

# Expression of Semliki Forest Virus Proteins from Cloned Complementary DNA. II. The Membrane-spanning Glycoprotein E2 Is Transported to the Cell Surface without Its Normal Cytoplasmic Domain

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**ABSTRACT** The E2 protein (422 amino acid residues long) of Semliki Forest virus is a spanning membrane protein which is made in the rough endoplasmic reticulum of the infected cell and transported to the cell surface. The cytoplasmic domain of this protein comprises 31 amino acid residues. We introduced deletions of various sizes into the gene region encoding this part of the protein molecule and analyzed the transport behavior of the mutant proteins. The deletions were made using exonuclease digestions of cloned cDNA encoding the E2 protein. When the mutated DNA molecules, engineered into an expression vector, were introduced into nuclei of baby hamster kidney 21 cells, membrane proteins with cytoplasmic deletions were expressed and routed to the cell surface in the same way as the wild-type protein. This suggests that the cytoplasmic domain of the E2 protein does not carry information that is needed for its transport from the rough endoplasmic reticulum to the cell surface.

Proteins with many different destinations are synthesized on polysomes bound to the endoplasmic reticulum (RER) (22). Some proteins become secreted from the cell, while others are routed to the cell surface or to various intracellular organelles. All of these proteins possess an N-terminal signal sequence that allows translocation of the polypeptide chain across the membrane of the RER. This translocation may be completed, giving rise to a luminal protein (e.g., a secretory protein), or may be incomplete, resulting in a membrane-spanning protein. In the latter case, the translocation process is arrested by a hydrophobic peptide that anchors the polypeptide in the lipid bilayer. Exactly how different luminal and membrane-bound proteins made in the RER are sorted out from one another and transported to their correct destinations is still an enigma.

The mechanisms for this process are likely to involve receptors for structural features of the proteins. By analogy with the signal peptide required for cotranslational insertion of polypeptides into the lumen of the RER, these could be represented either by short segments (transport signals) of the polypeptide chain, or by more general features of the protein as a whole. In the case of lysosomal proteins, for instance, it

is known that phosphomannosyl groups are necessary for correct delivery (13). For a plasma membrane protein which spans the lipid bilayer, a putative transport signal must be located in at least one of its three topological domains, these being the extracytoplasmic, the intramembranous and the cytoplasmic segments of its polypeptide chain. In the latter case, for instance, one might envisage a sorting and transport mechanism based on an interaction with cytoplasmic elements.

We are studying the transport of the membrane glycoproteins of Semliki Forest virus (SFV)<sup>1</sup> from their site of synthesis in the RER to the plasma membrane (PM), where they are recruited into virus particles. SFV has three membrane glycoproteins (E3, 66 amino acids; E2, 422 amino acids; and E1, 438 amino acids), two of which (E3 and E2) are made as a precursor protein (p62) (9). During infection, a single mRNA species of 4.1 kilobase (kb) directs the synthesis of first the capsid protein (C, 167 amino acids) and then the two mem-

<sup>1</sup> *Abbreviations used in this paper:* PM, plasma membrane; SFV, Semliki Forest virus; and SV, simian virus; cDNA, complementary DNA.

brane proteins p62 (E3 + E2) and E1. The two membrane proteins have an N-terminal signal sequence that initiates their translocation across the endoplasmic reticulum membrane. However, this process is not completed for either of the two chains; a hydrophobic stretch of amino acids, close to their C-termini, arrests translocation and anchors the polypeptides in the lipid bilayer such that 31 amino acid residues of the p62 chain and two amino acids of the E1 chain remain on the cytoplasmic side of the rough endoplasmic reticulum membrane. These two membrane glycoproteins form a complex with each other in the RER and are then transported via the Golgi apparatus to the PM. At a late stage during their transport the p62 protein is cleaved to E3 and E2. The E2 protein represents most of the p62 polypeptide chain, including the membrane-spanning and the cytoplasmic domains.

We have recently cloned and sequenced the cDNA transcribed from the SFV 4.1 kb mRNA (7, 8). When the cDNA was engineered into a Simian Virus (SV)40-derived expression vector and introduced into nuclei of baby hamster kidney (BHK) cells, we observed expression of all the structural proteins of the SFV (14, 15, 18). In this study, we used our cDNA expression system to analyze the importance of the cytoplasmic domain of the p62/E2 protein for its transport to the cell surface. (In many of our analyses we cannot distinguish between E2 and its precursor form p62. In these cases we will use the designation p62/E2 in the text. On the cell surface we assume only E2 to be present.) Several deletions were made in the gene region coding for the cytoplasmic domain of this protein. The truncated proteins were expressed in BHK cells and their intracellular migration was compared with that of the intact protein using immunofluorescence techniques. All p62/E2 protein mutants were found to be routed to the cell surface, thus suggesting that the structure of the cytoplasmic domain is not important for the transport of this protein from the RER to the PM.

