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# Deletion analysis of the internal signal-anchor domain of the human asialoglycoprotein receptor H1

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**The human asialoglycoprotein receptor H1 is a single-spanning membrane protein with the amino terminus facing the cytoplasm and the carboxy terminus exposed on the exoplasmic side of the plasma membrane. It has been shown earlier that the transmembrane segment, residues 38-65, functions as an internal signal directing protein synthesis to the endoplasmic reticulum and initiating membrane insertion. This process is co-translational and mediated by signal recognition particle (SRP). To identify subsegments within this region containing the signal information, we prepared deletion mutants at the level of the cDNA and analysed them in a wheat germ *in vitro* translation system with microsomes as the target membrane. Insertion and membrane anchoring were judged by the glycosylation of the protein, its resistance to exogenous protease and the extent to which it can be extracted from the microsomes by alkaline treatment. It was found that very small deletions already reduce the stability of membrane anchoring. However, nearly half of the transmembrane domain can be deleted, both from the amino-terminal and from the carboxy-terminal side, without completely abolishing membrane insertion. Several mutants, although not inserted, still interact with SRP. The results support the notion that the main feature of a signal sequence is a hydrophobic stretch of sufficient length (10-12 residues in our sequence), and indicate that recognition by SRP is not sufficient for membrane insertion.**

*Key words:* asialoglycoprotein receptor/internal signal sequence/leader peptide/signal recognition

## Introduction

Most secretory and plasma membrane proteins are targeted to the endoplasmic reticulum (ER) membrane by an amino-terminal signal or leader peptide (reviewed by Walter *et al.*, 1984; Wickner and Lodish, 1985). Cleavage of the leader sequence by signal peptidase on the luminal surface of the ER and anchoring by a hydrophobic 'stop transfer' segment near the carboxy terminus confers an N-out/C-in orientation to the majority of single-spanning membrane proteins studied (e.g. glycophorin, VSV G protein).

Several membrane proteins have been characterized which are inserted into the bilayer with the opposite N-in/C-out orientation (i.e. amino terminus cytoplasmic, carboxy terminus exoplasmic). None possesses a cleaved leader sequence at the amino terminus. By recombinant DNA techniques, it could be shown for several of them that their membrane-spanning segment provides the signal directing them to the ER. The transmembrane domains of influenza virus neuraminidase (Bos *et al.*, 1984), the asialoglycoprotein receptor (Spiess and Lodish, 1986), the transferrin receptor (Zerial *et al.*, 1986) and the HLA-DR invariant chain (Lipp and Dobberstein, 1986a,b), fused in front of sequences of normally cytoplasmic proteins are sufficient to target them to the ER, to initiate their translocation and to anchor them in the membrane.

Structurally, these sequences resemble the amino-terminal leader peptides in that they consist of a stretch of uncharged, hydrophobic amino acids following a hydrophilic, positively charged segment. However, with ~20 apolar amino acids, the hydrophobic segments are consistently longer than those found in leader peptides (8-12 residues; Watson, 1984; von Heijne, 1985). With only few exceptions (e.g. neuraminidase), they are preceded by a sizable hydrophilic domain of up to 60 amino acids and, therefore, are considered internal signal sequences.

The lack of primary structure homology between signal peptides (Watson, 1984) and the analysis of a large number of mutants (reviewed by Garoff, 1985) suggest that signal recognition is not based on sequence specificity. Recent work by several groups (Mize *et al.*, 1986; Kaiser *et al.*, 1987; Zerial *et al.*, 1987) has shown that unrelated hydrophobic sequences can functionally replace both amino-terminal leader peptides and internal signal/anchor sequences.

In this work, we have analysed the internal signal/anchor of the human asialoglycoprotein (ASGP) receptor (Ashwell and Harford, 1982; Schwartz, 1984). This hepatic cell surface protein mediates endocytosis of glycoproteins containing terminal galactose residues. Two different but homologous receptors, H1 and H2 (with 58% identical amino acids), have been cloned and sequenced (Spiess *et al.*, 1985; Spiess and Lodish, 1985). Previously, we have studied the membrane insertion of the receptor H1 in an *in vitro* transcription-translation system with dog pancreas microsomes as target membranes (Spiess and Lodish, 1986). Insertion was found to be co-translational and dependent on signal recognition particle (SRP). Deletion of the transmembrane domain abolished membrane insertion, while the sequences preceding and following this segment were dispensable. In fusion constructs with tubulin, it was shown that the membrane-spanning domain is sufficient to initiate translocation even of normally cytoplasmic sequences across the microsomal membrane.

Here, we have attempted to identify in detail the subsegments of this domain that are responsible for targeting the protein to the ER and for anchoring it in the membrane. By progressively deleting sequences from either end of the membrane-spanning segment, we have asked whether these two topogenic functions are encoded in separate or overlapping segments, and what distinguishes internal signal/anchor sequences from transient leader peptides.

