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Fusion Function of the Semliki Forest Virus Spike Is Activated by Proteolytic Cleavage of the Envelope Glycoprotein Precursor p62

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The precursor protein p62 of the prototype alphavirus Semliki Forest virus (SFV) undergoes during transport to the cell surface a proteolytic cleavage to form the mature envelope glycoprotein E2. To investigate the biological significance of this cleavage event, single amino acid substitutions were introduced at the cleavage site through mutagenesis of cDNA corresponding to the structural region of the SFV genome. The phenotypes of the cleavage site mutants were studied in BHK cells by using recombinant vaccinia virus vectors. Nonconservative substitutions completely abolished p62 cleavage. Uncleaved p62 was transported with normal kinetics to the cell surface, where it became accessible to low concentrations of exogenous trypsin. The proteolytic cleavage of envelope glycoprotein precursors has been shown to activate the membrane fusion potential of viral spikes in several virus families. Here we demonstrate that the fusion function of the SFV spike is activated by the cleavage of p62. Cleavage-deficient p62 expressed at the cell surface did not function in low-pH-triggered (pH 5.5) cell-cell membrane fusion; however, cleavage of the mutated p62 with exogenous trypsin restored the fusion function. We discuss a model for SFV assembly and fusion where p62 cleavage plays a crucial role in the stability of the multimeric association of the viral envelope glycoproteins.

The infection cycle of enveloped animal viruses requires during maturation the envelopment of the nucleocapsid with a lipid membrane and during entry of the newly infected cell the release of the nucleocapsid into the cytoplasm. An attractive model suggests that the disassembly of the enveloped particle is initiated via virus-host cell membrane fusion. In some virus families, this fusion event appears to be tightly regulated by two activation steps (11, 33, 59): (i) the proteolytic cleavage of a spike protein precursor during virus maturation which induces a low-pH-sensitive conformation of the fusion domain and (ii) an acid-induced conformational change of the spike, which triggers the fusion of the virus and host membranes and thereby the release of the nucleocapsid into the cytoplasm.

The prototype of a cleavage-activated, low-pH-triggered fusion glycoprotein is the hemagglutinin of the influenza virus (61). The spike precursor protein is cleaved late during virus assembly by a trypsinlike host enzyme, which cleaves after a pair of basic amino acids and is normally responsible for the processing of prohormones (14, 49). Virus entry is by receptor-mediated endocytosis. The cleaved hemagglutinin undergoes a conformational change in the acidic endosomal compartments, releasing the hydrophobic fusion domains which mediate the virus-cell membrane fusion and the release of the nucleocapsid into the cytoplasm. The cleavage of the hemagglutinin is essential for productive virus infection but not particle formation. Other examples of cleavage-activated fusion glycoproteins are found in the paramyxoviruses (37, 41), retroviruses (34), and coronaviruses (51, 52).

Semliki Forest virus (SFV) is a member of the *Togaviridae*, a family of small, enveloped RNA viruses. The envelope is modified by two virally encoded transmembrane glycoproteins, E1 and E2, which remain associated as heterodimers during virus assembly (17, 44). In the mature particle, three copies of the E1-E2 heterodimer are thought to form the hexameric spike (16). Alphaviruses enter by receptor-mediated endocytosis into newly infected cells (25, 32). The penetration of the nucleocapsid into the cytoplasm takes place in the endosomal compartments via low-pH-induced virus-host membrane fusion (57–60). The virus receptor or the fusion domain has not been clearly assigned to any of the two spike glycoproteins, but there is good indirect evidence that the E1 protein is the fusogen. (i) E1 particles essentially free of E2 generated by protease digestion are fusogenic and infectious (39). (ii) Sindbis virus variants which differ in their optimal fusion pHs have amino acid changes in E1 (4). (iii) Upon exposure to low pH, the E1 protein undergoes a conformational change, which promotes the protease resistance of this protein (24) and exposes previously buried disulfide bonds at the surface of the molecule (40).

The mature E2 glycoprotein originates from the cleavage of the precursor protein p62 late during transport from the endoplasmic reticulum to the plasma membrane. It probably occurs during or after exit of the viral protein from the trans-Golgi network but before arrival at the cell surface (9). The cleavage is mediated by a trypsinlike host enzyme with specificity for dibasic residues (14, 49). The conservation of the p62 cleavage site (8, 50) and the efficient processing of the spike precursor among alphaviruses suggest that the conversion of p62 to E2 is crucial for virus maturation or infectivity. Thus, it has been proposed that p62 cleavage triggers budding possibly by promoting the lateral interaction between the mature spike heterodimers at the cell surface (7, 21, 46).

