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 NH_2-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 (ΔC 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). The degree of processing varies between different ΔC mutants. Mutant proteins retaining 20 or more of the hydrophobic amino acid residues can span the membrane like the parent IγCAT protein and are not proteolytically processed.

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 NH_2 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)[1] 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 NH_2 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 NH_2-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, 1986*a*).

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, 1986*a*). The single hydrophobic segment in Iγ contains the signal for membrane insertion as well as for membrane anchoring. The NH_2-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, 1986*b*).

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, 1986), the hepatic glycoprotein receptor (Chiacchia and Drickamer, 1984), and the sucrase–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 Iγ can determine cleavage by signal peptidase. Upon deletion of the hydrophilic NH_2 terminus of Iγ, 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, 1986*b*).

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 Iγ 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, ^{7}mGpppA, and DNA sequencing reagents were from Pharmacia Fine Chemicals, Freiburg, FRG. L-[^{35}S]Methionine was from Amersham Buchler GmbH, Braunschweig, FRG.

DNA Preparation and Sequencing

Small-scale plasmid preparations were done as described by Haeuptle 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 (Norrander et al., 1983). Sequencing was done as described by Sanger et al. (1977).

Construction of ΔC Mutants

Plasmid Iγcat was previously described (Lipp and Dobberstein, 1986*b*). It codes for IγCAT, a fusion protein consisting of the 72 NH_2-terminal amino acid residues of Iγ and the entire CAT protein. The plasmid is a derivative of pDS 5 which allows transcription of the Iγcat sequence from a T5 promoter (Stueber et al., 1984).

Plasmid Iγcat was linearized by Pst I 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 $MgCl_2$, 12 mM $CaCl_2$, 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 (Legerski 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 and used for transformation. Selected ΔC clones were analyzed for the size of the deletion by restriction analysis and tested for an open reading frame by in vitro transcription and translation.

In Vitro Transcription and Translation

Plasmids were transcribed in vitro by *E. coli* RNA polymerase in the presence of ^{7}mGpppA, 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-asn-leu-thr-*N*-methylamide to a final concentration of 30 μM (Lau et al., 1983; Lipp and Dobberstein, 1986*b*).

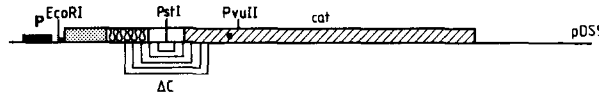

Figure 1. Outline of pIγcat and the strategy for generating the ΔC deletion clones. Regions coding for protein are boxed. The membrane-spanning region derived from Iγ is indicated by loops, the cytoplasmically located segment by dots. Sequences derived from cat are indicated by slanted lines. The position of a potential site for N-linked glycosylation is indicated by an asterisk. Deletions were made from the Pst I site using exonuclease Bal 31.

Posttranslational Assays

Proteinase K treatment of microsomal membranes and carbonate extraction, at pH 11, were done as previously described (Lipp and Dobberstein, 1986*b*). Proteins were separated by SDS-PAGE (Laemmli, 1970) and labeled proteins visualized by fluorography using EN^{3}HANCE (New England Nuclear, Dreiech, FRG).

Results

Construction of Plasmids Coding for the ΔC Mutants

We previously described the plasmid Iγcat which codes for a fusion protein consisting of the 72 NH_2-terminal amino acid residues of Iγ followed by the cytoplasmic protein CAT (Lipp and Dobberstein, 1986*b*; 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 Iγcat at its unique Pst I 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. Iγcat 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, 1986*b*).

IγCAT was previously shown to be a glycosylated type II membrane protein (Lipp and Dobberstein, 1986*b*). In the absence of RM, IγCAT 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, 1986*b*; Fig. 2). All protein is digested if membranes are solubilized by detergent before proteinase K digestion (Lipp and Dobberstein, 1986*b*). Digestion in the presence of detergent serves as a control for protease-resistant fragments.

