pH-Induced Alterations in the Fusogenic Spike Protein of Semliki Forest Virus

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ABSTRACT The spike glycoproteins of Semliki Forest virus mediate membrane fusion between the viral envelope and cholesterol-containing target membranes under conditions of mildly acidic pH (pH < 6.2). The fusion reaction is critical for the infectious cycle, catalyzing virus penetration from the acidic endosome compartment. To define the role of the viral spike glycoproteins in the fusion reaction, conformational changes in the spikes at acid pH were studied using protease digestion and binding assays to liposomes and nonionic detergent. A method was also developed to prepare fragments of both transmembrane subunit glycopolypeptides of the spike, E1 and E2, which lacked the hydrophobic anchor peptides. Unlike the intact spikes the fragments were monomeric and therefore useful for obtaining information on conformational changes in individual subunits. The results showed that both E1 and E2 undergo irreversible conformational changes at the pH of fusion, that the conformational change of E1 depends, in addition to acidic pH, on the presence of cholesterol, and that no major changes in the solubility properties of the spikes takes place. On the basis of these findings it was concluded that fusion involves both subunits of the spike and that E1 confers the stereo-specific sterol requirement. The results indicated, moreover, that acid-induced fusion of Semliki Forest virus differs in important respects from that of influenza virus, another well-defined model system for protein-mediated membrane fusion.

The plasma membrane of a cell and the membrane of an enveloped animal virus form a double barrier that must be crossed by the virus genome to initiate replication. A number of animal viruses are now known to use the constitutive endocytic pathway of the cell and membrane fusion as the means of passing these membrane barriers and gaining access to the cell cytoplasm (reviewed in references 22, 24, 26, and 43). This entry pathway is best characterized for Semliki Forest virus (SFV)[1], an alphavirus. SFV binds to the cell plasma membrane, is internalized in coated vesicles, and fuses its membrane with that of the prelysosomal endosome compartment, releasing the nucleocapsid into the cytoplasm. Endosomes have been shown to have a pH range of 5.0–5.5 (11, 29, 30, 34, 35), and it is the acidic pH that specifically triggers the virus fusion reaction.

SFV fusion can also be triggered in vitro by lowering the pH and using cell plasma membranes or artificial liposomes as target membranes (36, 40, 41). Such in vitro studies have demonstrated that the fusion reaction is rapid (<10 s), nonleaky, has a threshold pH of 6.2, and is strictly dependent on the presence of cholesterol or other 3β-OH sterols in the target membrane (21, 40). It is mediated by the virus spike protein, a heterotrimer of two transmembrane glycopolypeptides, E1 (50,786 mol wt) and E2 (51,855 mol wt), and a peripheral glycopolypeptide, E3 (11,369 mol wt). While all of the structural proteins of SFV have been cloned and sequenced (13, 14), and the spike protein expressed from cloned cDNA shown to be fusion-competent (23), the mechanism of fusion and the role of the individual components are unknown.

In this paper we have characterized the conformational change occurring in the SFV spike at mildly acidic pH by monitoring its protease sensitivity and amphiphilic properties. It was found that the ectodomains of both E1 and E2 undergo irreversible changes that correlate with the pH of fusion, and that the change in E1 requires, in addition to acid pH, the presence of cholesterol.

[1] *Abbreviations used in this paper*: DTBP, dimethyl-3,3′-dithiobispropionimidate; HA, hemagglutinin; MES, 2,(*N*-morpholino)ethane sulfonic acid; PMSF, phenylmethylsulfonyl fluoride; SFV, Semliki Forest virus; STI, soybean trypsin inhibitor; TCA, trichloroacetic acid; TX100, Triton X-100; TX114, Triton X-114.

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MATERIALS AND METHODS

Virus and Cells: SFV was propagated from a plaque-purified stock in baby hamster kidney cells as previously described (18). Virus labeled with [^{3}H]uridine or [^{35}S]methionine was prepared essentially as before (16, 19).

Proteinase K Digestion and Fragment Purification: The ectodomain fragments of E1 and E2 (termed E1* and E2*) were prepared as follows: unlabeled SFV (50 μg protein) and ~1 × 10^7 cpm of ^{35}S-labeled SFV were mixed in phosphate-buffered saline (PBS) containing calcium and magnesium at 4°C. Proteinase K (Boehringer-Mannheim Diagnostics, Inc., Houston, TX) and precondensed Triton X-114 (TX114, Fluka A.G., Basel, Switzerland) (3) were added to a final concentration of 100 μg/ml and 0.5%, respectively, and the mixture incubated on ice for 60 min. The solution was then made 0.5 mM in phenylmethylsulfonyl fluoride (PMSF), 2% in aprotinin, and 1 mg/ml in bovine serum albumin (BSA) (all from Sigma Chemical Co., St. Louis, MO) and incubated 10 min on ice to inactivate protease. TX114 was then added to 2%, and the detergent phase pelleted as described (3), but without a sucrose cushion; this detergent wash was repeated twice. The resulting aqueous phase was added to 0.5 ml packed volume concanavalin A–Sepharose (Sigma Chemical Co.) that had been prewashed once with basic buffer (130 mM NaCl, 20 mM Tris pH 7.4, 1 mM $CaCl_2$, and 1 mM $MnCl_2$) and once with basic buffer containing 1% aprotinin, 1 mg/ml BSA, and 0.5% Triton X-100 (TX100) (buffer 1). After binding at room temperature for 1 h with gentle shaking, the mixture was poured into a 1-ml column and eluted sequentially with 5 ml buffer 1, 5–10 ml buffer 2 (buffer 1 without detergent), 5 ml buffer 3 (basic buffer plus 100 μg/ml BSA), and finally 5 ml buffer 4 (buffer 3 plus 500 mM α-methyl mannoside). 0.5-ml fractions were collected and 10 μl of each counted in 4 ml Hydrofluor (National Diagnostics, Inc., Somerville, NJ). The glycoprotein peak was pooled and dialyzed overnight vs 20 mM 2,(*N*-morpholino)ethane sulfonic acid (MES)-130 mM NaCl, pH 7 containing 25 μM PMSF. Aliquots were then frozen at −70°C until use. Recovery of ^{35}S-labeled material from the column ranged from 70–100%, and 1 × 10^7 cpm of SFV starting material yielded 1–2 × 10^6 cpm of ectodomain fragments.

