Mutants of the Membrane-binding Region of Semliki Forest Virus E2 Protein. II. Topology and Membrane Binding

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Abstract. The p62/E2 protein of Semliki Forest virus (SFV) is a typical transmembrane glycoprotein, with an amino-terminal lumenal domain, a transmembrane (hydrophobic) domain, and a carboxy-terminal cytoplasmic domain (or tail). Our hypothesis has been that the membrane-binding polypeptide region (membrane anchor) of this protein consists of both the transmembrane domain and the adjacent positively charged peptide, Arg-Ser-Lys, which is part of the cytoplasmic domain. We have investigated three anchor mutants of the p62 protein with respect to both their disposition and their stability in cell membranes. The construction of the three mutants has been described (Cutler, D. F., and H. Garoff, *J. Cell Biol.*, 102:889–901). They are as follows: A1, changing the basic charge cluster from Arg-Ser-Lys(+2) to Gly-Ser-Glu(−1); A2, replacing an Ala in the middle of the hydrophobic stretch with a Glu; A3, changing the charge cluster from Arg-Ser-Lys(+2) to Gly-Ser-Met(0). All three mutants retain the transmembrane configuration of the wild-type p62. In a cell homogenate they have a cytoplasmic domain that is accessible to protease. In living cells an anti-peptide antibody specific for the cytoplasmic tail of p62 reacts with the tails of both wild-type and mutant p62s following its introduction into the cytoplasm. All three mutant proteins have Triton X-114 binding properties similar to the wild-type p62. However, when the membranes of cells expressing the three mutants or the wild-type p62 protein are washed with sodium carbonate, pH 11.5, three to four times as much mutant protein as wild-type p62 is released from the membranes. Thus the stability in cell membranes of the three mutant p62 proteins is significantly reduced.

THE Semliki Forest virus (SFV)[1] p62/E2 protein is a typical example of the transmembrane glycoproteins that span the membrane once, with a large NH_2-terminal lumenal domain, a transmembrane domain (followed by two closely spaced basic amino acids), and a small COOH-terminal cytoplasmic domain (8, 14, 20). This topology is most likely generated co-translationally by halting transfer through the membrane. As discussed in the preceding paper, it has been our working hypothesis that the region containing the transmembrane (hydrophobic) domain and the charge cluster, from now on referred to as membrane-binding region or anchor, is responsible for the establishment and maintenance of this topology. To test this hypothesis, we have made a series of point mutations in the cDNA encoding the SFV structural proteins (see the preceding paper). These mutations change the basic charge cluster which follows the p62 spanning region from +2 (wild-type Arg-Ser-Lys) to 0 (SFV-A3, Gly-Ser-Met) or −1 (SFV-A1, Gly-Ser-Glu) or introduce a glutamic acid into the middle of the hydrophobic stretch (SFV-A2). We have already shown (see the preceding paper) that the proteins made from the DNA encoding these mutants are of the predicted size and are glycosylated. The membrane association of all three mutants was functionally unaltered as assayed by cell surface transport and ability to promote cell–cell fusion.

In this paper, we investigate the transmembrane topology and membrane binding of the mutants directly. Since there is no loss of functional membrane association for the charge cluster mutants of p62, we expect the hydrophobic stretch and not the charge cluster to play the major role in membrane–protein interaction. However, the precise topology of this interaction might be defined by the charge cluster. For example, A3, which has completely lost its charge cluster, might have passed through the bilayer and might be bound to the lumenal face of the membrane via the hydrophobic stretch without retaining a cytoplasmic domain. If a single negatively charged amino acid could act to maintain normal topology, then A1 (with the charge cluster replaced by Glu) might still span the bilayer. If A1 were still transmembrane, then transfer of A2 (with a Glu replacing an Ala in the hydrophobic stretch) might be halted earlier, and/or stabilized in a new position by the introduced glutamic acid. A2 would

[1] *Abbreviation used in this paper:* SFV, Semliki Forest virus.

then be shifted upstream in the membrane. Although not as hydrophobic as the region thought to span the bilayer, the region immediately upstream consists mainly of uncharged and hydrophobic amino acids (see Fig. 1 of the preceding paper). Alternatively, the charge cluster may only serve to increase stability in the membrane and to provide a sharp distinction between membrane and cytoplasmic domains. In this case, A1 and A3 should not have a large effect on topology, being manifested instead in a decreased capacity for membrane binding, possibly coupled to a minor repositioning of the mutant polypeptide chains in the membrane. Moreover, since the primary interaction between lipid bilayer and protein is most likely through the hydrophobic stretch of the latter, we would also expect A2 to show similar tendencies.

Our results show that all three mutants still span the bilayer, with a reagent-accessible cytoplasmic tail. We also demonstrate that all three have a reduced membrane-binding capacity compared to wild-type p62.

Materials and Methods

All methods not detailed here are described in the preceding paper.

Antibody Microinjection Experiments

The method for these experiments and the characterization of the anti-peptide antibody are detailed elsewhere (Roman L., and H. Garoff, manuscript in preparation). Briefly, the nuclei of 100 BHK-21 cells were injected (18) with pS-S-SFV (see preceding paper) containing wild-type or mutant p62-encoding sequences. After 6 h at 37°C, the cells were injected with affinity-purified anti-peptide antibody at 0.8 mg/ml or with a monoclonal anti-E2 antibody that reacts against the lumenal domain of E2 (Roman, L., unpublished data) at 0.8 mg/ml. These cells were then fixed in methanol and stained with fluorescent second antibodies. Surface staining with the same two antibodies was performed (see above) on cells that had been microinjected with DNA as above and then gluteraldehyde fixed at 6 h. For surface staining, the anti-peptide antibody was diluted 100-fold, the monoclonal antibody 50-fold.

Preparation of Microsomes

5-cm dishes of transfected cells were placed on ice and rinsed (twice) with ice-cold phosphate-buffered saline. 1 ml phosphate-buffered saline with Trypsin (0.05%) and EDTA (0.02%) was added and the cells rocked at 37°C for 5 min. The trypsinized cells were pelleted at 2,000 rpm at 4°C for 3 min, followed by two washes with sucrose buffer (0.25 M sucrose, 10 mM Tris, pH 7.5, 1 mM $MgCl_2$). The cells were then suspended in 30 μl sucrose buffer per 5-cm dish starting material. The suspension was firmly passed through a 25-μl Hamilton syringe nine times, diluted to 200 μl/dish with sucrose buffer, and passed once through a 100-μl Hamilton syringe. Nuclei were spun out in a microfuge at 4°C, 3 min, and the "microsomal" preparation was removed to a fresh tube.