Selected ΔC clones were subjected to the same analysis

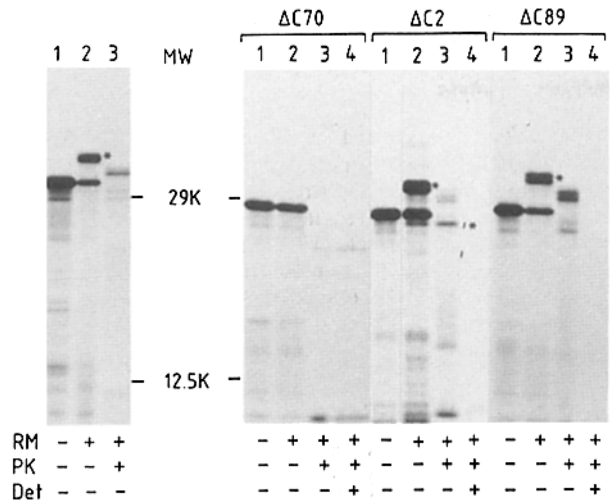

Figure 2. In vitro translation and membrane insertion of IγCAT and of selected ΔC deletion derivatives. ΔC mutant plasmids were transcribed by *Escherichia coli* RNA polymerase. The resulting mRNA was translated in the wheat germ cell-free system in the absence (lanes *1*) or presence (lanes *2*, *3*, 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 IγCAT protein and suggests that ΔC89 is glycosylated and spans the membrane as IγCAT.

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

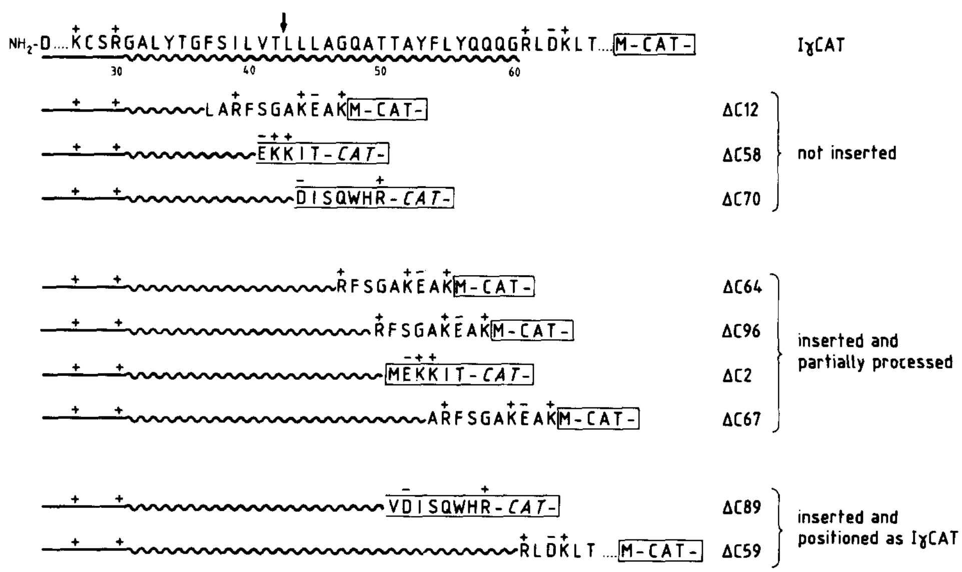

Figure 3. Outline and amino acid sequences of the hydrophobic segments and their flanking regions in IγCAT and in the ΔC mutants. The NH_2-terminal cytoplasmic segments are underlined, the hydrophobic segments derived from Iγ are indicated by wavy lines, and CAT by a closed box. When the deletion extended into the CAT sequence, this is indicated by an open box. The numbers below the IγCAT sequence indicate amino acid residues from NH_2-terminal end. The arrow indicates the potential signal-peptidase cleavage site which is used in ΔN-IγCAT. 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 membrane or are proteolytically processed and translocated; and clones coding for proteins that span the membrane as IγCAT.

