Biosynthesis, Maturation, and Acid Activation of the Semliki Forest Virus Fusion Protein

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The Semliki Forest virus spike protein has a potent membrane fusion activity which is activated *in vivo* by the low pH of endocytic vacuoles. The spike protein is composed of two transmembrane subunits, E1 and E2, plus E3, a peripheral polypeptide. Acid-induced conformational changes in the E1 or E2 subunits were analyzed by using monoclonal antibodies specific for the acid-treated spike protein. E1 and E2 reacted with the antibodies after treatment of wild-type or mutant virus at the pH of fusion. The E1 conformational change resembled fusion in its requirement for both low pH and cholesterol. Pulse-chase analysis and intracellular pH treatment were then used to determine the ability of the newly synthesized spike to undergo acid-induced conformational changes. p62, the precursor to E2 and E3, was shown to undergo a pH-dependent conformational change similar to that of E2 and was sensitive to acid very soon after biosynthesis. In contrast, a posttranslational maturation event was required for the conversion of E1 to the pH-sensitive form. E1 maturation occurred fairly late in the exocytic pathway, after the virus spike had passed the medial Golgi but before incorporation of the spike into a new virus particle.

Membrane fusion is used by enveloped animal viruses to infect host cells. Many of these viruses exploit the cell's constitutive endocytic activity to gain access to an intracellular compartment in which fusion occurs. Such viruses bind to cell surface receptors, are endocytosed in coated pits and coated vesicles, and are delivered to prelysosomal endosomes (reviewed in references 23, 24, and 57). Viral fusion is specifically triggered by the mildly acidic endosomal pH (pH 6.5 to 5.0), which is also critical for endosome function during receptor-ligand internalization and intracellular sorting (reviewed in reference 37). Membrane fusion results in the release of the viral genome into the cytoplasm and the beginning of viral replication. Our studies focus on the fusion reaction and endocytic entry of Semliki Forest virus (SFV), the alphavirus for which this pathway was first delineated (15).

SFV fusion is mediated by the viral spike glycoprotein, a heterotrimer of two transmembrane glycopolypeptides, E1 (50,786 daltons [Da]) and E2 (51,855 Da), and a peripheral glycopolypeptide, E3 (11,369 Da) (reviewed in references 44 and 47). It occurs with a pH threshold of about 6.2 and requires the presence of cholesterol or a 3 β -hydroxysterol in the target membrane (21, 56). Previous work has demonstrated that upon treatment at the fusion pH, irreversible conformational changes rapidly occur in both the E1 and E2 subunits. The conformational change in the E1 subunit can be detected by the increased resistance of the acid form of E1 to trypsin digestion (22). In contrast, the acid conformation of E2 has an increased sensitivity to trypsin digestion (10, 22). Soluble ectodomains of E1 and E2 which lack the transmembrane anchor can be prepared by proteolytic cleavage and undergo pH-dependent conformational changes similar to those of intact E1 and E2 (22). Notably, the purified

E1 ectodomain requires both low pH and cholesterol to shift to the trypsin-resistant form.

While it is clear that the SFV spike protein catalyzes low pH-dependent membrane fusion, the role of subunit associations and assembly in this process is undefined. In this article, conformation-specific monoclonal antibodies (MAbs) have been used to further characterize the acid-dependent changes in E1 and E2. E2 and E3 are initially synthesized as a precursor, p62, which is proteolytically processed intracellularly during its transit to the plasma membrane (9, 35). By using specific MAb reagents, the acid sensitivities of pulse-labeled p62 and E1 were examined. We identified a posttranslational maturation step which is required for the conversion of newly synthesized E1 to a fusion-competent form.

MATERIALS AND METHODS

Viruses and cells. Wild-type (wt) and *fus-1* SFV were propagated, radiolabeled, and purified as previously described (25) in baby hamster kidney (BHK-21) cells.

Antibodies. MAbs to SFV spike polypeptides were raised by intrasplenic injection of BALB/c mice with 7 to 10 μ g of spike protein purified by Triton X-114 precipitation (3). Six months after the initial injection, mice were boosted by the intrasplenic injection of 40 μ g of purified spike rosettes prepared as described before (16), with or without acid treatment. Spleen cells were fused with the NSO myeloma line 3 days after the boost (11) or were used to repopulate the spleens of sublethally irradiated mice (11, 59) and then fused after an additional boost (E2a MAbs only). Hybrids were initially screened by enzyme-linked immunosorbent assay against Triton X-114-purified SFV spike proteins adsorbed to 96-well microtiter dishes. E2a MAbs were screened by using a solid-phase radioimmunoassay against acid and neutral pH-treated spike proteins. The subunit and conformation specificities of selected positive clones were determined by immunoprecipitation of acid or neutral pH-treated, Triton X-114-purified SFV spike proteins. Antibody subclass

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was determined by using an enzyme immunoassay. Hybrid lines were subcloned twice in soft agar (59). Antibodies were purified by growing hybrid cells lines in serum-free medium (Ex-Cell 300; JR Scientific, Woodland, Calif.), fractionating the antibody-containing supernatant by ammonium sulfate precipitation (14), and dialyzing the antibody-containing fraction into a buffer of 20 mM MES (morpholinoethanesulfonic acid)–130 mM NaCl (pH 7.0). The antibody concentration was determined (32), and the approximate titer was checked by immunoprecipitation.

Effect of MAbs on SFV infectivity. Neutralization was assayed by mixing fivefold dilutions of purified antibody (starting at a concentration of from 120 to 350 µg/ml) with an SFV stock containing about 200 PFU per sample and incubating for 2 h at room temperature and then overnight at 4°C (5). Infectious virus was then estimated by plaque assay (25). The rabbit antispikes antiserum showed 95% plaque reduction at a dilution of 1:2,500. Control nonspecific mono- or polyclonal sera showed no neutralization activity.

