Signal and Membrane Anchor Functions Overlap in the Type II Membrane Protein IγCAT

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Abstract. IγCAT is a hybrid protein that inserts into the membrane of the endoplasmic reticulum as a type II membrane protein. These proteins span the membrane once and expose the NH2-terminal end on the cytoplasmic side and the COOH terminus on the exoplasmic side. IγCAT has a single hydrophobic segment of 30 amino acid residues that functions as a signal for membrane insertion and anchoring.

The signal–anchor region in IγCAT was analyzed by deletion mutagenesis from its COOH-terminal end (AC mutants). The results show that the 13 amino acid residues on the amino-terminal side of the hydrophobic segment are not sufficient for membrane insertion and translocation. Mutant proteins with at least 16 of the hydrophobic residues are inserted into the membrane, glycosylated, and partially proteolytically processed by a microsomal protease (signal peptidase).

Our data suggest that in the type II membrane protein IγCAT, the signals for membrane insertion and anchoring are overlapping and that hydrophilic amino acid residues at the COOH-terminal end of the hydrophobic segment can influence cleavage by signal peptidase.

From this and previous work, we conclude that the function of the signal-anchor sequence in IγCAT is determined by three segments: a positively charged NH2 terminus, a hydrophobic core of at least 16 amino acid residues, and the COOH-terminal flanking hydrophilic segment.

SECRETORY and membrane proteins are inserted into the membrane of the endoplasmic reticulum (ER) by a mechanism requiring the interaction with signal recognition particle (SRP) and docking protein (DP) or SRP receptor (for review see Walter et al., 1984; Wickner and Lodish, 1985; Rapoport and Wiedmann, 1985). Membrane proteins can span the membrane either once or several times. Those which span the membrane once can expose either the COOH terminus (type I membrane proteins) or the NH2 terminus (type II membrane proteins) on the cytoplasmic side of the membrane. Our aim is to characterize the signals that determine the orientation of proteins spanning the membrane once.

The invariant chain (Iγ) of class II histocompatibility antigens is a glycosylated type II membrane protein. It spans the membrane of the ER once and exposes its 30 NH2-terminal amino acid residues on the cytoplasmic side whereas the COOH-terminal portion, comprising 156 amino acid residues, lies on the exoplasmic side of the membrane (Claesson et al., 1983; Strubin et al., 1984; Lipp and Dobberstein, 1986a).

Neither Iγ nor other type II membrane proteins have a cleavable signal sequence. Membrane insertion of Iγ is nevertheless dependent on SRP and DP (Lipp and Dobberstein, 1986a). The single hydrophobic segment in Iγ contains the signal for membrane insertion as well as for membrane anchoring. The NH2-terminally located 72 amino acid residues of Iγ, when fused to the cytoplasmic protein chloramphenicol-acetyltransferase (CAT), translocate the CAT portion to an exoplasmic location (Lipp and Dobberstein, 1986b).

Other type II membrane proteins, such as the neuraminidase of influenza virus (Bos et al., 1984; Markoff et al., 1984), the transferrin receptor (Schneider et al., 1984; Zerial et al., 1986), the asialoglycoprotein receptor (Holland et al., 1984; Spiess and Lodish, 1985), the hepatic glycoprotein receptor (Chiaccia and Drickamer, 1984), and the sucrose–isomaltase receptor (Semenza, 1986) also have single hydrophobic segments which function in ER membrane targeting and anchoring. Zerial et al. (1986) recently showed that it is the hydrophobic character, rather than the distinct amino acid sequence of the transmembrane segment, that is important for the insertion and membrane anchoring of a mutant transferrin receptor. However, it is not the hydrophobic segment alone that

1. Abbreviations used in this paper: AP, acceptor peptide; CAT, chloramphenicol-acetyltransferase; DP, docking protein; ER, endoplasmic reticulum; Iγ, human invariant chain; RM, rough microsome; SRP, signal recognition particle; TE, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA.

