A Tripartite Structure of the Signals that Determine Protein Insertion into the Endoplasmic Reticulum Membrane

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Abstract. Multilineage colony stimulating factor is a secretory protein with a cleavable signal sequence that is unusually long and hydrophobic. Using molecular cloning techniques we exchanged sequences NH_2- or COOH-terminally flanking the hydrophobic signal sequence. Such modified fusion proteins still inserted into the membrane but their signal sequence was not cleaved. Instead the proteins were now anchored in the membrane by the formerly cleaved signal sequence (signal–anchor sequence). They exposed the NH_2 terminus on the exoplasmic and the COOH terminus on the cytoplasmic side of the membrane.

We conclude from our results that hydrophilic sequences flanking the hydrophobic core of a signal sequence can determine cleavage by signal peptidase and insertion into the membrane. It appears that negatively charged amino acid residues close to the NH_2 terminal side of the hydrophobic segment are compatible with translocation of this segment across the membrane. A tripartite structure is proposed for signal–anchor sequences: a hydrophobic core region that mediates targeting to and insertion into the ER membrane and flanking hydrophilic segments that determine the orientation of the protein in the membrane.

SECRETORY and membrane proteins are translocated across or inserted into the membrane of the ER by a mechanism involving a signal sequence, signal recognition particle, and docking protein (signal recognition particle receptor) (Walter and Lingappa, 1986; Lipp and Dobberstein, 1986*b*; Zerial et al., 1986; Bos et al., 1984; Holland et al., 1984; Sakaguchi et al., 1984). Two functions of a signal sequence can be distinguished: (*a*) targeting to the ER membrane and (*b*) insertion into the membrane. Uncleaved signal sequences can mediate membrane insertion and anchoring. Such proteins span the membrane once and can expose the NH_2 terminus either on the exoplasmic (type I) or on the cytoplasmic (type II) side of the membrane. The signal sequence in these proteins functions in targeting as well as in anchoring in the membrane (signal-anchor- [SA][1] proteins) (Blobel, 1980; Spiess and Lodish, 1986; Lipp and Dobberstein, 1986*a*). Signal sequences can then be considered to have "common" features for ER targeting and "private" features which determine cleavage by signal peptidase or membrane insertion in a type I or type II orientation. We want to define the "private" features in a signal sequence by converting secretory proteins into SA–membrane proteins.

A common theme for the targeting function of a signal sequence is a continuous stretch of apolar and neutral amino acid residues (von Heijne, 1985; Kaiser et al., 1987). The length of the hydrophobic segment in a signal sequence can vary between eight and more than 20 amino acid residues. The signal sequence can be cleaved upon translocation across the ER membrane or can remain on the polypeptide chain (von Heijne, 1985). If the hydrophobic segment of a signal sequence is of sufficient hydrophobicity and is not cleaved, it can anchor the protein in the membrane (Lipp and Dobberstein, 1986*a*, 1988; Zerial et al., 1987; Spiess and Handschin, 1987). However, the hydrophobic segment alone does not determine the membrane topology of SA-proteins. Experimental evidence suggests that the hydrophilic regions flanking the hydrophobic segment determine signal cleavage and membrane translocation or, in the absence of signal cleavage, insertion in a type I or type II orientation. Thus, a type II membrane protein was converted into a secretory protein when either the NH_2- or the COOH-terminal sequences flanking the hydrophobic segment of a signal sequence were altered (Lipp and Dobberstein, 1986*a*, 1988). Similarly, the SA–membrane protein cytochrome P-450 was converted into a secretory protein by exchanging an NH_2-terminal acidic amino acid residue for two basic ones (Szczesna-Skorupa et al., 1988). By comparison of sequences flanking hydrophobic SA-segments NH_2-terminally, a prevalence of positively charged amino acid residues can be observed in type II proteins whilst type I SA-proteins mainly contain negative and uncharged residues in this segment (von Heijne, 1986*a*,*b*; Lipp and Dobberstein, 1986*a*, 1988; Williams and Lamb, 1986; High and Tanner, 1987; Szczesna-Skorupa et al., 1988).

We chose the haematopoietic growth factor multilineage

1. *Abbreviations used in this paper*: CAT, chloramphenicol acetyltransferase; Mu-CSF, multilineage colony stimulating factor; SA, signal-anchor.

colony stimulating factor (Mu-CSF) (Metcalf, 1987) as a model protein to determine the requirements for converting a secretory protein into a SA-membrane protein. The cleaved signal sequence of Mu-CSF is unusually hydrophobic and lacks charged amino acid residues (Fung et al., 1984; Yokota et al., 1984). A cDNA clone was isolated from a T cell library encoding a Mu-CSF species with a highly charged NH_2-terminal extension of the precursor polypeptide (Dunn et al., 1985). We speculated that if signal cleavage could be prevented, the hydrophobic segment might be converted to a SA-segment. We found that modifications of the segments flanking the hydrophobic signal sequence converted Mu-CSF to a SA-membrane protein with a type I orientation.

Materials and Methods

Materials

Restriction endonucleases, T4 DNA polymerase and ligase SI nuclease exonuclease Bal 31, and proteinase K were from Boehringer Mannheim Diagnostics, Inc. (Houston, TX). DNA sequencing reagents were from Pharmacia Fine Chemicals (Freiburg, FRG). L-[^{35}S]methionine, *L*-[^{3}H]leucine, and adenosine 5'-([gamma-^{35}S]thio)triphosphate were from Amersham (Buchler, Braunschweig). Wheat germ was obtained from General Mills (Minneapolis, MN). The acceptor peptide benzoyl-Asn-Leu-Thr-*N*-methylamide was a generous gift from E. Bause, Köln. Oligodeoxynucleotides were synthesized by Bryan Sproat (European Molecular Biology Laboratory, Heidelburg).

Cloning of lMu-CSF cDNA

A cDNA library was prepared using mRNA isolated from the murine T cell line LB3 6 h after stimulation with concanavalin A (Gough et al., 1985). The library was screened for Mu-CSF clones with 20-mer oligonucleotides complementary to the known Mu-CSF mRNA sequences as hybridization probes (Fung et al., 1984; Yokota et al., 1984). To select for clones having the entire coding region, the probes used for hybridization were complementary to sequences surrounding the translational start and stop codons. The nucleotide sequence of the clone with the longest insert, pMu2A1, was determined by the dideoxy chain termination method. This clone was found to encode the sequence for "long" Mu-CSF (lMu-CSF).

For in vitro transcription, the cDNA for lMu-CSF was subcloned into pDS plasmids (Stueber et al., 1984).