## Results

### *In vitro translation and membrane insertion*

We have shown earlier (Spiess and Lodish, 1986) that residues 38-65 of the human ASGP receptor H1 (the boxed sequence in Figure 1A) are sufficient to target proteins to the ER and to initiate membrane insertion. Deletion mutants that lack portions

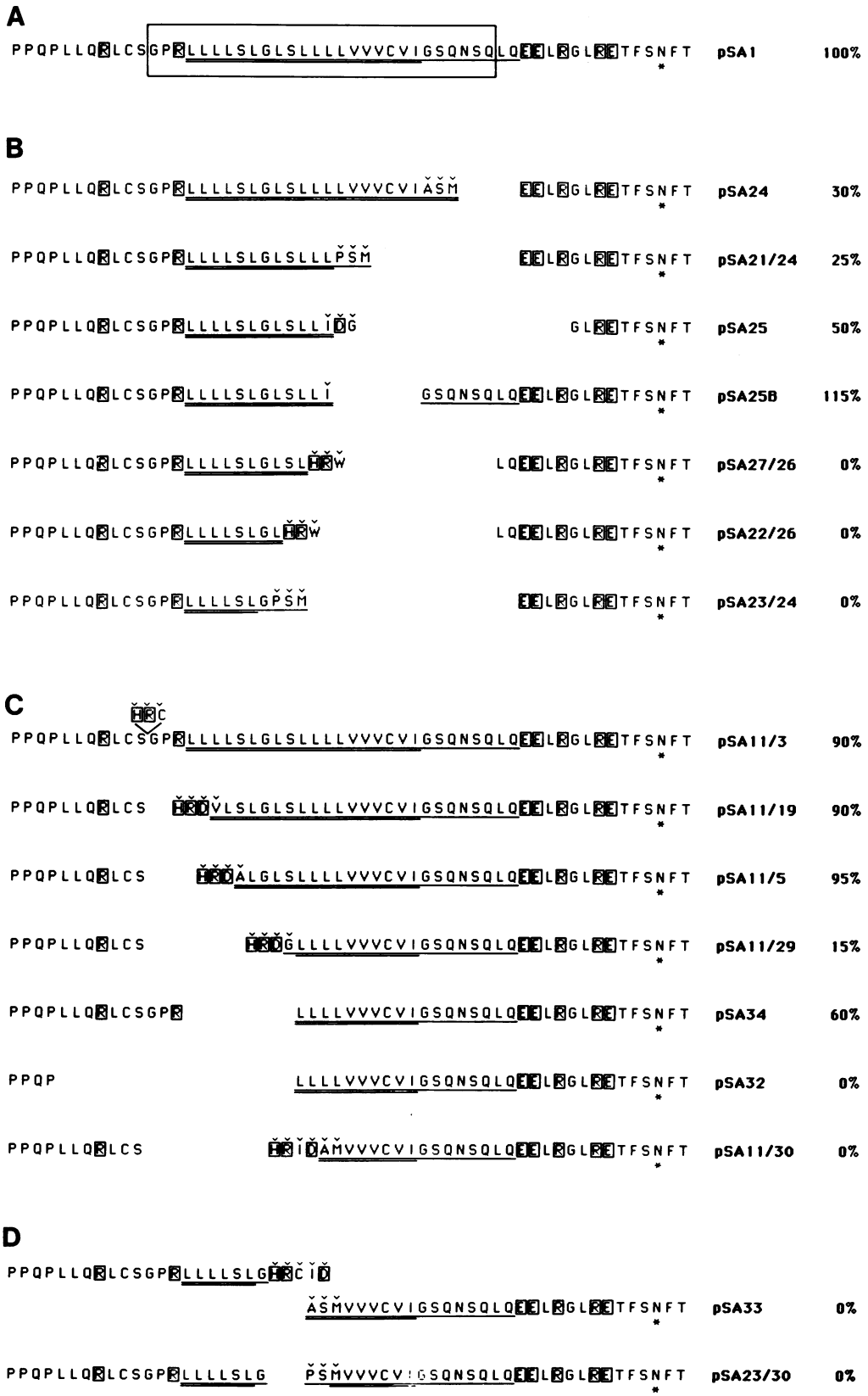


**Fig. 1.** Summary of the wild-type and mutant sequences of the internal signal–anchor of the ASGP receptor H1. Residues 27–81 of the wild-type ASGP receptor H1 (**A**) and the corresponding mutant sequences (**B–D**) are shown. The region of the wild-type sequence that is known to be sufficient for membrane insertion is framed (residues 38–65). The long stretch of uncharged amino acids in this domain is underlined, its hydrophobic sequence is underlined twice. Ile, Val, Leu, Phe, Cys, Met, and Ala are considered hydrophobic based on the hydropathy indices assigned by Kyte and Doolittle (1982). Interspersed uncharged residues were assumed not to interrupt a longer hydrophobic stretch. Charged residues are boxed, and the potential site for N-linked glycosylation (at position 79 in the wild-type protein) is indicated by an asterisk. New residues introduced by the insertion of linker sequences into the cDNA are marked by arrow heads. The efficiencies of membrane insertion (determined as described in the text and in Figure 3) are listed in percent (rounded to multiples of 5%) of that of the wild-type sequence.