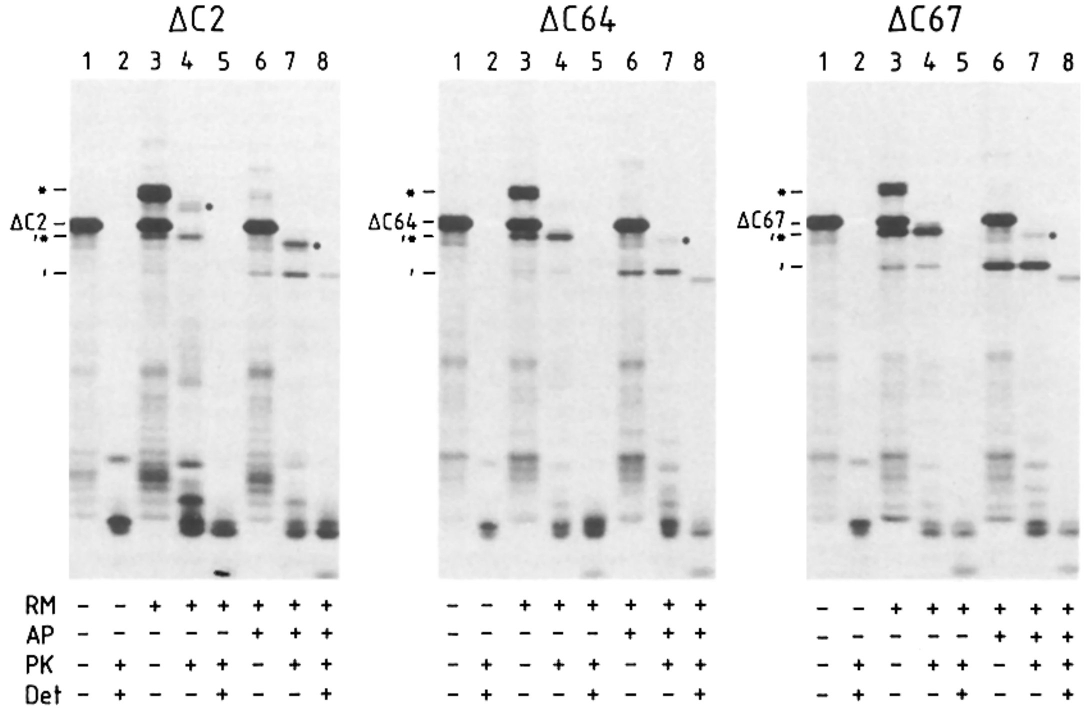

Figure 4. Characterization of proteolytically processed ΔC mutants. Selected ΔC transcripts (ΔC2, ΔC64, and ΔC67) were translated in the wheat germ cell-free system without additions (lanes *1* and *2*), in the presence of RM (lanes *3–8*), or of RM and an acceptor peptide (*AP*) which competes for N-glycosylation (lanes *6–8*). After translation, samples were treated with proteinase K (*PK*) (lanes *4* and *7*) or with proteinase K and the detergent NP-40 (*Det*) (lanes *2*, *5*, and *8*). Glycosylated forms are indicated by an asterisk. Forms processed by a microsomal protease are indicated by a shill, those generated by the proteinase K treatment by a dot.

deletions were subcloned into phage M13–derived plasmid mp18 (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.

ΔC 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 Iγ-derived hydrophobic segment. 12 of these hydrophobic amino acid residues constitute the cleaved signal sequence of ΔN-IγCAT as shown previously (Lipp and Dobberstein, 1986*b*). The cleavage site for signal peptidase in ΔN-IγCAT is indicated by an arrow in Fig. 3.

In the second group of ΔC 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 ΔC proteins that span the membrane as IγCAT. No proteolytic processing can be observed. ΔC89 retained 20 amino acids of the hydrophobic segment. Note that the identical number of residues is retained in ΔC2 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 ΔC Fusion Proteins Become Glycosylated and Proteolytically Processed

ΔC 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 (ΔC2, ΔC64, and ΔC67), were selected for further analysis. After translation in the absence or presence of RM, proteinase K was used to determine the degree of translocation.

In the absence of RM, a single major polypeptide was synthesized in each case (ΔC2, ΔC64, and ΔC67) (Fig. 4, lanes *1*). Proteinase 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*) ΔC 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*) ΔC* polypeptides which are ~3 kD larger than the ΔC polypeptides. The shift in molecular mass is consistent with N-glycosylation at one site without proteolytic processing; (*c*) ΔC'* polypeptides which are ~2 kD smaller than the ΔC polypeptides. They are proteolytically processed and glycosylated (see below); (*d*) ΔC' polypeptides which are ~4 kD smaller than the ΔC ones. They are proteolytically processed by a microsomal protease (signal peptidase) (see below).

