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Evidence for the loop model of signal-sequence insertion into the endoplasmic reticulum

(protein translocation/vesicular stomatitis virus glycoprotein/signal cleavage mutation)

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ABSTRACT **The insertion of proteins into the endoplasmic reticulum is mediated by short hydrophobic domains called signal sequences, which are usually cleaved during insertion. We previously constructed DNAs encoding vesicular stomatitis virus glycoproteins with N-terminal extensions preceding the signal sequence and showed that these extensions allowed normal signal-sequence function and cleavage *in vivo*. To analyze signal sequence topology during membrane insertion, we generated a point mutation that blocks cleavage of these signal sequences. After expressing these proteins in HeLa cells, we used proteolysis of microsomal membranes to determine that the N terminus of the signal sequence and the C terminus of each protein remain on the cytoplasmic side of the endoplasmic reticulum after insertion. This result indicates that the proteins were inserted in a looped configuration. Extending this finding, we were able to reverse the orientation of such a mutant protein by deleting its normal C-terminal transmembrane and cytoplasmic domains. In addition to demonstrating that a signal sequence can function as a membrane anchor, these findings show that except for the presence of a cleavage site, the cleaved signal sequence of a type I transmembrane protein is structurally and functionally equivalent to the noncleaved signal sequences of type II transmembrane proteins.**

Segregation of membrane and secretory proteins by translocation across the endoplasmic reticulum (ER) plays a fundamental role in organizing the complex biochemical events that are essential to cell function. Efforts to understand the signals that initiate and mediate translocation have led to the identification in many proteins of N-terminal presequences known as signal sequences. These sequences are recognized by a protein–RNA complex (signal-recognition particle, SRP) that acts to temporarily arrest translation and, by binding to its receptor in the ER, facilities translocation by directly juxtaposing the protein-synthesizing apparatus with the membrane of the ER (1). By a process that is still poorly understood, the nascent peptide is extruded across the lipid bilayer, where a peptidase cleaves the signal from the protein (2).

Because the interaction between the signal sequence and the ER was specific, Blobel and Dobberstein (3, 4) postulated that the binding of the ribosome to the membrane of the ER initiates the formation under the ribosome of a transient pore through which the nascent peptide chain is actively threaded. Direct insertion of the N terminus across the membrane could explain the ultimate orientation of type I membrane proteins, which have their N termini directed into the lumen of the ER and their C termini in the cytoplasm. It does not, however, readily explain the orientation of the increasing number of membrane proteins (type II) now known to be anchored in the opposite orientation (for a review, see ref. 5). These proteins include the asialoglycoprotein receptor (6, 7), HLA-DR invariant chain (8), the transferrin receptor (9), and influenza virus neuraminidase (10).

To provide a unifying model of translocation explaining the topology of type I, type II, and multispanning proteins, three groups have proposed that signal sequences might be inserted as a loop (or helical hairpin) into the ER with the N terminus of the signal sequence remaining on the cytoplasmic side (11–13). The nascent chain would then be threaded through the membrane and appear as a loop on the other side during the insertion process. If the signal sequence were cleaved from the growing peptide, the N terminus would be released into the lumen and a type I protein would be generated, provided that a membrane anchor was present elsewhere in the polypeptide chain. If the signal sequence were not cleaved, the loop model predicts that the signal would remain in the membrane and act as an N-terminal anchor, generating a type II membrane protein with the C terminus free in the lumen.

One of the main distinguishing features between the two models is the location and orientation of the signal sequence within the membrane during insertion. If the signal sequence were threaded directly through the membrane, it should lie free within the extracytoplasmic space after cleavage. If, however, the protein were looped into the membrane, the signal sequence would span the membrane with the N terminus on the cytoplasmic side of the ER. Unfortunately, cleaved signals are generally short hydrophobic stretches of 15–30 amino acids that are rapidly cleaved and not easily detected.