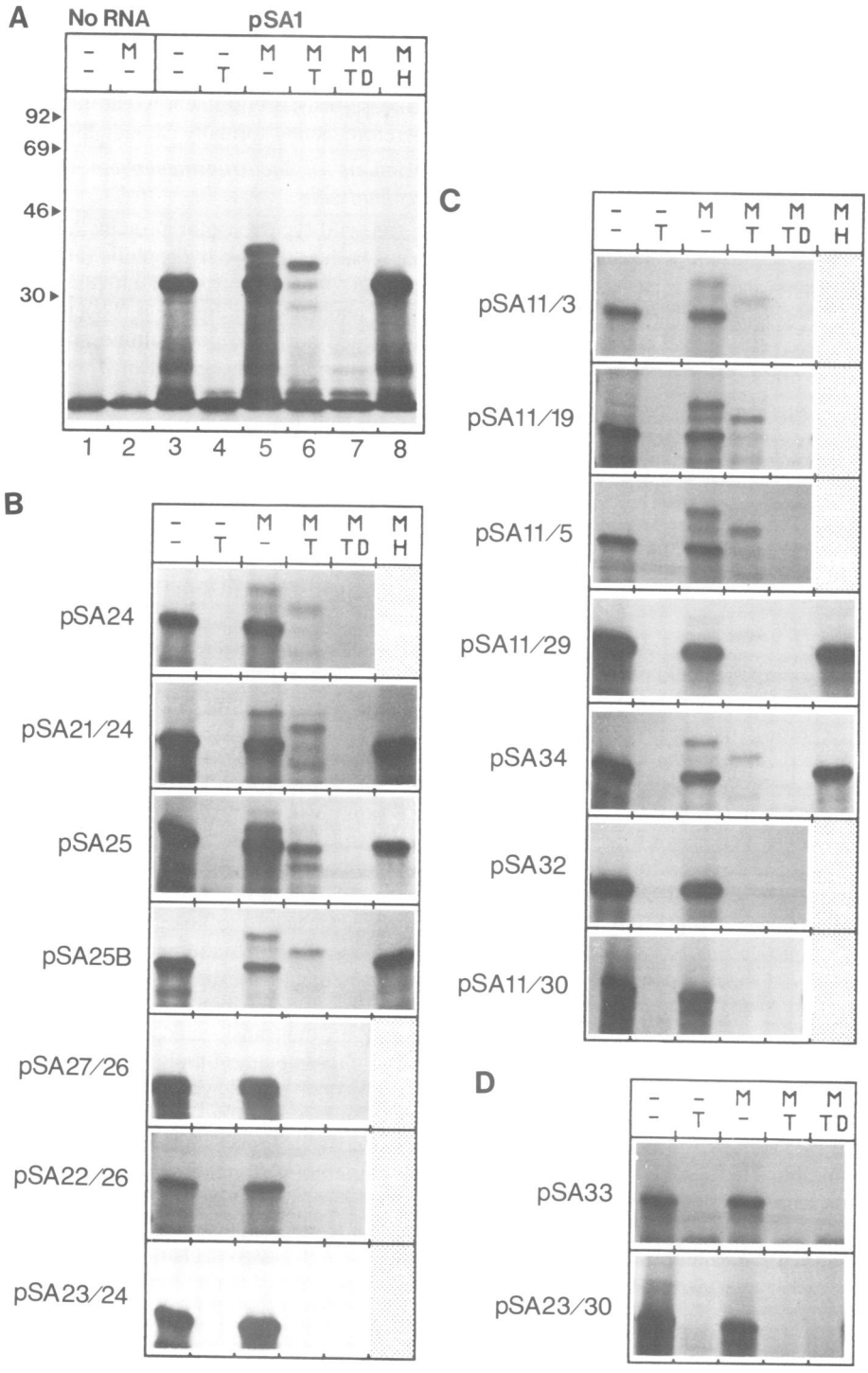


**Fig. 2.** *In vitro* translation and membrane insertion of wild-type and mutant ASGP receptor proteins. RNAs derived from the indicated plasmids were translated in a cell-free wheat germ translation system. Fluorographs of the translation mixture after SDS–PAGE are shown. M, microsomes present during translation; T, post-translational incubation with trypsin; D, detergent NP-40 present during trypsin digestion; H, Endo H digestion of the final products (gray areas: corresponding experiments not done). The position of marker proteins and their mol. wts in kd are indicated in **panel A**.

of this segment were prepared *in vitro* by linearizing the receptor cDNA either with *Bam*HI at codons 60/61 or with *Pst*I at codons 32/33 (i.e. on either side of the membrane-spanning domain) and digesting with exonuclease *Bal*31. *Cla*I linkers were inserted and the plasmids were re-ligated to the circular form. From a collection of such truncated cDNAs, suitable 5′ and 3′ portions, relative to the *Cla*I site, were combined to yield a series of constructs with progressive deletions from either side into the region coding for the transmembrane domain and with an open reading frame across the fusion point (see Materials and methods, Figure 6). The sequences of the resulting deletion proteins in the region of interest are listed in Figure 1.

