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The Membrane-Spanning Segment of Invariant Chain (Iγ) Contains a Potentially Cleavable Signal Sequence

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Summary

The human invariant chain (Iγ) of class II histocompatibility antigens spans the membrane of the endoplasmic reticulum once. It exposes a small amino-terminal domain on the cytoplasmic side and a carboxy-terminal, glycosylated domain on the exoplasmic side of the membrane. When the exoplasmic domain of Iγ is replaced by the cytoplasmic protein chloramphenicol acetyltransferase (CAT), CAT becomes the exoplasmic, glycosylated domain of the resulting membrane protein IγCAT*. Deletion of the hydrophilic cytoplasmic domain from IγCAT gives rise to a secreted protein from which an amino-terminal segment is cleaved, most likely by signal peptidase. We conclude that the membrane-spanning region of Iγ contains a signal sequence in its amino-terminal half and that hydrophilic residues at the amino-terminal end of a signal sequence can determine cleavage by signal peptidase.

Introduction

Translocation of proteins across the membrane of the endoplasmic reticulum (ER) requires signal sequences and specific receptors that recognize them (see recent reviews by Hortsch and Meyer, 1984; Walter et al., 1984; Rapoport and Wiedmann, 1985; Wickner and Lodish, 1985). Signal sequences have been found at the amino-terminal end of precursors for secretory and transmembrane proteins. In many cases they are cleaved during their translocation across the membrane by a specific protease (signal peptidase). Signal sequences are quite variable in length, ranging from 16 to more than 50 amino acid residues (von Heijne, 1983). They all have a central core of hydrophobic amino acid residues, and most of them have a positively charged amino-terminal segment (von Heijne, 1985). Signal sequences on nascent polypeptides are recognized by the signal recognition particle (SRP), a ribonucleoprotein complex that mediates the interaction with the membrane by the selective binding to docking protein (or SRP receptor) (Walter et al., 1981b; Meyer et al., 1982; Gilmore et al., 1982).

Membrane proteins are also inserted into the ER membrane by an SRP-mediated mechanism (Anderson et al., 1983; Rottier et al., 1985; Spiess and Lodish, 1986; Lipp and Dobberstein, 1986). Those spanning the membrane once have either the carboxyl terminus (type I membrane proteins) or the amino terminus (type II membrane proteins) exposed on the cytoplasmic side. Membrane insertion of type I membrane proteins most likely proceeds in a manner very similar to that of secretory proteins (Lingappa et al., 1978). Type I membrane proteins are usually synthesized with a cleavable signal sequence and, in contrast to secretory proteins, are held in the membrane by a "stop transfer" sequence. Examples of type I membrane proteins are the vesicular stomatitis virus G protein and class I and class II histocompatibility antigens (Lingappa et al., 1978; Dobberstein et al., 1979).

Of the type II membrane proteins so far investigated, all are synthesized without a cleavable signal sequence. The neuraminidase of influenza virus (Bos et al., 1984), the invariant chain (Ii or Iγ) of class II histocompatibility antigens (Claesson et al., 1983; Strubin et al., 1984; Long, 1985; Lipp and Dobberstein, 1986), the transferrin receptor (Schneider et al., 1984), and the asialoglycoprotein receptor (Chiacchia and Drickamer, 1984; Holland et al., 1984; Spiess and Lodish, 1986) all belong to this class of membrane proteins. Some steps in their membrane insertion must be similar to that of secretory and type I membrane proteins, as an SRP- and docking protein–dependent membrane insertion has been demonstrated for some of them (Spiess and Lodish, 1986; Lipp and Dobberstein, 1986). Membrane insertion might occur in a loop-like fashion as this scheme can most easily explain how the different membrane topologies of membrane proteins are achieved (Engelman and Steitz, 1981). As type II membrane proteins contain only a single stretch of hydrophobic amino acid residues, this might function as a signal for membrane insertion as well as a membrane anchor (Markoff et al., 1984; Spiess and Lodish, 1986). To identify and characterize this sequence, we tested membrane insertion of the human invariant chain (Iγ) and several deletion and fusion proteins derived from it in a cell-free membrane insertion system.

Iγ is a typical type II membrane protein (Claesson et al., 1983; Strubin et al., 1984; Lipp and Dobberstein, 1986). It exposes 30 amino-terminal residues on the cytoplasmic side, spans the membrane between residues 30 and 60, and exposes a large carboxy-terminal domain on the exoplasmic side. This domain has two sites for the addition of N-linked carbohydrate units. Membrane insertion of Iγ requires SRP and docking protein (Lipp and Dobberstein, 1986). As the amino-terminal, cytoplasmic domain is hydrophilic and shows no resemblance to a signal sequence, it has been proposed that the membrane-spanning region, or part of it, functions as an internal, uncleavable signal sequence (Dobberstein et al., 1983; Claesson et al., 1983; Lipp and Dobberstein, 1986).

We demonstrate here that the membrane-spanning region of Iγ is composed of a potentially cleavable signal sequence fused to part of a membrane anchor, which together with the cytoplasmic domain determine the orientation of Iγ in the ER membrane. Deletion of the cytoplasmic domain exposes the signal sequence at the amino terminus of the membrane-spanning region, resulting in cleavage of this otherwise uncleaved signal.

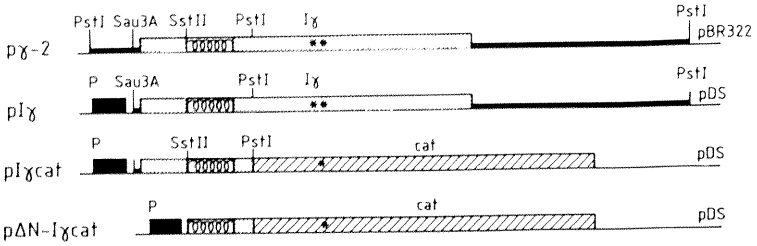

Figure 1. Outline of the Relevant Coding Regions in pγ-2, pIγ, pIγcat and pΔN-Iγcat

pγ-2, Iγ cloned into the PstI site of pBR322 (Claesson et al., 1983). pIγ, the complete Iγ coding and all of its 3′ noncoding sequence was cloned behind the T5 promoter (P) in the pDS5 expression vector. pIγcat, the portion downstream of the PstI site in pIγ was replaced by the chloramphenicol acetyltransferase (*cat*) gene resulting in an in-frame fusion protein. pΔN-Iγcat, the segment between the Sau3A and SstII sites of pIγcat coding for the cytoplasmic domain was deleted. A new ATG initiation codon right in front of the membrane-spanning segment is provided by the vector (see Figure 4A). The regions coding for protein are boxed. The membrane-spanning region of Iγ is indicated by loops; the hydrophilic domains by dots. *cat*-derived sequences are indicated by slanted lines. The position of N-linked glycosylation sites in Iγ and the potential N-linked glycosylation site in CAT protein are indicated by an asterisk. Relevant cleavage sites for restriction endonucleases are also indicated.

