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Role of Spike Protein Conformational Changes in Fusion of Semliki Forest Virus

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The alphavirus Semliki Forest virus (SFV) and a number of other enveloped animal viruses infect cells via a membrane fusion reaction triggered by the low pH within endocytic vesicles. In addition to having a low pH requirement, SFV fusion and infection are also strictly dependent on the presence of cholesterol in the host cell membrane. A number of conformational changes in the SFV spike protein occur following low-pH treatment, including dissociation of the E1-E2 dimer, conformational changes in the E1 and E2 subunits, and oligomerization of E1 to a homotrimer. To allow the ordering of these events, we have compared the kinetics of these conformational changes with those of fusion, using pH treatment near the fusion threshold and low-temperature incubation to slow the fusion reaction. Dimer dissociation, the E1 conformational change, and E1 trimerization all occur prior to the mixing of virus and cell membranes. Studies of cells incubated at 20°C showed that as with virus fusion, E1 trimerization occurred in the endosome before transport to lysosomes. However, unlike the strictly cholesterol-dependent membrane fusion reaction, the E1 homotrimer was produced in vivo during virus uptake by cholesterol-depleted cells or in vitro by low-pH treatment of virus in the presence of artificial liposomes with or without cholesterol. Purified, lipid-free spike protein rosettes were assayed to determine the requirement for virus membrane cholesterol in E1 homotrimer formation. Spike protein rosettes were found to undergo E1 oligomerization upon exposure to low pH and target liposomes and showed an enhancement of oligomerization with cholesterol-containing membranes. The E1 homotrimer may represent a prefusion complex that requires cholesterol to carry out the final coalescence of the viral and target membranes.

Membrane fusion is a ubiquitous cellular function that occurs both intracellularly as part of endocytosis and exocytosis and during cell-cell fusion processes such as myoblast formation and sperm-egg fusion (32, 41, 42). Membrane fusion is also the mechanism used by enveloped viruses to introduce their genomes into the host cell. The fusion reactions of such enveloped viruses have been used extensively to analyze the molecular events that occur during membrane fusion (reviewed in references 2, 21, 32, and 42). One of the best-characterized viruses for such studies is the enveloped alphavirus Semliki Forest virus (SFV) (reviewed in reference 12). This virus infects cells by binding to a plasma membrane receptor, being endocytosed in coated pits and vesicles, and being delivered to prelysosomal endosomes. Upon exposure to the mildly acidic pH in endosomes, the spike protein of the virus changes to a conformation that triggers fusion of the virus and endosome membranes. This fusion reaction releases the virus nucleocapsid into the cytoplasm to begin the virus replication process. It is now clear that many elements of this overall entry pathway are used by a wide variety of enveloped and nonenveloped viruses (2, 21, 32).

SFV fusion is mediated by the virus spike protein, a heterotrimer which contains two transmembrane glycopolypeptides, E1 (50,786 Da) and E2 (51,855 Da), and a peripheral glycopolypeptide, E3 (11,369 Da) (reviewed in references 28 and 31). E2 and E3 are initially synthesized as a precursor, p62, which is cleaved during the transport of the spike through the secretory pathway to the plasma membrane. On the surface of the virus particle, the spikes are further organized into higher-order trimers consisting of $(E1/E2/E3)_3$ (35). Upon exposure to the fusogenic pH of 6.2 or below, a number of conformational changes occur in the viral spike protein. These fall into three classes. The first involves individual conformational changes in either the E1 or the E2 subunit, detected by the exposure of previously masked antigenic epitopes (14, 23, 38), alterations in the sensitivity of the E1 or E2 subunit to protease digestion (4, 13), or exposure of cysteine residues (1, 25). The second class involves the dissociation of the normally tight E1-E2 dimer complex, detected by either antibody coprecipitation assays or sucrose gradient sedimentation (36). This dissociation is thought to be reversible (36), and its pH dependence is dramatically acid shifted in spike proteins containing uncleaved p62 (18, 27). The last type of conformational change is the formation of an oligomer of E1, which occurs during endocytosis of virus by cells (38).

As first demonstrated in a virus-liposome system (39), cholesterol is absolutely required for the low-pH-triggered fusion of SFV with membranes. Fusion is maximal at a sterol-to-phospholipid ratio of 0.5 (39) and requires the sterol 3β-hydroxyl group (15). The SFV cholesterol requirement does not appear to be related to membrane fluidity, since nonfluidizing cholesterol analogs substitute for cholesterol as long as they contain the critical 3β-hydroxyl group (15). The role of cholesterol in fusion may involve a direct interaction of 3β-hydroxysterol with the SFV E1 spike subunit (13). One piece of evidence for such an interaction is that a proteolytically truncated form of E1 requires both low pH and a 3β-hydroxysterol in order to undergo its conformational change (13, 14). In addition to the in vitro data, our laboratory has developed an in vivo system to examine the role of cholesterol, based on cholesterol-depleted mosquito cells (26). Such cholesterol-depleted cells are resistant to SFV infection. This block in infection is specifically due to a block in the fusion of SFV with the cholesterol-depleted host cell endosome membrane (26).

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There is thus evidence from several sources for the occurrence of distinct conformational changes in the SFV spike protein upon low-pH treatment. To achieve a better understanding of the fusion process, we set out to order the conformational changes during the fusion reaction. In addition, given the key role of cholesterol as a component of the target membrane, we wished to analyze the potential involvement of sterol in these conformational changes. Our results demonstrate that dimer dissociation, exposure of acid-specific E1 epitopes, and formation of the E1 oligomer all occur prior to membrane fusion, as measured by the mixing of the virus and target membranes. In contrast, exposure of an acid-specific E2 epitope appears to be a postfusion event that is not required for membrane mixing. Additionally, our data indicate that efficient E1 oligomerization requires low pH and is enhanced by a target membrane but can occur in the absence of cholesterol in either the virus or target membranes. Further molecular events, presumably involving cholesterol, may then lead to the final membrane-mixing step of fusion.

While the manuscript was in preparation, studies were published which demonstrated that the E1 oligomer consists of an E1 homotrimer that can be formed in vitro during virus-liposome fusion (37) and which examined the order of the conformational changes during the fusion of SFV with liposomes (3).

MATERIALS AND METHODS

Virus and cells. The SFV used in these experiments was a plaque-purified isolate which was propagated and radiolabeled with [^{35}S]methionine by using BHK-21 cells as previously described (16). Purified virus, used for the fluorescence-dequenching (FdQ) fusion assays and spike rosette purification described below, was prepared by banding on tartrate gradients (11).

BHK-21 cells were cultured at 37°C in Dulbecco's modified Eagle medium (DME) containing 5% fetal calf serum, 100 U (each) of penicillin and streptomycin per ml, and 10% tryptose phosphate broth. C6/36 cells, a clonal cell line derived from *Aedes albopictus* (10), were grown at 28°C in DME containing 100 U (each) of penicillin and streptomycin per ml and either 10% heat-inactivated fetal calf serum or 10% delipidated heat-inactivated fetal calf serum for cholesterol depletion, as previously described (26). The cholesterol depletion conditions reduce both free and esterified cholesterol to levels less than 2% of those of control cells (26, 30). The cells have been maintained under cholesterol-depleted conditions for over a year.