Protease Protection Experiments

Five dishes of cells were used for each probe. 30 h after transfection, cells were labeled for 20 min followed by a 5-min chase. A microsomal preparation was performed (see above) and the cell homogenates were split into three. Samples were incubated for 30 min at 20°C with proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, IN) (2 mg/ml), or proteinase K and Triton X-100 (1%), or neither. Phenylmethylsulfonyl fluoride was then added to 2 mM. Samples without detergent were then spun at 100,000 *g* for 1 h and the membrane pellet was solubilized in immunoprecipitation buffer. The sample with detergent was adjusted to immunoprecipitation buffer (see previous paper). Immunoprecipitation was followed by gel electrophoresis (9) and fluorography.

Carbonate Experiments

40 h after transfection, 5-cm dishes of cells were homogenized to give a microsomal preparation. This procedure was modified by diluting each sample to 300 μl before the final syringing. 300 μl of the sodium carbonate or NaOH solution (7, 15) was then added to the postnuclear supernatant and the mix passed once more through a 100-μl Hamilton syringe, and left on ice for 20 min. Membranes were pelleted by a 100,000 *g* spin for 1 h at 4°C in cellulose nitrate tubes (Beckman Instruments Inc., Palo Alto, CA) of diameter 3/16 × 1-21/32 inches through a 25% sucrose cushion (50 μl). The tubes were spun in an SW50 rotor using the appropriate adaptor. The supernatant was removed, trichloroacetic acid precipitated, and dissolved in sample buffer for gel electrophoresis. The pellet was rinsed with distilled water and dissolved directly in sample buffer.

Triton X-114 Partitioning

40 h after transfection, 5-cm dishes of cells (2 dishes/probe) were placed on ice and washed twice with ice-cold phosphate-buffered saline. 0.5 ml of cold saline made 1% with Triton X-114 (4) was added and left for 5 min. The cells were solubilized by pipetting up and down with a Gilson pipetman, and insoluble material was removed by spinning 5 min at 4°C in a microfuge. The supernatant was heated to 37°C for 5 min, spun at 1,000 rpm at 37°C for 5 min, and the supernatant and pellet (aqueous and detergent) phases were separated (4). The aqueous phase was trichloroacetic acid precipitated and solubilized in sample buffer. The detergent phase was solubilized in sample buffer with added SDS (final concentration of 6%).

Results

Transmembrane Topology of the SFV-A Mutants

The question of topology has been investigated using two complementary approaches. Both were designed to test the mutated p62 proteins for a cytoplasmic domain that was still accessible to reagents. The first approach involved labeling of the cytoplasmic tail by microinjection of an anti-E2 COOH-terminal peptide antibody into living cells expressing the mutant protein—a nonperturbing approach. The second method was the classical protease–protection experiment; isolation of microsomes from transfected cells and digestion of their cytoplasmic face with a protease followed by immunoprecipitation and electrophoretic analysis. The two independent approaches have different advantages. The microinjection experiment reveals the topology in vivo, and moreover avoids the possibility of potential artefacts arising during microsome isolation. On the other hand, a protease–protection experiment can give the approximate size of the cytoplasmic domain and moreover can reveal how much heterogeneity of topology for each mutant p62 exists. Together, the two approaches, in vivo and in vitro, should provide a comprehensive answer to this important question.

In Vivo Topology

The in vivo topology of the mutants was investigated by microinjecting, within a delineated region of a coverslip, the nuclei of all the BHK-21 cells present (~100 cells) with pS-S-SFV containing wild-type or mutant DNAs. pS-S-SFV is a plasmid where transcription of the SFV cDNA is driven from the SV40 early promoter (see preceding paper and reference 9). 6–7 h were allowed for expression of viral proteins to peak (18) and then the cells within the same area were again microinjected with either a monoclonal anti-E2 lumenal domain antibody (control) or a polyclonal anti-E2 cytoplasmic tail antibody. This antibody only recognizes the cytoplasmic domain of p62/E2 (Roman, L., and H. Garoff, manuscript in preparation). The cells were incubated for half an hour and then fixed/permeabilized and stained with a rhodamine-conjugated second antibody. As a further control, microinjected cells were fixed after 6–7 h and the surface stained with either of the two antibodies. The results (Fig. 1) show that the anti–COOH-terminal peptide antibody does stain all three mutants from the cytoplasmic side of intracellular membranes (cells injected with antibody) (*a*), but not from the outside of

