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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

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The Semliki Forest virus spike glycoproteins E1 and p62 form a heterodimeric complex in the endoplasmic reticulum (ER) and are transported as such to the cell surface. In the mature virus particle, the heterodimeric association of E1 and E2 (the cleavage product of p62) is maintained, but as a more labile and acid-sensitive oligomer than the E1-p62 complex. The E3 peptide forms the N-terminal part of the p62 precursor and carries the signal for the translocation of p62 into the lumen of the ER. The question of whether E3 is also important in the formation and stabilization of the E1-p62 heterodimer has been addressed here with the aid of an E3 deletion mutant cDNA. In this construct, the entire E3 was replaced with a cleavable, artificial signal sequence which preserved the membrane topology of an authentic E2. The E3 deletion, when expressed via a recombinant vaccinia virus, abolished heterodimerization of the spike proteins. It also resulted in the complete retention of E1 in the ER and almost total inhibition of E2 transport to the plasma membrane. The oligomerization and transport defect of E1 expressed from the E3 deletion mutant could be complemented with a wild-type p62 provided from a separate coding unit in double infections. These results point to a central role of E3 in complex formation and transport of the viral structural components to the site of budding. In conjunction with earlier work (M. Lobigs and H. Garoff, J. Virol. 64:1233–1240, 1990; J. Wahlberg, W. A. M. Boere, and H. Garoff, J. Virol. 63:4991–4997, 1989), the data support a model of spike protein oligomerization control of Semliki Forest virus assembly and disassembly which may be mediated by the presence of E3 in the uncleaved p62 precursor and release of E3 after cleavage.

Many viral membrane proteins form oligomeric complexes, and there is increasing evidence that the oligomerization event is important in virus assembly (21a, 36). Oligomerization usually occurs in the endoplasmic reticulum (ER), and unassembled proteins often fail to be transported out of the ER to the site of virus formation. Here we have examined the molecular interactions of oligomer formation and their effect on the cellular location of the Semliki Forest virus (SFV) spike glycoproteins.

SFV belongs to the alphavirus family, a group of small, enveloped animal viruses. The biology of SFV has been extensively studied as a model for virus assembly (13). The viral structural proteins are translated from a common coding unit on a subgenomic messenger (26S RNA) in the order capsid, p62 (E3 plus E2), and E1. During cotranslational processing of the polyprotein precursor, the capsid protein is autoproteolytically cleaved and remains in the cytoplasm (35). p62 and E1 are both integral membrane proteins with lipid-binding peptide anchors at their C-terminal ends. A stretch of apolar residues at the N-terminus of p62 functions as a signal peptide in the translocation of p62 into the lumen of the ER (H. Garoff, D. Huylebroeck, A. Robinson, U. Tillman, and P. Liljeström, J. Cell Biol., in press). Interestingly, the signal of p62 is not removed by signal peptidase cleavage, but instead becomes translocated itself and glycosylated. p62 is cleaved to E3 and E2 in a post-Golgi compartment late in virus maturation (8). After cleavage, the SFV E3 remains noncovalently associated with the spike protein heterodimer E1-E2 and is found in mature virus particles (15). In other alphaviruses, E3 is released into the culture fluid and not found on mature virions (33).

The integral membrane proteins E1 and p62 form a complex in the ER which is efficiently transported to the plasma membrane (40, 42). Coprecipitation and sedimentation analyses demonstrated a marked resistance to acid-induced dissociation of the p62 heterodimer, whereas mature E1-E2 heterodimers of viral particles dissociated under mildly acidic conditions (40). A strict correlation between p62 cleavage and activation of the acid-triggered fusion function of the SFV spike has been established which may be mediated by the weakening of the oligomeric interactions after cleavage of p62 (29).

Here we examine whether E3 plays a role in virus assembly other than as signal peptide for p62 and as cleavable precursor peptide in the regulation of the fusion activity. With the aid of an E3 deletion mutant, we show that E3 has an important role in heterodimer formation of the viral spike proteins and that E1 is retained in the ER unless complexed with its heterodimeric partner.

MATERIALS AND METHODS

Cells and virus. BHK-21 cells were grown in BHK medium (GIBCO) supplemented with 5% fetal calf serum (FCS). Cos-7 cells (17) were propagated in Dulbecco minimal essential medium (DMEM) containing 10% FCS. vvSFVwt, a recombinant vaccinia virus expressing the wild-type (wt) SFV structural proteins under control of the vaccinia virus 7.5-kilobase (7.5K) early-late promoter has been described (29). Construction of recombinant vaccinia viruses vvSFVΔE3 and vvSFVΔE1 by using plasmids p7.5KSFVΔE3 and p7.5KSFVΔE1, respectively, and virus propagation were done as described before (20, 24, 29).

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Oligonucleotide site-directed mutagenesis. Site-directed in vitro mutagenesis was done by the method of Kunkel et al. (28). A 1,355-base-pair (bp) *Eco*RI fragment containing the sequence encoding the SFV capsid, E3, and part of E2 was subcloned from pSVSSFV (25) into M13 mp9. The phage was grown in *Escherichia coli* RZ1032 in the presence of uridine to give uracil-containing single-stranded template DNA. The mutating oligonucleotide was a 99-mer: 5′ - G A AGTGTTGCGACACGCTGGCTTGTGCCTGTGGAAG TAGCAGAAGTAGCAGGAGAAGCAGCAGGCGCAT GGCGGATCCCGGGGCGGACCACTCTTCGGA-3′, which has 21 and 19 complementary bases at the 3′ and 5′ ends, respectively, and which introduces an additional *Bam*HI site (underlined). The oligonucleotide was purified by electrophoresis on an 8% polyacrylamide sequencing gel and excision of the largest detectable band, followed by elution of the DNA from the gel slice. The phosphorylated oligonucleotide and template DNA were hybridized at a 10:1 molar ratio.

