Membrane Fusion Process of Semliki Forest Virus I: Low pH–induced Rearrangement in Spike Protein Quaternary Structure Precedes Virus Penetration into Cells

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Abstract. The Semliki Forest virus (SFV) directs the synthesis of a heterodimeric membrane protein complex which is used for virus membrane assembly during budding at the surface of the infected cell, as well as for low pH–induced membrane fusion in the endosomes when particles enter new host cells. Existing evidence suggests that the E1 protein subunit carries the fusion potential of the heterodimer, whereas the E2 subunit, or its intracellular precursor p62, is required for binding to the nucleocapsid. We show here that during virus uptake into acidic endosomes the original E2E1 heterodimer is destabilized and the E1 proteins form new oligomers, presumably homooligomers, with altered E1 structure. This altered structure of E1 is specifically recognized by a monoclonal antibody which can also inhibit penetration of SFV into host cells as well as SFV-mediated cell–cell fusion, thus suggesting that the altered E1 structure is important for the membrane fusion. These results give further support for a membrane protein oligomerization-mediated control mechanism for the membrane fusion potential in alphaviruses.

ALPHAVIRUSES, such as Semliki Forest virus (SFV)[1] and Sindbis virus, are enveloped animal viruses which mature by budding at the plasma membrane (PM) of infected cells and enter new cells by an acid-induced membrane fusion process inside the endosomal compartment (Kielian and Helenius, 1986; Schlesinger and Schlesinger, 1986). Because of their simple structure and efficient replication these viruses represent useful systems for studying the molecular mechanisms of membrane budding and fusion. SFV directs the synthesis of three structural proteins, the capsid protein and two transmembrane glycoproteins, p62 and E1 (Garoff et al., 1982). The capsid protein associates in the cell cytoplasm with the viral RNA genome into nucleocapsids (NCs). The two membrane proteins oligomerize soon after synthesis in the ER into p62E1 heterodimers and are then transported to the cell surface in order to take part in the budding process (Ziemiecki et al., 1980; Wahlberg et al., 1989). In the released virus particles the heterodimers are clustered into groups of three, each representing a spike-like projection on the virus surface (Vogel et al., 1986; Fuller, 1987). Furthermore, the original p62E1 heterodimer is processed by a limited proteolytic attack 66 residues from the NH₂ terminus of p62 to form mature E2E1 complexes. This cleavage occurs at a very late stage during cell surface transport of the heterodimer and is probably mediated by a host protease (de Curtis and Simons, 1988).

There exists substantial evidence that the envelopment of the NC at the cell surface is driven by the binding of the cytoplasmic protein domains of E2 (p62) to the NC. The proximity of these two structures has been demonstrated in virus particles by chemical cross-linking, their structural complementarity has been shown by immunological techniques, and the binding has been verified biochemically in in vitro assays (Garoff and Simons, 1974; Vaux et al., 1988; Metsikkö and Garoff, 1990). The last studies have furthermore emphasized the possible importance of the trimerization reaction for the formation of the spike structures as a way to obtain strong multivalent binding of membrane proteins to the NC. The entry functions of the virus (that is, receptor binding and membrane fusion activity) are, on the other hand, carried by the E1 subunit of the heterodimer. The most direct evidence for this was obtained in studies using virus particles from which essentially all of the E2 subunits had been digested by trypsin. Such particles were shown to be able to infect cells almost as efficiently as the control virus (Omar and Koblet, 1988). In addition, the hemagglutinating activity of the virus has been shown to be a specific function of the E1 subunit (Helenius et al., 1976).

The actual mechanism by which E1 catalyzes membrane fusion is not yet solved. This problem is of particular interest as the E1-mediated membrane fusion process involves several differences as compared with that of the extensively studied hemagglutinin molecule (HA) of orthomyxoviruses as well as those of many other viral fusion proteins. First, the E1 fusion protein is made as part of a protein heterodimer as opposed to a homooligomer in the other cases. Second,

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in contrast to most other viral fusion proteins, the E1 protein of the alphaviruses is not processed by a cleavage reaction. This host-mediated cleavage reaction of a fusion protein seems to represent a fundamental control mechanism by which many viruses prevent activation of their entry functions during the assembly phase of the virus life cycle. Without such a control mechanism fusion proteins might cause aberrant fusion inside the mildly acidic compartments of the biosynthetic transport pathway (Anderson and Orci, 1988). Third, E1-catalyzed membrane fusion requires the presence of cholesterol in the acceptor membrane (White and Helenius, 1980; Kielian and Helenius, 1984). This absolute restriction in lipid composition has not been observed for other viral fusion proteins. Apart from these unique features of the SFV fusogen it is well established that it requires induction by mildly acidic pH (Helenius et al., 1980). By analogy with the low pH–induced changes of HA it is believed that the acidic pH alters the E1 structure into a form that is competent to perform a membrane fusion reaction. The fact that E1 changes into a trypsin-resistant form during entry into acidic endosomes and exposes new immunological epitopes as a result of low pH treatment might reflect this conformational change (Edwards et al., 1983; Helenius et al., 1985; Kielian and Helenius, 1985; Kielian et al., 1986, 1990).

In the accompanying paper (Salminen et al., 1992) and in recent published reports we have shown that, despite the lack of E1 fusion protein processing, SFV still exerts a protein cleavage-mediated control of entry functions, similar to other viruses (Wahlberg et al., 1989; Lobigs and Garoff, 1990; Lobigs et al., 1990). This control is, however, directed by the p62 protein with which the E1 protein is associated. Thus, in the intracellular p62E1 form of the complex, the fusion function of E1 cannot be activated by mildly acidic conditions. This is not possible until after formation of the E2E1 complex through p62 cleavage. The control mechanism appears to involve a p62 cleavage-facilitated disruption of the heterodimeric association by mildly acidic pH. This suggests that the heterodimer has to dissociate before the E1 can be induced by low pH to convert into its fusion-active form.

In this work we have followed the structure of the viral membrane spike proteins during SFV entry into BHK-21 cells, and have found that the E2E1 heterodimeric association is indeed destabilized soon after the virus enters the endocytic pathway. Furthermore, we found that the E1 subunit reorganizes itself into higher oligomeric forms, probably homooligomers. These show increased trypsin resistance and expose unique epitopes recognized by a monoclonal anti-E1 antibody which is able to inhibit virus penetration into cells as well as SFV-mediated cell–cell fusion.

Materials and Methods

Virus, Cells, and Monoclonal Antibodies

Stocks of SFV were propagated in BHK-21 cells as described earlier (Wahlberg et al., 1989). BHK-21 cells were grown in Glasgow minimal essential medium supplemented with 10% tryptose phosphate broth, 5% FCS, and 2 mM glutamine (GIBCO Laboratories Life Technologies Ltd., Paisley, Scotland). For the internalization experiments the cells were either grown in 60-mm dishes or on 18 × 18-mm coverslips in 35-mm dishes.