Virus fusion assays. Virus fusion with the plasma membrane of BHK cells was evaluated by using virus labeled with octadecylrhodamine (R18), a fluorescent fatty acid. The assay followed the dequenching of fluorescence upon fusion of R18-labeled virus with the plasma membrane (7, 33). Purified SFV was labeled with R18 (Molecular Probes, Eugene, Oreg.) at a ratio of 50 pmol/nmol of viral phospholipid. In a typical labeling reaction, 7.3 μl of a 6.8 mM solution of R18 in absolute ethanol was injected into 1 ml of phosphate-buffered saline (PBS) containing 2 mg of SFV protein and incubated in the dark at room temperature for 60 min. Unincorporated R18 was removed by filtration on a Sephadex G-50 column, and the labeled virus was centrifuged at 12,000 × *g* for 5 min at 4°C to remove aggregates. Recovery of virus was estimated by addition of trace amounts of [^{35}S]methionine-labeled SFV, and recovered virus averaged about 50%. The self-quenching of R18-labeled virus, determined by comparing fluorescence in the presence and absence of 1% Nonidet P-40 (NP-40), ranged from 78 to 90% for all experiments.

For the R18 fusion assay, 35-mm plates of BHK cells were washed twice at 4°C with cold Hanks–10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer, pH 6.8 (HH 6.8), and chilled on ice for 5 min (7). R18-labeled SFV (50 to 60 μg) in 500 μl of HH 6.8 was added and allowed to bind on ice in the dark for 1 to 1.5 h, and then the cells were washed twice in HH 6.8. About 10 to 12% of the added R18-labeled SFV bound to the cells. Fusion was induced by treating the cells and bound virus for the indicated time with HH 6.8–10 mM MES (morpholinoethanesulfonic acid), pH 6.0, at either 37 or 4°C. Cells were then washed twice with ice-cold HH 6.8, scraped into 1.8 ml of this buffer, and kept on ice until the fluorescence was quantitated. Fluorescence measurements were performed on a Perkin-Elmer LS 5B fluorometer with excitation and emission wavelengths of 560 and 590 nm, respectively. Readings were taken at room temperature while the mixture was stirred. After being read, the cells were lysed with 1% NP-40 for 30 min at room temperature in the dark, and the total cell-associated fluorescence was measured. The FdQ value is the amount of fluorescence measured before the addition of NP-40 as a percentage of the total cell-associated fluorescence.

The background FdQ value at time zero of R18-labeled virus bound to cells ranged from 10 to 26%, the same range as that of virus in the absence of cells. This background FdQ value was subtracted from the readings for each experiment. In control experiments, R18-labeled virus was acid treated at either 37 or 0°C in the absence of target membranes. The FdQ value of these samples was within the range of that of untreated virus, indicating that the FdQ value seen after low-pH treatment of cell-bound virus reflected virus fusion with the plasma membrane.

A second fusion assay measured the resistance of fused virus to removal by protease digestion (40). [^{35}S]methionine-labeled SFV was bound to 35-mm plates of BHK cells on ice for 60 min in binding medium (RPMI with no bicarbonate but containing 0.2% bovine serum albumin [BSA] and 10 mM HEPES, pH 6.8). About 35% of the added virus radioactivity bound to the cells. Fusion was triggered by treatment with pH medium (binding medium plus 10 mM MES, adjusted to pH 6.0) for the indicated time at either 37 or 4°C. Unfused virus was removed by digestion with proteinase K. Fusion was expressed as the percentage of the bound-virus radioactivity that was resistant to protease. The background proteinase K-resistant virus at time zero ranged from 7 to 16% of the bound virus and was subtracted for each experiment.

Monoclonal antibody assays of conformational changes in the E1 and E2 subunits. Conformational changes in the E1 and E2 spike protein subunits were assayed by immunoprecipitation with previously characterized acid-conformation-specific monoclonal antibodies (MAb) (14). For these assays, [^{35}S]methionine-labeled SFV in binding medium was prebound on ice to 35-mm plates of BHK cells for 60 min and pH treated at 37 or 4°C with pH medium. The cells were then washed in PBS and lysed, and an aliquot was immunoprecipitated as described elsewhere (14) by using either MAb E1a-1 or MAb E2a-1 to recognize the acid conformation of E1 or E2, respectively. A parallel aliquot was precipitated with a rabbit polyclonal antispike antibody to determine the total spike protein present. Following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography (14), precipitations were quantitated by densitometry on preflashed exposures of gels with a Molecular Dynamics class A computing densitometer with Quantity One software from PDI Corp. (Huntington

Station, N.Y.). E1 or E2 conversion is expressed as the percentage of the total spike protein subunit precipitated by the conformation-specific MAb.

Assay of E1-E2 dimer dissociation. The amount of spike protein present in an E1-E2 dimer was evaluated by antibody coprecipitation (36). For this assay, [^{35}S]methionine-labeled SFV was bound to cells and pH treated as described above for various times at either 37 or 4°C. Cells were washed with PBS and lysed in a buffer containing 1% NP-40, 50 mM Tris (pH 7.4), 100 mM NaCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 μg of pepstatin per ml, 25 μg of leupeptin per ml, and 1 mg of BSA per ml. The nuclei were pelleted by 10 min of centrifugation in a Microfuge, and the samples were preabsorbed with preimmune rabbit serum and Zysorbin, a fixed *Staphylococcus aureus* preparation (Zymed Laboratories, San Francisco, California), after which aliquots were precipitated with MAb E1-1, a MAb against the E1 subunit (14). The samples were washed twice with low-salt buffer, twice with high-salt buffer, and once with 10 mM Tris, pH 7.4, as described by Wahlberg et al. (36). Samples were analyzed by electrophoresis and densitometry as described above. The percentage of dimer dissociation was expressed as 100 × (1 − E2 precipitated by MAb E1-1/total E2). Total E2 present in the reaction mixture was determined by precipitation of an identical aliquot with a rabbit polyclonal antibody against the SFV spike protein. Similar assays used MAb E2-3, a MAb against E2 (14), and evaluated the amount of E1 coprecipitated with E2.

Assay of E1 homotrimer formation in vivo. Formation of an E1 homotrimer during endocytic uptake of virus was assayed by a method similar to that described by Wahlberg and Garoff (38). In brief, [^{35}S]methionine-labeled SFV was bound at pH 7.2 to 7.4 to 60-mm plates of BHK cells or C6/36 cells for 60 to 90 min, and the cells were then warmed in binding medium at the indicated temperature to permit endocytosis. At each time point, BHK cells were treated with proteinase K to remove noninternalized virus (38). As the nonendocytosed virus bound to C6/36 cells is not removed by such protease digestion (26), these cells were simply washed with binding medium and placed on ice to terminate endocytosis. All cell samples were then lysed as described elsewhere (38), and an aliquot was centrifuged on a 5- to 20% sucrose gradient for 22 to 24 h at 4°C (38). The gradients were fractionated, and the radioactivity was quantitated. As indicated, selected gradient fractions were immunoprecipitated with a rabbit anti-spike protein antibody and analyzed by SDS-PAGE and fluorography to identify the viral proteins present.

For assays of E1 homotrimer formation during plasma membrane fusion, radiolabeled virus was bound as described above and the plasma membrane-bound virus was treated with pH medium at pH 6.0 for the indicated times at either 37 or 0°C. Cells were then washed with binding medium, lysed, and evaluated by sucrose gradient centrifugation. The total cell-associated virus was analyzed, without removing nonfused virus by proteinase K digestion.

For some experiments, the amount of radioactivity in the spike protein monomer, dimer, and E1 homotrimer peaks was quantitated with nonlinear-curve-fitting software (PeakFit; Jandel Scientific, San Rafael, Calif.). Similar results were obtained when the area was quantified by cutting out and weighing each peak.