Second-strand synthesis with Sequenase (U.S. Biochemical Corp.) in the presence of T4 DNA ligase was done as described before (37). Competent *E. coli* JM109 cells were transformed with the mutagenesis mixture, and 20 plaques were screened by restriction enzyme analysis with *Bam*HI, yielding three mutants. The correct mutant sequence was verified by dideoxy sequencing in a region delineated by unique *Nae*I and *Nco*I sites. The 488-bp *Nae*I-*Nco*I fragment encompassing the E3 deletion mutation was subcloned into the vaccinia virus recombination vector p7.5KSFV (29), and the resulting plasmid, p7.5KSFVΔE3, was used in vaccinia virus recombination.

Plasmid pGEM-SFVΔE1 (a gift from S. Lusa and P. Liljeström, Center for Biotechnology, Karolinska Institute, Stockholm) contains the sequence of the structural genome of SFV with a stop codon, TAG, at nucleotide 9937 at the 5′ end of the E1 sequence, introduced by site-directed mutagenesis. The SFV coding region was excised as a 4,004-bp *Bam*HI fragment and subcloned into the vaccinia virus recombination plasmid p7.5K131A (a gift from H. Stunnenberg, European Molecular Biology Laboratory, Heidelberg).

Transfection and immunofluorescence staining of Cos cells. Subconfluent Cos-7 cells grown on glass cover slides in 35-mm petri dishes were transfected by the DEAE-dextran method. Plasmid DNA (5 μg) was suspended in 250 μl of transfection medium (Eagle minimal essential medium [EMEM] without bicarbonate, buffered with 25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.15]) and mixed dropwise with an equal volume of DEAE-dextran in transfection medium (1 mg/ml, pH 7.25). After 30 min at room temperature, the DNA-DEAE-dextran mixture was added to the cell monolayer which had been washed twice with transfection medium. The cells were incubated for 30 min at room temperature, washed twice with transfection medium, and DMEM supplemented with 10% FCS and 0.1 mM chloroquine was added to minimize lysosomal degradation of the DNA. After 3 h at 37°C, the chloroquine-containing medium was replaced with DMEM supplemented with 10% FCS and 0.1 mM sodium butyrate (18). After 2 days at 37°C, the cells were fixed with 3% paraformaldehyde and stained as described before (38). Monoclonal antibodies 8.139 (anti-E1) and 5.1 (anti-E2) (2) were used as the first and sheep anti-mouse immunoglobulin G (IgG) fluoresceine (Biosys, Compiégne, France) as the second antibody in indirect immunofluorescence staining.

Metabolic labeling, immunoprecipitation, and electrophoresis. Metabolic labeling of recombinant vaccinia virus-infected BHK cells has been described before (29). Immunoprecipitation with monoclonal antibodies by using protein A-Sepharose was done as described before (40). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was done on 1.0-mm-thick 10% polyacrylamide gels (acrylamide-bisacrylamide, 30:0.8) with 5% stacking gels and a discontinuous buffer system. Fluorography was done with 1 M sodium salicylate (4), and quantitation of radioactivity was done as described before (40).

Quantitation of viral membrane proteins at the cell surface. Pairs of infected cells in 35-mm dishes were pulse-labeled and chased for different time periods. For surface labeling, the cells were first washed with cold phosphate-buffered saline (PBS) and then incubated with 0.5 ml of PBS containing 0.5 mg of sulfosuccinimidyl 6-(biotinamido)hexonate (Pearse) per ml for 30 min at 4°C (Brändli et al., unpublished data). The surface labeling reaction was stopped by a wash with PBS containing 50 mM lysine. After this, one dish of each pair was treated with protease K in PBS (0.8 mg/ml) for 60 min at 4°C to remove membrane proteins at the cell surface. Further digestion was inhibited by adding phenylmethylsulfonyl fluoride (Sigma Chemical Co.) to a final concentration of 0.2 mg/ml. After incubation for 20 min on ice, the medium was removed and the cells were lysed with lysis buffer (40) in parallel with the nondigested cell samples and used for immunoprecipitation (40).

For isolation of the biotinylated fraction, the immunoprecipitated membrane proteins were released from the immunoabsorbent by incubating twice in 100 μl of a elution solution containing 1% SDS, 0.2 M Tris hydrochloride (pH 8.8), 5 mM EGTA (ethylene glycol tetraacetic acid), and 1 mM methionine for 2 min at 95°C. After this, the immunoabsorbent was washed with 300 μl of lysis buffer (40) containing 3% Nonidet P-40. Eluates and wash solution were then pooled, and the biotinylated molecules were precipitated by adding streptavidine agarose (Sigma) (40 μl of 50% streptavidine agarose per 400 μl of sample). Incubation was for 20 min at 4°C. Streptavidine agarose pellets were first washed as was normally done for immunoprecipitates (40) and then, in addition, with 1% SDS. The samples were prepared for SDS-PAGE by heating for 5 min at 95°C in the appropriate sample buffer (40).

EndoH assays. Endoglycosidase H (endoH) digestion was done in sodium citrate buffer (50 mM, pH 5.5) at 37°C for 16 h. The viral spike glycoproteins were immunoprecipitated (as above), and the washed immune complexes bound to protein A-Sepharose were treated with 10 mU of endoH (Seikagaku Kogyo Co., Tokyo, Japan) in a volume of 200 μl. After enzyme treatment, the samples were washed once with 10 mM Tris hydrochloride (pH 7.5) prior to the addition of SDS-PAGE sample buffer.

N-terminal radiosequencing. BHK cells in 60-mm petri dishes were infected with vvSFVwt or vvSFVΔE1 at a multiplicity of 20. At 6 h after infection, the monolayers were washed with PBS, and valine-free EMEM was added for 1 h. Metabolic labeling was done for 1 h in valine-free EMEM containing 60 μCi of [^{3}H]valine (Amersham) per ml. After two washes with PBS, the label was chased for 2 h by the addition of EMEM containing five times the normal concentration of valine. The E2 glycoprotein was immunoprecipitated, and the immune complexes were boiled for 2 min in 50 μl of 1.5% SDS to release E2 from the protein A-Sepharose beads. The protein was sequenced on an Applied Biosystems 470A protein sequencer.

