Membrane Fusion Process of Semliki Forest Virus II: Cleavage-dependent Reorganization of the Spike Protein Complex Controls Virus Entry

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Abstract. The envelope of the Semliki Forest virus (SFV) contains two transmembrane proteins, E2 and E1, in a heterodimeric complex. The E2 subunit is initially synthesized as a precursor protein p62, which is proteolytically processed to the mature E2 form before virus budding at the plasma membrane. The p62 (E2) protein mediates binding of the heterodimer to the nucleocapsid during virus budding, whereas E1 carries the entry functions of the virus, that is, cell binding and low pH-mediated membrane fusion activity. We have investigated the significance of the cleavage event for the maturation and entry of the virus. To express SFV with an uncleaved p62 phenotype, BHK-21 cells were transfected by electroporation with infectious viral RNA transcribed from a full-length SFV cDNA clone in which the p62 cleavage site had been changed. The uncleaved p62E1 heterodimer was found to be used for the formation of virus particles with an efficiency comparable to the wild type E2E1 form. However, in contrast to the wild type virus, the mutant virus was virtually noninfectious. Noninfectivity resulted from impaired uptake into cells, as well as from the inability of the virus to promote membrane fusion in the mildly acidic conditions of the endosome. This inability could be reversed by mild trypsin treatment, which converted the viral p62E1 form into the mature E2E1 form, or by treating the virus with a pH 4.5 wash, which in contrast to the more mild pH conditions of endosomes, effectively disrupted the p62E1 subunit association. We conclude that the p62 cleavage is not needed for virus budding, but regulates entry functions of the E1 subunit by controlling the heterodimer stability in acidic conditions.

The spreading of enveloped animal viruses between cells is dependent on their ability to mature by budding at the membranes of the infected cells and to enter new cells by the process of membrane fusion. To perform these functions these viruses use different membrane proteins. Proteins located at the internal side of the membrane, like the M protein of orthomyxo-, paramyxo-, and rhabdoviruses, as well as the N-terminal part of the gag precursor protein of retroviruses, seem to play a major role during virus assembly, whereas other proteins forming spike-like surface projections carry cell-binding and membrane fusion functions. A control mechanism ensures that assembly and entry functions do not interfere with each other. Typical for this regulation is that the functions required for virus entry are not activated before the final stage of maturation has been reached and the virus is released from the cell (Simons and Garoff, 1980; Dubois-Dalcq et al., 1984; Stegmann et al., 1989; Kielian and Jungerwirth, 1990; White, 1990; Pettersson, 1991). For most enveloped viruses a host cell–mediated limited proteolysis of the viral fusion protein initiates this activation process. In some cases the cleavage generates a fusion protein that is activated upon receptor binding, thereby resulting in fusion directly with the target cell surface (e.g., HIV-1). In other cases (e.g., influenza virus), further activation of the fusion protein is required in the acidic surroundings of the target cell endosome after endocytosis, and the virus fuses with the endosomal membrane (Wellink and van Kammen, 1988; Hesekstra and Kok, 1989; Marsh and Helenius, 1989; Stegmann et al., 1989; Kielian and Jungerwirth, 1990).

In contrast to the strategies described above, a dissimilar control of entry function activation is used by the alphaviruses. This group of viruses specifies the synthesis of a heterodimeric membrane protein unit, which carries both assembly and entry functions. Virus maturation involves a proteolytic processing of the spike heterodimer, but the cleaved subunit is not the assigned fusion protein. Therefore it is of interest to study how the expression of the budding and entry functions of the alphavirus spike are regulated, and especially to establish which functions are served by the proteolytic processing. To answer these questions we have investigated the assembly and entry mechanisms of the Semliki Forest Virus (SFV). The envelope proteins of SFV are made as a p62E1 heterodimer, which is converted into the

1. Abbreviations used in this paper: EMEM, Earles minimum essential medium; mL, mutant L; pfu, plaque-forming units; PM, plasma membrane; SFV, Semliki Forest virus.
E2EI form late in virus maturation (Ziemiecki et al., 1980; de Curtis and Simons, 1988; Wahlberg et al., 1989). Several experimental results suggest that the p62 (E2) subunit plays the major role in virus budding, whereas the E1 represents the entry protein of this virus (Garoff and Simons, 1974; Väänänen, 1981; Kielian and Helenius, 1985; Omar and Koble, 1988; Vaux et al., 1988; Boggs et al., 1989; Metsäkä and Garoff, 1990). On the basis of our earlier studies we have suggested a membrane protein oligomerization-mediated control mechanism for the activation of the entry functions of the SFV E1 protein (Wahlberg et al., 1989; Lobigs et al., 1990a, b). Central to this model is the requirement of E1 to disrupt its heterodimeric interaction before it can be activated. As this is easily achieved by mild acid treatment of the mature E2EI form, but not of the precursor p62EI form, it follows that activation cannot occur before the virus enters the endosome of a potential host. Several experimental results support this model. First, the higher tolerance to low pH treatment of the precursor form compared with the mature heterodimer has been verified using isolated complexes (Wahlberg et al., 1989). Second, the mature heterodimers of the entering virus particles have been followed and shown to undergo rapid dissociation and reformation of a new E1 oligomeric structure (see accompanying paper [Wahlberg and Garoff, 1992]). Third, a cleavage-deficient variant of the heterodimer has been obtained through in vitro mutagenesis of subgenomic SFV cDNA, and shown to be inactive in promoting cell–cell fusion at the normal pH optimum of 5.5 when expressed on the surface of tissue culture cells. However, when using pH 4.5 treatment, which also causes dissociation of the precursor form compared with the mature heterodimer, extensive polykaryon formation is observed (Lobigs and Garoff, 1990; Lobigs et al., 1990a).