After proteinase K digestion the molecular mass of ΔC2* was reduced by ~2 kD. This indicates that it spans the membrane and exposes a 2-kD segment on the cytoplasmic side. For ΔC64* and ΔC67* similar membrane-spanning forms could not clearly be detected. Instead, the amount of endogenously processed ΔC'* forms increased after the proteinase K treatment. Usually only ~50% of the membrane-translocated protein is protected against exogenously added

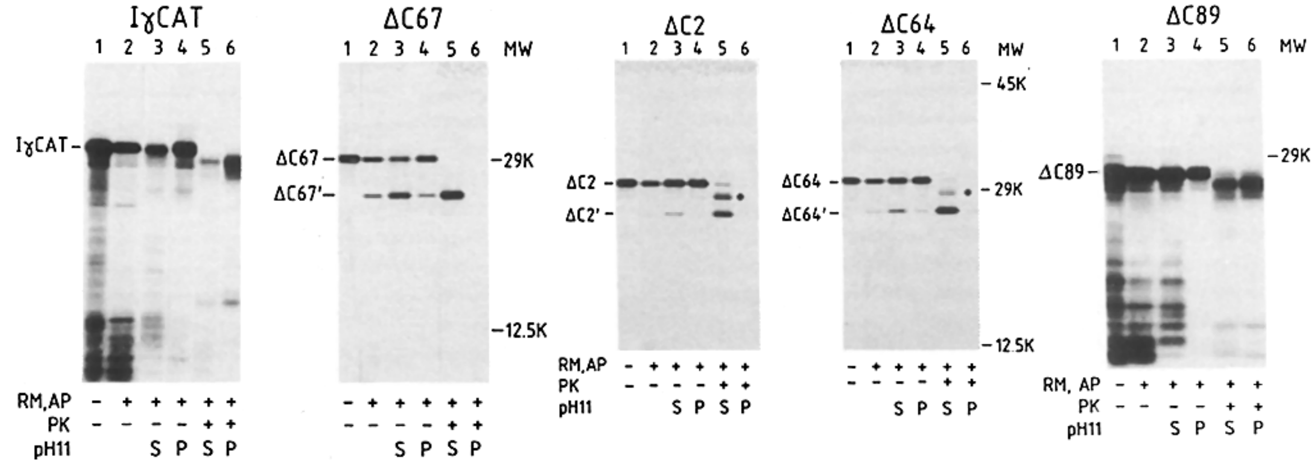

Figure 5. Carbonate extraction of IγCAT, the group II proteins ΔC67, ΔC2, ΔC64, and the group III protein ΔC89 from RM membranes. IγCAT and ΔC transcripts were translated in the wheat germ cell-free system in the absence (lanes *1*) and presence of RM and acceptor peptide for N-glycosylation (*AP*) (lanes *2–6*). After translation, 50% of the samples were treated with proteinase K (*PK*) (lanes *5* and *6*). Samples were centrifuged through a cushion containing carbonate at pH 11. The pellet (*P*) (lanes *4* and *6*) and the supernatant (*S*) fractions (lanes *3* and *5*) were characterized by SDS-PAGE. Proteins processed by an RM protease are indicated by a dash.

proteinase K due to unsealed vesicles. We suspect that ΔC64 and Δ67 proteins from which proteinase K has removed the cytoplasmically exposed segment are posttranslationally processed by signal peptidase. Thus, no membrane-integrated protein (ΔC*) can be detected after the proteinase K treatment. This interpretation is supported by our previous finding that removal of the NH_2-terminal segment from IγCAT led to signal peptidase cleavage in ΔN-IγCAT (Lipp and Dobberstein, 1986*b*).

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 IγCAT polypeptide for N-linked glycosylation (Lipp and Dobberstein, 1986*b*).