Results

Protein segments that perform a particular function can be identified by their deletion or addition to unrelated proteins. We used this approach to localize and characterize the region in Iγ that is responsible for membrane insertion. Deletions and fusions were made at the DNA level after cloning of Iγ cDNA into an expression vector. Messenger RNA was transcribed from these plasmids and translated in a cell-free system. The resulting proteins were tested for their ability to insert into microsomal membranes (Blobel and Dobberstein, 1975; Stueber et al., 1984).

Construction of Expression Plasmids pIγ, pIγcat, and pΔN-Iγcat

We have shown previously that cDNA sequences cloned behind the strong T5 promoter in pDS5 can be transcribed very efficiently by E. coli RNA polymerase (Stueber et al., 1985). When transcription is performed in the presence of the cap analog 7mGpppA, the resulting mRNA can be translated efficiently in eukaryotic cell-free systems. We have observed, however, that a stretch of GC residues at the 5′ end of a cDNA negatively affects expression of the resulting RNA (unpublished observation). The Iγ cDNA construct (pγ-2) had been GC tailed and was inserted into the PstI site of pBR322 (Claesson et al., 1983). We deleted the 5′ GC tail and cloned Iγ cDNA, or part of it, into the polylinker site of pDS5 or p6/5R (see Experimental Procedures for details). pIγ contains the entire Iγ coding region behind the T5 promoter (Figure 1). pIγcat is an in-frame fusion between the 5′ region of Iγ encoding the cytoplasmic, membrane-spanning segment plus 12 amino acids of the exoplasmic portion of Iγ and the gene encoding the cytoplasmic protein chloramphenicol acetyltransferase (CAT). The CAT protein contains one potential site for the addition of N-linked oligosaccharide 36 amino acid residues downstream of its original initiator methionine. In ΔN-IγCAT, the entire hydrophilic, cytoplasmic segment from Iγ was deleted. The new initiator methionine is provided by the vector and is located in front of the hydrophobic segment.

In Vitro Translation and Membrane Insertion of Iγ

When pIγ was transcribed by E. coli RNA polymerase and the resulting mRNA translated in the wheat germ cell-free system, a single polypeptide species of 27 kd was obtained (Figure 2, lane 1). This is the expected molecular weight for nonglycosylated Iγ (Claesson et al., 1983). When rough microsomes (RM), derived from dog pancreas, were added to the translation system, a higher molecular weight species of 33 kd appeared. This increase of 6 kd in molecular weight is consistent with the addition of two oligosaccharides to the two N-glycosylation sites. The 33 kd form Iγ* was reduced in molecular weight by about 2 kd when proteinase K was used to remove the cytoplasmically exposed domain (Figure 2, lanes 2 and 3). When protease digestion was performed in the presence of the detergent NP 40, Iγ* was digested. These data suggest that Iγ* is integrated into the membrane and exposes 20–30 amino acid residues on the cytoplasmic side and a 30 kd domain on the exoplasmic side of the membrane. The identity of Iγ and its glycosylated form was confirmed by immunoprecipitation with antibodies raised against the amino-terminal 72 (anti-IγN) or against the carboxy-terminal 144 (anti-IγC) residues of Iγ. As shown in Figure 2, lanes 5, 6, 8, and 9, these antibodies recognize glycosylated and nonglycosylated forms of Iγ. No protein could be precipitated with anti-IγN antibody when the cytoplasmic domain was removed from membrane-integrated Iγ* by protease digestion (Figure 2, lane 7). As the antibody is directed against the amino-terminal portion of Iγ, the data directly demonstrate that the amino terminus is located on the cytoplasmic side and is accessible to the protease. With anti-IγC antibody, the processed form of Iγ is readily detectable, demonstrating an exoplasmic location of the carboxy-terminal portion of Iγ (Figure 2, lane 10).

Membrane Insertion of IγCAT

An analysis of membrane insertion was performed for Iγ-CAT and CAT as described above for Iγ. CAT was expressed from pDS5. IγCAT was synthesized in the absence of microsomal membranes as a 34 kd protein (Figure 3, lane 1) and in the presence of microsomal membranes as a 37 kd protein called IγCAT* (Figure 3, lane 2).