Pilot experiments demonstrated that proteinase K (PMSF treated) did not bind to concanavalin A. The TX100 concentrations of the fractions and final dialysate were measured by absorbance at 276 nm. The detergent concentration of the dialyzed preparation was estimated to be <0.001%.

Immunoprecipitation: SFV before or after proteinase K digestion was diluted into Nonidet P-40 buffer (10 mM Tris pH 7.5, 1% Nonidet P-40, 0.15 M NaCl, 2 mM EDTA, and 1 mg/ml BSA). 100-μl aliquots were reacted with a rabbit anti–E1 or anti–E2 antiserum (45), or with a nonspecific antiserum for 30 min at room temperature. 50 μl of a 10% suspension of fixed *S. aureus* (Zysorbin, Zymed Labs, San Francisco, CA) (vol/vol in PBS plus 1 mg/ml BSA and 0.5% TX100) was added, and the mixture shaken for 30 min at room temperature. The Zysorbin was pelleted, washed twice with Nonidet P-40 buffer, once with Nonidet P-40 buffer containing 0.5 M NaCl, and once with water. Samples were resuspended in 30 μl sample buffer containing SDS, and those to be reduced and alkylated were treated as described under gel electrophoresis.

Cross-linking Experiments: The detergent phase of undigested virus or the aqueous phase of proteinase K–cleaved virus was diluted in 0.15 M NaCl. 100-μl aliquots were mixed with 100 μl freshly prepared 200 mM triethanolamine pH 8.2 containing 2–3 mg/ml dimethyl-3, 3′-dithiobispropionimidate (DTBP, Pierce Chemical Co., Rockford, IL) or 0.5–6 mg/ml dimethyl suberimidate (Pierce Chemical Co.) (12, 45). Samples were incubated 1–2 h at room temperature, precipitated with trichloroacetic acid (TCA), the precipitates dissolved in sample buffer, and reduced and alkylated as described. DTBP samples were dissolved in sample buffer at 37°C to prevent cleaving the cross-linker in the nonreduced samples (38).

Gel Filtration: Lectin-purified E1* and E2* were sized by chromatography on a 65 × 1.3-cm Sephadex G-75 column at room temperature. A 0.25-ml sample containing ~6 × 10^4 cpm of ^{35}S-labeled ectodomains, 100 μg/ml BSA, 25 μM PMSF, and 1 absorbance unit (605 nm) of blue dextran in PBS without calcium and magnesium was applied to the column. The sample was eluted with PBS without calcium and magnesium containing 100 μg/ml BSA and 25 μM PMSF, and 0.5-ml fractions were collected. The blue dextran peak was located by measuring absorbance at 605 nm. Radioactivity was determined by counting 300-μl aliquots in 4 ml of Hydrofluor. 100-μl samples of the peak fractions were TCA-precipitated and analyzed by gel electrophoresis after reduction and alkylation. Recovery of radioactivity from the column was 82%, of which 66% eluted in the major peak, from fractions 62–82. The column was further calibrated by chromatographing human gamma globulin (Miles Laboratories Inc., Naperville, IL) and ovalbumin (Sigma Chemical Co.) in PBS without calcium and magnesium. Protein elution was monitored by absorbance at 280 nm. The void volume determined by human gamma globulin coincided exactly with that found by blue dextran.

Gel Electrophoresis: Samples for gel electrophoresis were concentrated by precipitation in TCA (10% final concentration), washed in ice-cold acetone to remove detergent, and dissolved in Laemmli sample buffer (2). Disulfide bonds were reduced and alkylated as described (27), using concentrations of 8 mM dithiothreitol and 24 mM iodoacetamide. High and low molecular weight standards (Sigma Chemical Co.) were reduced and alkylated before electrophoresis. Modified Laemmli gels (2) were prepared with an acrylamide gradient from 7.5 to 15%. Fluorography was performed essentially by the method of Chamberlain (5) using 1 M sodium salicylate (pH 7) in 30% methanol. The ^{35}S-gel bands were cut out using the fluorogram as a guide; radioactivity in individual bands was quantitated as described (37).

Liposome Preparation and Binding Assays: Liposomes for binding studies were formed from sterol/phosphatidylethanolamine/phosphatidylcholine/sphingomyelin/phosphatidic acid in the molar ratio 1.5:1:1:1:0.2 (21, 40), or ~1 cholesterol per 2 phospholipid molecules. Cholesterol and epicholesterol were incorporated as indicated, and liposome floatation on sucrose gradients to assay binding was performed as described (21). For the E1* protease-resistance assay, liposomes with an increased sterol concentration (3:1:1:1:0.375 molar ratio) were used, corresponding to ~1 cholesterol per phospholipid molecule. Lipids were from Sigma Chemical Co. or Avanti Polar-Lipids, Inc. (Birmingham, AL).