The monoclonal antibodies UM 8.139 (anti-E1), UM 8.47 (anti-E1'), UM 8.64 (anti-E1'), and UM 5.1 (anti-E2) were all used as mouse ascites prepa-

rations (Boere et al., 1984). The IgG fraction from the UV 8.64 ascites preparation was purified using the ImmunoPure® (A/G) IgG Purification Kit (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions. The monoclonal antibody OKT-9 reacting with the human transferrin receptor (anti-TR) was provided by T. Ebel in our laboratory and used as mouse ascites fluid.

Growth and Purification of Radioactively Labeled Virus

[35S]Methionine-labeled SFV (150,000–500,000 cpm/μl, 1–2 × 10^{11} plaque-forming units [pfu]/ml) was prepared by infecting confluent monolayers of BHK-21 cells in 75-cm^2 bottles (Costar Corp., Cambridge, MA) with SFV (10 pfu/cell) in 1 ml MEM supplemented with 10 mM Hepes, 2 mM glutamine, and 0.2% BSA (GIBCO). After 1 h adsorption in 37°C, 9 ml MEM with 0.2% BSA was added and the incubation was continued. At 6 h after infection the cells were labeled for 15 h at 37°C with [35S]methionine (1,000 Ci/mmol; Amersham International, Amersham, UK) at a final concentration of 200 μCi/ml in methionine-free MEM supplemented with 1% FCS. The virus-containing medium was cleared from cell debris by centrifugation at 2,500 g for 15 min at 4°C. The supernatant was applied to the top of a two-step sucrose density gradient (1 ml 55% [wt/wt] and 3 ml 20% [wt/wt] sucrose in 50 mM Tris-HCl [pH 7.4], 100 mM NaCl, and 0.5 mM EDTA [TNE]) in an SW40 tube, and centrifuged at 30,000 rpm for 90 min at 4°C in an ultracentrifuge (model L8-60M; Beckman Instruments, Inc., Fullerton, CA). After collecting the gradients from below with a fraction collector (model 023 Micro Fraction Collector, Gilson Co., Inc., Worthington, OH), the fractions containing radioactive virus were pooled and diluted in TNE. The purified, labeled virus stock was plaque titrated using carboxymethyl cellulose in the overlay and the plaques were visualized after staining with crystal violet. The protein concentration in the sample was determined according to Lowry et al. (1951). The number of virus particles in the sample was calculated using MW 48.8 × 10^6 for the total protein of one virus particle (Fuller, 1987).

Binding and Internalization Assays

The assays for [35S]methionine-labeled SFV binding to, and endocytosis into, BHK cells were performed essentially as described (Marsh and Helenius, 1980; Helenius et al., 1985). Typically, 80% confluent BHK cells in 60-mm dishes were washed and preincubated with cold MEM containing 0.2% BSA, 10 mM Hepes, and 2 mM glutamine for 20 min on ice. The radiolabeled virus (10 pfu/cell) was bound to the cells in 0.5 ml MEM, pH 7.4, for 1 h on ice with continuous shaking. Free virus particles were removed by washing the cells twice with cold MEM. To assay for bound virus particles (0 min sample) the cells were solubilized at this stage in 400 μl of lysis buffer (1% NP-40, 50 mM Tris [pH 7.4], 150 mM NaCl, 2 mM EDTA, and 10 μg/ml PMSF). Endocytosis of bound virus was initiated by adding prewarmed (37°C) medium and incubating at 37°C. At different time points the cells were cooled on ice, the medium was removed, and 2 ml of cold PBS containing 0.5 mg/ml proteinase K (Bethesda Research Laboratories, Gaithersburg, MD) was added, followed by an incubation on ice for 45 min. In control experiments this treatment removed 96% of the virus particles that had been bound to the surface of the cell and kept on ice. The reaction was stopped by adding 2 ml cold PBS containing 30 mg/ml BSA (Sigma Chemical Co., St. Louis, MO) and 1 mM PMSF. The cells were pelleted (400 g for 3 min at 4°C), washed once with PBS containing 0.2% BSA and 1 mM PMSF, and solubilized in 400 μl lysis buffer. Nuclei were removed (2,000 g for 5 min at 4°C) and the radioactivity in the solubilized cell samples was measured in a scintillation counter. The lysates were used for immunoprecipitation analyses, trypsin digestions, and sedimentation analyses as described below. For the experiments using lysosomotrophic agents, 25 μM monensin (Sigma Chemical Co., St. Louis, MO) was present during all incubation steps (Marsh et al., 1982).

Analysis of Solubilized Cell and Virus Samples

The samples used for these analyses were (a) [35S]methionine-labeled virus particles solubilized in lysis buffer at pH 7.4, (b) [35S]methionine-labeled virus particles treated with 20 mM Na-succinate buffer, pH 5.5, for 10 min at 4°C followed by solubilization in lysis buffer and neutralization, and (c) solubilized BHK cells containing bound as well as internalized [35S]methionine-labeled SFV particles.

Sucrose gradient centrifugation. The 150-μl samples were loaded on gradients consisting of 5–20% (wt/wt) sucrose in TNE, pH 7.4, containing

0.1% NP-40 and 10 μg/ml PMSF, and centrifuged in an SW40 rotor at 39,000 rpm for 24 h at 4°C. The gradients were fractionated and the radioactivity was measured from each collected fraction as described (Wahlberg et al., 1989). The fractions containing peaks of viral protein radioactivity were pooled and analyzed using the assays described below.

Immunoprecipitation analysis. Solubilized SFV particles or cells with virus were diluted in lysis buffer and used in a final volume of 100–500 μl for the analyses. Precleaning using rabbit anti-mouse IgG was done for 30 min at 4°C and the incubations with the monoclonal antibodies for 16 h at 4°C using protein A Sepharose (Pharmacia LKB Biotechnology, Uppsala, Sweden) as carrier (Wahlberg et al., 1989). Precipitates were solubilized in SDS-PAGE sample buffer for 2 min at 70°C and the proteins were resolved on a 10% SDS-PAGE gel (Cutler and Garoff, 1986). For visualization of ^{35}S-labeled proteins we used fluorography with 1 M sodium salicylate (Chamberlain, 1979). Quantitation of the bands in the gels was done by cutting these out and counting the radioactivity as described earlier (Wahlberg et al., 1989).

Trypsin digestions. The appearance of the trypsin-resistant form of E1 during entry of SFV was assayed as described earlier (Helenius et al., 1985). At various times after starting the 37°C incubation virus cell samples were solubilized and treated with a final concentration of 200 μg/ml trypsin (Sigma Chemical Co.) for 10 min at 37°C. The reaction was stopped by adding a threefold excess of soybean trypsin inhibitor (Boehringer Mannheim Corp., Indianapolis, IN), and samples were used for immunoprecipitation. The trypsin-resistant fraction of E1 was recovered by immunoprecipitation with a mixture of the monoclonal antibodies anti-E1, anti-E1', and anti-E1" and subjected to SDS-PAGE analysis.