WT: GSEEW SAPLITAMCVLA -----E3----RHRR SVSQHFNVYKATR

ΔE3: GSEEW SAPGSAMRLLLLLLLLLLPQAQA SVSQHFNVYKATR

FIG. 1. Replacement of the SFV E3 peptide with a cleavable, artificial signal sequence, and N-terminal radiosequencing of mutant and wt E2. The wt amino acid sequence (single-letter code) of E3 is partially shown at the top, with the cleavage region of the capsid protein at the N-terminus of E3 and that of the E2 protein after a pair of basic residues at the C terminus of E3. E3 was deleted and replaced with 23 residues predicted to function as a cleavage signal sequence of E2 in mutant SFVΔE3. The putative apolar translocation signals in wt and SFVΔ3 are underlined. To ensure the autoproteolytic cleavage of the capsid, the three N-terminal residues of E3 were maintained in the mutant construct. At the C-terminal end of the hypothetical signal sequence of SFVΔE3, small, uncharged amino acids were introduced at the −1 and −3 positions, fulfilling the predicted requirements for signalase cleavage. Note the two valines at positions 2 and 8 at the N terminus of E2. Peaks of radioactivity were seen at these positions in N-terminal radiosequencing of [^{3}H]valine-labeled, immunoisolated E2 from vvSFVwt- and vvSFVΔE3-infected cells (bottom).

RESULTS

Construction of an E3 deletion mutant cDNA. For the functional analysis of E3 in virus assembly, a mutant cDNA of the SFV structural genome was constructed by the precise deletion of the coding sequence for E3. Site-directed mutagenesis on M13 mp9 DNA containing the coding sequence for the SFV subgenomic RNA was performed with a long, synthetic oligonucleotide (99-mer; see Materials and Methods). This was designed to replace the sequence of the E3 gene with a sequence encoding 23 residues which agrees with the predicted requirements for a cleavable translocation signal (39) to obtain normal processing and membrane insertion of E2 (Fig. 1). The three N-terminal amino acids of E3 (the first two of which are strictly conserved among alphaviruses [7]) were maintained to ensure cotranslational cleavage of the capsid protein. In addition, a *Bam*HI site was introduced for convenient screening for mutant phage.

Synthesis and processing of the spike proteins in vvSFVΔE3-infected cells. When the E3 deletion mutant cDNA was expressed in BHK cells via a recombinant vaccinia virus (vvSFVΔE3), two glycoproteins, E1 and E2, were immunoprecipitated with their respective monoclonal antibodies, but no p62 was seen, consistent with the deletion of E3 (Fig. 2, lanes 7 to 12). The E2 protein from the mutant construct was resolved as a doublet in SDS-PAGE. Neither of the two bands showed exact comigration with the wt E2. When chased for 4 h after pulse-labeling, the slower-migrating form of the E2 from SFVΔE3 seemed to predominate (see Fig. 4, lane 7).

To test whether the two forms of E2 in vvSFVΔE3-infected cells differed in glycosylation from each other and the wt, we treated infected cells with tunicamycin (1 μg/ml) for 3 h prior to a pulse and maintained the drug during a 2-h chase (data not shown). The inhibition of glycosylation resulted in a single form of E2, which, however, could not be compared in its electrophoretic mobility with the unglycosylated wt E2, since tunicamycin prevents p62 cell surface transport and thereby also its cleavage (14).

When treated with endoH, the E2 doublet from the mutant construct was reduced to a single faster-migrating band, which coincided with an endoH-sensitive form of the wt E2 (see Fig. 4; data discussed in more detail below). E1 synthesized in vvSFVΔE3-infected cells appeared identical to the wt form in its electrophoretic mobility in SDS-PAGE (Fig. 2). Together the data show that in cells expressing the E3 deletion construct, the spike proteins E1 and E2 are produced, but the carbohydrate processing of E2 may differ from that of the wt.

In addition, we performed N-terminal radiosequencing to verify that signal peptidase cleavage of E2 from vvSFVΔE3 occurred at the predicted site (Fig. 1). Recombinant vaccinia virus-infected cells were labeled with [^{3}H]valine, and E2 was immunoisolated. Wt and mutant E2 proteins were subjected to 13 cycles of Edman degradation, and the products of each cycle were assayed for the presence of ^{3}H radioactivity (Fig. 1). Peaks were found in cycles 2 and 8 in both the SFV wt and SFVΔE3 E2 proteins. The presence of valine at the positions expected from the predicted amino acid sequence confirms the efficient and correct removal of the signal peptide of E2 in mutant vvSFVΔE3.

The SFV E3 peptide is required for the heterodimeric association of the spike glycoproteins. Previous work has demonstrated that the p62 precursor exists exclusively as an E1-p62 heterodimer on the basis that (i) the two glycoproteins can be coimmunoprecipitated from lysates of virus-infected cells and (ii) they sediment as 4.5S complexes in sucrose gradients in the presence of Triton X-100 (40). In the mature virus particle, where p62 has been cleaved to E3 and E2, E1 and E2 also exist in a heterodimeric, albeit less acid stable, form (40). To test whether E3 provides some important requirement for the formation and stability of the spike oligomer, coimmunoprecipitation assays were performed on lysates from recombinant vaccinia virus-infected cells expressing the E3 deletion mutation.