In this work we present novel genetic evidence in strong support of our model of the SFV entry function activation. We have introduced the mutation (Arg<sub>56</sub>→Leu), with cleavage-deficient p62 phenotype, into a complete cDNA copy of the SFV genome, and used this for the production of the corresponding mutant RNA to transfect cells. We show that virus particles with uncleaved p62 protein are formed normally. However, the particles are noninfectious. This is due partly to inefficient uptake and partly to the inability of the p62EI heterodimer to undergo changes in tertiary and quaternary structure required for the activation of the fusion function of the virus. The block in penetration of the virus can be overcome by a pH 4.5 wash of cell-bound virus. This pH treatment is also shown to cause subunit dissociation of the viral p62EI heterodimer and induction of p62EI-mediated cell–cell fusion.

Materials and Methods

DNA Constructions

Generation of the p62 cleavage site mutant L (mL) has been described (Lobigs and Garoff, 1990). Construction of the full-length cDNA clone of SFV (pSP6-SFV4) is described elsewhere (Liljestrom et al., 1991). To construct the pSP6-SFV4/ml clone, a fragment carrying the mL sequence was excised from the vaccinia virus recombinant plasmid p7.5KSFV (Lobigs and Garoff, 1990) and substituted for the corresponding wild-type fragment of the pSP6-SFV4.

Cells, Viruses, and Antibodies

BHK-21 cells were grown in BHK medium (Glasgow minimum essential medium supplemented with 10% tryptose phosphate broth, 5% FCS, 2 mM glutamine, and 20 mM Hepes (Gibco Laboratories, Life Technologies Ltd., Paisley, Scotland) in 75-cm² bottles (Costar Corp., Cambridge, MA) in a 37°C, 5% CO₂ incubator. Cells used for electroporation were tryptophan-starved, suspended in BHK medium, and stored on ice.

Preparation of radioactively labeled wild type (SFV4) and mutant (mL) viruses was as follows: In vitro transcribed RNA was mixed with 2 x 10⁷ cells in 2 ml PBS and electroporation was carried out (Liljestrom et al., 1991). After electroporation the cells were diluted in 15 ml of BHK medium, placed in a 75-cm² bottle and incubated at 37°C for 6 h before labeling. At this point cells were washed once with PBS (with Ca²⁺ and Mg²⁺) and the medium was replaced with 9 ml of methionine-free MEM (Gibco) supplemented with 1% FCS, 2 mM glutamine, and 20 mM Hepes, containing [³⁵S]methionine (1,000 Ci/ml; Amersham International, Amersham, UK) at 100 μCi/ml, and the labeling was continued for 15 h. The virus was purified as previously described (Wahlberg et al., 1989).

The monoclonal antibodies UM 8.139 (anti-E1), UM 5.1 (anti-E2), and UM 5.1 (anti-E2) and their properties have been described elsewhere (Boere et al., 1984). The UM 8.139 (anti-E1) antibody is useful to study the heterodimeric association. It coprecipitates the E2 (p62) subunit of solubilized cells and viruses (Wahlberg et al., 1989). The anti-E" antibody reacts with the large oligomeric structure of E1, found in infected cells shortly after virus internalization, and involved in the fusion reaction (see Wahlberg and Garoff, 1992). The monoclonal antibody OKT-9 against the human transferrin receptor (anti-TR) was used as mouse ascites fluid and was provided by T. Ebel in the laboratory.

Virus Maturation Analysis

For pulse-chase experiments, cells transfected by electroporation (5 x 10⁸ cells) were resuspended in 10 ml of BHK medium, split into 35-mm dishes (2 ml/dish), and incubated at 37°C. At 7 h after electroporation cells were washed once with 2 ml of PBS (with Ca²⁺, Mg²⁺), which was replaced with 2 ml of labeling medium (methionine-free MEM without FCS). After 30 min incubation the medium was replaced with 0.5 ml of labeling medium containing 100 μCi/ml [³⁵S]methionine. Cells were pulsed for 15 min, washed once with PBS, and chased in 1.5 ml complete Earles minimal essential medium (EMEM), supplemented with 10-fold excess of cold methionine, for the times indicated. After the chase, growth medium was collected. The cells were rinsed with an additional 0.5 ml of PBS, which was combined with the chase medium. For preparation of cell lysates the monolayers were solubilized in 200 μl NP-40 buffer (1% NP-40, 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM EDTA, 1 mM PMSF). Viral proteins were immunoprecipitated from the lysates with a combination of the anti-E1 and anti-E2 monoclonals. Complete virus particles were immunoprecipitated from the culture medium with anti-E2 monoclonal antibody. The immunocomplexes were brought down using Pansorb cells (Calbiochem Corp., La Jolla, CA) in PBS (10% wt/vol). Immunoprecipitates were washed (intact virus precipitations were washed in the absence of detergent) and prepared for gel electrophoresis (10% SDS-PAGE) in nonreducing conditions, gels were prepared for fluorography, and the radioactivity was quantitated essentially as described before, except that 1 M sodium salicylate was used as an enhancer (Chamberlain, 1979; Wahlberg et al., 1989).