Fusion Assays

Fusion of SFV at the PM was done by using a variation of the procedure described in White et al. (1980). Confluent BHK cells grown on coverslips were washed with MEM, pH 7.4, and left on ice for 20 min in MEM. SFV (3 pfu/cell) was bound to the cells in 50 μl MEM, pH 6.5, containing 200 μM chloroquine (Sigma Chemical Co.) for 1 h on ice at 4°C. Unbound virus particles were removed by washing the cells twice with PBS while kept on ice. Each coverslip was then placed on a 50-μl drop of MEM without bicarbonate, supplemented with either 20 mM MOPS (pH 7.4), 20 mM MES (pH 6.0), or 20 mM Na-succinate (pH 5.5), and incubated for 1 min at 37°C. The low pH flash was stopped by adding an excess of pH 7.4 medium (0.2 ml) under each coverslip. These were then washed in warm pH 7.4 medium and transferred to dishes containing 3 ml MEM, pH 7.4, with 0.2% BSA and incubated at 37°C for 3 h. 200 μM chloroquine was present throughout all incubations described above. In the antibody inhibition experiments 1:10 dilutions of the ascites preparations of the antibodies anti-E1, anti-E1', anti-E1", anti-E2, and anti-TR were added to the different incubations, as described in the figure legends. BSA (1 mg/ml) was present during some of the antibody treatments. After the final 3-h incubation the cells were fixed in ice-cold methanol for 6 min (de Curtis and Simons, 1988) and prepared for indirect immunofluorescence staining of the infected cells, essentially as described previously (Lobigs and Garoff, 1990) using the anti-E2 monoclonal antibody in combination with sheep anti-mouse IgG fluorescein (FITC) (Biosys, Compiègne, France).

Cell–cell fusion was induced by treatment of infected cells with buffers of varying pH. BHK cells were infected with SFV (100 pfu/cell). 4 h after infection the cells were washed and the coverslips placed on a 50-μl drop of MEM titrated to pH 7.4, 6.0, or 5.5 with the buffers described above. Monoclonal antibodies were included as 1:10 dilutions of the ascites preparations or as a purified IgG fraction (350 μg/ml) and incubation was done for 1 min at 37°C. After washing with pH 7.4 medium the coverslips were incubated further for 2 h in complete BHK medium at 37°C, then fixed in ice-cold methanol and analyzed for the formation of polykaryons using phase-contrast.

Results

Rearrangement of the Membrane Protein Tertiary Structure

To study the entry of SFV into cells we used [^{35}S]methionine-labeled virus which was bound to BHK cells at 0°C for 1 h. The bound virus particles were then allowed to enter the cells by incubation at 37°C for various times. Virus particles that had not entered during the incubation at 37°C were re-
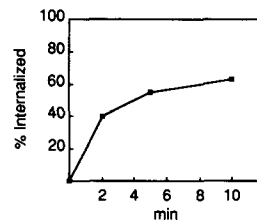

Figure 1. The kinetics of SFV uptake into BHK cells. [^{35}S]Methionine-labeled SFV (10 pfu/cell) was bound to BHK cells at 0°C. After incubation in 37°C for the indicated times the uninternalized virus particles were removed by treatment with 0.5 mg/ml proteinase K and the radioactivity in the cell lysates was measured. The uptake is expressed as the amount of proteinase K–resistant radioactivity in percent of the total amount of labeled material bound to the cells.

moved by proteinase K digestion before cell samples were solubilized and measured for ^{35}S radioactivity (Helenius et al., 1980). The kinetics of the SFV uptake are shown in Fig. 1. 40% of the bound particles had entered the cells after 2 min incubation at 37°C, and by 10 min 63% was internalized. This corresponds to ~1 pfu/cell.

Using this virus internalization protocol we first followed the fate of the viral spike protein oligomer E2E1 by performing sedimentation analyses in sucrose density gradients of cell samples that had been solubilized in an NP-40-containing buffer. These analyses enable the separation of different oligomeric forms of the spike subunits. Controls in Fig. 2 *a* show the separation of intact E2E1 heterodimers from solubilized virus (sedimentation coefficient of ~4.5 S) and the dissociated subunits that derive from virus that has been treated with an acidic (pH 5.5) buffer (Simons et al., 1973; Wahlberg et al., 1989). Analysis of the solubilized spike proteins in virus particles that had been bound to cells and kept at 0°C showed that these migrated as the heterodimers in the control sample (Fig. 2 *b*). However, this sedimentation profile underwent major changes upon 37°C incubation of the cell-bound virus. Already after a 2-min incubation a considerable fraction of the spike proteins was distributed partly into monomers and partly into forms that were larger than the dimeric forms (Fig. 2 *c*). This shift became even more pronounced in the cell sample that was incubated with virus for 5 or 10 min (Fig. 2, *d* and *e*, and quantitation in Fig. 6). After longer times of incubation (20 and 30 min) the monomeric and dimeric material disappeared, whereas the larger oligomers were still clearly visible as a distinct peak (Fig. 2, *f* and *g*). Fig. 2 *h* shows a control experiment that was done to exclude an artefactual reorganization of the SFV spike oligomer in the sample during solubilization. Here virus particles were mixed with a cell solubilizate in vitro and then analyzed in a sucrose gradient. The result shows the presence of dimeric material only. Fig. 3 shows analyses by SDS-PAGE of the pooled fractions corresponding to each peak region of the gradient (Fig. 2 *c*) where the spike subunits were found (large oligomer, dimer, monomer regions). These indicate that the dimer region contained both E1 and E2 subunits (lane *4*), whereas the monomer region contained mainly E2 (lane *5*) and the large oligomer contained only E1 (lane *3*). The trace amounts of faster migrating material also found in the monomer region represented E1 protein fragments corresponding to the lumenal domain of this protein. It reacted with the E1 antibody and could not be precipitated with TX-114 (data not shown). These sedimentation analyses suggest that the E2E1 heterodimer in the virus particle is rearranged during virus uptake. The original E2E1 interaction is dis-
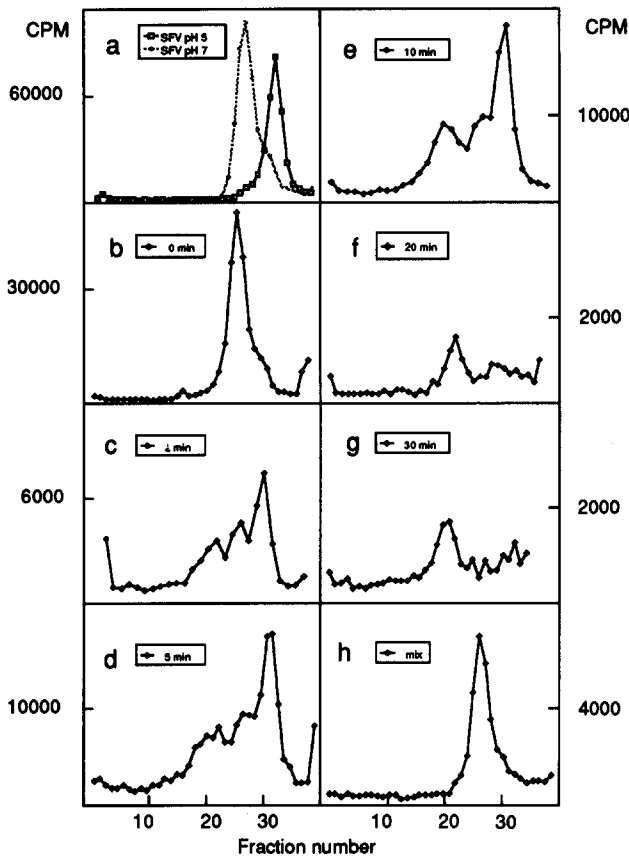
Figure 2. Sedimentation profiles of solubilized, [³⁵S]methionine-labeled membrane proteins from virus that had been bound to (*b*) or bound and internalized into BHK cells (*c–g*). The samples were run in a 5–20% (wt/wt) sucrose gradient for 24 h at 39,000 rpm and at 4°C in a SW40 rotor. Top fraction to the right. Control sample analyses in *a* represent unbound virus particles that were treated with buffers of neutral or acid pH before solubilization. Control sample in *h* shows free virus particles that were mixed with a solubilized cell sample before sedimentation.
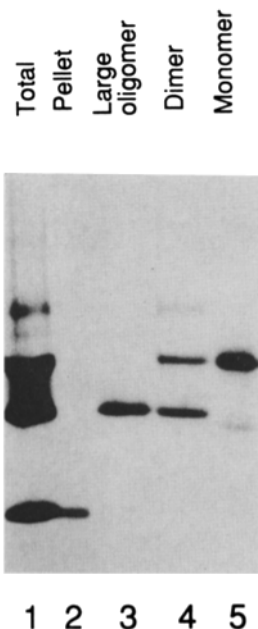
rupted and E1 forms new oligomers, whereas E2 stays in a monomeric form.