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determines membrane disposition of a protein. Previously, we have shown that hydrophilic sequences preceding the hydrophobic segment in ly can determine cleavage by signal peptidase. Upon deletion of the hydrophilic NH2-terminus of ly, a cleavage site formerly hidden in the middle of the hydrophobic segment became accessible for signal peptidase and led to complete translocation of this protein (Lipp and Dobberstein, 1986b).

Here we investigate the contribution of the COOH-terminal half of the hydrophobic segment and of its flanking sequences to membrane insertion, orientation, and processing by signal peptidase. We show that the signals for membrane insertion and membrane anchoring in ly overlap and that the amino acid residues adjacent to the COOH-terminal side of the hydrophobic segment can determine cleavage by signal peptidase and, as a consequence, integration into or translocation across the membrane. A tripartite structure is suggested for the signal-anchor sequence.

Materials and Methods

Materials
Restriction endonucleases, T4 DNA ligase, nuclease Bal 31, and proteinase K were from Boehringer Mannheim GmbH, Mannheim, FRG. Escherichia coli RNA polymerase, 7mGpppA, and DNA sequencing reagents were from Pharmacia Fine Chemicals, Freiburg, FRG. L-[35S]Methionine was from Amersham Buchler GmbH, Braunschweig, FRG.

DNA Preparation and Sequencing
Small-scale plasmid preparations were done as described by Haemptple et al. (1986). For large-scale plasmid preparations, the alkaline lysis method of Birnboim and Doly (1979) was used (Maniatis et al., 1982).

Eco RI-Pvu II fragments of the ΔC plasmids were cloned into phage M13-derived plasmid mp18 (Norramber et al., 1983). Sequencing was done as described by Sanger et al. (1977).

Construction of ΔC Mutants
Plasmid Iycat was previously described (Lipp and Dobberstein, 1986b). It codes for Iycat, a fusion protein consisting of the 72 NH2-terminal amino acid residues of Iy and the entire CAT protein. The plasmid is a derivative of pDS5 which allows transcription of the Iycat sequence from a T5 promoter (Stueber et al., 1984).

Plasmid Iycat was linearized by PstI restriction enzyme and exonuclease Bal 31 was used at 0.3 U/μg DNA to digest between 50 and 115 nucleotides from the ends. Digestions were carried out for 2–10 min at 37°C in 20 mM Tris-HCl, pH 8.0, 12 mM MgCl2, 12 mM CaCl2, 600 mM NaCl, 1 mM EDTA. Aliquots were removed after 1-min intervals. The reactions were stopped by EGTA at a final concentration of 50 mM (Lagersténi et al., 1978). The samples were diluted sixfold with TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA), extracted twice with phenol, and the DNA was precipitated with ethanol. To estimate the degree of digestion, 10% of the DNA was cut with Xho I and Pvu II restriction enzymes and analyzed by electrophoresis. We found that under the above conditions 60–80 bp/min were deleted. For the construction of the ΔC mutants, DNA from the 2-min digestion was ligated with appropriate deletions (AC) were further analyzed by in vitro transcription, translation, and membrane-insertion assays.

In Vitro Transcription and Translation
Plasmids were transcribed in vitro by E. coli RNA polymerase in the presence of 7mGpppA, and the resulting capped mRNA was translated in the wheat germ cell-free system as described by Stueber et al. (1984). In some of the translations, SRP and microsomal membranes from dog pancreas were included to test for membrane insertion (Blobel and Dobberstein, 1975; Walter and Blobel, 1980).

Glycosylation of asparagine residues was blocked by the addition of the acceptor peptide (AP) benzoyl-asy-leu-thr-N-methylamide to a final concentration of 30 μM (Lau et al., 1983; Lipp and Dobberstein, 1986b).