Protease Assays of the Acid-induced Conformational Change: 150-μl aliquots of virus containing ~1 × 10^5 cpm [^{35}S]methionine virus and 5 μg viral protein in MES-saline were adjusted to the indicated pH with 0.5 N HCl, incubated 10 min at 37°C, neutralized by the addition of 0.5 N NaOH, and kept on ice until protease digestion. TPCK-trypsin (Sigma Chemical Co.) then was added to a final concentration of 100 μg/ml in PBS, with 1% TX100 present as indicated. After incubation for 10 min at 0°C or 37°C, the digestion was stopped by adding a threefold excess of soybean trypsin inhibitor (STI, Sigma Chemical Co.). After 10 min on ice, the samples were acid-precipitated and processed for gel electrophoresis as above.

E2* and E1* were similarly digested, except that the samples contained 100 μg/ml BSA as carrier, and ~1.5 × 10^4 cpm of lectin-purified fragments. Some of the samples were pH-treated in the presence of cholesterol or epicholesterol liposomes at a concentration of 1 mM lipid.

RESULTS

While the entry pathway of SFV is the best-defined of any enveloped animal virus, the fusion reaction has not been characterized at the molecular level. Our approach to this problem was to expose the spike glycoproteins of SFV to decreasing pH, monitor irreversible changes in their conformation by biochemical means, and relate these changes to the known properties of SFV fusion. Towards this end, it proved crucial to have water-soluble fragments comprising the ectodomains of the two major glycopolypeptide subunits of the spike, E1 and E2. Such molecules made possible the analysis of the individual responses of the subunits to acid.

Preparation of Soluble Ectodomain Fragments of E1 and E2

When SFV was solubilized with TX100 and treated with proteinase K at 4°C, partial proteolysis of all of the structural proteins was observed (Fig. 1). The E1 glycopolypeptide was cleaved within the first 10 min to a form which in nonreducing SDS PAGE gels migrated slightly faster than authentic E1 (see arrow). E2 was digested more slowly to a fragment with similar migration as the E1-derived fragment. Concurrent with the disappearance of E1 and E2, two distinct low molecular weight bands appeared (arrow heads). The capsid protein (*C*) was, moreover, rapidly cleaved into two fragments termed C1* and C2*, the properties of which will be discussed elsewhere.

The temperature during the protease digestion was critical; while at 4°C, 82% of the viral protein remained TCA-precipitable after 5 min of digestion and 73% after 60 min, the viral proteins were all rapidly cleaved to acid-soluble form if the

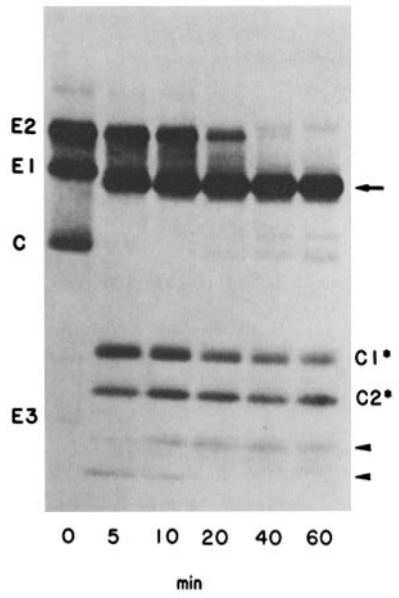

FIGURE 1 Time course of digestion of SFV by proteinase K. [^{35}S]-Methionine-labeled SFV was digested with 100 μg/ml proteinase K in 0.5% TX100 at 4°C. At the indicated time, proteinase was inactivated with PMSF and aprotinin, and samples were then TCA-precipitated and resolved by SDS PAGE under nonreducing conditions. For the time 0 sample, proteinase K was pretreated with inhibitors. The ectodomain fragments of E1 and E2 (arrow) and the presumed transmembrane domains (arrowheads) are indicated. Capsid protein is cleaved within the first 5 min of digestion to two fragments termed C1* and C2*.

temperature was elevated to 37°C. The partial cleavages were not specific to proteinase K; similar digestion patterns resulted using either subtilysin or pronase. The detergent TX100 could be replaced with TX114 or octylglucoside without changes in digestion pattern.

The co-migrating E1- and E2-derived fragments could be separated from each other on SDS polyacrylamide gels simply by reducing the samples before electrophoresis (Fig. 2*B*, compare lanes *1* and *2*). Two distinct bands were then seen with apparent molecular weights of 48 kD and 41 kD (arrows). The substantial decrease in electrophoretic mobility of the 48-kD fragment band after reduction was consistent with a similar change in intact E1 which allows the separation of nonreduced E1 from E2 although they have similar molecular weights (~51 kD and 52 kD, respectively) and migrate together after reduction (reference 45; Fig. 2*A*, compare lanes *1* and *2*). The observation that the 48-kD fragment appeared early during digestion with proteinase K suggested that it was, indeed, derived from E1. Conversely, the 41-kD band appeared in concert with the digestion of E2. These assignments were confirmed by immunoprecipitation with antibodies specific for E1 or E2 (Fig. 3). The 48-kD fragment was consequently named E1* and the 41 kD fragment E2*. The small molecular weight fragments were not precipitated by the antibodies and could not be positively identified.

The water-soluble nature of E1* and E2* was demonstrated using TX114 partitioning, a method that allows the separation of amphiphilic and soluble proteins on the basis of detergent binding (3). Nondigested E1 and E2 and the small peptides generated during proteolyis partitioned into the detergent phase (Fig. 2*B*, lane *3*). In contrast, E1* and E2* were recovered in the aqueous phase (Fig. 2*B*, lane *4*), indicating that they did not possess large hydrophobic moieties.

