E genes, but not expression of E by itself, results in secretion of subviral particles containing properly glycosylated envelope proteins. The data presented here provide evidence that the TBE prM and E proteins are intrinsically capable of self organizing in the absence of other viral components into regular lipid-containing structures which, after undergoing a maturation process analogous to that of whole virions, attain structural and functional characteristics very similar to those of the virion envelope.

Earlier studies with TBE virus have suggested that E proteins on the virion surface are arrayed as a matrix of dimers (2, 12), and it can be inferred from the X-ray crystal structure that these lie flat in an orientation parallel to the viral membrane (35). Preliminary studies (34a) indicate that a virion of appropriate size can be modeled with an icosahedral surface lattice consisting of 90 protein E dimers, and preliminary cryo-electron microscopy studies with immature virions (6a) appear to support this model. Since RSPs and virions undergo similar

TABLE 1. Comparison of properties of RSP and virus

| Characteristic | RSP | Virus |
|---|---|---|
| Physical properties | | |
| Diameter | ~30 nm | ~50 nm |
| Density | 1.14 g/cm$^3$ | 1.19 g/cm$^3$ |
| Composition | | |
| Viral proteins | E, M | E, M, C |
| Oligosaccharide type | Complex | Mostly complex |
| Lipid membrane | Yes | Yes |
| Nucleic acid | Little or none | Viral RNA |
| Oligomeric state of protein E at: | | |
| pH 8.0 | Dimeric | Dimeric |
| pH 6.0 | Trimeric | Trimeric |
| pH threshold of dimer-to-trimer transition | 6.5 | 6.5 |
| Functional activity | | |
| Specific HA activity[a] | 128 | 128 |
| Fusion activity | Yes | Yes |

[a] HA titer at a protein E concentration of 1 μg/ml.

structural rearrangements, it is reasonable to speculate that their envelope proteins are organized in a similar way. Because of their smaller surface area, however, RSPs could accommodate only 30 dimers or 20 trimers per particle, corresponding to a T=1 arrangement. The question of symmetry in RSPs and virions is currently under investigation by using image reconstructions from cryo-electron micrographs (6a).

In an earlier study, Konishi et al. (23) isolated extracellular subviral particles produced in HeLa cells infected with vaccinia virus recombinants expressing the prM and E genes from JEV. The JEV-derived particles, like those reported here, contained mature E and M proteins, were sensitive to detergent treatment, and possessed HA activity. Unlike the TBE virus-derived particles, however, the JEV subviral particles were reported to have a diameter of only about 20 nm. Therefore, it remains to be seen whether they and other flavivirus subviral particles are structurally analogous to the TBE virus RSPs.

Since RSPs and virions apparently use the same pathway for intracellular transport, oligosaccharide processing, and proteolytic activation of biological functions, RSPs represent an easily manipulable model system for investigating the role of envelope protein interactions in viral assembly, transport, and maturation. Equally important, however, is the fact that envelope proteins in RSPs interact and function as they would on the virion envelope, making them extremely suitable for studying the relationship between low-pH-induced rearrangements and the mechanism of membrane fusion. They offer the advantage that they can be used to evaluate mutations that knock out essential functions and would thus be lethal when engineered into an infectious clone. Furthermore, the ability to suppress the cleavage of prM will allow the role of prM in these processes to be examined with immature RSPs.

At present, TBE virus RSPs represent an attractive model system for studying structure-function relationships in viral envelope glycoproteins, because of the combination of their organization and functionality, amenability to site-directed mutagenesis, and the availability of a high-resolution glycoprotein structure to guide the interpretation of the results.

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