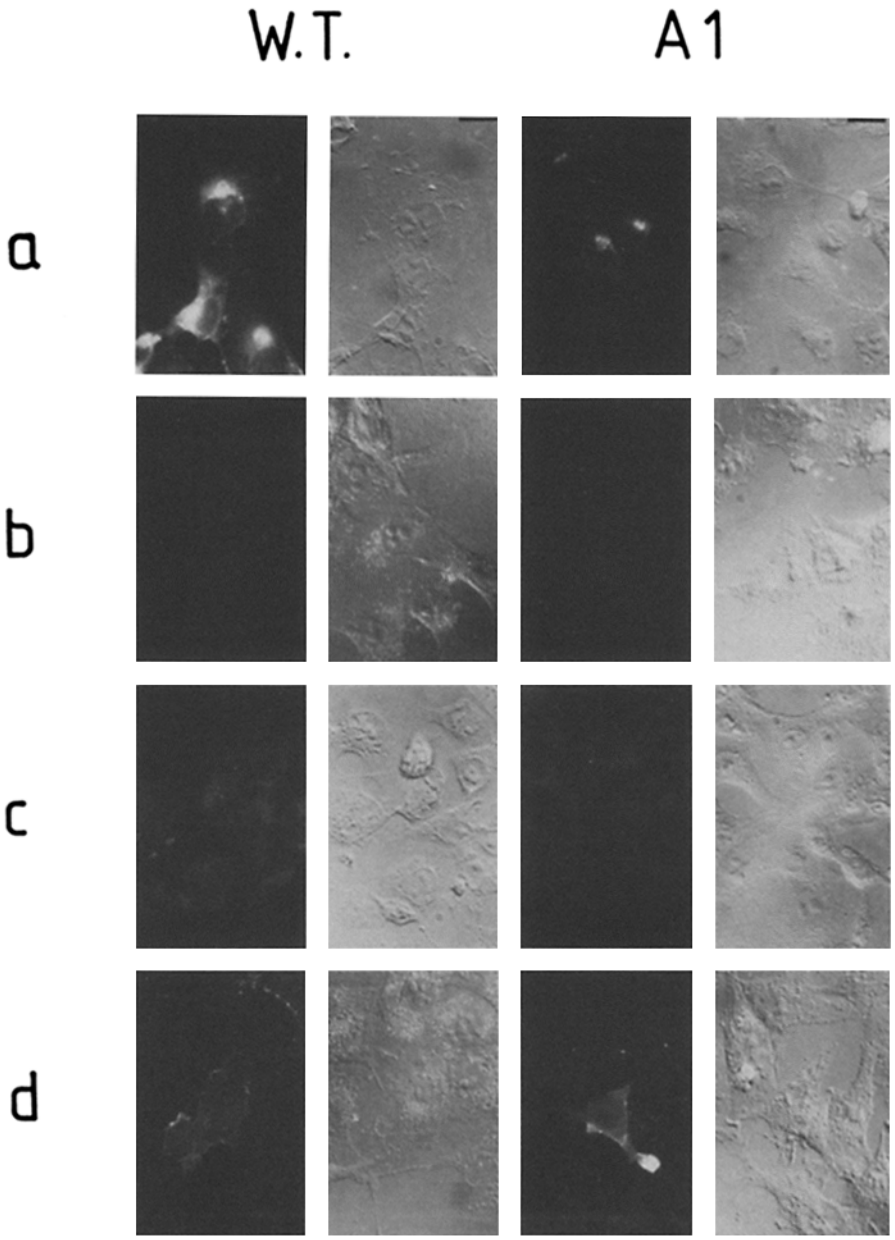


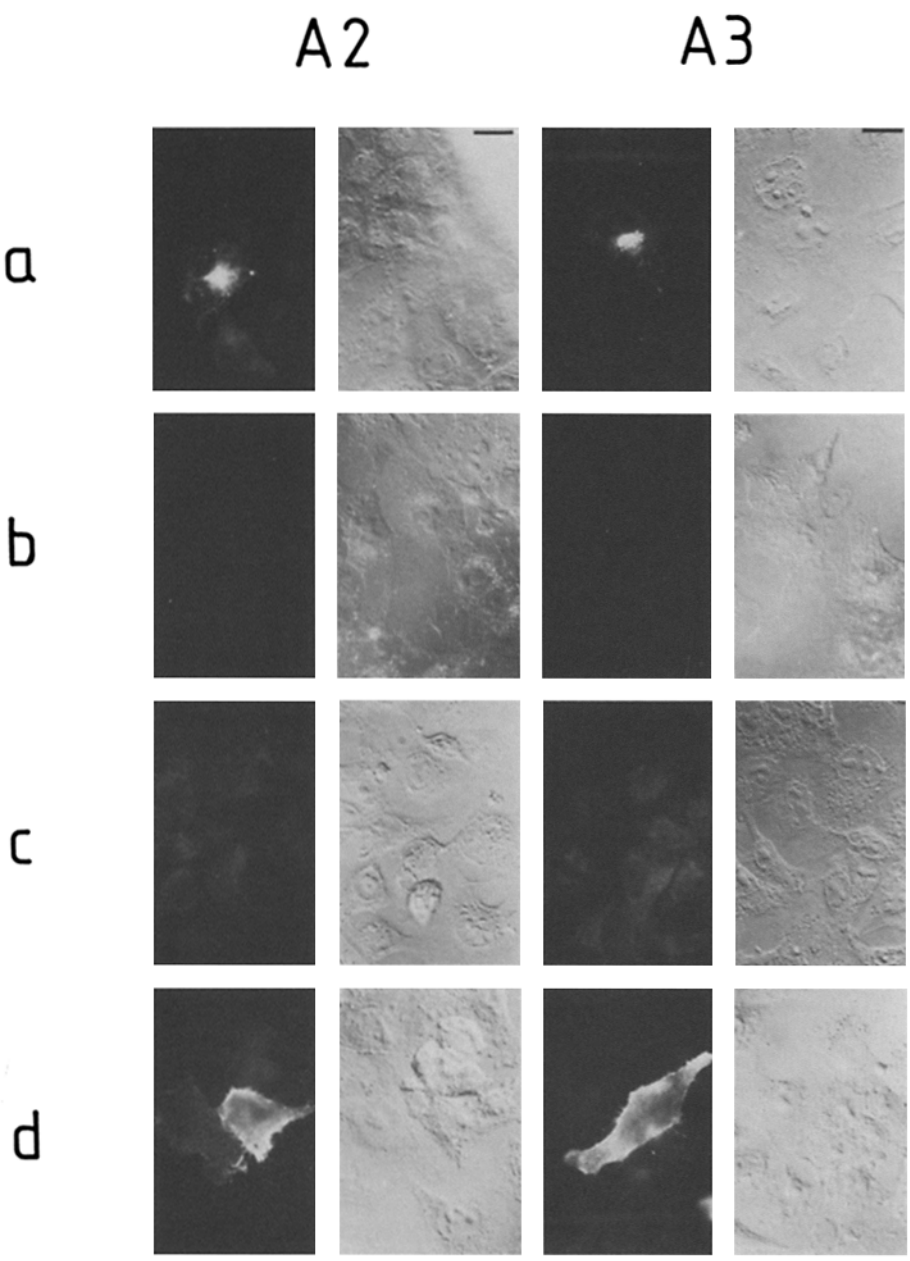
Figure 1. In vivo topology of the SFV-A mutants. The nuclei of BHK-21 cells were microinjected with pS-S-SFV (see preceding paper) encoding the wild-type (*WT*) or mutant (*A1*, *A2*, and *A3*) p62 proteins. 6 h later the cells were microinjected with an anti-cytoplasmic tail anti-peptide antibody (*a*) or with an anti-lumenal domain antibody (*c*). Alternatively, 6 h after microinjection of DNA, cells were fixed and then stained on the surface with anti-cytoplasmic tail antibody (*b*) or anti-lumenal domain antibody (*d*). In all cases, staining with a fluorescent second antibody followed. Immunofluorescent and corresponding Nomarski micrographs are shown. Bar, 20 μm. The left-hand panel shows the wild-type and A1 p62 proteins. The right-hand panel (*facing page*) shows the A2 and A3 p62 proteins.

cells (*b*). The anti-lumenal domain antibody only stains from the outside of cells (*d*) and not from the cytoplasm (*c*). The pattern of internal staining concentrated in the perinuclear region, seen using the anti–COOH-terminal antibody is similar to that obtained in infected cells (Roman, L., and H. Garoff, manuscript in preparation). Thus, the concentration of spike glycoprotein in the Golgi apparatus observed during infection (12) can also be detected after microinjection (Fig. 1 *a*). The brightness of fluorescence seen after microinjection of the anti-peptide antibody (Fig. 1 *a*) varies considerably from cell to cell and from experiment to experiment, both for the three mutants and for the wild-type p62/E2 proteins. The level of background staining after microinjection of antibody was consistently higher than that seen for surface staining (compare Fig. 1, *b* with *c*). We conclude from these experiments that a significant fraction of each population of mutant p62 proteins has the wild-type transmembrane topology in vivo.