To test these deletion constructs for their ability to be inserted into the ER membrane, an *in vitro* transcription–translation–insertion system was used. mRNA was transcribed from the cDNA by SP6 RNA polymerase (Melton and Krieg, 1984) and translated in a wheat germ cell-free translation system with [$^{35}$S]methionine in the absence or presence of dog pancreas

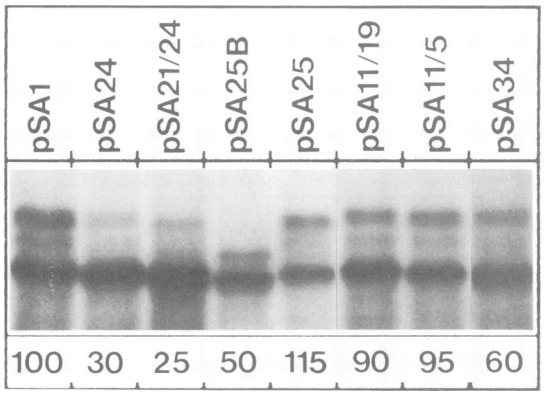


**Fig. 3.** Quantitation of insertion efficiency of mutant ASGP receptor proteins. Wild-type (pSA1) and mutant proteins were translated simultaneously in the presence of microsomes and analysed by SDS−PAGE and fluorography. By densitometrical scanning of several exposures of the fluorographs, the ratio of the glycosylated (and hence inserted) material to the total full-length protein synthesized was determined. This ratio, expressed in percent of that of the wild-type protein, was taken as the insertion efficiency. A representative experiment is shown with the insertion efficiencies (rounded to multiples of 5%) indicated below the lanes. The values for all the mutant constructs are listed in Figure 1.

microsomes. RNA prepared from the plasmid pSA1, which contains the wild-type cDNA, was used as the control (see Figure 2A, and Spiess and Lodish, 1986). In the absence of membranes, a 34-kd polypeptide is translated (Figure 2A, lane 3) which can be completely digested with trypsin (lane 4). In the presence of microsomes, a fraction of the protein synthesized (typically between 25 and 50%) migrates as two species of higher apparent mol. wt on SDS−PAGE (lane 5), a major form of 40 kd and a minor one of 37 kd. They correspond to polypeptide chains correctly inserted into the membrane, i.e. with the carboxy-terminal domain translocated into the lumen of the microsomes and glycosylated at one or both of the two potential sites for N-linked glycosylation (at positions 79 and 147), and with the amino terminus exposed on the outside of the microsomes. This conclusion is based on the following observations. By digestion with endo-$\beta$-N-acetylglucosaminidase H (Endo H), an enzyme that removes all but the first sugar residue of the N-linked oligosaccharide moiety added in the ER, the larger species are converted back to the 34-kd form (lane 8). The two glycosylated species are resistant to exogenous protease except for a portion of ~3 kd which corresponds to the size of the amino-terminal domain of the receptor (lane 6). The protease resistance is abolished when the microsomal membrane is disrupted with detergent (lane 7).

The deletion mutants were similarly analysed (Figure 2B−D). Their ability to be inserted into the ER membrane is judged from the fraction of the protein that is glycosylated and becomes protease resistant. Absolute insertion efficiencies varied somewhat from one experiment to the other, but the relative values within an experiment were constant. For this reason, the insertion efficiencies of the mutants were determined as a percentage of that of the wild-type receptor (pSA1) translated in the same experiment. A representative experiment for the quantitation of insertion is shown in Figure 3. The values obtained are listed in Figure 1. Zero percent insertion efficiency was assigned to those constructs for which no glycosylated proteins could be detected on fluorographs even after very long exposures.

Mize *et al.* (1986) have reported that the IgG heavy chain anchor domain in different fusion proteins could initiate translocation of both amino- and carboxy-terminal domains across the ER membrane. In our assay, mutant proteins inserted in the opposite orientation to that of the wild-type receptor would remain undetected, since they would not be glycosylated and the small protease-resistant fragment would lack methionine residues. We therefore cannot formally exclude this rather unlikely possibility.

*Deletions of the transmembrane segment from its carboxy-terminal side*

In the wild-type receptor protein, the transmembrane segment that separates the two hydrophilic (amino-terminal and carboxy-terminal) domains consists of a hydrophobic stretch of 19 residues (twice underlined in Figure 1). Together with the following eight uncharged but quite polar amino acids (once underlined), they form an uncharged segment of 27 residues that is flanked by a positively charged arginine residue on the amino-terminal side and by two negatively charged glutamic acid residues on the carboxy-terminal side. In a first deletion mutant, pSA24, the uncharged but polar octapeptide between these two negative charges and the hydrophobic stretch is deleted and replaced by the quite hydrophobic tripeptide Ala-Ser-Met, caused by the insertion of the *ClaI* linker into the cDNA (indicated by arrow heads in Figure 1). The hydrophobic stretch is thereby extended from 19 to 22 residues, while the uncharged segment is shortened from 27 to the same 22 amino acids. As is shown in Figure 2B, this mutant is still inserted into the microsomal membrane, although with an efficiency of only approximately one third of that of the wild-type.