We developed a strategy for determining whether a signal sequence is inserted as a loop into the ER. The strategy built upon our earlier finding that random hydrophilic amino acid sequences of 20, 61, or 102 amino acids could be appended N-terminal to the vesicular stomatitis virus (VSV) glycoprotein (G) signal sequence without interfering with translocation, cleavage of the signal peptide, or subsequent transport to the plasma membrane (14). VSV G protein is a type I membrane protein with a cleaved 16 amino acid N-terminal signal sequence and a 20 amino acid membrane-spanning domain near the C terminus (15). We reasoned that the extended signal sequences might provide markers that would be readily detected in a proteolysis assay if they were, in fact, exposed on the cytoplasmic side of the ER during insertion. Our results demonstrate that a signal sequence is inserted as a loop into the ER. Further, we show that, except for the presence of a signal cleavage site, a type I signal sequence is functionally and structurally equivalent to a type II signal sequence.

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Abbreviations: ER, endoplasmic reticulum; VSV G protein, vesicular stomatitis virus glycoprotein; Endo H, endoglycosidase H.

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MATERIALS AND METHODS

Plasmid Constructions. The plasmid constructs used in this paper are depicted in Fig. 1. The constructs encoding VSV G-protein precursors with N-terminal extensions of 20, 61, and 102 amino acids in the pTZ18U vector have been described (14). To generate the signal-sequence cleavage mutants, oligonucleotide-directed mutagenesis was performed with single-strand phagemid DNA by the method of Zoller and Smith (16). Template DNA was produced from the pTZ vector with the helper phage M13K07 (17). The oligodeoxynucleotide 5′-GGTGAACTTCCGGTTCACCCCAA-3′, prepared by the DNA-synthesis service of Yale Medical School, was used to generate the change TGC to CGG (Cys to Arg) at the signal-sequence cleavage site and to generate a *Hpa* II site (CCGG) in VSV G-protein DNA. Mutated DNAs were screened by differential hybridization to ^{32}P-labeled primer and for the presence of a new *Hpa* II restriction site. DNA sequences were confirmed by the chain-terminator method (18).

The starting material for the truncated G (TG) constructs was the *Hin*dIII–*Bam*HI fragment from pSV2TG encoding TG (19), which was cloned into the *Hin*dIII–*Bam*HI site of the Bluescript vector SK+ (Stratagene, San Diego, CA) to create pTGT7. By exchanging *Kpn* I fragments (which encode the N-terminal two-thirds of the protein) from pGint61 and pGint61*, the DNA segments encoding the N-terminal extension of 61 amino acids with and without the signal-sequence cleavage mutation were introduced into pTGT7 to generate pTGint61 and pTGint61*.

Expression, Labeling, Immunoprecipitation, and Endoglycosidase H Digestion. The method for the infection and transfection is based on the procedure of Fuerst *et al.* (20), with only minor modifications. HeLa cells on 35-mm dishes (≈5 × 10^5 cells per dish) were infected with vTF-7 at a multiplicity of 10 plaque-forming units per cell. The virus was allowed to adsorb for 30 min in phosphate-buffered saline solution (10 mM NaH_2PO_4/10 mM Na_2HPO_4/150 mM NaCl, pH 7.2) at room temperature with gentle rocking. The inoculum was removed and Dulbecco's modified Eagle's medium (DMEM) with 5% fetal bovine serum was added. One hour after infection, 250 μl of a suspension of calcium phosphate-precipitated DNA (10 μg), prepared as described (21), was added. Before metabolic labeling, cells were depleted of methionine by incubation in methionine-free DMEM for 15 min. Labeling was for 30 min with [^{35}S]methionine (100 μCi/ml, 1 μCi = 37 kBq), 5 hr after infection. Cells were harvested and immunoprecipitated as described (22). Endoglycosidase H (Endo H) digestion of immunoprecipitated proteins was as described (23).