Figure 2 shows a typical immunoprecipitation profile of the three SFV glycoproteins E1, E2, and p62 and a set of higher-molecular-weight bands which correspond to glycoprotein complexes and remain resistant to SDS dissociation (9, 21). The wt spike proteins p62 and E1 essentially quantitatively coimmunoprecipitated with either the anti-E1 or anti-E2 antibody after short chase intervals (Fig. 2, lanes 2 and 3; compare with lane 1). In contrast, in vvSFVΔE3-infected cells, coimmunoprecipitation of E1 or E2 with the heterologous antibody was never detected (Fig. 2, lanes 8, 9,

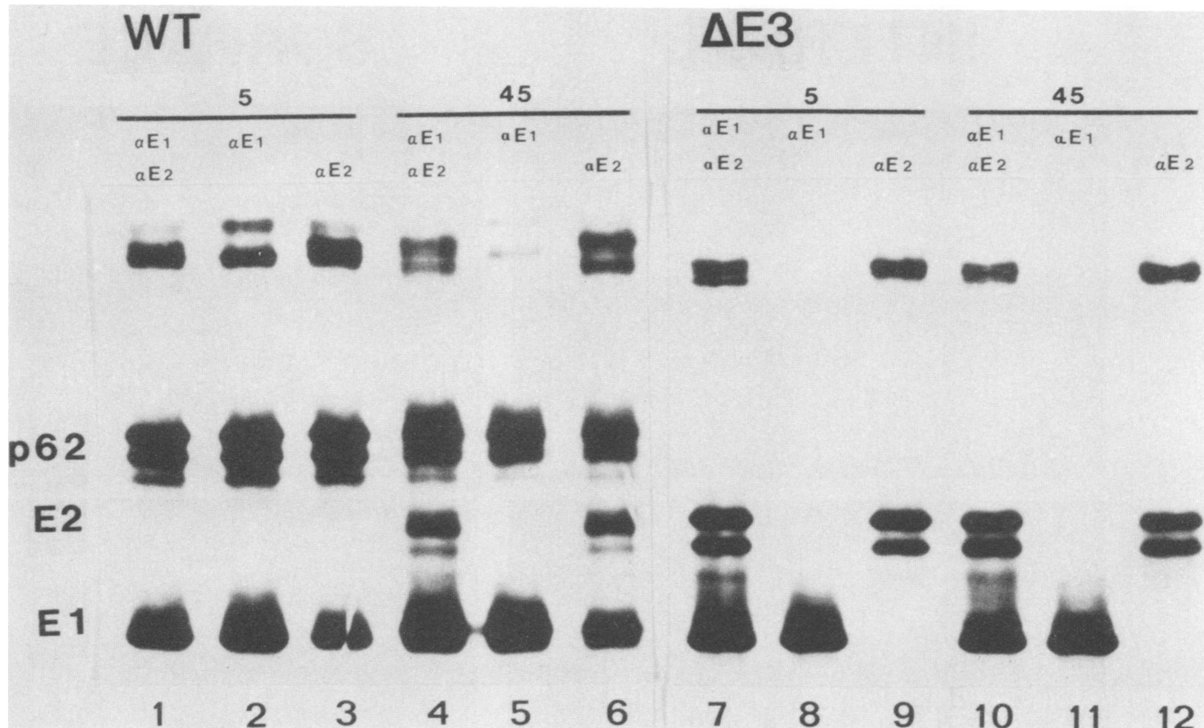

FIG. 2. Coimmunoprecipitation of the SFV spike proteins is abolished in the absence of E3. BHK cells (1.5×10^6 cells) were infected with recombinant vaccinia virus vvSFVwt or vvSFVΔE3 and pulsed for 15 min with [^{35}S]methionine, and the label was chased for 5 or 45 min. The cells were lysed in 1% Nonidet P-40 buffer, and the lysate, after a round of incubation with rabbit anti-mouse IgG and protein A-Sepharose, was divided into three portions. The spike proteins were immunoprecipitated with two monoclonal antibodies (8.139 [anti-E1] and 5.1 [anti-E2]) in combination or separately and analyzed by SDS-PAGE under nonreducing conditions to separate E1 and E2.

11, and 12). This suggests that in the absence of E3, the spike glycoproteins do not form heterodimeric complexes at any time after synthesis.

It can be noted that with the onset of p62 cleavage (Fig. 2, 45-min chase), the wt E1 and E2 glycoproteins did not coimmunoprecipitate as heterodimers. Thus, E2 was never seen in immunoprecipitates with the anti-E1 antibody (Fig. 2, lane 5), and the decrease in the fraction of E1 precipitating with the anti-E2 antibody corresponded to the fraction of p62 cleaved to E2 (Fig. 2, lane 6). Apparently the cleavage of p62 destabilizes the heterodimer sufficiently to prevent coimmunoprecipitation. A strict correlation between p62 cleavage and the abolition of E1-E2 coimmunoprecipitation could be established by coimmunoprecipitation assays of p62 cleavage site mutants (29) after cleavage of p62 with exogenous trypsin (Lobigs and Garoff, unpublished). Accordingly, in vaccinia virus, expression of the SFV structural protein E1-p62 but not E1-E2 heterodimer can be demonstrated with the monospecific antibodies. This points to a clear difference in the association of the E1-E2 heterodimer in virus particles and cDNA expression studies. Apparently the spike-nucleocapsid interaction in the virion induces the formation of a more stable oligomer.

Intracellular location of the spike proteins expressed from SFVΔE3. The formation of oligomeric complexes has been implicated to play an important role in the export of membrane proteins out of the ER. To test whether the defect in heterodimer formation in the E3 deletion mutant influences the intracellular location of the spike proteins, we first performed immunofluorescence surface staining for E1 and E2. Immunofluorescence labeling was done in Cos cells transfected with simian virus 40-based wt or mutant expression plasmids by the DEAE-dextran method. This system was chosen instead of the vaccinia virus infection because the latter gave high background staining. Figure 3 shows that in SFVΔE3-transfected cells, E2 but not E1 was expressed at the cell surface. When the cells were permeabilized with Triton X-100, both glycoproteins gave a strong reticular staining pattern characteristic of the ER. Thus, the absence of surface staining of E1 was apparently not the result of a reduced rate of synthesis but of a defect in its exocytic transport. The wt staining pattern was as previously described (25) and consistent with the surface expression of both E1 and E2.