Shortening the hydrophobic stretch to only 12 residues and insertion of the tripeptide Pro-Ser-Met in pSA21/24 retains about the same efficiency of insertion as for pSA24.

A surprising result is found for pSA25 which has a hydrophobic segment of the same length as that of pSA21/24, but lacks a few more polar amino acids including the two flanking glutamic acid residues; instead the linker sequence provides an aspartic acid residue to terminate the uncharged stretch. This mutant protein is inserted into the membranes more efficiently than the two preceding ones, about half as efficiently as the wild-type. However, the translocated protein appears as a single additional band whose apparent mol. wt is 3 kd higher than that of the unmodified polypeptide chain, suggesting that it is glycosylated only once. In fact, the glycosylation site at position 79 (indicated by an asterisk in Figure 1) is separated by only very few residues from the point of deletion. Probably, it is this site which is no longer glycosylated in pSA25, either because it is located so close to the membrane surface that it is sterically inaccessible for the transfer of the oligosaccharide precursor to the asparagine residue or because it is not recognized as a glycosylation site due to an altered conformation of the polypeptide in this region. Indeed, if the complete polar sequence, residues 60−71, of the wild-type protein is restored, as in pSA25B, the potential site at position 79 is used again and a twice glycosylated protein is produced, 6 kd larger than the unmodified polypeptide. Although the hydrophobic sequence is truncated to only 12 residues, this mutant protein is inserted into the microsomes very efficiently, even somewhat better than the wild-type receptor.

Upon shortening the apolar domain to 10, eight and six residues (mutants pSA27/26, pSA22/26 and pSA23/24, respectively), membrane insertion is lost. A hydrophobic segment of only six residues is extremely short, even for cleaved leader peptides. Apolar domains of 8−10 residues, however, are not unusual for amino-terminal signals (Watson, 1984), but very rarely are they immediately flanked by charged residues on both sides, as is the case in pSA27/26 and pSA22/26.