In this study, we have addressed whether cleavage of the spike precursor protein activates the fusion function of the alphavirus spike similarly to the cleavage activation of fusion proteins in other virus families (as described above). We have employed in vitro mutagenesis to introduce single amino acid substitutions at the p62 cleavage site. Here we demonstrate that cleavage-deficient p62 does not mediate cell-cell membrane fusion after low-pH treatment. The fu-

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sion potential can be restored after mild trypsin digestion of cell surface-expressed p62.

MATERIALS AND METHODS

Cells and virus. BHK-21 cells were grown in BHK medium (GIBCO Laboratories) supplemented with 5% fetal calf serum. Human TK-143 cells were grown in Eagle minimal essential medium (EMEM) containing 10% fetal calf serum. Wild-type (wt) vaccinia virus (strain WR) and the temperature-sensitive mutant *ts*7 (10) were a gift from H. Stunnenberg, European Molecular Biology Laboratory, Heidelberg, Federal Republic of Germany, and were propagated on BHK cell monolayers.

Plasmids. The vaccinia virus recombination plasmid p7.5K-HBsAg was provided by H. Stunnenberg. The hepatitis B surface antigen was excised with *Xho*I and *Sal*I. The religated plasmid p7.5K was cut with *Bgl*II, and the complete SFV structural genome, excised as a 4,004-base-pair *Bam*HI fragment from pL2-SFV (36), was inserted under the control of the 7.5K vaccinia virus early-late promoter.

For the construction of the p62 cleavage site mutants, a 362-base-pair *Xho*I-*Nco*I fragment encompassing the p62 cleavage site was replaced in the vaccinia virus recombination plasmid p7.5KSFV with the corresponding mutant fragment. The same approach was taken to subclone the cleavage site mutations into the simian virus 40-based expression vector pSVSSFV (27).

Preparation of recombinant vaccinia virus. Homologous recombination using the vaccinia virus temperature-sensitive mutant *ts*7 and subsequent bromodeoxyuridine selection were performed according to the procedure of Kieny et al. (26) and as described elsewhere (20). Recombinants were screened for the expression of the SFV structural proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of pulse-labeled infected-cell lysates (as described below), twice plaque purified on TK-143 cells, and amplified on BHK cells. Crude, high-titer virus stocks were prepared as described previously (30).

Oligonucleotide site-directed mutagenesis. A 1,355-base-pair *Eco*RI fragment containing the SFV p62 cleavage site was subcloned from plasmid pSVSSFV (27) into M13mp9. The mutating oligonucleotides were 5′-CGACACGCTCTC CCGGTGTC (for mutE), 5′-CGACACGCTGAGCCGGTG TC (for mutL), and 5′-CGACACGCTCTTCCGGTGTC (for mutK). In vitro mutagenesis was by the gapped-duplex approach (29), using the site-directed mutagenesis kit manufactured by Boehringer GmbH. Mutants were screened by single-track dideoxy sequencing by using the M13 sequencing primer (Boehringer), and positives were resequenced entirely in the region delineated by the *Nco*I and *Xho*I sites, which contains the p62 cleavage site and which was subcloned into the expression vectors. Dideoxy sequencing was with Sequenase (United States Biochemical Corp.) as outlined in the protocol of the manufacturer.

Metabolic labeling. BHK cell monolayers were infected with recombinant vaccinia virus at a multiplicity of 10. At 8 h after infection, the cells were washed twice with phosphate-buffered saline (PBS) and starved with methionine-free EMEM for 0.5 h. The cells were then pulsed with methionine-free EMEM containing [^{35}S]methionine (1,000 Ci/mmol; Amersham Corp.) at a final concentration of 100 μCi/ml. Following two washes with PBS, the label was chased by the addition of EMEM containing 10 times the normal concentration of methionine. Pulse and chase periods were as described below. The monolayers were solubilized in Nonidet P-40 buffer (1% Nonidet P-40, 50 mM Tris hydrochloride [pH 7.4], 150 mM NaCl, 2 mM EDTA), and the SFV spike glycoproteins were immunoprecipitated with monoclonal antibodies anti-E1 8.139 and anti-E2 5.1 (3). Immunoprecipitation, SDS-PAGE, and fluorography were performed as described previously (56).

Treatment of cells with exogenous trypsin. After the pulse-chase treatment, the cell monolayers were washed twice with PBS, cooled on ice for 5 min, and incubated with trypsin (Boehringer) in PBS (15 μg/ml) for 0.5 h on ice. After the protease digestion, the monolayers were washed with PBS and incubated with soy bean trypsin inhibitor (SBTI; Boehringer) in PBS (100 μg/ml) on ice for 10 min. The cells were then lysed in Nonidet P-40 buffer containing 20 μg of phenylmethylsulfonyl fluoride (Sigma Chemical Co.) per ml.