Determination of Heterodimer Subunit Dissociation in Low pH

For pH treatment, 2.5 μl of purified virus stock (1 x 10⁹ plaque-forming units [pfu]) was mixed with 50 μl of the different pH buffers (20 mM Na-succinate, 150 mM NaCl, 1 mM EDTA for pH 4.5, 5.0, and 5.5; 20 mM MES for pH 6.0 and 6.5; and 20 mM MOPS for pH 7.4) and incubated on ice for 10 min. The viral proteins were then solubilized in the same buffer, with 1% NP-40. This sample was neutralized by adding 500 μl of lysis buffer (see above), and immunoprecipitation of the virus proteins was carried out using the anti-E1 antibody. The protein samples were analyzed in SDS-PAGE as above.

Digestion of Virus with Exogenous Trypsin

Trypsin treatment of the mL virus was carried out either in MEM or in the pH 7.4 buffer. 1 x 10⁶ pfu/ml of virus was incubated with trypsin (Boehringer Mannheim Corp., Indianapolis, IN), 15 μg/ml for 30 min on ice. After protease digestion, soybean trypsin inhibitor (Boehringer Mannheim Corp.) was added to 100 μg/ml final concentration for 10 min on ice. This incubation mixture was used directly for experiments. For the partial trypsin digestion of the mL virus, 1.5 μg/ml of trypsin was used. At different
Figure 1. Maturation of the mL and SFV4 viruses. BHK-21 cells were transfected with the full-length RNA transcripts derived from cDNA clones of the pSP6-SFV4 and pSP6-SFV4/mL. Cells were pulse labeled with [35S]methionine and chased for the times indicated (1-5 h). Cell lysates were analyzed for labeled viral membrane proteins by immunoprecipitation with anti-E1 and anti-E2 monoclonal antibodies. Culture medium was analyzed for the production of virus progeny by immunoprecipitation with anti-E2 antibody, and samples were analyzed by SDS-PAGE gels. For quantitation, bands corresponding to the membrane proteins p62, E2, and E1 were cut out and the radioactivity was measured by liquid scintillation counting.

time points aliquots were drawn from the digestion and mixed with soybean trypsin inhibitor (300 μg/ml final concentration). A portion of the samples was analyzed by SDS-PAGE.

Fusion of the Virus with the Plasma Membrane

BHK-21 cells were grown on 18 x 18-mm coverslips in 35-mm dishes in BHK medium to ~70-80% confluence. The dishes were cooled on ice and the cells were washed once with 2 ml PBS (with Ca++ and Mg++) and once with 2 ml binding medium (EMEM; pH 6.5, 0.2% BSA). Virus was diluted in the binding medium and applied to cells in 0.5-ml volume. Virus was allowed to bind for 1.5 h at 0°C with frequent shaking. Unbound virus was removed and replaced with 2 ml of cold binding medium. To induce fusion of the virus with the plasma membrane (PM), coverslips with cell-bound virus were dipped into EMEM adjusted to pH 5.5 or 4.5 at 37°C for 30 s unless stated otherwise, and placed thereafter into warm complete EMEM, pH 7.4, supplemented with 0.2% BSA for a further 4 h. In most experiments chloroquine (Sigma Chemical Co., St. Louis, MO) at 200 μM concentration was present in all solutions to prevent virus entry via endocytosis.

Cell–Cell Fusion Assay

Cell–cell fusion was induced by treatment of mL-infected cells with buffers of varying pH and the ability of monoclonal antibodies to prevent this fusion was studied as described in the accompanying paper (Wahlberg and Garoff, 1992). BHK cells were infected with trypsin-treated mL virus (25 pfu/cell) and incubated 5 h before the low pH (pH 5.5 and 4.5) treatments. The monoclonal antibodies anti-E1’ and anti-TR were included in the low pH buffers as 1:10 dilutions of ascites preparations.