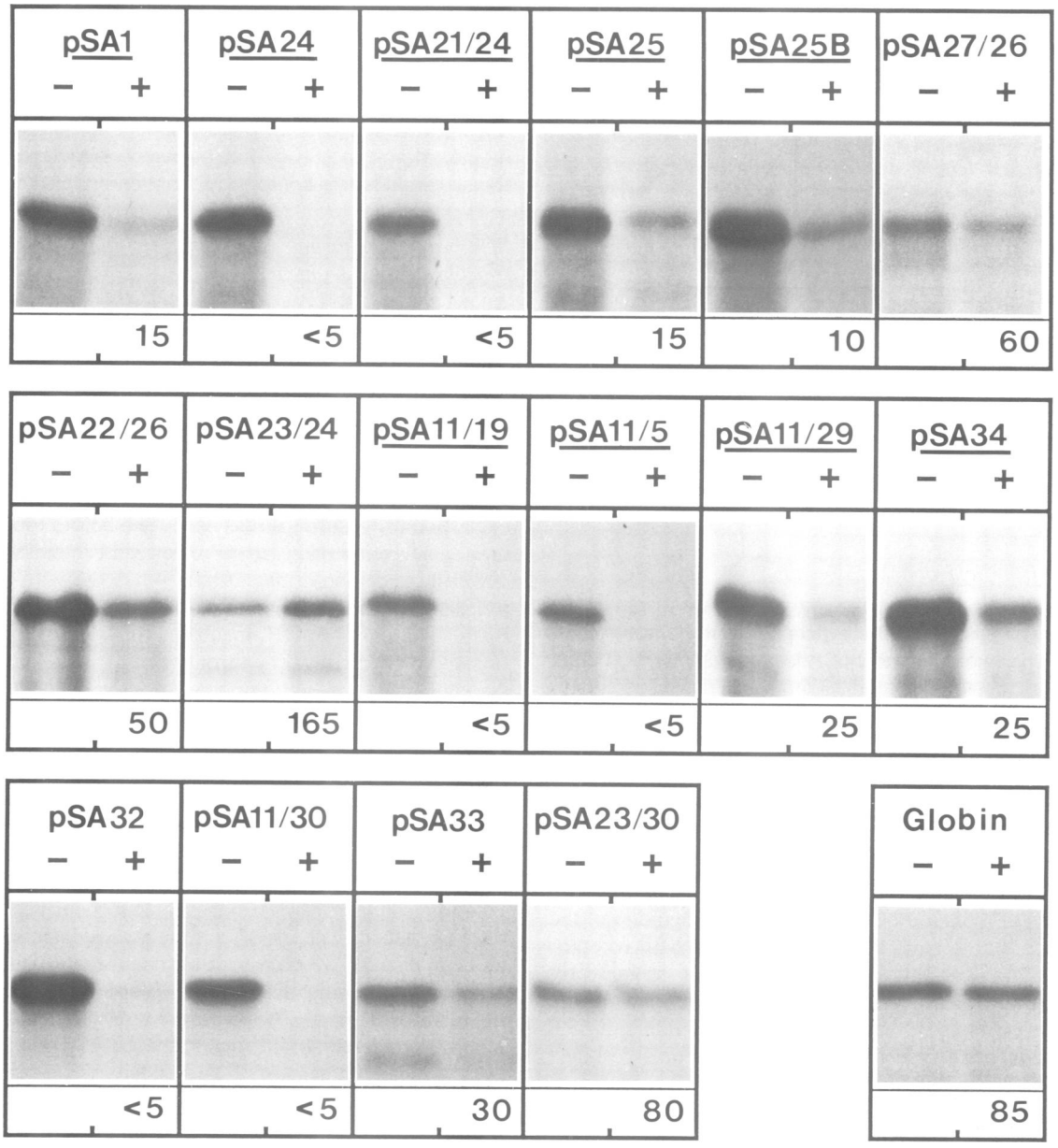


**Fig. 4.** Effect of SRP on the translation of wild-type and mutant ASGP receptor proteins in the absence of microsomes. RNA derived from the indicated plasmids were translated in the absence of microsomes without (−) and with (+) SRP. Fluorographs of the translation products after SDS−PAGE are shown. Synthesis of the full-size proteins was quantitated by scanning varying exposures of the autoradiographs. Protein synthesis in the presence of SRP is indicated in percent (rounded to multiples of 5) of that in the absence of SRP. Rabbit globin mRNA was translated in parallel as a negative control. The constructs that were found to be inserted into the membrane in the previous experiments (Figure 2) are underlined.

*Deletions of the transmembrane segment from its amino-terminal side*

In a second set of deletions (listed in Figure 1C and analysed in Figure 2C), the transmembrane segment of the receptor was shortened from its amino-terminal end. pSA11/3, which contains an insertion of the tripeptide His-Arg-Cys between residues 37 and 38, is inserted into the microsomes nearly as efficiently as the wild-type protein. This is not unexpected, since the inserted amino acids are still outside of the sequence that was shown to function as a signal if fused to tubulin. More surprising is the finding that also the deletion mutants pSA11/19 and pSA11/5 are inserted with equal efficiency. In these constructs the hydrophobic stretch is shortened by two and four residues, respectively, and the sequence Gly-Pro-Arg preceding it is changed to His-Arg-Asp, resulting in a negative charge immediately in front of the apolar domain. Only when five more amino acids are deleted, leaving a stretch of only 10 hydrophobic residues, in pSA11/29, is the insertion efficiency reduced 5- to 10-fold.

That the hydrophilic flanking sequence, nevertheless, has a dramatic influence on membrane insertion is evident from the comparison of pSA11/29, pSA34 and pSA32. All three have the same hydrophobic domain of 10 amino acids. Insertion of pSA34, which has the original amino-terminal flanking sequence, is approximately four times as efficient as that of pSA11/29, while pSA32 is not inserted at all. In this last construct, the hydrophobic segment comes to lie just adjacent to the proline-rich segment of residues 25−30, Pro-Pro-Pro-Pro-Gln-Pro. It is conceivable that this sequence assumes a conformation incompatible with any of the steps involved in membrane insertion (see below).

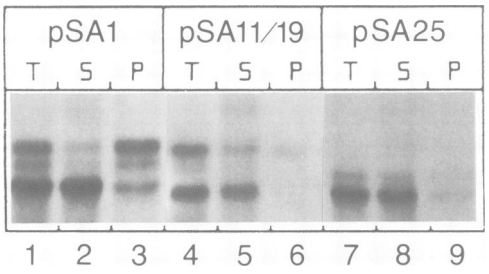


**Fig. 5.** Characterization of membrane anchoring by alkaline extraction. RNA derived from the indicated plasmids was translated in the presence of microsomes. The translation mixture was adjusted to pH 11, incubated on ice for 10 min, and then centrifuged through an alkaline sucrose cushion (as described in Materials and methods). The supernatant (S), the pellet (P) and an equivalent aliquot of the total translation mixture were analysed by SDS–PAGE and fluorography.

pSA11/30, a deletion construct with a hydrophobic segment of only eight residues, is not incorporated into the microsomal membrane either.

*Interruption of the hydrophobic domain*

Of the constructs described above, only those are inserted into the microsomes which possess a hydrophobic stretch of at least 10 residues. The two constructs pSA33 and pSA23/30 contain hydrophobic segments of 15 and 13 amino acids, respectively, which are, however, interrupted either by a cluster of charged residues (pSA33) or by polar but uncharged amino acids including a proline (Figures 1D and 2D). That neither of the two is inserted may suggest that the hydrophobic stretch must be continuous to initiate or to allow membrane insertion.

*Interaction of mutant proteins with SRP*

The assay described above and shown in Figure 2, analyses the overall process of membrane insertion, from recognition of the signal sequence by SRP and the 'docking' to the SRP receptor to the actual translocation process, which is still poorly understood. In the wheat germ *in vitro* translation system, it is possible to test the first step separately, because binding of SRP to the signal sequence arrests translation. Elongation is resumed only when the arrested complex binds to the SRP receptor on the ER membrane. In the absence of microsomes, translation arrest by SRP thus results in a dramatic decrease of the synthesis of the full-size protein in comparison to an identical translation without added SRP. It has been shown previously that the synthesis of the wild-type ASGP receptor, but not that of a mutant lacking the entire transmembrane segment, is strongly inhibited by SRP in the absence of membranes (Spiess and Lodish, 1986).