Assay of E1 homotrimer formation in vitro. For these assays, [^{35}S]methionine-labeled virus was pH treated in the presence or absence of various types of artificial liposomes, followed by gradient analysis. Liposomes contained a molar ratio of 1:1:1:0.3 phosphatidylcholine/phosphatidylethanolamine/sphingomyelin/phosphatidic acid (15). As indicated, liposomes also contained cholesterol or epicholesterol in a molar ratio of 1.5 mol of sterol to 3.3 mol of phospholipid. All liposomes were used at a final lipid concentration of 1 mM and were made in a buffer containing 20 mM MES (pH 7.0), 130 mM NaCl, and 0.2% BSA (MES buffer), as previously described (39). Radiolabeled virus was diluted into the MES buffer, liposomes were added as indicated, and the reaction mixture was preincubated for 10 min at 37°C. Some samples were then adjusted to pH 5.5 by the addition of a predetermined volume of 0.5 N acetic acid, and the incubation was continued for an additional 10 to 15 min at 37°C. Samples were then either neutralized and solubilized in 1% NP-40 or treated for 10 min on ice with 1% NP-40 followed by neutralization (36). Aliquots of the samples were centrifuged on 5 to 20% sucrose gradients as described above, fractionated, and counted. The amount of radioactivity in the homotrimer, dimer, and monomer peaks was quantitated as described above. Aliquots of the samples were also analyzed by immunoprecipitation with polyclonal rabbit antispike antiserum, or with MAb E1a-1, followed by SDS-PAGE. Selected gradient fractions were concentrated by precipitation with trichloroacetic acid and analyzed by SDS-PAGE.

When indicated, homotrimer formation was also assayed by gel electrophoresis after samples had been heated in SDS-sample buffer at 30°C for 2 min (37). The proportion of E1 in the homotrimer band migrating at approximately 150 kDa was compared with the total E1 by densitometry. For assay by gel electrophoresis, the samples were prepared in MES buffer without BSA.

Lipid-free spike protein preparations. Purified SFV spike proteins were prepared by detergent solubilization and sucrose gradient sedimentation as previously described (9). This method yields octameric spike protein complexes termed rosettes that contain E1 and E2 associated by their transmembrane domains. Rosettes are water soluble and free of viral nucleocapsid, virus membrane lipids, and detergent. The rosettes were dialyzed into 20 mM MES–130 mM NaCl, pH 7.0, and frozen at −80°C until used.

To assay acid-induced conformational changes in the rosettes, they were mixed with liposomes or MES buffer as described above for the virus-liposome experiments, treated at either pH 7.0 or pH 5.5 for 10 min at 37°C, neutralized, and solubilized with 1% NP-40 for 30 min at 37°C. Aliquots of the samples were analyzed by sucrose gradient centrifugation or by precipitation with polyclonal rabbit antispike antiserum or MAb E1a-1. Selected gradient fractions were concentrated by precipitation with trichloroacetic acid and analyzed by SDS-PAGE.

RESULTS

In all of the experiments performed to determine the kinetics of SFV fusion or spike protein conformational changes, virus was bound to the plasma membrane of BHK cells and acid treated at the cell surface with buffer at pH 6.0 at either 37 or 0°C. Thus, the virus was in a receptor-bound state, only virus active in binding was assayed, and the time course of low-pH effects was assayed in the absence of the in vivo processes of virus endocytosis and endosome acidification. Preliminary studies demonstrated that fusion at pH 6.0, slightly below the virus fusion threshold of pH 6.2 (12), was slower than that seen at pH 5.5 and should more readily resolve the steps of the reaction. In addition, SFV fusion with the plasma membrane of BHK cells at temperatures ranging from 0 to 37°C has been shown to result in productive infection (3), indicating the biological relevance of this fusion system.

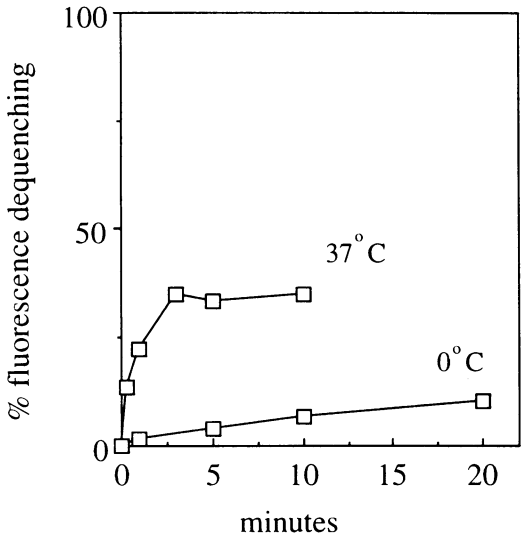

FIG. 1. Kinetics of SFV fusion with BHK cell plasma membranes. R18-labeled SFV was prebound to BHK cells on ice for 90 min, and virus-plasma membrane fusion was triggered by treatment at pH 6.0 for the indicated times at 37 or 0°C. Fusion of R18-labeled virus was quantitated by measuring the resultant dequenching of R18 fluorescence, expressed as a percentage of total dequenching. The data shown are the averages for two experiments at each temperature.

Kinetics of SFV-membrane fusion at 37 and 0°C. To analyze fusion of SFV with BHK cell target membranes, we followed the mixing of virus and cell membranes by the FdQ of virus labeled with the fluorescent fatty acid R18 (27, 33). As shown in Fig. 1, the kinetics of fusion at 37°C were rapid, with a half-life ($t_{1/2}$) of about 40 s and a final extent of ~35% FdQ. In contrast, at 0°C virus fusion occurred at a much lower rate, with a $t_{1/2}$ of about 5.8 min and an extent of ~11% at 20 min. A lag of ~1 min was observed before the start of fusion at 0°C. Fusion was also assayed by a previously characterized method that measures the resistance of fused virus to removal by protease digestion (40). At either 37 or 0°C, the kinetics of fusion were similar with the two assays (data not shown).

Thus, fusion of SFV with the plasma membrane could be dramatically slowed by low temperature and intermediate pH conditions, making this system amenable to detailed analysis of spike protein conformational changes.

Correlation of SFV fusion with conformational changes in the E1 and E2 spike protein subunits. The kinetics of spike protein conformational changes were next determined during SFV-plasma membrane fusion at both low and physiological temperatures (Fig. 2). The dissociation of the E1-E2 heterodimer (36) was assayed by coprecipitation with subunit-specific MAbs (14). Dimer dissociation occurred with a $t_{1/2}$ of ~15 s at 37°C and a $t_{1/2}$ of ~22 s at 0°C (Fig. 2A and B). Essentially all of the spike protein present in the 0°C reaction dissociated before fusion was observed. Thus, even at 0°C, dimer dissociation occurred much more rapidly and completely than fusion and without a detectable lag phase.

The irreversible conversion of E1 and E2 to acid-specific conformations was assayed by precipitation with previously described acid-conformation-specific MAbs (14). Exposure of the acid-specifc E1 epitope had a $t_{1/2}$ of ~43 s at 37°C and a $t_{1/2}$ of ~3.6 min at 0°C (Fig. 2C and D). MAb E1a-1 reactivity at 0°C thus appeared to occur before the actual fusion event, which had a $t_{1/2}$ of about 5.8 min at 0°C. The maximum extent of conversion at either temperature was about 35% of the total E1 protein present. A lag of about 30 s was observed for MAb E1a-1 conversion at 0°C.