Microsome Isolation and Proteolysis with Trypsin. HeLa cells on 60-mm dishes (≈1.5 × 10^6 cells per dish) were infected with vTF-7, transfected, and labeled as described above. Cells were scraped from the dish and resuspended in 5 mM Tris Cl/15 mM NaCl, pH 7.4. Cells were broken by Dounce homogenization. Microsomes were isolated by sedimentation through 10% (wt/vol) sucrose in Tris/saline (50 mM Tris Cl/150 mM NaCl, pH 7.4) in a Beckman SW41 rotor at 38,000 rpm for 4 hr at 4°C. Samples were resuspended in Tris/saline and divided into three aliquots. One aliquot was mock-treated and the remaining samples were incubated for 30 min at 37°C with trypsin (100 μg/ml; Worthington) in the presence or absence of Nonidet P-40 (1%, vol/vol). Digestion was stopped with the addition of soybean trypsin inhibitor (100 μg/ml). Samples were diluted with equal volumes of a solution containing 1% Nonidet P-40, 0.4% deoxycholate, 66 mM EDTA, and 10 mM Tris Cl (pH 7.4) and centrifuged to remove nuclei and debris. Samples were immunoprecipitated and analyzed by $NaDodSO_4$/PAGE in gels containing 10% acrylamide and 0.13% *N,N′*-methylenebisacrylamide. Because of the low and variable yields of protein after proteolysis, the amount of radiolabeled protein analyzed was adjusted to give approximately equal band intensities on fluorograms.

RESULTS

Generation of a Mutation That Blocks Signal-Sequence Cleavage. Comparison of a large number of signal sequences has shown that the last amino acid preceding the cleavage site is always small and uncharged (24). In the VSV G-protein precursor this residue is a cysteine. We reasoned that the substitution of the cysteine with a large, charged amino acid, arginine, would block cleavage of the signal sequence. This mutation was generated by oligonucleotide mutagenesis and introduced into plasmids encoding G proteins with N-terminal extensions of 20, 61, and 102 amino acids preceding the normal signal sequence of 16 amino acids (Fig. 1*A*). Without this mutation at the cleavage site, these extended signals function normally *in vivo* and are cleaved correctly (14). The plasmids encoding mutated G proteins with N-

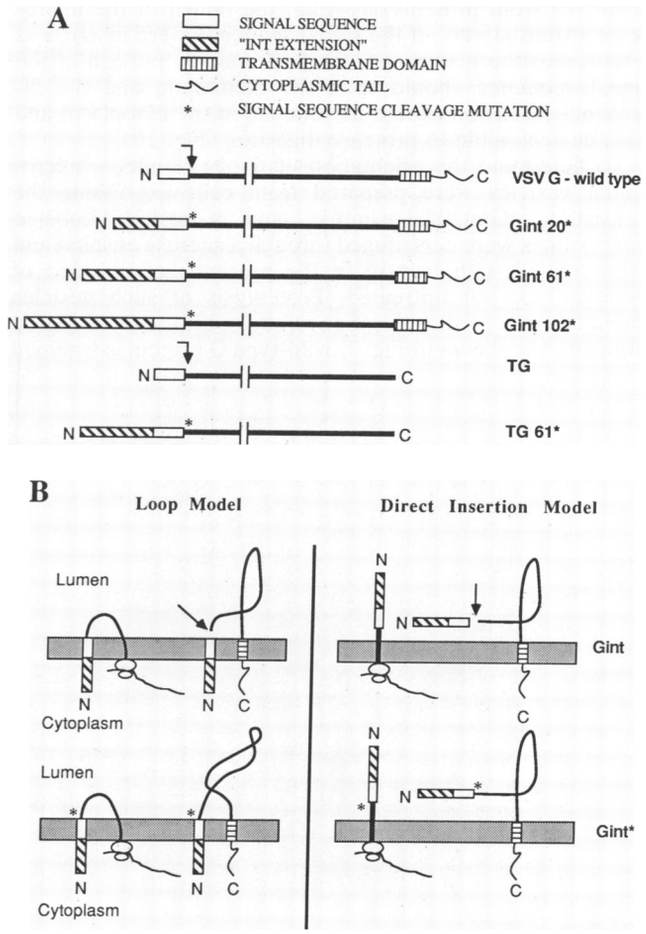

FIG. 1. (*A*) Schematic diagrams of VSV G mutant proteins with N-terminal extensions and with signal-sequence cleavage mutation (*). Also depicted are the constructs encoding truncated G (TG) and TGint61*. TG and TGint61* are constructs in which sequences encoding the normal transmembrane and cytoplasmic tail of VSV G were deleted. (*B*) Models for signal-sequence insertion into the ER. In the loop model, the extended signal sequences should be exposed on the cytoplasmic surface of the ER and be accessible to digestion by proteases. In the direct insertion model, the extended signal sequence should be translocated into the lumen of the ER and thus protected from digestion from the cytoplasmic side.

terminal extensions and the point mutation blocking cleavage (*) are designated pGint20*, pGint61*, and pGint102*.