## MATERIALS AND METHODS

**Enzymes:** Restriction endonuclease *Bgl*II, *Pst*I, and *Pvu*I were purchased from New England Biolabs (Beverly, MA) and *Clal* from Boehringer Mannheim (Mannheim, W. Germany). *Bam*HI and *Hind*III were gifts from V. Pirrotta (European Molecular Biology Laboratory [EMBL]). The exonuclease *Bal* 31 and the Kleenow fragment of *Escherichia coli* DNA polymerase were obtained from New England Biolabs. T4 DNA ligase was provided by F. Winkler (EMBL) and T4 polynucleotide kinase by H. Lehrach (EMBL).

**Oligonucleotides:** The dodecanucleotide containing the recognition sequence for the *Clal* restriction endonuclease was bought from Collaborative Research (Lexington, MA).

**Enzyme Reactions:** Endonuclease digestion were performed as recommended by the manufacturers.

Exonuclease digestions were performed using 2.5 U of *Bal*31 for 20  $\mu$ g linearized plasmid DNA in 100  $\mu$ l of a buffer containing 20 mM Tris, pH 8.0, 12 mM MgCl<sub>2</sub>, 12 mM CaCl<sub>2</sub>, 1 mM EDTA, and 300 mM NaCl. The exact length of incubation (~17 min at 30°C) was monitored by analyzing the reduction in size of the *Bal*31-treated DNA on an agarose gel as compared to suitable DNA length standards. At the end of the exonuclease reaction the sample was extracted with phenol and the DNA precipitated in ethanol.

Filling in of 5' sticky ends using DNA polymerase and all four desoxynucleotide triphosphates was performed as described in Bahl et al. (2). Ligation of the dodecanucleotide to linearized and blunt ended DNA molecules with T4 DNA ligase was performed using a 100-fold molar excess of linker molecules over plasmid molecules as reported before by Bahl et al. (2).

**Electrophoresis of DNA Fragments:** Separation of DNA fragments to be used for our constructions was done on a 0.8% low gelling temperature agarose gel. Appropriate bands were cut out and the agarose with the DNA was melted and used as such in the ligation reaction (14).

**Transformation of *E. coli*:** Strain DH1 was transformed with DNA in melted agarose as described in Villa-Kamaroff et al. (27).

**Extraction of Plasmid DNA:** Small scale plasmid preparations (~30  $\mu$ g DNA), used for restriction endonuclease screening purpose only, were obtained as described before by Holmes and Quigley (12). Plasmid DNA to be used for expression analyses were made in larger quantities (200–300  $\mu$ g) using the alkaline extraction procedure described by Birboim and Doly (3).

**Screening of *Bal* 31-treated Plasmids:** The plasmids were first screened for the presence of the recognition sequence of the *Clal* restriction endonuclease inserted at the deletion site (see Fig. 1) and then, for the size of the deletion, by analysing the fragments after a second digestion with *Pst*I. The latter endonuclease has four cleavage sites in pSV 2-SFV (8, 18). Two of these are located within the coding region for the SFV proteins: one resides 550 bp upstream from the *Pvu*I site that is about in the middle of the region coding for the cytoplasmic domain of the p62/E2 protein, and the other 841 bases further upstream in the p62 gene. Molecules that are deleted from the *Pvu*I site and recircularized with the *Clal* oligonucleotide will, upon a double digestion with *Clal* and *Pst*I, show a *Clal-Pst*I fragment of <550 bp and an intact 847 bp *Pst*I-*Pst*I fragment, or no small *Clal-Pst*I fragment at all, and a shortened 847 bp fragment depending on whether the deletion extends beyond the *Pst*I site at the gene-region coding for the cytoplasmic protein domain.

**Nucleotide Sequence Determination:** Deleted DNA molecules were cleaved with *Hind*III endonuclease at the unique *Hind*III site in the stop translation oligonucleotide (see Fig. 1). The staggered 5' ends were labeled with <sup>32</sup>P using  $\gamma$ -<sup>32</sup>P ATP and polynucleotide kinase as outlined in Maxam and Gilbert (16). The *Hind*III linearized DNA was incubated with *Eco*RI which cuts at two places, at the junction between the pBR 322 and the SV 40 DNA (see Fig. 1), and, depending on the extent of the deletion, 900–950 bp upstream from the unique *Hind*III site, that is, in the coding region of the p62 protein (not shown in Fig. 1, see reference 9). The 900–950 bp *Hind*III-*Eco*RI fragment was used for sequencing as described by Maxam and Gilbert (16).

**Introduction of DNA into Cell Nuclei:** Plasmid DNA was introduced into cell nuclei of BHK cells using either the calcium phosphate precipitation method (14) or microneedle injection (26).