In Vitro Topology of the SFV-A Mutants

In vivo and in the presence of E1, the SFV-A mutants of p62 appear to show unaltered topology. For the in vitro assay, p62 was analyzed in the absence of E1. The rationale for this was that E1 may stabilize E2 in the membrane through interactions between the two halves of the heterodimer spike complex and thus mask differences between wild-type and mutant p62. For this purpose we used the pL1-SFV-d9 plasmids. In these plasmids, the portion of the SFV cDNA encoding E1 and the carboxyl half of the 6K peptide are deleted. The 6K peptide is normally synthesized after E2 and before E1. When expressed from pL1-SFV-d9, the normally occurring p62-6K cleavage ensures that p62 with an authentic COOH-terminus is produced (see preceding paper for details).

COS-7 cells were transfected with pL1-SFV-d9 or the same plasmid containing the three mutants. These cells express the SV40 T-antigen endogenously and therefore promote repli-



cation of the pL1 plasmids and transcription of the SFV cDNAs from the SV40 late promoter. 30 h after transfection, the cells were pulse-labeled and a microsomal fraction was prepared. The microsomes were divided into three aliquots and incubated either with proteinase K, or proteinase K plus Triton X-100, or with neither. After incubation, the remaining p62 was immunoprecipitated, electrophoresed on SDS polyacrylamide gels, and fluorographed. The fluorograph (Fig. 2) shows that for the wild-type and all three mutants a shift in molecular weight, presumably due to loss of the protease-accessible cytoplasmic domain, occurs when protease is added (compare lanes 1, 4, 7, and 10 with 2, 5, 8, and 11). In the presence of both proteinase K and Triton X-100, the p62 is completely digested (lanes 3, 6, 9, and 12). The size of the change in molecular weight in each case is similar. The shaving of the cytoplasmic domain is complete with each mutant, as with the wild type; all polypeptide chains of each population are transmembrane. Thus both in vivo and in vitro the three SFV-A mutants are clearly transmembrane with a cytoplasmic domain accessible to reagents.

Membrane Binding of the SFV-A Mutants

Two biochemical assays for the membrane-binding capacity of the mutant and wild-type p62s have been used. The partitioning of the proteins between aqueous and detergent (Triton X-114) phases assays for general hydrophobicity. The second assay is of release of p62 from cellular membranes when treated with the protein perturbant sodium carbonate at pH 11.5.

Triton X-114 Binding of the SFV-A Mutants

The partitioning of protein between an aqueous phase and Triton X-114 has been proposed as an operational distinction between membrane-bound and unbound proteins (4). More recently, it has been suggested that partitioning measures the general surface hydrophobicity of proteins (3). If this property

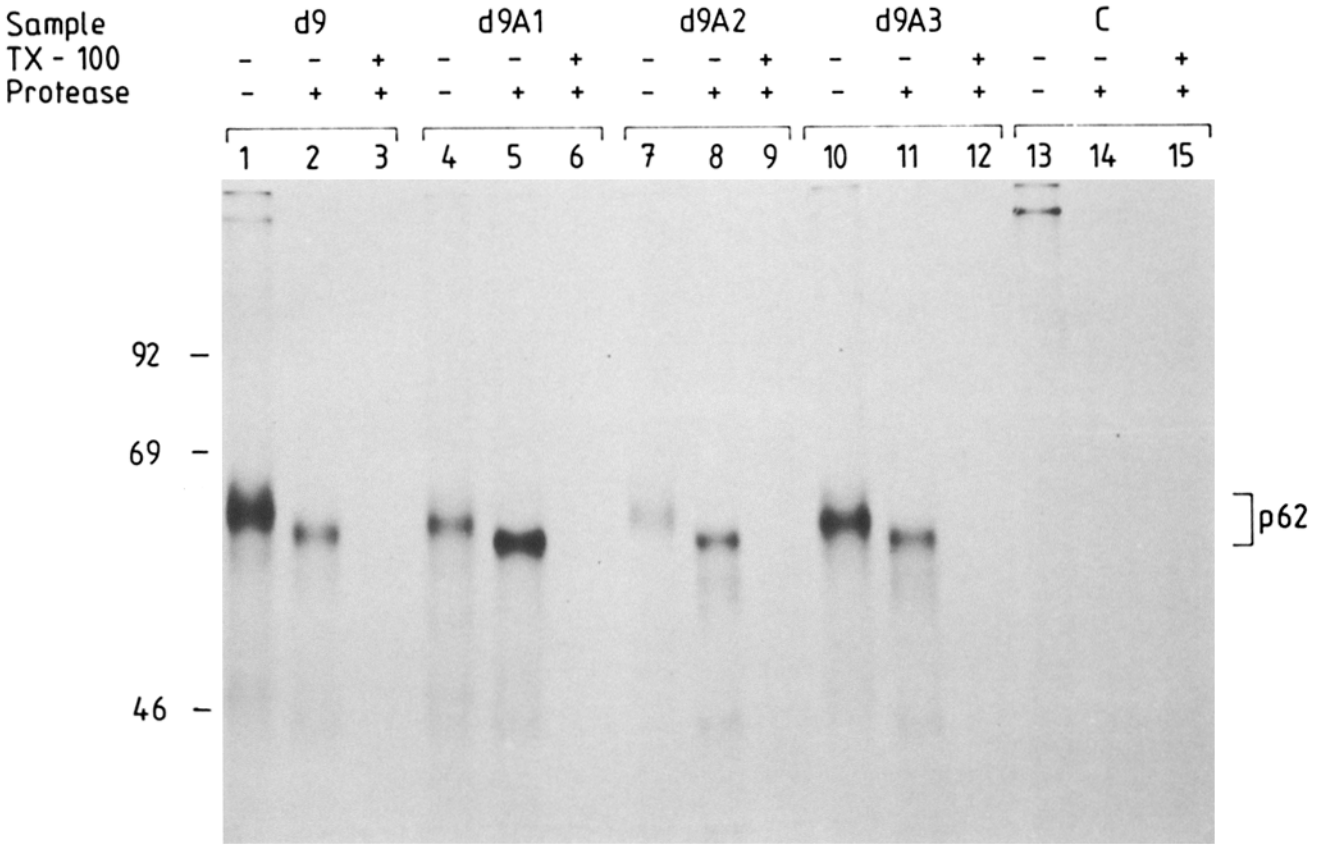


Figure 2. In vitro topology of the SFV-A mutants. COS-7 cells were transfected with the mutant and wild-type forms of pL1-SFV-d9 (d9A1, d9A2, d9A3, and d9 respectively). 30 h later, cells were labeled, homogenized, and samples were incubated with proteinase K, proteinase K plus Triton X-100, or neither. Samples were then immunoprecipitated with affinity-purified anti-E2 antibody and electrophoresed on a 10% SDS polyacrylamide gel which was fluorographed. Samples transfected and presence (+) or absence (−) of Triton X-100 and proteinase K are indicated in lane headings. *C* indicates nontransfected cells. Input from ~1.5 × 10^6 cells is loaded on each lane. Migration of molecular mass standards (kD) is shown.

