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Different Membrane Anchors Allow the Semliki Forest Virus Spike Subunit E2 to Reach the Cell Surface

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The Semliki Forest virus spike subunit E2, a membrane-spanning protein, was transported to the plasma membrane in BHK cells after its carboxy terminus, including the intramembranous and cytoplasmic portions, was replaced by respective fragments of either the vesicular stomatitis virus glycoprotein or the fowl plague virus hemagglutinin. The hybrid proteins were constructed by cDNA fusion. Upon a transient expression they could be localized at the cell surface by immunofluorescence with specific antibodies directed against any of the protein fragments.

The spike subunits E1 and E2 of the Semliki Forest virus (SFV) envelope are integral membrane proteins. They are translocated into the lumen of the rough endoplasmic reticulum during translation. E1 and p62 (the precursor of E2 and E3) are anchored in the membrane of the rough endoplasmic reticulum with a spanning hydrophobic segment, leaving a small portion of the molecules extending into the cytoplasm. This orientation of the subunits is maintained during their transport to the plasma membrane (for a review see reference 5). Putative transport signals have been studied by using cDNA expression in BHK cells. E2 contains all of the information necessary for its own surface transport (12) even when its cytoplasmic portion is deleted (4). After additional deletion of the intramembranous peptide, the truncated E2 could not be detected at the cell surface or in the culture medium (Henrik Garoff, European Molecular Biology Laboratory, personal communication). The present study shows a restoration of the surface transport of the truncated E2 by fusion with C-terminal fragments of the vesicular stomatitis virus (VSV) glycoprotein (G) or the fowl plague virus (FPV) hemagglutinin (HA) containing the respective membrane anchor sequences. When expressed from cDNA, the hybrid proteins were transported to the plasma membrane, suggesting that different membrane anchors enable the surface transport of E2.

This study is based on a transient expression analysis of cDNAs which were inserted into a simian virus 40 expression vector similar to pSV-2 (17). Circular cDNAs were introduced into BHK cells by either calcium phosphate transfection or microneedle injection. Expression was monitored either by separating proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western immunoblotting or by indirect immunofluorescence.

A map of all the E2 derivatives analyzed is shown in Fig. 1a. Complete E2 was expressed from a vector, pSV-SFV, which is identical to pSVS-SFV described in reference 11. Truncated E2d5 was expressed from the plasmid pSV2-SFVd5 (Fig. 2), which was provided by Henrik Garoff (unpublished data), and is similar in construction to the vectors described in reference 4. The hybrid proteins E2d5-G and E2d5-HAF were expressed from the vectors pSV-SFVd5-G and pSV-SFVd5-HAF, which code for C-terminal fragments of VSV G or FPV HA, respectively (Fig. 2). The correct structure of the constructs was confirmed by cDNA restriction analysis or sequencing (data not shown).

The proteins coded by the new constructs were first analyzed by Western immunoblotting 36 h after transfection of BHK cells with the vector DNAs described above. Cell lysates and culture media were characterized separately by SDS-PAGE. Proteins were transferred to nitrocellulose filters and stained with a specific anti-E2 serum. A similar cDNA expression study followed by Western immunoblotting has already been carried out with the complete E2 spike subunit (11) and with truncated forms of E2 lacking different portions of its cytoplasmic domain (4). All constructs were detected at the cell surface in immunofluorescence experiments, and in all cases a heterogeneity of protein bands representing the precursor p62 and the processed E2 was observed by SDS-PAGE (4). In experiments in which E1 was present at the cleavage stage, most of the p62 was processed into E2 (11), whereas in the absence of E1 a significant portion of p62 remained uncleaved (4, 11). It was therefore suggested that the aggregation of p62 with E1 may be required for the correct cleavage of p62 (4). This cleavage to form the final products E2a and E3 takes place near the cell surface at a late stage during the intracellular transport of the SFV spike (25). Thus, this cleavage can be used to monitor the intracellular transport of E2.