Virus infection of MAb-secreting hybrid cell lines was assayed by infecting cells in serum-free medium at a multiplicity of 1 PFU/cell, incubating for 24 h, and evaluating cell lysis from virus production.

Immunofluorescence. Purified SFV (0.5 µg) was bound for 60 min on ice to approximately 10⁶ BHK cells plated on cover slips. Some of the cultures were warmed to 37°C to permit endocytosis of bound virus. The cells were washed in ice-cold phosphate-buffered saline (PBS), fixed in freshly prepared 3% formaldehyde in PBS, quenched in 50 mM NH₄Cl in PBS, and permeabilized with 0.1% Triton X-100. Preparations were stained with unpurified hybridoma culture supernatants, followed by a rhodamine isothiocyanate-labeled goat antiserum against mouse immunoglobulin G (IgG), IgA, and IgM (Organon Teknika-Cappel, Malvern, Pa.). Cells were examined on a Zeiss fluorescence microscope and photographed with Kodak TMAX 400 film.

Liposome experiments. For the ectodomain experiments, liposomes were prepared in buffer (20 mM MES [pH 7.0], 130 mM NaCl, 100 µg of bovine serum albumin [BSA] per ml) as described previously (56), at a molar ratio of 1 sterol per 1 phospholipid molecule. Ectodomains of E1 and E2, termed E1* and E2*, were prepared by digestion of virus at 4°C with proteinase K in Triton X-114 and purification by concanavalin A-Sepharose chromatography (22). Liposomes (final concentration, 1 mM lipid) and radiolabeled ectodomains (~50,000 cpm per sample) were mixed and treated for 10 min at 37°C at either pH 7 or pH 5.5. The samples were neutralized and assayed for resistance to trypsin (22) and for susceptibility to immunoprecipitation by the acid-specific MAbs. Immunoprecipitation was performed in the presence of Triton X-100 to disrupt the liposomes. In some experiments, ectodomains were preacidified for various times, followed by addition of liposomes at either neutral or acidic pH.

To assay the effect of MAbs on viral fusion, [³H]uridine-labeled SFV was used to follow the fusion of virus with liposomes containing RNase A (21, 56). Purified MAbs were added to the fusion reaction mix at concentrations of 10 and 60 µg/ml, and then fusion was assayed by incubation for 15 min at pH 7.0, 6.2, 5.9, or 5.6, followed by neutralization and acid precipitation of the labeled RNA.

Immunoprecipitation and gel electrophoresis. All immunoprecipitations were performed at neutral pH. Antigen-MAb complexes were formed by coinubation for 1 h at 4°C and precipitated by shaking for 30 min with fixed *Staphylococcus aureus* (Zymed Labs, San Francisco, Calif.), which was

coated with a rabbit antiserum against mouse IgG (Organon Teknika-Cappel, Malvern, Pa.). Some samples were reacted with a rabbit antiserum raised to purified spike protein rosettes (15, 16) and precipitated with uncoated Zysorbin. Precipitates were washed four times with a mixed-detergent micelle buffer (48) and once with PBS and suspended in a Tris buffer (6) to which 2× sodium dodecyl sulfate (SDS) sample buffer (2) was added, followed by heating at 95°C. Samples were electrophoresed on modified 10% Laemmli gels (2). Sample reduction and alkylation (where indicated) and gel fluorography with sodium salicylate were performed as described previously (22). Radioactivity in viral protein bands was directly quantitated (26). The ability of an MAb to precipitate intact virus was assayed by eliminating detergent from both the antibody-binding and washing steps. The conformation-insensitive MAbs against both E1 and E2 reacted in immunoblot analysis, whereas none of the acid-specific antibodies reacted.

Biosynthetic labeling experiments. Dishes (35 mm) of confluent BHK cells were infected with SFV at a multiplicity of 100 PFU per cell, and the infection was allowed to proceed for a total of 4 to 5 h. The cells were then washed twice with methionine-deficient modified Eagle medium (MEM) containing 10 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.0), incubated in this medium for 15 min, and pulse-labeled with 50 to 200 µCi of [³⁵S]methionine per ml in this medium for the indicated time. They were then washed twice with MEM and chased in MEM containing a 10-fold excess of unlabeled methionine. This chase protocol was demonstrated to block further incorporation of label into acid-precipitable protein (data not shown). For some experiments, the chase was performed in the presence of carbonylcyanide *m*-chlorophenylhydrazone (CCCP) to block transport from the rough endoplasmic reticulum (RER) to the Golgi (39, 50). In this case, after the pulse the cells were incubated for 10 min on ice in chase medium containing 50 µg of CCCP per ml and then incubated in the same medium for 2 h at 37°C.

At the conclusion of the experiment, cells were harvested by washing twice with ice-cold PBS and lysing in PBS containing 1% Triton X-100, 1 mM EDTA, 1 µg of pepstatin per ml, 1 mg of BSA per ml, 1 mM phenylmethylsulfonyl fluoride, and 10 µg of aprotinin per ml. Nuclei were pelleted by spinning at maximum speed for 10 min in a microfuge at 4°C. The supernatant was then analyzed by immunoprecipitation and gel electrophoresis as described above.

Intracellular pH treatment. For some experiments, infected radiolabeled cells were treated just before harvesting with ionophores at low pH to acidify intracellular compartments. Nigericin was used at a concentration of 10 µM in a buffer consisting of 130 mM KCl, 10 mM MES, 10 mM HEPES, 5 mM NaCl, 0.2% BSA, 0.9 mM CaCl₂, and 0.5 mM MgCl₂, at pH 7 or 5.5 (51). CCCP was used at a concentration of 20 µM in pH medium (RPMI 1640 without bicarbonate, plus 10 mM MES, 10 mM HEPES, and 0.2% BSA, pH 7 or 5.5). Ionophore medium warmed to 37°C was added to cells floating in a 37°C water bath for the indicated time. The ionophores were obtained from Calbiochem.