Results

Construction of Plasmids Coding for the ΔC Mutants
We previously described the plasmid Iycat which codes for a fusion protein consisting of the 72 NH2-terminal amino acid residues of Iy followed by the cytoplasmic protein CAT (Lipp and Dobberstein, 1986b; Fig. 1). The hydrophobic membrane-spanning segment of 30 amino acid residues is located within the segment of 72 amino acids. To delete portions from the COOH-terminal end of the hydrophobic segment, we cut the plasmid Iycat at its unique PstI restriction site, 36 bases downstream from the region, coding for the hydrophobic segment. Exonuclease Bal 31 was used to delete stepwise from either end of the linearized DNA (Fig. 1). Mutant plasmids were characterized by restriction map analysis to determine the size of the deletions. Plasmids with appropriate deletions (ΔC) were further analyzed by in vitro transcription, translation, and membrane-insertion assays.

ΔC Mutants Result in Three Topologically Different Groups of Proteins
An in vitro transcription-translation membrane translocation system was used to analyze membrane insertion and orientation of the ΔC mutant proteins. Iycat cDNA as well as its ΔC deletion derivatives were inserted behind a phage T5-derived promoter (Stueber et al., 1984). After transcription by E. coli RNA polymerase, the resulting mRNA was translated in a wheat germ cell-free system in the presence or absence of dog pancreas microsomal (RM) membranes (Lipp and Dobberstein, 1986b).

Iycat was previously shown to be a glycosylated type II membrane protein (Lipp and Dobberstein, 1986b). In the absence of RM, Iycat is synthesized as a 34-kD protein and in the presence of RM, as a 37-kD glycosylated form. Proteinase K digestion removes a segment of ~2 kD, indicating the cytoplasmic location of this segment (Lipp and Dobberstein, 1986b; Fig. 2). All protein is digested if membranes are solubilized by detergent before proteinase K digestion (Lipp and Dobberstein, 1986b). Digestion in the presence of detergent serves as a control for protease-resistant fragments. Selected ΔC clones were subjected to the same analysis.
In vitro translation and membrane insertion of IyCAT and of selected ΔC deletion derivatives. ΔC mutant plasmids were transcribed by Escherichia coli RNA polymerase. The resulting mRNA was translated in the wheat germ cell-free system in the absence (lanes 1) or presence (lanes 2, 3, and 4) of microsomal membranes (RM). Membrane translocation or insertion was assayed by treatment with proteinase K without (lanes 3) or with (lanes 4) the detergent NP-40. Glycosylated forms are indicated by an asterisk. Proteins processed by a microsomal protease (signal peptidase) are indicated by a shill.

and, according to their membrane interaction, classified into three groups. The analysis of one member of each group is shown in Fig. 2.

**Group I.** In the absence (Fig. 2, lane 1) as well as in the presence (lane 2) of RM, ΔC70 is synthesized as a 28-kD protein. This protein is digested upon addition of proteinase K. Therefore, the protein is not translocated across RM membranes and does not become glycosylated. The protein portion of ~25 kD in lanes 3 and 4 is not protected by the membranes. It is resistant to proteinase K digestion even in the presence of detergent and is most likely derived from CAT. CAT protein is known to be very resistant to proteinase K digestion.

**Group II.** In the absence of RM, ΔC2 is synthesized as a 29-kD protein. In the presence of RM, several additional forms appear: a larger one, which is most likely due to glycosylation (*); and smaller ones, probably due to proteolytic processing () or proteolytic processing and glycosylation (*). After treatment with proteinase K, several different molecular mass forms are found protected (Fig. 2, lane 3). A more detailed analysis of this group of proteins is given below (see Fig. 4).

**Group III.** In the absence of RM, ΔC89 is synthesized as a 28-kD protein. In the presence of RM, a form with a molecular mass 3 kD higher appears, the size of which is reduced by ~2 kD after treatment with proteinase K. This analysis pattern is the same as that obtained for authentic IyCAT protein and suggests that ΔC89 is glycosylated and spans the membrane as IyCAT.

Several other ΔC proteins were analyzed. They were placed in the first group if no processing and no protection against proteinase K was observed; in the second group if membrane insertion, glycosylation, and proteolytic processing occurred; and in the third group if membrane integration and glycosylation, but no proteolytic processing, occurred (see Fig. 3).