Subcloning of Mu-CSF cDNA into In Vitro Transcription Plasmids

Standard molecular cloning techniques were used as described by Maniatis et al. (1982). The in vitro transcription vectors pDS5 and pDS6 were described by Stueber et al. (1984). DNA sequences were determined by applying the chain termination technique to the double-stranded pDS plasmids as described by Chen and Seeburg (1985). 20-mer primers complementary either to the T5 promoter region or to the sequence 3' of the Pvu II site in the CAT gene were used.

plMC-SS. The Eco RI–Hind III fragment of ~360 bp that contains the 5' sequences coding for the signal peptide was isolated from pMu2A1 (Dunn et al., 1985) and subcloned into pDS6 cut with Eco RI and Hind III. The 5' GC-tail had to be deleted to obtain efficient in vitro expression of the cDNA. This was accomplished by opening the vector at the Xho I site 5' of the T5 promoter and digesting with the exonuclease Bal 31, followed by restriction with Hind III. Bal 31–Hind III fragments of 160–200-bp length were isolated from a gel and ligated into pDS6 that had been cut with Pst I, blunt-ended, and cut with Hind III. A clone efficiently expressing the intact signal sequence was selected by in vitro transcription and translation. The 5' sequences of the Mu-CSF insert of this clone were determined by DNA sequencing (see Fig. 1 *A*).

plMu-CSF. The Eco RI fragment containing the entire Mu-CSF coding sequence was isolated from pMu2A1. After filling in the Eco RI overhangs followed by Hind III restriction, the Hind III–Eco RI fragment coding for the mature part of Mu-CSF was isolated and ligated into plMC-SS that had been cut with Hind III (3' of the signal peptide sequence) and Pvu II (in the CAT sequence).

plMC-CAT. plMC-SS was opened with Hind III (3' of the segment coding for the signal sequence), filled in with T4 DNA polymerase in the presence of all four deoxynucleotide triphosphates, cut with Pvu II (in the CAT sequence), and religated at a dilution favoring circularization of the vector. Colonies were screened for expression of a Mu-CSF signal sequence/CAT fusion protein by in vitro transcription and translation of vector DNA isolated by a minipreparation technique. One clone was selected and its DNA sequence at the ligation site determined (see Fig. 1, *B* and *C*).

psMu-CSF. plMu-CSF was cut with Bam HI and the 369-bp fragment coding for the NH_2-terminal sequences of lMu-CSF as well as the large vector fragment (3784 pb) were isolated from an agarose gel. The 369-bp fragment was digested with Sau 3A. The resulting 307-bp Sau 3A–Bam HI fragment was isolated from a gel and ligated to the 3,784 bp of the Bam HI–cut vector, thus restoring the sequences coding for the COOH-terminal 80% of the signal sequence and the entire mature part of Mu-CSF. This "short" Mu-CSF construct psMu-CSF, when transcribed *in vitro*, results in a mRNA in which translation is initiated at the AUG that corresponds to the first methionine of the authentic pre-Mu-CSF described by several groups (see Fig. 1 *A*; Fung et al., 1984; Miyatake et al., 1985; Yokota et al., 1984).

psMC-CAT. An 828-bp Sau 3A–Xba I fragment coding for the COOH-terminal 80% of the Mu-CSF signal sequence and all of the CAT sequences fused to it was isolated from plMC-CAT. A 2,505-bp Xba I–Bam HI fragment lacking the CAT sequences was prepared from pDS5. The two fragments were ligated and transformed into bacteria. Resulting colonies were screened for plasmids expressing a shortened form of lMC-CAT. This sMC-CAT starts at the same AUG as sMu-CSF (Fig. 1).

All constructs were verified by sequence analysis.

In vitro Transcription and Translation

Plasmids were transcribed in vitro with *Escherichia coli* RNA polymerase and the resulting mRNA was translated in the wheat germ cell-free system as described by Stueber et al. (1984). Membrane insertion and translocation were assayed by the addition of dog pancreas microsomes (Warren and Dobberstein, 1978) and SRP (Walter and Blobel, 1980) to the translation mixture. In some experiments, *N*-linked core glycosylation was inhibited by the addition of the acceptor tripeptide benzoyl-Asn-Leu-Thr-*N*-methylamide to a final concentration of 30 μM (Lau et al., 1983; Bause et al., 1983).

COOH-terminal truncation of newly synthesized polypeptides was achieved by the addition of complementary oligodeoxynucleotides to the translation mixture as described by Haeuptle et al. (1986).

Posttranslational Assays

Protection from proteinase K digestion was assayed as described by Blobel and Dobberstein (1975).

Membrane integration was tested by treatment with carbonate at pH 11 (Fujiki et al., 1982) as follows: after translation in the presence of microsomes, the translation mixture was diluted sixfold with 0.1 M Na_2CO_3, pH 11, and spun through a cushion of 0.25 M sucrose, 0.1 M Na_2CO_3, pH 11. The top layer and pellet were collected, while the sucrose cushion was discarded. Proteins from the top layer were precipitated with 10% TCA. Both the TCA and the carbonate pellet were dissolved in sample buffer and analyzed by SDS-PAGE. For carbonate-extracted samples, the equivalent of two times the amount of translation mixture was loaded on an SDS-gel as for untreated aliquots.

Immunoprecipitations were performed as described by Lipp and Dobberstein (1986*a*). The anti-CAT antibody was a kind gift from H. Bujard, Heidelberg.

Other Methods

SDS-PAGE was carried out by using either 10–15% polyacrylamide gradient gels (Maizel, 1969) or 22% polyacrylamide/6 M urea gels (Haeuptle et al., 1986). Fluorography of the gels was performed using EN_3HANCE according to the instructions provided by the company (New England Nuclear, Boston, MA).