Endoglycosidase H treatment. [³⁵S]methionine-labeled pulse-chase samples for endoglycosidase H analysis were prepared by immunoprecipitation as above, and the proteins were eluted off the Zysorbin by boiling for 3 min in 0.05 M Tris (pH 6.7)–10 mM 1, 10-phenanthroline–200 µg of BSA per ml–1% SDS. After the Zysorbin was pelleted, samples of the eluates were treated with endoglycosidase H in a final concentration of 60 mM citrate buffer, pH 5.0, for 1 h at

TABLE 1. MAbs against the SFV spike protein

MAb	Specificity	Subclass
E2-1	E2	IgG2a
E2-2	E2	IgG2a
E2-3	E2	IgG2a
E1-1	E1	IgG1
E1-2	E1	IgG2a
E1a-1	Acid E1	IgG1
E1a-2	Acid E1	IgG2b
E1a-3	Acid E1	IgG1
E2a-1	Acid E2	IgG1
E2a-2	Acid E2	IgG2a
E2a-3	Acid E2	IgG1
E2a-4	Acid E2	IgG2a

37°C. SDS sample buffer (2) was added, and the samples were analyzed on 12.5% nonreducing gels. Endoglycosidase H was a kind gift of Paul Atkinson.

Budding assays. Cells were infected with SFV and pulsed for 10 min with [³⁵S]methionine as described above. Plates were placed on ice after the pulse, washed three times with MEM plus 10 mM HEPES, pH 7 (medium 1), and chased as usual for the indicated time. The chase medium was then harvested, the plates were washed on ice with 0.5 ml of medium 1 at pH 8.0 to elute bound virus, and the combined media were layered over a step gradient consisting of 1 ml of 50% sucrose and 3 ml of 20% sucrose (wt/wt in 50 mM Tris, 100 mM NaCl [pH 7.4]). The gradients were spun in an SW41 rotor for 90 min at 40,000 rpm, 4°C, and 10 250-μl fractions were collected from the bottom of the tube. For each time point, the trichloroacetic acid (TCA)-precipitable counts in the virus peak (fractions 1 to 5) were determined. Purified ³⁵S-labeled SFV was sedimented under these conditions, and ~45% of the input counts were recovered in the lower 10 fractions.

RESULTS

Characterization of MAbs against the SFV spike protein.

Anti-SFV MAbs of four different specificities were identified (Table 1). The first two groups contained antibodies which were monospecific for either the E1 (E1-1 and E1-2) or E2 (E2-1, -2, and -3) spike subunits, without differentiating the acid and neutral conformations. The last two groups of antibodies recognized E1 (E1a-1, -2, and -3) or E2 (E2a-1, -2, -3, and -4) only after treatment at a pH of about 6.2 or below.

None of these MAbs showed neutralization activity, including those which could immunoprecipitate intact virus particles (E2-1, E2-2, and E1-1). The hybrid cell lines secreting MAbs E2-2, E1-2, E1a-2, and E1a-3 were tested and found to be fully susceptible to virus infection. This result implies that the MAbs also do not neutralize virus within the endosome, where the virus spike undergoes its conformational change. Endocytosed antigens and secreted MAbs have been demonstrated to interact within intracellular compartments of hybridoma cells (58), but if this interaction occurred for the SFV spike protein, it was apparently not sufficient to block fusion activity. In addition, none of the E1 MAbs, including those which bound specifically to the acid form of E1, had any effect on the fusion of virus with liposomes.

The pH dependence of conversion to reactivity with the

FIG. 1. pH dependence of E2 conversion in wt and *fus-1* mutant SFV, assayed by MAb precipitation. Samples of ³⁵S-labeled wt (A) or *fus-1* (B) virus were incubated for 10 min at 37°C in buffer of the indicated pH, neutralized, and dissolved in 1% Triton X-100 in PBS. Samples were then precipitated with MAb E2a-3 with a nonimmune (N/I) serum or with a rabbit antiserum directed against the spike protein (Con) and processed for gel electrophoresis and fluorography.

acid-specific MAbs was determined for wt SFV and *fus-1*, a previously isolated SFV mutant with a more acidic fusion threshold (25). Conformational changes in both the E2 and E1 subunits of the *fus-1* mutant begin at approximately pH 5.3 (22, 46). The pH dependence of E2 conversion in wt and *fus-1* virus was analyzed by precipitation with acid-specific MAb E2a-3 (Fig. 1) or MAb E2a-1 (data not shown). In agreement with the protease results and fusion studies, the antibodies revealed an irreversible acid-dependent conformational change in E2 with a pH threshold of approximately 6.7 for wt virus and 5.3 for the *fus-1* mutant. Similar precipitation studies with the E1 acid-specific MAbs revealed a conformational change in E1 with a pH threshold of about 6.2 to 6.5 for the wt and 5.3 for the *fus-1* mutant (not shown). When assayed by trypsin sensitivity, the efficiency of the conformational change was shown to vary from ~30% with wt to 12 to 20% with the *fus-1* mutant (22; Kielian, unpublished observations). The efficiency of MAb precipitation of acid-treated spike proteins was within this range or higher. The reasons for this incomplete conversion are unclear but could involve differences in conditions in vitro versus in vivo, where the change appears more complete (46).

Although the specificity of the antibodies is to the acid conformation of either subunit, variable amounts of the other subunit were observed to coprecipitate in these experiments. The two subunits are joined by tight noncovalent associations (44, 47), making them difficult to separate. This coprecipitation was most apparent when isolated virus was acid treated, while in contrast, one subunit was precipitated from the ectodomain, biosynthetically labeled proteins, or endocytosed virus samples (see below). We have not analyzed the precipitation pattern of acid-treated spike proteins disrupted in detergent before neutralization; such samples show less coprecipitation with other antibodies (55).