Figure 4 shows the effect of SRP on the translation of our deletion mutants. As expected, the wild-type protein and all the constructs that are inserted into the microsomal membrane (underlined in Figure 4) clearly interact with SRP and are efficiently arrested in translation, while the synthesis of globin remains nearly the same in the presence of SRP, as is expected for a cytoplasmic protein. However, there is no further correlation between the insertion efficiency and the susceptibility to translation arrest by SRP. The synthesis of some of the constructs that are not inserted into microsomes is only little affected (that of pSA23/24 even appears repeatedly to be somewhat stimulated, for reasons we do not understand). Other mutants, in particular pSA32 and pSA11/30, are strongly inhibited in translation by SRP. Clearly, SRP does recognize their signal sequences and binds to them. Their structures, thus, seem to interfere with one of the subsequent steps in membrane association and translocation.

Even with the constructs which strongly interact with SRP (including the wild-type receptor) at least 50% of the protein is not translocated and is completed as a soluble polypeptide outside of the microsomes. Coupling between signal recognition and translocation seems not to be as stringent in this cell-free system as it is *in vivo*.

*Membrane anchoring is affected by partial deletion of the transmembrane segment*

All the deletion proteins that are still inserted into the microsomes span the lipid bilayer in a manner similar to that of the wild-type receptor (Figure 2): the carboxy-terminal domain is translocated into the lumen of the microsomes and is inaccessible to externally added protease, while the amino-terminal domain is exposed on the outside and readily digested by protease. To characterize the association of the shortened transmembrane sequences of some of the deletion constructs, we used an alkaline extraction procedure that has been used before to analyse membrane integration of *in vitro* synthesized proteins (Fujiki *et al.*, 1982; D.Anderson *et al.*, 1983). When the wild-type receptor translated in the presence of microsomes is adjusted to pH 11 by the addition of NaOH, incubated for 10 min at 0°C and fractionated by centrifugation through an alkaline sucrose cushion, the unglycosylated 34-kd polypeptide is, as expected for a soluble protein, almost quantitatively recovered in the supernatant (Figure 5, lane 2). The two glycosylated species of 37 and 40 kd, on the other hand, are pelleted with the membranes (lane 3), confirming their stable integration in the lipid bilayer. In contrast, the glycosylated translation products of the mutants are clearly less stably anchored in the membrane. At least 50% of the translocated protein of pSA11/19, whose hydrophobic domain is shortened by only two residues, are reproducibly extracted into the supernatant (lanes 4–6). Of the glycosylated material derived from pSA25, which has a transmembrane anchor of only 12 hydrophobic amino acids, even more is released from the microsomes (lanes 7–9). To ensure stable association of the protein with the membrane under the harsh conditions used, a hydrophobic segment of ~19 residues, corresponding to the thickness of the apolar core of the lipid bilayer, seems to be critical.

## Discussion

*The membrane anchor*

Our results show that all the ASGP receptor mutants deleted within the signal/anchor domain that are inserted into the membrane assume a transmembrane topology, with the amino terminus exposed in the cytoplasm and the carboxy terminus translocated into the ER lumen, even if their hydrophobic domains consist of as few as 10 amino acids.

Previously, it has been shown for 'stop transfer' anchor domains that hydrophobic segments of 8–16 residues, either truncated natural anchor sequences (Adams and Rose, 1985a; Davis *et al.*, 1985) or artificial apolar sequences (Davis and Model, 1985), are sufficient to terminate translocation of the growing polypeptide across the membrane and to anchor the resulting protein in a transmembrane orientation. All these proteins as well as mutants with anchor sequences interrupted by a charged amino acid (Adams and Rose, 1985b; Cutler and Garoff, 1986; Cutler *et al.*, 1986) were found to be less stably integrated in the bilayer

than the corresponding wild-type proteins and were partially extractable under alkaline conditions.

Not unexpectedly, pSA25 with a hydrophobic segment of only 12 residues is also quite effectively extracted by alkaline treatment which does not affect the membrane association of the wild-type receptor. More surprising is the observation that the membrane integration even of pSA11/19 lacking only two residues of the apolar segment is significantly less stable. Cutler *et al.* (1986) showed that changing the charged residues flanking the anchor segment of the Semliki Forest virus E2 protein on the cytoplasmic face of the bilayer destabilized its membrane association. A similar situation may pertain to pSA11/19, since the flanking Arg of the wild-type receptor is changed to the cluster His-Arg-Asp. Alternatively, anchoring stability might be very sensitive to the length of the hydrophobic domain. Statistically, the length of the apolar stretch of the receptor H1, 19 residues, is the lower limit for transmembrane segments of either orientation in single-spanning membrane proteins (19–25 residues).

