JOURNAL OF VIROLOGY, Dec. 1989, p. 4991–4997
0022-538X/89/124991-07$02.00/0

Vol. 63, No. 12

The Heterodimeric Association between the Membrane Proteins of Semliki Forest Virus Changes Its Sensitivity to Low pH during Virus Maturation

JOHANNA M. WAHLBERG,[1]* WIL A. M. BOERE,[2] AND HENRIK GAROFF[1]

Department of Molecular Biology, Center for Biotechnology, The Karolinska Institute, Huddinge University Hospital, K87, S-141 86 Huddinge, Sweden,[1] and Laboratory of Microbiology, State University of Utrecht, 3511 GG Utrecht, The Netherlands[2]

Received 23 May 1989/Accepted 25 August 1989

The budding and the fusion processes of the enveloped animal virus Semliki Forest virus serve the purpose of transporting its nucleocapsid, containing its genome, from the cytoplasm of an infected cell into that of an uninfected one. We show here that, in the infected cell, the viral membrane (spike) proteins p62 and E1 are organized as heterodimers which are very resistant to dissociation in acidic conditions. In contrast, the mature form of the heterodimer, E2E1, which is found in the virus particle and which is generated by proteolytic processing of p62, is very prone to dissociate upon treatment with mildly acidic buffers. We discuss the possibility that this difference in behavior of the intracellular precursor form and the mature form of the spike protein complex represents an important regulatory mechanism for the processes involving membrane binding around the nucleocapsid during budding and membrane release from the nucleocapsid at the stage of virus fusion.

The function of the membrane of enveloped viruses is to transport the encapsidated viral genome from the cytoplasm of the infected cell into that of an uninfected one. This is accomplished through the budding, targeting, and fusion events of these viruses (6, 18; K. Simons and S. D. Fuller, *in* R. Burnett and H. Vogel, ed., *Biological Organization: Macromolecular Interactions at High Resolution*, in press; M. Sommerfeld and M. Marsh, Drug Targetting Rev., in press). In the case of many viruses, budding takes place at the plasma membrane whereas fusion occurs after entry into the acidic milieu of the endosome (10, 17, 28). There are still many open questions in the processes of virus budding and entry. Perhaps the most interesting ones concern the regulation of the different molecular interactions which are involved in these events. One example is how the membrane-nucleocapsid interaction can be enforced during budding and released after fusion (25). Another is how the fusion activity can be induced during virus entry but avoided at the stage of virus maturation.

The spike proteins of many viruses become arranged into oligomeric structures soon after synthesis and are proteolytically processed just before being incorporated into the viral envelope during budding (6; S. M. Hurtley and A. Helenius, Annu. Rev. Cell Biol., in press). Different functional properties of the precursor and mature form of the oligomeric complex, as well as the varying sensitivity of the two forms toward dissociation and conformational changes in surroundings of mildly low pH, could possibly help to explain some of the paradoxes in virus assembly and disassembly. With these possibilities in mind we have undertaken a detailed study about the properties of the membrane protein oligomer of Semliki Forest virus (SFV).

SFV represents an alphavirus which matures by budding at the plasma membrane and enters new host cells through membrane fusion in the endosome (10, 18). Recent electron microscopic studies of vitrified SFV samples have shown that its spike projections are arranged in an icosahedral surface lattice in which the triangulation number equals 4 (8, 26, 27). This suggests that the virus membrane contains 80 spikes, each of which consists of three copies of the membrane proteins E2 (52,000) and E1 (49,000) and the peripheral protein E3 (10,000). Biochemical analyses have demonstrated that the viral membrane can be solubilized by nonionic detergents into E2E1 heterodimeric structures (24, 29). The morphological and the biochemical analyses together suggest that the SFV spike is organized as a trimer of the membrane protein heterodimer E2E1. The way by which the small E3 molecule is bound to the spike is not known.

During synthesis in the endoplasmic reticulum the E2 and E3 proteins are made as a common precursor, p62 (22). In this the E3 forms an N-terminal extension to the membrane-bound E2 part. The intracellular spike proteins also form dimeric complexes, which probably represent p62E1 heterodimers and thus precursors for the mature E2E1 forms found in the virus particle (3, 12, 13, 21, 30). The p62 protein is cleaved into its mature form when it arrives at the plasma membrane (5, 9, 23).

In the present work we have used coprecipitation analyses with anti-E1 and anti-E2 monoclonal antibodies and sedimentation analyses in sucrose gradients to demonstrate that (i) the intracellular membrane proteins indeed form p62E1 complexes and thus represent precursor forms to the mature E2E1 heterodimer, and (ii) the mature heterodimer becomes dissociated by treatment with mildly acidic buffers whereas the precursor form is very resistant to such treatments. On the basis of these results, we postulate that the acid stability of the p62E1 heterodimer represents an important factor during virus assembly in the infected cell and that the acid sensitivity of the E2E1 association represents an equally important one during virus disassembly at the entry stage.

* Corresponding author.

MATERIALS AND METHODS

Monoclonal antibodies. The monoclonal antibodies anti-E1 8.139, anti-E1 8.47, and anti-E2 5.1, which have been prepared against complete SFV, were all used as mouse ascites preparations (2).

Preparation of SFV. Stocks of the prototype strain of SFV were prepared in baby hamster kidney (BHK-21) cells, as described previously (14). The final virus pellet was suspended in a buffer containing 50 mM Tris (pH 7.4) and 100 mM NaCl and stored in small aliquots at −70°C. Bulk preparation of virus was done as described by Kääriäinen et al. (14).