has been significantly altered in any of the mutants by changes made in or around the most hydrophobic portion of p62 (8) this could clearly affect its detergent-binding properties which may in turn reflect its membrane-binding capacity. Accordingly, COS-7 cells were transfected with the pL1-SFV-d9 series of plasmids, left for 40 h, and then solubilized in a solution of Triton X-114. Partitioning was effected, and supernatant, pellet, and input fractions electrophoresed on an SDS polyacrylamide gel. The proteins were transferred to nitrocellulose and incubated with a mixture of anti-E2 and anti-capsid polyclonal antibodies followed by a peroxidase-conjugated second antibody. The partitioning of capsid was monitored to provide a control cytoplasmic protein. The blot was stained using the peroxidase/diaminobenzidine/H_2O_2 reaction. To ensure that there were no differences between p62s produced in the presence and absence of E1 40 h after transfection, wild-type pL1-SFV DNA was transfected in parallel and blotted. The blot (Fig. 3) reveals that all the p62s partition approximately equally between pellet (detergent phase: lanes 2, 5, 8, and 11) and supernatant (aqueous phase: lanes 3, 6, 9, and 12). No major differences are seen. Capsid partitions mainly into the aqueous phase.

The same experiment has been performed with the mutant proteins expressed from pL1-SFV A1, A2, and A3 (data not shown). In these analyses, most of the p62 was found to be cleaved to E2 as in the case of the wild-type pL1-SFV (see above). The remaining p62 protein and the E2 form of the mutants partitioned in the Triton X-114 assay as the corresponding wild-type protein forms shown in lane 1 of Fig. 3. The p62 proteins partitioned about equally in both phases whereas the E2 form was almost quantitatively found in the detergent phase. Presumably, the relatively hydrophilic E3 part of p62 (the E3-E2 precursor; references 8 and 14) forces this protein into the aqueous phase to some extent. A surprising difference between pL1-SFV and pL1-SFV-d9 lies in the amount of capsid found: much less capsid is found in the presence of E1 than in its absence. This is a consistent and repeatable observation (Cutler, D., and P. Melancon, unpublished observations) for which we have no explanation. Moreover, capsid in this system runs as a doublet (both these bands disappear when anti-capsid antibody is not used). This may be due to partial posttranslational modification (e.g., reference 17).

Carbonate Extraction of SFV-A Mutants

Many protein perturbants have been used to extract proteins from membranes. The differential release of proteins from membranes following the use of such reagents may reflect heterogeneity in the association of those proteins with the membrane (15). To look for differences in membrane binding between the wild-type and the A-mutants of the SFV-p62 protein we have therefore used an extraction procedure using

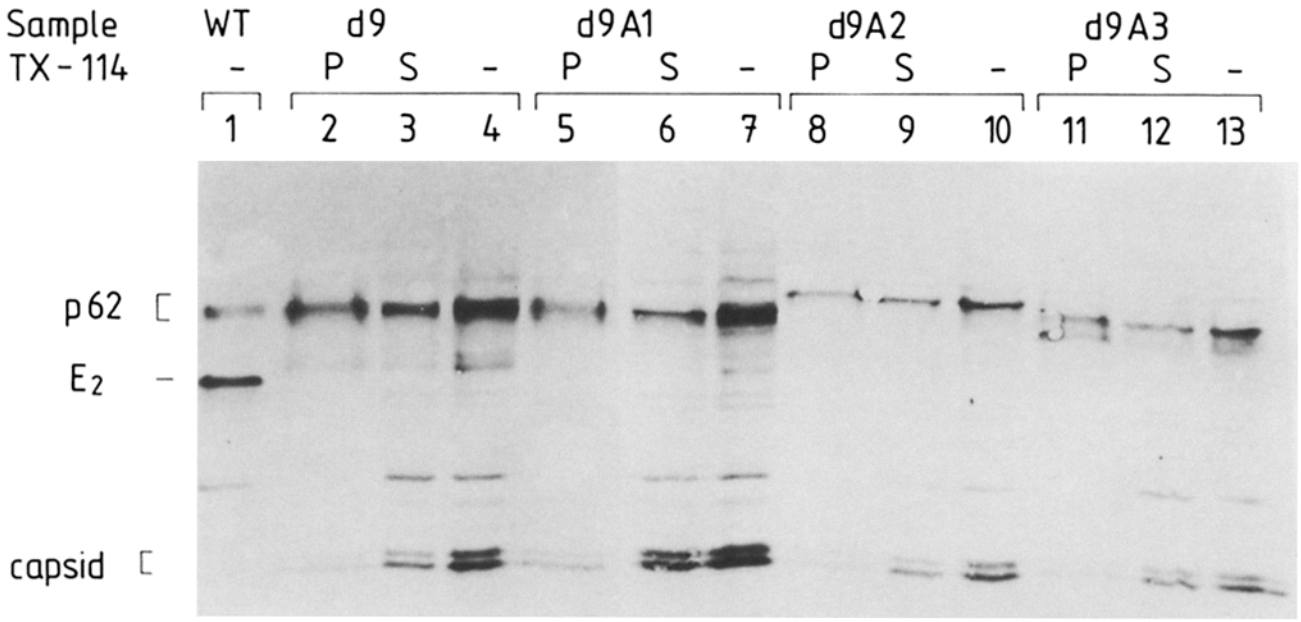


Figure 3. Triton X-114 binding of SFV-A mutants. Two 5-cm dishes of COS-7 cells were transfected with wild-type and mutant p62s in pL1-SFV-d9 (d9, d9A1, d9A2, and d9A3, respectively) and with the wild-type p62 in pL1-SFV (*WT*). After 40 h, cells were lysed with Triton X-114. One-half of each sample was partitioned before electrophoresis to give aqueous (*S*, supernatant) or detergent (*P*, pellet) phases and the other half electrophoresed directly (−) on a 10% SDS polyacrylamide gel. The proteins were transferred to nitrocellulose, and incubated with a mixture of anti-E2 and anti-capsid IgG fractions followed by peroxidase-conjugated sheep anti-rabbit IgG. The filter was stained with the H_2O_2/diaminobenzidine reaction. All of each sample was loaded onto this gel.

one of the most convenient of these reagents, sodium carbonate (7).