To determine whether the mutation blocked cleavage of the signal sequence, expression of the mutant proteins was carried out in a transient expression system based on recombinant vaccinia virus expressing the bacteriophage T7 RNA polymerase (20). Cells infected with recombinant vaccinia virus were transfected with plasmids encoding the mutated genes preceded by a T7 promoter. As can be seen in Fig. 2, pGint20*, pGint61*, and pGint102* (lanes 6–8) encode proteins of progressively greater length, commensurate with the predicted molecular weights of these proteins with non-cleaved and extended signal sequences. In contrast, the corresponding proteins lacking the signal cleavage mutation (lanes 1–3) are all cleaved to a protein the size of wild-type VSV G.

Insertion of Mutant Proteins with Blocked Signal Cleavage Sites. Because we had generated uncleaved signal sequences preceded by N-terminal extensions, it was possible to determine the orientation of the signal sequence in the membrane by using proteolysis of microsomes (25). If the signal sequence were directly inserted through the membrane, the signal sequence and the N-terminal extension should be protected from proteolysis within the lumen of the microsome. Alternatively, if the signal sequence were inserted as a loop into the membrane, the hydrophobic portion of the signal sequence should span the membrane and the N-terminal extension should be retained in the cytoplasm and thus be accessible to protease digestion (Fig. 1*B*).

To determine the orientation of the N terminus, microsomal vesicles were prepared from cells expressing the constructs pGint20*, pGint61*, and pGint102*. Labeled microsomes were centrifuged through a sucrose cushion and then treated with trypsin in the presence or absence of detergent or left untreated. Proteolysis of such vesicles should result in the digestion of cytoplasmically exposed domains. As shown in Fig. 3, wild-type G protein is trimmed

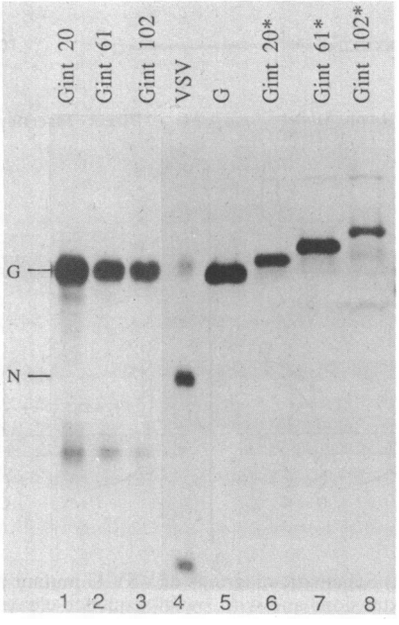

FIG. 2. Expression of mutated VSV G proteins. HeLa cells were infected with a recombinant vaccinia virus encoding T7 RNA polymerase and transfected with plasmid DNA. Mutated VSV G proteins with N-terminal extensions (lanes 1–3) and with a mutation blocking signal-sequence cleavage (lanes 6–8) were labeled, 5 hr after infection, for 30 min with [^{35}S]methionine (100 μCi/ml), lysed, immunoprecipitated, and analyzed by NaDodSO$_4$/PAGE. Positions of VSV G protein and nucleocapsid protein (N) are indicated at left.