In contrast to the rapid exposure of the MAb E1a-1 epitope, exposure of the acid-specific E2 epitope detected by MAb E2a-1 occurred much more slowly. The $t_{1/2}$ of the E2 reaction was at least 6 min at 37°C, and it had a maximum extent of conversion of about 26% of the total E2 after 10 min of pH treatment at 37°C (Fig. 2E). No MAb E2a-1 reactivity was detectable after pH treatment at 0°C for 20 min, a time point at which about 11% FdQ had occurred (Fig. 2F). Thus the spike protein conformational change detected by MAb E2a-1 appears to occur considerably later than the fusion of the virus with the target membrane.

Kinetics of formation of the E1 homotrimer during SFV fusion. Wahlberg and Garoff (38) have described the formation of an E1 homotrimer during endocytic uptake of SFV in BHK cells. We monitored the kinetics of E1 homotrimer formation by pH 6.0 treatment of cell-bound virus at 0 or 37°C (Fig. 3). At each time point, the cells with bound virus were lysed, the lysate was centrifuged on a sucrose gradient, and the amount of spike protein radioactivity in the homotrimer peak was determined. At 37°C, homotrimer formation was found to occur with a $t_{1/2}$ of ~24 s and thus showed kinetics similar to those of fusion (Fig. 3A). The amount of total spike protein radioactivity found in the E1 trimer peak reached a maximum of about 34% after 1 min at 37°C. When fusion was slowed by incubation at 0°C, homotrimer formation was similarly slowed and occurred with a $t_{1/2}$ of ~3.3 min and a final extent of ~28% (Fig. 3B). Fusion at this temperature had a $t_{1/2}$ of about 5.8 min. Thus, both the exposure of the MAb E1a-1 epitope and the conversion of E1 to the homotrimer form showed somewhat faster kinetics than those of fusion and occurred before the final membrane-mixing event.

Formation of the E1 homotrimer in endosomes. If the formation of the E1 homotrimer is important in the infectious fusion of SFV in vivo, it should occur within the endosome compartment where productive entry of SFV takes place (20). SFV can be trapped within the endosome by incubation of cells at 20°C (20), a temperature that inhibits fusion of endosomes with lysosomes. Under these conditions, fusions of both the wild type and a pH-shift mutant of SFV occur and result in virus infection (16, 20). We allowed radiolabeled SFV to be endocytosed by BHK cells for 45 min at 37°C or for 2 h at 20°C. E1 homotrimer formation was then assayed by sucrose gradient centrifugation, and the exposure of the MAb E1a-1 epitope was assayed by immunoprecipitation. At 37°C, in agreement with the results obtained in previous studies (38), the predominant peak of spike protein radioactivity was in the homotrimer (Fig. 4A). Little monomer peak was observed, consistent with the rapid degradation of E2 in lysosomes (20). Assay of the 20°C sample (Fig. 4B) revealed that the E1 homotrimer was efficiently formed within the endosome compartment. A more prominent monomer peak containing primarily E2 was found in the 20°C sample, in agreement with the inhibition of lysosomal delivery at 20°C. E1 was efficiently immunoprecipitated by MAb E1a-1 after endocytosis at either temperature (data not shown), demonstrating the effective acidification of virus in endosomes. The generation of monomer, E1 homotrimer, and MAb E1a-1 reactivity were all blocked by treatment with monensin (22), which dissipates endosomal acidity (data not shown).

E1 homotrimer formation does not require cholesterol in vivo. Our previous work demonstrated that SFV fusion both in vitro and in vivo requires the presence of cholesterol in the target membrane (15, 26). As part of these studies, we

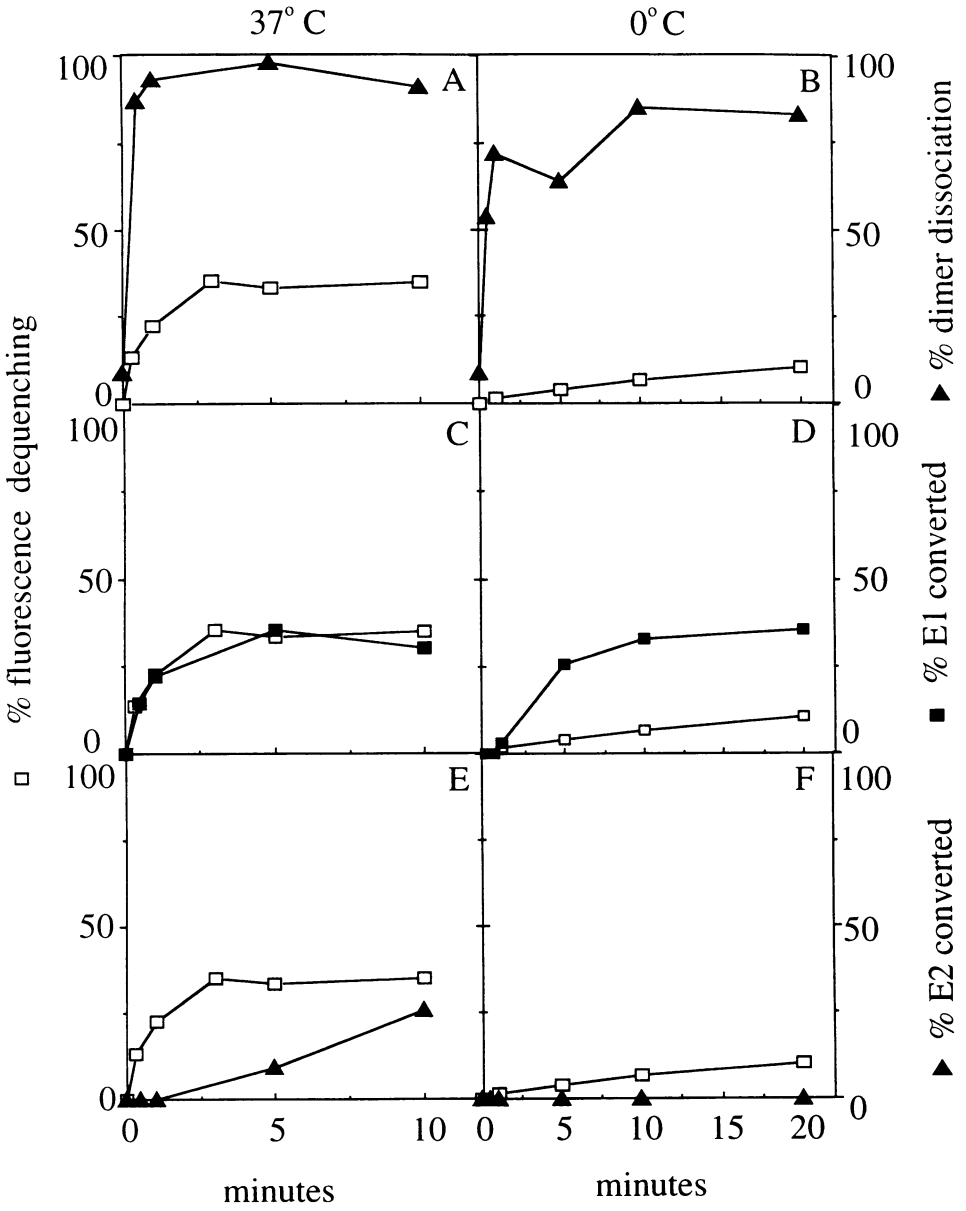

FIG. 2. Comparison of the kinetics of SFV spike protein conformational changes and virus-plasma membrane fusion. [^{35}S]methionine-labeled SFV was prebound to BHK cells on ice for 90 min, and fusion was triggered by treatment at pH 6.0 for the indicated times at 37°C (A, C, and E) or 0°C (B, D, and F). The dissociation of the E1-E2 dimer was measured by coprecipitation of E2 by MAb E1-1 against the E1 subunit (▲) (A and B). The acid-dependent conformational change in E1 was quantitated by immunoprecipitation with an acid-conformation-specific MAb against E1, MAb E1a-1 (■) (C and D). The acid-dependent conformational change in E2 was quantitated by precipitation with an acid-conformation-specific MAb against E2, MAb E2a-1 (▲) (E and F). Immunoprecipitates were analyzed by SDS-PAGE, fluorography, and densitometry. MAb precipitations are expressed as percentages of the total spike protein subunit, as determined by precipitation with a polyclonal rabbit antispike antiserum. Data shown are the averages for two experiments each. For comparison, in each panel the kinetics of SFV fusion determined by the R18 assay are shown (□) (data obtained from Fig. 1).