Immunofluorescence

To perform indirect immunofluorescence, infected cell monolayers on glass coverslips were rinsed twice with PBS with Ca++ and Mg++ and fixed in cold (−20°C) methanol for 6 min on ice. After fixation, the methanol was removed and the coverslip was washed three times with PBS. Unspecific antibody binding was blocked by incubation at room temperature with PBS containing 0.5% gelatin and 0.25% BSA. The blocking buffer was removed and replaced with the same buffer containing primary antibody. After 30 min at room temperature the reaction was stopped by washing three times with PBS. Binding of secondary antibody (FITC-conjugated sheep anti-mouse; BioSys, Compiègne, France) was done as for the primary antibody. After three washes with PBS and one rinse with water the coverslip was drained and mounted in Moviol 4-88 (Hoechst; Frankfurt am Main, Germany) containing 2.5% DABCO(1,4-diazobicyclo-[2.2.2]-octane).

Results

The Rate of the mL Virus Maturation Parallels That of the Wild Type

The cDNA fragment containing the p62 cleavage-deficient mutation (mL) was isolated from a vaccinia virus recombinant plasmid and inserted into the full-sized SFV cDNA clone pSP6-SFV4 (Lobigs and Garoff, 1990). To analyze the phenotype of this mutant, RNA was transcribed from the pSP6-SFV4/mL cDNA template and electroporated into BHK-21 cells. Wild type cDNA transcript was used as a control. Transfected cells were pulse labeled for 15 min and chased for 1-5 h. After each time point, cultures were analyzed for cell-associated viral membrane proteins and for mature particles by immunoprecipitation. Cell lysates were reacted with a mixture of anti-E1 and anti-E2 antibodies, and a corresponding volume of culture supernatant with anti-E2 monoclonal antibody. The labeled samples were analyzed in 10% SDS-PAGE under nonreducing conditions to separate the E2 and E1 proteins (Fig. 1).

The results showed that the p62 protein derived from the wild type cDNA (SFV4) was correctly processed to E2 (and E3, not visible on the gel) in the cells, showing >90% cleavage after 1 h of chase. The membrane proteins E2 and E1 were efficiently chased into new virus particles, which started to appear in the culture medium after 1 h of chase. The virions
Figure 3. Immunofluorescence analysis of cells infected with SFV4, mL+T, and mL virus infected via endosomes, or after direct fusion with the PM. Virus was bound at 0°C to the cells on glass coverslips, which were placed directly, or after a 30-s low pH (5.5 or 4.5) wash at 37°C, into neutral medium for 4 h at 37°C. Chloroquine was included in all the incubation media where indicated. Cells were then processed for immunofluorescence using anti-E2 monoclonal as the primary antibody. Bars represent 60 μm.

The mL Virus Is Noninfectious, but Can Be Activated by Trypsin Cleavage

To investigate the behavior of the mL virus in cell entry, we first tested purified mL virus by a standard plaque titration assay. No plaque formation was observed, indicating that a block in steps leading to productive infection existed (not shown). An obvious reason for the lack of infectivity of the mL virus was the uncleaved form of the p62E1 heterodimer. Since we had previously shown that mL forms of the p62E1 complex expressed at the cell surface can be cleaved and activated to induce cell–cell fusion if treated with exogenous trypsin, we tested whether mild trypsin treatment of the virus would restore infectivity (Lobigs and Garoff, 1990). When radioactively labeled mL virus was incubated in the
The mL Virus Shows Deficiency in Uptake into Cells

Possible reasons for the noninfectious phenotype of the mL virus could be inefficient uptake and an inability to penetrate into the cytoplasm through fusion with the endosomal membrane. To address first of these possibilities, we carried out binding and internalization analyses with the viruses. Labeled SFV4 and mL virus (25 pfu/cell) were bound to BHK-21 cells on glass coverslips at 0°C for 1.5 h using pH 6.5 medium. The coverslips were then placed into neutral medium for incubation at 37°C for 4 h, and the cells were processed for immunofluorescence staining with the anti-E2 monoclonal antibody (Fig. 3). At 2.5 pfu/cell, the SFV4 virus infected ~50% of the cells (top row, left), whereas <0.1% of infected cells were observed with the mL virus (bottom row, left; mL titer was measured as mL+T virus). As expected, the mL+T virus was capable of infection with about the same frequency as the wild type virus (middle row, left).

The Spike Heterodimer of the mL Virus Shows Impaired Ability to Convert into a Fusion Active Form

We have recently shown that the E2E1 oligomeric structure of the SFV particle is reorganized during endocytosis (see Wahlberg and Garoff, 1992). This reorganization is induced by low pH in the endosomes and involves the dissociation of the E2E1 heterodimer, as well as the formation of higher ordered oligomers of E1. These latter forms appear to be required for virus penetration, since a monoclonal antibody (anti-E1') specific for the new E1 oligomer inhibits this process. To follow possible rearrangements in the p62E1 structure of the mL virus, we used a similar internalization protocol at 20°C as described in the preceding section. Immunoprecipitation analyses carried out with the anti-E1 monoclonal showed that 82% of the E1 subunits from the internalized mL virus were still in complex with p62 after a 60-min incubation at 20°C. In comparison, almost all of the E2E1 heterodimers from internalized SFV4 control virus and the mL+T virus had dissociated (Fig. 5, middle). Analysis of the samples with the anti-E1' antibody revealed that only ~10% of the E1 subunits from the mL virus cell sample exposed this epitope. In contrast, ~80% of the E1 subunits in the SFV4 and mL+T virus cell samples reacted with this

Figure 4. The effect of pH on the binding of SFV4 and mL virus particles to BHK-21 cells. [3S]methionine-labeled virus particles (25 pfu/cell) were adsorbed to the cells at 0°C for 1 h at the indicated pH. Unbound particles were removed and the radioactivity in the cell lysates was measured by scintillation counting. The data represent the mean of at least three experiments and are given as the percentage bound of the total amount added to the cells.