Growth and purification of radioactively labeled virus. Confluent monolayers of BHK-21 cells in 60-mm dishes (Nunc) were infected with SFV using 50 PFU/cell in 1 ml of minimal essential medium (MEM) supplemented with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 10 mM glutamine, and 2 μg of dactinomycin (Sigma) per ml. At 1 h postinfection (p.i.) the medium was replaced with MEM containing 0.2% bovine serum albumin (GIBCO), and cells were further incubated at 37°C. At 4 h p.i. the medium was replaced with methionine-free MEM containing 150 μCi of [^{35}S]methionine (Amersham, >800 Ci/mmol) per ml, and incubation was continued for 60 min. The cells were washed twice with MEM containing 100 times excess methionine and then incubated in MEM with 150 mg of methionine per liter at 37°C. The medium was collected at 8.5 h p.i. and layered on top of a 10 to 55% (wt/wt) sucrose gradient in MNTE {20 mM MES [2-(N-morpholino)ethanesulfonic acid; Sigma], 30 mM Tris, 100 mM NaCl, 1.25 mM EDTA, pH 7.4} at 4°C. Centrifugation was done at 30,000 rpm in an SW40 rotor at 4°C for 90 min in a Beckman L8-60M ultracentrifuge. The gradients were collected from below with a fraction collector (Gilson model 023 Micro Fraction Collector), and radioactivity was measured in a liquid scintillation counter (LKB 1214 Rackbeta, LKB Wallac) using Emulsifier Safe (Packard). The fractions containing radioactive virus were pooled and then pelleted through a 50% (wt/vol) glycerol cushion by centrifugation in an SW40 rotor at 30,000 rpm for 90 min at 4°C. The virus pellet was suspended in MNTE buffer and stored at −70°C.

Labeling of viral proteins in infected cells. BHK-21 cells in 35-mm dishes (Nunc) were infected with 50 PFU per cell in 0.5 ml as described above. At 4 h p.i. the cells were labeled with 100 μCi of [^{35}S]methionine per dish in 500 μl of methionine-free medium for 5 min at 37°C. The cells were washed twice with prewarmed MEM with 100-fold excess methionine, followed by a chase in complete MEM at 37°C for various times. The cell samples were then transferred to ice and homogenized according to Quinn et al. (20). Briefly, cells were scraped into cold phosphate-buffered saline supplemented with protease inhibitors (per ml: 10 μg of phenylmethylsulfonyl fluoride, 1 μg of leupeptin, 1 μg of aprotinin, 0.7 μg of pepstatin, and 1 μg of antipain; Sigma), followed by a wash with a buffer containing 10 mM Tris (pH 8.0) and 1 mM $MgCl_2$. The wash was repeated with the same buffer (phosphate-buffered saline) supplemented with 40 μg of phenylmethylsulfonyl fluoride per ml. Cells were finally homogenized by pulling the suspension 25 times through a needle (0.6 by 25 mm). Nuclei were removed by centrifugation at 6,000 rpm for 5 min at 4°C (Eppendorf centrifuge 5415).

Treatment of virus particles and infected-cell homogenates with buffers of different pH. A mixture of cold and labeled SFV (2 μg of protein total) was added to 500 μl of a solution containing 10 mM Tris (pH 8.0), 1 mM $MgCl_2$, and 40 μg of phenylmethylsulfonyl fluoride per ml. This solution was then adjusted to the desired pH by the addition of pretitrated volumes of 1 N HCl. The pH of the cell homogenates (500 μl) was adjusted similarly. All samples were incubated on ice for 10 min and then solubilized by adding Nonidet P-40 (NP40; octylphenylpolyethylene glycol; Fluka) to 1%. At this step the NaCl concentration was also corrected to 150 mM. Some acid-treated virus samples were readjusted to pH 7.4 and incubated for an additional 10 min on ice before solubilization. For sedimentation analyses the virus sample was diluted into 500-μl volumes of MNTE buffer at pH 5.8, 6.2, 6.6, 7.0, 7.4, and 7.8 before incubation and solubilization.

Analysis of solubilized cell and virus samples. (i) Sedimentation analysis. Solubilized cell or virus samples (500 μl) were loaded on 5 to 20% (wt/wt) sucrose gradients in MNTE buffer (pH 7.4) containing 0.1% Triton X-100. Centrifugation was done in a Beckman SW40 rotor at 39,000 rpm for 23 h at 4°C. Gradients were fractionated from the bottom, and the fractions were analyzed for radioactivity in a liquid scintillation counter. One-tenth (30 μl) of some of the fractions were analyzed further by immunoprecipitation, cross-linking, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Pelleted material was solubilized in 50 μl of SDS-PAGE sample buffer (0.1 M Tris hydrochloride [pH 8.8], 0.5 M sucrose, 0.02% bromphenol blue, 5 mM EDTA [pH 8.0], 10 mg of methionine per ml, and 4% SDS) by heating at 70°C for 5 min.

(ii) Immunoprecipitation. The samples used for immunoprecipitation were (i) NP40-solubilized virus particles, (ii) NP40-solubilized homogenates of infected cells, and (iii) sucrose-containing fractions with sedimented viral proteins. For our standard reactions we used 150 μl of each of the first two. The sucrose-containing samples (30 μl) were diluted to 100 μl with an immunoprecipitation buffer containing 1% NP40, 50 mM Tris (pH 7.4), 150 mM NaCl, and 2 mM EDTA. All the samples that had been treated with acidic buffers were neutralized before immunoprecipitation. The immune analyses started out with precleaning steps, as follows. One microliter of commercially available rabbit anti-mouse immunoglobulin G was added, and the mixture was incubated for 5 min on ice. This was followed by a 2-h incubation at 4°C with 40 μl of a 1:1 (vol/vol) slurry of protein A-Sepharose (Pharmacia). After unspecifically reacted products were spun down, the supernatant was mixed with monoclonal antibodies against SFV. One microliter of each antibody preparation was added together with 1 μl of rabbit anti-mouse immunoglobulin G. Incubation was done first for 10 min on ice and then for an additional 16 h at 4°C in the presence of 40 μl of protein A slurry. A second round of immunoprecipitation was done by adding a new volume of antibody to the cleared supernatant of the first reaction together with slurry and incubating for 16 h at 4°C. Pellets were washed twice with a solution containing 0.2% NP40, 10 mM Tris hydrochloride (pH 7.5), 150 mM NaCl, and 2 mM EDTA, twice with a solution containing 0.2% NP40, 10 mM Tris hydrochloride (pH 7.5), 500 mM NaCl, and 2 mM EDTA, and finally once with a solution containing 10 mM Tris hydrochloride (pH 7.5). The precipitates were then solubilized by heating for 2 min at 70°C in SDS-PAGE sample buffer. Viral polypeptides were resolved on a 10% SDS-polyacrylamide gel and quantitated as described below.