**SDS Polyacrylamide Electrophoresis and Immunoblotting:** BHK cells that had been transfected by the precipitation method were incubated for 30 h to allow expression of the viral proteins and then processed for SDS gel electrophoresis. The cells were lysed at 0°C in a buffer containing protease inhibitors and 0.5% Triton X-114 and the nuclei removed by centrifugation at 15,000 g for 3 min (14). After raising the temperature to 30°C for 3 min, the membrane proteins partitioned preferentially into the detergent phase that was separated as a small oily pellet from the larger aqueous phase (supernatant) by slow centrifugation (4). For SDS gel electrophoresis the Triton X-114 pellet was solubilized directly in sample buffer (17). Blotting of proteins onto nitrocellulose paper after fractionation by SDS gel electrophoresis and subsequent detection of E2/p62 specific bands using rabbit anti-E2 antibodies and anti-rabbit antibodies conjugated to horseradish peroxidase were carried out according to the procedures described by Burnette (6) as modified by Burke et al (5).

**Indirect Immunofluorescence:** Viral proteins expressed in the BHK cells were visualized by both surface and internal staining (1).

## RESULTS

### Strategy of Engineering

The gene region coding for the cytoplasmic domain of the p62/E2 protein was deleted from the expression plasmid pSV 2-SFV (Fig. 1) (15). This plasmid contains the complete coding region for the structural proteins of SFV. It was first cleaved at a site downstream from the cytoplasmic gene region and then treated with an exonuclease until various lengths of this region were removed. In a second step we inserted at the deletion site a synthetic oligonucleotide which encodes stop codons in all three reading frames (19). This ensures prompt termination of translation after the deleted p62 protein has been read by the ribosomes on the mRNA molecule.

### Deletion of the Gene Region Coding for the Cytoplasmic Domain of the p62 Protein

The plasmid pSV 2-SFV was cleaved partially with *Pvu*I. This endonuclease has two recognition sites in the plasmid molecule, one in the ampicillinase gene of the pBR 322

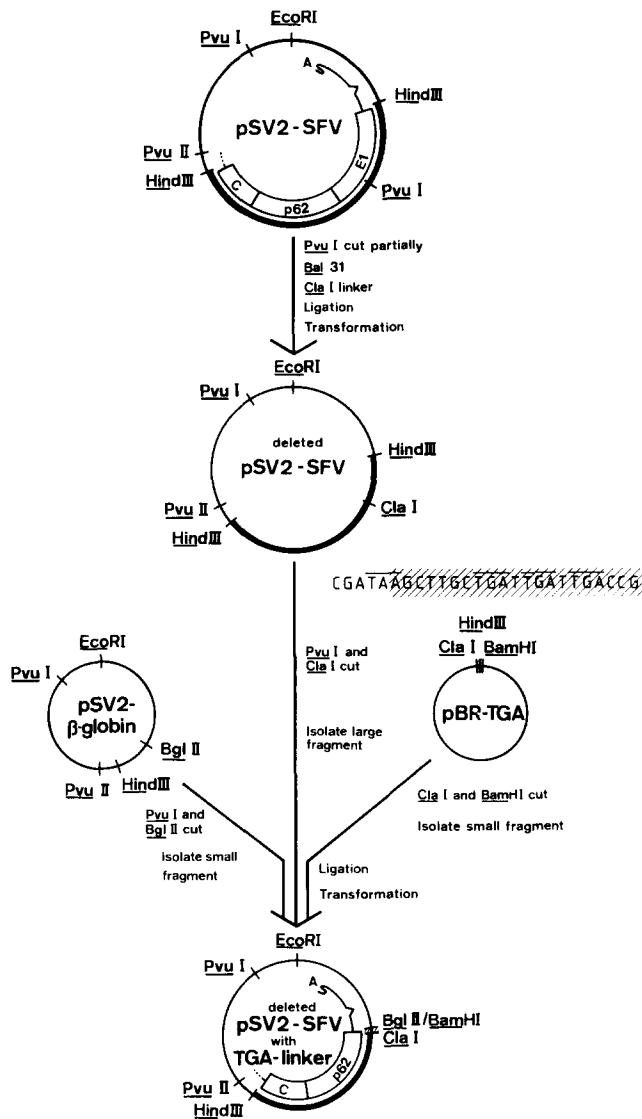


FIGURE 1 Deletion of the gene-region coding for the cytoplasmic domain of the p62/E2 protein—a construction scheme. pSV 2-SFV represents the plasmid molecule that we used before for expression of the structural proteins of SFV (15). In this molecule the coding sequences for the SFV proteins (C, p62, and E1; the thick line in the circle) is inserted between two *Hind*III sites, downstream from the SV 40 early promoter (between the sites for *Pvu*II and *Hind*III) and upstream from the SV 40 sequences encoding the small t intron and the polyadenylation signal for early SV 40 mRNAs (between the sites for *Hind*III and *Eco*RI). The sequence between *Pvu*II and *Eco*RI is derived from plasmid pBR 322 and includes the origin for plasmid replication and the gene for ampicillin resistance. The vector portion of pSV 2-SFV is derived from the plasmid pSV 2-beta-globin (18). Plasmid pBR-TGA contains the 21 bp stop translation oligonucleotide between the sites for *Hind*III and *Bam*HI (19). The plasmid pSV 2-SFV was cleaved partially with *Pvu*I that cleaves in the E1 gene and in the ampicillinase gene of the pBR 322 sequences. Using the exonuclease *Bal*31, DNA was digested until the gene-region encoding the cytoplasmic domain of the p62/E2 protein was completely or partially removed. The deleted molecules were circularized with a *Cla*I linker oligonucleotide and used for transformation of *E. coli*. On the basis of mapping with restriction endonucleases *Cla*I and *Pst*I four molecules were selected to have the desired deletions in the gene region encoding the cytoplasmic protein domain of p62/E2. Through a three-fragment ligation the 26 bp *Cla*I-*Bam*HI fragment (from plasmid pBR-TGA [19]) encompassing the stop translation oligonucleotide (the hatched part of the