developed conditions to cholesterol deplete the C6/36 mosquito cell line and maintain it in culture for indefinite periods of time in the absence of sterol (26). Such cells show normal virus binding, endocytosis, and E1 acid conversion as assayed by MAb E1a-1, but the fusion of the virus with the endosome membrane is blocked (26). We used these cells to ascertain whether cholesterol is required in vivo for the formation of the E1 homotrimer. Virus was bound to control and cholesterol-depleted C6/36 cells and endocytosed at the normal culture temperature of 28°C, and the formation of the E1 homotrimer was assayed by sucrose gradient centrifugation (Fig. 5). All of the cell-associated virus radioactivity was assayed, as we have not found conditions that allow removal of nonendocytosed virus from C6/36 cells. The E1 homotrimer was formed in both the control and cholesterol-depleted cells (Fig. 5). The spike protein radioactivity in the homotrimer peak was compared with the total spike protein radioactivity. After 30 min of endocytosis in either cell type, the percentage of homotrimer

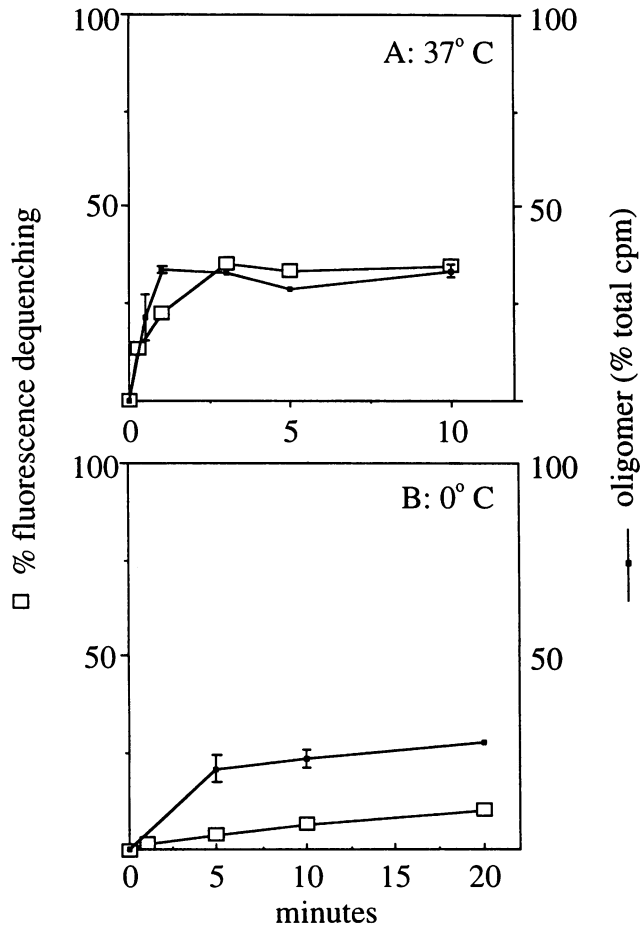

FIG. 3. Kinetics of E1 homotrimer formation during SFV-plasma membrane fusion. [^{35}S]methionine-labeled SFV was prebound to BHK cells on ice for 90 min, and fusion was triggered by treatment at pH 6.0 for the indicated times at 37°C (A) or 0°C (B). Cells were washed and lysed, and then cell lysates were analyzed by centrifugation on 5 to 20% sucrose gradients. The amount of E1 homotrimer was then determined as described in Materials and Methods. Data shown are the averages and standard deviations (error bars) for three experiments. For comparison, in each panel the kinetics of SFV fusion determined by the R18 assay are shown (□) (data obtained from Fig. 1).

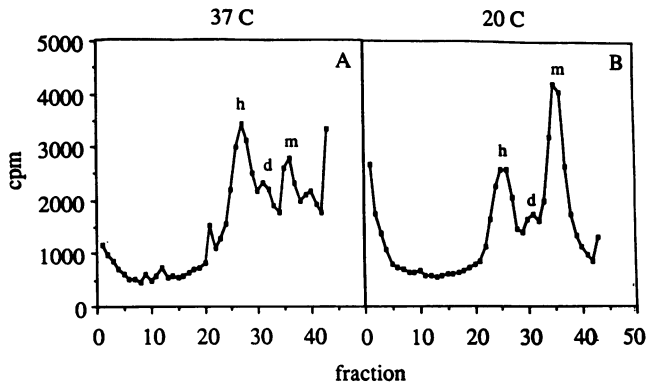

FIG. 4. Formation of the E1 homotrimer during endocytosis in BHK cells at 37 and 20°C. [^{35}S]methionine-labeled SFV was prebound to BHK cells on ice for 60 min, after which the cells were washed once to remove unbound virus and warmed to 37°C for 45 min (A) or to 20°C for 2 h (B). Noninternalized virus was removed by proteinase K digestion, and the samples were lysed; aliquots were then analyzed by centrifugation on 5 to 20% sucrose gradients. The positions of spike protein dimer (d), monomer (m), and E1 homotrimer (h) are indicated. The data are representative examples from two experiments each.

was 32% ± 1% for cholesterol-containing cells and 24% ± 3% for cholesterol-depleted cells (average for three experiments). Pooled fractions from each of the three peaks shown in both panels of Fig. 5 were analyzed by gel electrophoresis. The homotrimer peaks contained E1 exclusively, the dimer peaks consisted of both E1 and E2, and the monomer peaks consisted of predominantly E2 (data not shown). Aliquots of the same cell lysates were also assayed by MAb E1a-1 precipitation and were shown to have efficiently converted to the E1 acid conformation (data not shown). All of these changes in the SFV spike protein were blocked by the addition of monensin (data not shown). Thus, the formation of the E1 homotrimer was pH dependent but relatively cholesterol independent in this in vivo system.

