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

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Summary

The human invariant chain (Ly) 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 Ly is replaced by the cytoplasmic protein chloramphenicol acetyltransferase (CAT), CAT becomes the exoplasmic, glycosylated domain of the resulting membrane protein Ly/CAT*. Deletion of the hydrophilic cytoplasmic domain from Ly/CAT 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 Ly 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 Ly) 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 (Chiocchia 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 Stahlitz, 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 (Ly) and several deletion and fusion proteins derived from it in a cell-free membrane insertion system.

Ly 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 Ly 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 Ly 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 Ly 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 uncleavable signal.
the exoplasmic portion of ly and the gene encoding the region in ly that is responsible for membrane insertion. Deletions and fusions were made at the DNA level behind the strong T5 promoter in pDS5 can be transcribed 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

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 in a eukaryotic cell-free system. We used this approach to localize and characterize the region in ly that is responsible for membrane insertion. Deletions and fusions were made at the DNA level after cloning of ly 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).

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 ly that is responsible for membrane insertion. Deletions and fusions were made at the DNA level after cloning of ly 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).

In Vitro Translation and Membrane Insertion of ly

When ply 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 ly (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 ly* 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, ly* was digested. These data suggest that ly* 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 ly and its glycosylated form was confirmed by immunoprecipitation with antibodies raised against the amino-terminal 72 (anti-lyN) or against the carboxy-terminal 144 (anti-lyC) residues of ly. As shown in Figure 2, lanes 5, 6, 8, and 9, these antibodies recognize glycosylated and nonglycosylated forms of ly. No protein could be precipitated with anti-lyN antibody when the cytoplasmic domain was removed from membrane-integrated ly* by protease digestion (Figure 2, lane 7). As the antibody is directed against the amino-terminal portion of ly, the data directly demonstrate that the amino terminus is located on the cytoplasmic side and is accessible to the protease. With anti-lyC antibody, the processed form of ly is readily detectable, demonstrating an exoplasmic location of the carboxy-terminal portion of ly (Figure 2, lane 10).

Membrane Insertion of lyCAT

An analysis of membrane insertion was performed for lyCAT and CAT as described above for ly. CAT was expressed from pDS5. lyCAT 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 lyCAT* (Figure 3, lane 2).
The Signal Sequence in Invariant Chain

Figure 2. In Vitro Translation and Membrane Insertion of Iy

RNA derived from pIycat or pDN5 was translated in the wheat germ cell-free system in the presence or the absence of RM. Membrane insertion was tested by treatment with proteinase K (PK) or PK and the detergent NP40. Addition of RM, PK, and NP40 is indicated at the bottom of each lane.

Membrane Insertion and Glycosylation of ΔN-IyCAT

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 Iy 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 IyCAT would affect its membrane insertion and topology.

The cytoplasmic domain of IyCAT was deleted and the initiator methionine was placed in front of the membrane spanning segment. The amino-terminal sequences of IyCAT and AN-IyCAT as deduced from the DNA sequences are shown in Figure 4A. When RNA derived from pAN-Iycat was translated in the wheat germ cell-free system, a single polypeptide of 29 kd was synthesized, AN-IyCAT (Figure 4B, lane 1). This was, as expected, about 3 kd smaller than the IyCAT 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-IyCAT. Both of these forms were resistant to proteinase K, indicating that they were inserted into or translocated across microsomal membranes (Figure 4B, lanes 2 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.
\( \Delta N-I\gamma CAT \) 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). \( I\gamma CAT \) and \( \Delta N-I\gamma CAT \) were translated in the presence of microsomes with and without the acceptor peptide Asn-Leu-Thr. The size of \( I\gamma CAT \) 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 kDa (Figure 5A). We can conclude that nonglycosylated \( I\gamma CAT \) 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 \( \Delta N-I\gamma CAT \) was synthesized in the presence of RM, three polypeptides accumulated: \( \Delta N-I\gamma CAT \), a 2 kDa larger form, and a 1 kDa smaller form. In the presence of the acceptor peptide, the larger form disappeared and the amount of the smaller form, \( \Delta N-I\gamma CAT' \), increased (Figure 5B, lanes 2 and 3). \( \Delta N-I\gamma CAT' \) 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 \( \Delta N-I\gamma CAT' \) was generated by a proteolytic cleavage, most likely by signal peptidase.

To determine the site of cleavage in the proteolytically processed forms of \( \Delta N-I\gamma CAT \), the positions of leucine in the amino-terminal regions of \( \Delta N-I\gamma CAT \) and membrane-inserted \( \Delta N-I\gamma CAT'' \) were determined. \( \Delta N-I\gamma CAT \) was translated in the absence or presence of RM with \(^{3}H \)leucine as label. As \( \Delta N-I\gamma CAT \) is essentially the only protein synthesized from p\( \Delta N-I\gamma CAT \)-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 py-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 \( \Delta N-I\gamma CAT \) 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 \( \Delta N-I\gamma CAT \) (see insert in Figure 6B). Taking into account the size reduction of about 1 kDa by the processing
The Signal Sequence in Invariant Chain

Proteolytically Processed Δ N- I yCAT 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 Δ N- I yCAT, 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.