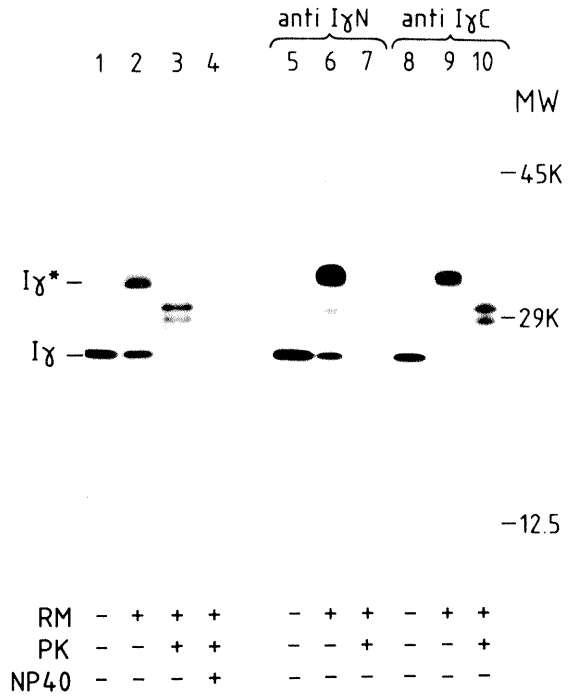

Figure 2. In Vitro Translation and Membrane Insertion of Iγ

pIγ was transcribed in the presence of the cap analog 7mGpppA by E. coli RNA polymerase. The resulting mRNA was translated in the wheat germ cell-free system in the absence (lanes 1, 5, and 8) or presence (lanes 2, 3, 4, 6, 7, 9, and 10) of RM. The membrane topology of Iγ was determined by treatment with proteinase K (PK) (lanes 3, 7, and 10) or PK and the detergent NP40 (lane 4). Proteins were separated by SDS-PAGE and visualized by autoradiography. Lanes 1–4 show total protein synthesized. Samples characterized in lanes 5–7 were immunoprecipitated with an antibody raised against the amino-terminal 72 amino acid residues of Iγ (anti-IγN); in lanes 8–10, with an antibody against the carboxy-terminal portion of Iγ (anti-IγC).

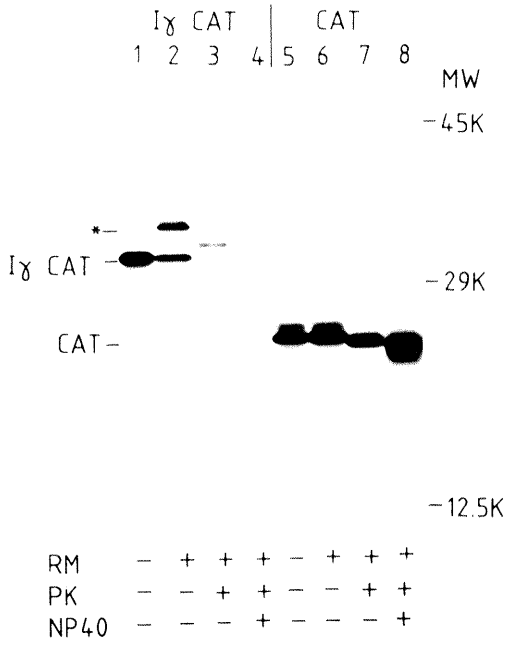

Figure 3. In Vitro Translation and Membrane Insertion of IγCAT and CAT Protein

RNA derived from pIγcat or pDS5 was translated in the wheat germ cell-free system in the absence or the presence of RM. Membrane insertion was tested by treatment with proteinase K (PK) and NP40. Addition of RM, PK, and NP40 is indicated at the bottom of each lane.

The increase in molecular weight is consistent with the addition of one N-linked oligosaccharide to the CAT-derived portion. There is one potential site for N-linked glycosylation in the CAT protein. After protease digestion in the presence of microsomal membranes, IγCAT* is reduced in molecular weight by about 2 kd, suggesting that it exposes 20–30 amino acid residues on the cytoplasmic side (Figure 3, lanes 2 and 3).

CAT protein obtained after transcription–translation from pDS5 is not modified by the added microsomes. As expected, no shift in molecular weight can be seen (Figure 3, lanes 5 and 6). CAT protein was very resistant to protease digestion even in the presence of NP40 (Figure 3, lanes 7 and 8). IγCAT, in contrast, was very sensitive to added protease. This might reflect a difference in conformation between the free CAT protein and the CAT-derived portion in IγCAT. The location of CAT outside of the membrane vesicles can be demonstrated by sedimenting the membranes by centrifugation. CAT protein is then found in the supernatant (data not shown). We conclude from the data obtained with IγCAT and CAT that the signal for membrane insertion must be located within the first 72 amino acid residues of Iγ. To localize this signal more precisely, we deleted the first 30 residues of IγCAT.

Membrane Insertion and Glycosylation of ΔN-IγCAT

In all secretory proteins the cleavable signal for membrane translocation is located at the amino-terminal end of the precursor polypeptide. The main feature of this signal appears to be its hydrophobicity. In Iγ the only hydrophobic stretch of amino acid residues that resembles a signal sequence is located in the membrane spanning region about 30 amino acid residues away from the amino-terminal initiator methionine. We asked whether removal of the 30 amino-terminal residues in IγCAT would affect its membrane insertion and topology.

The cytoplasmic domain of IγCAT was deleted and the initiator methionine was placed in front of the membrane-spanning segment. The amino-terminal sequences of Iγ-CAT and ΔN-IγCAT as deduced from the DNA sequences are shown in Figure 4A. When RNA derived from pΔN-Iγcat was translated in the wheat germ cell-free system, a single polypeptide of 29 kd was synthesized, ΔN-IγCAT (Figure 4B, lane 1). This was, as expected, about 3 kd smaller than the IγCAT protein (Figure 4B, lane 1). In the presence of microsomes, two new protein bands appeared, one about 1 kd smaller and one 2 kd larger than ΔN-IγCAT. Both of these forms were resistant to proteinase K, indicating that they were inserted into or translocated across microsomal membranes (Figure 4B, lanes 3 and 4). We suspected that the smaller molecular weight form was generated by signal peptidase cleavage without concomitant glycosylation and that the larger molecular weight form was glycosylated and cleaved by signal peptidase. These possibilities were tested.