Given the structure and amino acid sequence of the SFV spike glycopolypeptides (13), the molecular weights of the fragments, and their solubility properties, we concluded that E1* and E2* corresponded to the *N*-terminal ectodomains of E1 and E2, and that the small peptides were probably derived from the transmembrane domains. The molecular weight of E1* (48 kD) indicated that it lacked the entire transmembrane anchor of E1 and possibly an additional 10–15 residues. The presence of [^{35}S]methionine in the transmembrane peptide first generated by proteinase K suggested that the E1 cleavage site was located N-terminal to the last methionine in position 402, or at least 12 amino acids from the membrane-spanning

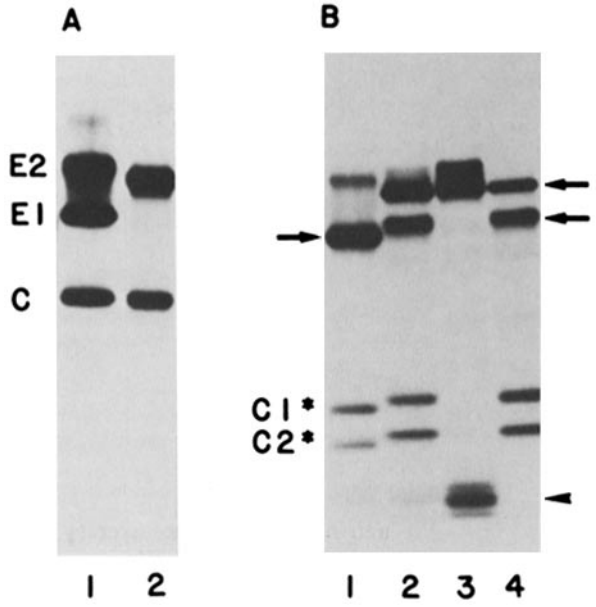

FIGURE 2 The electrophoretic mobility and solubility properties of the E1 and E2 ectodomains. (*A*) Intact viral proteins were electrophoresed either without (lane *1*) or with (lane *2*) reduction and alkylation. E1 and E2 co-migrate after reduction. (*B*) A 60-min proteinase K digest of virus in TX114 was electrophoresed either without (lane *1*) or with (lanes *2–4*) reduction and alkylation. The complete digestion mixture is shown in lanes *1* and *2*. After TX114—partitioning, the detergent phase (lane *3*) was overloaded to demonstrate the partitioning of putative transmembrane domains (arrowhead) and nondigested E1 and E2. The aqueous phase (lane *4*) contained the ectodomains (arrows) and capsid fragments.

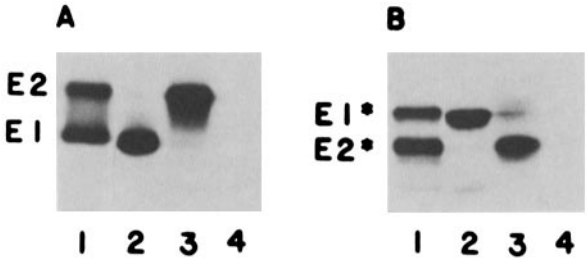

FIGURE 3 Immunoprecipitation of E1* and E2* with specific antibodies. Nondigested SFV (panel *A*) or SFV digested with proteinase K for 60 min (panel *B*) were either TCA-precipitated (lane *1*) or immunoprecipitated with an anti-E1 (lane *2*), anti-E2 (lane *3*), or nonspecific rabbit antiserum (lane *4*). Immune complexes were absorbed to *S. aureus* and processed for gel electrophoresis. Samples in *A* were electrophoresed without reduction; samples in *B* were reduced and alkylated.

segment. E2* probably lacked both the C-terminal polar domain (31 residues) of E2 and the transmembrane region that contains two methionine residues. Judging from its apparent molecular weight of 41 kD, it too may have lost ~10–15 residues from the extramembraneous side.

Purification of E1* and E2*

After proteinase K digestion, E1* and E2* were present in a mixture of protein fragments, protease, detergent, and protease inhibitors. To purify the ectodomains, the aqueous phase generated by TX114 partitioning (see above) was reacted with concanavalin A–Sepharose which binds intact E1 and E2 (28). The affinity column was first eluted with detergent-containing buffer to ensure complete removal of capsid fragments and protease. After extensive washing to remove detergent, a glycoprotein fraction was eluted in buffer containing α-methyl mannoside (Fig. 4). In addition to E1* and E2*, which constituted the major components, this fraction contained variable amounts of a 56-kD band. It may correspond to the anchor-free fragment of the p62 precursor glycoprotein, which was sometimes present in our virus preparations. In addition, the glycoprotein peak occasionally contained smaller peptide fragments that we assume to be derived from further proteolysis of E1 and E2.

E1* and E2* Are Monomers

Chemical cross-linking and gel filtration were next used to determine whether E1* and E2* remained associated with each other or occurred as monomers in solution. In the intact virus, E1 and E2 are present as heterodimers, which can be cross-linked to a 100-kD protein with dimethyl suberimidate (12) or DTBP (45), and react with cross-linker even after solubilization in nonionic detergent (45). We used both of these cross-linkers on TX114-solubilized and phase-separated viral spikes and proteinase K fragments. Fig. 5 shows an experiment with DTBP, a cross-linker that is cleavable by reduction. In addition to the monomer bands of E1 and E2, a 100-kD band was seen after cross-linking (compare lanes *1* and *2*) which was lost when the sample was reduced with

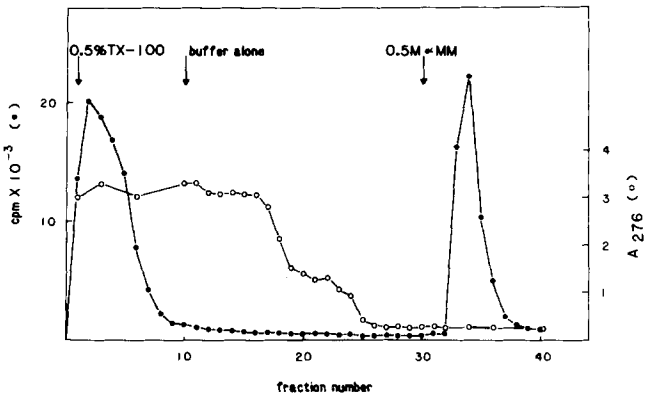

FIGURE 4 Affinity purification of E1* and E2* on concanavalin A–Sepharose. SFV was digested with proteinase K and the ectodomains separated from noncleaved E1 and E2 and transmembrane domains by TX114 partitioning. This aqueous phase was bound to concanavalin A–Sepharose and sequentially eluted with TX100-containing buffer, buffer alone, and buffer containing 0.5 M α-methyl mannoside. [^{35}S]Methionine-labeled viral protein was monitored by liquid scintillation counting of an aliquot of each fraction, and TX100 by absorbance at 276 nm.