(iii) SDS-PAGE. Samples were centrifuged (15,000 rpm, 1 min) at room temperature before being applied to polyacrylamide gels composed of a 10% separating gel and a 5% stacking gel (4). In some experiments we used a separating

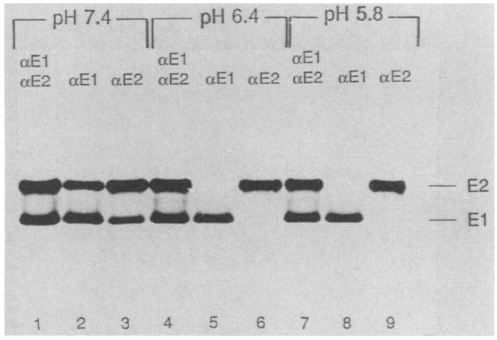

FIG. 1. Immunoprecipitation analyses of the membrane proteins of virus particles after treatment with a buffer of neutral or low pH. Lanes 1 to 3 show [^{35}S]methionine-labeled SFV which has been solubilized in NP40 under neutral conditions and then immunoprecipitated with the monoclonal antibodies anti-E1 (αE1) and anti-E2 (αE2). Lanes 4 to 9 show virus samples which have been incubated for 10 min on ice in a pH 6.4 buffer (lanes 4 to 6) or pH 5.8 buffer (lanes 7 to 9) before immunoprecipitation. Analyses shown in lanes 1, 4, and 7 include anti-E1 and anti-E2 together, those in lanes 2, 5, and 8 were done with anti-E1 only, and those in lanes 3, 6, and 9 were done with anti-E2 only. The precipitates were analyzed on a 10% SDS-polyacrylamide gel which was processed for fluorography.

gel composed of a 5 to 10% polyacrylamide gradient. The gel was fixed in 10% tricholoroacetic acid–30% methanol–10% acetic acid for 30 min at room temperature and then treated for fluorography with En^{3}Hance (Du Pont). The gel was dried and exposed to a Kodak XAR 5 film at −70°C. For quantitation of the [^{35}S]methionine in protein bands, these were cut out and solubilized in Protosol (Du Pont) according to the instructions of the manufacturer, followed by scintillation counting. Protein ratios were calculated after correction for their content of methionine residues.

RESULTS

Monoclonal antibodies which precipitate intact heterodimers. In previous studies on the solubilized SFV glycoprotein complex it was necessary to fix the E2E1 glycoprotein association by chemical cross-linking before these could be analyzed with E1- and E2-specific antibodies (21, 29). This was due to the strongly dissociating effect of the polyclonal antibody preparations used in these studies. However, as the effect of cross-linking is relatively inefficient (about 45%), we decided to investigate whether any of the monoclonal antibodies described by Boere and coworkers (2) would be less dissociative and precipitate intact complexes directly from a solubilized virus sample. We found two monoclonal preparations, anti-E1 8.139 (referred to as anti-E1 in the text) and anti-E2 5.1 (referred to as anti-E2), which coprecipitated both subunits from NP40 lysates of SFV (Fig. 1, lanes 1 to 3). The specificity of the monoclonal antibodies has been shown before by immunoblot analysis of unreduced SFV proteins separated by SDS-PAGE (2). This was further confirmed in this work by the specific precipitation of only the homologous antigen from a solubilized membrane preparation in which the subunits have been dissociated from each other by treatment with acidic buffers (see Fig. 1, lanes 8 and 9, and section on dissociation below).

Quantitation of E2E1 heterodimers in the virus membrane. Quantitation analyses of the total [^{35}S]methionine-labeled immunoprecipitate in which both antibodies have been used showed that E1 and E2 are present in the viral membrane in an almost equimolar ratio (E1/E2 = 1.05) (Fig. 1, lane 1). Similar analyses of the immunoprecipitates obtained with anti-E1 alone show that about 70% of the E2 subunits coprecipitate with E1 (see Fig. 1, lane 2, and the summary of quantitations obtained with anti-E1 in Fig. 8). Using anti-E2, the coprecipitating E1 fraction was found to represent about 50% (Fig. 1, lane 3). The decreased yield of complexes which were precipitated with anti-E2 compared with that of anti-E1 was probably due to a dissociative effect of anti-E2 on the E2E1 complex, as both subunits are present in equal amounts in the solubilized virus samples. We conclude that at least 70% of the E1 and E2 subunits are forming hetero-oligomeric complexes. These have to represent heterodimers, as no higher oligomeric structures have been demonstrated in solubilized virus samples (21, 29).

Coprecipitation analysis of virus samples treated with acidic buffers. To analyze the possible dissociative effect of mildly low pH on the E2E1 association, [^{35}S]methionine-labeled virus samples were treated with either pH 6.4 or 5.8 buffers for 10 min at 0°C. The samples were then solubilized and used for precipitation analyses with anti-E1 and anti-E2 (Fig. 1, lanes 4 to 9). No visible bands which would correspond to coprecipitated material were seen in the anti-E1 and anti-E2 precipitations of either the pH 6.4- or the pH 5.8-treated material. Quantitation of ^{35}S radioactivity in the gel showed 2% and 1% of complexes (see Fig. 8). Thus, the heterodimeric glycoprotein association appears to be very sensitive to low pH.