This was further corroborated by coimmunoprecipitation of E2 and E1 using a monoclonal anti-E1 antibody (UM 8.139). We used this assay earlier to demonstrate the destabilizing effect of mildly acidic pH on the E2E1 interaction in virus particles (Wahlberg et al., 1989). As shown in Fig. 4, the anti-E1 antibody coprecipitated almost quantitatively the E2 subunit from solubilized virus particles (lanes *1* and *2*) as well as from solubilized cells that had viruses bound to the cell surface (lanes *5* and *6*). However, after a 2-min incubation of cells and virus at 37°C only a subfraction (24%) of the E2 subunits could be coprecipitated with the anti-E1 antibody (lane *8*) and after 10 min even less (8%) could be coprecipitated (lane *12*). The quantitation of the fraction of heterodimers found in this experiment is shown in Fig. 6.

The E1 Subunit Changes Its Antigenicity during Internalization

The immunoprecipitation analyses described above also showed that the reactivity of the E1 subunit for the monoclonal antibody anti-E1 decreased with prolonged incubation times of virus and cells. In Fig. 5 we show that concomitantly with this reduction in reactivity the E1 protein exposed a new epitope for another monoclonal anti-E1 antibody called anti-E1″ (UM 8.64). After 10 min at 37°C almost 80% of the E1 proteins of internalized virus had obtained this new conformation (quantitation is shown in Fig. 6). This anti-E1″ antibody didn't significantly react with mature (Fig. 5, lane *2*) or newly synthesized (not shown) E1 proteins, nor with E1 from virus treated with low pH buffer at 4°C (lane *4*). It clearly differs from the anti-E1′ antibody (UM 8.47) de-

Figure 3. Protein analyses by SDS-PAGE of the peak regions from the gradient in Fig. 2 *c*. "Large oligomer" represents pooled fractions 19–22, "dimer" represents fractions 24–27, and "monomer" represents fractions 29–32. Sample analyzed in lane *1* represents 10% of the original sample used for the sedimentation analysis. Sample analyzed in lane *2* represents 10% of the pellet resulting from the centrifugation. The pooled peak fractions in lanes *3–5* represent 16% of respective pools.
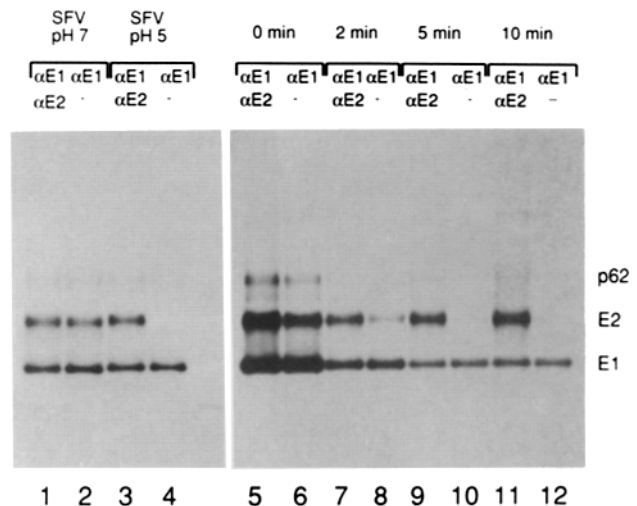

Figure 4. Immunoprecipitation analyses of the E2E1 association during SFV uptake. [³⁵S]Methionine-labeled SFV was bound to BHK cells for 1 h at 0°C (0 min) and incubated at 37°C for the indicated times (2, 5, or 10 min). Cell and virus samples were then treated with 0.5 mg/ml proteinase K at 0°C. After this the cells were solubilized and immunoprecipitated with anti-E1 and anti-E2 together (αE1/αE2) or with anti-E1 alone (αE1) and analyzed on a 10% SDS-PAGE gel which was processed for fluorography. Immunoprecipitation analyses shown in lanes *1–4* represent controls and correspond to free virus particles treated with buffers of pH 7 and 5.
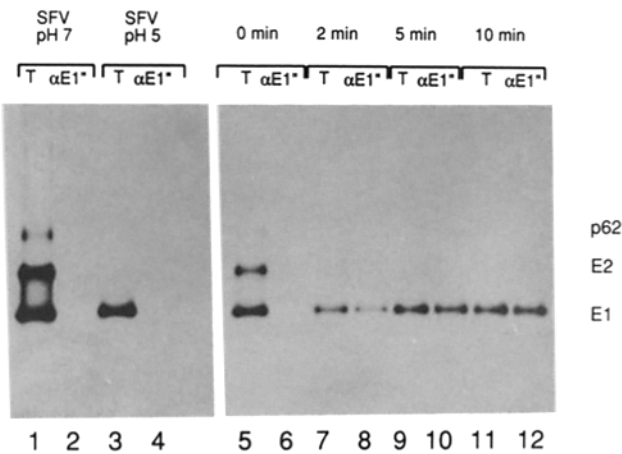
Figure 5. The appearance of a new epitope on E1 during virus uptake. Cell samples with virus which had been allowed to be internalized for different times (0, 2, 5, or 10 min) were solubilized and used for immunoprecipitation with a mixture of the monoclonal antibodies anti-E1 and anti-E1″ to follow the total (*T*) E1 protein or with the anti-E1″ alone (*αE1*″) to follow only E1 with the anti-E1″ epitope. Immunoprecipitates were dissolved in SDS sample buffer and run on a 10% SDS-PAGE gel. The control samples (analyzed in lanes *1–4*) have been treated as described in the legend to Fig. 4.

scribed previously, which reacts specifically with E1 in virus particles after treatment with a low ph buffer (Wahlberg et al., 1989) but not with E1 in virus that has entered cells (not shown).