Figure 5. Analysis of the membrane proteins of internalized SFV4, mL, and mL+T viruses. BHK-21 cells were incubated with [3S]methionine-labeled virus (25 pfu/cell) in a 20°C water bath for 60 min to allow efficient internalization. Uninternalized virus was removed by incubating cells on ice in the presence of protease K (0.5 mg/ml) for 45 min. Solubilized cell lysates were used for immunoprecipitation. Precipitates were analyzed on SDS-PAGE and bands were cut out for quantitation by scintillation counting. (Top) The total amount of E1 internalized during 60 min incubation at 20°C. Bars represent E1 protein precipitated with the mixture of monoclonal antibodies anti-E1, anti-E1', and anti-E1". (Middle) Amount of membrane protein heterodimers after internalization, given as percentage of p62 or E2 coprecipitating with the anti-E1 antibody. Amount at start of infection (dark bars) and after incubation for 60 min at 20°C (light bars). (Bottom) The exposure of the anti-E1' epitope after 60 min incubation at 20°C. Bars represent fraction of E1 subunits reacting with anti-E1' monoclonal antibody.
brane fusion activity. This is probably a direct consequence of the impaired ability of the mL spike to convert to the anti-E1' antibody (Fig. 5, bottom). These results were further confirmed by sucrose gradient velocity sedimentation analyses of viral membrane protein oligomers in the solubilized virus cell samples (not shown). Thus, the p62E1 heterodimer of mL virus was severely inhibited in its conversion into a fusion-competent form during uptake into cells.

**The mL Virus Is Unable to Induce Membrane Fusion at pH 5.5**

To test the capacity of the mL virus to penetrate into the cell cytoplasm by membrane fusion, we made use of a previously described assay in which SFV is induced to fuse directly with the PM by a brief low pH treatment (White et al., 1980). By using this assay it had been shown that the pH threshold for SFV fusion at the PM is 6.2, with optimum at pH 5.8. These pH conditions probably reflect those that normally elicit virus fusion within endosomes. To study whether mL virus could fuse with the PM at such conditions, cells with bound virus (2.5 pfu/cell) on coverslips were subjected to a 30-s, pH 5.5 wash at 37°C and subsequently placed into neutral medium for incubation at 37°C for 4 h. Acid-induced fusion inside endosomes was prevented by using chloroquine (200 μM) in all incubations (Helenius et al., 1982). The cultures were then analyzed for infected cells by immunofluorescence staining of viral proteins. The results shown in Fig. 3 clearly demonstrated that the mL virus, in contrast to the SFV4 virus, was unable to induce membrane fusion at the PM at pH 5.5. Thus, in addition to inefficient virus uptake, the mL virus also had a major deficiency in its membrane fusion activity. This is probably a direct consequence of the impaired ability of the mL spike to convert to the anti-E1" antibody reactive form described above.

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**The mL Virus Can Induce Membrane Fusion at a Lower pH, Which Also Causes p62E1 Dissociation**

In earlier studies we showed that the cell-associated p62E1 complexes were able to induce cell–cell fusion if incubated in conditions where the pH was low enough (pH 4.5) to cause disruption of the heterodimeric interactions. These conditions were ~1 pH unit lower compared with the ones required for the dissociation of, and optimal fusion by, the mature E2E1 complex. Therefore, it was of interest to study whether a similar correlation existed between the stability and the fusion function of the p62E1 complex in the mL virus membrane, with the possibility of virus penetration at the PM in more acidic pH.

To establish the pH threshold for the disruption of the p62E1 heterodimer interactions, we performed in vitro coimmunoprecipitation analyses. Labeled, purified mL and SFV4 viruses were treated with buffers of decreasing pH on ice. After 10 min of incubation samples were solubilized by adding NP-40 into the sample (% final concentration), and the viral proteins were then precipitated from neutralized samples using the anti-E1 monoclonal antibody. In the case of the SFV4 virus, stoichiometric amounts of the E2 protein coprecipitated with the E1 at pH 6.5 or higher (Fig. 6). At pH 6.0, most of the heterodimer was already dissociated, indicating that the pH threshold for this event lies between pH 6.5 and 6.0. At pH 5.5 and lower, the wild type spike heterodimer was completely dissociated. By contrast, the mL virus heterodimer was more resistant toward acid treatment since even at pH 5.5 no dissociation was apparent. At pH 4.5 all the p62E1 heterodimers had dissociated, suggesting that at least 1 pH unit more acidic conditions were needed for this event to take place.