In these tests the samples were incubated in a low-salt buffer (10 mM Tris) and the solubilization was done with NP40. These conditions were used in all coprecipitation analyses in this work, as they corresponded to those used for the preparation of the cell homogenates (see below). However, we have in additional tests with SFV particles also compared the pH effect of this buffer system with that of the isotonic MNTE buffer (see Materials and Methods) and found no differences. Similarly, the use of Triton X-100 instead of NP40 during solubilization gave the same results. We also found that incubations for 1 or 10 min at either 0 or 37°C all resulted in about 70% heterodimers if a pH 7.4 buffer was used, and complete dissociation resulted with a pH 5.8 buffer. These control analyses show that it is the acidity of the buffer alone and no other factors that causes the dissociation of the heterodimer.

Sedimentation analyses of acid-treated virus. The dissociative effect of low pH on the spike heterodimer was also studied by sedimentation analyses in sucrose gradients. In these experiments the virus was treated with a series of different pH values before solubilization and centrifugation. The sedimentation profiles are shown in Fig. 2. With decreasing pH a gradual shift from a faster-sedimenting peak into a slower one was observed. At a pH of 6.6, about half of the material was shifted into the slower-sedimenting fraction (see quantitation in Fig. 3). Using immunoprecipitation and cross-linking analyses, we have shown that the faster-sedimenting peak contains E2E1 heterodimers, in agreement with earlier findings (24, 29), and the slower one contains E2 and E1 monomers (data not shown). Note that about 19% of the subunits of this sample were found in the monomer fraction already at neutral conditions. This material is possibly derived from heterodimers which have undergone dissociation during sample processing or migration in the sucrose gradient. Thus, these results show that the NP40-solubilized glycoprotein heterodimer becomes gradually dissociated into monomers when treated with buffers of de-

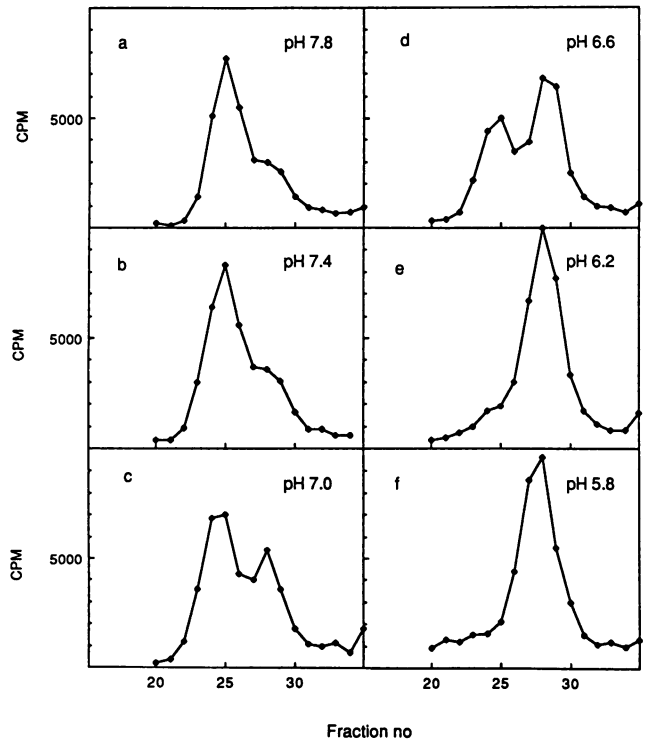

FIG. 2. Sedimentation profiles of solubilized SFV membrane proteins after treatment with buffers of different pH. [^{35}S]methionine-labeled virus was incubated in a buffer with a pH as indicated, solubilized with NP40, and then layered on a 5 to 20% (wt/wt) sucrose gradient containing Triton X-100. The gradient was run for 24 h at 4°C in an SW40 rotor at 39,000 rpm. Fractions were collected from below, and radioactivity was measured in a liquid scintillation counter. Bottom of the gradient is to the left. Only the peak regions of the profiles are shown. In all gradient profiles the missing fractions showed close to base-line radioactivity. The pellet fractions contained the capsid protein.

creasing pH values. They clearly confirm the earlier results obtained by immunoprecipitation.

A monoclonal antibody which precipitates acid-dissociated E1. During our initial screening for E2E1 coprecipitating monoclonal antibodies, one preparation, anti-E1 8.47 (called anti-E1′ in the text), was shown to have very poor reactivity toward native subunits. However, when tested against acid-dissociated membrane proteins, efficient E1 reactivity was found. This is shown by the sequential immunoprecipitation analyses in Fig. 4. Most of the E1 subunits (but no E2 proteins) were brought down with the anti-E1′ antibody if the virus sample had been treated with pH 5.8 buffer before solubilization (Fig. 4, lanes 6, 7, and 8), whereas very little reactivity was seen with the control sample (lanes 3, 4, and 5). We conclude that the E1 subunit exposes an epitope for anti-E1′ after it has been dissociated from E2 by treatment with mild acid.

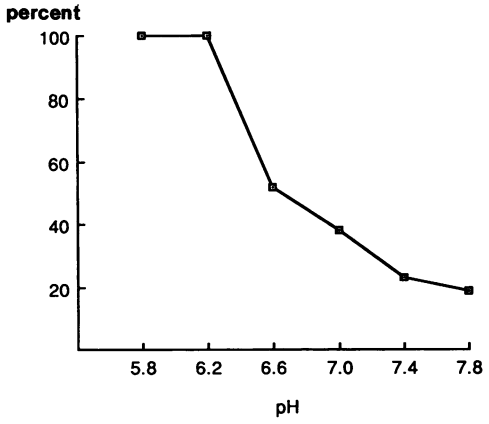

FIG. 3. Dissociation of the E2E1 heterodimer with decreasing pH. Experimental points represent the fractions (in percent) of nonassociated E1 and E2 subunits in virus samples which have been treated with buffers of different pH. The data are derived from Fig. 2.