In vitro requirements for E1 homotrimer formation. We next wished to determine whether the E1 homotrimer could be detected after in vitro acidification in the presence and absence of artificial target membranes. Radiolabeled virus was mixed with 1 mM liposomes containing either a molar ratio of one cholesterol per two phospholipids or phospholipids alone. Alternatively, virus was mixed with liposome dilution buffer containing MES, saline, and BSA, as described in Materials and Methods. Samples were treated at low or neutral pH at 37°C and then analyzed by centrifugation on sucrose gradients. A prominent peak in the homotrimer position resulted when virus was low pH treated in the presence of a target membrane (Fig. 6A and B). Consistent with the lack of a cholesterol requirement observed in our in vivo C6/36 experiments, no requirement for cholesterol was observed in vitro. The percentage of the total spike protein radioactivity migrating as homotrimer was 32% ± 11% (average for four experiments) for cholesterol-containing target membranes and 39% ± 2%

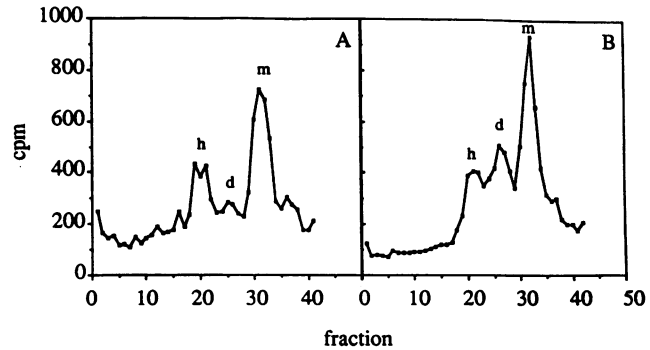

FIG. 5. Effect of cholesterol on E1 homotrimer formation in vivo. [^{35}S]methionine-labeled SFV was prebound to control (A) or cholesterol-depleted (B) C6/36 cells on ice. The cells were then washed once to remove unbound virus and warmed to 28°C for either 30 min (A) or 45 min (B). The samples were lysed, and aliquots were analyzed by centrifugation on 5 to 20% sucrose gradients. The data are representative examples from three experiments. The positions of spike protein dimer (d), monomer (m), and E1 homotrimer (h) are indicated.

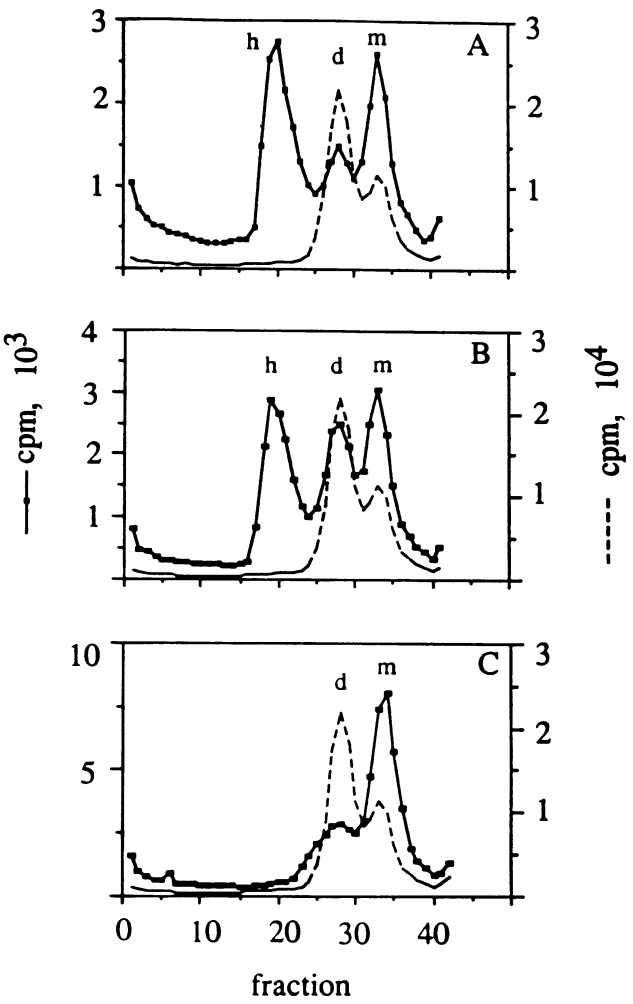

FIG. 6. Formation of the E1 homotrimer in vitro. [^{35}S]methionine-labeled virus was treated at pH 5.5 for 10 to 15 min in the presence of cholesterol-containing liposomes (A) or sterol-free liposomes (B) or in MES buffer without liposomes (C). Samples were then solubilized and analyzed by centrifugation on 5 to 20% sucrose gradients. In each panel, the solid line shows the sedimentation pattern of the pH 5.5-treated virus and the dashed line shows the sedimentation pattern of a control sample of virus incubated with cholesterol-containing liposomes at pH 7.0. Representative data from four experiments each are shown. The positions of spike protein dimer (d), monomer (m), and E1 homotrimer (h) are indicated.

(average for three experiments) for sterol-free target membranes. SDS-PAGE analysis of the homotrimer peaks shown in Fig. 6A and B confirmed that they were composed exclusively of E1 (data not shown). Similar homotrimer formation, averaging 34% in two experiments, was obtained with target liposomes containing epicholesterol, a cholesterol analog with a 3α-hydroxyl group that does not permit fusion (15) (data not shown). In contrast, little E1 homotrimer was observed when the virus was low pH treated in the absence of a target membrane (Fig. 6C). The decrease in homotrimer observed on the gradients was not due to an increase in the amount of aggregated material that pelleted in the gradient (data not shown). The overall recovery of virus radioactivity from the virus sample treated at low pH in buffer was reproducibly somewhat lower, from 68 to 76% that of the virus treated at low pH in the presence of cholesterol liposomes. The exposure of the MAb E1a-1 epitope was assayed in aliquots of the samples shown in Fig. 6, and results were found to be similar in all three low-pH-treated samples (data not shown).

The reversibility of the gradient patterns was assayed by acid treating the three sample types and either neutralizing before solubilization or solubilizing with NP-40 and then neutralizing (36) (data not shown). The amount of spike protein dimer was slightly increased and the amount of monomer was decreased when the samples were neutralized prior to solubilization. This agrees with previous results obtained by Wahlberg and Garoff (36), who found that the dissociation of the dimer into spike protein monomers was reversible under similar conditions. However, the amount of E1 homotrimer was not affected by the order of the neutralization and solubilization steps. Thus, the formation of the E1 homotrimer was not reversible.

The E1 homotrimer is resistant to mild treatment with SDS and can be assayed by gel electrophoresis (37). By using this assay, formation of the E1 homotrimer had been found to be independent of the presence of a target membrane (37). We used the gel electrophoresis assay to quantitate E1 homotrimer as a percentage of the total E1. The percentage of homotrimer is thus higher with this assay, since homotrimer is compared with E1 rather than with total spike protein as in the gradient assay. The percentage of homotrimer was 78% ± 11% with cholesterol liposomes (four experiments), 52% ± 13% with sterol-free liposomes (three experiments), and 24% ± 13% for virus in buffer (four experiments). Thus, in our hands, the proportion of E1 migrating as a homotrimer was significantly enhanced by the presence of target membranes and was further enhanced by the inclusion of cholesterol in the target membrane.

Conformational changes in lipid-free spike protein rosettes. The in vitro results above indicate that the E1 homotrimer was most efficiently formed during treatment of virus in the presence of target membranes with sterol. The virus used in these experiments, however, contained cholesterol in its membrane, having been propagated in cholesterol-containing BHK cells (19). To assay E1 homotrimer formation in the absence of cholesterol, lipid-free spike protein rosettes were prepared by previously characterized methods. These complexes contain about eight spike proteins associated via their hydrophobic domains, are free of detectable lipid from the virus membrane, and are soluble in the absence of detergent (9). Such rosette preparations were treated at pH 5.5 with buffer alone, cholesterol-containing liposomes, or sterol-free liposomes; dissociated with NP-40; and assayed by gradient sedimentation for production of E1 homotrimer (Fig. 7). Under all three conditions, some rosettes were incompletely dissociated and sedimented to the bottom of the gradient (Fig. 7, fractions 1 through 5). A peak with migration similar to that of the E1 homotrimer was observed in the cholesterol-containing-liposome sample (Fig. 7B, fractions 16 through 21) and contained 22% of the dissociated radioactivity migrating in the upper half of the gradient. A smaller peak in this position, containing 11% of the dissociated radioactivity, was observed in the sterol-free-liposome sample (Fig. 7C). This peak was not present when rosettes were acidified in the absence of a target membrane (Fig. 7A). The putative E1 homotrimer peak from the sample presented in Fig. 7B was analyzed by SDS-PAGE and shown to contain exclusively E1 (Fig. 8, lane 9). Thus, in the absence of virus cholesterol, the E1 homotrimer was formed during acidification in the presence of a target membrane, and its formation was enhanced by the presence of cholesterol.