portion of the molecule and another in the E1 gene ~500 bp downstream from the 3' end of the p62 gene (Fig. 1) Conditions of digestion were chosen such that production of linearized molecules was maximized. The restricted DNA molecules were incubated with the exonuclease *Bal* 31 to remove 500–600 bp from each end. This corresponds to a partial or complete removal of the gene region encoding the cytoplasmic domain of the p62/E2 protein in such DNA molecules that have been restricted at the *Pvu*I site in the E1 gene.

The shortened DNAs were polished at their ends using T4 DNA polymerase and linked to a dodecanucleotide containing the recognition sequence of the *Cla*I enzyme. This facilitated the subsequent screening of deletion mutants and allowed us to insert the stop translation oligonucleotide in a convenient way (see below). The linear plasmid molecules were isolated through electrophoresis in a low gelling temperature agarose gel, circularized with T4 DNA ligase and used for transformation of *E. coli*.

50 colonies were picked and grown up for small-scale plasmid preparation. The plasmids were characterized with respect to their deletions using restriction endonucleases. 41 clones contained a unique *Cla*I site that was always located in the E1 gene, never in the ampicillin gene. This was expected since we selected for ampicillin-resistant bacterial colonies. Using a *Pst*I-*Cla*I double digestion (see Materials and Methods) we found four plasmid molecules with deletions of the gene region coding for the cytoplasmic domain of the P62/E2 protein.

### Insertion of the Stop Translation Oligonucleotide

The stop translation oligonucleotide was excised from the plasmid pBR-TGA as a 26 bp *Cla*I-*Bam*HI fragment and isolated by electrophoresis on a low gelling temperature agarose gel. This fragment includes 5 bp from pBR322 in addition to the synthetic portion (21 bp) that has been inserted between the *Hind*III and the *Bam*HI sites of the plasmid (Fig. 1) (19). The 5 bp sequence adds a fourth stop codon (TAA) into the *Cla*I-*Bam*HI fragment.

The stop translation oligonucleotide was first linked at the end of the shortest p62 gene through a three-fragment ligation involving the 28 bp *Cla*I-*Bam*HI fragment from pBR-TGA (fragment I), the larger *Pvu*I-*Cla*I fragment from the pSV 2-SFV molecule with the largest deletion (fragment II), and the smaller *Pvu*I-*Bgl*II fragment from plasmid pSV 2-beta-globin (fragment III). Fragment II contains the SV 40 early promoter, SFV sequences (the capsid and the deleted p62 gene), and pBR322 DNA including the origin for plasmid replication and part of the ampicillinase gene. The rest of the ampicillinase gene and the SV 40 sequences specifying the small t intron and the polyadenylation signals for the RNA are all encoded by the fragment III. Fragments II and III were both isolated by electrophoresis in an agarose gel together with fragment I and the ligation was done in the presence of melted agarose. Transformation and screening for ampicillin-resistant colonies resulted in the isolation of the desired plasmid clone designated pSV 2-SFV d-1. The d-1 stands for the largest deletion in the p62 gene. This plasmid differs from the original pSV 2-SFV plasmid in three respects: (a) it contains a deleted

sequence) was linked to the deleted p62/E2 gene. In this step the E1 gene was left out from the final construct. The designation TGA-linker refers to the stop translation oligonucleotide.

p62 gene that is (b) followed by a stop translation oligonucleotide, and (c) it is lacking the E1 gene completely. The plasmids pSV 2-SFV d-2, 3, and 4 were constructed by joining the larger *PvuI-ClaI* fragments of respective deleted pSV 2-SFV molecules with the smaller *PvuI-ClaI* fragment of the plasmid pSV 2-SFV d-1.

### Nucleotide Sequencing of the Deleted p62 Genes

Each mutant gene was sequenced using 5' end-labeling at the *HindIII* site of the stop translation oligonucleotide and base specific cleavage reactions as described by Maxam and Gilbert (16). A common sequence representing part of the *HindIII* recognition site as well as that of *ClaI* was found next to the labeled end in each mutant. This was followed by the p62 gene sequence starting at the point of the deletion for the respective mutant. In one case we used an upstream *AvaI* site for sequencing downstream all the way across the stop translation oligonucleotide (H. Garoff, unpublished observations). This sequence confirmed the structure of the stop translation oligonucleotide as well as its proper insertion in the DNA molecule.