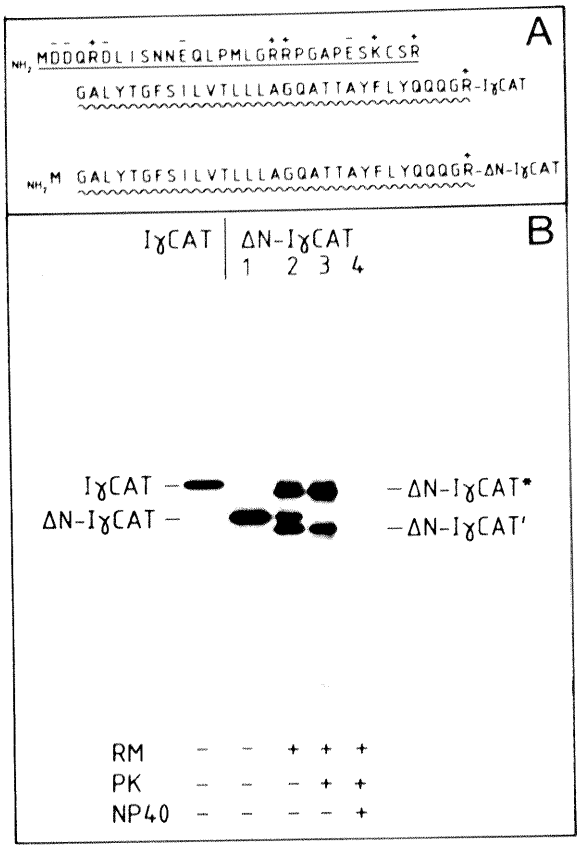

Figure 4. In Vitro Translation and Membrane Insertion of ΔN-IγCAT

(A) Amino acid sequences of the amino-terminal hydrophilic (cytoplasmic) and hydrophobic segments of IγCAT and ΔN-IγCAT. The cytoplasmic segment of IγCAT is underlined, and the hydrophobic segments are indicated by a wavy line. (B) mRNA transcribed from pΔN-Iγcat was translated in the wheat germ cell-free system in the absence or presence of RM. Membrane insertion and topology was tested by treatment with proteinase K (PK) and NP40. Components were added as indicated below the lanes. IγCAT translated in the wheat germ cell-free system is shown for comparison.

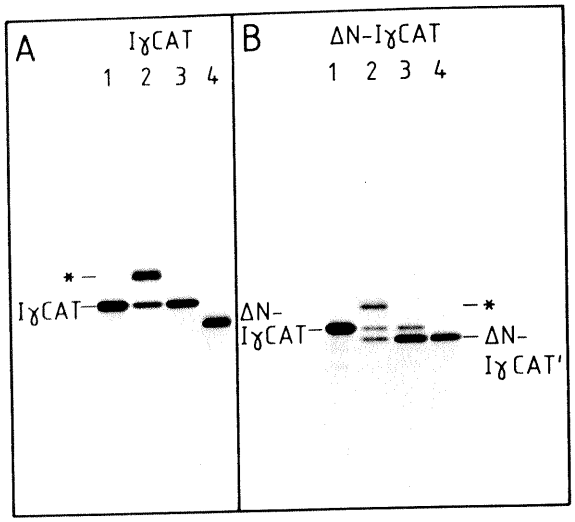

Figure 5. Membrane Insertion of Glycosylated and Nonglycosylated IγCAT and ΔN-IγCAT

mRNA derived from plγcat and pΔN-Iγcat was translated in the wheat germ cell-free system in the absence (lane 1) or presence of RM (lane 2) and in the presence (lanes 3 and 4) of RM and acceptor peptide for N-linked glycosylation. After translation, proteinase K was used to cleave protein segments accessible from the outside of RM (lane 4). The asterisks indicate glycosylated forms of IγCAT and ΔN-IγCAT.

ΔN-IγCAT Becomes Proteolytically Processed and Glycosylated

To detect the signal peptide cleavage of a glycosylated protein on a polyacrylamide gel it is necessary to block its glycosylation, but still allow membrane insertion to occur. Addition of N-linked oligosaccharides onto nascent polypeptides can be blocked by including synthetic acceptor peptides in an in vitro membrane insertion assay (Bause, 1983; Lau et al., 1983). IγCAT and ΔN-IγCAT were translated in the presence of microsomes with and without the acceptor peptide Asn-Leu-Thr. The size of IγCAT synthesized in the presence of RM and acceptor peptide was indistinguishable from that made in the absence of RM. When proteinase K was used to digest its cytoplasmically exposed domain, the size was reduced by about 2–3 kd (Figure 5A). We can conclude that nonglycosylated IγCAT synthesized in the presence of RM and acceptor peptide is inserted into the membrane in the same way as its glycosylated form and that no signal sequence is cleaved during membrane translocation (Figure 5A, lanes 3 and 4; cf., Figure 3, lanes 2–4). When ΔN-IγCAT was synthesized in the presence of RM, three polypeptides accumulated: ΔN-IγCAT, a 2 kd larger form, and a 1 kd smaller form. In the presence of the acceptor peptide, the larger form disappeared and the amount of the smaller form, ΔN-IγCAT', increased (Figure 5B, lanes 2 and 3). ΔN-IγCAT' was also found to be protected against exogenous proteinase K (Figure 5B, lane 4). This suggested to us that the larger form was glycosylated and proteolytically processed and that ΔN-IγCAT' was generated by a proteolytic cleavage, most likely by signal peptidase.

To determine the site of cleavage in the proteolytically processed forms of ΔN-IγCAT, the positions of leucine in the amino-terminal regions of ΔN-IγCAT and membrane-inserted ΔN-IγCAT*' were determined. ΔN-IγCAT was translated in the absence or presence of RM with [^{3}H]leucine as label. As ΔN-IγCAT is essentially the only protein synthesized from pΔN-Iγcat-derived mRNA, the complete translation mixture was subjected to automated Edman degradation. As seen in Figure 6A, leucine residues are found at the positions 3, 10, 13, 14, and 15, as predicted from the sequence deduced from pγ-2 cDNA (Claesson et al., 1983). The initiator methionine is probably removed during or shortly after translation (Kozak, 1983).