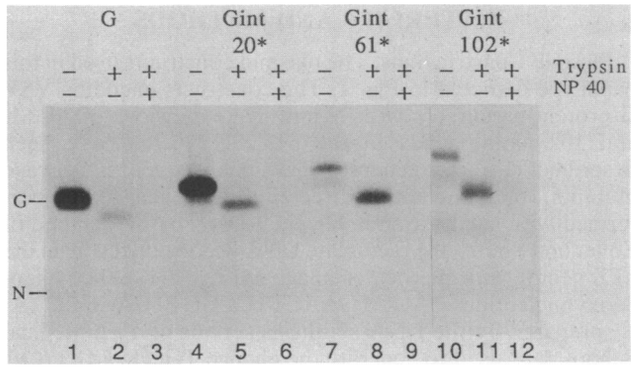

FIG. 3. Proteolysis of microsomes containing Gint* mutant proteins. Infected HeLa cells transfected with DNA encoding VSV G mutant proteins with N-terminal extensions of the signal sequence and with the signal-sequence cleavage mutation (Gint20*, Gint61*, and Gint102*) were labeled, 5 hr after infection, with [^{35}S]methionine. Cells were broken in a Dounce homogenizer in hypotonic Tris/saline (5 mM Tris Cl/15 mM NaCl, pH 7.4) and pelleted through a 10% sucrose/Tris/saline (50 mM Tris Cl/150 mM NaCl, pH 7.4) cushion. Microsomes were treated with trypsin (100 μg/ml) with or without detergent [1% Nonidet P-40 (NP 40)] or left untreated at 37°C for 20 min. Samples were immunoprecipitated. Because of low yields after proteolysis, sample volumes were adjusted so that roughly equivalent amounts of radiolabeled protein were analyzed by NaDodSO$_4$/PAGE.

only slightly with trypsin; this is known to result from proteolytic removal of the 29 amino acids in the cytoplasmic domain (25). In contrast, protease digestion of proteins with uncleaved signal sequences, Gint20*, Gint61*, and Gint102*, gives larger mobility shifts. The calculated molecular weight shifts indicate the exposure of additional cytoplasmic domains composed of the N-terminal extensions on each of the Gint* constructs. Note that the digested Gint* proteins are slightly larger (lanes 5, 8, and 11) than the digested wild-type protein. This is consistent with protection of the uncleaved, hydrophobic portion of the signal sequence as an N-terminal transmembrane domain. These data indicate that the N-terminal extensions are exposed on the cytoplasmic side of the membrane along with the normal cytoplasmic domain and that the remainder of the protein is inside the vesicles. We therefore conclude that the Gint* proteins were inserted as loops into the ER such that the N-terminal extensions remained on the cytoplasmic side with the normal signal sequence forming a transmembrane domain.

Reversal of the Membrane Orientation of VSV G Protein. A logical prediction from the findings described above is that deletion of the C-terminal membrane anchor from the Gint* mutant proteins would completely reverse the membrane orientation of the mutant protein as compared to wild-type, since the new C-terminus would be released into the lumen of the ER and the N-terminus would remain in the cytoplasm (13). It was also possible, however, that the uncleaved signal sequence of VSV G protein would not be hydrophobic enough or long enough to anchor the protein securely in the absence of the C-terminal anchor.

To test this prediction, we used a previously described (19) mutant G protein, with a C-terminal deletion of the transmembrane and cytoplasmic domains, that we call truncated G (TG). Although this protein is inserted and translocated into the ER, it is not anchored and is secreted. We recombined this deletion mutant into Gint61* to create TGint61* (Fig. 1*A*). If the signal sequence of VSV G were able to function as a membrane anchor, TGint61* should be anchored in the membrane with its N terminus in the cytoplasm and its C terminus in the lumen of the ER (a type II orientation).

A proteolysis protection assay was performed on TG and TGint61*. Because TG lacks the transmembrane domain and cytoplasmic tail of VSV G, it behaves as a secreted protein and, as expected, is completely protected from proteolysis within the microsomes (Fig. 4, lanes 1 and 2). TGint61*, on the other hand, has the internalized noncleaved signal sequence and shows a decrease in apparent molecular weight after digestion (lanes 4 and 5). We conclude that TG61* has a short N-terminal sequence on the cytoplasmic side of the ER. Thus, the membrane topology of the mutant protein must be reversed as compared to wild-type G protein.

Proteins with Signal Cleavage Mutations Are Translocated but Are Not Transported. To determine whether lack of signal-sequence cleavage would alter transport of the mutant proteins, we first looked for expression of these mutant proteins on the cell surface. No surface membrane expression was visible by indirect immunofluorescence, although the proteins were detectable in internal membranes of permeabilized cells (data not shown). We also examined acquisition of Endo H resistance. Because the oligosaccharide modifications that result in Endo H resistance occur in the Golgi compartment (26), the rate at which glycoproteins acquire Endo H resistance can be used to measure the rate of transport to this compartment. VSV G protein normally acquires Endo H resistance with a half-time of 15 min (23).