Using the nucleotide sequence data it was possible to deduce the primary structures for the cytoplasmic domains of each mutant protein (Fig. 2). The p62/E2 mutant encoded in pSV 2-SFV d-1 has lost almost the whole of its original 31 amino acids long cytoplasmic domain, only the basic Arg-Ser-Lys sequence adjacent to the membrane spanning segment is left. The stop translation oligonucleotide adds eight amino acid residues at the C-terminal end of this mutant protein. The pSV 2-SFV d-2 and d-3 sequences yield cytoplasmic domains with seven and twelve original amino acid residues, respectively, and seven additional ones. The pSV 2-SFV d-4 molecule encodes a cytoplasmic domain comprising 15 original and six additional amino acid residues.

### Characterization of Mutant Proteins by Electrophoresis in SDS Polyacrylamide Gels

Subconfluent monolayer cultures of BHK cells were transfected with the four deleted DNA molecules by the calcium phosphate precipitation method. After incubation for 30 h at 37°C, cells were lysed and membrane proteins were extracted with Triton X-114. The four samples were run on an SDS gel together with two controls, namely, a Triton X-114 extract from SFV-infected cells and Triton X-114 extract from cells transfected with the wild-type DNA, the pSV 2-SFV. p62/E2 proteins were detected using an immunoblotting technique. In an earlier report, we have shown that when SFV proteins are expressed from the pSV 2-SFV DNA, the E2 protein reaches the cell surface without the E1 protein which remains in the RER (15). Under these circumstances (14) very little of the p62 protein appears to be processed to E2, most of it being incorrectly cleaved into a protein that in SDS gel analyses migrates somewhat slower than authentic E2. Apparently the p62 protein needs to be complexed with E1 in order for correct processing to occur. The band pattern of p62/E2 proteins expressed from pSV 2-SFV has been described in detail in the preceding report and is shown in Fig. 3, lane 1, for comparison. Three bands are seen: one band which co-migrates with the smallest forms of the p62 protein of the infected cell lysate (Fig. 3, lanes 2 and 9), a second band that is the most intense one and that migrates slightly more slowly than E2 and a third band that co-migrates with E2. It is evident that our deletion mutants which all lack the E1 gene express essentially the same pattern of p62/E2 proteins as the pSV 2-SFV DNA (Fig. 3, lanes 3-8). However the whole band pattern is shifted towards lower molecular weights, exactly as would be expected from the extent of the deletions. The pSV 2-SFV d-1, 2, 3, and 4 DNAs should express proteins which contain 11, 14, 19, and 20 amino acid residues, respectively, in their cytoplasmic domains in con-