Trypsin treatment of microinjected cells was as follows: cell monolayers were washed twice with PBS and cooled on ice for 5 min; ice-cold trypsin in PBS (0.5 μg/ml) was added, and the cells were incubated on ice for 10 min; and the monolayers were then washed with ice-cold BHK medium containing 5% fetal calf serum and incubated with fresh BHK medium in a 37°C incubator for 20 min before being processed for acid-induced fusion and immunofluorescence staining.

Microinjection, fusion, and immunofluorescence staining. Circular plasmid DNA at a concentration of 1 μg/ml was injected into the nuclei of subconfluent BHK cells grown on glass cover slips essentially as described previously (27, 28, 53). A Zeiss automated injection system was used, and glass capillaries were from Eppendorf. Injected cell monolayers were incubated overnight before being tested for viral spike protein-mediated low-pH-induced cell-cell fusion. Following two washes with PBS, fusion medium (EMEM without bicarbonate, containing 10 mM sodium succinate, pH 5.5) at 37°C was added for 60 s. The fusion medium was replaced with BHK medium containing 5% fetal calf serum, and the cells were returned to a 37°C incubator for 2 h to allow polykaryon formation. Immunofluorescence staining was essentially as described previously (53). A mixture of two monoclonal antibodies (anti-E1 8.139 and anti-E2 5.1) was used for surface staining with either sheep anti-mouse immunoglobulin G fluorescein or goat anti-mouse immunoglobulin G rhodamine (Biosys, Compiégne, France) as second antibodies.

RESULTS

Mutagenesis of the proteolytic cleavage site of the spike precursor p62. During virus maturation, the spike precursor p62 is proteolytically cleaved to E2 and E3. At the cleavage site of p62, the consensus sequence R-X-R/K-R ↓ (with cleavage at the position of the arrow), which is also present at the cleavage sites of spike precursors of a number of other virus families (50), is found (22). To inhibit cleavage of p62, we introduced a conservative (R→K in mutK) or nonconservative (R→L in mutL and R→E in mutE) substitution at the −1 position of the cleavage site consensus sequence (Fig. 1). Site-directed mutagenesis using synthetic oligonucleotides was performed on M13mp9 DNA containing the structural genes of SFV (as described in Materials and Methods). Mutant fragments were subcloned into vaccinia virus recombinant vectors or a simian virus 40-based expression vector for the phenotypic analysis of the cleavage site mutants.

Expression of the structural proteins of SFV in BHK cells via recombinant vaccinia virus vectors. The vaccinia virus

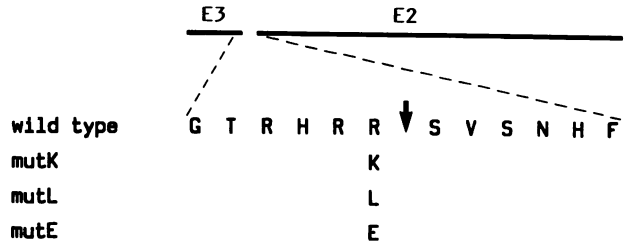

FIG. 1. Spike precursor cleavage site mutants. The SFV spike precursor p62, which is proteolytically processed to E3 and E2, is drawn schematically at the top. The amino acid sequence at the p62 cleavage site region of the wt virus is shown below, with the vertical arrow indicating the cleavage site (22). The amino acid substitutions at the −1 position in the three cleavage site mutants are listed. The single-letter amino acid code is used.

expression system was used to study the phenotypes of the p62 cleavage site mutants in vivo. Vaccinia virus vectors which had the wt or mutant SFV subgenomic cDNA inserted under the control of the 7.5K vaccinia virus early-late promoter were constructed. In pulse-chase experiments (15-min pulse, chase as indicated in Fig. 2), the correct synthesis and processing of the wt construct was ascertained. The spike proteins E1 and p62/E2 were immunoprecipitated with monoclonal anti-E1 and anti-E2 antibodies from recombinant vaccinia virus-infected cell lysates and analyzed by SDS-PAGE (Fig. 2, lanes 1 to 3). The two spike proteins E1 and p62/E2 are similar, if not identical, in size to those synthesized in SFV-infected cells and are seen to undergo a number of well-characterized maturation events (19). Thus, E1 is converted to a higher-molecular-weight form because of the addition of a complex oligosaccharide (5). After a 10-min chase (Fig. 2, lane 1), only the immature form of E1 was seen; both forms were present after a 45-min chase (Fig. 2, lane 2); and only the mature form remained after a 100-min chase (Fig. 2, lane 3). The spike precursor p62 appeared as a doublet shortly after synthesis (Fig. 2, lane 1). The lower band disappeared after longer chase intervals, with a slightly diffuse higher-molecular-weight band remaining. After a 45-min chase (Fig. 2, lane 2), processing of p62 to E2 was clearly visible, and the ratio of E2 to p62 increased with longer chase intervals. The mature E2 also migrated as