When the immunofluorescence labeling of E2 from wt and SFVΔE3-transfected cells was compared, the surface staining of the wt appeared to be somewhat stronger. It was also noted that after permeabilization of the cells, the internal staining of wt E2 was complemented with surface staining visible as tight delineation of the plasma membrane; this was not apparent for E2 from SFVΔE3. In summary, the immunofluorescence labeling suggests that the deletion of E3 results in the retention of E1 in the ER, whereas E2 is at least partially transported to the cell surface.

To confirm these results, the resistance to endoH digestion of the wt and mutant spike proteins expressed in BHK cells via recombinant vaccinia viruses was examined. Figure 4 (lanes 1 and 2) shows that almost all E1 from the wt construct was chased to an endoH-resistant form. This is consistent with the processing of its single asparagine-linked glycosylation site to a complex oligosaccharide during transport of the p62-E1 heterodimer to the plasma membrane. The small fraction of endoH-sensitive E1 (the hardly visible, faster-migrating band in Fig. 4, lane 2) probably reflects the

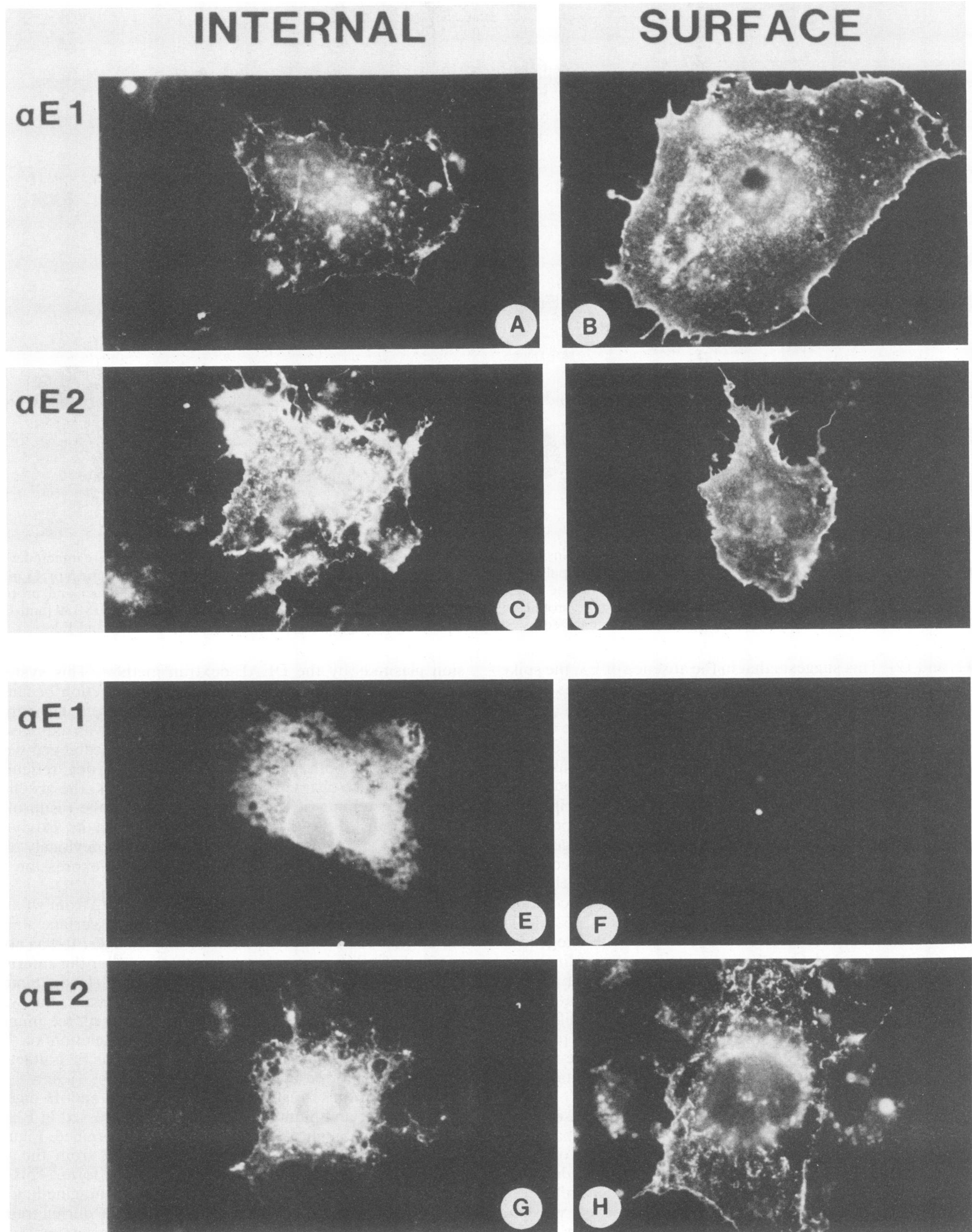

FIG. 3. Cellular location of E1 and E2 by indirect immunofluorescence analysis. Cos cells were transfected with SFV wt (A-D) or SFVΔE3 (E-H) simian virus 40-based expression plasmids, fixed 2 days after transfection, and stained for E1 or E2 with or without permeabilization of the plasma membrane to give internal or surface staining.