The positions of leucine residues in the membrane-translocated forms of ΔN-IγCAT were similarly determined. As RM in the in vitro assay do not translocate all chains, some cytoplasmic forms remained (see inserts in Figures 6A and 6B). Leucine residues were found at positions 1, 2, 3, and 13 (Figure 6B). Larger peaks at positions 3 and 10 are consistent with the presence of some unprocessed ΔN-IγCAT (see insert in Figure 6B). Taking into account the size reduction of about 1 kd by the processing

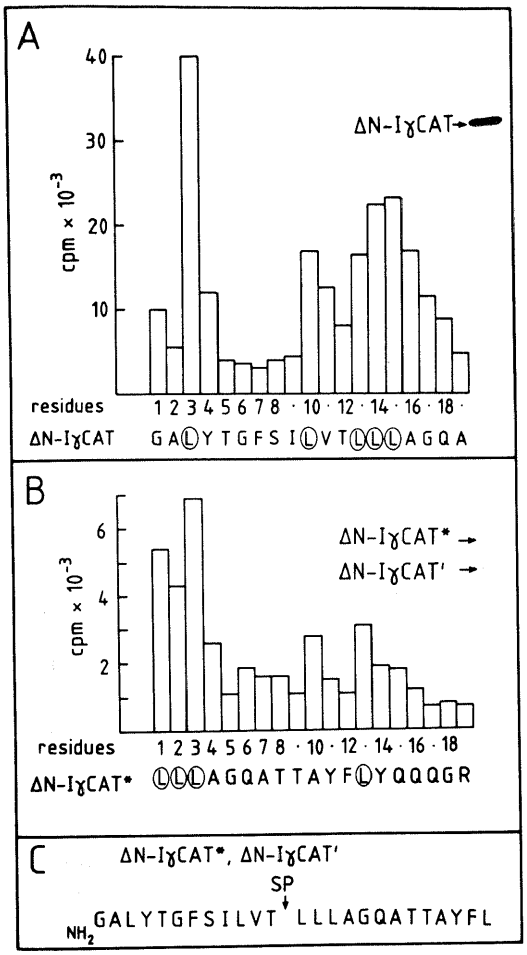

Figure 6. Site of Cleavage of ΔN-IγCAT by RM-Specific Protease (Signal Peptidase)

RNA transcribed from pΔN-Iγcat was translated in the absence or presence of RM with [^{3}H]leucine as label. Proteins were characterized by SDS-PAGE and autoradiography (see inset in [A], ΔN-IγCAT synthesized in the absence of RM; in [B], ΔN-IγCAT* and ΔN-IγCAT′ glycosylated and nonglycosylated forms inserted into RM). Protein samples as shown in the figures were subjected to automated Edman degradation, and [^{3}H]leucine label was determined for each sequenator cycle. The known amino acid sequence of the amino terminus of Iγ is given below the graph (A). The positions of leucine residues experimentally determined are encircled. Leucine residues as shown in (B) are consistent with a cleavage in front of the 3 leucine residues at positions 13, 14, and 15 of ΔN-IγCAT. The cleavage site (SP) in ΔN-IγCAT* and ΔN-IγCAT′ are indicated in (C).

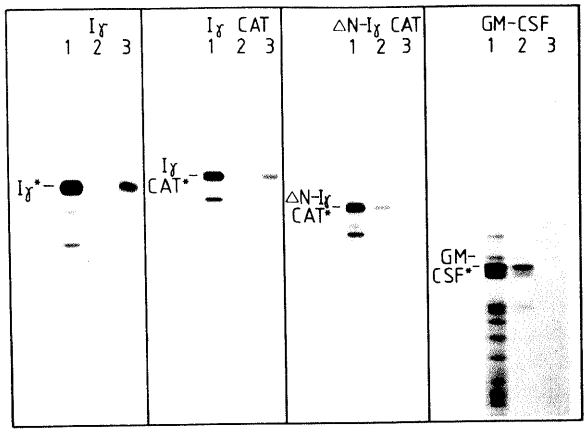

Figure 7. Membrane Association of Iγ, IγCAT, ΔN-IγCAT, and GM-CSF

RNA transcribed from pIγ, pIγcat, pN-Iγcat, and pGM-CSF was translated in a wheat germ cell-free system in the presence of RM (lane 1). Membranes were sedimented by centrifugation after completion of translation and subjected to carbonate treatment, which releases proteins not integrated into the lipid bilayer. After centrifugation the supernatant fractions containing soluble proteins (lane 2) and the pellets (lane 3) were characterized by SDS-PAGE and fluorography. The positions of the glycosylated forms of Iγ, IγCAT, ΔN-IγCAT, and the secretory protein GM-CSF are indicated by asterisks.

step and the fact that 3 leucine residues in a row are found at positions 13, 14, and 15 in authentic ΔN-IγCAT, we conclude that processing has occurred between amino acid residues 12 and 13 (Figures 6B and 6C).

Proteolytically Processed ΔN-IγCAT Is Translocated into the Lumen of Microsomal Vesicles

With the proteolytic removal of 12 of the 30 hydrophobic amino acid residues in the membrane-spanning region of ΔN-IγCAT, the question arose as to whether the processed protein was still anchored in the membrane or whether it was now released into the lumen of the microsomal vesicles as is the case for secretory proteins. We used the extractability with carbonate as a criterion for membrane integration. Treatment of RM with carbonate at pH 11 releases proteins that are not integrated into the lipid bilayer as well as proteins present in the lumen of microsomal vesicles.

ΔN-IγCAT was translated in the presence of RM. Membranes were isolated by centrifugation through a sucrose cushion and resuspended in carbonate buffer. Solubilized components were then separated from membranes by centrifugation. Proteins in the membrane pellet and supernatant were analyzed by SDS-PAGE and autoradiography. Membrane-spanning proteins, Iγ and IγCAT, and the secretory protein, mouse granulocyte-macrophage colony stimulating factor (GM-CSF), were used as control (Gough et al., 1985). As is shown in Figure 7, Iγ* and IγCAT*, as expected for membrane-spanning proteins, were found in the membrane fraction. Both ΔN-IγCAT* and the GM-CSF* were found essentially in the soluble, carbonate-released fraction. Thus ΔN-IγCAT* is released after the proteolytic processing into the lumen of the microsomal vesicles. Proteolytic processing, as described above for ΔN-IγCAT, was also obtained for ΔN-Iγ, a protein that lacks the amino-terminal 30 residues of Iγ (data not shown).

Discussion

Our results show that the membrane-spanning segment of the type II membrane protein Iγ contains a potentially cleavable signal sequence. This signal sequence is located in the amino-terminal half of the membrane-spanning segment, and it is cleaved when the preceding cytoplasmic domain is removed. All properties known to identify a signal sequence and a cleavage by signal peptidase can be demonstrated.