The Exposure of the Anti-E1″ Epitope Correlates with E1 Becoming Resistant Toward Trypsin and with the Formation of Its Higher Oligomeric Form

The E1 subunit has earlier been shown to become trypsin resistant both in virus particles that enter cells and in virions that have been treated with a low pH buffer (Helenius et al., 1985; Kielian and Helenius, 1985). To see how the generation of the trypsin-resistant form of E1 correlated with the generation of the anti-E1″ epitope, we performed E1 immunoprecipitation reactions with cell samples that had been in-
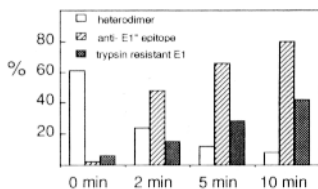

Figure 6. Quantitation of the fraction of viral membrane proteins that remain as heterodimers (□), and the amount of E1 protein that carries the Anti-E1″ epitope (▨), and is trypsin resistant (▦) in virus particles that have been allowed to enter into BHK cells for different times. The quantitation was done by measuring the radioactivity in the bands cut out from SDS-PAGE gels corresponding to autoradiographs as shown in Figs. 4, 5, and 7. All values represent the mean of three experiments. The heterodimer fraction was calculated as the amount of E2 protein that was precipitable with the anti-E1 antibody. The anti-E1″ epitope-carrying fraction corresponds to the amount of E1 protein that was reactive toward the anti-E1″ antibody (percentage of total E1 in the sample that was precipitated with a mixture of monoclonal antibodies against E1). The trypsin-resistant fraction represents the amount of E1 protein that was precipitable with the mixture of monoclonal antibodies against E1 (percentage of total E1 in the sample).

cubated with virus for different times, solubilized, and then treated with trypsin, using the anti-E1″ antibody alone as well as a mixture of anti-E1 antibodies reacting with all forms of E1. The quantitation in Fig. 6 shows that up to 45% of E1 of the cell-bound virus became resistant with increasing times of incubation. Fig. 7 *a* shows that the material precipitated with the anti-E1″ antibody is also trypsin resistant. Thus, the trypsin-resistant E1 form that appears during entry seems to correspond to the E1 that presents the anti-E1″ epitope. To see how the trypsin-resistant and anti-E1″ antibody binding form of E1 was migrating in the sucrose density gradients, sedimentation analyses were performed and the material in the peak fractions of a separation as shown in Fig. 2 *c* was digested with trypsin and analyzed by immunoprecipitation. The results shown in Fig. 7 *b* demonstrate that only the E1 of the higher oligomers corresponds to the trypsin-resistant and anti-E1″ antibody binding form.

The Changes in the Quaternary and Tertiary Structure of SFV Spike Protein Are Induced by Low pH in Endosomes

To assess the importance of the acidic milieu of the endosomes for the observed alteration in SFV membrane protein structure, we analyzed the effect of monensin treatment of cells on these changes. Monensin treatment is known to raise the pH in intracellular organelles (Marsh et al., 1982). For this reason cells with bound virus were incubated at 37°C in the presence of 25 μM monensin before solubilization. Viral proteins were then analyzed for the E2E1 heterodimer association using the coprecipitation assay with the anti-E1 antibody for reactivity toward the anti-E1″ antibody and for trypsin resistance. Fig. 8 *a* shows that the presence of monensin caused E2 to coprecipitate with E1 and most of the E1 to stay trypsin-sensitive and unreactive to anti-E1″. Thus we conclude that the observed tertiary and quaternary changes in E1 structure require the low pH which the virus encounters when entering the endosomes of the cell. This was further confirmed by indirect immunofluorescence analyses of cells incubated with SFV (Fig. 8 *b*). The anti-E1″ antibody gave a punctal staining pattern which probably represented the endosomal structures - (*left*). This staining was completely abolished when 25 μM monensin was present during the incubation (*right*). The control anti-E1 antibody showed no difference in reactivity towards the E1 subunits of internalized virus particles in the presence or absence of monensin (not shown).

The Rearranged Form of E1 Is Involved in the Low pH–induced Fusion and Penetration Processes

If the structural alterations in the SFV spike, as described above, are important for virus entry and hence membrane fusion, then the binding of the anti-E1″ antibody to the new form of E1 might be expected to interfere with these processes. To test this we first made use of a previously described experimental protocol which allowed virus penetration to occur solely at the cell surface (White et al., 1980). Virus particles were bound to cells and penetration was induced directly at the PM by a 1-min incubation at 37°C in a pH 7.4, 6, or 5.5 buffer. Concomitantly, the activation of fusion in the endosomes was inhibited by the presence of 200 μM chloroquine. The efficiency of virus penetration was es-
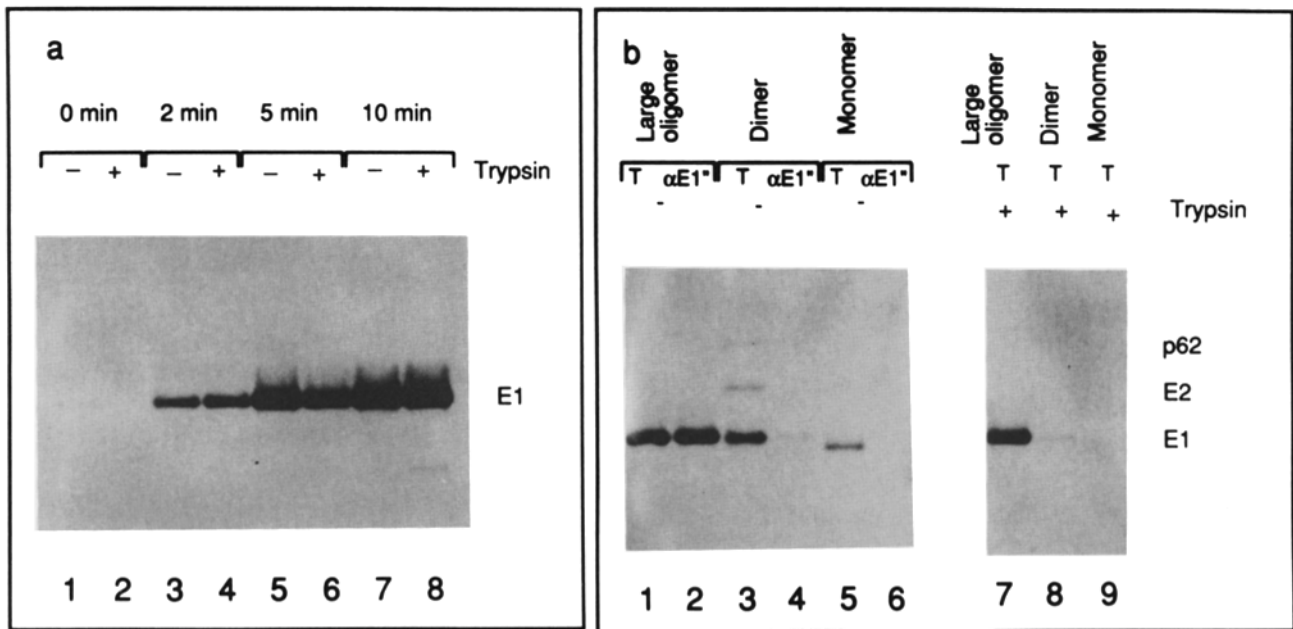
Figure 7. (*a*) Analysis of trypsin-resistant E1 in SFV after uptake. [³⁵S]Methionine-labeled SFV was allowed to be internalized for various times 0–10 min) into BHK cells. The samples were then solubilized and treated with trypsin and the resistant fraction was recovered by immunoprecipitation using the monoclonal antibody anti-E1″. Samples analyzed in lanes *1, 3, 5,* and *7* represent controls that have not been trypsin treated. The immunoprecipitates were analyzed on 10% SDS-PAGE. (*b*) Correlation of reactivity toward the anti-E1″ monoclonal antibody and trypsin resistance with the SFV spike protein material found in the peak regions (fractions defined in Fig. 3) of the sedimentation analyses shown in Fig. 2 *c.* Portions of pooled samples were reacted either with a mixture of anti-E1, anti-E1′, and anti-E1″ (*T*) or with anti-E1″ alone (lanes *1–6*), or treated with trypsin followed by precipitation with a mixture of the monoclonal antibodies against E1 (lanes *7–9*).