G proteins with N-terminal extensions (with or without the signal cleavage mutation) were pulse-labeled with [^{35}S]methionine for 30 min and "chased" for 30 min. As can be seen in Fig. 5, VSV G proteins with normal signal cleavage (G, Gint20, Gint61, and Gint102), are comprised of mainly Endo H-resistant glycoprotein, indicating normal transit to the Golgi compartment. In contrast, the mutant G proteins with blocked signal-sequence cleavage (Gint20*, Gint61*, and Gint102*) are completely Endo H-sensitive, indicating that these proteins are not transported to the Golgi compartment. In separate experiments, with chases up to 3 hr long, no Endo H-resistant protein was detected (data not shown).

DISCUSSION

The purpose of the study reported here was to examine the topology of N-terminal signal-sequence insertion into the ER of a eukaryotic cell. The critical aspect of our approach was to use random N-terminal extensions of 20, 61, or 102 amino acids as markers for the N terminus of the signal sequence on the VSV G protein. Previously, we demonstrated that these extensions, which were chosen because they are not especially hydrophobic or hydrophilic, do not in any way interfere with the rates of synthesis, translocation, cleavage, or subsequent transport of VSV G protein (14). When cleavage of

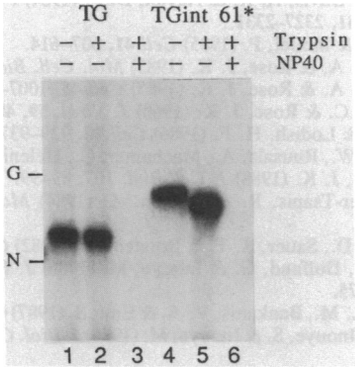

FIG. 4. Proteolysis of microsomes containing TG and TGint61* proteins. Microsomes from HeLa cells expressing truncated G (TG) and TGint61* were prepared and treated with trypsin as described in Fig. 3 legend.

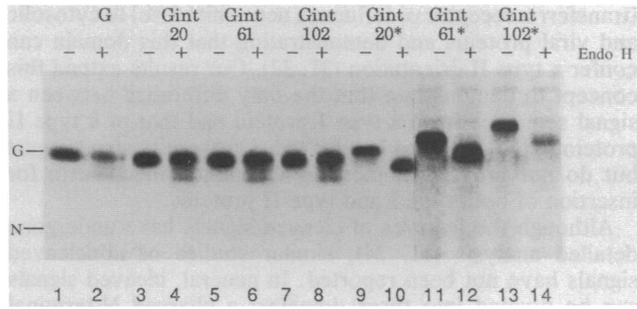

FIG. 5. Acquisition of Endo H resistance. HeLa cells expressing DNA of pGint and pGint* constructs were pulse-labeled with [^{35}S]methionine for 30 min and "chased" by incubation in medium containing 2 mM unlabeled methionine for 30 min. Cells were lysed and immunoprecipitated. Equal portions were incubated with (+) or without (−) Endo H and analyzed by NaDodSO$_4$/PAGE.

these extended signal sequences was blocked by changing one amino acid at the normal signal cleavage site, we found that the N-terminal extensions (as well as the C terminus) were exposed on the cytoplasmic side of the ER, resulting in a looped conformation of the protein. These findings provide direct evidence for the loop model of protein insertion into the ER of eukaryotic cells.

Two groups have provided direct evidence for the loop model of signal-sequence insertion into the bacterial membrane (27, 28). Bacterial systems, however, are not known to have a signal-recognition particle (SRP)-dependent insertion mechanism and thus may not be representative of insertion into the ER. In a study related to ours, Perara and Lingappa (29) studied insertion into the ER of a hybrid protein in which α-globin was placed N-terminal to the signal sequence of preprolactin. They found that the signal was cleaved and that both domains flanking the signal sequence were at least partially translocated into the ER. Because the signal sequence was cleaved, and because globin translocation may occur after cleavage, this study could not address the question of signal-sequence topology during insertion. Thus the ultimate disposition of globin might not reflect the functional orientation or conformation of the signal sequence during translocation of prolactin. Because we did not detect any translocation of the extended signal sequences of VSV G, it is clear that the mode of insertion of these signal sequences into the ER must occur by a loop mechanism.