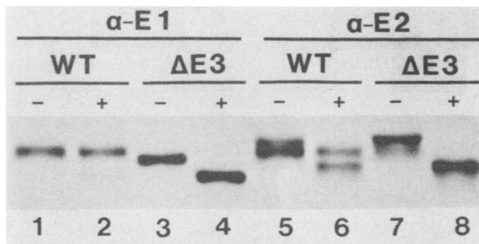

FIG. 4. Acquisition of resistance to endoH of the spike proteins of the wt and E3 deletion mutant. BHK cells (1.5×10^6 cells) were infected with recombinant vaccinia virus vvSFVwt or vvSFVΔE3 and pulsed for 15 min with [^{35}S]methionine, and the label was chased for 2 h. Cell lysates were divided into two portions, and the SFV spike proteins were immunoprecipitated with an anti-E1 or anti-E2 monoclonal antibody. The immune complexes, suspended in 50 mM sodium citrate, were incubated with endoH (+) or mock-treated (−) for 16 h at 37°C, and the spike proteins, after reduction with 5% 2-mercaptoethanol, were analyzed by SDS-PAGE.

fact that some E1 molecules can undergo carbohydrate processing into a complex form which remains endoH sensitive (19, 30).

The deletion of E3 from the p62 precursor conferred complete endoH sensitivity to the E1 glycoprotein (Fig. 4, lanes 3 and 4). Accordingly, E1 appeared to be retained in the ER, since its carbohydrate moiety failed to be trimmed by the mannosidases in the *cis*-Golgi compartment and modified by the addition of terminal sugars to give rise to a complex and endoH-resistant form (27). It can also be noticed that E1 from the wt construct had a slightly higher apparent molecular weight than E1 from the mutant construct. Such a difference can be seen when the sugar unit of E1 matures by sialylation in the *trans*-Golgi compartment on its way to the cell surface (19). The lack of this modification in SFVΔE3 is also highly indicative of ER retention of the mutant E1 (compare Fig. 4, lanes 1 and 3).

In contrast to what was described for E1, the endoH assay of the E2 protein gave no clear answer about its cellular location (Fig. 4). The pulse-labeled E2 protein which was expressed from vvSFVwt and chased for 4 h showed two closely migrating bands which, after endoH treatment, shifted to two faster-migrating forms. In the mutant construct, E2 also appeared as a doublet. These did not exactly comigrate with the wt E2 bands, and after endoH treatment they shifted to a single band comigrating with the faster endoH-sensitive wt E2 molecule. These results could be explained by the occupancy of the two acceptor sites on the E2 chain by either one or two sugar units, both of which are endoH sensitive in mutant SFVΔE3 whereas one of the two mature sugar units on the wt E2 is resistant (19, 31). This could mean that in contrast to the wt E2, most of the E2 from vvSFVΔE3 is not transported out of the ER. Thus, we were unable to corroborate the surface expression of E2 seen by immunofluorescence staining with this biochemical assay.

In order to solve this problem, two additional biochemical assays, protease K digestion and biotin labeling, were used to study the appearance of pulse-labeled viral membrane proteins directly at the cell surface. For this purpose, several dishes with cultures of BHK cells were infected in parallel with recombinant vaccinia viruses containing the wt or mutant form of the p62 gene and pulse-labeled. Figure 5A shows the quantitation of the total and surface-exposed fractions of E2 and E1 membrane proteins expressed from the wt construct at different time points after the pulse-labeling. The total amount of E2 and E1 membrane proteins increased during the initial chase time due to E2 formation by p62 cleavage. After 60 min of chase, there was a rapid decrease in the amount of viral membrane proteins owing to degradation. This made it difficult to measure the cell surface appearance of the viral proteins exactly. However, by comparing the values with and without protease K digestion, it is evident that almost half of the pulse-labeled membrane proteins were found at the cell surface with this assay. Although the E2 and E1 proteins were not quantitated separately in this experiment, the autoradiographs of the SDS-PAGE analysis demonstrate that both subunits were about equally sensitive to protease K (data not shown). In the biotin-streptavidin assay, a somewhat smaller fraction of E2 and E1 membrane protein was found at the cell surface (Fig. 5A). In contrast to the results obtained with the wt construct, we were not able to find any significant amount of the E2 and E1 subunits made from SFVΔE3 at the cell surface with either assay (Fig. 5B). Thus, these results confirm our earlier finding about the retention of the E1 membrane protein in the ER; furthermore, they show clearly that E2 is also defective in intracellular transport when made from the E3 deletion variant.

Interestingly, the SFVΔE3-made E2 and E1 subunits appeared to be much more stable during the chase than those of the wt. One possible explanation for this difference is that (wt derived) E1 and E2 subunits are internalized efficiently at the cell surface and degraded in lysosomes, whereas (mutant derived) intracellularly retained subunits are excluded from this pathway and therefore remain intact for longer time periods.

Complementation of the transport defect of E1 from SFVΔE3. Our results, so far, indicate that the deletion of E3 abolishes heterodimerization of the SFV spike proteins and, in turn, changes their transport phenotypes, with E1 being strictly retained in the ER. To confirm the requirement of oligomer formation in the export of E1 out of the ER, we examined whether the transport defect of E1 in mutant SFVΔE3 could be complemented with a wt p62 expressed from a separate coding unit. Double-infection experiments in BHK cells were done with the recombinant vaccinia viruses vvSFVΔE3, expressing the E3 deletion mutation, and vvSFVΔE1, which encodes a wt p62 but no E1 due to a stop codon introduced at the 5′ end of the E1 gene (see Materials and Methods). To maximize the rescue of the transport defect of E1, the cells were infected with an excess of vvSFVΔE1 and pulsed for 20 min, and the label was chased as indicated in the legend to Fig. 6.

Figure 6A shows that in the double-infection experiment, the SFV spike proteins E1, E2, and p62 were produced, with an excess of p62 over E1 and E2. The E1 derived from vvSFVΔE1 appeared to heterodimerize efficiently with the p62 synthesized from vvSFVΔE1, as judged by the relative amounts of p62 and E1 coimmunoprecipitated by the anti-E1 antibody. Oligomerization occurred rapidly, as most of E1 was already complexed with p62 after a 20-min pulse, without a visible increase in oligomerization after 1 and 2 h of chase.