As shown in Fig. 2A and B, an acid-specific MAb against E1 gave negligible staining of cell surface-bound SFV unless the bound virus was first treated at low pH. In contrast, staining with a conformation-insensitive MAb such as E2-1 was strongly positive with either acid-treated or untreated samples (not shown). When the cells with bound virus were warmed to 37°C, endocytosis of virus resulted, and the intracellular exposure to endosomal acidity converted E1 to a form which could then interact with acid-specific MAb. Within 2 min of the shift to 37°C, staining by the MAb was detected in small peripheral fluorescent granules (Fig. 2c).

FIG. 2. Immunofluorescence staining of bound and internalized SFV with MAb E1a-1. Purified SFV was bound to the surface of BHK cells on cover slips for 60 min on ice. The cells were then fixed either directly (a), after treatment for 1 min at 37°C with pH 5.5 medium (b), or after allowing virus to be internalized by warming the cultures to 37°C for 2 min (c) or 20 min (d). Fixed and permeabilized preparations were then stained with MAb E1a-1, followed by rhodamine-labeled rabbit antiserum against mouse immunoglobulins. Bars, 20 μ M.

Staining was brighter after longer times of endocytosis and also showed a shift of the bulk of the acid-positive virus to a more perinuclear region (Fig. 2d). This presumably represents the transfer of endocytosed virus from early peripheral endosomes to later endosomes and lysosomes located more centrally in the cell (37, 49). Staining patterns similar to those in Fig. 2 were obtained with the E2 acid-specific antibodies, but the staining was somewhat variable.

In previous studies, wt and *fus-1* SFV were used to follow the kinetics of endosome acidification, using either the intracellular viral fusion reaction or E1 protease resistance as assays for endosomal pH (26, 46). In agreement with these results, precipitation by MAb E1a-1 demonstrated that endosomes became more acidic with time, rapidly reaching a pH of <6.2, the wt fusion threshold, and then more gradually becoming acidified to the *fus-1* mutant threshold of pH 5.3 (data not shown). The E2 acid-specific MAbs were somewhat variable in precipitating E2 from endocytosed virus (data not shown).

Conformation of the acid form of the SFV spike protein. The acid-specific MAbs recognized domains on E2 and E1 which were hidden in the neutral conformation of the molecule but became exposed after low pH treatment. We tested whether other treatments which might result in partial or complete unfolding of the spike protein would allow binding of these MAbs. Reduction of the spike protein by treatment

with β -mercaptoethanol at 37°C permitted its recognition by the acid-specific E1 (Fig. 3A) and E2 (not shown) MAbs. When the spike protein was extensively denatured by exposure to SDS, the epitopes recognized by these MAbs were lost, although the spike could still be precipitated by a polyclonal rabbit antiserum (Fig. 3B). Thus, both the E1 and E2 acid-specific MAbs were directed against conformational epitopes, not primary sequence determinants.

Ectodomains of E1 and E2, termed E1* and E2*, can be prepared by proteinase K digestion of virus in nonionic detergent (22). Such ectodomains are monomeric, have lost their membrane-spanning sequences, and retain the ability to undergo acid-induced conformational changes. However, E1* differs strikingly from E1 in that it requires both cholesterol and low pH in order to convert to the trypsin-resistant form (22). Purified ectodomains were therefore used to define the requirements for recognition by the E1 MAbs. A conformation-insensitive MAb, E1-1, did not require cholesterol or low pH in order to precipitate the E1 ectodomain (data not shown). In contrast, E1* could only be precipitated by MAbs E1a-1 and E1a-2 after acidification in the presence of cholesterol-containing liposomes, conditions which also resulted in E1* trypsin resistance (Fig. 4, lanes 3). Neutral pH or low pH plus epicholesterol liposomes did not result in either trypsin resistance or immunoreactivity (lanes 1, 2, and 4). Thus, the MAbs appear to detect a

FIG. 3. Effect of protein conformation on recognition by MAbs. (A) 35 S-labeled SFV was reduced by treatment of intact virus with 1% β -mercaptoethanol (+ β -ME) at pH 7 for 2 h at 37°C, followed by quenching with a twofold excess of iodoacetamide for 1 h at 37°C. The virus was then dissolved in 1% Triton X-100 in PBS containing BSA (1 mg/ml), and samples were precipitated with either rabbit polyclonal antibody (Ab), nonimmune serum (N/I), or the indicated MAbs and then processed for gel electrophoresis and fluorography. Reduced E1 and E2 comigrated on SDS-polyacrylamide gels. (B) 35 S-labeled SFV was denatured by treatment with 1% SDS at 37°C for 15 min, the SDS was diluted to <0.01% (a concentration which did not interfere with the MAb reaction) by the addition of 1% Triton X-100 in PBS, and samples were precipitated with the indicated antibodies, followed by electrophoresis and fluorography.

conformational change similar or related to that demonstrated by the protease sensitivity assay.

Using the MAb reagents, we were able to follow the kinetics of the cholesterol requirement. E1* showed significant conversion when the protein was acidified for up to 5 min in the absence of cholesterol as long as cholesterol liposomes were then added to the protein at acid pH. No conversion was seen when the protein was treated with acid and cholesterol liposomes were added at neutral pH (data not shown).

Characterization of low pH sensitivity of p62. The conformation of the SFV spike protein during transit through the RER and Golgi and the ability of the newly synthesized spike

FIG. 4. Cholesterol dependence of the E1 conformational change. Ectodomains of the E1 and E2 subunits, E1* and E2*, were mixed with either epicholesterol liposomes (lanes 1 and 2) or cholesterol liposomes (lanes 3 and 4). Samples were then incubated at either pH 5.5 (lanes 1 and 3) or pH 7.0 (lanes 2 and 4) for 10 min at 37°C. Samples were neutralized, and the conformational change was assayed in one set by digestion with trypsin (100 μ g/ml) for 10 min at 37°C. The reaction was stopped by the addition of a threefold excess of soybean trypsin inhibitor, and the samples were acid precipitated and processed for gel electrophoresis. Identical, nondigested samples were immunoprecipitated in 0.25% Triton X-100 in PBS with MAb E1a-1 or E1a-2. Control 1 (C1) shows ectodomains digested with premixed trypsin and trypsin inhibitor. Control 2 (C2) shows ectodomains immunoprecipitated with the rabbit antiserum against the SFV spike protein. All samples were reduced and alkylated prior to gel electrophoresis.