The procedure was first titrated using a form of p62 that has been truncated so as to completely lose its transmembrane and cytoplasmic domains. This mutant is expressed from SFV d5 cDNA (one of the d-series [9]) which ends with a stop translation linker 87 base pairs before the region encoding the transmembrane region of p62 (Garoff, H., unpublished results). We expected that a form of p62 which has completely lost its membrane-binding polypeptide region should give a measure of the maximum possible difference between the wild-type p62 and mutant p62s with nonfunctional membrane anchors. Accordingly, microsomal membranes isolated from COS-7 cells transfected with pL1-SFV-d5 were treated with carbonate solutions at different pHs between 10.5 and 11.5 or with NaOH (100 mM final concentration). The membranes were pelleted and the supernatant and pellet monitored for p62 release by Western blotting. The results (Fig. 4*A*) show that following an extraction at pH 10.5, the lumenal protein (d5) is mostly found (70%) in the membrane pellet but that with more stringent conditions, the distribution between pellet and supernatant can be reversed (compare lanes 1 and 2 with 7 and 8). This effect is even more dramatic for the two forms of the capsid protein (shown in the lower part of the Fig. 4; note also the differential release of the two capsid forms). This implies that the assay can be very sensitive to small changes in a particular protein and therefore ideal for assaying the A-mutants. The observation that even at pH 11.5 30% of the d5 form of p62 remains in the membrane pellet could be accounted for by some aggregation of the truncated d5 protein. Alternatively, it could suggest the existence of a weak interaction (hence partial extraction) between the d5 protein and the cellular membrane. If p62 A-mutants, in addition to the transmembrane interaction described above, also display this lumenal interaction (or aggregation), we would therefore expect to release at best 70% of the protein in those mutants where the transmembrane binding region has been destroyed. (It should be noted that a partial extraction as observed for d5 has been seen for many other proteins which are not integral to the membrane; see, for instance, proteins of the endoplasmic reticulum in reference 7 and ovalbumin in reference 16.)

The conditions used in Fig. 4*A* were then applied to microsomes prepared from cells transfected with the two controls, pL1-SFV-d5 and pL1-SFV-d9, and the three A-mutants in the d9 form. These experiments are shown as a peroxidase-stained blot (Fig. 5). This and another experiment have been quantitated (Table I). Greater than 90% of the wild-type p62 is retained in the membrane fraction after this treatment, but 30% or less of the d5-p62 is pelleted. The three SFV-A mutants fall midway between these two extremes, with 30–40% of the protein being released. The magnitude and direction of differences between the mutants differ from experiment to experiment and therefore do not appear to be significant. Thus all three A-mutants are destabilized in the membrane when compared to the wild type.

Discussion

In this paper, the topology and stability in cellular membranes of three mutants of the membrane-binding region of SFV-E2 have been analyzed. The three mutants have been previously shown to be associated with the membrane in a functionally unaltered way (see preceding paper). Here the nature of that association has been analyzed.

The topology of all three SFV-A mutants of p62 is unaltered from wild type. All three span the bilayer, having reagent-accessible cytoplasmic domains both when expressed as a complete p62/E1 spike or as p62 alone. The data from both