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, ΔC′ proteins were found protected. In addition, proteins smaller than the ΔC proteins by ∼2 kD (dot) were protected (Fig. 4, lanes *6* and *7*). These proteins span the membrane and expose a segment of ∼2 kD on the cytoplasmic side (Fig. 4, lanes *7*). We conclude that unglycosylated 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, 1986*b*). Processing of ΔC2 by the microsomal peptidase occurred, most likely, very close to the NH_2 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 IγCAT 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 NH_2-terminal hydrophilic segment by proteinase K has an effect on the stable integration of these proteins in the membrane.

IγCAT 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*, shows the analysis of 5 μl of the translation mixture. Small amounts of proteolytically processed 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 proteinase K; another aliquot of identical volume was left untreated. Membranes from both samples were pelleted and extracted with carbonate at pH 11. After sedimentation of the membranes, proteins in the supernatant and the pellet were analyzed by SDS-PAGE and autoradiography. As expected, the membrane protein IγCAT was found largely in the pellet fraction after the carbonate treatment (Fig. 5, lanes *3* and *4*). Also ΔC67, ΔC2, ΔC64, and ΔC89 proteins were found to a large extent in the pellet fraction (Fig. 5, lanes *3* and *4*). The processed ΔC′ forms accumulated in the supernatant, suggesting removal of the hydrophobic segment by the signal peptidase (Fig. 5, lanes *3*).

After proteinase K treatment, IγCAT and ΔC89 were still found in the pellet fraction (Fig. 5, lanes *5* and *6*). In contrast, none of the ΔC proteins of group II accumulated after the proteinase K treatment in the pellet fraction (Fig. 5, lanes *5* and *6*; cf. ΔC67, ΔC2, and ΔC64). ΔC group II proteins, shortened either by ~2 kD (ΔC) or by ~4 kD (ΔC′), were found in the supernatant fraction. Two types of proteolytic cleavages must have occurred. (*a*) ΔC′ forms most likely originate from processing by signal peptidase, as these forms are already present before the proteinase K treatment (Fig. 5, lanes *3* and *5*; cf. ΔC67, ΔC2, and ΔC64). The amount of processed ΔC′ proteins drastically increased after the proteinase K treatment. This is particularly evident with ΔC2′ and ΔC64′. We suspect that the posttranslational removal of the cytoplasmic segment from these proteins resulted in an increased accessibility to signal peptidase and, as a consequence, in increased cleavage. (*b*) ΔC proteins that are processed by the proteinase K can be seen for ΔC2 and ΔC64. They are indicated in Fig. 5 by a dot (lanes *5*). These proteins are ~2 kD smaller than the uncleaved ΔC proteins. They are expected to have lost their cytoplasmically located hydrophilic NH_2 terminus but still retain most of the hydrophobic segment. Nevertheless, they are found in the supernatant, suggesting that the hydrophilic NH_2-terminal portion is crucial for a stabile membrane integration of these proteins.

Discussion

The Signal for Membrane Insertion and Anchoring in IγCAT

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

Our results show that the 13 NH_2-terminal residues of the hydrophobic segment in IγCAT are not sufficient for membrane insertion or translocation (see ΔC70, Fig. 3). We have previously shown that 12 of these amino acid residues constitute a cleavable signal sequence in ΔN-IγCAT (Lipp and Dobberstein, 1986*b*). This indicates that the functional signal sequence in IγCAT 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 NH_2-terminal region of the mature protein (Moreno et al., 1980; Abrahamsen et al., 1985; Lehnhardt et al., 1987).

The amino-terminally located 16 amino acids of the hydrophobic segment in IγCAT 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-IγCAT. 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 IγCAT 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 IγCAT 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 NH_2-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 NH_2-terminal end of IγCAT was deleted (Lipp and Dobberstein, 1986*b*). The resulting protein, ΔN-IγCAT, 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 lumenal 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-spanning 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 cytoplasmic segment, ΔC2 became carbonate extractable from microsomal membranes, whereas ΔC89 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 NH_2-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 NH_2-terminal side can determine whether a protein is anchored in the membrane or translocated (Lipp and Dobberstein, 1986*b*). 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 NH_2-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 NH_2-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 NH_2-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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