When the fusion potential of the mL virus with the PM was analyzed using the pH 4.5 wash instead of the standard pH 5.5 wash, it became apparent that this treatment resulted in successful infection (Fig. 3, lower panel, right). We thus concluded that the pH 4.5 treatment, which facilitated the dissociation of the p62E1 heterodimer, also activated the membrane fusion function of the mL virus El subunit. This finding was confirmed by the observation that mL virus-infected cells could be induced to form polykaryons at pH 4.5 but not at pH 5.5, as in the case of the wild type (data not shown). The anti-E1" monoclonal antibody, which was shown to inhibit penetration and fusion of the wild type virus at pH 5.5 (see Wahlberg and Garoff, 1992), also inhibited the formation of polykaryons between mL-infected cells when present during the pH 4.5 flash (data not shown). This suggested that the new El structure with the anti-E1" epitope was formed after treatment of the cell-associated p62E1 complex with pH 4.5 buffers. The control antibody (anti-TR) did not inhibit polykaryon formation in these conditions (data not shown).

Since the mL virus was virtually noninfectious in physiological conditions, we wanted to test further whether the low pH wash had any effect on the infectivity of the mL virus during normal endocytic uptake. A similar experiment as for the fusion test was carried out without chloroquine. Interestingly, in this case 5–10 times more infected cells were observed with the mL virus, suggesting that the pH 4.5 treatment rendered the virus infectious (Fig. 7). The increased infectivity obtained using this protocol might be partly explained by an additional low pH effect in the endosomes. Indeed additional entry experiments using the mL virus at the PM, in which the pH 4.5 flash in the presence of chloroquine was followed by additional incubations at low pH, showed increased numbers of infected cells. A twofold increase was observed when using a 1–5-min additional incubation at pH 5.5 and a fourfold increase was seen when the pH 4.5 flash was prolonged to 5 min (data not shown).

**A Subfraction of Mature Heterodimers on the Surface of the mL Virus Are Sufficient for Virus Entry**

The poor capacity of the mL virus to enter cells allowed us to investigate what fraction of mature spike protein heterodi-
mers would be required for virus entry via endosomes or through the PM. For this analysis the mL virus was exposed to very mild trypsin treatment for brief periods of time (Fig. 8). Quantitation of the partial digestions showed that under these conditions 10% of the p62 protein was converted to the E2 form after a 0.5-min digestion, 50% after 7 min, and 70% after 10 min. Aliquots of the virus digestion mixtures (25 pfu/cell) were tested for infectivity both after normal entry through endosomes and in the virus-PM fusion assay. Immunofluorescence analysis showed that the virus sample, in which 10% of the p62 subunits were cleaved to the E2 form, infected ~20% of the cells when particles had entered from the endosomal compartment. A gradual increase in infectivity was seen with the more completely digested samples, and the infectivity reached ~80% when 50% of the p62 proteins were digested (data not shown). The virus sample with only 10% mature heterodimers was also able to infect cells via the PM route after the pH 5.5 wash, corresponding to the conditions required for the wildtype virus fusion. However, the infection frequency was about fourfold lower (~5%) when entry was through the PM. These results point to the fact that not all of the viral spikes need to be activated to obtain membrane fusion and subsequent infection.

Discussion

In this work we have analyzed the importance of the p62 protein cleavage for the assembly and entry of SFV. Using a cleavage-deficient mutant form of the p62 protein (mL), we found that virus particles matured from transfected cells, but these virions were noninfectious. Quantitation showed that the mL virus particles formed at the surface of the infected cells as efficiently as the wild type virus. This shows unequivocally that the p62E1 precursor form of the heterodimer can express all those spike protein functions that are necessary for virus budding.

The reason for the noninfectious phenotype of the mL virus was found to be partly dependent on inefficient uptake into new cells. Although reduced binding of mL virus to cells was observed, it could not alone explain this phenotype. Initially we measured the binding of the mL virus at neutral pH and found it to be ~25% of that of the control virus. At slightly lower pH the binding capacity of the mL improved considerably, being almost equal to that of SFV4. However, in subsequent incubations at 37°C, clearly less of the bound mL virus was actually internalized than the control virus. The major reason for the noninfectious phenotype of the mL virus was evidently its almost complete inability to penetrate the endosomal membrane by fusion. The fact that a defect at this step existed was shown by the failure of the mL virus to fuse at the PM when using the same pH 5.5 wash which readily gave the control SFV4 genome access to the cell cytoplasm. Also in support of this was the apparent inability of the mL virus to rearrange into the new E1 forms that are typical for the wildtype virus during entry.

Incorporation of uncleaved spike precursor protein into alphavirus particles has been shown in two recent reports, but in contrast to our results these virus particles were found to be fully infectious. Presley and Brown (1989) reported the production of Sindbis virus particles containing a high percentage of uncleaved p62 protein in experiments using monensin. The infectivity of this virus variant, however, can be explained by our results with mL virus in which only a small portion of the heterodimers were converted to the mature form, suggesting that virus particles need only a subset of mature spikes for entry. Russel and co-workers have also reported the isolation of a Sindbis virus variant that carries uncleaved p62 protein (Russel et al., 1989). However, this virus variant contained a mutation at the p62 cleavage site, changing it into an acceptor site for N-linked glycosylation. As this region is so important in regulating the heterodimer stability and thereby also the E1 protein functions, it is highly possible that insertion of a huge sugar unit in this location somehow compensates for the lack of cleavage by destabilizing the p62E1 complex (see also Lobigs et al., 1990b).