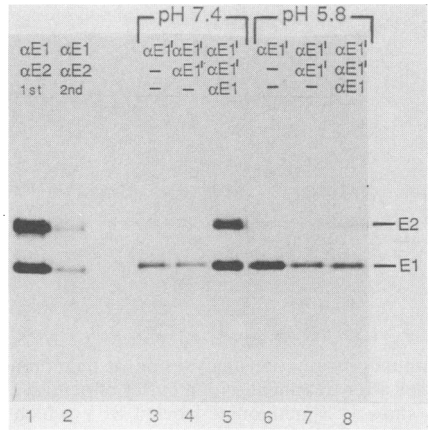

FIG. 4. Sequential immunoprecipitation of SFV membrane proteins solubilized at neutral and low pH with different anti-E1 antibodies. [^{35}S]methionine-labeled SFV was treated with buffers of pH 5.8 and 7.4, solubilized in NP40, and precipitated sequentially first with anti-E1′ (αE1′) in lanes 3 and 6, then a second time with αE1′ (lanes 4 and 7), and finally with anti-E1 (αE1) in lanes 5 and 8. Lanes 1 and 2 show the total amount of proteins precipitable with anti-E1 and anti-E2 (αE2) antibodies together in two subsequent precipitations. The precipitates were run on a 10% SDS-polyacrylamide gel.

The acid-induced dissociation of E2E1 is reversible in the viral membrane. The possible reversibility of the acid-induced dissociation of the E2E1 complex in the viral membrane was tested by reneutralization of a pH 5.8-treated virus sample before solubilization and analysis for oligomers. Reincubation in neutral buffer reconstituted the heterodimeric structure (Fig. 5). The membrane proteins migrate as dimers in a sucrose gradient (Fig. 5A), anti-E1 coprecipitates E2 together with the E1 subunit (Fig. 5B, lane 3), and the anti-E1′ antibody has lost most of its reactivity (Fig. 5B, lanes 1 and 2).

The E2 precursor protein p62 exists as a p62E1 heterodimer. We have shown before that the E2 precursor protein p62 of infected cells is found as a E2E1-sized protein oligomer after solubilization with a nonionic detergent (30). However, the subunit composition of this apparent dimer is not known. To settle this question we decided to analyze the intracellular complexes with the anti-E1 and anti-E2 monoclonal antibodies. Cells were pulse-labeled for 5 min with [^{35}S]methionine and chased with excess cold methionine for only 15 min. During this time no cleavage of p62 occurred. The cells were then homogenized, solubilized, and reacted with the monoclonal antibodies (Fig. 6). Reaction with anti-E1 and anti-E2 together resulted in a complete precipitation of all the intracellular subunits (Fig. 6, lane 1).

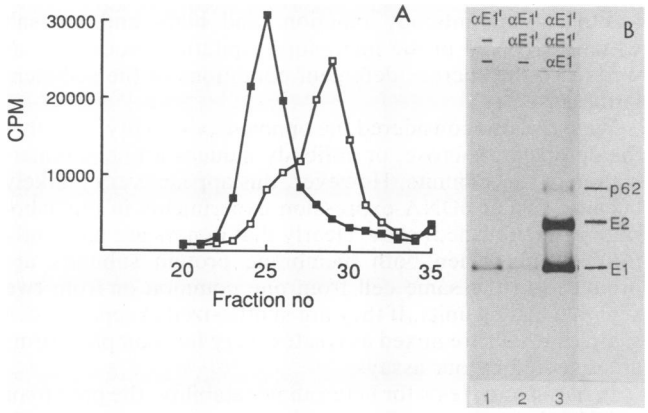

FIG. 5. Sedimentation and antibody precipitation analysis of pH 5.8-treated SFV membrane proteins which have been reneutralized before solubilization. (A) Sedimentation profiles of [^{35}S]methionine-labeled SFV treated with a pH 5.8 buffer (□) and with first a pH 5.8 buffer and then a pH 7.4 buffer (■) before solubilization. Sucrose gradients and running conditions were as for Fig. 2. Only the peak fractions are shown. Bottom of the gradient is to the left. (B) Sequential immunoprecipitation of the reneutralized sample in panel A with first anti-E1′ (αE1′, lane 1), then a second time with αE1′ (lane 2), and finally with anti-E1 (αE1, lane 3). The precipitates were run on a 10% SDS-polyacrylamide gel.

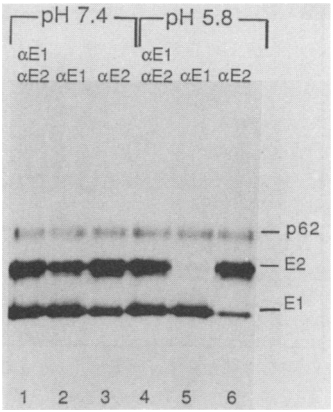

FIG. 7. Immunoprecipitation analyses of cell-derived p62, E2, and E1 membrane proteins after treatment with neutral or acidic buffers. Two dishes of BHK cells were infected with SFV and processed as described in the legend to Fig. 6, with the exceptions that the chase was longer (60 min) to allow cleavage of p62 and the homogenates were only treated with neutral and pH 5.8 buffer before antibody reactions. Lanes 1 to 3 show precipitations from the sample incubated in the neutral buffer, and lanes 4 to 6 show precipitations from the acid-treated sample. The membrane proteins p62, E2, and E1 are indicated.