Results

Cloning of cDNA Coding for Mu-CSF and MC-CAT Proteins

Mu-CSF stimulates the proliferation and differentiation of

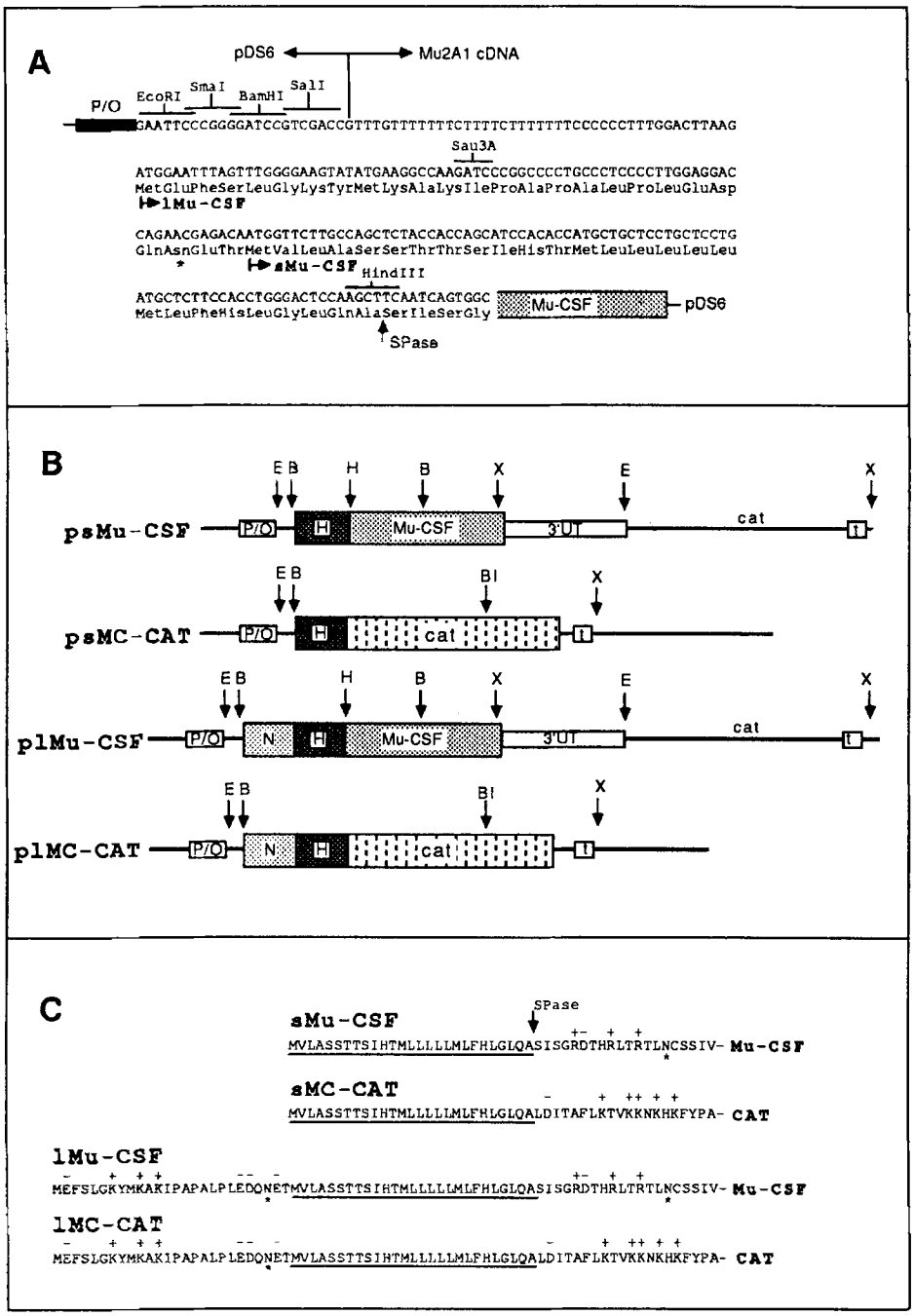

Figure 1. (*A*) Nucleotide sequence of the 5′ region of the Mu2A1 cDNA subcloned into the polylinker region of pDS 6. The deduced amino acid sequence is aligned underneath. The translational start sites for lMu-CSF and sMu-CSF, respectively, are indicated. Note that in psMu-CSF the upstream AUG responsible for the NH_2-terminal extension in lMu-CSF is removed by deleting a Bam HI-Sau 3A fragment from the cDNA (see Materials and Methods). Restriction sites relevant for the subcloning described in Materials and Methods are indicated. *P/O* indicates the promoter/operator sequences for *E. coli* RNA polymerase. The arrow designated *SPase* points at the signal peptidase cleavage site. The asterisk indicates a potential *N*-glycosylation site. (*B*) Schematic outline of Mu-CSF and MC-CAT cDNAs cloned into the in vitro transcription vector pDS6. Coding sequences derived from multilineage colony stimulating factor (*Mu-CSF*) or chloramphenicol acetyl transferase (*cat*) are represented by large boxes. *H*, hydrophobic core of the signal sequence; *N*, hydrophilic NH_2-terminal extension; *3′ UT*, the 3′-untranslated region of Mu-CSF; *P/O*, promoter/operator sequences containing the transcription start site; and *t*, transcription terminator. The following restriction sites are indicated: *B*, Bam HI; *Bl*, Bal I; *E*, Eco RI; *H*, Hind III; *X*, Xba I. Bl indicates the site of truncation by an oligonucleotide. (*C*) NH_2-terminal amino acid sequences of Mu-CSF and MC-CAT proteins. The differences in the hydrophilic sequences flanking the hydrophobic segment at its COOH and NH_2 terminus are shown. Charged amino acid residues are indicated. Potential *N*-glycosylation sites are marked with asterisks. *SPase*, signal peptidase cleavage site.

haematopoietic cells such as granulocytes, macrophages, eosinophils, megakaryocytes, erythroid, and mast cells (Metcalf, 1987). Sequence analysis of Mu-CSF and of cDNA coding for mouse Mu-CSF (Fung et al., 1984; Yokota et al., 1984) suggests that Mu-CSF is a secreted protein with a cleavable signal peptide of 27 uncharged amino acid residues. This signal peptide is exceptionally long and hydrophobic if compared to those of other secretory proteins (von Heijne, 1985). It has all the features of a membrane-spanning sequence, except that it is cleaved. We constructed four fusion proteins containing the signal sequence of Mu-CSF but differing in the regions flanking the signal sequence (Fig. 1, *B* and *C*).

During the course of screening a cDNA library complementary to mRNA from a stimulated murine T cell line (see Materials and Methods) a Mu-CSF cDNA clone, pMu2A1, with a novel sequence at its 5′ end was isolated (Fig. 1 *A*). The additional segment in this clone does not represent an extension into the 5′ noncoding region of the short Mu-CSF (sMu-CSF) mRNA (Fung et al., 1984; Yokota et al., 1984), but rather diverges from the latter at an apparent splice site internal to its sequence. Intriguingly, this additional 5′ exon includes two in-frame translational initiation codons (Fig. 1 *A*, *arrows*). It is expected that in a mRNA corresponding to this cDNA, the first AUG would initiate translation, since in 95% of eukaryotic mRNAs translation begins at the AUG closest to the 5′ end of the mRNA (Kozak, 1984). Moreover, the nucleotides surrounding this AUG conform

well to the consensus sequence noted by Kozak (1984). Translation initiating at this AUG would give rise to a pre-Mu-CSF molecule that is elongated at the NH_2-terminus of the signal peptide by 26 amino acids with clusters of positively and negatively charged residues (Fig. 1, *A* and *C*). This long Mu-CSF (lMu-CSF) contains a sequence with rather exceptional features. It is 53 amino acids long with a hydrophobic core of 27 residues and a highly charged hydrophilic NH_2-terminal extension. On average, signal sequences are ~20 amino acid residues long with a hydrophobic core of 8–12 residues and a short hydrophilic NH_2-terminus with only one positively charged amino acid (see von Heijne, 1985).

Plasmid plMu-CSF was used to construct psMu-CSF which codes for authentic Mu-CSF initiating at the ATG immediately NH_2-terminal to the hydrophobic part of the signal sequence (Fig. 1, *A* and *C*, see Materials and Methods).

To test the topogenic effect of sequences flanking the hydrophobic core of a signal sequence on the COOH-terminal side, we constructed plasmids plMC-CAT and psMC-CAT. In these plasmids the mature COOH-terminal portions of lMu-CSF and sMu-CSF were exchanged for the COOH-terminal 181 amino acids of the bacterial cytoplasmic protein chloramphenicol acetyltransferase (CAT) (see Materials and Methods; Fig. 1, *B* and *C*). CAT protein had previously been shown to be translocated across microsomal membranes if fused to a signal-anchor (SA) sequence (Lipp and Dobberstein, 1986*a*) or a cleavable signal sequence (Ibrahimi and Gentz, 1987). The CAT-derived portion of lMC-CAT encodes no site for *N*-glycosylation. By contrast, the mature part of Mu-CSF contains four sites for *N*-linked glycosylation. The NH_2-terminal, hydrophilic extension contains one potential site for *N*-glycosylation very close to the hydrophobic segments of lMu-CSF and lMC-CAT (*asterisks* in Fig. 1 *C*). All cDNAs were cloned into the in vitro expression vector pDS6 (Fig. 1, *B*; Stueber et al., 1984).