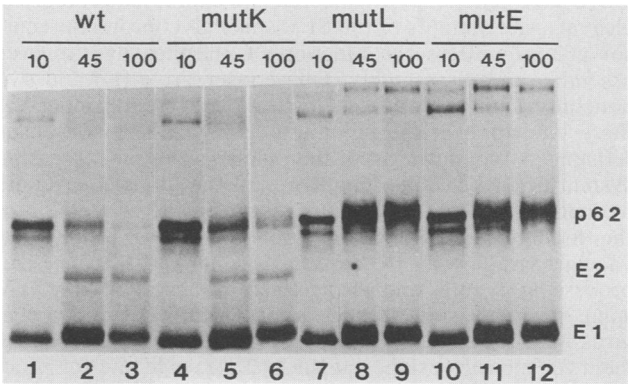

FIG. 2. Spike precursor cleavage phenotypes of the wt and p62 mutants. BHK cells were infected with recombinant vaccinia virus and pulse-labeled for 15 min, and the label was chased for 10, 45, or 100 min. The spike glycoproteins were immunoprecipitated and resolved by electrophoresis on a 10% SDS–polyacrylamide gel.

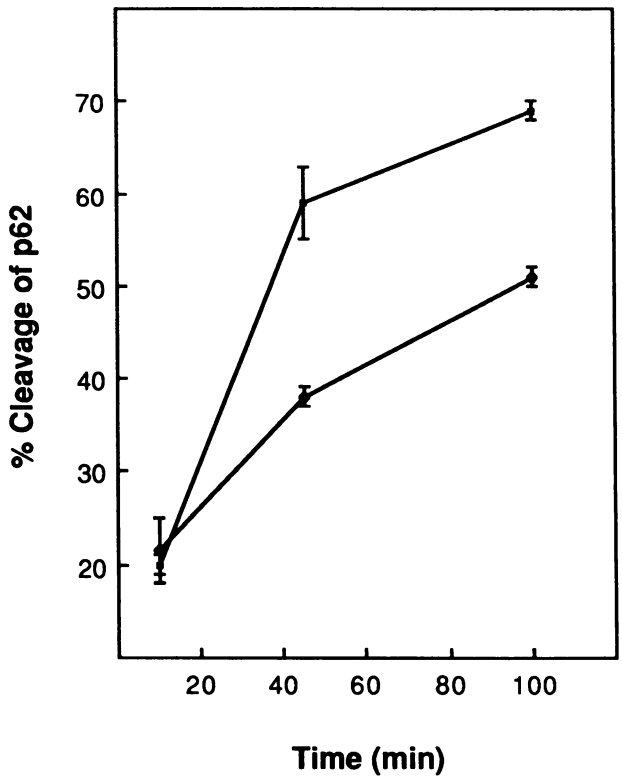

FIG. 3. Cleavage kinetics of p62 in wt and mutK recombinant vaccinia virus-infected cells. Pulse-chase experiments in wt (■) or mutK (◆) recombinant vaccinia virus-infected BHK cells were performed as described in the legend to Fig. 2. After fluorography, the radiolabeled bands corresponding to p62 and E2 were excised from dried gels and solubilized in Protosol (DuPont Co.). The radioactivity was measured by liquid scintillation counting. After adjusting for the number of methionines in p62 and E2, the cleavage of p62 was calculated as the counts per minute in the E2 band divided by the sum of the counts per minute in the p62 and E2 bands and was expressed as a percentage. Error bars represent the results from two separate experiments from which the average was plotted.

a slightly diffuse band. The second cleavage product E3 is not visible on these gels. A cluster of bands was commonly seen in the 100,000-molecular-weight range (Fig. 2). These bands probably correspond to heterodimers of the spike glycoproteins, which can be converted to their monomeric forms if SDS-PAGE is performed under reducing conditions (even though these conditions did not resolve E1 and E2).