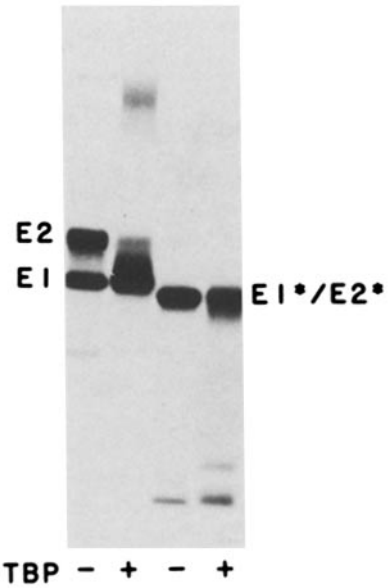

FIGURE 5 Reaction of E1/E2 and E1*/E2* with DTBP, a cleavable cross-linker. Triton X114 partitioning was used to separate E1 and E2 from untreated virus and E1* and E2* from proteinase K–digested virus. Part of each sample was reacted with DTBP as indicated for 1 h at room temperature. Samples were then TCA-precipitated and processed for gel electrophoresis without reduction.

dithiothreitol (data not shown). In contrast, E1* and E2* showed virtually no cross-linked species when reacted with DTBP under the same conditions (lanes *3* and *4*), suggesting that unlike E1 and E2, E1* and E2* were present mainly as monomers. Cross-linking with dimethyl suberimidate gave the same result.

Gel filtration studies were also consistent with the monomeric nature of E1* and E2*. When lectin-purified, radiolabeled E1* and E2* were passed over a Sephadex G-75 column, most of the radioactivity eluted as a broad peak centered around the position of ovalbumin (~45 kD) (Fig. 6). Given the apparent molecular weights of E1* and E2* on SDS PAGE, this was the position expected of monomeric fragments. Dimers or higher oligomers of the ectodomains would have eluted in the void volume of the column (see elution profile of blue dextran, Fig. 6). SDS PAGE and fluorography of the fractions showed that the peak observed in fractions 50–61 consisted mainly of the 56-kD contaminant, the main peak (fractions 62–82) of E1* and E2*, and the trailing material (fractions 82–100) of E1* and E2* with some smaller fragments.

Acid-induced Changes in Hydrophobicity of E2*

The water-soluble ectodomain of the influenza virus fusion protein (a 208-kD bromelain fragment of the hemagglutinin molecule; 4) is known to undergo a major conformational change at acidic pH. It exposes a new hydrophobic moiety that renders the fragment amphiphilic and able to bind detergent, lipid, or another bromelain fragment of hemagglutinin (8, 33). The change correlates with fusion activity and is thought to reflect the analogous interaction between the spike glycoprotein and the target membrane during fusion (8, 33, 43). To determine whether a similar change occurs in E1* and/or E2*, their partitioning was determined in TX114 buffered to different pHs. As shown in Fig. 7, it was observed

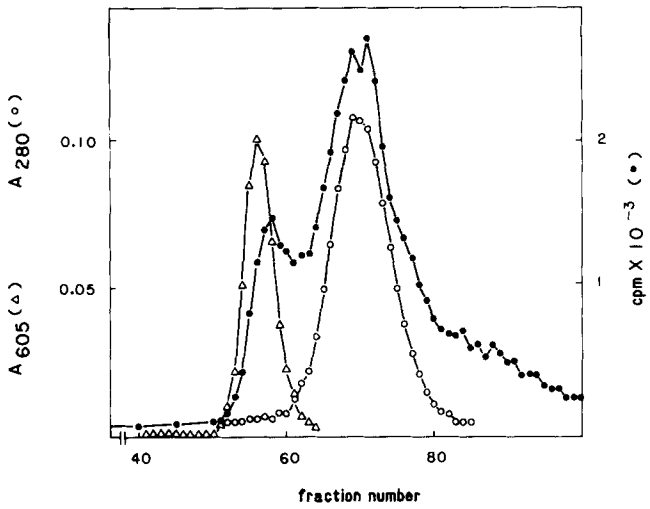

FIGURE 6 Gel filtration of E1* and E2*. Lectin-purified E1* and E2* were chromatographed on a Sephadex G-75 column in PBS containing BSA and 25 μM PMSF. The elution of ^{35}S-labeled virus protein was determined by liquid scintillation counting, and the elution of tracer blue dextran in the same column run was determined by absorbance at 605 nm. In a separate column run, the elution of ovalbumin was determined by absorbance at 280 nm.