In Vitro Translation and Membrane Translocation of sMu-CSF

Plasmid psMu-CSF was transcribed with *E. coli* RNA polymerase and the resulting mRNA was translated in wheat germ lysate supplemented with rough microsomes derived from dog pancreas ER membranes. Since the mature portion of sMu-CSF contains only a single methionine residue, the commonly used label [^{35}S]methionine was replaced by [^{3}H]leucine to obtain an evenly labeled protein. sMu-CSF contains four potential sites for *N*-glycosylation. To obtain migration as a single band on SDS polyacrylamide gels, *N*-linked core glycosylation was prevented by the addition of an acceptor peptide for *N*-linked glycosylation. Such synthetic peptides have been shown to efficiently compete as substrates for oligosaccharyl transferase (Bause, 1983; Lau et al., 1983). Fig. 2 (lane *1*) shows that translation of sMu-CSF mRNA in the absence of microsomes results in a precursor protein with a relative molecular mass of 18 kD termed pre-sMu-CSF. When microsomes and acceptor peptide were added to the translation assay, membrane translocation and signal cleavage became apparent by the appearance of a second, faster migrating band representing proteolytically processed Mu-CSF (Fig. 2, lane 2). This band was protected from proteinase K digestion in the absence, but not in the presence of detergent, suggesting that it had segregated into the microsomal lumen (Fig. 2, lanes *3* and *4*).

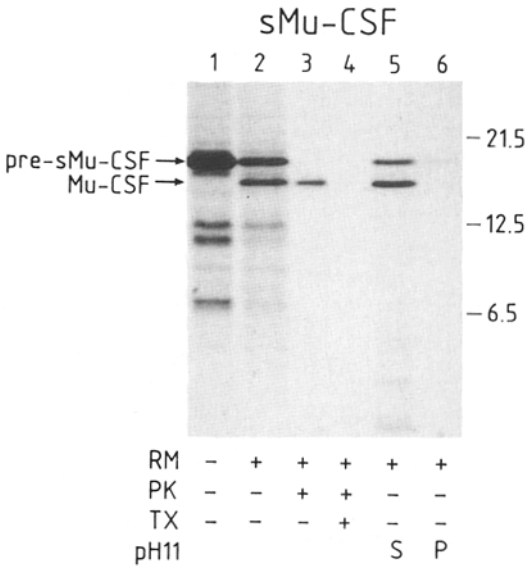

Figure 2. Translation and translocation of sMu-CSF. A wheat germ cell-free system containing [^{3}H]leucine was primed with sMu-CSF mRNA either in the absence (lane *1*) or presence (lanes *2–6*) of microsomes (*RM*) derived from dog pancreas and an acceptor peptide for *N*-linked glycosylation. Aliquots of the proteins translated in the presence of RM were assayed for protease protection by incubation with proteinase K (*PK*) either in the absence (lane *3*) or presence (lane *4*) of the detergent Triton X-100 (*TX*). Other aliquots were extracted with carbonate at pH 11. Solubilized material (lane 5, *S*) was separated from membrane-bound proteins (lane 6, *P*) by centrifugation. Proteins were analyzed on a 22% SDS polyacrylamide gel. Precursor multi-CSF (pre-sMu-CSF) and processed, mature multi-CSF (Mu-CSF) are indicated. Relative molecular masses are given in kilodaltons at the right hand side.

The cytoplasmically located precursor and the translocated mature Mu-CSF both represent soluble proteins as suggested by their extractability with carbonate at pH 11 (Fig. 2, lanes *5* and *6*). Such a treatment opens the microsomal vesicles and releases all proteins that are not embedded in the membranes (Fujiki et al., 1982). From the results presented in Fig. 2, we conclude that Mu-CSF, as expected, behaves as a typical secretory protein despite its rather unusual signal peptide.

The Fusion Protein sMC-CAT Is Inserted into Microsomal Membranes

When sMC-CAT, consisting of the Mu-CSF signal peptide fused to the cytoplasmic protein CAT, was assayed in the in vitro translocation system in the presence of microsomes and SRP no proteolytically processed sMC-CAT could be observed (Fig. 3, lane *2*). Treatment with proteinase K revealed a low molecular mass doublet (*M*) that migrated close to the dye front (Fig. 3, lanes *3* and *4*). The upper of the two bands was digested by proteinase K in the presence of detergent, suggesting protection of this peptide by the membranes. Note that on the same type of gel no such low molecular weight

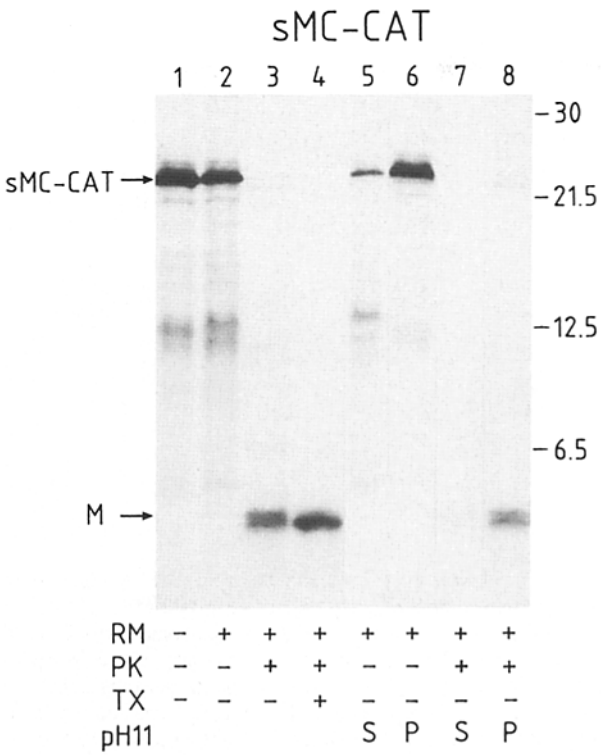

Figure 3. Translation and translocation of sMC-CAT. The experiment was done as described in Fig. 2 for Mu-CSF, except that [^{35}S]methionine was used as a label and an additional aliquot of protein translated in the presence of RM was treated with proteinase K (*PK*) before carbonate extraction at pH 11. Lanes *7* and *8* represent the soluble (*S*) or membrane-bound (*P*) fraction of the protease protected polypeptides. *M*, low relative molecular mass fragment resistant to protease digestion and cosedimenting with microsomal membranes.

material resistant to protease is observed for Mu-CSF, as shown in Fig. 2.