FIG. 5. Kinetics of intracellular pH treatment of p62. SFV-infected BHK cells were pulse-labeled for 5 min with [35 S]methionine, chased for 5 min, and treated for the indicated time with pH 5.5 buffer containing 10 μ M nigericin. Samples were then harvested, immunoprecipitated, and analyzed by gel electrophoresis. (A) Pulse-labeled cell lysate (lys) or purified 35 S-labeled SFV (SFV) precipitated with a rabbit antispike antiserum. (B) pH-treated cell lysates precipitated with E2a-3, an acid-specific MAb. Controls in panel B were infected cells treated with nigericin buffer at pH 7 for 4 min and labeled SFV treated at pH 5.5 or 7.0 before immunoprecipitation.

to undergo acid-induced conformational changes were examined by pulse-chase analysis of SFV-infected cells. The most direct way to assay for acid-induced changes in the nascent subunits would have been to pH treat a detergent lysate of pulse-labeled cells. However, the E1 conformational change does not occur in Triton X-100, as assessed by trypsin analysis or acid-specific MAb reactivity (Kielian, unpublished observations). Preliminary data also showed that the change in p62 was more reproducible in the absence of detergent. Ionophores were therefore used to equilibrate intracellular compartments to a defined pH (13, 30, 51). Nigericin, which will exchange potassium for protons, and CCCP, which will allow proton equilibration without a counter-ion requirement, were used in pH treatments at 37°C (51). In a control experiment (data not shown), identical conditions of nigericin treatment were found to dissipate the lysosomal accumulation of acridine orange, a weak base which requires the low pH environment of lysosomes for its selective concentration (30, 37).

To assay for low pH-dependent conformational changes in p62, SFV-infected cells were briefly pulsed and chased so that only the p62 precursor and not the E2 product would be labeled. Cells were then treated for increasing times at pH 5.5 with ionophore, shifted back to neutral pH medium, harvested, and precipitated with E2a-3, a MAb which recognizes the acid conformation of E2 (Fig. 5). Within 1.5 min of treatment at pH 5.5, a proportion of intracellular p62 converted to a form which reacted with the acid-specific MAb. Reactivity appeared to be maximal by 2 min and decreased somewhat after 5 min, possibly due to proteolysis of the intracellular protein by lysosomal enzymes. The kinetics shown in Fig. 5 reflect both the time needed for the ionophore to equilibrate intracellular pH and the time required to trigger the conformational change in p62. Previous studies reported that ionophore equilibration under these conditions is essentially complete within 1 min (51). It therefore appears that the conformational change in p62 was complete within about 1 min and was irreversible within the time span of our experiments. The efficiency of p62 conversion ranged from 17 to 40% of the total p62 present. Given the intracellular location of p62 (9, 35), its acid conversion also demonstrated that the ionophores were equilibrating intracellular pH.

FIG. 6. pH dependence of the conformational change in p62. SFV-infected BHK cells were labeled and chased as described in the legend to Fig. 1 and treated for 3 min at the indicated pH with buffers containing 10 μ M nigericin. Cells were harvested and precipitated with either a rabbit antispikes antiserum (Rab), nonspecific MAb (NI), or E2a-3, an acid-specific MAb.

Next we assayed the pH dependence of the conformational change in p62 by treating cells for 3 min with nigericin buffers at different pHs, followed by precipitation with MAb E2a-3. As shown in Fig. 6, reactivity with the MAb began to increase after pH 6.7 treatment and was maximal by pH 5.5. The pH threshold for p62 was thus similar to that of E2 assayed with this antibody, although the pH dependence of E2 is sharper (Fig. 1).

Lastly, we asked how soon after its biosynthesis p62 was sensitive to acid conversion. After a 5-min pulse, cells were chased for various times and treated with nigericin buffers at pH 5.5 or 7, and the p62 conformational change was assayed by E2a-3 precipitation (Fig. 7). The protein was acid sensitive immediately after biosynthesis (0 min of chase), and maximal conversion was observed within 2.5 min of chase. In some experiments, higher background precipitation was seen in the pH 7 sample immediately after biosynthesis (compare 0 min pH 7 lane with other pH 7 lanes in Fig. 7). This probably represents exposure of the MAb-reactive epitope in nascent unfolded p62 (see Fig. 11 below).

Biosynthetic studies of E1 conformation. We next examined the conformation of newly synthesized E1. Using similar pulse-chase analysis of SFV-infected cells, we found that E1 was recognized by a conformation-insensitive MAb (E1-1) immediately after biosynthesis (Fig. 8). This pool of radio-labeled E1 was gradually lost from the cells as virus was released by budding (see 60- and 90-min time points). In contrast to results with MAb E1-1, newly synthesized E1 was not recognized by E1a-1, an acid-specific MAb, even in the absence of chase. Shorter pulse times (5 min) or precipitation with MAb E1a-2 gave similar results. Thus, although the acid MABs recognized "unfolded" or reduced E1, the reactive epitope was not detectably exposed during biosynthesis of the E1 molecule (see also Fig. 11 below). We

FIG. 7. Time course of the acid sensitivity of newly synthesized p62. SFV-infected BHK cells were labeled for 5 min with [35 S] methionine, chased for the indicated time, and treated for 3 min at 37°C with nigericin-containing buffer at pH 7 (—) or 5.5 (+). Cells were harvested and precipitated with E2a-3, an acid-specific MAB.