Spike protein rosettes were also treated at acidic or neutral pH in the presence of buffer or liposomes and then assayed for

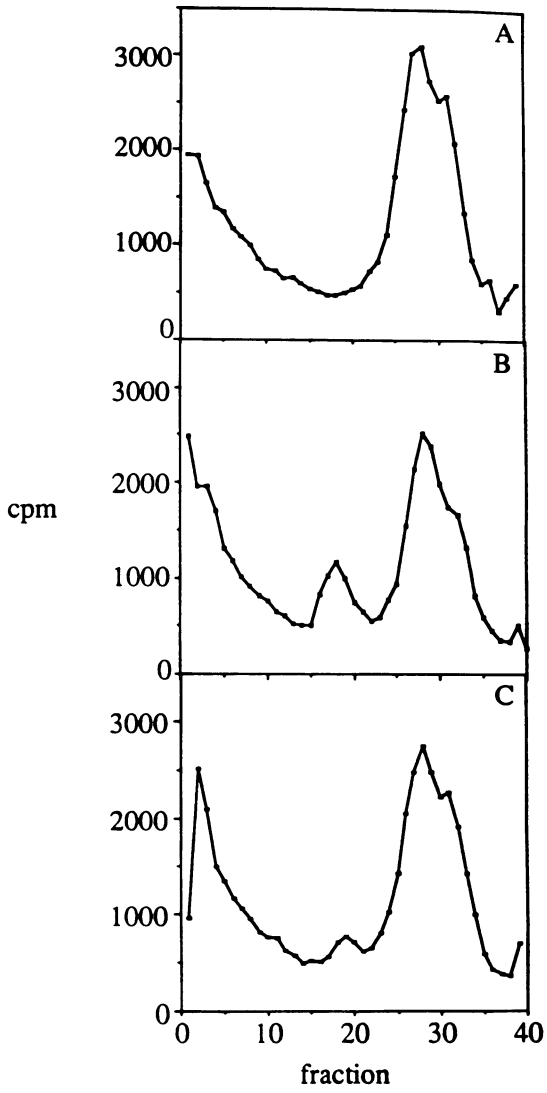

FIG. 7. E1 oligomerization with lipid-free spike protein rosettes. Purified radiolabeled spike protein rosettes were mixed with MES buffer (A), cholesterol-containing liposomes (B), or sterol-free liposomes (C), treated at pH 5.5 for 10 min at 37°C, neutralized, and solubilized; aliquots of the samples were then analyzed by gradient centrifugation. Representative data from two experiments each are shown.

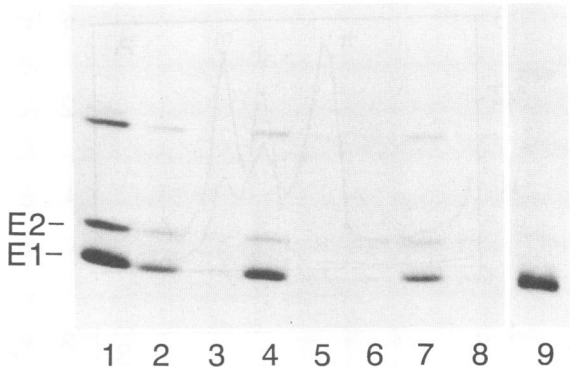

FIG. 8. Low-pH-dependent conformational changes in lipid-free spike protein rosettes. Purified radiolabeled spike protein rosettes were mixed with cholesterol-containing liposomes (lanes 1, 4, 5, and 6), sterol-free liposomes (lanes 7 and 8), or MES buffer (lanes 2 and 3) and treated either at pH 5.5 (lanes 2 through 5, 7, and 8) or at pH 7.0 (lanes 1 and 6) for 10 min at 37°C. The samples were then neutralized, solubilized, and immunoprecipitated with either polyclonal rabbit antispike antiserum (lane 1), MAb E1a-1 (lanes 2, 4, 6, and 7), or a MAb to an unrelated protein (lanes 3, 5, and 8). Lane 9 shows the migration of a sample of the pooled oligomer peak from the gradient in Fig. 7B. The more slowly migrating bands in lanes 1 through 8 are incompletely dissociated rosettes.

reactivity with MAb E1a-1 (Fig. 8). This E1 acid-conformational change was most efficient in the presence of cholesterol-containing liposomes (Fig. 8, lane 4) but also occurred after pH treatment in either buffer alone (lane 2) or sterol-free liposomes (lane 7). Thus, in the absence of virus cholesterol, low pH alone could induce the change in conformation recognized by mAb E1a-1, but this conformational change was enhanced by the presence of cholesterol-containing target membranes.

DISCUSSION

In this study, we have analyzed the kinetics and order of spike protein conformational changes during SFV fusion and evaluated the role of cholesterol in the formation of the E1 homotrimer. Our results and those of others suggest a model for the changes in fusion protein structure leading up to membrane fusion. First, following low-pH treatment, the E1-E2 dimer dissociates. The E2 subunit rapidly changes to a trypsin-sensitive conformation and much later, after fusion, adopts a conformation recognized by MAb E2a-1. The E1 subunit rapidly undergoes conformational changes resulting in trypsin resistance, reactivity with MAb E1a-1, and a homotrimeric configuration. Following further cholesterol-dependent events, the virus and target membranes fuse and the mixing of the two bilayers ensues.

The kinetic studies presented here monitored the low-pH-dependent fusion of SFV with the plasma membrane of BHK cells and used a fluorescence assay that monitored the dilution of R18 incorporated into the virus membrane. Our results indicated that fusion could be dramatically slowed by low temperature and intermediate pH conditions. These slower conditions for fusion enabled us to monitor the kinetics of the spike protein conformational changes. The extent of SFV fusion at 0°C was much lower than the extent at 37°C. SFV thus behaved quite differently than did influenza virus, which showed an increased fusion extent at low temperature due to inhibition of acid-induced inactivation of fusion (34).

There have been some concerns about the R18 dequenching assay for virus fusion. Under some conditions with influenza virus, R18 was previously reported to exchange with the target membrane at neutral pH in the absence of fusion (43). Our SFV experiments showed no significant exchange of the R18 probe during incubation of the labeled virus with the cells at pH 7.0. In addition, minimal dequenching was observed when virus was treated at low pH in the absence of a target membrane.