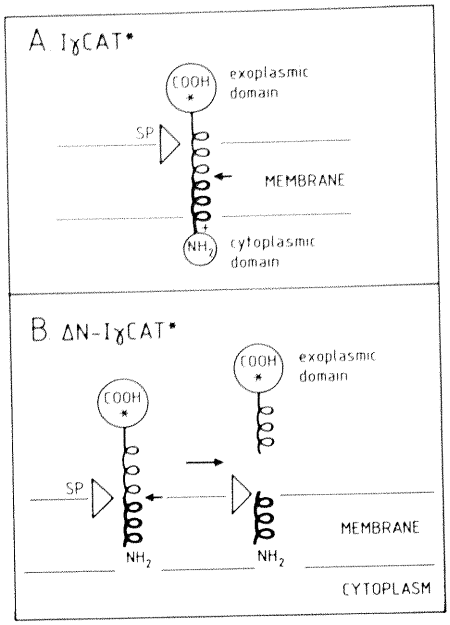

Figure 8. Proposed Topology of the Hydrophobic Segment of IγCAT and ΔN-IγCAT Protein within the ER Membrane

(A) IγCAT spans the membrane and exposes a domain of about 30 amino acid residues on the cytoplasmic side of the ER membrane. The charged amino acid residues close to the cytoplasmic side of the membrane are suggested to restrict vertical mobility of the membrane-spanning segment.

(B) ΔN-IγCAT, during its initial stage of membrane insertion, also spans the membrane with its hydrophobic segment. However, as no charged amino acid residues are present at the extreme amino-terminal end, the hydrophobic segment has some freedom to change its topology across the membrane. Part of the hydrophobic segment might now be pulled into the lumen of the ER membrane, and a former cryptic site for signal peptidase cleavage might become accessible to the active center of signal peptidase.

First, the cleavage occurs concomitant with insertion into the ER membrane as is typical for cleavable signal sequences of presecretory proteins (Blobel and Dobberstein, 1975).

Second, the cleaved segment is located at the amino-terminal end of the deletion protein ΔN-IγCAT. It is 13 amino acid residues long and composed entirely of hydrophobic or uncharged residues. Signal sequences can vary in length from about 15 to over 60 residues. The only structural element identified so far for a signal sequence is its hydrophobic core, usually 8–12 residues long. It is followed by a more polar region 5–7 residues long, which is thought to define the cleavage site for signal peptidase. Thus, a "minimal" signal sequence would be composed of an 8 residue hydrophobic core followed by a 5 residue region conferring cleavage specificity (von Heijne, 1983, 1985). The segment cleaved from protein ΔN-IγCAT would be consistent with such a minimal length signal sequence.

Finally, the amino acid residues around the cleavage site in membrane-translocated ΔN-IγCAT*′ are consistent with cleavage by signal peptidase. Based on a sequence comparison of 78 eukaryotic signal sequences, von Heijne found that only small neutral residues are found at the site of cleavage (−1 position) and that only small neutral and uncharged ones are found at the −3 position, that is 3 amino acid residues in front of the signal peptidase cleavage site (von Heijne, 1983). In the segment cleaved from ΔN-IγCAT, threonine, a small neutral amino acid, is found at the −1 position, and leucine, an uncharged amino acid, at the −3 position. Both of these residues fulfill the above described criteria for a signal peptidase cleavage site. Thus, place (RM) and time of cleavage (cotranslational), hydrophobic character of the cleaved segment, and property of the cleavage site demonstrate that ΔN-IγCAT contains a signal sequence at its amino terminus which is cleaved upon membrane insertion by signal peptidase.

How can we possibly explain how the deletion of the cytoplasmic, hydrophilic segment from IγCAT reveals a cleavable signal sequence in a formerly membrane-spanning region? To us the most plausible explanation is that the position of the hydrophobic segment in the membrane is different in IγCAT and ΔN-IγCAT. Signal peptidase is known to be an integral membrane protein not exposed on the cytoplasmic side of RM (Jackson and Blobel, 1977; Lively and Walsh, 1983; Evans et al., 1986). As in many secretory proteins, the cleavage site for signal peptidase is surrounded on either side by 1 or even 2 charged amino acid residues. It is reasonable to assume that the active center of this enzyme is located close to the exoplasmic side of the ER membrane, not within the membrane. We propose that the removal of the cytoplasmic, hydrophilic segment from IγCAT allows the hydrophobic segment to shift its position within the ER membrane. Most likely it positions itself more toward the exoplasmic side. Hence, a potential signal peptidase cleavage site becomes accessible to the active center of signal peptidase (see Figure 8B).

It has been noted previously in type I membrane proteins that a deletion of the charged amino acid residues flanking the membrane-spanning region does not affect the overall topology (Zuninga and Hood, 1986; Cutler et al., 1986). In the case of E_2 glycoprotein of Semliki Forest virus, it has been shown that mutation of the basic amino acid residues at the cytoplasmic side of the membrane-spanning segment reduces the stability of the mutant protein in the membrane (Cutler et al., 1986).

When the membrane-spanning regions of type I and type II membrane proteins are compared, no obvious structural difference can be found. In both types of membrane proteins these regions comprise a stretch of 20 to 30 hydrophobic amino acid residues that is flanked on the cytoplasmic side by positively charged amino acid residues. In type I membrane proteins the segment spanning the membrane does not appear to participate in the initial stage of membrane insertion. Type I membrane proteins usually have cleavable signal sequences that initiate the membrane translocation of the amino-terminal half of the protein. The membrane-spanning region, in its position close to the carboxy-terminal end, seems only to function in anchoring the protein in the membrane. Yost et al. placed the membrane-spanning segment of the murine surface immunoglobulin heavy chain close to the amino-

terminal end of a fusion protein (Yost et al., 1983). In this position the segment did not provide the signal function for membrane insertion. As, however, a hydrophilic segment of about 40 amino acid residues precedes the membrane-spanning segment, the question still remains as to whether a membrane-spanning region from a type I membrane protein, when placed into the appropriate surrounding, can also initiate translocation across the ER membrane. It is well conceivable that certain hydrophilic sequences preceding a hydrophobic segment play a crucial role in exposing a potential signal for membrane insertion. Up to now no special structural features, besides hydrophobicity, are known to be crucial for the function of a signal sequence.