FIG. 8. Precipitation of newly synthesized E1 by MABs. SFV-infected BHK cells were pulse-labeled with [35 S]methionine for 15 min and then chased for the indicated amount of time. At each time point, the cells were lysed in detergent, and duplicate samples were immunoprecipitated with either E1-1, an MAB which recognizes both the acid and neutral forms of E1, or E1a-1, an acid-specific MAB. Where indicated, cells were treated for 1 min with pH 5.5 medium before detergent lysis. Immunoprecipitates were then processed for gel electrophoresis and fluorography. The E1-1 portion of the gel has been overexposed to permit visualization of any E1a-1-reactive material.

treated one set of pulse-labeled cells briefly with medium of pH 5.5 to convert E1 which had reached the cell surface (Fig. 8). Upon surface acid treatment, E1 was recognized by MAB E1a-1, and the earliest time after the beginning of the pulse when it could be recognized was about 25 min. As increasing amounts of E1 arrived at the cell surface, they became accessible to acid conversion. This rate of transport to the plasma membrane is similar to previously published results (60).

We then asked at what point after biosynthesis E1 was competent to undergo the acid-induced conformational change. SFV-infected cells were pulse-labeled, chased for various times, and treated for 4 min at 37°C with buffers of pH 5.5 or 7 containing nigericin or CCCP (Fig. 9). Cells were harvested, and duplicate samples of the cell lysates were precipitated with either a polyclonal antispikes antibody or with MAB E1a-1. Precipitation by the polyclonal antibody quantitated the amount of E1, p62, and E2 present and indicated that in this experiment p62 cleavage was observed beginning at 15 min of chase. Precipitation with the acid-specific MAB demonstrated that the newly synthesized E1 was not acid sensitive after a 10-min pulse and either a 0- or 5-min chase. After 15 min of chase, E1 converted when the cells were treated at low pH with ionophore (15 min, lanes c and d), but at that time point E1 was also accessible to low-pH treatment at the cell surface in the absence of ionophore (15 min, lane a). After 5 min of further chase, increased amounts of both total and cell surface E1 were converted by acid pH treatment (20 min, lanes a, c, and d). Slightly more E1 was precipitated when acidified in the presence of the ionophores (15 and 20 min, lanes c and d) compared with surface treatment (lane a). It is not clear whether this difference is significant, but it might represent a form of E1 which is still intracellular but competent to undergo acid conversion. This experiment also demonstrates that the time course of maturation of E1 to the acid-responsive form closely parallels the time course of p62 cleavage.

Subcellular localization of the E1 maturation step. We were interested in further defining the point in E1 biosynthesis and transport at which E1 becomes sensitive to acid. We first examined the kinetics of budding of newly synthesized virus proteins. The data in Fig. 8 and 9 suggested that release of E1 in virions occurred after E1 maturation. We directly analyzed virus protein assembly into particles by pulse-labeling cells, chasing for various times, and following the

FIG. 9. Time course of the maturation of newly synthesized E1 to an acid-convertible form. SFV-infected BHK cells were pulse-labeled with [35 S]methionine for 10 min and chased for the indicated amount of time. At each time point, identical plates were treated for 4 min at 37°C with (lanes a) pH 5.5 medium, (lanes b) 10 μ M nigericin at pH 7, (lanes c) 10 μ M nigericin at pH 5.5, or (lanes d) 20 μ M CCCP at pH 5.5, as described in Materials and Methods. Cells were then lysed, and duplicate samples were immunoprecipitated with either rabbit antispike antiserum (A) or MAb Ela-1 (B). Control (Con) shows purified [35 S]SFV bound to the cell surface and treated at pH 5.5 before lysis.

release of label into gradient sedimentable virus (Fig. 10). Newly synthesized viral proteins were found in virus particles after 45 min of chase but not after 15 or 20 min, the time of E1 maturation. Although the label released in virus could represent either capsid or spike proteins, neither of these viral components was incorporated at the early chase times. Therefore, maturation appeared not to be dependent on virus budding.

E1 maturation apparently occurred at a point between synthesis and budding and with kinetics similar to those of p62 cleavage. After initial synthesis and translocation into the RER, the spike protein dimer is transported to the Golgi complex, where the simple carbohydrate chains can be modified to complex type (28). This carbohydrate modification confers resistance to digestion with the enzyme endoglycosidase H and thus is diagnostic of transport through the medial Golgi. p62 cleavage is believed to occur in the trans-Golgi network after acquisition of endoglycosidase H resistance (9). We used endoglycosidase H digestion to ask whether acid-converted E1 had traversed the medial Golgi. In addition, since transport from the RER to the Golgi requires ATP, we used CCCP treatment to uncouple oxidative phosphorylation and trap newly synthesized E1 in the RER (39, 50). As shown in Fig. 11A, about 60% of total pulse-labeled E1 became endoglycosidase H resistant after 25 min of chase in the absence of a CCCP block. When the chase was performed in the presence of CCCP, even after 2 h of chase E1 was still fully endoglycosidase H sensitive, demonstrating that the block in transport was complete. p62 and E2 do not become completely endoglycosidase H resistant at any time, since even mature E2 contains one high-mannose carbohydrate chain (17, 44).

CCCP treatment and endoglycosidase H digestion were then used to analyze the acid-reactive form of E1 (Fig. 11B). We first followed the kinetics of E1's acquisition of endoglycosidase H resistance and found that they preceded maturation (data not shown). However, since the two time courses were close, the endoglycosidase H sensitivity of mature precipitated E1 was determined to ask directly whether mature E1 had passed the medial Golgi. After 25 min of chase in the absence of CCCP, a proportion of E1 became

acid sensitive and was precipitated with MAb Ela-1. We analyzed this immunoprecipitated E1 and found that it was largely endoglycosidase H resistant, although about 40% of total labeled E1 was still endoglycosidase H sensitive at this time point (Fig. 11A). The acid sensitivity of the CCCP-treated samples was then analyzed, and the RER form of E1 appeared to be unable to undergo the acid-induced conformational change (Fig. 11B). These data place the E1 maturation step after transport from the RER to the Golgi and suggest that maturation occurs after the medial Golgi.