As the signal sequence of Mu-CSF is very hydrophobic, we suspected that in sMC-CAT it might be integrated in the membrane. To determine membrane association of sMC-CAT and peptide M, we analyzed their membrane association by subjecting microsomes to carbonate extraction at pH 11. The high pH treatment was carried out either before or after protease treatment. As shown in Fig. 3 (lanes *5* and *6*) the majority of the precursor protein sediments with the membranes, i.e., it behaves as an integral membrane protein. Fragment M also behaved as integral membrane protein in that it was not extractable by carbonate at pH 11 (Fig. 3, lanes *7* and *8*). Artefactual precipitation of sMC-CAT by the high pH treatment was excluded in a control experiment where microsomes were added after completion of translation; neither in the absence of microsomes nor upon their posttranslational addition did high pH treatment cause sedimentation of precursor sMC-CAT (data not shown).

From these data we conclude that no signal cleavage occurred and that the unusually long and apolar signal peptide anchored the sMC-CAT fusion protein in the microsomal membrane in a type I orientation. This is evidenced by the membrane integration of the low molecular mass peptide M that represents the NH_2-terminal hydrophobic segment of sMC-CAT. The COOH-terminal CAT sequences are exposed on the cytoplasmic side and thus accessible to protease digestion.

In Vitro Transcription, Translation, and Translocation of lMu-CSF and lMC-CAT

Plasmids plMu-CSF and plMC-CAT were transcribed as above and the resulting mRNAs translated in a wheat germ cell-free system supplemented with microsomes. Membrane topology was analyzed by the protease protection assay.

lMu-CSF. As shown in Fig. 4 (lane *1*), translation of lMu-CSF mRNA gave rise to a major band of ~22 kD. When microsomes and acceptor peptide blocking *N*-linked glycosylation were added to the wheat germ cell-free system containing [^{35}S]methionine no faster migrating band could be observed indicating that no signal cleavage had occurred (lane *2*). Protease treatment of the microsomes resulted in a protected polypeptide of ~6 kD (lane *3*, band *lM*). Protection of this peptide from proteolysis was mediated by the microsomes as the band disappeared when detergent was added to the reaction. The size of the lM peptide and its strong labeling with [^{35}S]methionine suggest that it represents the NH_2-terminal part of lMu-CSF as five of the six

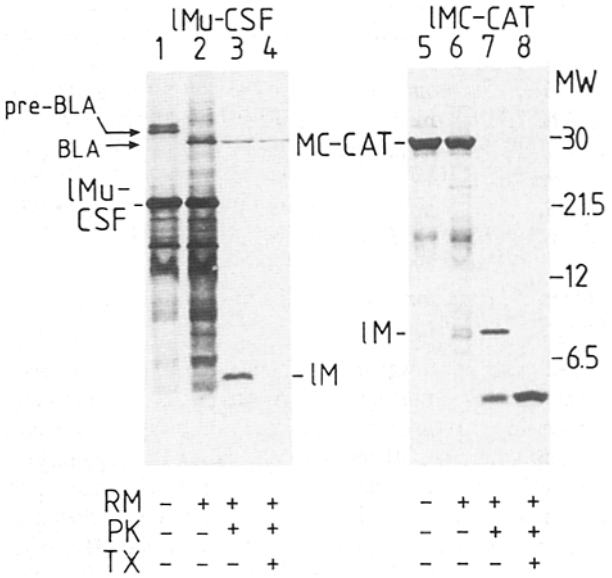

Figure 4. In vitro translation and membrane insertion of lMu-CSF and lMC-CAT. Messenger RNAs coding either for lMu-CSF (lanes *1–4*) or for lMC-CAT (lanes *5–8*) were translated in a wheat germ cell-free system supplemented with rough microsomes and acceptor peptide for *N*-linked glycosylation (*RM*, lanes *2–4* and *6–8*). Membrane translocation or insertion was analyzed for by a protection assay; after translation, microsomes were treated with proteinase K (*PK*) in the absence (lanes *3* and *7*) or presence (lanes *4* and *8*) of Triton X-100 (*TX*). Proteins were analyzed on a 22% polyacrylamide gel. The bands representing full-length lMu-CSF (~22 kD) and lMC-CAT (~30 kD) are indicated. *lM*, low molecular mass peptides protected from proteolysis. Precursor (*pre-BLA*) and mature β-lactamase (*BLA*) are also indicated as they provide a convenient internal control for the processing capacity of the RM used in this experiment. Relative molecular masses are given in kilodaltons.

methionines in lMu-CSF are within the NH_2-terminal 50 amino acid residues. The protein presumably exposes its major COOH-terminal portion to the cytoplasm and a small segment containing the NH_2 terminus to the microsomal lumen. As only one methionine residue is present in mature Mu-CSF we also used [^{3}H]leucine as a label. In addition to the band lM a small amount of lMu-CSF was found to be processed to the size of mature Mu-CSF (data not shown).

The 30-kD band in Fig. 4 (lanes *3* and *4*) represents β-lactamase that is also encoded on the pDS plasmids (Stueber et al., 1984). The efficient processing of pre-β-lactamase to β-lactamase serves as a convenient internal marker showing that the signal peptidase was active (Fig. 4, cf. lanes *1* and *2*).

lMC-CAT. lMC-CAT synthesized in the absence of microsomes migrated as a 30-kD protein (Fig. 4, lane *5*). When microsomes and acceptor peptide were added to the translation mixture the same size polypeptide was observed (lane *6*). Protease protection of lMC-CAT revealed a prominent low relative molecular mass band of 8 kD that was protected in the absence but not in the presence of detergent (Fig. 4, lane *7*, band *lM*). As judged by its size this band probably represents the NH_2 terminus of lMC-CAT inserted into microsomes in a type I orientation. The fact that for lMC-CAT the protected lM band is larger than for lMu-CSF could be explained by the availability of different cleavage sites for proteinase K on the two different COOH-terminal polypeptide portions (mature CSF vs. CAT).

The Fragment lM Arises from the NH_2-Terminal End of the lMC-CAT Precursor

The results described above suggest that the majority of the precursors of lMu-CSF and lMC-CAT have the hydrophilic NH_2 terminus translocated across the membrane; the hydrophobic segment serves as a membrane anchor and the COOH-terminal residues of the mature proteins remain in the cytoplasm and therefore accessible to proteinase K digestion.