A common step has been proposed for the early stage of membrane insertion of secretory and membrane proteins (Dobberstein et al., 1983; Spiess and Lodish, 1986; Lipp and Dobberstein, 1986). This was based largely on the finding that both of these types of proteins require SRP and docking protein for their membrane insertion. Here, we show that a type II membrane protein can be converted into a secretory protein by removal of the cytoplasmic segment. This directly demonstrates that the signal for membrane insertion of these two types of proteins can be the same. Further deletion into the carboxy-terminal half of the Iγ hydrophobic segment is required to elucidate whether the cleaved signal sequence contains all the information for membrane insertion. It is conceivable that the functional signal sequence extends over the cleaved signal sequence into the adjacent hydrophobic part. For some secretory protein it has been observed that the cleavable signal sequence is not sufficient for membrane insertion. In the case of staphylococcal protein A, sequences of the amino-terminal part of the mature protein are required for membrane insertion and correct processing (Abrahmsen et al., 1985).

SRP can arrest elongation of presecretory and type II membrane proteins after 70 or even more amino acid residues have been polymerized (Walter and Blobel, 1981a; Meyer et al., 1982; Lipp and Dobberstein, 1986; Lipp et al., unpublished data). These domains are then inserted into the ER membrane by a yet unknown mechanism. As the amino terminus of a type II membrane protein has to remain on the cytoplasmic side, the formation of a loop during membrane insertion has been proposed. In the case of a secretory protein, signal peptidase would be able to act as soon as the loop appears on the exoplasmic side. An initial interaction of basic residues in a signal sequence with the phosphates of the membrane lipids was originally proposed by Inouye for the lipoprotein of E. coli (Inouye et al., 1977). Our results rule out an essential role of these basic residues in ER membrane insertion. The ΔN-IγCAT protein does not contain any charged amino acid residues preceding the hydrophobic segment. It is nevertheless translocated across the ER membrane and processed.

The rules that define the cleavage site for signal peptidase in presecretory proteins are not yet fully understood. Von Heijne points out that the type of amino acids at the −1 and −3 position in front of the site of cleavage are important in assigning a cleavage site. Here we show that sequences at the very beginning of a signal sequence can also influence cleavage by signal peptidase. In the case of Iγ, these charged residues can prevent cleavage by signal peptidase. The variability in the length and in the amount of charged amino acid residues at the amino terminus of a signal sequence has not as yet been explained. Mutation and deletion experiments have clearly shown that charged residues are not essential for membrane insertion. In the light of our findings, we propose that the charged amino acids at the amino terminus of signal sequences function in the alignment of signal sequences in the ER membrane such that signal peptidase can cleave at a very specific site with high fidelity. Our prediction is that removal of charged residues from the amino-terminal end of the signal sequences can lead to an altered or less specific signal peptidase cleavage.

Experimental Procedures

Materials

Restriction enzymes, T4 DNA ligase, micrococcal nuclease S1, reverse transcriptase, and proteinase K were from Boehringer Mannheim. DNA sequencing reagents were from Pharmacia. L-[^{35}S]methionine, L-[^{3}H]leucine, and adenosine 5′-([γ-^{35}S]thio) triphosphate were from Amersham. Wheat germ was obtained from General Mills, California. The acceptor peptide benzoyl-Asn-Leu-Thr-N-methylamide was a generous gift from E. Bause, Cologne.

Plasmid Constructions

Standard molecular cloning techniques, as described by Maniatis et al. (1982), were used. The cDNA clone pγ-2, containing the entire coding region of the human invariant chain cloned into the PstI site of pBR322, was obtained from P. A. Peterson's laboratory, Uppsala, Sweden (Claesson et al., 1983). The expression plasmids pDS5, pDS6, and pDS5/3 have been described previously (Stueber et al., 1984). They allow efficient transcription by E. coli RNA polymerase of cDNAs cloned behind the strong T5 promoter P25. Figures 9A and 9B summarize the construction of the fusion and deletion plasmids described below.

plγcat

pγ-2 was digested with PstI, and the 317 bp fragment containing the 5′ end and the 860 bp fragment containing the 3′ end of the Iγ coding region were isolated. The 317 bp fragment coding for the Iγ cytoplasmic domain, the membrane-spanning segment and 12 amino acid residues of the exoplasmic domain, was cleaved by Sau3A to remove the 5′ GC tail. The 234 bp Sau3A–PstI fragment was isolated and cloned into BamHI/PstI-cut pDS5. This results in an in-frame fusion of the 5′ end of Iγ to the *cat* gene.

plγ

Initial attempts to clone the complete Iγ coding region into pDS5 failed. When expressed, this region is probably lethal to the bacterium. To repress transcription from the T5 promoter/operator (P/O) in bacteria, we cloned the *lac i* repressor between the *bla* gene and the T5 P/O. This plasmid is called pRIγcat. For the construction of plγ, pRIγcat was linearized by PstI and the 860 bp PstI fragment, coding for the carboxy-terminal domain of Iγ, was ligated into this site. Transformants containing the 860 bp fragment were screened for expression of immunoprecipitable Iγ chain after in vitro transcription–translation.

pΔN-Iγcat

To delete the cytoplasmic domain from IγCAT, the 950 bp SstII–XbaI fragment from plγcat was isolated and ligated at the XbaI site of BamHI/XbaI cut p6/5R. The protruding ends at the BamHI and the SstII sites were blunted with S1 nuclease and ligated. As a result, a new ATG initiation codon is placed just in front of the membrane-spanning segment of Iγ. The construction was confirmed by DNA and amino acid sequence analyses (see Figure 4A).