The protein banding pattern of the expression products described in this work is shown in Fig. 1b. Specific proteins could not be detected in the culture medium, which indicates that at most only a small fraction of the proteins is secreted from the cells. Complete degradation of the secreted products in the culture medium is highly unlikely, since membrane-bound hybrid proteins whose extracellular domains protrude into the culture medium were detectable in significant amounts. E2 expressed from pSV-SFV in the presence of E1 was found in the cell lysate. It comigrated with the E2 synthesized during SFV infection (Fig. 1b, arrowhead), indicating that the cDNA expression product is cleaved efficiently into the final form, E2. Traces of the precursor p62 were only visible on the original filter. For the membrane-anchorless mutant E2d5 expressed from pSV-SFVd5 in the absence of E1, only forms which migrated much slower than processed E2 were detected, and they probably represent forms of p62 (Fig. 1, asterisk). Thus, E2d5 may escape cleavage because it does not reach the cell surface. For the hybrid molecules E2d5-G and E2d5-HAF expressed

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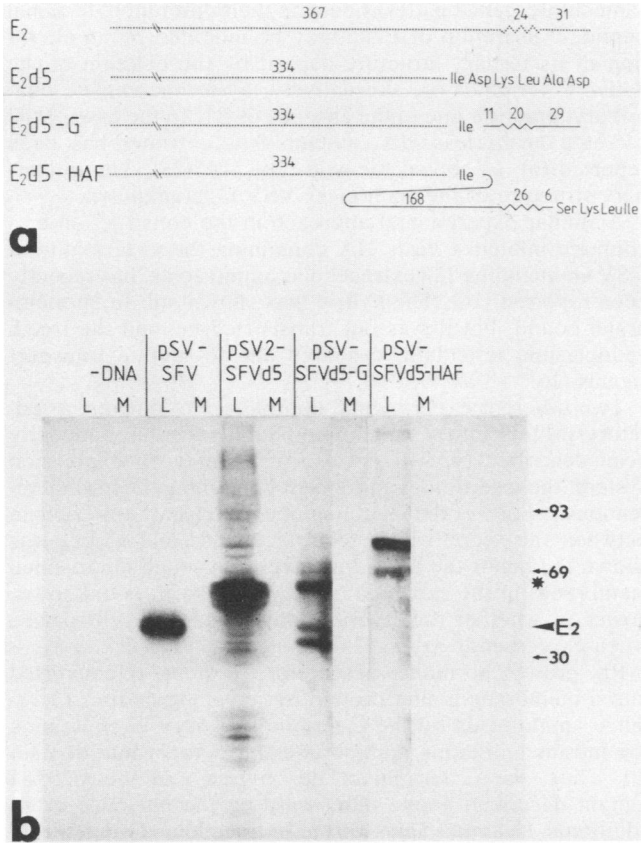

FIG. 1. (a) Map of the E2 derivatives. Portions of E2 are represented by a dotted line, and fragments of VSV G and FPV HA are shown by a solid line. The intramembranous peptides are shown by a zigzag line. New amino acids introduced by cloning are shown by their abbreviations. The lengths of the extracellular, intramembranous, and cytoplasmic domains are given as the number of amino acids. (b) Western immunoblotting analysis of the cDNA expression products. After 36 h of calcium phosphate transfection of BHK cells with the vectors indicated, Triton X-114 cell lysates (L) or the culture media (M) were analyzed by SDS-PAGE. Untransfected cells were used as a control (−DNA). The migrations of p62 and E2 isolated from SFV-infected BHK cells are indicated by an asterisk and an arrowhead, respectively. Molecular weight standards are indicated on the right ($\times 10^3$). BHK cells were grown as described previously (8). Semiconfluent cells were transfected with vector DNA by a calcium transfection procedure (7) modified as described by Kondor-Koch et al. (11). After 36 h of incubation at 37°C, the culture medium (which had been changed to serum-free medium after 30 h) was analyzed after precipitation with 10% trichloroacetic acid. Triton X-114 cell lysates were prepared as described previously (11). Trichloroacetic acid extracts and Triton X-114 detergent phases (for pSV2-SFVd5 whole-cell Triton X-114 lysates) were processed by SDS-PAGE (10% acrylamide) (14). Proteins were blotted onto nitrocellulose filters (3) and detected with a specific rabbit anti-E2 serum (kindly provided by H. Garoff) and a peroxidase-conjugated sheep anti-rabbit IgG (supplied by the Institut Pasteur, Paris) (2).