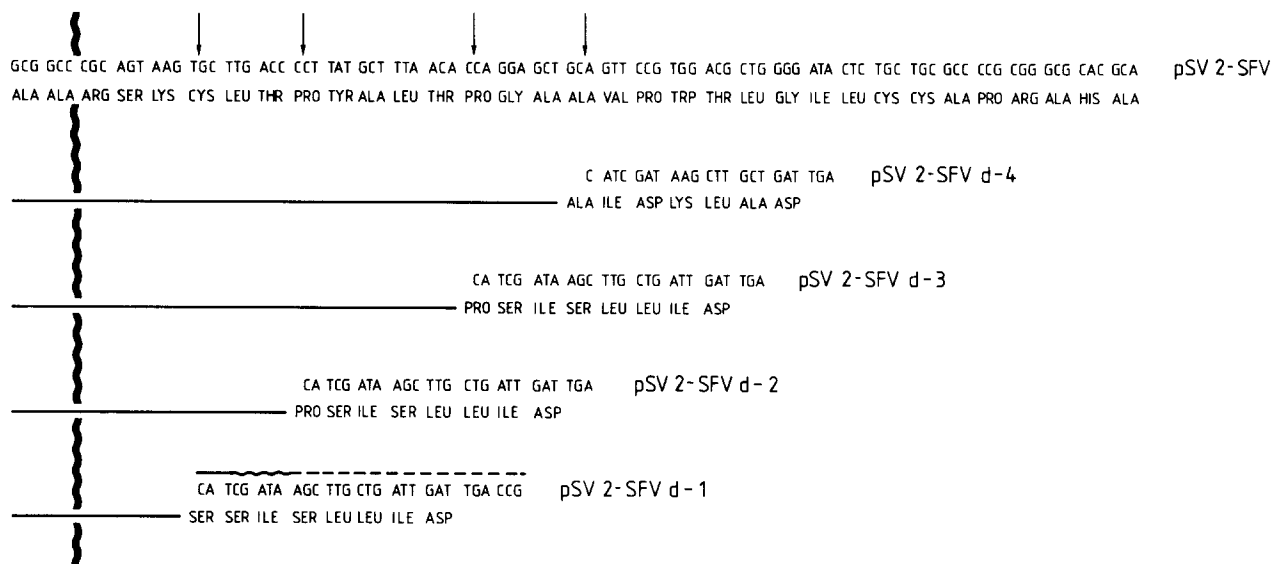


FIGURE 2 Nucleotide sequences of the gene region encoding the cytoplasmic domains of the p62/E2 proteins in the plasmid pSV 2-SFV and in the deleted plasmids pSV 2-SFV d-1, 2, 3, and 4. The deduced amino acid sequences are shown below the base sequences. The arrows indicate the extent of the gene deletion (from 3' to 5'). The *ClaI* linker and the 26 bp *ClaI-BamHI* fragment that contains the stop translation oligonucleotide adds various amino acid residues to the deleted p62/E2 protein depending on which reading frame is used and when the first stop codon is encountered. In pSV 2-SFV d-1 is depicted the origin of the extra base sequences; the first three nucleotides below the straight line originate from the *ClaI* linker, the next five ones below the wavy line from pBR 322, and the rest represent the synthetic stop translation oligonucleotide (19).

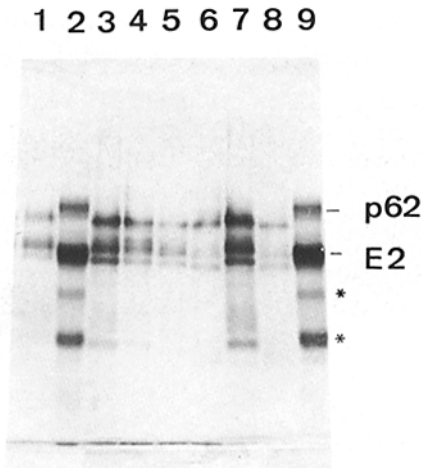


FIGURE 3 Immunoblot analysis of E2 and its precursor p62 expressed from pSV 2-SFV and the deletion derivatives pSV 2-SFV d-1, 2, 3, and 4. Lysates of BHK 21 cells transfected with respective DNAs were extracted with Triton X-114, and the detergent phase was run on a 10% acrylamide gel containing SDS. Proteins were then blotted onto a nitrocellulose filter and E2 and its precursor p62 labeled using rabbit anti E2 followed by peroxidase-conjugated sheep anti-rabbit IgG. The labeled bands were visualized by means of the peroxidase reaction using diaminobenzidine as substrate. Lane 1 shows the proteins expressed from pSV 2-SFV while lanes 3–6 show the proteins expressed from the deletion derivatives pSV 2-SFV d-1, 2, 3, and 4, respectively. Lanes 7 and 8 show the proteins expressed from the largest (pSV 2-SFV d-4) and the smallest (pSV 2-SFV d-1) deletion molecules, clearly indicating the size difference between the two. Lanes 2 and 9 show control samples from SFV-infected cells. p62 and E2 proteins are indicated on the sample from SFV-infected cells (lane 8). The stars indicate bands that represent p62/E2 degradation products. These increased with every freeze/thawing of this sample (compare an earlier analysis of the same sample in Fig. 4*b* of the preceding paper).

trast to the 31 residues present in the same domain of the wild-type protein.

#### Cell-surface Expression of Mutant Proteins

The cellular location of the unmodified and mutant p62/E2 proteins was studied by immunofluorescence labeling of BHK cells following microinjection of the four deleted DNAs and the original wild-type DNA, pSV 2-SFV, into cell nuclei. Two times 50 cells were injected with each sample, incubated for 8 h, and then processed for both internal and surface labeling. As shown in Fig. 4, *f–j*, all four deleted DNAs express E2 protein on the cell surface as does the pSV 2-SFV DNA. Inside the cell both the mutant and wild-type proteins showed the staining pattern typical of polypeptides made in the RER and then transported to the cell surface via the Golgi complex (Fig. 4, *a–e*). Thus, in addition to plasma membrane labeling, both a reticular pattern extending throughout the cell cytoplasm and a strong perinuclear fluorescence were also apparent. Previous studies have shown that these structures correspond to the RER and Golgi regions, respectively (11).

To compare the rates by which the different mutant and wild-type proteins reached the plasma membrane, cells mi-

croinjected with the various DNAs were incubated at 37°C for 1, 2, 4, and 6 h prior to fixation and surface labeling. In all cases, the earliest detectable surface fluorescence was seen after a 2-h incubation. This suggests that both the wild-type p62/E2 and the mutant p62/E2 polypeptides containing cytoplasmic deletions are transported to the cell surface with similar rates.