Cleavage phenotypes of the p62 cleavage site mutants. BHK cells were infected with recombinant vaccinia virus and pulse-labeled for 15 min, and the label was chased for intervals of 10 to 100 min. The spike proteins were immunoprecipitated and analyzed by SDS-PAGE (Fig. 2). In mutL and mutE, the cleavage of p62 was completely abolished. Even overexposures of the fluorograms did not reveal any E2. The uncleaved p62 was chased to a diffuse, higher-molecular-weight molecule, which probably represents the sialylated form of p62. In contrast, the conservative amino acid substitution in mutK did not inhibit the processing of p62. E2 became visible after a 45-min chase (Fig. 2, lane 5) and accumulated with longer chase intervals (Fig. 2, lane 6). However, reduced p62 cleavage kinetics of mutK compared with that of the wt can be noted. Quantitation shows 38 and 51% conversion, respectively, of p62 to E2 after 45- and 100-min chases in mutK, compared with 59 and 69%, respec-

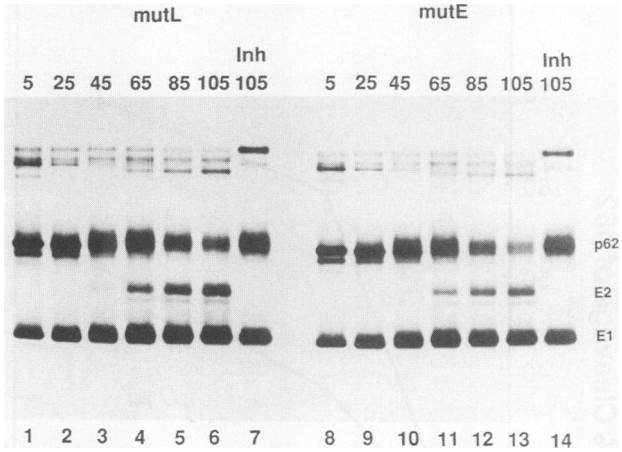

FIG. 4. Cleavage of mutL and mutE with exogenous trypsin. BHK cells were infected with recombinant vaccinia virus mutant mutL or mutE, pulse-labeled for 10 min, and chased for time periods as indicated (min). After the chase intervals, the monolayers were incubated with trypsin (15 μg/ml) for 0.5 h on ice, followed by inactivation of the protease with SBTI (as described in Materials and Methods). In lanes 7 and 14 (marked by Inh), SBTI was present during the trypsin treatment. The spike glycoproteins were immunoprecipitated from cell lysates and resolved by electrophoresis on a 10% SDS–polyacrylamide gel.

tively, in the wt (Fig. 3). These results demonstrate that dibasic residues at the p62 cleavage site are absolutely necessary for the maturation of p62 to E2.

Cleavage-deficient p62 can be cleaved with exogenous trypsin. In mutL and mutE, the consensus sequence at the p62 cleavage site was changed at the −1 position, resulting in cleavage inhibition. However, two basic residues constituting potential trypsin cleavage sites remained in the p62 cleavage region at the −2 and −4 positions (Fig. 1). The accessibility of these to exogenous trypsin was examined. Recombinant vaccinia virus-infected cells were pulse-chased (10-min pulse, chase as indicated in Fig. 4), and the monolayers were incubated on ice for 30 min in the presence of a low concentration of trypsin (15 μg/ml), followed by inactivation of the protease with SBTI. The fluorogram shows that the cleavage of p62 can be restored with the addition of exogenous trypsin. After a 45- to 60-min chase interval (Fig. 4, lanes 3, 4, 10, and 11), p62 first became accessible to cleavage by exogenous trypsin. It was processed to an E2 molecule with an identical electrophoretic mobility to that of the wt form (data not shown) but which should have at least one additional amino acid at the amino-terminal end (an L in mutL or an E in mutE). With longer chase periods, increasing amounts of p62 were cleaved. The trypsin assay clearly established that the cleavage-deficient p62 is transported to the cell surface. After a 100-min chase, approximately 50% of the pulse-labeled p62 was susceptible to cleavage by exogenous trypsin. The residual p62 represents an intracellular pool which becomes susceptible to trypsin processing after detergent solubilization of the monolayers (data not shown). The addition of SBTI concomitantly with the protease inhibited the enzyme activity, showing that the cleavage of p62 at the cell surface was entirely due to the presence of trypsin (Fig. 4, lanes 7 and 14).

Cleavage of the mutL and mutE p62 with exogenous trypsin was first seen after a somewhat longer chase interval than cleavage of the wt p62 by the endogenous enzyme (Fig. 2). This is consistent with the idea that the endogenous enzyme is active in an intracellular compartment at a late stage of the exocytotic pathway but before arrival of the glycoprotein at the cell surface.