It was important to determine that low-pH-treated SFV does not continue to fuse after transfer to a neutral pH environment, since all of our kinetic studies were based on low pH treating the cell-bound virus and stopping the reaction by a shift to neutral pH at various times. Our results suggest that both fusion and the spike protein conformational changes are inhibited by the shift to neutral pH, since both partial fusion

and partial conversion of the spike protein were observed after short periods of pH treatment. This finding also agrees with the elegant experiments recently published by Bron et al. (3), who used a continuous assay system to monitor SFV fusion with target liposomes lacking virus receptors. They demonstrated that fusion and spike protein conformational changes required exposure to acidic pH and that both processes were inhibited by a shift to neutral pH. Although the two fusion systems are based on different target membranes, one containing and the other lacking receptors, the overall results are in agreement. Similar to results obtained with the liposome fusion system, our low-temperature experiments indicated a lag period between exposure of virus to low pH and the actual onset of fusion. Although we did not study the lag period extensively, it was clear that the conformational changes in the spike dimer and E1 were taking place prior to fusion and that a lag occurred even with a biological target membrane containing the virus receptor. In agreement with the liposome fusion results of Bron et al. (3), our results suggest that further reorganization of protein and/or lipid components of the membrane is required for the final fusion event.

Studies of the conformational changes in the SFV spike at 0°C showed that the dissociation of the E1-E2 dimer was the first low-pH-dependent event detected, with a very rapid $t_{1/2}$ of about 22 s even at 0°C. Although the first description of this conformational change emphasized its reversibility (36), we found only a low level of reversion to spike protein dimers. Presumably this is because the E1 dissociated from the dimer was complexed irreversibly into homotrimers, removing it from the pool of monomers that could reassociate into dimers (Fig. 6). Interestingly, dimer dissociation has a pH threshold of about 7.0, considerably higher than that of fusion (36). Flynn et al. (5) and Meyer et al. (23) have described a very early, pH-independent conformational change that occurs at the plasma membrane upon warming of the virus-receptor complex. Given its high pH threshold, it is possible that dimer dissociation represents the conformational change recognized by the MAb binding assays of Flynn et al. (5). Analysis of p62-containing spikes with impaired dimer dissociation demonstrated that dissociation is critical to the occurrence of further conformational changes in E1, including E1 homotrimer formation (27). The rapid kinetics of dimer dissociation are in keeping with this requirement (Fig. 2 and 3) (3).

A variety of conformational changes have been observed following low-pH treatment of the E2 subunit. Very rapidly after low-pH treatment, E2 converts to a more trypsin-sensitive conformation (4, 13). This E2 alteration has a pH threshold of 6.2 and a $t_{1/2}$ of about 5 s at 37°C (13). E2 trypsin sensitivity is not due to dimer dissociation, since it is also observed upon low-pH treatment of a proteolytically truncated form of E2 that is monomeric (13). Following the protease studies, we isolated MAbs that specifically recognized the acid-treated E2 or E1 subunit (14). As shown here, however, the antibodies to E2 recognize a low-pH-induced conformational change that occurs considerably later than the fusion reaction (Fig. 2). The kinetics of this conformational change suggest that it could be involved in the acid inactivation of the spike protein, which has been shown to occur considerably later than fusion (3). Taken together, our results indicate that E2 in the spike protein dimer first dissociates from E1, that there are early changes in E2 prior to the fusion event that result in trypsin sensitivity, and that following fusion there is a reorganization of the E2 subunit to a form that is recognized by our E2 acid-specific MAb, E2a-1.

In these and previous studies, we have demonstrated that E1 undergoes low-pH-dependent conformational change(s) leading to recognition by acid-conformation-specific MAbs (Fig. 2) (14) and increased trypsin resistance (13). By using both of these assays, the E1 conformational change has been shown to occur during the endocytic uptake of SFV (8, 14, 26, 29) and with more rapid kinetics than those of membrane fusion (Fig. 2) (3, 13). In addition to E1's changes in antigenicity and protease sensitivity, low-pH treatment causes E1 to oligomerize into a homotrimer (37, 38). Homotrimer formation occurs early in virus endocytosis (38), within the endosome compartment known to be the site of virus fusion (Fig. 4). The results obtained by Wahlberg et al. (37) and Bron et al. (3), as well as those reported here (Fig. 3), indicate that the E1 homotrimer is formed after pH treatment but before the membrane-mixing event of fusion. Interestingly, another acid-specific MAb, E1″, recognizes the homotrimer and blocks both fusion and infection (37, 38). These data concerning the importance of E1 conformational changes in fusion agree with structural evidence suggesting that E1 is the fusogenic spike subunit. E1 contains a highly conserved apolar region suggested to be the virus fusion peptide (6), and mutations within this region can shift the pH threshold of fusion or completely block fusion (17). In addition, protease-treated viruses containing only the E1 subunit are fusogenic (24). All of this evidence thus suggests a central role of E1 in the final fusion reaction.

The SFV fusion reaction is striking in its requirement for cholesterol or a 3β-hydroxysterol in the target membrane (15, 26, 39). In this paper, we have addressed the involvement of cholesterol in the formation of the E1 homotrimer and in MAb E1a-1 reactivity. By using purified virus, homotrimer formation was shown to be relatively independent of cholesterol in vitro (Fig. 6) (37). Wahlberg et al. previously reported that homotrimer formation also occurs efficiently after low-pH treatment of isolated virus (37). Using either sucrose gradient or SDS-PAGE analysis, we found that the formation, or recovery, of E1 homotrimer appeared to be enhanced by the presence of a target membrane, even if that membrane lacked cholesterol and could not support the final membrane-mixing step of virus fusion. In keeping with our previous results, reactivity of virus E1 with MAb E1a-1 did not show a significant target membrane or cholesterol requirement (13, 26).

To address the role of virus membrane cholesterol in the E1 conformational change, we then examined membrane-free spike protein rosettes (Fig. 7 and 8). Both the expression of the E1 homotrimer and reactivity with MAb E1a-1 were enhanced by acidification of rosettes in the presence of cholesterol-containing target membranes. Similar to our findings with virus, efficient homotrimer expression was dependent on target membrane addition. Thus, the E1 homotrimer could form in the absence of the virus membrane, but formation appeared most efficient when cholesterol-containing target membranes were present.

A requirement for cholesterol was previously observed for the acid conformational change in a proteolytically truncated form of E1 termed E1* but not for that in full-length viral E1 (13, 14). E1* contains most of the ectodomain of E1 without the transmembrane domain and is a monomeric soluble protein purified free of the virus membrane (13). The enhancement of rosette E1 conformational changes by cholesterol suggests that at least part of the striking cholesterol dependence of the E1* conformational change may be due to the absence of virus membrane cholesterol from these preparations as well.

The conformational change detected by MAb E1a-1 occurred with kinetics similar to those of E1 homotrimer formation. Given the similarities of their kinetics, it is possible that the antibody, trypsin, and homotrimer assays are detecting the

same conformational change in E1, as has been suggested for MAb E1″ (3, 37). However, since the assays most probably recognize changes in tertiary protein structure (trypsin sensitivity and MAb reactivity) versus changes in quaternary protein structure (E1 homotrimerization), it is difficult to say that the MAb E1a-1 conformational change represents a recognition of the homotrimer. To date, the assays closely correlate.

Our results order several previously described conformational changes in the fusogenic SFV spike protein. While none of these conformational changes is strictly dependent on the presence of cholesterol in the target membrane, cholesterol was found to enhance E1 homotrimer formation and MAb E1a-1 reactivity, especially of virus spikes assayed in the absence of the cholesterol-containing virus membrane. Our results suggest a model in which E1 forms an acid-dependent complex, stabilized by the target membrane and requiring cholesterol for further conformational changes leading to fusion. Thus, the E1 homotrimer and MAb E1a-1 conformational change may be involved in the formation of such a prefusion complex. Our further studies of the conformational changes in E1 during fusion will focus on the unique aspects of the interaction of E1 with bilayers containing cholesterol.

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