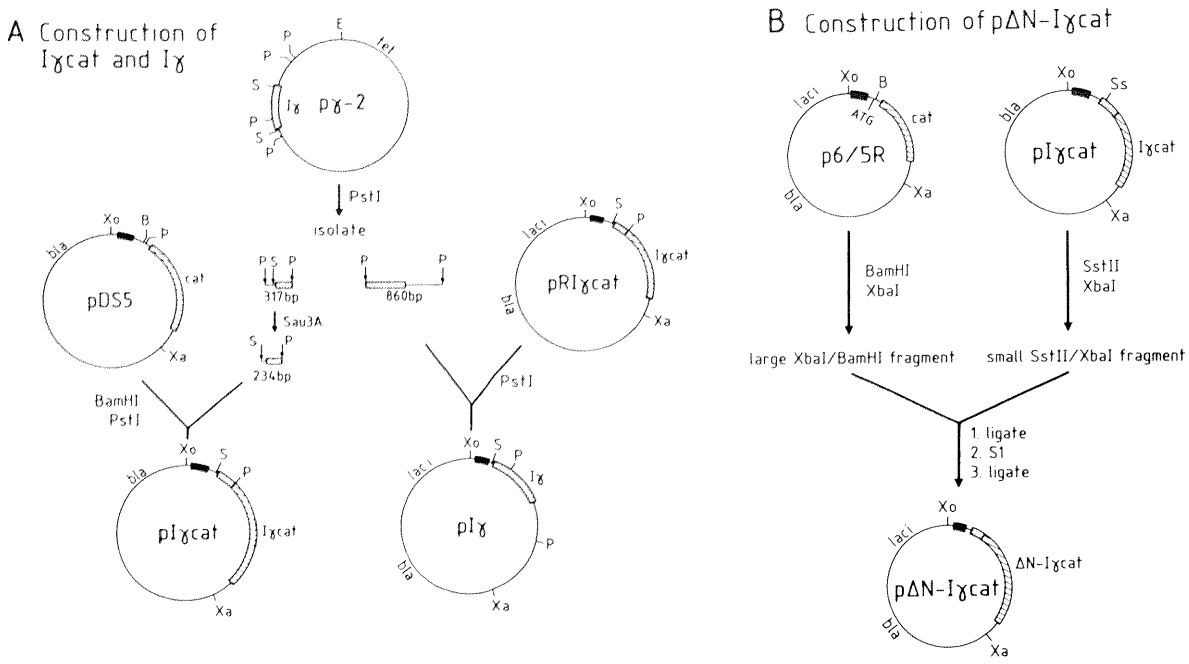

Figure 9. Summary of the Construction of Iγ Deletion and Fusion Plasmids

(A), Construction of pIγcat and pIγ; (B), construction of pΔN-Iγcat. Symbols are as follows: box, coding sequence; dotted box, Iγ-derived sequence; box with slanted lines, *cat*-derived sequence; black box, T4 promoter. The location of genes coding for lac repressor (*lac i*), β-lactamase (*bla*) and tetracycline (*tet*) are indicated. Abbreviations for restriction sites are as follows: B, BamHI; P, PstI; S, Sau3A; Ss, SstII; Xo, XhoI; Xa, HbaI. For details see text on constructions.

p6/5R

To repress transcription from the T5 promoter the *lac i* gene was inserted between the *bla* gene and the T5 P/O region of pDS5/3 (Stueber et al., 1984).

Antibodies Against Iγ Domains

To raise antibodies against the amino- and the carboxy-terminal domains of Iγ, fusion proteins of β-galactosidase and parts of Iγ were produced in bacteria and used as antigens to raise antibodies in rabbits.

From a PstI digest of pγ-2, the 317 bp fragment coding for the amino-terminal 72 amino acids of Iγ and the 860 bp fragment coding for the exoplasmic carboxy-terminal domain of Iγ were isolated. Each of the fragments was inserted into the PstI site of the bacterial expression vector pEX1 (Stanley and Luzio, 1984).

Fusion proteins expressed in NF1 bacteria were separated on preparative SDS–polyacrylamide gels (7% acrylamide; Laemmli, 1970). Protein bands were visualized by KOAc precipitation, and fusion proteins were eluted from gel slices. Two rabbits were immunized with each of the two fusion proteins. Antibodies against the amino terminus of Iγ (anti-IγN) and its carboxyl terminus (anti-IγC) were obtained. They reacted with authentic Iγ chains synthesized by human Raji cells (data not shown).

Immunoprecipitations

After translation and posttranslational assays, antigens in a 25 μl aliquot were solubilized by adding Nonidet-P40 (NP40) to 0.5%. Then 1 μl of either anti-IγN or anti-IγC antiserum was added and the mixtures incubated for 15 min at 4°C. Forty microliters of a 1:1 slurry of protein A–Sepharose (equilibrated in 0.2% NP40, 10 mM Tris–HCl [pH 7.5], 150 mM NaCl, and 2 mM EDTA) was added to each sample, and incubation continued for 60 min at 4°C. Beads were sedimented by centrifugation and washed three times with 0.2% NP40, 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 2 mM EDTA, twice with 0.2% NP40, 10 mM Tris–HCl (pH 7.5), 500 mM NaCl, and 2 mM EDTA, and once with 10 mM Tris–HCl (pH 7.5). Sample buffer for SDS-PAGE was added to the sedimented beads, and antigens were analyzed by SDS-PAGE and fluorography.

In Vitro Transcription and Translation

Plasmids were transcribed in vitro by E. coli RNA polymerase, and the resulting mRNA was translated in a wheat germ cell-free system as described by Stueber et al. (1984). To test for membrane translocation, rough microsomes from dog pancreas were included in the translation (Blobel and Dobberstein, 1975). Glycosylation onto asparagine residues was blocked by the addition of the acceptor peptide benzoyl-Asn-Leu-Thr-N-methylamide to a final concentration of 30 μM (Lau et al., 1983; Bause, 1983).

Posttranslational Assays

To test translocation of in vitro–synthesized proteins across, or their insertion into, the ER membrane, accessibility to proteinase K was used. A 10 μl aliquot of a translation mixture containing rough microsomes was incubated for 10 min at 25°C with either 0.3 mg/ml of proteinase K or 0.3 mg/ml of proteinase K and 0.5% NP40. Further proteolysis was stopped by the addition of phenylmethylsulfonyl fluoride (PMSF) to 0.1 mg/ml, and the sample was further characterized by SDS-PAGE (Laemmli, 1970) and fluorography or, where indicated in the figure, by immunoprecipitation. To remove secretory and peripheral membrane proteins, rough microsomes were subjected to a carbonate wash with 0.1 M Na_2CO_3, pH 11 (Fujiki et al., 1982).

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