To demonstrate that it is indeed a segment close to the NH_2 terminus that anchors lMC-CAT in the membrane we truncated the protein at the COOH-terminal end. If the NH_2 terminus of lMC-CAT were translocated across the membrane, the size of the protease-protected lM-fragment would be the same whether the COOH terminus was truncated or not. However, if it was the COOH terminus that was luminally exposed, then the size of the protease-protected lM fragment should decrease. Truncated polypeptides were obtained by translating the corresponding mRNA in the presence of a 20-mer oligodeoxynucleotide complementary to the mRNA sequence located 180 bp 5′ of the stop codon (see Bal I restriction site, Fig. 1 *B*). We previously had shown that oligodeoxynucleotides added to the wheat germ translation system give rise to COOH-terminally truncated peptides. Presumably a RNase H–like activity endogenous to the wheat germ lysate cuts the mRNA at the site of RNA/DNA hybrid formation (Haeuptle et al., 1986; Minshull and Hunt; 1986). Translocation of lMC-CAT mRNA in the presence of the Bal I oligodeoxynucleotide, should therefore shorten the resulting peptide by the COOH-terminal 60 amino acid residues giving rise to an ~19 kD polypeptide. Such a polypeptide was observed when truncated lMC-CAT (ΔlMC-CAT) was synthesized in the wheat germ cell-free system in

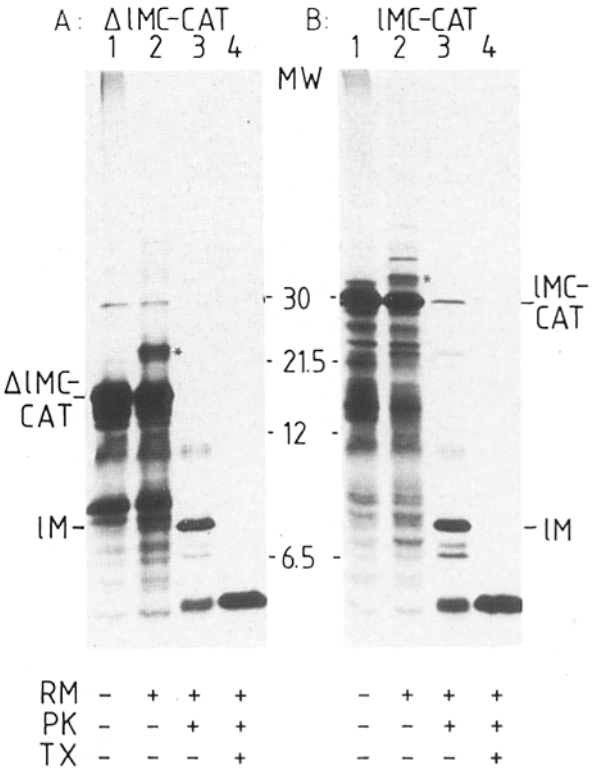

Figure 5. Membrane insertion of lMC-CAT that is truncated at its COOH-terminal end. Messenger RNA encoding lMC-CAT was translated either in the absence (*B*, lanes *1–4*) or presence (*A*, lanes *1–4*) of an oligodeoxynucleotide complementary to the mRNA sequences located 180 bp 5′ of the stop codon in the cat cDNA (*Bl* in Fig. 1 *B*). In the wheat germ system, DNA/RNA hybrids are cleaved by an endogenous RNase H–like endonuclease activity. The truncated mRNA codes for a polypeptide species shortened at its COOH-terminus by 60 amino acid residues. Translation of full-length lMC-CAT and its truncated form ΔlMC-CAT was performed in the absence (lanes *1*) or presence (lanes *2–4*) of rough microsomes (*RM*). Protein translocation and membrane insertion were analyzed for by posttranslational treatment of the microsomes with proteinase K (*PK*) in the absence or presence of detergent (*TX*). Proteins were analyzed on 22% polyacrylamide gels. *lM* shows the position of the low molecular mass, protease-protected band. The asterisks mark bands that represent *N*-glycosylated forms of membrane-inserted ΔlMC-CAT and lMC-CAT.

the absence or presence of membranes (Fig. 5 *A*, lanes *1* and *2*). After digestion with proteinase K the same 8-kD peptide was found protected for both full-length and truncated lMC-CAT (Fig. 5, *A* and *B*, lanes *3*, band *lM*). This demonstrates that the COOH terminus of the lMC-CAT polypeptides is exposed on the cytoplasmic side while the NH_2 terminus is on the exoplasmic side.

The NH_2-Terminal Hydrophilic Segment of lMC-CAT Is Glycosylated

The oligosaccharyl-transferase that transfers high mannose oligosaccharides onto asparagine residues of nascent polypeptides is known to be located in the ER lumen (Kornfeld and Kornfeld, 1985). Therefore, *N*-glycosylation is a convenient marker for luminal disposition of a protein or parts

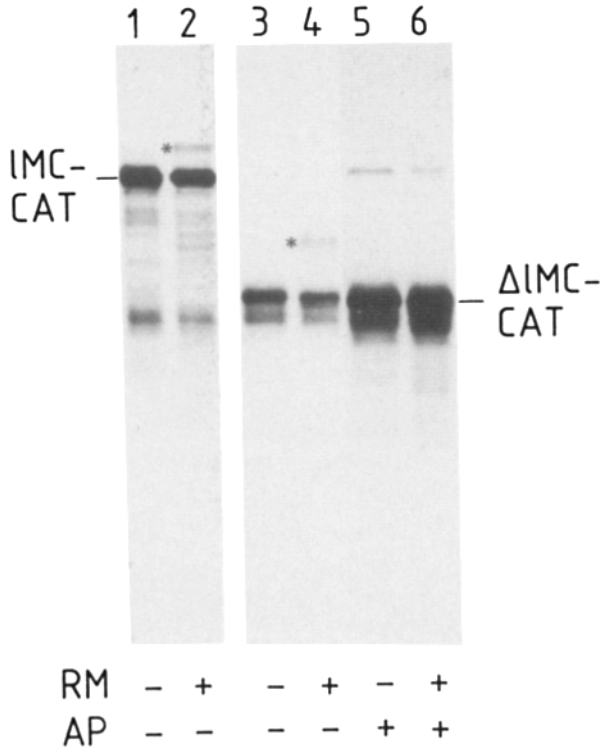

Figure 6. *N*-glycosylation of lMC-CAT and ΔlMC-CAT. lMC-CAT (lanes *1* and *2*) and ΔlMC-CAT (lanes *3–6*) are synthesized in the absence (lanes *1*, *3*, and *5*) or presence (lanes *2*, *4*, and *6*) of RM either without (lanes *1–4*) or with (lanes *5* and *6*) the addition of an acceptor peptide (*AP*) that inhibits *N*-glycosylation. All samples were immunoprecipitated with an anti-CAT antiserum and analyzed on a 22% polyacrylamide gel. Asterisks indicate the *N*-glycosylated bands as identified by their absence in those samples containing acceptor peptide.

of it. lMC-CAT contains a single potential site for *N*-glycosylation and this is located NH_2-terminally to the membrane spanning segment. When membrane translocation of lMC-CAT and of ΔlMC-CAT was analyzed, we observed a band ~3 kD larger than the precursor that only appeared upon translation in the presence of microsomes (Fig. 5, *A* and *B*, lane *2*; Fig. 6, lanes *2* and *4*, see bands marked with asterisks). The increase in relative molecular mass is consistent with the addition of one core oligosaccharide to the proteins.

To demonstrate glycosylation more clearly, transfer of *N*-linked core oligosaccharides to ΔlMC-CAT was inhibited by the acceptor peptide for *N*-glycosylation. Subsequently, newly synthesized polypeptides were immunoprecipitated with an anti-CAT antibody. The results are shown in Fig. 6. Only when ΔlMC-CAT was translated in the presence of microsomes but in the absence of the acceptor peptide was the higher relative molecular mass band representing glycosylated precursors detectable (lane *4* band marked by an asterisk). We conclude that lMC-CAT can be glycosylated and that its NH_2 terminus is translocated into the lumen of microsomal vesicles.