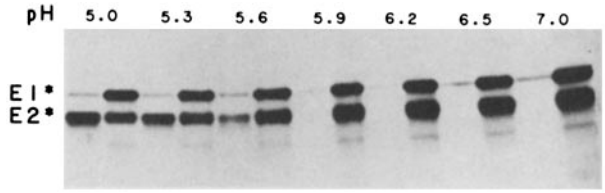

FIGURE 7 The pH dependence of ectodomain fragment partitioning in TX114. SFV was digested with proteinase K for 30 min on ice and protease inhibitors added. The mixture was partitioned in TX114 to separate the aqueous phase containing E1* and E2*. Aliquots of the aqueous phase were adjusted to 1% TX114, treated at the indicated pH for 10 min at 37°C, and separated into a detergent pellet and aqueous supernatant by centrifugation through a sucrose cushion at the indicated pH. Samples were electrophoresed after reduction and alkylation. For each pH, the left lane is the detergent pellet and right lane the aqueous supernatant. Partitioning was similar if performed after neutralization.

that a significant fraction of E2* partitioned into the detergent phase at pH 5.6 or lower, while E1* remained in the aqueous phase from pH 7.0 to pH 5.0. Binding to liposomes (prepared from cholesterol/phosphatidylethanolamine/phosphatidylcholine/sphingomyelin/phosphatidic acid, 1.5:1:1:1:0.2) was assayed by incubating liposomes together with lectin-purified E1* and E2* at pH 5.0 or 7.0. Liposomes were then isolated from unbound protein by floatation on sucrose gradients (21). No significant association of either fragment could be detected by this technique, nor did the exposure of the fragments to liposomes at different pH modify their subsequent partitioning in TX114.

Taken together the results indicated that E1* did not become amphiphilic when exposed to acid. In contrast, E2* did seem to become rather more hydrophobic, but the change was not as clear-cut and complete as that observed with influenza bromelain fragment of hemagglutinin. It also occurred at a pH of 0.6 units below the threshold for SFV fusion, which is pH 6.2. It thus seemed doubtful that the change in E2* was directly involved in the fusion activity of the spikes.

Changes in Protease Sensitivity of E1 and E2

To obtain evidence for a relevant acid-induced conformational change, we turned to the intact viral protein. Using trypsin and other proteolytic enzymes, we hoped to detect altered exposure of sites for protease cleavage in viral spikes after treatment at acid pH. All digestions were performed at neutral pH, and thus were expected to reflect only irreversible changes.

Virus particles labeled with [^{35}S]methionine were treated with buffers of varying pH, neutralized, digested with trypsin, and subjected to SDS PAGE without reduction. The digestions were performed either at 37° or at 4°C, and in the presence or absence of TX100. When the trypsin digestion was performed at 4°C in TX100, it was found that prior acid treatment made E2 totally trypsin sensitive with a pH threshold around pH 6.2 (Fig. 8). E1, on the other hand, remained relatively resistant to trypsin under these digestion conditions, being reduced only by ~25% after pH 5 treatment. Similar effects on E2 were observed when TX100 was omitted (not shown), in agreement with the previous finding of Edwards et al. (9) that E2 in the closely related Sindbis virus becomes trypsin sensitive after low pH treatment.

When trypsin digestion was performed at 37°C in the presence of TX100, a totally different pattern of protease sensitivity emerged (Fig. 8). Irrespective of prior pH treatment, E2 was totally trypsin sensitive. In contrast, while fully digested in its pH 7 form, E1 was converted to a trypsin-resistant form with a pH threshold of ~ 6.2. About 30% of the total E1 was converted by a 10-min pH 5 treatment at 37°C.

Acid treatment thus caused a change in the SFV spike glycoprotein, which modified the trypsin sensitivity of both E1 and E2. Similar results were obtained using either chymotrypsin or pronase, suggesting that a rather extensive change in spike structure may have occurred. Importantly, the pH dependence of the changes in both E1 and E2 corresponded closely to the pH dependence of SFV fusion, having a midpoint at ~pH 6.0. The kinetics of the conversion were also similar to the known rate of fusion. To determine this,

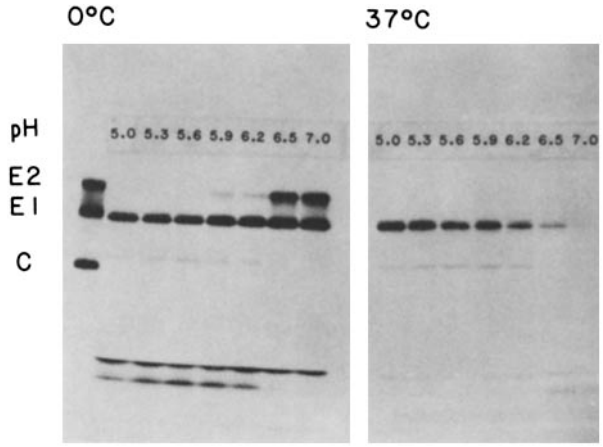

FIGURE 8 Trypsin sensitivity of viral proteins after low pH treatment. Aliquots of intact virus in MES-saline buffer were treated at the indicated pH for 10 min at 37°C, neutralized, and then digested with 100 μg/ml trypsin in 1% TX100 for 10 min at 0°C or 37°C. The reaction was terminated by the addition of STI and the samples processed for gel electrophoresis without reduction. Control lane at left shows virus reacted with premixed trypsin and STI.

viruses were treated at pH 5.6 for varying times before neutralization and trypsin digestion. Digestion was performed either at 0°C or 37°C in the presence of TX100, the samples subjected to SDS PAGE, and the ^{35}S present in the E1 and E2 bands directly quantitated after fluorography. The conversion in each case was virtually complete after 10 s of acidification. We have previously shown that SFV fusion takes ~10–15 s (41). It appeared that the irreversible conformational changes detected by protease sensitivity reflected the fusion activity of the protein.

Changes in Protease Sensitivity of E1 and E2**

The changes in the protease sensitivity of E1 and E2 did not necessarily mean that both underwent conformational changes at acidic pH. It was possible that a change in one polypeptide could lead to altered accessibility of the other polypeptide within the dimeric complex. By exposing E1* and E2* to low pH and then determining their trypsin sensitivity at 4° and 37°C, it was possible to assay the pH-induced changes of the individual polypeptides in solution.