Fusion phenotypes of p62 cleavage site mutants. The fusion of the virus and host cell membranes constitutes an important biological function in SFV entry. It is acid activated and occurs in the early endosomal compartment at a pH below 6.2 (57–60). To test whether p62 cleavage is important for fusion, we examined the fusion after low-pH treatment between neighboring cells expressing the SFV spike proteins. Because of the early cytopathic effect observed in recombinant vaccinia virus-infected cells, the distinction between polykaryons and aggregates of rounded cells could not be made accurately. Therefore, we chose a simian virus 40-based expression system with the vector pSVSSFV, which upon injection into the nucleus expresses the SFV structural proteins and induces polykaryon formation after low-pH treatment (27). The three cleavage site mutations were subcloned into pSVSSFV, and the plasmid DNA was microinjected into the nuclei of neighboring BHK cells. At 16 h after injection, the monolayers were treated with or without low concentrations of trypsin at 0°C and fusion was triggered by a 60-s incubation with pH 5.5 medium. The expression of the spike proteins was confirmed by double immunofluorescence staining, and areas of positive cells were examined for polykaryon formation (Fig. 5). Cell-cell fusion assays of the cleavage site mutants were repeated at least three times.

The fusion phenotypes are summarized in Table 1. Fusion correlated strictly with p62 cleavage. In mutK, where correct cleavage of p62 took place (Fig. 2), polykaryon formation was observed after acid treatment (Fig. 5c and d). As expected, fusion was not seen prior to acid treatment (Fig. 5a and b). In the p62 cleavage-deficient mutants mutL and mutE, low-pH treatment failed to activate the fusion function (Fig. 5e, f, i, and j), although the spike proteins were expressed on the plasma membrane as shown by surface staining. However, when the microinjected monolayers were treated with trypsin prior to pH 5.5 treatment, fusion activity was restored (Fig. 5g, h, k, and l), as demonstrated by large areas of immunofluorescence corresponding to polynucleated cells. This clearly shows that cleavage activation of p62 is important for the acid-induced fusion activity of the SFV spike heterodimer.

DISCUSSION

We have genetically engineered and expressed proteolytic cleavage site mutants of the SFV spike glycoprotein precursor p62 to address the function of p62 cleavage in virus assembly and disassembly. One conservative (K) and two nonconservative (E and L) substitutions were introduced at the −1 position of the ubiquitous cleavage site consensus sequence. We have used the vaccinia virus expression system to analyze the phenotypes of the p62 cleavage site mutants. Similar to a previous report on the expression of the Sindbis virus structural proteins via a recombinant vaccinia virus vector (43), we have confirmed that the size, processing events, and intracellular transport of the SFV spike proteins were similar, if not identical, to those in virus-infected cells. We have confirmed the strict requirement for dibasic residues at the p62 cleavage site for processing by the trypsinlike host enzyme. As noted by others (15, 38), an R→K substitution can somewhat reduce the efficiency of cleavage by the protease. Uncharged or acidic residues at the cleavage site completely abolished processing of p62.