**Amino Acid Sequences of the Hydrophobic Segments and Their Flanking Regions in ΔC Mutant Proteins**

To determine the extent of the deletions in the ΔC mutant proteins, the Eco RI–Pvu II fragments (see Fig. 1) of the ΔC

![Figure 3. Outline and amino acid sequences of the hydrophobic segments and their flanking regions in IyCAT and in the ΔC mutants. The NH2-terminal cytoplasmic segments are underlined, the hydrophobic segments derived from Iγ are indicated by wavy lines, and CAT by a closed box. When the deletion extended into the CAT sequence, this is indicated by an open box. The numbers below the IyCAT sequence indicate amino acid residues from NH2-terminal end. The arrow indicates the potential signal-peptidase cleavage site which is used in ΔN-IyCAT. Clones are grouped according to their association with the membrane: clones coding for proteins that are not inserted into RM membranes; clones coding for proteins that either span the membranes or are proteolytically processed and translocated; and clones coding for proteins that span the membrane as IyCAT.](https://www.jcb.org/content/jcb/185/1/101/F3.large.jpg)
deletions were subcloned into phage M13-derived plasmid mpl8 (Norrander et al., 1983) and relevant segments were sequenced by the dideoxy method (Sanger et al., 1977). The deduced amino acid sequences are shown in Fig. 3.

AC proteins of the first group are neither inserted into the membrane nor processed (Fig. 3), nor is synthesis affected by SRP (data not shown). These proteins retained up to 13 amino acid residues of the Iy-derived hydrophobic segment. 12 of these hydrophobic amino acid residues constitute the cleaved signal sequence of AN-IyCAT as shown previously (Lipp and Dobberstein, 1986b). The cleavage site for signal peptidase in AN-IyCAT is indicated by an arrow in Fig. 3.

In the second group of AC proteins, 16-23 amino acid residues of the hydrophobic segment are retained. These proteins either span the membrane or become proteolytically processed and translocated. Thus, it can be concluded that 16 amino acids are sufficient for membrane insertion and anchoring.

The third group comprises AC proteins that span the membrane as IyCAT. No proteolytic processing can be observed. AC89 retained 20 amino acids of the hydrophobic segment. Note that the identical number of residues is retained in AC2 which is however partially processed by a microsomal peptidase. This suggests that amino acid residues flanking the hydrophobic segment at the COOH-terminal side can determine proteolytic cleavage.

**Group II AC Fusion Proteins Become Glycosylated and Proteolytically Processed**

AC proteins of the second group appeared to become glycosylated as well as proteolytically processed. As the degree of processing varied quite drastically between different members of this group, three clones (AC2, AC64, and AC67), were selected for further analysis. After translation in the absence or presence of RM, protease K was used to determine the degree of translocation.

In the absence of RM, a single major polypeptide was synthesized in each case (AC2, AC64, and AC67) (Fig. 4, lanes 1). Protease K digestion of these proteins resulted in small polypeptide fragments (Fig. 4, lanes 2). In the presence of RM, four major size classes of polypeptides were synthesized (Fig. 4, lanes 3): (a) AC polypeptides which are not inserted into the membrane and are thus identical to those synthesized in the absence of RM (Fig. 4, lanes 1 and 3); (b) AC* polypeptides which are ~3 kD larger than the AC polypeptides. The shift in molecular mass is consistent with N-glycosylation at one site without proteolytic processing; (c) AC* polypeptides which are ~2 kD smaller than the AC polypeptides. They are proteolytically processed and glycosylated (see below); (d) AC** polypeptides which are ~4 kD smaller than the AC ones. They are proteolytically processed by a microsomal protease (signal peptidase) (see below).