in the absence of E1, heterogeneously migrating forms were observed. For E2d5-G, this pattern was very similar to that observed for complete E2 (11) or for the truncated membrane-bound forms of E2 (4) when expressed in the absence of E1. This is consistent with the primary structure of E2d5-G, which is only 27 amino acids shorter than complete E2, and suggests surface transport of those hybrid molecules from which E3 has been cleaved. The protein banding pattern of E2d5-HAF is consistent with the significant rise in molecular weight expected from the introduction of 168 extracellular amino acids from FPV HA which include two putative carbohydrate attachment sites (18). Because no specific antisera directed against E3 were available, further characterization of the multiple migrating forms regarding the cleavage of E3 was not possible.

Appearance of the proteins at the plasma membrane was monitored by surface immunofluorescence of unpermeabilized cells with different antisera (Fig. 3). E2 was detected at the plasma membrane as previously described (11, 12). E2d5 has never been observed at the cell surface (H. Garoff, personal communication). In cells permeabilized with Triton X-100, the internal fluorescence showed a Golgi-like and an endoplasmic reticulum-like staining (13) of structures around the nucleus and a network throughout the cytoplasm which was not detected near the cell surface. The same pattern was observed upon expression of the mutant E2d5′ (data not shown). This construct was expressed from a vector similar to pSV-SFVd5-HAF in which the *Sal*I-*Hin*dIII restriction fragment coding for the C terminus of FPV HA was inserted in the wrong orientation (Fig. 2b). The expression product was a truncated E2 similar to E2d5 which was expected to contain 26 extra C-terminal amino acids, as opposed to the 6 extra amino acids of E2d5. Neither of the anchorless constructs could be detected at the plasma membrane. Surface expression was clearly observed for the hybrid proteins E2d5-G with anti-E2 serum and E2d5-HAF with anti-E2 serum (data not shown) and anti-FPV HA serum. This indicates that the latter antiserum recognizes domains in the HA2 region. Upon permeabilization of the cells with Triton X-100, E2d5-G could also be detected internally with antibodies specifically directed against the cytoplasmic domain of VSV G. These data suggest that the primary structure of the hybrid proteins and their orientation in the plasma membrane are as expected from the cDNA fusion.

At present, detailed protein structure data are available for only one of the three membrane proteins used in this study, FPV HA (23). Thus, it is not known how the truncation of E2 and its fusion with foreign peptides alter its conformation. The mapping of putative transport signals in these proteins is severely hampered by this fact, since a lack of transport can be due to either a lack of signal or a cryptic signal. A cryptic signal is a part of a protein (modified or unmodified) which has lost the potential to be recognized as a signal as a result of a structural alteration of the protein in which it occurs.

The observation that E2d5 was not secreted into the culture medium in detectable amounts differs from those made for other viral envelope proteins. Anchorless mutants of influenza virus HA (strains other than FPV) (6, 21) and of VSV G (19, 20) are secreted out of the cells into the culture medium, although at a slow rate for the latter molecules. Thus, these mutants still contain the information needed for their export to the cell surface. In the case of E2d5 this information is either cryptic or lost. It is unlikely that the presence of six new C-terminal amino acids in E2d5, which were introduced by the construction of the mutant, alters the intracellular expression of E2d5 dramatically, since the same six amino acids were also attached to one of the membrane-bound C-terminal deletions of E2 described by Garoff et al. (4). No differences in expression were observed when this deletion mutant was compared with similar E2 mutants to which different peptides had been attached (4). Similarly, proteins from the expression of E2d5 and E2d5′ gave the