Quantitation of the amounts of E1 and p62 showed that they were represented in an almost equimolar ratio (E1/p62 = 1.1). When anti-E1 was used separately, close to complete coprecipitation of both subunits was obtained (Fig. 6, lane 2). Quantitation analysis showed that the fraction of p62E1 complexes was 86% (see Fig. 8). Thus, the p62 precursor protein exists almost exclusively as a p62E1 heterodimeric complex. Similar analysis with the E2 antibody resulted in a lower fraction of complexes (Fig. 6, lane 3). Apparently, anti-E2 induced some p62E1 dissociation in the same way as with the mature E2E1.

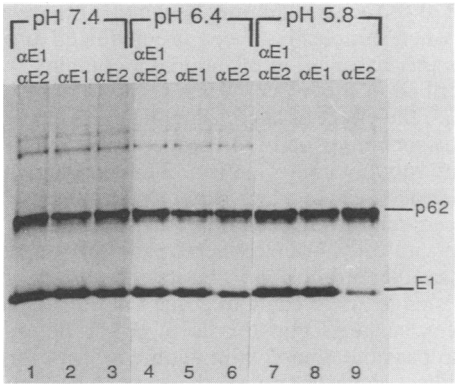

FIG. 6. Immunoprecipitation analyses of cell-derived p62 and E1 membrane proteins after treatment with a buffer of neutral or low pH. Three dishes with BHK cells were infected with SFV. At 4 h p.i. proteins were labeled with [^{35}S]methionine for 5 min and then chased with excess cold methionine for 15 min. After this, cells from each dish were scraped together and homogenized. One sample was incubated in a neutral buffer, another one in a pH 6.4 buffer, and a third one in a pH 5.8 buffer. Each sample was then divided into three portions for reactions with antibodies and subsequent electrophoresis on a 10% SDS-polyacrylamide gel. Lanes 1 to 3 show immunoprecipitation analyses of SFV proteins treated at neutral pH. Lanes 4 to 6 show samples treated with pH 6.4 buffer, and lanes 7 to 9 show others treated with pH 5.8 buffer. Anti-E1 (αE1) and anti-E2 (αE2) were combined in the analyses shown in lanes 1, 4, and 7. Anti-E1 was used alone in analyses shown in lanes 2, 5, and 8. Anti-E2 analyses are shown in lanes 3, 6, and 9. The figure represents a fluorograph. p62 and E1 proteins are indicated. The large-sized material seen in lanes 1 to 6 probably represents SDS-resistant p62E1 heterodimers.

The p62E1 heterodimer is more resistant to low-pH-induced dissociation than is E2E1. To test the possibility that the precursor complex is more resistant toward acid treatment than is the mature one, we treated the p62- and E1-containing homogenate described above with pH 6.4 and 5.8 buffers before immunoprecipitation. The analysis with the anti-E1 showed that almost all (80 to 90%) of the p62 and E1 subunits were still in hetero-oligomeric associations in both the pH 6.4- and the pH 5.8-treated samples (see Fig. 6, lanes 5 and 8, and quantitation in Fig. 8). This is in marked contrast to the virus sample which, after corresponding treatments, was almost completely dissociated. Lanes 6 and 9 in Fig. 6 show the analysis of the 15-min-chased cell sample with anti-E2. A significant coprecipitation was seen also in this reaction. However, compared with the anti-E1 assay, clearly less heterologous subunit was brought down. Most likely, this again reflects the property of anti-E2 to partially dissociate the heterodimeric complex.

We also prepared a 5-min-pulse-labeled and 60-min-chased cell sample which we treated and analyzed in the same way as the 15-min-chased cell sample described above. In this sample, part of the p62 was cleaved into the E2 form, thereby offering the possibility to compare the features of the p62E1 and E2E1 forms of the heterodimer in the same sample. The results of the immunoprecipitation analyses are shown in Fig. 7. One can see that the p62 subunit followed the E1 antigen very faithfully in the anti-E1 coprecipitation assay both in the sample treated with the neutral buffer and in the pH 5.8-treated sample (Fig. 7, lanes 2 and 5, and Fig. 8). In contrast, an almost complete separation of the mature E2 subunit from the E1 subunit was observed when the sample was incubated in the acid buffer (compare E2 and p62 bands in lanes 1 and 5). Thus, in contrast to the E2E1

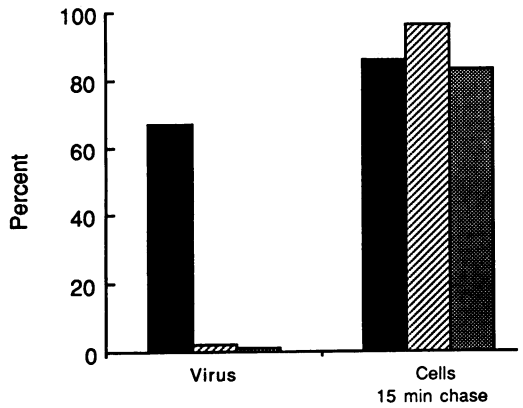

FIG. 8. Quantitation of the heterodimer fraction in virus and cell samples which have been incubated in buffers of different pH: ■, pH 7.4; ▨, pH 6.4; ▩, pH 5.8. The columns represent the heterodimer fractions (p62E1 and E2E1) obtained by the anti-E1 immunoprecipitations shown in Fig. 1 and 6. The values for E2E1 complexes of virus particles represent the mean of six experiments, and those for cellular p62E1 complexes represent the mean of three experiments.

association, the p62E1 association appears to be remarkably resistant to low-pH treatment.

The conclusion above was also corroborated by the results from sedimentation analyses of the 60-min-chased cell sample which had been treated with pH 5.8. Most of the material migrated in a position corresponding to viral membrane monomers, with a shoulder in the dimer region. SDS-PAGE analyses showed that the monomer peak contained only E2 and E1 subunits, whereas the material in the shoulder consisted of p62 subunits together with part of the E1 protein (but no E2) (data not shown). Apparently, most of the uncleaved p62 protein of the sample remained as an acid-resistant p62E1 heterodimer, whereas the mature E2E1 heterodimers were dissociated by the pH 5.8 treatment.