Lastly, the RER form of the p62 in CCCP-treated cells was analyzed for its acid sensitivity and found to be reactive with MAb E2a-3 even in the absence of low pH treatment (Fig. 11C; see also Fig. 7 above). Thus, the epitope which was hidden in the post-RER forms of p62 and E2 was available for MAb binding in newly synthesized p62. Since oligomerization has been shown to occur in the RER during a CCCP block (6), the exposure of the E2a-3-reactive epitope is presumably not due to p62 monomers. However, the dimerization of p62 with E1 was not evaluated in this experiment.

DISCUSSION

MAbs to the SFV spike protein. From these MAb studies and our previous protease assays (22), we conclude that E2 and E1 undergo irreversible acid-induced conformational changes upon exposure to the threshold pH for fusion. The MAb assay of these conformational changes has several advantages over the trypsin assays we developed previously. It is more quantitative and reproducible when used on cell-associated spike proteins and made possible the localization of converted E1 within the cell. In addition, the acid-specific antibodies have enabled the biosynthetic analysis of E1 and E2. In particular, the conformational change in p62 cannot be analyzed by the trypsin assay (54). In the future, the MAbs should serve as useful reagents for assaying pH-induced conformational changes in vector-expressed SFV proteins.

To our knowledge, these low pH-specific MAbs are the first such antibodies to be described for the SFV E1 and E2 proteins. Several previously reported MAbs to Sindbis virus

FIG. 10 Virus protein assembly into particles. The release of newly synthesized virus protein in virions was determined by using a gradient centrifugation assay. (A) Sedimentation of purified [35 S]SFV on a sucrose step gradient (1 ml of 50% sucrose and 3 ml of 20% sucrose, wt/wt), centrifuged and fractionated as described in Materials and Methods. The TCA-precipitable counts in the lower 10 fractions were determined. The pooled counts in fractions 1 to 5 were defined as the virus peak for subsequent experiments. (B) SFV-infected BHK cells were pulse-labeled with [35 S]methionine for 10 min and chased for the indicated times. The chase medium containing released virus was centrifuged on a sucrose gradient identical to that in panel A, and the TCA-precipitable counts in the virus peak were determined for each chase time.

E1 have an increased binding to E1 in acid-treated virions, but this seems to be a steric effect, since the MABs react well with detergent-solubilized, neutral E1 (45). Our MABs E1-1 and E1-2 showed a similar pattern of reactivity. A recent study described an MAB which reacted specifically with E1 after it was dissociated from E2 by acid treatment (55). However, the epitope detected by this MAB was lost if the virus sample was neutralized prior to detergent solubilization, while our MABs detected irreversible conformational changes.

Acid conformation of the SFV spike protein. It is striking that the purified ectodomain of E1 requires both low pH and

FIG. 11. Localization of E1 maturation. SFV-infected BHK cells were pulse-labeled for 5 min with [35 S]methionine and chased for either 25 min at 37°C (–CCCP) or 2 h at 37°C in the presence of 50 μ g of CCCP per ml (+CCCP). Some samples were then treated with nigericin buffers at pH 7 or 5.5 for 3 min at 37°C to acid-treat intracellular spike protein. The cells were harvested and precipitated with either polyclonal antibody (rabbit antispike) or acid-specific MABs (E1a-1 or E2a-3). Immunoprecipitates were digested with endoglycosidase H (Endo H) as indicated.

cholesterol liposomes to change conformation. The difference between ectodomain and intact E1 could be that the membrane of the virus particle is providing sterol when the intact virus particle is treated at low pH. However, we have assayed lipid-free rosettes (16) of the intact spike protein, and they show no sterol requirement for E1 conversion (Marzullo and Kielian, unpublished results). One explanation is that E1 carries associated cholesterol and that this sterol is lost during purification of the ectodomains. This explanation is probably oversimplified, however, since the fusion of intact untreated virus with artificial liposomes requires cholesterol or 3 β -hydroxysterol in the liposome target membrane (21, 56).

A conformation of E1 and E2 similar to that of the acid form was induced when the neutral molecule was treated with reducing agents. Interestingly, Kaluza et al. (18, 19) reported that a rabbit polyclonal antiserum raised to unglycosylated SFV spike proteins also reacted with the reduced forms of E1 and E2, but not with the native spike protein. Thus, epitopes which are hidden in the mature, neutral conformation of E1 and E2 become exposed upon reduction or acid treatment. Omar and Koblet demonstrated that upon acid treatment, SFV E1 exposes previously hidden disulfide bonds which can be labeled with radioactive sulfite (41). These molecular rearrangements may well be secondary to the conformational changes involved in fusion, and to date there is no convincing evidence for a more mechanistic effect of protein reduction or disulfide bond exposure. It is interesting, however, that a class of bacterial cytolysins which act by binding cholesterol in eucaryotic cell membranes show a requirement for thiol reduction for lysis. This is believed to be due to a single critical cysteine residue in their cholesterol-binding site (38, 52).

Acid treatment of intracellular spike proteins. We have tested the acid sensitivity of newly synthesized spike protein subunits by using ionophore permeabilization to pH treat the intracellular protein. While some investigators have reported that a drop in extracellular pH has relatively little effect on intracellular pH in the absence of ionophore (51), recent studies indicate that some acidification of at least the cytoplasmic compartment may occur (7, 33). We did observe some acid conversion of intracellular p62 during pH treatment in the absence of ionophore, but the amount was

variable. All our experimental pH treatments therefore contained ionophore to ensure that intracellular compartments were being treated uniformly at the desired pH.

Intracellular acidification is known to inhibit both the endocytic and exocytic pathways (7, 33). Our experiments would not be affected by acid inhibition of protein transport, since pulse-labeled protein was chased for various times under neutral pH conditions and acid pH treatment with ionophore was only used to convert the protein at the time of harvest.