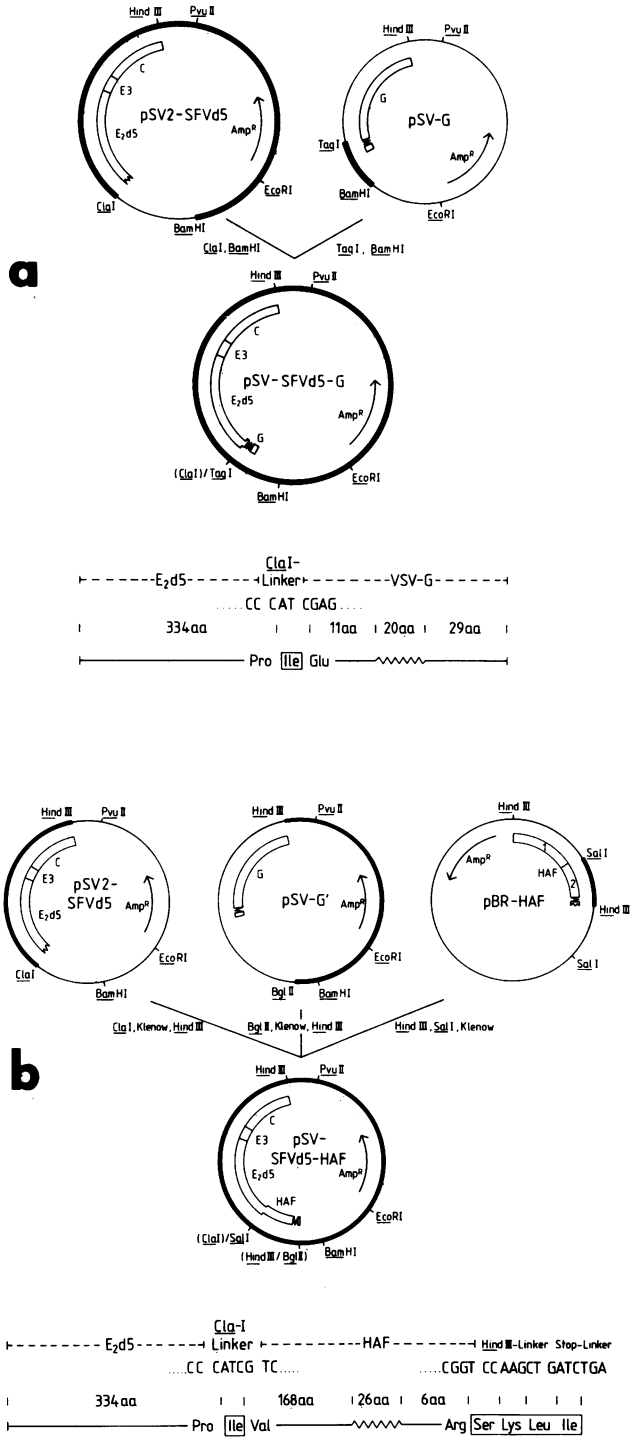

FIG. 2. Construction scheme of pSV-SFVd5-G (a) and pSV-SFVd5-HAF (b). The DNA fragments represented by heavy lines were ligated to form the final vectors. Protein-coding regions are shown by boxes, and intramembranous peptides are shown by zigzag lines. The origin of the different DNA fragments is indicated at the bottom of panels a and b together with the deduced composition of the hybrid proteins. All sequences are shown as derived from the respective publications (see references 4, 11, 18, and 19). pSV2-SFVd5 was a gift from H. Garoff (unpublished data) and is constructed like the vectors described in reference 4. pSV-G and pSV-G' contain the complete VSV G-coding cDNA followed by a stop-linker fragment similar to that described by Kondor-Koch et al. (11), all of which is cloned into a vector derived from pSV-2 (provided by Mulligan and Berg [17]) lacking splice signals. pBR-HAF is identical to pBR-FPV4 (provided by Porter et al. [18]) and contains cDNA coding for most of the FPV HA. Constructs were made by standard procedures (15). C, SFV capsid protein; aa, amino acids.

same fluorescence patterns despite their different C-terminal peptides. Secretion of E2d5 may be inhibited by an alteration of its tertiary structure caused by the deletion of the native C terminus or, alternatively, by an incomplete quaternary structure due to the absence of E1. In the case of the secreted anchorless HA, assembly as a trimer has been reported (6), as shown for wild-type HA (23). The quaternary structure of the anchorless VSV G is unknown.