When trypsinization was performed at 4°C, no change in E1* was seen irrespective of pH treatment, thus conforming with the behavior of intact E1 (Fig. 9). Although in general somewhat more sensitive to trypsin, E2* resembled E2 in showing a clear-cut increase in sensitivity below pH 6.2 (Fig. 9). The digestion patterns of E1* and E2* were not affected by the presence of nonionic detergent. Interestingly, we found that the 56-kD fragment, which we assume to be derived from the p62 precursor of E2 and E3, proved sensitive to trypsin at all pH values tested. This result agrees with the trypsin sensitivity of metabolically labeled p62 as assayed in cell lysates (Wagner, K., A. Helenius, and M. Kielian, unpublished results).

When trypsinization was performed at 37°C, the initial results suggested a difference between the fragment E1* and

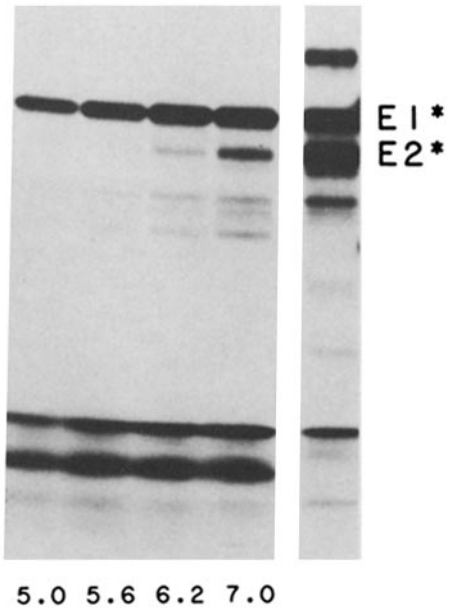

FIGURE 9 Trypsin sensitivity of isolated E1* and E2* after pH treatment. Lectin-purified E1* and E2* were diluted in MES-saline buffer, treated at the indicated pH for 10 min at 37°C, neutralized, and digested with trypsin at 0°C in 1% TX100. Samples were then processed as in Fig. 8, and reduced and alkylated. Control lane at right shows ectodomains treated with premixed trypsin/STI.

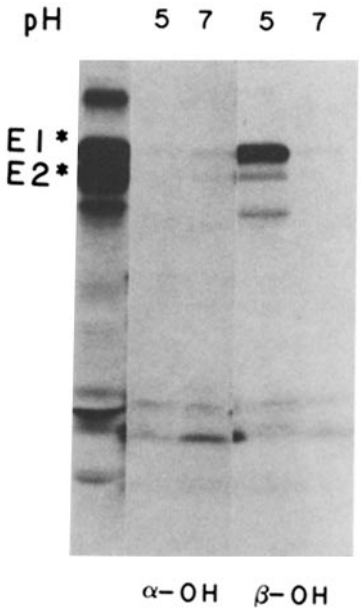

FIGURE 10 Trypsin resistance of E1* acidified in the presence of cholesterol-containing liposomes. Lectin-purified E1* and E2* were diluted in MES-saline buffer to a final concentration of 100 μg/ml BSA and 1 mM lipid as either cholesterol (β-OH) or epicholesterol (α-OH) containing liposomes. Samples were pH-treated 10 min at 37°C, neutralized, and then digested for 10 min with trypsin at 37°C in the absence of detergent. Reduced and alkylated samples were then analyzed by SDS PAGE. Control lane at left shows ectodomains treated with premixed trypsin/STI.

intact E1. Both E2* and E1* were completely cleaved at 37°C, regardless of prior pH treatment. No trypsin-resistant E1* was observed. One difference between the experiments, however, was the presence or absence of membranes. The pH treatment of the lectin-purified fragments was therefore repeated in the presence of cholesterol or epicholesterol-containing liposomes. The change to trypsin resistance could now be reproduced with the E1* fragment (Fig. 10, lanes *4* and *5*), and it proved absolutely dependent on the presence of cholesterol. The fusion-inactive epimer, epicholesterol, was inactive in promoting this conformational change (Fig. 10, lanes *2* and *3*). Further studies showed that the cholesterol had to be present in a lipid membrane; it had no activity if added alone in aqueous buffer or dispersed in TX100 (not shown). The limited aqueous solubility of cholesterol (15) may have affected these results. After pH treatment, the trypsin digestion could be performed in the presence of TX100 without changing the outcome.

The conformational change in E1* detected by trypsin digestion was thus found to depend both on exposure to a pH $\leq$ 6.2 and on the presence of cholesterol. The results implied that there is a stereospecific interaction with cholesterol, which may explain the requirement for 3β-OH sterols observed in SFV fusion.

The virus membrane is known to contain cholesterol derived from the plasma membrane of the host animal cell (25). It is thus possible that the virus itself was the source of cholesterol in the conversion of intact E1 (Fig. 8). The limited availability of sterol may explain the finding that not all of the viral E1 was detected as trypsin-resistant.

DISCUSSION

The fusion activity of animal viruses depends on specific virally encoded membrane proteins. The proteins must be integrated into a bilayer membrane in order to be active, but no other viral components are needed (10, 23, 32, 42). The influenza hemagglutinin (HA), which is the best characterized of the fusion factors, undergoes a conformational change at acidic pH which exposes a previously concealed hydrophobic domain of the trimeric spike glycoprotein. Several lines of

evidence suggest that this domain contains the apolar, highly conserved N-terminal sequences of the HA2 subunits (7, 8, 33). It is likely that this hydrophobic moiety binds to the target membrane, and thus facilitates the close approach of the two membranes (7, 8, 33, 43).