The internalization and penetration defects of the mL virus could be reversed by limited trypsin digestion, which converted the p62E1 phenotype to that of the wild type (E2E1).
The penetration defect of the mL virus could also be circumvented by considerably lowering the pH of the treatment of the cell-bound virus. This clearly correlates with simultaneous disruption of the spike oligomer structure and dissociation of E1 from p62, because similar in vitro treatment of mL virus caused E1 to separate from the p62 protein. Taken together, these results strongly support a mechanism for the activation of SFV fusion function, where the E1 needs to disrupt its heterodimeric association before it can be activated by the low pH to catalyze membrane fusion. It means that mL virus particles could accomplish infection only if transported to an endocytic compartment with pH <5.0, which induces the reorganization steps obligatory for fusion. Consequently, many mL particles may have been lost due to degradation, since internalized virions in a late endosomal (prelysosomal endosome) compartment with sufficiently low pH to cause heterodimer disruption would be very close to arrival in the lysosomes (Kielian et al., 1986; Schmid et al., 1989; Park et al., 1991). The few positive cells that were occasionally seen in the immunofluorescence analysis may thus represent cells infected by the mL virus successfully penetrating from the prelysosomal endosome or from the lysosomal compartment.

When the effect of the low pH (pH 4.5) wash on the mL virus infectivity was analyzed without blocking entry through endosomes by a lysosomotropic agent, we consistently observed 5–10 times more infected cells. This was surprising in view of the almost complete lack of untreated mL virus infection via the endosomes. These results suggest that, in addition to promoting direct penetration of the mL virus at the cell surface, the lower pH wash also caused priming of some of the particles to perform successful virus penetration after uptake into the natural acidic environment of the endosomes. Similar results were recently reported for the influenza virus. A brief exposure to threshold pH values for fusion triggered an irreversible conformational change of the influenza virus spike trimmer complex, rendering the spike fusion competent even at elevated pH. This change also enhanced virus binding to the cells (Stegmann et al., 1990). Similar structural reorganizations of the viral spike, which may represent priming for fusion competence, have also been reported for the Sindbis virus and the HIV-1 after receptor binding (Flynn et al., 1990; Moore et al., 1990). The priming reaction may alter the conformation of the spike oligomer sufficiently to allow for an initial interaction of the fusion peptide with the cell membrane. This interaction could potentially be required for internalization, and may be a prerequisite for the fusion reaction triggered by the low pH in the endosomes, or sufficient for fusion at the PM.

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References

Boere, W. A. M., T. Harmsen, J. Vinje, B. J. Benaisa-Trouw, C. A. Kraaijeveeld, 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.

Boggs, H. M., C. S. Hahn, J. H. Strauss, and D. E. Griffin. 1989. Low pH-dependent Sindbis virus-induced fusion of BHK cells: differences between strains correlate with amino acid changes in the E1 glycoprotein. Virology. 169:485–488.

Chamberlain, J. P. 1979. Fluorescent detection of radioactivity in polyacrylamide gels with water-soluble sulfur, sodium salicylate. Anal. Biochem. 98:132–135.

de Curtis, L., 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.

Dubois-Dalq, M., R. V. Holmes, and B. Renier. 1984. Assembly of enveolated RNA viruses. Springer Verlag, Vienna. 235 pp.

Flynn, D. C., W. J. Meyer, W. M. Chaillet, Jr., and R. E. Johnston. 1990. A conformational change in Sindbis virus glycoproteins E1 and E2 is detected at the plasma membrane as a consequence of early virus-cell interaction. J. Virol. 64:3645–3653.

Garoff, H., and K. Simons. 1974. Location of the spike glycoproteins in the Semliki Forest virus membrane. Proc. Natl. Acad. Sci. USA. 71:3988–3992.

Helenius, A., M. Marsh, and J. White. 1982. Inhibition of Semliki Forest virus penetration by lysosomotropic weak bases. J. Gen. Virol. 58:47–61.

Hoekstra, D., and J. W. Kok. 1989. Entry mechanisms of enveloped viruses. Implications for fusion of intracellular membranes. Biosci. Rep. 9:273–305.

Kielian, M., and A. Helenius. 1985. pH-induced alterations in the fusogenic spike protein of Semliki Forest virus. J. Cell Biol. 101:2284–2291.

Kielian, M., and S. Jungerwirth. 1990. Mechanism of enveloped virus entry into cells. Mol. Biol. Med. 7:17–31.

Kielian, M. C., S. Keränen, L. Kääriäinen, and A. Helenius. 1984. Membrane fusion mutants of Semliki Forest virus. J. Cell Biol. 98:139–145.