A similar experimental approach in the construction of a chimeric influenza virus HA containing the C terminus of VSV G, including 124 extracellular amino acids, has recently been reported (16). The hybrid was shown still to be membrane bound, but it was not transported beyond the rough endoplasmic reticulum, probably due to cryptic transport signals (16).

Two different experimental approaches to convert a secretory protein into a membrane-bound form have recently been described (9, 24). By use of an in vitro expression system, the insertion of a fragment containing the intramembranous peptide of the immunoglobulin M (IgM) heavy chain between the secretory proteins lactamase and globin was shown to anchor the hybrid molecules in rough microsomal membranes in the expected orientation (24). It is unknown, however, whether this hybrid would reach the cell surface when expressed in vivo.

Rat growth hormone, a secretory protein, is converted into a membrane-bound protein by replacing its four C-terminal amino acids by the C terminus of VSV G containing the intramembranous peptide and the cytoplasmic domain (9). Thus, the C termini of this hybrid and the E2d5-G mutant described above differ only by the presence of an additional 11 amino acids of the extracellular G domain in E2d5-G. The membrane-bound growth hormone is transported to the Golgi complex but not to the plasma membrane in COS-1 cells (9). It is not known whether a surface transport signal is absent or cryptic in this protein.

The present study shows the restoration of surface transport of the membrane-anchorless mutant E2d5 in vivo by the fusion of C-terminal fragments of VSV G and FPV HA containing the cytoplasmic domains, the intramembranous peptides, and parts of the extracellular domains of these proteins. For SFV E2 (4) and VSV G (20), deletion studies of the cytoplasmic domains have shown that the truncated molecules are still transported to the cell surface as long as the complete intramembranous peptide is present. This is consistent with the results obtained with the hybrid E2d5-HAF, in which the cytoplasmic domain (of HA) is incomplete (Fig. 2) owing to partial cloning of the cDNA (18). These data suggest that it is not the cytoplasmic domain that contains the signals for surface transport of the protein molecules. Instead, specific surface transport signals may be located in the newly introduced extracellular or intramembranous domains of the proteins. The latter is suggested by the hybrid E2d5-G because the remaining extracellular part of VSV G is very short (11 amino acids). In the hybrid E2d5-HAF, 168 amino acids of the extracellular domain of FPV HA are present. This construct shows that the position of the intramembranous peptide relative to E2d5, when compared with wild-type E2 and E2d5-G, is not critical for