Although unrelated to amino acid sequence, size, and overall structure, the SFV spike glycoprotein is similar to HA in its general activities. It binds the virus to cell surface receptors, it causes efficient acid-activated membrane fusion, and it contains the neutralizing antigenic epitopes (reviewed in reference 22). SFV-induced fusion occurs, however, at a somewhat higher pH than influenza, it is more rapid, and it is absolutely dependent on the presence of cholesterol or other 3β-OH containing sterols in the target membrane (21, 40). Unlike HA, the spike protein of SFV does not have distinct hydrophobic peptide sequences located outside of the transmembrane regions (6, 31). E1 does, however, contain a highly conserved internal uncharged stretch of 17 residues that has been suggested to play a role in fusion (6, 13, 31).

The results reported here indicate that, like HA, the SFV spike glycoprotein undergoes conformational changes at the pH of fusion. Irreversible alterations were detected in both of the major subunits of the spike, E1 and E2. The changes in E2 render it more sensitive to digestion by trypsin and other proteolytic enzymes, as recently reported for the E2 of the related Sindbis virus (9). E1 becomes, in contrast, more resistant to trypsin, and this change appears to depend both on exposure to acid and on the presence of cholesterol. Apparently one or more critical cleavage sites needed for full degradation are masked in the low pH form by a mechanism that depends on a molecular interaction between E1 and cholesterol.

The changes in E1 and E2 correlate quite well with the known properties of SFV fusion (pH-dependence, kinetics, and sterol requirement), and thus probably reflect the alteration in the protein that makes it fusion active in vitro. There are, moreover, indications that the alterations in E1 and E2 are relevant to SFV entry into cells. We have shown that E1's conversion to trypsin resistance occurs in baby hamster kidney cells during endocytic uptake and infection, and that it is blocked by monensin, an entry inhibitor that elevates the pH in endosomes (17; Kielian, M., M. Marsh, and A. Helenius, manuscript in preparation). To detect the corresponding conversion of intracellular E2 has proven more difficult because of its sensitivity to cellular proteases even at neutral pH. The evidence to date for the biological relevance of the E2 conversion has been obtained using *fus*-1, an SFV mutant that requires a lower pH for fusion than wild type SFV (20). When we assayed E2's trypsin sensitivity in *fus*-1, we observed that the pH threshold of the conformational change was also shifted to a more acidic value (Kielian, M., M. Marsh, and A. Helenius, manuscript in preparation).

The acid-induced changes in the SFV spike glycoproteins differed in important respects from those observed for influenza HA. We found no convincing evidence from studies with E1* and E2* for the exposure of new hydrophobic moieties that would allow the SFV spike to bind to the target membrane in a nonspecific hydrophobic manner. Instead of HA-like nonspecific insertion, SFV fusion may, in fact, depend on a stereospecific protein interaction with cholesterol. The differences between influenza and SFV fusion illustrated by the results show that, in considering viral membrane fusion, one should not think in terms of a single unifying mechanism. The fusion proteins identified so far in various enveloped viruses are diverse in general structure and amino acid sequence, and thus may function according to a variety of principles that can be determined only by molecular analysis of the individual systems.

One of the reasons why the HA of influenza is relatively well understood compared with other virus spike glycoproteins is the availability of the isolated ectodomain. The bromelain HA fragment has allowed detailed functional and structural studies that would be impossible with the intact amphiphilic protein (8, 33, 44). We have shown here that it is feasible to derive the corresponding soluble ectodomain fragments from SFV spikes, and that they can be used to characterize the functional properties of the spike. After numerous attempts at making such fragments, the critical parameters that allowed cleavage of both major transmembrane subunits at a site close to the membrane without further cleavage of the ectodomain were found to be low temperature and the presence of solubilizing concentrations of nonionic detergent during digestion. While the ability of the ectodomain fragments to undergo conformational changes at low pH appeared to be retained, the intermolecular association between E1 and E2 was not. Their monomeric nature suggested that the interactions between E1 and E2 in the intact spike are concentrated in or near the transmembrane domain. Our results suggest, moreover, that the conformation of the ectodomains is largely independent of the membrane anchoring segments, and that cleavage may preferentially occur at the interphase between the transmembrane domain and the ectodomain. The soluble ectodomain fragments contained the oligosaccharide chains, which enabled their binding to concanavalin A, while the C-terminal hydrophobic peptides of both E1 and E2 appeared to contain the fatty acyl groups (Wagner, K., A. Helenius, and M. Kielian, unpublished results; see also reference 1). The fate of the third component of the spike, E3, was difficult to visualize on our gels owing to its weak labeling with [^{35}S]-methionine (cf. Fig. 1). It is unlikely, however, that E3 plays a critical role in membrane fusion because it is not found in the closely related Sindbis virus (39).

The results reported here raise challenging issues in understanding the significance of cholesterol in SFV fusion. Although optimal fusion requires as much as 1 cholesterol per 2 phospholipids, the sterol structural requirements do not appear to correlate with known effects of sterol on phospholipid condensation or membrane phase transition (21). Yet, cholesterol appears active in promoting the conformational change in E1* only when presented within the context of a lipid bilayer. Furthermore, if E1* has a stereospecific cholesterol binding site, the sterol must be removed from the bilayer after binding, as E1* does not remain liposome-associated. Future studies must address the molecular nature of the sterol-E1 interaction, and the possible role of the conserved uncharged sequence in E1. It cannot be ruled out that both E1 and E2 are required in concert to mediate stable membrane binding, and thus the possible cooperative interaction of the dimer with membranes must be investigated. Finally, it will be important to determine if cholesterol is really a prerequisite for entry during an in vivo infection. If so, it is conceivable that this requirement might be exploited in the design of specific antiviral agents against toga- and other cholesterol requiring viruses.

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