It can also be noted in Fig. 6A that during the chase intervals, almost complete processing of the E1 molecules to the sialylated form took place, indicative of the export of E1 from the ER to the *trans*-Golgi compartment (see above). The same conclusion can be drawn when the resistance of E1 to endoH digestion was followed in pulse-chase experiments of double-infected cells (Fig. 6B). While after a 20-min pulse E1 was endoH sensitive, the majority was chased to an endoH-resistant form after 60 and 120 min. This conclusively

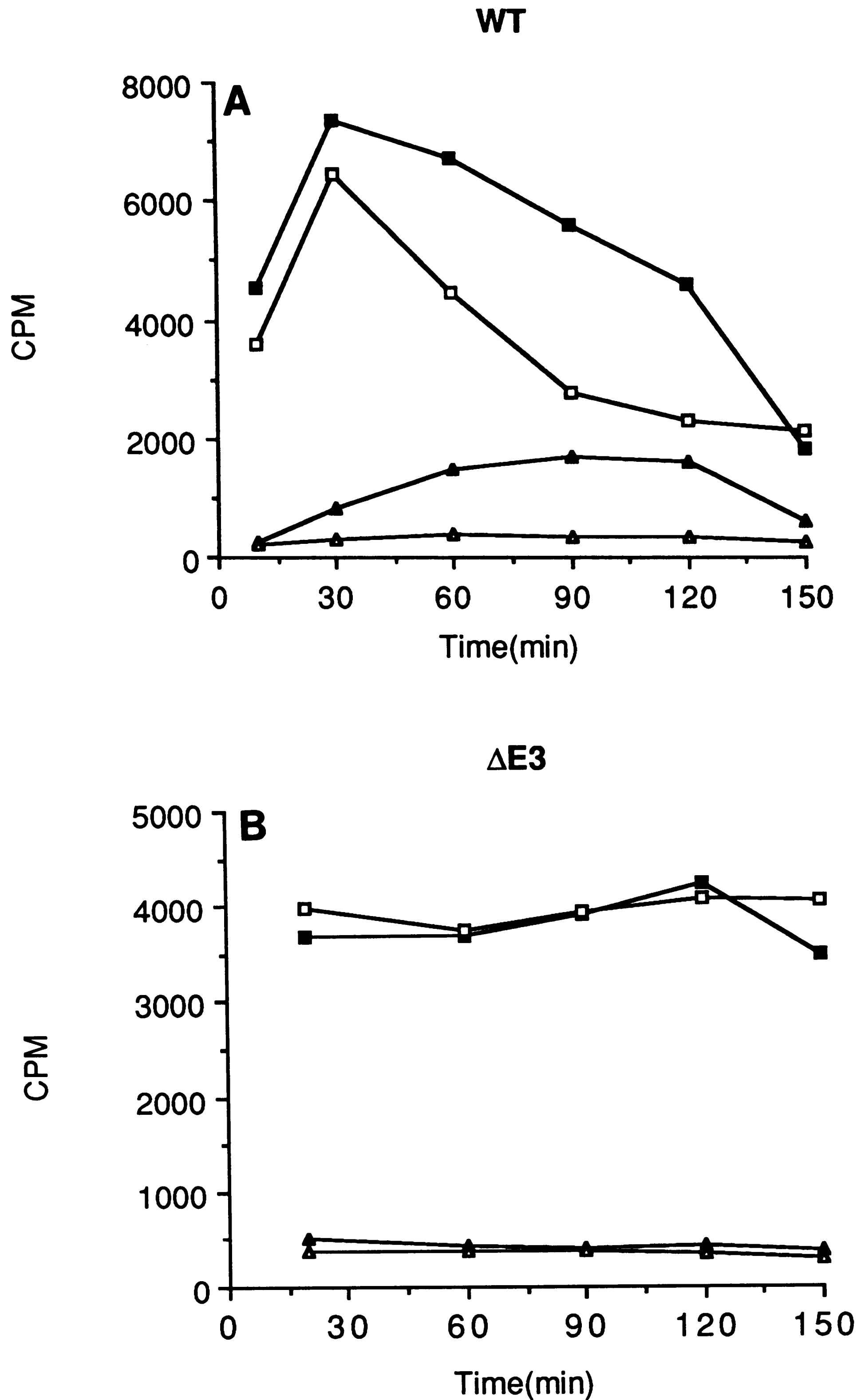

FIG. 5. Expression of viral membrane proteins on the surface of cells infected with vvSFVwt and vvSFVΔE3. Several dishes with BHK cells were infected with vvSFVwt or vvSFVΔE3, pulsed for 15 min with [^{35}S]methionine, and chased in pairs for 20, 60, 90, 120, or 150 min. Cell surface proteins were then biotinylated, and one dish for each pair was, in addition, treated with protease K. The cells of all dishes were lysed, and the total SFV membrane protein was first recovered by immunoprecipitation. The total membrane protein sample was further solubilized in SDS-containing buffer, and one half was incubated with streptavidine agarose for specific precipitation of biotinylated SFV membrane proteins. All samples were then analyzed by SDS-PAGE. The gel was processed for autoradiography and bands containing E1 and E2 membrane proteins were quantitated for radioactivity. Symbols: ■, total viral membrane protein; □, membrane proteins left after protease K treatment of cells; ▲, biotinylated fractions of viral membrane proteins; △, biotinylated fraction of viral membrane proteins after protease K treatment.