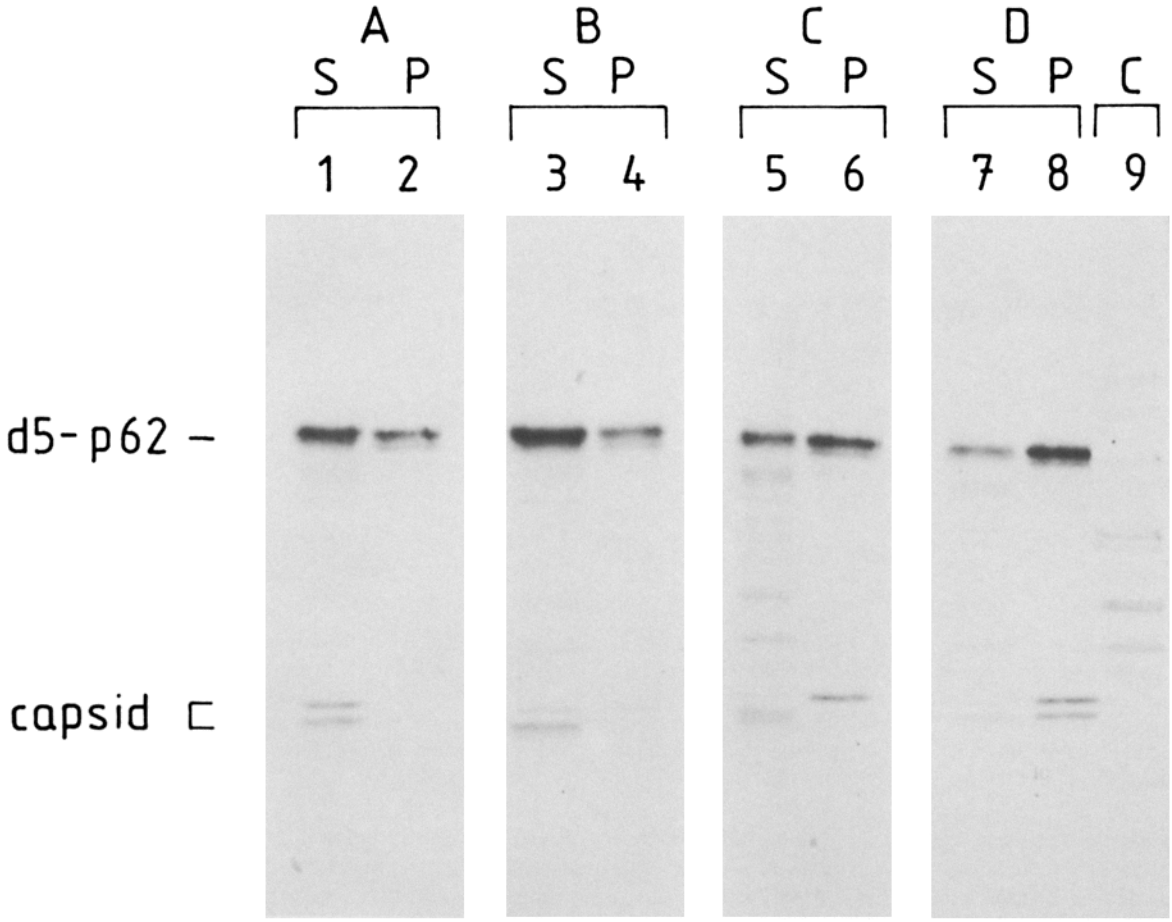


Figure 4. Membrane binding of d5. Four dishes of COS-7 cells were transfected with pL1-SFV-d5; microsomal fractions were made and mixed with an equal volume of (*A*) 200 mM Na carbonate, pH 11.5; (*B*) 200 mM NaOH; (*C*) 200 mM Na carbonate, pH 11; or (*D*) 200 mM Na carbonate, pH 10.5. Membranes were pelleted and pellet (*P*) and supernatant (*S*) fractions electrophoresed on a 10% SDS polyacrylamide gel. Protein was transferred to a nitrocellulose filter which was incubated with anti-E2 and anti-capsid antibodies (polyclonal IgG fractions) followed by peroxidase-conjugated sheep anti-rabbit IgG, and stained with diaminobenzidine/H_2O_2. Each pair of tracks equals input from one 5-cm dish of cells. *C* indicates nontransfected cells, input from half of one 5-cm dish.

the protease protection and antibody injection experiments imply that the topology of the mutants is the same as that of the wild-type p62. However, small differences between them might be beyond the resolution of these assays. There are two problems involved with the protease–protection assay: (*a*) the very small percentage shifts in molecular weight to be analyzed (the wild-type cytoplasmic domain is ~5% of unglycosylated p62); and (*b*) the aberrant migratory behavior resulting from changes in and around the hydrophobic portion of transmembrane glycoproteins (e.g., the aberrant migration of A2 [discussed in the preceding paper] and of Vesicular Stomatitis virus glycoprotein mutants [1]). However, within these limitations it is clear that each mutant has a protease-accessible domain (or tail) that is approximately the same size as that of wild-type p62. It is also clear that all molecules of each mutant population have such a tail.

The importance of the COOH-terminal region of the p62 protein for membrane binding is clearly shown by the fact that ~70% of the anchorless d5 form can be extracted from transfected cell membranes with a carbonate buffer at pH 11.5, whereas p62 with a wild-type membrane-binding region remains almost quantitatively membrane bound. The introduction into the p62 membrane-binding region of three different point mutations designed to alter either the hydrophobic domain or the charge cluster is shown to significantly weaken this interaction. This suggests that both of these features are critical for efficient membrane binding of the p62 protein. It is possible in theory that the A1, A2, and A3 mutations in the membrane-binding region affect instead the postulated interaction of the lumenal domain with the cellular membrane, which could account for the behavior of the d5 form of p62. We find this interpretation unlikely, because it would require that the interaction between the unaltered transmembrane domain of p62 and the lipid bilayer would only be partially (~70%) responsible for membrane anchoring, the rest of this function being provided by the postulated lumenal interaction. Moreover, all integral membrane proteins studied so far show quantitative co-pelleting with the membranes in such assays (7, 15).

In summary, and with the reservations discussed above, we have shown in this and in the preceding paper that the A-mutants of p62/E2 are all translocated, glycosylated, and reach the cell surface with similar efficiency to each other and wild type. Once on the cell surface they are all capable of promoting qualitatively wild-type cell–cell fusion. The topology both in vivo and in vitro of the mutants is indistinguishable from that of wild-type p62 but the stability of their association with the membrane has been reduced.

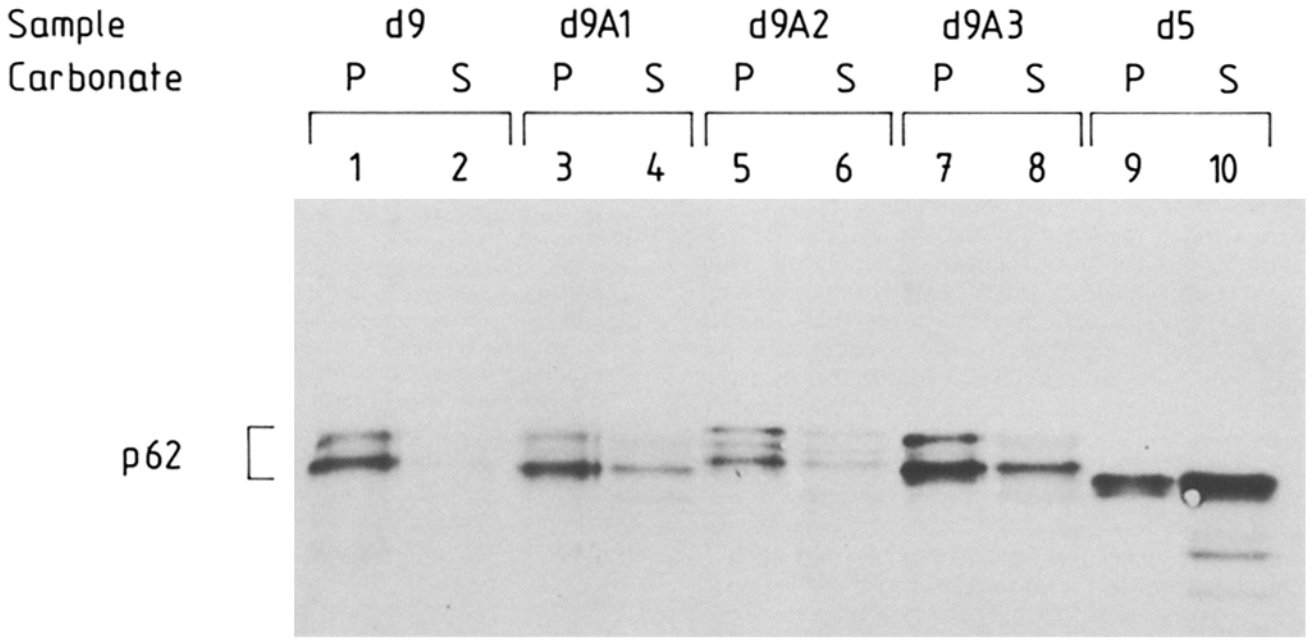


Figure 5. Membrane binding of the SFV-A mutants. COS-7 cells (one 5-cm dish/probe) were transfected with the pL1-SFV-d9 plasmid series (*d9*, *d9A1*, *d9A2*, *d9A3*) and pL1-SFV-d5 (*d5*). Microsomal preparations (cell homogenates) were mixed with an equal volume of Na carbonate, pH 11.5; the membranes were pelleted and supernatant (*S*) and pellet (*P*) fractions electrophoresed on a 10% polyacrylamide gel. Protein was transferred to a nitrocellulose filter which was incubated with an anti-E2 antibody (affinity-purified IgG), then a peroxidase-conjugated sheep anti-rabbit IgG, and stained with diaminobenzidine/H_2O_2.