Fragments lM Are Membrane Integrated

Protease protection of the ~6- and 8-kD peptides lM suggested that most of the newly synthesized lMu-CSF and lMC-CAT is inserted into microsomal membranes in a type I orientation. To test membrane integration of the lM-fragments, we extracted the microsomes with carbonate at pH 11. Fig. 7 shows that most of lMu-CSF and lMC-CAT synthesized in the presence of microsomes cosedimented with the membranes (lanes *1*, *2*, *5*, and *6*). In control experiments (results not shown here) we confirmed that neither of the two precursor proteins could be sedimented when microsomes were omitted or added posttranslationally. After protease digestion, the protected fragments lM resisted extraction with carbonate and were recovered in the membrane pellet (lanes *3*, *4*, *7*, and *8*).

Discussion

A Cleavable Signal Sequence Can Become the Membrane-spanning Segment of an SA-Membrane Protein

We have demonstrated that the hydrophobic, cleavable signal sequence of Mu-CSF can direct SA-type membrane insertion when the flanking amino acid sequences are changed. Conversion is observed by changes at both the COOH- and the NH_2-terminal side of the hydrophobic segment. The results can be summarized as follows (Fig. 8). (*a*) sMu-CSF is a secretory protein with a rather long apolar signal sequence. When pre-sMu-CSF is translocated across microsomal membranes the apolar signal peptide of 27 residues is cleaved. This finding confirms previously published results where multi-CSF cDNA transfected into cells gave rise to

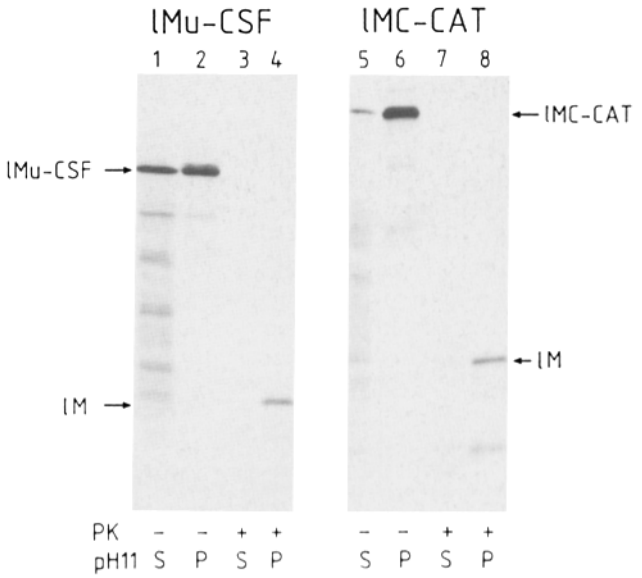

Figure 7. Resistance to membrane extraction at pH 11. After translation of lMu-CSF (lanes *1–4*) and lMC-CAT (lanes *5–8*) in the presence of rough microsomes (lanes *1–8*), some aliquots were digested with proteinase K (*PK*; lanes *3*, *4*, *7*, and *8*). All samples were then treated with carbonate at pH 11 and spun through a sucrose cushion. Carbonate-extractable material was recovered in the supernatants (*S*, lanes *1*, *3*, *5*, and *7*), membrane bound polypeptides was recovered in the pellets (*P*, lanes *2*, *4*, *6*, and *8*). *lM*, low relative molecular mass protease protected polypeptides.

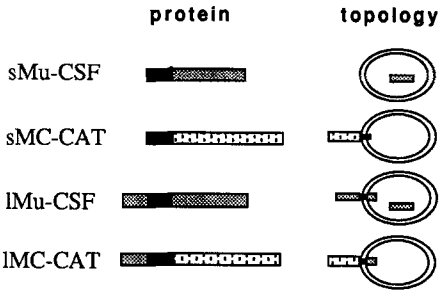

Figure 8. Diagrammatic presentation of the fusion proteins and their inferred membrane topology. The black boxes indicate the hydrophobic region of the fusion proteins. The NH_2 terminus is on the left and the COOH terminus on the right. The topology of the proteins in microsomal vesicles is indicated on the right hand side.

secreted and functional protein (Fung et al., 1984; Yokota et al., 1984). (*b*) Exchange of the mature portion of sMu-CSF for part of the cytoplasmic protein CAT resulted in a membrane spanning protein (sMC-CAT). Although the apolar signal sequence and its consensus cleavage site (−3 to −1) had not been modified by the construction no signal cleavage was observed for sMC-CAT. The protein spans the membrane in a type I orientation. (*c*) lMu-CSF has a hydrophilic extension at the NH_2-terminal side of the hydrophobic segment. It is membrane inserted in a type I orientation. A small proportion of lMu-CSF is translocated across microsomal membranes and processed by signal peptidase to authentic Mu-CSF. This was detected when [^{3}H]leucine was used as a label (data not shown). A secreted form of Mu-CSF was also found when lMu-CSF was expressed in cos cells (Dunn et al., 1985). Whether a membrane integrated form of lMu-CSF exists also in vivo has not yet been investigated. (*d*) Finally, the fusion protein lMC-CAT was inserted into membranes as a SA-membrane protein with a type I orientation.

From these results we conclude that modifications in the COOH- or NH_2-terminal sequences flanking a hydrophobic signal peptide can convert a secretory protein into a SA-membrane protein with type I orientation. These and previously published results (Lipp and Dobberstein, 1986*a*, 1988; Szczesna-Skorupa et al., 1988) strongly suggest that the topological signal for ER membrane insertion resides in three segments, the hydrophobic core of the signal sequence and the flanking hydrophilic amino acid residues.

Hydrophilic Sequences Flanking the Hydrophobic Core of a Signal Peptide Determine Cleavage by Signal Peptidase

lMu-CSF, sMC-CAT, and lMC-CAT represent proteins in which segments flanking the hydrophobic core of the Mu-CSF signal sequence were replaced by unrelated amino acid sequences. The proteins were inserted into the membrane but cleavage by signal peptidase did not or did only very inefficiently occur. This confirms our previous observation that the hydrophilic amino acid residues flanking the hydrophobic core of a signal sequence determine the site of cleavage by signal peptidase (Lipp and Dobberstein, 1986*a*, 1988). Previously a type II membrane protein was converted into a secretory protein by either removing the hydrophilic amino acid residues preceding the hydrophobic segment or changing the sequences adjacent to it. We conclude therefore that two parameters specify the site of signal peptidase cleavage. Firstly, potential cleavage sites can be defined by the −3, −1 rule of von Heijne (1983). This rule states that neutral amino acid residues are prevalent in positions −3 and −1 NH_2-terminal to a signal peptidase cleavage site. Aromatic, charged, and large polar residues are essentially absent from these positions. Usually many sites are found in a polypeptide that conform to this −3,−1 rule. The second parameter therefore might be the accessibility of one of these sites to signal peptidase. The results presented here and previously suggest that the actual cleavage site is selected by the hydrophilic sequences flanking the hydrophobic core of a signal sequence (Lipp and Dobberstein, 1986*a*, 1988). It is well-conceivable that in each polypeptide that is inserted into the membrane only a small segment is accessible to signal peptidase. If no potential cleavage site is found in this segment, cleavage can not occur and the polypeptide is either completely translocated across the membrane or inserted as a SA-membrane protein.