timated after 3 h of further incubation at 37°C by screening for infected cells using indirect immunofluorescence staining of newly synthesized spike proteins. Under conditions (3 pfu/cell and pH 5.5 flash) in which virtually all of the cells could be infected via the PM route (Fig. 9 *i*), we could demonstrate a significant inhibition when the anti-E1″ monoclonal antibody was present during the flash (Fig. 9 *j* and Table I). This inhibitory effect was also evident after a wash with a pH 6.0 buffer (Fig. 9 *f*). However, as pH 6.0 is close to the pH threshold for SFV fusion, the overall efficiency of SFV entry via the pM was lower. Without antibody the efficiency was ~50% of the efficiency of infection after a pH 5.5 treatment (Fig. 9, *e* and *i*). At both pH conditions the antibody had to be present during the low pH treatment in order to cause inhibition. No inhibition of infection was observed if the anti-E1″ antibody was present during binding of the virus particles to the PM and then removed before the low pH flash (Fig. 9 *b*). In contrast, the control monoclonal antibodies anti-E1 and anti-E2 (neutralizing antibodies; UM 8.139 and UM 5.1; Boere et al., 1984) inhibited infection when present before as well as during the low pH treatment (Fig. 9, *c, g, k*; Table I). Additionally, the anti-TR antibody did not decrease the number of infected cells if present during any of the incubations (Fig. 9, *d, h, l*; Table I). No inhibition was observed for either a rabbit anti–mouse IgG preparation or a monoclonal antibody against the VSV G protein (not shown).

To relate the inhibitory effect of the anti-E1″ antibody on SFV infectivity more directly to the actual fusion process, we studied its effect on the SFV-mediated cell–cell fusion

event (White et al., 1981). A brief wash of SFV-infected cells with a buffer of pH 6 or 5.5 induced massive polykaryon formation (Table I; Fig. 9 *m*). The presence of the anti-E″ or the anti-E1 antibody during the low pH treatment prevented the formation of polykaryons (Fig. 9 *n* and *o*), whereas the anti-TR, did not inhibit the cell–cell fusion (Fig. 9 *p* and Table I). Inhibition was observed both by the anti-E1″ ascites preparation and by a IgG preparation (350 µg/ml) purified from the anti-E1″ ascites preparation (not shown). We conclude that the anti-E1″ antibody can interfere with virus penetration at the stage of membrane fusion, suggesting that the alterations in the spike proteins that we have observed are important for virus entry.

Discussion

In this work we have shown that the heterodimeric E2E1 protein complex of the SFV spike undergoes a major change in both its quaternary and tertiary structure when entering the acidic milieu of the endosome in a cell. The heterodimeric interactions are interrupted and the E1 subunits are organized into new oligomers, whereas the E2 subunits appear to remain monomeric. Concomitant with the change in tertiary structure of the spike proteins, the E1 subunit undergoes conformational alterations as detected by the exposure of the anti-E1″ epitope and by the previously described increased resistance of E1 towards trypsin digestion (Helenius et al., 1985).

The exact composition of the new E1 oligomer is presently under investigation. In preliminary experiments we have
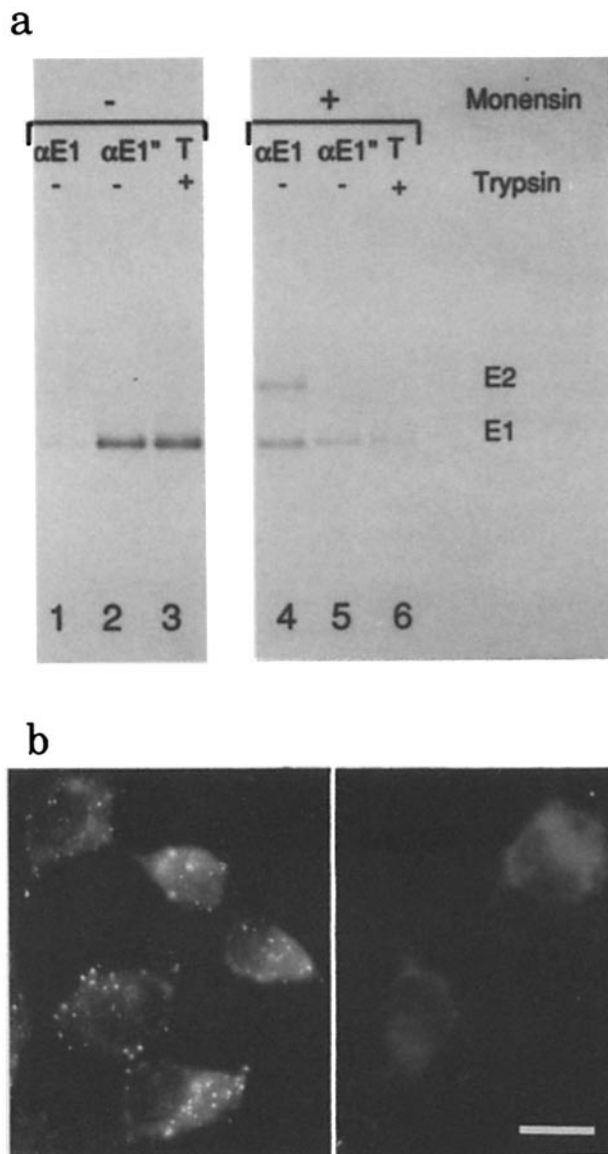
a

b

Figure 8. Effect of monensin on changes in the quaternary and tertiary structure of E1 during virus uptake. (a) SFV particles were allowed 10 min internalization into BHK cells in the absence (lanes 1–3) or presence (lanes 4–6) of 25 μM monensin. Cells were solubilized and the samples were used for immunoprecipitation with anti-E1 (lanes 1 and 4) or anti-E1″ (lanes 2 and 5), or digestion with trypsin before immunoprecipitation with a mixture of the monoclonal antibodies against E1 (lanes 3 and 6). (b) Immunofluorescence staining of BHK cells into which SFV has been allowed to enter (1.5 h, 20°C) in the absence (left) or presence (right) of 25 μM monensin. The cells were stained using a combination of the anti-E1″ antibody and sheep anti–mouse-conjugated FITC. Bar represents 15 μm.