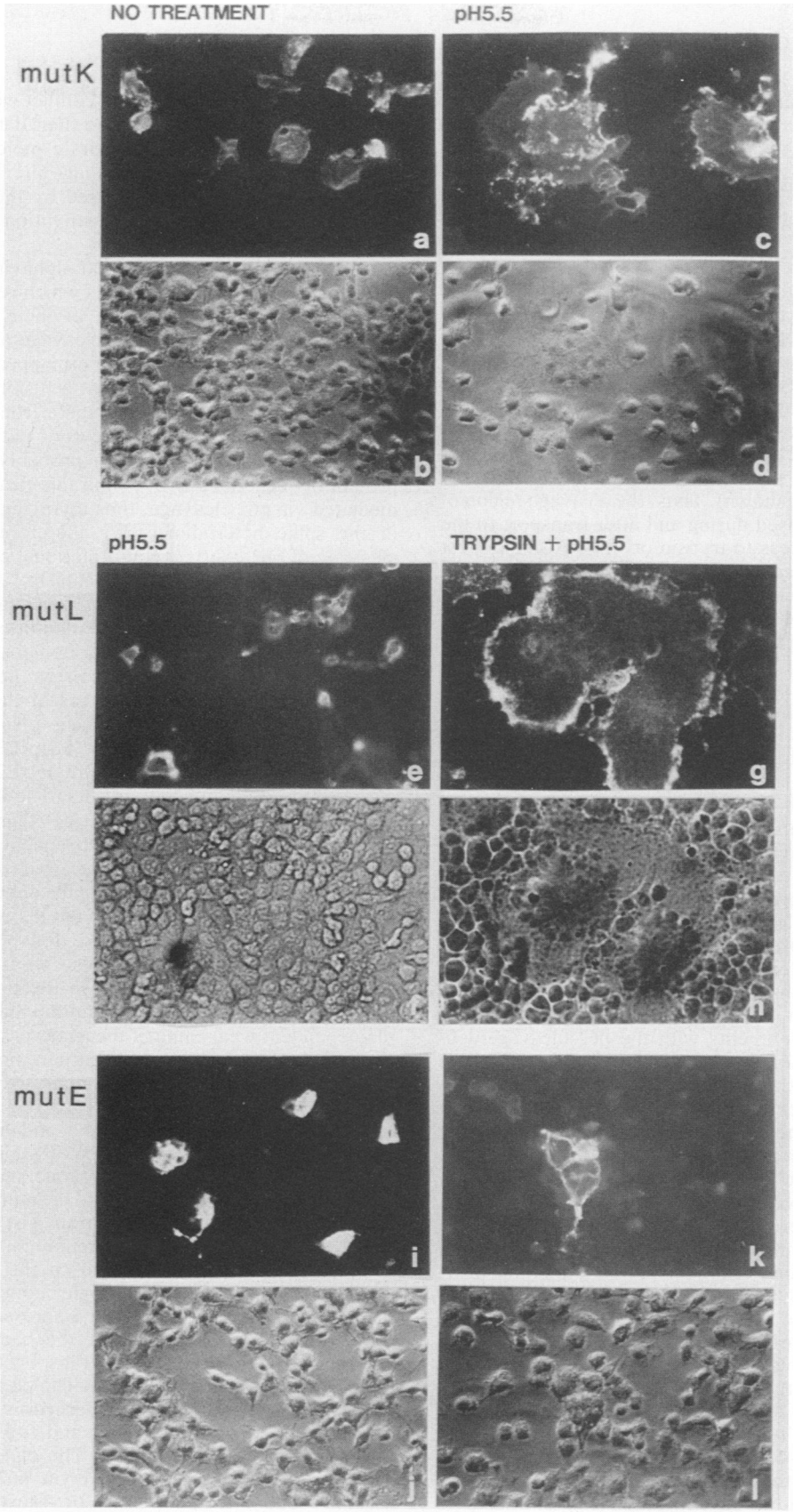

FIG. 5. Fusion phenotypes of p62 cleavage site mutants. Neighboring BHK cells were microinjected with plasmid DNA and incubated at 37°C for 16 h. Fusion was triggered by a 60-s incubation with pH 5.5 medium with or without prior treatment of the monolayers with trypsin (0.5 μg/ml) for 15 min on ice (as described in Materials and Methods). After a further 2-h incubation at 37°C, double immunofluorescence staining was performed. Areas showing surface expression of the spike glycoproteins were photographed by fluorescence (top panel in each pair) and phase-contrast (bottom panel in each pair) microscopy.

TABLE 1. Fusion phenotypes of p62 cleavage site mutants

Mutant	Fusion after treatment:		
	None	pH 5.5	Trypsin + pH 5.5
mutK	−	+	ND[a]
mutL	ND	−	+
mutE	ND	−	+

[a] ND, Not determined.

Surface expression of the spike precursor p62 was demonstrated with exogenous trypsin, which mimicked the activity of the host enzyme to generate the mature spike protein E2. The cleavage probably occurred at one of the two remaining basic residues at the mutated p62 cleavage site. The resultant E2 was relatively resistant to further digestion with trypsin at an at-least-10-fold-higher enzyme concentration (data not shown). Thus, the cleavage region of p62 appears to be exposed during and after transport to the cell surface, giving access to trypsin or the trypsinlike host enzyme. In many strains of ortho- and paramyxoviruses, uncleaved fusion protein precursors are exposed at the cell surfaces and can be activated with exogenous trypsin (18, 37). Spike precursor cleavage site mutants of retroviruses (34, 42) and ortho- and paramyxoviruses (23, 41) display a similar sensitivity to exogenous trypsin of surface-exposed uncleaved precursors. Interestingly, a requirement for cleavage in the activation of the biological functions of the spike, but not in particle formation, can be noted.