After protease K digestion the molecular mass of AC2* was reduced by ~2 kD. This indicates that it spans the membrane and exposes a 2-kD segment on the cytoplasmic side. For AC64* and AC67* similar membrane-spanning forms could not clearly be detected. Instead, the amount of endogenously processed AC* forms increased after the protease K treatment. Usually only ~50% of the membrane-translocated protein is protected against exogenously added
proteinase K due to unsealed vesicles. We suspect that ΔC64 and ΔC67 proteins from which proteinase K has removed the cytoplasmically exposed segment are posttranslationally processed by signal peptidase. Thus, no membrane-integrated protein (AC*) can be detected after the proteinase K treatment. This interpretation is supported by our previous finding that removal of the NH2-terminal segment from IyCAT led to signal peptidase cleavage in ΔN-IyCAT (Lipp and Dobberstein, 1986b).

To distinguish between glycosylation and proteolytic processing, membrane insertion was carried out under conditions in which N-linked glycosylation was inhibited by an AP (Lau et al., 1983). This AP, benzoyl-asn-leu-thr-N-methylamide, competes efficiently with the nascent IyCAT polypeptide for N-linked glycosylation (Lipp and Dobberstein, 1986b).

When mRNA from ΔC2, ΔC64, and ΔC67 clones was translated in the presence of RM and AP, the ΔC* and ΔC'* proteins disappeared. This showed that they were N-glycosylated. The amounts of ΔC and, in particular, ΔC' forms increased. We conclude that ΔC' proteins are proteolytically processed and that the ΔC'* proteins represent the glycosylated forms of ΔC'. ΔC' forms are ~4 kD smaller than the ΔC proteins and most likely are derived from cleavage by signal peptidase. The degree of proteolytical processing differed considerably between the three ΔC mutant proteins. The highest amount of processing (ΔC') was found with ΔC67.

When nonglycosylated and membrane-inserted proteins were digested with proteinase K, AC' proteins were found protected. In addition, proteins smaller than the AC proteins by ~2 kD (dot) were protected (Fig. 4, lanes 6 and 7). We conclude that nonglycosylated ΔC proteins of group II can either be translocated across the membrane or integrated in the membrane. However, the amount of membrane-integrated ΔC64 and ΔC67 protein seems to be drastically reduced after the proteinase K treatment (compare proteins marked by a dot in lanes 4 and 7). Concomitantly, the amount of endogenously processed ΔC' proteins is increased.

In experiments where proteinase K treatment was done on the nonglycosylated and membrane-inserted proteins in the presence of detergent, a protease-resistant protein was found (Fig. 4, lanes 8). The molecular mass of the resistant fragment was the same in each case and is identical to that of authentic CAT protein. CAT is known to be resistant against proteinase K (Lipp and Dobberstein, 1986b). Processing of ΔC2 by the microsomal peptidase occurred, most likely, very close to the NH2 terminus of authentic CAT and thus resulted in the same size of protease-resistant protein, irrespective of the presence of detergent (Fig. 4, lanes 7 and 8).

Membrane Integration of Processed and Unprocessed ΔC Group II and III Proteins

The hydrophobic segments in the ΔC proteins of group II and III are shortened to between 16 and 23 amino acid residues when compared to the IyCAT protein (see Fig. 3). We asked whether the reduced number of hydrophobic amino acid residues is still sufficient to anchor the proteins in the membrane. Membrane integration was tested by the extractability with carbonate at pH 11. Treatment of membranes with carbonate at pH 11 is known to release most proteins that are not integrated into the lipid bilayer of the membrane (Fujiki et al., 1982). We also asked whether removal of the NH2-terminal hydrophilic segment by proteinase K has an effect on the stable integration of these proteins in the membrane.

IyCAT and the ΔC proteins of group II (ΔC67, ΔC2, and ΔC64) and group III (ΔC89) were synthesized in the absence or presence of microsomal membranes and AP (Fig. 5, lanes 1 and 2). The analysis of 5 μl of the translation mixture shows that the ΔC' forms can be seen in the samples containing microsomes (Fig. 5, lanes 2; ΔC67, ΔC2, and ΔC64). The AP was included to detect the processed ΔC' forms. One aliquot (20 μl) of the assay containing microsomes was treated with pro-
The Signal for Membrane Insertion and Anchoring in IyCAT

Using a deletion analysis, we tested the importance of the COOH-terminal portion of the hydrophobic segment in IyCAT to membrane insertion, anchoring, processing, and translocation of mutant proteins.