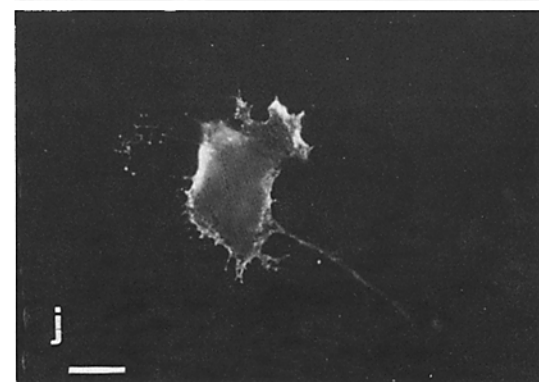
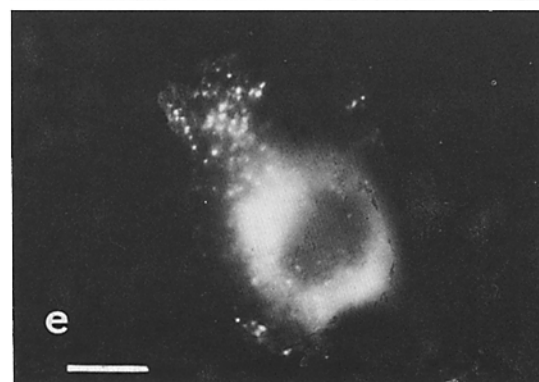
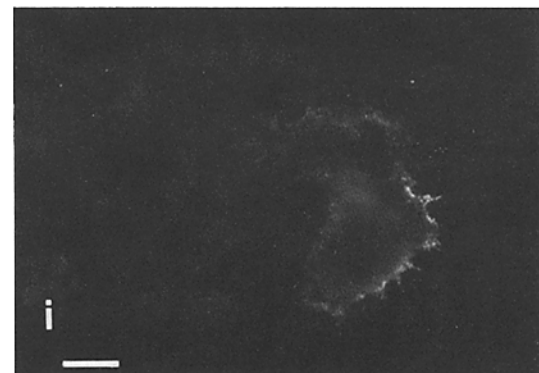
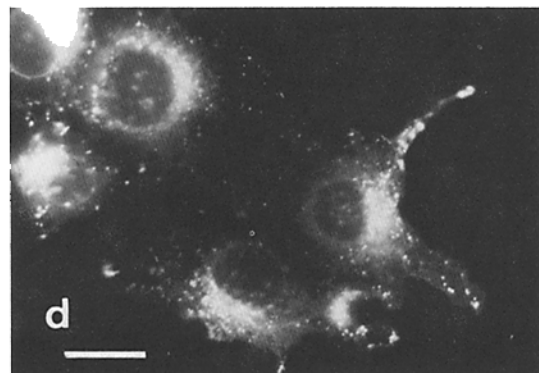
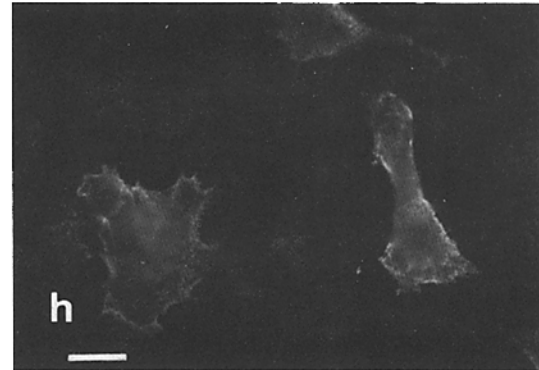
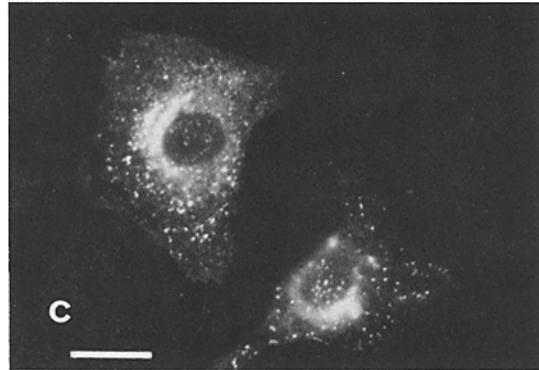
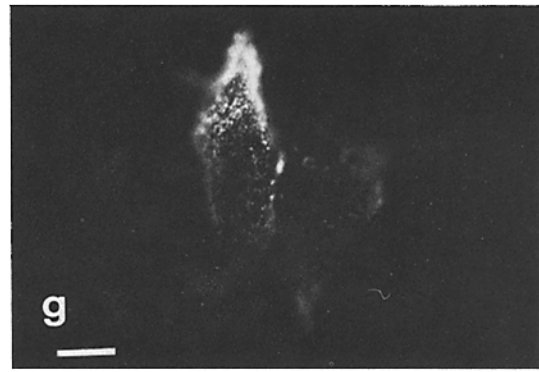
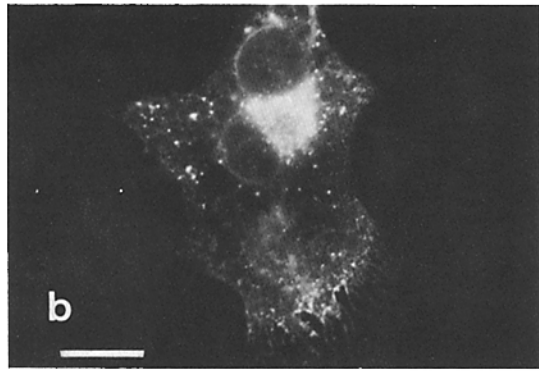
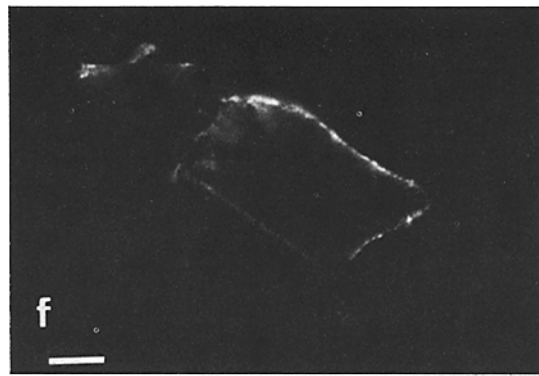
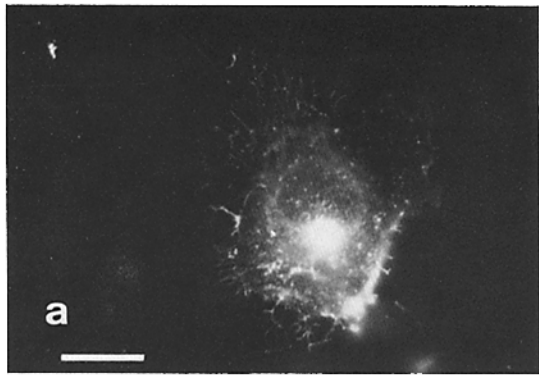
#### DISCUSSION