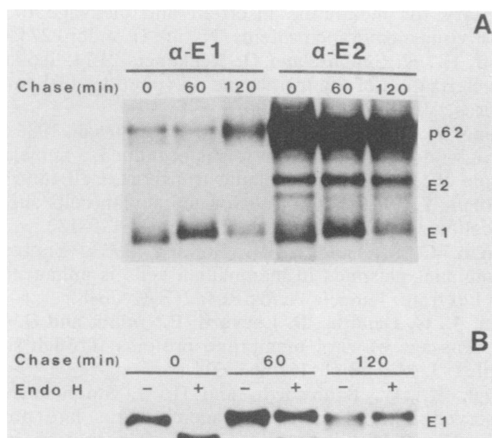

FIG. 6. Complementation of the heterodimerization and transport defects of E1 from SFVΔE3 in double infections. BHK cells (1.5×10^6) were double infected with vvSFVΔE3 and vvSFVΔE1 at multiplicities of 5 and 15, respectively, and pulsed with [^{35}S]methionine for 20 min, and the label was chased as indicated. Following lysis with Nonidet P-40 buffer, the spike proteins were immunoprecipitated with an anti-E1 or anti-E2 monoclonal antibody, and one-half of the precipitate was analyzed for coimmunoprecipitation of the spike proteins by SDS-PAGE under nonreducing conditions (A). The second half of the anti-E1 antibody precipitation was treated (+) or mock-treated (−) with endoH, and the proteins, after reduction, were resolved by SDS-PAGE (B).

shows that the ER retention of E1 in mutant SFVΔE3 can be efficiently complemented when a wt p62 is provided as a partner for heterodimer formation.

DISCUSSION

In the functional characterization of the SFV E3 peptide, we have taken the "reductionist" approach by entirely deleting the E3 portion of the spike precursor p62 and replacing it with a cleavable, artificial signal sequence to generate a translocation-competent, authentic E2. The substitution in the E3 deletion mutant of polyleucine for the wt signal promoted efficient translocation of E2 in accordance with the previously described effectiveness of this idealized signal sequence (22, 41). Translocation of E2 into the lumen of the ER could be monitored by the addition of endoH-sensitive carbohydrates and the absence of unglycosylated products. In addition, immunofluorescence labeling showed characteristic ER staining and some surface expression of E2. To promote correct leader peptidase cleavage of the idealized signal sequence, we followed the −1, −3 rule of von Heijne (39) and verified the formation of an authentic N-terminus of E2 by radiosequencing.

In this article we report on the requirement for the presence of the E3 peptide in the p62 precursor for spike protein heterodimerization in the ER. Spike complex formation was monitored in a coimmunoprecipitation assay, which previously demonstrated a strong, noncovalent oligomerization of E1 and p62 and a somewhat weaker interaction between E1 and E2 in the virion spike (40). Spike protein heterodimerization was abolished in the E3 deletion mutant but could be restored when p62 was provided from a separate coding unit in double infections. This strongly supports the idea that p62 but not E2 can initiate complex formation with E1 in the ER. Whether E3 carries the contact site for the molecular interaction of p62 and E1 or only induces an oligomerization-competent conformation of p62 cannot be concluded from this work. As a working model, one could envisage that p62 consists of two relatively independent domains, E2 and E3, linked by a protease-accessible hinge region. In the spike complex, E3 interacts with E1, inducing and stabilizing the oligomer and controlling the cleavage-activated and acid-inducible fusion function of E1 (29). Nevertheless, it cannot be excluded that the interaction, or additional interactions, between the SFV spike components is concentrated in other regions, such as the transmembrane domains, as has been suggested on the basis that soluble ectodomains generated by protease digestion of intact virus were monomeric (23).

The E3-mediated heterodimerization in the ER was absolutely required for the transport of E1 to the plasma membrane. The transport defect of E1 in SFVΔE3 was demonstrated by the absence of immunofluorescence surface staining of E1, the failure to detect E1 on the cell surface with protease K and biotinylation assays, and the failure of the molecule to be converted to its endoH-resistant form. These results are consistent with those of an earlier study on SFV p62 deletion mutants (34). Also, E2 appeared to be inhibited in its routing to the cell surface. However, the fact that this protein can be detected on the surface by immunofluorescence suggests that the transport block is not complete, and some E2 can reach the cell surface with time.

The evolution of an absolute requirement for heterodimer formation in the surface transport of E1 reported here may reflect on the possible detrimental effect of the passage of a monomeric E1 to the cell surface. Thus, one could imagine that the mildly acidic milieu encountered in parts of the exocytic pathway (1, 3, 32) would trigger the fusion function of an unprotected E1. The mechanism for ER retention of E1 from mutant SFVΔE3 is beyond the scope of this study. ER to Golgi transport appears to be a major quality control step for the exclusion of misfolded proteins from the exocytic pathway (21a). By analogy with the well-characterized models of oligomerization-controlled spike protein export from the ER of the influenza virus (5, 16, 21) and vesicular stomatitis virus (10, 11) homotrimers, the transport defect of the mutant E1 could result from aggregation and/or interaction of monomeric E1 with ER-resident proteins. The transport defect of E1 was, however, not irreversible, since E1 retained its capacity to assemble into oligomers when p62 was provided in double infections. This relatively rapid heterodimer formation of E1 and p62 expressed from separate coding units points to an efficient subunit recognition of the spike components, which in a natural infection are encoded by the same message and probably heterodimerize instantaneously after translocation into the lumen of the ER. The oligomerization kinetics of the influenza virus hemagglutinin (5, 16) and vesicular stomatitis virus G protein (10) homotrimers have been reported to have a half-life of 7.5 and 6 to 10 min, respectively.

In contrast to the rather clearcut location properties of the E1 membrane protein of SFVΔE3, the exact intracellular distribution of the E2 subunit remains elusive. The bulk of this material could possibly also be retained in the ER. This could be due to the folding of E2 into a transport-incompetent conformation because of the lack of E3 or of heterodimerization. Earlier studies have shown that the intact p62 protein can reach the cell surface, but since this conclusion was based on immunofluorescence studies alone, it must be regarded with caution (6, 12, 26).

In summary, the data presented in this article point to the important role of the SFV E3 peptide in the heterodimeriza-

tion of the viral spike proteins in the ER and to an oligomerization-controlled transport mechanism of the spike proteins to the site of virus budding. In conjunction with the function of E3 as a signal sequence of p62 and as a cleavable precursor peptide in the regulation of the virus-cell membrane fusion, E3 can be regarded as a spike protein domain initiating and controlling the assembly of the virus from its individual components.

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