Although our findings do not rule out direct insertion of some signal sequences, they do demonstrate that the loop model of signal-sequence insertion is correct in at least some cases. Loop insertion of the signal sequence may have an important function in directing protein folding as well. The structure of the hemagglutinin protein of influenza virus suggests that, during its biogenesis, it might be tethered as a loop by the signal sequence and the normal transmembrane domain (30). Depending on the timing of signal-sequence cleavage, this tethering of the N terminus might play an important role in the determination of protein folding.

Our studies suggested that the hydrophobic portion of the VSV G-protein signal sequence was capable of serving as a membrane anchor because the N-terminal extensions preceding it were cytoplasmic. This was demonstrated clearly by elimination of the normal C-terminal anchor from one of the proteins anchored at both N and C termini. This left the protein anchored only by its N-terminal, uncleaved signal. In this case, the truncated G protein was effectively converted into a type II membrane protein since its orientation was reversed relative to the wild-type protein. That the signal sequence of type II transmembrane proteins controls the orientation of the protein has been clearly demonstrated by fusing the signal sequence of a type II membrane protein

(transferrin receptor or influenza neuraminidase) to cytosolic and viral proteins and demonstrating that this domain can confer a type II orientation (31, 32). Our results extend this concept to demonstrate that the only difference between a signal sequence from a type I protein and that of a type II protein is the ability to be cleaved. Our results also suggest but do not prove that there is a common mechanism for insertion of both type I and type II proteins.

Although the features of cleaved signals have undergone detailed analysis (11, 24), similar studies of noncleaved signals have not been reported. In general, cleaved signals can be divided into three domains: a charged N-terminal domain of variable length, a central hydrophobic region measuring 12–20 amino acids in length, and a more polar C-terminal region that governs the cleavage site. Although most noncleaved signals are notable for very long stretches of noncharged residues (sometimes numbering more than 25; ref. 9), the fact that the signal sequence of VSV G (which has a hydrophobic core of only 11 amino acids) can confer a type II membrane topology suggests that the length of the hydrophobic core does not play a critical role in translocation. It is possible, however, that longer hydrophobic stretches are important for providing a stable anchor function or for allowing subsequent diffusion and transport to the cell surface.

It is not entirely surprising that the signal sequence of VSV G protein can function as an anchor. Davis and Model (33) demonstrated that a continuous hydrophobic domain as short as 12 residues shows considerable membrane association. Other studies have shortened transmembrane domains or interrupted them with highly charged residues and demonstrated that hydrophobic regions containing uncharged stretches as short as 8 residues can act as membrane anchors (34, 35), although the actual spanning regions are presumably longer than this. Although it is quite possible that some of the N-terminal anchor function resides somewhere within the N-terminal extensions, this seems unlikely, as the extensions were originally selected because of their hydrophilicity. The longest stretch of noncharged amino acids is only 5 residues long in these extensions (14).

The inability of the proteins with signal-sequence cleavage mutation to be transported is consistent with previous results showing that even very small mutations near the N terminus of VSV G protein (deletion of four amino acids) can block transport (36). We suspect that membrane anchoring of the N terminus interferes with the folding and trimerization of VSV G protein, features that have been shown to be essential for intracellular transport (37, 38).

An important part of our approach was the generation of signal-sequence cleavage mutations in proteins with N-terminal extensions preceding the signal. Such mutations have not been described for animal cell proteins, although they have been generated in bacteria and yeast (39–43). Alteration of the native signal cleavage site can result in the cleavage of the signal sequence at an adjacent site (44). We were fortunate that the protein we chose to study and the alteration that we selected resulted in almost complete inhibition of signal-sequence cleavage.

Although the loop model appears to explain how cleaved and uncleaved signal sequences generate proteins of differing orientations, it does not explain how the orientations of proteins such as the NB protein of influenza (which is a type I single-spanning transmembrane protein without a cleaved signal sequence) are attained (45). It is not clear whether the loop model can be modified to explain this topology or whether a completely different mechanism of insertion applies to such proteins. This is a problem that can be approached by utilizing a strategy similar to the one we employed here.

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