Kielian, M. C., M. Marsh, and A. Helenius. 1986. Kinetics of endosome acidification detected by mutant and wild-type Semliki Forest virus. EMBO (Eur. Mol. Biol. Organ.) J. 5:3103–3109.

Klek, H. D., R. Rott, M. Orlich, and J. Bödlorn. 1975. Activation of influenza A viruses by trypsin treatment. Virology. 68:426–439.

Liljestrom, P., S. Lusa, D. Hylebroeck, and H. Garoff. 1991. In vivo mutagenesis of a full-length cDNA clone of Semliki Forest Virus: The small 6000-molecular-weight membrane protein modulates virus release. J. Virol. 65:4107–4113.

Lobigs, M., and H. Garoff. 1990. Fusion function of the Semliki Forest virus spike is activated by proteolytic cleavage of the envelope glycoprotein p62. J. Virol. 64:1233–1240.

Lobigs, M., J. M. Wahlberg, and H. Garoff. 1990a. Spike protein oligomerization control of Semliki Forest virus fusion. J. Virol. 64:5214–5218.

Lobigs, M., H. Zhao, and H. Garoff. 1990b. Function of Semliki Forest virus E3 peptide in virus assembly: replacement of E3 with an artificial signal peptide abolishes spike heterodimerization and surface expression of E1. J. Virol. 64:4346–4355.

Marsh, M., and A. Helenius. 1989. Virus entry into animal cells. Adv. Virus Res. 36:107–151.

Metsäkki, K., and H. Garoff. 1990. Oligomers of the cytoplasmic domain of the p62/E2 membrane protein of Semliki Forest virus bind to the nucleocapsid in vitro. J. Virol. 64:4678–4683.

Moore, J. P., A. A. McKeating, R. A. Weiss, and Q. J. Sattensastou. 1990. Disassociation of gp120 from HIV-1 virions induced by soluble CD4. Science (Wash. DC). 250:1139–1142.

Omar, A., and H. Koblet. 1988. Semliki Forest virus particles containing only the E1 envelope glycoprotein are infectious and can induce cell-cell fusion. Virology. 166:17–25.

Park, J. E., J. M. Lopez, E. B. Chuet, and W. J. Brown. 1991. Identification of a membrane glycoprotein found primarily in the prelysosomal endosome compartment. J. Cell Biol. 112:245–255.

Petersen, R. F. 1991. Protein localization and virus assembly at intracellular membranes. Curr. Top. Immunol. Microbiol. 170:67–98.

Presley, J. F., and D. T. Brown. 1989. The proteolytic cleavage of PE2 to envelope glycoprotein E2 is norcetatively required for the maturation of Sindbis virus. J. Virol. 63:1975–1980.

Russel, D. L., J. M. Dalrymple, and R. E. Johnston. 1983. Sindbis virus mutations which coordinately affect glycoprotein processing, penetration, and virulence in mice. J. Virol. 63:1619–1629.

Schmid, S., R. Fuchs, M. Kielian, A. Helenius, and J. Mellman. 1989. Acidification of endosome subpopulations in wild-type Chinese Hamster Ovary cells and temperature-sensitive acidification-defective mutants. J. Cell Biol. 108:1291–1300.

Simons, K., and H. Garoff. 1980. The budding mechanism of enveloped animal viruses. J. Gen. Virol. 50:1–21.

Stegmann, T., R. W. Doms, and A. Helenius. 1989. Protein-mediated membrane fusion. Annu. Rev. Biophys. Biophys. Chem. 18:187–211.
Stegmann, T., J. M. White, and A. Helenius. 1990. Intermediates in influenza induced membrane fusion. EMBO (Eur. Mol. Biol. Organ.) J. 9:4231–4241.

Väänänen, P. 1981. Interaction of Togaviruses with red cells. Ph.D. thesis. University of Helsinki, Helsinki, Finland. 112 pp.

Vaux, D. J. T., A. Helenius, and I. Mellman. 1988. Spike-nucleocapsid interaction in Semliki Forest virus reconstructed using network antibodies. Nature (Lond.). 336:36–42.

Wahlberg, J. M., and H. Garoff. 1992. Membrane fusion process of Semliki Forest virus I. Low pH-induced rearrangement in spike protein quaternary structure precedes virus penetration into cells. J. Cell Biol. 116:339–348.

Wahlberg, J. M., W. A. Boere, and H. Garoff. 1989. The heterodimeric association between the membrane proteins of Semliki Forest virus changes its sensitivity to mildly acidic pH during virus maturation. J. Virol. 63:4991–4997.

Wellink, J., and A. van Kammen. 1988. Proteases involved in the processing of viral polyproteins. Arch. Virol. 98:1–26.

White, J., J. Kartenbeck, and A. Helenius. 1980. Fusion of Semliki Forest virus with the plasma membrane can be induced by low pH. J. Cell Biol. 87:264–272.

White, J. M. 1990. Viral and cellular membrane fusion proteins. Annu. Rev. Physiol. 52:675–697.

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.