*Table I. Percentage of Wild-type and Mutant p62s Retained in Cellular Membranes after Carbonate Treatment**

Experiment‡	d9	d9A1	d9A2	d9A3	d5
1§	93‖	67	66	57	21
2	91	63	69	69	32

* Carbonate experiments were quantitated by Western blotting of samples followed by incubation of blots with specific antibody followed by ^{125}I–protein A. Autoradiographs were scanned with a densitometer, peaks cut out, and weighed.

‡ Each experiment is a separate series of transfections.

§ Samples used for this blot are the same as those used in Fig. 5.

‖ Weight of pellet sample expressed as percent of sum of weights of pellet plus supernatant samples.

Thus our data indicate that, for p62/E2, (*a*) the hydrophobic stretch alone can stop translocation; (*b*) the charge cluster does appear to stabilize the protein–membrane interaction; (*c*) the type and/or number of charged amino acids may well be important for this function.

Because in the case of A3 (which appears to have unaltered topology), the next charged amino acid is 23 amino acids downstream from the hydrophobic stretch towards the carboxy terminus of E2, we believe that it is unlikely that charge is involved in arresting the transfer of p62 protein. Recently, other studies have shown that the deletion of the hydrophobic stretch, leaving the charge cluster and cytoplasmic domain intact, leads to loss of anchoring function in Vesicular Stomatitis virus glycoprotein (1) and the retroviral oncogene V-fms (13). In addition, in a prokaryotic system, Davis and Model (5) have shown that a synthetic hydrophobic stretch, inserted into the middle of the gene III protein of phage F1, can anchor that protein in the membrane. Although in the latter case the hydrophobic stretch was immediately followed by a charged amino acid, these results are consistent with our findings that a hydrophobic stretch is probably sufficient to cause anchoring of a membrane protein. It follows from this that the charge cluster is most likely not required as a stop transfer signal but may have some other role.

Our data suggest that the charge cluster acts as a stabilizing factor but that not any charged amino acid will fulfill this role (both A1, with a single Glu and A3, with no change cluster were both distinguishable from wild-type p62). The gene III protein system has also been used to investigate this problem. Davis et al. (6) have shown that in their membrane-binding assay, a gene III mutant which has lost its charge cluster (pND 418A) is associated with the membrane in a wild-type fashion. Unfortunately, since the gene III protein has no large cytoplasmic domain, no easy assay for its topology is possible. Moreover, although under their assay conditions, its membrane-binding capacity is not reduced, it could be that under more stringent assay conditions pND 418A would be less stable in the membrane than the wild-type protein. Another example of a membrane-spanning protein losing its charge cluster is that of the Friend Spleen Focus-Forming virus envelope protein gp65. This protein, initially detected on the cell surface, is slowly released from infected cells (11). Thus the charge cluster may play a role of varying importance in different proteins, ranging from undetectable through to essential for maintenance (but not necessarily establishment) of transmembrane topology. This may simply reflect the structures of the individual proteins concerned.

The results obtained with A2 (where a single charged amino acid has been introduced into the membrane-spanning region) raise some interesting questions. Since one glutamic acid alone cannot substitute for the wild-type charge cluster of E2, as demonstrated by A1, we might not expect to find A2 (with a single Glu introduced into the hydrophobic region) anchored upstream of its normal position in the membrane. Our results from the protease–protection experiments (with the reservations discussed above) concur with this expectation.

Thus one possible way of looking at A2 is as a protein with a very short (8 amino acids long) hydrophobic stretch (from the charge cluster to the introduced Glu). This is of course shorter than any natural membrane spanning region, most of which are 20 or more amino acids long. This length of 8 may be an underestimate since the charged side chain of an amino acid could remain outside the bilayer while the backbone is buried within it. When the lengths of the side chains of charged amino acids are taken into account as suggested by von Heijne (19), we reach an effective length of 8(+5) amino acids. To obtain the figure of 5 we have taken the largest possible estimate by adding the corrective values suggested by von Heijne (19) for the two charged amino acids that border the eight hydrophobic amino acids in this case. Other studies have also addressed the question of whether a shortened membrane-spanning region can still function as an anchor. Studies by Adams and Rose published recently (1) suggest that the transmembrane topology of the Vesicular Stomatitis virus glycoprotein is maintained by a hydrophobic stretch of 12 amino acids. Moreover, interrupting the hydrophobic stretch of the Vesicular Stomatitis virus glycoprotein with an Arg, leaving 11 uninterrupted hydrophobic amino acids, does not prevent transmembrane topology from being achieved (2). In their studies with the gene III protein, Davis et al. show that binding by the natural anchor is gradually lost as the hydrophobic domain is truncated, being impaired at 12 amino acids, and lost thereafter (6). Most elegantly, using a synthetic linker encoding hydrophobic amino acids inserted into gene III they show that 12 or 14 amino acids, depending on position within the protein, are sufficient for anchorage to at least some extent (5). This discussion ignores exactly how hydrophobic each stretch of uncharged amino acids is, or which charged amino acids are involved at which boundaries of the domain in each case, and what contributions these might make to the effective span length. However, it does suggest that it is possible for a considerably shortened hydrophobic stretch to act as an anchor, even if only in a partially successful fashion. It is also of interest that the minimum length requirement for both pro- and eukaryotic proteins appears to be similar. Normal membrane-spanning domains are thought to exist as alpha helices (10). Exactly how these shortened domains span the bilayer is not clear, possibly either in an extended structure or by burying the charges at either or both ends in the bilayer. In the case of the A2 mutation of p62, the region upstream of the introduced Glu is hydrophobic and so this might facilitate the latter solution.

Thus a length of hydrophobic and uncharged amino acids, considerably shorter than those spanning regions described in wild-type proteins, followed by a charge cluster, can act as an anchor. The stretch of hydrophobic amino acids spanning the bilayer can also act as an anchor without a following charge cluster. Our data concurs with that published by others. Moreover, we have added to this picture by showing in a eukaryotic system that although a full-length hydrophobic stretch without a charge cluster is fully effective as an anchor in vivo, it can be distinguished from the wild-type protein by a sufficiently stringent assay. We have also shown that a single Glu cannot substitute for the dibasic wild-type charge cluster of p62. Our results fit a model where (as described recently by Davis and Model [5]) the membrane-binding region halts translocation mainly by nonspecific (degenerate) hydrophobic interactions with the tunnel or the interior of the bilayer.

A further implication arises from our use of functional as well as structural assays; cell surface transport of membrane proteins and cell–cell fusion promoted by those proteins apparently do not require precise and stable relationships between the proteins and the membranes that they are buried in.

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