Membrane Orientation of a SA Segment Is Specified by the Hydrophilic Sequences Flanking an Apolar Segment

Hydrophobicity is certainly the most conspicuous feature in primary sequences known to be involved in targeting and topogenesis of membrane proteins. If this segment is deleted no membrane insertion occurs. In several approaches characterizing the signal(s) for membrane insertion hydrophobic segments were exchanged. They usually included some of the polar flanking sequences. The hydrophobic segment close to the NH_2 terminus of the transferrin receptor, a type II membrane protein, was exchanged for apolar segments from different origins. All the constructs, when transcribed and translated, resulted in type II membrane proteins (Zerial et al., 1987). In a different series of experiments each of the transmembrane segments of the multiple spanning membrane protein bovine opsin, including some of their polar flanking sequences, was individually placed behind the hydrophilic NH_2-terminus of the protein. The resulting fusion proteins were either membrane inserted in a type II orientation or entirely translocated to the microsomal lumen (Friedlander and Blobel, 1985; Audigier et al., 1987). Such experiments confirmed that hydrophobicity was essential for ER targeting and membrane insertion. However, they gave no clue as to what determines the final membrane orientation.

In our experiments all modifications were made precisely at the NH_2- or COOH-terminal end of the hydrophobic segment corresponding to the cleavable multi-CSF signal peptide. The resulting mutant proteins were either secreted or type I SA-membrane proteins. The question then arises as to why these proteins were inserted in a type I rather than type II orientation. Comparing SA-membrane proteins it appears that the imbalance of charged amino acid residues at the NH_2- and COOH-terminal side of the hydrophobic segment is the most conspicuous feature which distinguishes type I and type II SA-membrane proteins. Type II membrane proteins usually have positively charged amino acid residues at the NH_2-terminal side of the hydrophobic segment (von Heijne, 1986*a*). On the other hand type I membrane proteins

of the SA-type are characterized by the absence or low number of positively charged amino acid residues at the NH_2-terminal side of the hydrophobic segment. At present only a few SA-proteins of type I orientation are known (Sakaguchi et al., 1984; Williams and Lamb, 1986; High and Tanner, 1987). Cytochromes P-450, a family of microsomal membrane proteins, are type I SA-proteins. Their membrane insertion is dependent on signal recognition protein and docking protein (Sakaguchi et al., 1984). NH_2-terminal to the apolar segment there are either uncharged or acidic amino acid residues. Sakaguchi et al. (1987) found that a 29-amino acid long NH_2-terminal fragment of liver microsomal cytochrome P-450 containing the NH_2-terminal acidic residues was sufficient to promote membrane insertion when fused to the NH_2 terminus of interleukin-2 as a reporter protein. Szczesna-Skorupa et al. (1988) found that positive charges at the NH_2 terminus convert the type I membrane protein cytochrome P-450 either to a type II membrane protein or to a secretory protein. Von Heijne (1986*a*) observed that in bacterial inner membrane proteins basic residues are depleted in exoplasmic as compared to cytoplasmic domains.

All these results support the notion that negatively charged residues are more compatible with translocation than positively charged ones. The finding that the NH_2-terminal extension in lMu-CSF and lMC-CAT mainly confers type I membrane orientation supports this model. This NH_2-terminal extension contains a cluster of acidic residues adjacent to the apolar segment (Fig. 1 *C*). Comparison of sMu-CSF (secretory protein) with sMC-CAT (type I membrane protein) suggests that negatively charged residues on the NH_2-terminal flanking region of the hydrophobic segment are not a strict requirement for membrane insertion in the type I orientation. It is conceivable that an increase in, or redistribution of, positive charges at the COOH-terminal side of the hydrophobic segment can also lead to a SA type I membrane protein. A comparison of the sequence COOH-terminal to the signal sequence of sMu-CSF with that of sMC-CAT (Fig. 1 *C*) shows that the CAT portion contains more positively charged amino acid residues close to the hydrophobic segment than the mature Mu-CSF.

The effect of charged amino acid residues on the orientation of SA-membrane proteins needs further investigation. It is unclear which residues flanking the hydrophobic segment are relevant for determining the orientation in the membrane. In this respect it is interesting to mention that some of the signal-anchor segments in SA-membrane proteins are encoded by separate exons which include the charged amino acid residues on both sides of the hydrophobic segment (Koch et al., 1987). Besides an importance of the charged amino acid residues it is conceivable that the NH_2-terminal hydrophilic segments of SA-proteins require unfolding before translocation. These segments must be translocated posttranslationally as they precede the signal for membrane insertion. An unfolding step has been postulated for several types of proteins that are translocated posttranslationally (Eilers and Schatz, 1988; Park et al., 1988).

Functional Implications of the Conversion between Secretory and Membrane Proteins

As secretory and membrane proteins of opposite orientations can be converted to one another it is conceivable that such topological conversions are also found in the living cell. Indeed DNAs coding for the short and the long form of Mu-CSF were both isolated from cDNA libraries and, upon transfection studies, were shown to yield functional protein. sMu-CSF is the normally occurring, abundant species. The cDNA coding for lMu-CSF is most likely derived from a mRNA generated by the inclusion of an additional exon at the 5′ end of the canonical multi-CSF mRNA (N. Gough, unpublished observations). lMu-CSF mRNA could however not be traced by Northern blot analysis which leaves it unclear whether its expression is very low or nonexistent. Nevertheless, the fact that in an in vitro reconstituted system lMu-CSF displayed two topological phenotypes, i.e., a type I membrane protein and a low amount of mature, secretory protein, rendering speculations on in vivo implications challenging. Segregation of a single protein into different cellular compartments by the simple addition or deletion of peptide segments is not novel. Immunoglobulin heavy chains, for instance are known to exist as secretory and membrane-bound forms. These forms differ in a COOH-terminal hydrophobic segment that can anchor the heavy chain in the membrane (Alt et al., 1980). Such proteins are expressed from separate mRNAs. Could multi-CSF represent a case where two mRNAs differing at the 5′ end give rise to a secreted and a membrane protein? While the secretory form expresses the "normal" function of a colony stimulating factor we can only speculate about the potential function of a membrane bound form. One possibility would be autocrine regulation. Multi-CSF could gain access to the cytoplasm or nucleus of its producer cell by proteolytic release from the membrane-associated form. This is a particularly intriguing possibility in view of the highly significant homology on the amino acid level observed between a large region of multi-CSF and the x-lor gene product of HTLV-I, a transcriptional regulator (Gojobori et al., 1986).

In summary, our results suggest that the signal for anchoring SA-proteins in the membrane consists of a hydrophobic segment and its two hydrophilic flanking regions. Site directed mutagenesis of these flanking regions will allow further definition of the role that charged amino acid residues or folding play in determining the topology of SA-proteins in the membrane.

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