Our results show that the 13 NH₂-terminal residues of the hydrophobic segment in IyCAT are not sufficient for membrane insertion or translocation (see AC70, Fig. 3). We have previously shown that 12 of these amino acid residues constitute a cleavable signal sequence in ΔN-IyCAT (Lipp and Dobberstein, 1986b). This indicates that the functional signal sequence in IyCAT extends over the potentially cleaved signal sequence. It has also been found for some other proteins that the cleaved signal sequence is not always identical with the functional one, but extends into the NH₂-terminal region of the mature protein (Moreno et al., 1980; Abrahamsson et al., 1985; Lehnhardt et al., 1987).

The amino-terminally located 16 amino acids of the hydrophobic segment in IyCAT are sufficient to translocate the CAT portion to a luminal, membrane-bound or -soluble position (ΔC64, Fig. 3). The segment of 16 amino acid residues includes the 12 residues previously shown to constitute a cleavable signal sequence in ΔN-IyCAT. Also, with other proteins, it has been shown that at least 16 uncharged amino acid residues are necessary to span the membrane (Adams and Rose, 1985; Davis and Model, 1985). As the signal sequence in IyCAT comprises more than 12 amino acid residues and 16 residues are required for membrane anchoring, this indicates that the signals for membrane insertion and for membrane anchoring in IyCAT overlap.

The results with mutant protein ΔC89 show that 20 amino acid residues of the hydrophobic segment are sufficient to result in a type II membrane-spanning protein with no detectable proteolytic processing occurring (ΔC89, Fig. 3). Interestingly, in ΔC89, a negatively charged amino acid is flanking the COOH-terminal side of the hydrophobic segment. In all of the natural type II membrane proteins characterized so far, positively charged amino acids flank the hydrophobic segment on the COOH-terminal side. This shows that a negatively charged residue at this side of the hydrophobic segment is also compatible with membrane insertion and anchoring of a type II membrane protein.

Processing by an RM Protease, Signal Peptidase

Shortening of the 30-amino acid-long, hydrophobic segment to between 16 and 23 amino acid residues resulted in cleavage by a RM protease. The proteolytically processed proteins were completely translocated across the ER membrane. The cleavage is most likely performed by signal peptidase: it occurs during insertion of the protein into the membrane; it removes a segment of ~4 kD which includes the membrane-anchoring portion of the mutant proteins and thus must occur close to the NH₂-terminal end. Both of these events are consistent with signal peptidase cleavage. Sequence analysis is necessary to determine the exact site of cleavage. We previously observed a similar cleavage if the NH₂-terminal end of IyCAT was deleted (Lipp and Dobberstein, 1986b). The resulting protein, ΔN-IyCAT, was cleaved by signal peptidase between amino acid residues 12 (thr) and 13 (leu) of the hydrophobic segment, as indicated in Fig. 3 by an arrow. We suspect that in the ΔC deletions, a cryptic cleavage site becomes available to signal peptidase. Mechanistically, one could imagine that the shortened hydrophobic segment in the ΔC mutant proteins becomes stretched across the membrane. As a consequence, a potential cleavage site could become exposed to the luminal side, where signal peptidase is located (Jackson and Blobel, 1977).

Proteolytic processing of the group II mutant proteins not only depends on the length of the hydrophobic segment but also on the flanking amino acid residues. This is evident from a comparison of ΔC2 and ΔC89, both of which have the same length of hydrophobic segment but differ in the COOH-terminal hydrophilic amino acid residues. ΔC2 is partially cleaved, whereas ΔC89 is not. In ΔC2, positively charged residues are found more closely to the hydrophobic segment than in ΔC89. It is conceivable that a membrane-anchoring segment is stretched differently across the membrane if it is flanked by positively charged amino acid residues rather than by negative ones (Weinstein et al., 1982). Different membrane dispositions of ΔC2 and ΔC89 are also suggested from the analysis of proteinase K-processed ΔC2 and ΔC89 proteins. After removal of the cyto-
plasmic segment, ΔC2 became carbonate extractable from microsomal membranes, whereas ΔC9 did not.