Our results suggest that p62 cleavage plays a role in virus entry. The spike protein-induced and acid-triggered cell-cell membrane fusion in transient expression studies was clearly dependent on p62 cleavage. No polykaryon formation was observed after low-pH treatment when uncleaved p62 from mutL or mutE was expressed, but it could be induced when the mutant p62 was cleaved with exogenous trypsin. The SFV spike is therefore composed of cleavage-activated fusion glycoprotein heterodimers. This conclusion contrasts the suggestion of Brown and co-workers that p62 cleavage is not essential for fusion (31). Their interpretation was based on cell-cell fusion experiments with the p62 cleavage-deficient Sindbis virus *ts*20 mutant and pretreatment of Sindbis virus-infected cells with trypsin at 3 h after infection (31), which apparently blocks spike precursor cleavage (1). In fusion assays with the *ts*20 mutant at a nonpermissive temperature, the fusion activity was reduced relative to that in wt virus-infected cells. Nevertheless, a residual fusion activity remained, which could be correlated with the leakiness of this mutant, permitting some processing of the spike precursor. When fusion medium was added 7 h after infection to the trypsin-treated cells, efficient acid-induced polykaryon formation was noted. However, the investigators have not excluded the possibility that residual, normally processed glycoproteins are synthesized prior to the trypsin treatment and that these can mediate fusion. Moderate amounts of Sindbis virus glycoproteins can be seen at the cell surface as early as 2 h after infection, which is sufficient for the induction of cell-cell membrane fusion (13).

In a recent report (45), an interesting mutant of Sindbis virus with a change at the amino-terminal amino acid of E2, which introduced a novel glycosylation site and, importantly, abolished cleavage of p62, was described. The uncleaved spike precursor was incorporated into mature virions with normal growth characteristics in tissue culture cells. The fusion phenotype of this mutant has not been reported, but assuming that virus-cell membrane fusion is needed for virus entry, it would conflict with our results. An explanation which may reconcile their data with ours could be that the additional carbohydrate moiety in the Sindbis virus mutant, exactly at the cleavage site, substitutes for the structural change normally caused by the cleavage, which would be important for fusion activation (as described below).

Our present work suggests that alphaviruses are part of a growing number of virus families which regulate their disassembly via cleavage activation of their oligomeric fusion proteins (33). Spike precursor cleavage activation at a late stage during the surface transport appears to be a widespread mechanism to circumvent a fusion-inducing conformational rearrangement of the spike from occurring in the acidic compartments of the exocytotic pathway (2, 6). However, in SFV, the fusion function probably resides on the E1 protein (as described above) and the cleavage activation is mediated via p62 cleavage, thus involving different partners of the spike heterodimer. We therefore predict that the cleavage of p62 exerts a conformational effect on E1 via an oligomerization-controlled mechanism. According to our model, the mature E1-E2 spike protein heterodimer becomes sensitive to acid-induced dissociation after cleavage of p62, which allows the putative fusion domain on E1 to become exposed. Support for this comes from coimmunoprecipitation and cosedimentation analyses of the SFV spike heterodimer in buffers of decreasing pHs (56). A marked resistance to dissociation of the E1-p62 complex was observed, in contrast to the mature E1-E2 complex, which dissociated in mildly acidic buffers. The fact that the dissociation was occurring at a higher pH than that required for optimal fusion suggests that subsequent to dissociation a second low-pH-dependent change is needed. This may be related to additional changes in spike subunit conformation, resulting in the exposure of the putative fusion domain of E1 (12, 24). The cleavage-activated, low-pH-triggered fusion mechanism of influenza virus also envisages acid-induced conformational rearrangements in the tertiary and quaternary structures of the hemagglutinin homotrimer (61). A pH-dependent weakening of the trimeric structure as well as a rearrangement of the hemagglutinin monomers, exposing the well-characterized fusion domain, have been described previously (33, 61).

Several functions in the assembly and disassembly of SFV can now be associated with the p62/E2 spike protein. (i) The interaction between the cytoplasmic tail of p62 and the nucleocapsid in virus budding has been inferred (16, 48, 54). (ii) p62 is responsible for the transport of the spike heterodimer from the endoplasmic reticulum to the cell surface. Expressed alone, p62 is routed to the cell surface (27), whereas E1 expressed from a single coding unit is retained in the endoplasmic reticulum (35). Expression of p62 and E1 from separate coding units in the same cell results in heterodimerization and surface transport (M. Lobigs and H. Garoff, unpublished results). (iii) The cleavage of p62 to E2 may play a role in the lateral interaction between the spike heterodimers at the cell surface and regulation of the budding event (16, 21, 47, 55). (iv) The oligomerization of the spike heterodimer is controlled via p62 cleavage, which assures a stable E1-p62 dimer for transport via the acidic compartments of the exocytotic route and a much more acid-labile oligomer which can undergo fusion in the acidic compartments of the endocytotic pathway (56; our data). Thus, a picture emerges in which p62 plays a crucial role in the assembly and activation of disassembly of SFV, whereas

the E1 protein carries the critical signals for infection and fusion.

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