Δ N- I yCAT 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, I y and I yCAT, and the secretory protein, mouse granulocyte-macrophage colony stimulating factor (GM-CSF), were used as controls (Gough et al., 1985). As is shown in Figure 7, I y* and I yCAT*, as expected for membrane-spanning proteins, were found in the membrane fraction. Both Δ N- I yCAT* and the GM-CSF* were found essentially in the soluble, carbonate-released fraction. Thus Δ N- I yCAT* is released after the proteolytic processing into the lumen of the microsomal vesicles. Proteolytic processing, as described above for Δ N- I yCAT, was also obtained for Δ N- I y, a protein that lacks the amino-terminal 30 residues of I y (data not shown).

Discussion

Our results show that the membrane-spanning segment of the type II membrane protein I y 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.
Signal Sequence.

Comparison of 78 eukaryotic signal sequences, von Heijne, 1983. In the segment cleaved from ΔN-IyCAT, 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-IyCAT 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 IyCAT 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 IyCAT and ΔN-IyCAT. 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 IyCAT 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 F₉  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 differences 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 cytochemical-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-

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-IyCAT. 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-IyCAT would be consistent with such a minimal length signal sequence.

Finally, the amino acid residues around the cleavage site in membrane-translocated ΔN-IyCAT 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-IyCAT, 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-IyCAT contains a signal sequence at its amino terminus which is cleaved upon membrane insertion by signal peptidase.

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When the membrane-spanning regions of type I and type II membrane proteins are compared, no obvious structural differences 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 cytochemical-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 Iy 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 phosphatases 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-IyCAT 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 -9 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 Iy, 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 nucleases S1, reverse transcriptase, and proteinase K were from Boehringer Mannheim. DNA sequencing reagents were from Pharmacia, L-(+)-3′-methylthymidine, L-(+)-3H]uridine, and adenosine 5′-(32P)thio]triphosphate were from Amersham. Wheat germ was obtained from General Mills, California. The acceptor peptide benzoylAsn-Leu-Thr-N-methylamide was a generous gift from E. Bausa, Cologne.

**Plasmid Constructions**

Standard molecular cloning techniques, as described by Maniatis et al. (1982), were used. The cDNA clone py-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.

**pIy**

The 950 bp StuII-Xbal fragment from pIyCAT was isolated and ligated into BamHI/EcoRI cut pDS5. This results in an in-frame fusion of the 5′ end of Iy to the car gene.

**pIyCAT**

pIyCAT was digested with PstI, and the 317 bp fragment containing the 5′ and the 860 bp fragment containing the 3′ end of the Iy coding region were isolated. The 317 bp fragment coding for the Iy cytoplasmic domain, the membrane-spanning segment and 12 amino acid residues of the exoplasmic domain, was cleaved by Sau3AI to remove the 5′ GC tail. The 234 bp Sau3AI-PstI fragment was isolated and cloned into BamHI/PstI cut pDS5. This results in an in-frame fusion of the 5′ end of Iy to the car gene.

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Figure 9. Summary of the Construction of \( \Delta y \) Deletion and Fusion Plasmids

(A) Construction of \( \text{lycat} \) and \( \text{py} \); (B) construction of \( \text{pAN-lycat} \). Symbols are as follows: box, coding sequence; dotted box, \( \text{ly} \)-derived sequence; box with slanted lines, \( \text{cat} \)-derived sequence; black box, T4 promoter. The location of genes coding for lac repressor (\( \text{lac} i \)), \( \beta \)-lactamase (\( \beta \text{a} \)) and tetracycline (\( \text{tet} \)) are indicated. Abbreviations for restriction sites are as follows: B, BamHI; P, PstI; S, Sau3A; Ss, SstII; Xo, Xhol; Xa, Hsal. For details see text on constructions.

\( \text{p65SR} \)

To repress transcription from the T5 promoter the \( \text{lac} i \) gene was inserted between the \( \beta \text{a} \) gene and the T5 P/O region of \( \text{pDS5/3} \) (Stueber et al., 1984).

Antibodies Against \( \text{ly} \) Domains

To raise antibodies against the amino- and the carboxy-terminal domains of \( \text{ly} \), fusion proteins of \( \beta \)-galactosidase and parts of \( \text{ly} \) were produced in bacteria and used as antigens to raise antibodies in rabbits.

From a PstI digest of py-2, the 317 bp fragment coding for the amino-terminal 72 amino acids of \( \text{ly} \) and the 860 bp fragment coding for the exoplasmic carboxy-terminal domain of \( \text{ly} \) 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 SDSPolyacrylamide 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 \( \text{ly} \) (anti-\( \text{lyN} \)) and its carboxyl terminus (anti-\( \text{lyC} \)) were obtained. They reacted with authentic \( \text{ly} \) chains synthesized by human Raji cells (data not shown).

Immunoprecipitations

After translation and posttranslational assays, antigens in a 25 \( \mu \text{l} \) aliquot were solubilized by adding Nonidet-P40 (NP40) to 0.5%. Then 1 \( \mu \text{l} \) of either anti-\( \text{lyN} \) or anti-\( \text{lyC} \) 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 sedimemented 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 on asparagine residues was blocked by the addition of the acceptor peptide benzoyl-Asn-Leu-Thr-N-methylamide to a final concentration of 30 \( \mu \text{M} \) (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 \( \mu \text{l} \) 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&Os, pH 11 (Fujiki et al., 1982).

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