used cross-linking with glutaraldehyde and obtained some covalently linked oligomers of E1 as visualized by SDS-PAGE (Wahlberg, J., and H. Garoff, unpublished observations). We have also labeled host cells with [³⁵S]methionine before incubation with virus and analyzed whether any host proteins were coprecipitating with the E1 oligomers from cell lysates that had been fractionated by sedimentation in a sucrose gradient. No host proteins were detected as compared with a control cell sample lacking internalized virus. One possibility is that the E1 oligomer corresponds to an E1 homotrimer. Such a structure might be formed from the three heterodimers in a spike by the simultaneous disruption of the heterodimeric interactions and strengthening of intermolecular E1 interactions.
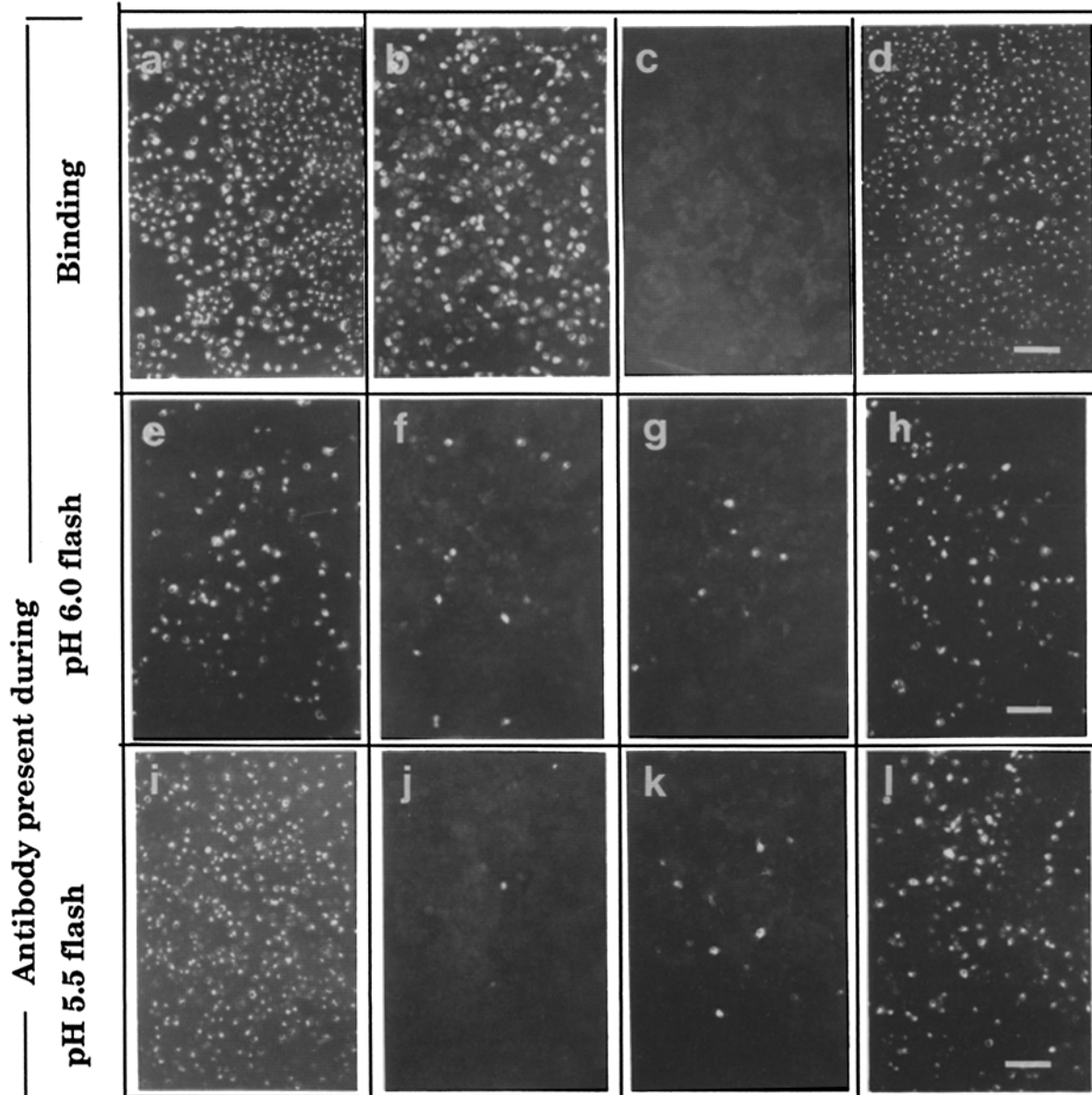
The fact that the monoclonal antibody anti-E1″ inhibited virus penetration in experiments where virus entry was manipulated to occur at the cell surface, as well as fusion of SFV-infected cells suggests that the new E1 oligomers represent important structures that are required for the membrane fusion step of the entry process. It is possible that the E1 oligomer represents a fusion-competent form which corresponds to the low pH–activated form of the influenza virus HA molecule observed at 0°C (Stegmann et al., 1990). In this fusion-competent HA the most prominent structural change appears to be the exposure of the hydrophobic peptide which constitutes the new NH₂-terminus after maturation cleavage of HA. This so-called fusion peptide has been suggested to play a critical role in initiating the membrane fusion process of orthomyxoviruses. In the case of the SFV E1 protein the hydrophobic peptide spanning amino acid residues 79–97 might have an analogous function. This finding is supported by the fact that this amino acid sequence is very conserved between alphaviruses and also by the recent characterization of a Sindbis virus variant which has a mutation close to the gene region encoding the putative fusion peptide of E1 (Garoff et al., 1980; Rice and Strauss, 1981; Dalgarno et al., 1983; Boggs et al., 1989). This mutation lowers the pH for fusion in in vitro assays from the normal value of 6.0 to 5.5. However, so far we have no information about whether this hydrophobic peptide is exposed in the new oligomeric form of the E1 protein.