Shortening of the hydrophobic segment in the group II ΔC proteins had a drastic effect on the stability of these proteins in the membrane as well as on cleavage by signal peptidase. This only became evident when the cytoplasmic segment of these proteins was removed by proteinase K treatment. All of the group II ΔC proteins became extractable with carbonate at pH 11 when their cytoplasmic segments were removed. Our results suggest that the hydrophilic cytoplasmic segment plays an important role in anchoring the proteins stable in the membrane. Mutants that changed the interaction of hydrophobic segments with the lipid bilayer have also been observed by Cutler et al. (1986). These authors found that mutations which shortened the hydrophobic transmembrane segment of the p62 protein of Semliki Forest virus reduced the stability of the mutant protein in the membrane (Cutler et al., 1986).

Deletion of the cytoplasmically exposed NH2-terminal segment from group II ΔC mutants resulted in further cleavage by signal peptidase. This became particularly evident when the amounts of ΔC' proteins recovered from proteinase K-treated and untreated membranes were compared (Fig. 4, lanes 6 and 7; and Fig. 5, lanes 3 and 5). In both sets of experiments, the amount of processed ΔC' protein increased after the proteinase K treatment, although to different extents. The amount of protection is dependent on the tight sealing of the vesicles and on complete inactivation of the proteinase K during sample preparation. In the experiment shown in Fig. 5, membranes were isolated after the proteinase K treatment and great care was taken to remove and inactivate proteinase K. We believe that isolation of the microsomal vesicles after the proteinase K treatment is the reason for the more quantitative recovery of processed ΔC proteins and the increased amount of endogenous processing (compare Figs. 4 and 5).

What Are the Structural Properties of a Signal–Anchor Sequence?

In this report, we show that a hydrophobic segment of a certain length is of crucial importance for a signal–anchor segment. This segment is not only required for membrane anchoring but also for membrane translocation. No mutant protein was obtained which was translocated but not anchored, at least in part, in the membrane.

Zerial et al. (1986) demonstrated recently that it is the hydrophobic character of the signal–anchor sequence and not its distinct amino acid sequence which is important for membrane insertion and anchor function. Similar findings were also made for signal sequences of secretory proteins. Many randomly chosen hydrophobic sequences were found to function in membrane translocation of an indicator protein (Kaiser et al., 1987).

It is, however, not the hydrophobic sequence alone which determines the function of a signal–anchor segment. In a previous publication, we demonstrated that the hydrophilic region flanking the hydrophobic segment on the NH2-terminal side can determine whether a protein is anchored in the membrane or translocated (Lipp and Dobberstein, 1986b). The hydrophilic region flanking the hydrophobic segment on the COOH-terminal side can also influence membrane translocation or anchoring. Here we show that this region can influence cleavage by signal peptidase and thus decide upon membrane insertion or translocation. All of the naturally occurring signal–anchor sequences have positively charged amino acid residues at their NH2-terminal end. Positively charged residues have been proposed to prevent proteins or parts of them from crossing the membrane (Weinstein et al., 1982; von Heijne, 1986). Negatively charged residues at this side of the hydrophobic segment do not seem to interfere with translocation of the NH2-terminal portion across the membrane (Haeuptle, M. T., N. Flint, N. M. Gough, and B. Dobberstein, manuscript submitted for publication).

From this and previous work, we conclude that three distinct segments constitute a signal–anchor sequence: (a) a positively charged NH2-terminal region, (b) a central segment of hydrophobic amino acid residues (at least 16 residues in length), and (c) a hydrophilic COOH-terminal portion.

We propose that the hydrophilic sequences flanking the hydrophobic core of a signal–anchor segment modulate its function by determining whether a protein is integrated into or translocated across a membrane (signal peptidase function) and which topological orientation (type I or type II) a protein has in the membrane (Haeuptle, M. T., N. Flint, N. M. Gough, and B. Dobberstein, manuscript submitted for publication).

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