Our results show that p62/E2 proteins with cytoplasmic deletions are expressed from the pSV 2-SFV d-1, 2, 3, and 4 DNAs and that these mutants become transported from their site of synthesis in the RER to the PM with rates similar to that of the wild-type protein. As the structures of the cytoplasmic domains of the mutant and the wild-type proteins vary substantially in both length and amino acid sequence, we find it unlikely that this domain of the p62/E2 protein carries any features essential for its intracellular sorting or its transport to the cell surface in BHK cells. The only common structure of the various cytoplasmic domains is the basic Arg-Ser-Lys sequence adjacent to the membrane-spanning peptide. Basic residues on the cytoplasmic side of the membrane-spanning segment are a feature common to all viral and plasma membrane glycoproteins so far studied (9, 22). It has been suggested that these residues, in combination with the membrane-spanning peptide, play a role in arresting the translocation of the polypeptide across the RER membrane during synthesis and/or anchoring it in the lipid bilayer (22). However, our results cannot rule out the possibility that these basic residues are also necessary for transport to the cell surface.

If the structural information for cell surface transport of the p62 protein is not localized in its cytoplasmic domain as suggested by our results, it has to be in its intramembranous region or in its large luminal portion. This might also be true for other viral glycoproteins such as the G protein of vesicular stomatitis and the hemagglutinin molecule of influenza virus. Deletions involving the whole of the membrane-spanning region as well as the cytoplasmic protein domain have been made for both membrane proteins using cloned cDNA (10, 21, 25). Expression of these protein mutants in cells derived from African Green Monkey kidney (COS1 cells and CV 1 cells, respectively) showed that they were effectively secreted.

A comparison of the primary structures of the cytoplasmic domain of the p62/E2 protein in the related SFV and Sindbis viruses shows a striking homology (20). This would imply that this protein domain does indeed have an important function, if not for transport to the cell surface, then possibly for virus assembly. It is thought that the viral nucleocapsid recognizes and binds to the cytoplasmic domain of the spike glycoproteins at the host PM and thereby surrounds itself with a viral envelope containing only virus-specific glycoproteins (24). There are several pieces of evidence for an interaction between the viral nucleocapsid and the spike glycoproteins both in the virus particle and at the PM of the infected cell (24). However, direct evidence that the cytoplasmic domain is involved is still lacking. Such evidence could, however, be obtained through the use of the wild-type and the mutant p62 proteins expressed from our DNA constructions

FIGURE 4 Indirect immunofluorescence analyses of BHK 21 cells microinjected with pSV 2-SFV (*a* and *f*), pSV 2-SFV d-1 (*b* and *g*), pSV 2-SFV d-2 (*c* and *h*), pSV 2-SFV d-3 (*d* and *i*), and pSV 2-SFV d-4 (*e* and *j*). The cells in *a–e* are processed for internal staining ( $\times 520$ ) of E2/p62 proteins and the remainder for surface staining ( $\times 384$ ). Bars, 20  $\mu\text{m}$ .



in complementation experiments involving mutants of SFV that are defective in the transport of their own glycoproteins to the cell surface (23). If such an SFV mutant could be made to mature using the wild-type p62 protein but not those possessing cytoplasmic deletions, it would demonstrate that the cytoplasmic domain is involved in the budding process.

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