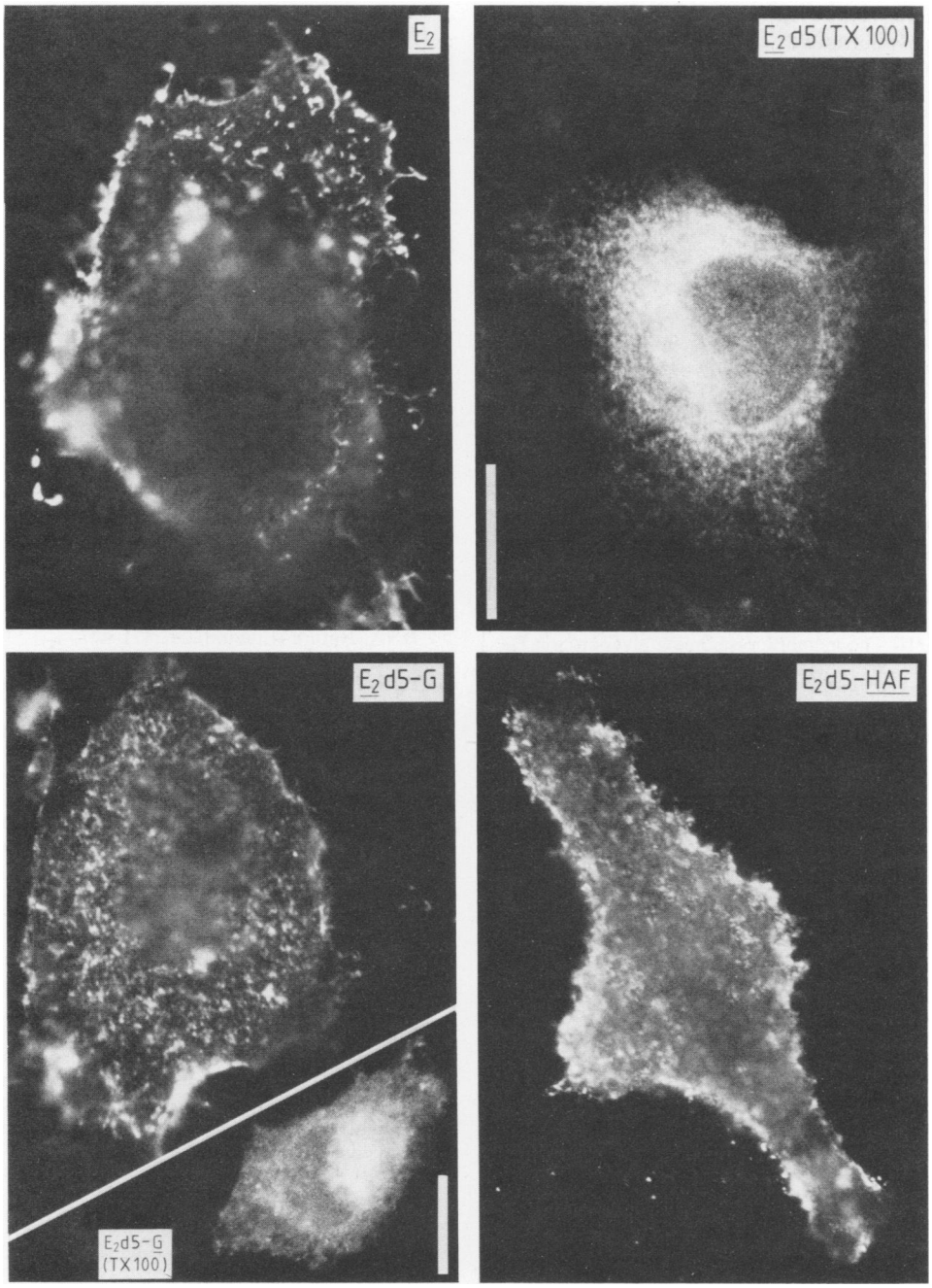

FIG. 3. Cell surface expression of the hybrid proteins E2d5-G and E2d5-HAF. For indirect immunofluorescent staining, cells were used either intact or after permeabilization with Triton X-100 (TX-100). Different antisera directed against the domains underlined in the figure were used. Vector DNAs at a concentration of 1 μg/μl were microinjected with glass capillaries into the nuclei of subconfluent BHK cells grown on cover slips. A de Fonbrune setup was used as previously described (22). After 14 h of incubation (or 36 h after transfection with pSV-SFVd5-HAF [see the legend to Fig. 1]), cells were fixed with paraformaldehyde and processed for indirect immunofluorescence (1). The different rabbit antisera used were directed against E2 (provided by Brian Burke, EMBL), FPV HA (provided by Karl Matlin, EMBL), or the 15 C-terminal amino acids of VSV G (provided by Thomas Kreis, EMBL). These antibodies were then coupled with rhodamine-conjugated sheep anti-rabbit antibodies (provided by Hilkka Virta, EMBL). The injected cells were photographed under UV light. Bars = 40 μm.

membrane binding. All the data can be summarized into a simple model. A membrane anchor peptide is essential for the transport of E2 to the plasma membrane. Removal of the anchor leads to complete translocation of the mutant into the endoplasmic reticulum lumen. Surface transport of the protein is inhibited because the necessary information is either cryptic or lost. Alternatively, a structurally altered anchorless protein may enter a different transport pathway (10), in which an inappropriate quaternary structure of the protein prevents secretion. When the anchorless E2d5 is fused to a new membrane anchor by the attachment of C-terminal fragments of either VSV G or FPV HA, these new anchors

stop the translocation of the hybrid proteins into the lumen of the rough endoplasmic reticulum and allow their surface transport as membrane-bound proteins.

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