Conformational changes in the p62 subunit. Unlike the E2 cleavage product, p62 cannot be assayed for an acid-induced conformational change by protease sensitivity, since both the acid and neutral forms of p62 are protease sensitive (54). The MAb studies described here thus provided the first direct evidence for an acid-dependent irreversible conformational change in p62. p62 was acid reactive very soon after synthesis, and the kinetics and pH dependence of the p62 conformational change were fairly similar to those found upon low pH treatment of mature E2 (10, 22). The efficiency of p62 conversion in our ionophore experiments was less than that of E2 in virions. This could represent a real difference or reflect the dissimilarity in the way in which the experiments were performed.

Previous studies by Presley and Brown showed that a change in susceptibility to iodination occurs during the biosynthesis of Sindbis virus pE2 (42). This alteration was blocked by monensin treatment, which dissipates intracellular pH gradients. Thus, pE2 may be affected by the mildly acidic pH of the Golgi complex (1, 37) during biosynthetic transport to the plasma membrane. In this light, it is interesting that the RER form of p62 accumulated by CCCP treatment appeared to be different than later biosynthetic forms, since it was reactive with MAb E2a-3 in the absence of prior low-pH-treatment. However, since p62 could well be altered for other reasons, the possible effect of Golgi acidification on the conformation of SFV p62 remains to be determined. Untreated p62 assayed after longer chase times was in the neutral conformation, arguing that the acidity, time of exposure to acid, or conditions of the Golgi was not sufficient to convert p62. Similarly, the influenza virus hemagglutinin precursor HA0 has an acid-dependent conformational change with a threshold of pH 6.0, but was shown to be in its neutral conformation during biosynthetic transport (4).

Maturation of the E1 subunit. In contrast to results with p62, a maturation step occurring late in the exocytic pathway was required to confer acid sensitivity upon newly synthesized E1. During biosynthetic transport, many posttranslational modifications occur in the spike protein (reviewed in references 44 and 47). Immediately after synthesis and RER insertion, E1 and p62 associate noncovalently to form a heterodimer (55, 60), are glycosylated, and may be assembled into higher-order oligomers (53), similar to other virus spike proteins (6, 12, 29). Further carbohydrate trimming and modification as well as fatty acylation take place upon transport to the Golgi compartment. Cleavage of p62 to E2 and E3 probably occurs during or after exit of the spike protein from the trans-Golgi network but prior to its insertion into the plasma membrane (9). The kinetics of E1 maturation argue that it is a relatively late event in exocytic transport, occurring at about the same time as p62 cleavage.

The cleavage of the precursors of myxo-, paramyxo-, and retrovirus fusion proteins is clearly required for both virus fusion and infection (36, 57). Similarly, recent studies using *in vitro* mutagenesis of the protease cleavage site directly

demonstrated that p62 cleavage is required for SFV fusion (31; Jain, DeCandido, and Kielian, submitted for publication). Two other studies evaluated the infectivity of pE2-containing Sindbis virus and found that they appeared to be fully infectious in tissue culture cells (42, 43). The membrane fusion activity of these virus preparations has not been directly assayed, nor has the possible proteolytic cleavage of pE2 by the host cell during endocytosis been evaluated. Presumably such cleavage or other effects of the mutation on spike conformation explain the infection.

The experiments described here demonstrate a correlation between the kinetics of p62 processing and the acquisition of E1 acid sensitivity. Although no direct evidence for a cause-effect relationship is yet available, the requirement for p62 cleavage for fusion argues that p62 cleavage may be necessary for activation of E1 acid sensitivity. Several alternative explanations are also possible. For example, if E1 must have cholesterol associated with it to be fusion active, the relatively cholesterol-poor environment in the RER and Golgi (8) might not encourage such an E1-cholesterol interaction, while the higher cholesterol content of the plasma membrane might permit it. Alternatively, Golgi processing steps such as carbohydrate modification and fatty acylation could be involved in conferring acid sensitivity. We are currently analyzing the E1 conformational change in the mutant spike proteins blocked in p62 cleavage to define which of these processes contribute to E1 maturation.

Role of acid-induced conformational changes in SFV fusion. Protease studies and MAb assays have demonstrated independent conformational changes in SFV E1 and E2 upon exposure to the pH of fusion (10, 22). The change in the E1 ectodomain is similar to virus fusion in its cholesterol requirements. What can these alterations tell us about the fusion mechanism of SFV and the roles of the two subunits? Ideally, one would like to express the subunits independently and evaluate fusion. Unfortunately, the subunits are only correctly folded and transported as a dimer (27, 55). Expression studies of p62 have demonstrated that it is transported to the plasma membrane in the absence of E1 and does not express fusion activity. In these experiments, however, p62 cleavage was aberrant, which might also affect fusion (27). In a recent study, protease digestion of SFV particles removed all detectable E2 and E3, while viral infection and fusion capabilities were retained (40). This suggests that E1 alone is sufficient for fusion. The role of p62/E2 may be in transport of the trimer to the plasma membrane, while the cleavage of p62 may serve to activate the fusogenic potential of the E1 subunit. The data to date and the E1-cholesterol requirement in particular suggests to us that the E1 subunit plays a central role. Thus, the maturation process we describe here may have evolved to prevent the occurrence of this critical E1 conformational change prior to insertion of the mature spike protein into the virus membrane.

ACKNOWLEDGMENTS

We thank Susan Buhl of the Cancer Center Hybridoma Facility (CA 13330) for expert instruction and assistance in the isolation of the MAbs. We also thank Matthew Scharff for helpful advice, John Bergman for initially suggesting the use of ionophores for intracellular pH treatment, and Pamela Stanley and Nancy Carrasco for comments on the manuscript.

This work was supported by grants to M.K. from the Public Health Service (GM-38743), the American Cancer Society (JFRA-219), and the Pew Memorial Trust, and by Cancer Center Core Grant NIH/NCI P30-CA13330. S.J. was supported by Public Health Service training grant T32 AI07183-10.

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