DISCUSSION

In the present work we have shown that the two membrane glycoproteins of SFV, p62 and E1, form a heterodimeric complex in the infected cell and that this matures into the E2E1 heterodimer of the virus membrane. Earlier studies have shown the E2E1 complex in the viral membrane, but these have not been able to clearly demonstrate the precursor complex (21, 29). Results supporting the existence of extensive complex formation between p62 and E1 come from the Sindbis virus (where p62 is called pE2). First, treatment of Sindbis virus-infected cells with polyclonal anti-E1 antibodies results in inhibition of pE2 cleavage (13). Second, treatment of Sindbis virus mutant *ts*20-infected cells (which transport uncleaved pE2 to the plasma membrane) with polyclonal anti-E2 antibodies is able also to immobilize E1 on the cell surface, as analyzed by photobleaching recovery measurements (12). Because of the new monoclonal antibodies available, especially the anti-E1 antibody, we were able in this work to provide direct quantitative results about this interaction.

The fact that a portion (20 to 30%) of the subunits in the SFV virus membrane could not be recovered as heterodimers either in our precipitation or sedimentation assay most likely reflects artificial dissociation during sample processing. The mature heterodimer is evidently less stable than the p62E1 oligomer, and therefore it might be sensitive to the solubilization, antibody reaction, and high- and low-salt washes involved in the immunoprecipitation procedure, as well as to the sucrose-detergent conditions of the sedimentation analyses.

We have also considered the opposite possibility, i.e., that the detergent, sucrose, or antibody induces artificial dimerization of the subunits. However, this appears very unlikely because recent cDNA expression experiments in our laboratory (unpublished) show clearly that dimers are efficiently formed only when both membrane protein subunits are produced in the same cell from one common or from two separate coding units. If they are synthesized in separate cell samples which are mixed as lysates, very few complex forms are detected by our assays.

In our in vitro tests for heterodimer stability, the precursor p62E1 oligomers of infected cells were shown to be stable when incubated in buffers at mildly low pH. In contrast, the mature E2E1 oligomers of virus particles were found to be extremely prone to dissociate under similar conditions. This effect was not caused by the dilution of the sample which took place when it was taken up in the acidic buffers, because a similar dilution in neutral conditions retained the complex forms. It is also unlikely that it represents an increased dissociative effect of the detergent at low pH because (i) the pH should have little influence on the micellar properties of nonionic detergents like NP40 (11) and (ii) the p62E1 precursor complex remains intact under the same conditions.

Earlier studies with SFV and Sindbis virus have shown that the mature heterodimer undergoes a conformational change upon incubation in acidic buffers (7, 15). In the case of SFV, the E2 protein becomes increasingly sensitive to trypsin digestion at 0°C when preincubated with buffers of pH 6.2 or below. When the digestion was done at 37°C instead of 0°C the E1 subunit became more resistant to digestion when samples had been preincubated at pH 6.2 or below. As the dissociation of the E2E1 complex, which we have described in this work, was already complete at a pH value of 6.2, the effect of decreasing pH seems to begin with subunit dissociation and to proceed with pronounced changes in subunit conformation. The separate nature of these two processes is further supported by the fact that the acid-induced subunit dissociation is a reversible phenomenon in the viral membrane, whereas the trypsin sensitivity changes have been reported to be irreversible ones (15).

The resistance toward acid treatment of the p62E1 precursor complex suggests that this form of the heterodimer is required to pass the complex, in an intact form, through the mildly acidic compartments of the exocytic pathway, i.e., the *trans*-Golgi. The exact pH of this organelle is not known, but it is most likely below the pH range of 6.6 to 7.0 which was found to be the threshold for dissociation of the mature E2E1 form of the heterodimer (1). Therefore, the proteolytic processing of the precursor complex probably does not occur until after the complex has passed through the *trans*-Golgi region. However, it still seems to take place before the heterodimer arrives at the plasma membrane. In a recent study, de Curtis and Simons (5) demonstrated that a significant fraction of the p62 protein became cleaved intracellularly when spike protein transport was inhibited by low temperature (19.5°C).

The pronounced acid sensitivity of the mature E2E1 complex suggests that this complex will be dissociated when the virus particle enters the endosome of a new cell and encounters the acidic milieu of this organelle (16, 19). This could represent the first event which is required for activa-

tion of the fusogenic activity of the spike complex. Final activation might require the additional conformational changes of individual subunits described by Kielian and Helenius (15).

One way to follow the putative heterodimer dissociation during infection could be to use the anti-E1′ antibody, which should be specific for acid-dissociated subunits. The fact that the exposition of the E1′ epitope coincides with the low-pH-induced E2E1 dissociation suggests that the latter phenotype has a role during the life cycle of SFV. The antibody was obtained by using complete, UV-inactivated SFV as antigen (2). It gives passive protection to mice against SFV infection, but does not neutralize virus in in vitro tests. Possibly, it cannot react with intact virus but only with dissociated E1 in such cells in which SFV has entered.

ACKNOWLEDGMENTS

We wish to thank Michael Baron, Peter Liljeström, Mario Lobigs, and Kalervo Metsikkö for critically reading the manuscript, Gunnar von Heijne for help with the figures, and Ingrid Sigurdson for typing.

This work was supported by the Swedish Medical Research Council, B88-12X-08272-01A, the Swedish National Board for Technical Development, 87-02750P, and the Swedish Natural Science Research Council, B-BV 9353-301.