It is interesting to note that the E1 oligomers carrying the anti-E1″ epitope are very efficiently expressed in virus that has been taken up into cells, but only to a very insignificant degree in free virus that has been treated with low pH buffers at 4°C. This is in clear contrast to the low pH–mediated disruption of the E2E1 interaction and the induction of E1 resistance to trypsin, both of which occur in vitro as well as in virus that has entered new cells. Apparently, acid treatment of SFV alone is not sufficient to induce all the conformational changes that take place in the viral spike proteins during entry. To accomplish these changes, simultaneous interaction with host membranes may be required. This might be related to the finding that the fusion process requires the presence of cholesterol or a 3β-hydroxysterol in the target membrane (White and Helenius, 1980; Kielian and Helenius, 1984). Recently, Flynn and co-workers have described several epitopes on the membrane proteins of Sindbis virus which react transiently with corresponding monoclonal antibodies. The exposure of this transient epitope also required prior binding of the virus particles to host membranes as well as incubation at 37°C (Flynn et al., 1990).

So far we and others have characterized several defined changes in the quaternary and tertiary structure of the alphavirus membrane proteins which appear to be important for virus penetration into new host cells. These include the p62 cleavage at the stage of virus maturation (Lobigs and Garoff, 1990; Lobigs et al., 1990), the exposure of unique

PLASMA MEMBRANE ENTRY

No antibody   αE1"   αE1   αTR

Binding

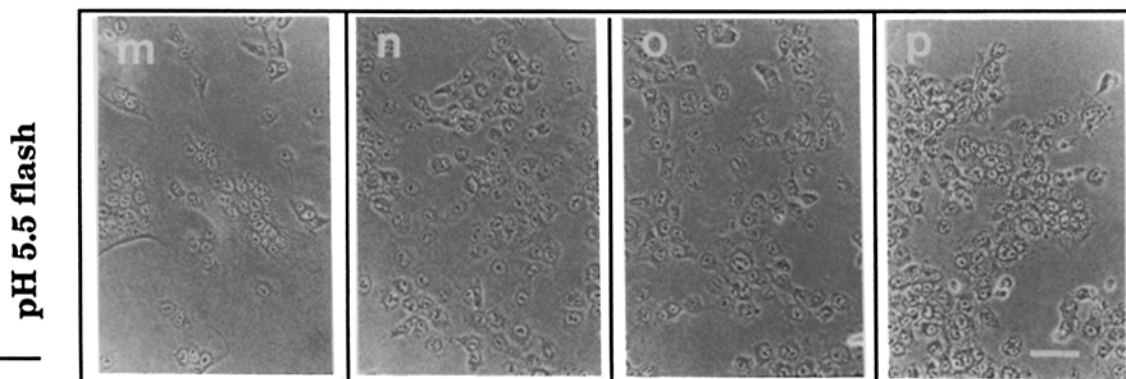Antibody present during

pH 6.0 flash

pH 5.5 flash

CELL-CELL FUSION

pH 5.5 flash

Table I. *Inhibition of Fusion Using Monoclonal Antibodies*

Antibody	% Infected cells*			Polykaryon formation‡		
	pH 7	pH 6	pH 5.5§	pH 7	pH 6	pH 5.5
—	0	50	100	—	+	+
αE1″	0	<5	<5	—	—	—
αE1	0	<5	<5	—	—	—
αE1′	0	ND	30	—	ND	—
αE2	0	<5	<5	—	—	—
αTR	0	50	80	—	+	+

* BHK cells were infected with SFV (3 pfu/cell) for 1 h at 0°C and incubated at the indicated pH values for 1 min at 37°C in the presence of the respective monoclonal antibodies and 200 μM chloroquine as described in Materials and Methods. The values represent the percentage of cells that had been infected through the PM.
‡ BHK cells were infected with SFV (100 pfu/cell) and analyzed for polykaryon formation 4 h after infection by incubating for 1 min at 37°C at the indicated pH values in the presence of the respective antibodies as described in Materials and Methods. −, no polykaryons; +, polykaryons formed; ND, not determined.
§ Experiments were done with or without 1 mg/ml BSA in the low pH incubation mixture.

epitopes transiently on virus particles that have been bound to the cell surface (Flynn et al., 1990), and the low pH–induced changes observed during virus uptake, such as disruption of the E2E1 interaction, reorganization of the E1 protein, exposure of the epitope for the anti-E1″ monoclonal antibody (this report), and the increased resistance of E1 toward trypsin digestion (Helenius et al., 1985). These results are in agreement with a model for the activation of the E1 fusion protein in which the E1 subunit disrupts its original heterodimeric association and builds up a new oligomer, presumably a homooligomer, consisting of structurally altered E1 subunits. Thus the E1 fusion protein uses first heterodimerization with p62 as a means to become incorporated into the viral envelope and then dissociation from the cleaved p62 (E2) and reorganization, possibly with itself, as a way to become activated. This process of fusion protein maturation and activation differs considerably from that among most other enveloped viruses. For instance, in the cases of orthomyxo-, paramyxo-, retro-, and coronaviruses the proteins carrying entry functions already form homooligomeric structures soon after being synthesized within the ER of the infected cell. These preformed oligomers are then incorporated into the viral envelope and activated for fusion by either limited proteolysis or acidic pH or both (reviewed in Stegmann et al., 1989; White, 1990).

The membrane protein oligomerization-mediated control mechanism of entry functions that we propose for alphaviruses might also be used by some other viruses, such as flaviviruses, bunyaviruses, and Rubella virus. In these cases the virus directs the synthesis of a heterodimeric membrane protein oligomer which apparently carries both assembly and entry functions (Wengler and Wengler, 1989; Persson and Pettersson, 1991; Baron, M. D., and K. Forsell, manuscript submitted for publication). Consequently, the control mechanisms of the SFV entry process that we have proposed are possibly not unique for alphaviruses, but are applicable to a whole group of different viruses.

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Figure 9. (a–l) Inhibition of virus penetration at the PM with the anti-E1″ antibody. Virus was bound to cells on coverslips at 0°C for 1 h and then treated with low pH buffers (pH 6.0 or 5.5) for 1 min to allow virus penetration at the PM. Chloroquine was present in the binding media and throughout all incubations to prevent virus penetration through the endosomal membrane. Infected cells, as a result of successful penetration at the PM were detected by indirect immunofluorescence staining for viral proteins. Monoclonal antibodies (αE1″, αE1, αTR) were present during the binding step in the experiments shown in the top panels (a–d) and then removed before the low pH treatment. In the middle panels (e–l) the indicated antibodies were only present during the low pH treatment (pH 6.0, e–h; pH 5.5, i–l). After further incubation at 37°C for 3 h, infected cells were stained with anti-E1 using FITC-conjugated sheep anti-mouse as second antibody. Bars represent 25 μm. (m–p) Inhibition of polykaryon formation with the anti-E1″ antibody. Infected cells were treated 5 h after infection for 1 min at 37°C with a buffer of pH 5.5 to induce cell–cell fusion. In (n–p) the indicated monoclonal antibodies were present during low pH treatment and then removed. Polykaryons were observed with phase-contrast after a further incubation for 2 h at 37°C. Bar represents 50 μm.

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