LITERATURE CITED

1. **Anderson, R. G. W., and L. Orci.** 1988. A view of acidic intracellular compartments. J. Cell Biol. **106**:539–543.
2. **Boere, W. A. M., T. Harmsen, J. Vinjé, B. J. Benaissa-Trouw, C. A. Kraaijeveld, and H. Snippe.** 1984. Identification of distinct antigenic determinants on Semliki Forest virus by using monoclonal antibodies with different antiviral activities. J. Virol. **52**:575–582.
3. **Bracha, M., and M. J. Schlesinger.** 1976. Defects in RNA+ temperature-sensitive mutants of Sindbis virus and evidence for a complex of pE2-E1 viral glycoproteins. Virology **74**:441–445.
4. **Cutler, D. F., and H. Garoff.** 1986. Mutants of the membrane-binding region of Semliki Forest virus E2 protein. I. Cell surface transport and fusogenic activity. J. Cell Biol. **102**:889–901.
5. **de Curtis, I., and K. Simons.** 1988. Dissection of Semliki Forest virus glycoprotein delivery from the trans-Golgi network to the cell surface in permeabilized BHK cells. Proc. Natl. Acad. Sci. USA **85**:8052–8056.
6. **Dubois-Dalcq, M., R. V. Holmes, and B. Rentier.** 1984. Assembly of enveloped RNA viruses. Springer Verlag, Vienna.
7. **Edwards, J., E. Mann, and D. T. Brown.** 1983. Conformational changes in Sindbis virus envelope proteins accompanying exposure to low pH. J. Virol. **45**:1090–1097.
8. **Fuller, S. D.** 1987. The T=4 envelope of Sindbis virus is organized by interactions with a complementary T=3 capsid. Cell **48**:923–934.
9. **Green, J., G. Griffiths, D. Louvard, P. Quinn, and G. Warren.** 1981. Passage of viral membrane proteins through the Golgi complex. J. Mol. Biol. **152**:663–698.
10. **Helenius, A., J. Kartenbeck, K. Simons, and E. Fries.** 1980. On the entry of Semliki Forest virus into BHK-21 cells. J. Cell Biol. **84**:404–420.
11. **Helenius, A., and K. Simons.** 1975. Solubilization of membranes by detergents. Biochim. Biophys. Acta **415**:29–79.
12. **Johnson, D. C., M. J. Schlesinger, and E. L. Elson.** 1981. Fluorescence photobleaching recovery measurements reveal differences in envelopment of Sindbis and vesicular stomatitis viruses. Cell **23**:423–431.
13. **Jones, K. J., R. K. Scupham, J. A. Pfeil, K. Wan, B. P. Sagik, and H. R. Bose.** 1977. Interaction of Sindbis virus glycoproteins during morphogenesis. J. Virol. **21**:778–787.
14. **Kääriäinen, L., K. Simons, and C.-H. von Bonsdorff.** 1969. Studies in subviral components of Semliki Forest virus. Ann. Med. Exp. Biol. Fenn. **47**:235–248.
15. **Kielian, M., and A. Helenius.** 1985. pH-induced alterations in the fusogenic spike protein of Semliki Forest virus. J. Cell Biol. **101**:2284–2291.
16. **Kielian, M., and A. Helenius.** 1986. Entry of alphaviruses, p. 91–119. *In* S. Schlesinger and M. J. Schlesinger (ed.), The Togaviridae and Flaviviridae. Plenum Publishing Corp., New York.
17. **Marsh, M., E. Bolzau, and A. Helenius.** 1983. Penetration of Semliki Forest virus from acidic pre-lysosomal vacuoles. Cell **32**:931–940.
18. **Marsh, M., and A. Helenius.** 1989. Virus entry into animal cells. Adv. Virus Res. **36**:107–151.
19. **Mellman, J., R. Fuchs, and A. Helenius.** 1986. Acidification of the endocytic and exocytic pathways. Annu. Rev. Biochem. **55**:663–700.
20. **Quinn, P., G. Griffiths, and G. Warren.** 1983. Dissection of the Golgi complex. II. Density separation of specific Golgi functions in virally infected cells treated with monensin. J. Cell Biol. **96**:851–856.
21. **Rice, C. M., and J. H. Strauss.** 1982. Association of Sindbis virion glycoproteins and their precursors. J. Mol. Biol. **154**: 325–348.
22. **Schlesinger, M. J., and S. Schlesinger.** 1986. Formation and assembly of alphavirus glycoproteins, p. 121–148. *In* S. Schlesinger and M. J. Schlesinger (ed.), The Togaviridae and Flaviviridae. Plenum Publishing Corp., New York.
23. **Schlesinger, S., and M. J. Schlesinger.** 1972. Formation of Sindbis virus proteins: identification of a precursor for one of the envelope proteins. J. Virol. **10**:925–932.
24. **Simons, K., A. Helenius, and H. Garoff.** 1973. Solubilization of the membrane proteins from Semliki Forest virus with Triton X100. J. Mol. Biol. **80**:119–133.
25. **Vaux, D. J. T., A. Helenius, and I. Mellman.** 1988. Spike-nucleocapsid interaction in Semliki Forest virus reconstructed using network antibodies. Nature (London) **336**:36–42.
26. **Vogel, R. H., S. W. Provencher, C.-H. von Bonsdorff, M. Adrian, and J. Dubochet.** 1986. Envelope structure of Semliki Forest virus reconstructed from cryo-electron micrographs. Nature (London) **320**:533–535.
27. **von Bonsdorff, C.-H., and S. C. Harrison.** 1978. Hexagonal glycoprotein arrays from Sindbis virus membranes. J. Virol. **28**:578–583.
28. **White, J. M., and A. Helenius.** 1980. pH-dependent fusion between the Semliki Forest virus membrane and liposomes. Proc. Natl. Acad. Sci. USA **77**:3273–3277.
29. **Ziemiecki, A., and H. Garoff.** 1978. Subunit composition of the membrane glycoprotein complex of Semliki Forest virus. J. Mol. Biol. **122**:259–269.
30. **Ziemiecki, A., H. Garoff, and K. Simons.** 1980. Formation of the Semliki Forest virus membrane glycoprotein complexes in the infected cell. J. Gen. Virol. **50**:111–123.