*The membrane insertion signal*

The length of the hydrophobic domain is also the most obvious difference between leader peptides and internal signal/anchor sequences. Our results show that all 19 hydrophobic residues of the transmembrane domain are not needed for membrane insertion: the apolar domain can be shortened to almost half its original length without abolishing insertion. In fact, one of the deletion constructs, pSA25B, with an apolar stretch of only 12 residues is inserted rather more efficiently than the wild-type receptor. These shortened sequences resemble amino-terminal leader peptides, except that they are located further within the polypeptide chain and lack a cleavage site for signal peptidase.

The hydrophobic segment can be shortened from either side without completely losing signal activity. This shows that the signal function does not reside specifically in the amino-terminal portion of the transmembrane domain that most resembles a leader peptide. The two deletions extending farthest from the amino- terminal (pSA25) and from the carboxy-terminal side of the membranous segment (pSA34) that are still inserted into microsomes have only two leucine residues (originally at positions 50 and 51) in common in the segment of amino acids 38–65. The two most extensive deletions that still strongly interact with SRP (pSA25 and pSA11/30) even have no sequence in common at all in this region. The signal information, therefore, is redundant and contained in both halves of the transmembrane domain. Our observations confirm earlier results which argue against sequence specificity in signal recognition, but rather suggest that a continuous hydrophobic peptide of sufficient length is essential. In our experiments, the shortest deletion mutants that are still inserted have apolar domains of 12 and 10 residues (pSA25 and pSA34, respectively), while a mutant with only eight hydrophobic residues (pSA11/30) is still bound by SRP but not inserted.

A large number of mutations in amino-terminal signals of both procaryotic and eucaryotic proteins have been analysed (reviewed by Garoff, 1985). Often elimination or persistance of signal activity correlates with the absence or presence of an uninterrupted hydrophobic segment of ~10 or more residues. The mutational analysis of the signal/anchor sequence of influenza virus neuraminidase by Sivasubramanian and Nayak (1987), who introduced three charges, singly and in combination, into the apolar domain, also confirms this concept with respect to membrane insertion. Furthermore, it has been shown by Mize *et al.* (1986)

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pSA24:     172 GTG ATC GCA TCG ATG GAG GAG 207
pSA21/24:  151 CTG CTG CCA TCG ATG GAG GAG 207
pSA25:     145 AGC CTC CTC ATC GAT GGG GGC 216
pSA25B:    145 AGC CTC CTC ATC GGA TCC CAA 186
pSA27/26:  145 AGC CTC CAT CGA TGG CTG CAG 201
pSA22/26:  139 GGC CTC CAT CGA TGG CTG CAG 201
pSA23/24:  136 CTG GGC CCA TCG ATG GAG GAG 207
pSA11/3:   106 TGC TCC CAT CGA TGC GGA CCT 117
pSA11/19:  106 TGC TCC CAT CGC GAT GTG CTC 132
pSA11/5:   106 TGC TCC CAT CGC GAT GCC CTG 138
pSA11/29:  106 TGC TCC CAT CGC GAT GGC CTC 150
pSA34:     112 GGA CCT CGC CTC CTG CTG 156
pSA32:      82 CCC CAG CCC CTC CTG CTG 156
pSA11/30:  106 TGC TCC CAT CGC ATC GAT GCG ATG GTG GTT 165
pSA33:     136 CTG GGC CAT CGA TGC ATC GAT GCA TCG ATG GTG GTT 165
pSA23/30:  136 CTG GGC CCA TCG ATG GTG GTT 165
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**Fig. 6.** The cDNA sequences of the ASGP receptor mutants in the altered regions. The nucleotide sequences of the mutant cDNAs are shown in the regions where they differ from the wild-type sequence. The numbers refer to the position of the first and the last nucleotide shown in the wild-type cDNA (with the numbering starting at the initiation codon; see Figure 2 in Spiess *et al.*, 1985). Underlined are the sequences introduced with the linkers. (pSA32 and pSA34 do not contain any new sequences; see text.)

that a 'stop transfer' sequence can function as an internal signal, and Zerial *et al.* (1987) demonstrated that foreign 'stop transfer' sequences as well as an artificial hydrophobic peptide can functionally replace the signal/anchor domain of the transferrin receptor. That probably any hydrophobic peptide of sufficient length is potentially recognized as a signal was also suggested by Kaiser *et al.* (1987). They showed that about one-fifth of essentially random sequences replacing the normal leader peptide of yeast invertase acted as functional translocation signals, and that these sequences are characteristically enriched in hydrophobic and depleted in charged residues.

Although hydrophobicity seems to be the main feature of the signal, the hydrophilic flanking sequences affect membrane insertion as well. This is particularly evident from constructs pSA11/29, pSA34 and pSA32, which have the identical apolar domain preceded by different hydrophilic sequences: pSA34 is inserted fairly efficiently, pSA11/29 just detectably and pSA32 not at all. The structural requirements for efficient insertion are not obvious. In pSA32 it is possibly the proline-rich segment (positions 25–30) that causes a conformation in the polypeptide incompatible with insertion. On the other hand, the positive charge preceding the hydrophobic domain in pSA1 does not seem to be essential, since pSA11/19 and pSA11/5 with a flanking aspartic acid residue are inserted almost as efficiently as the wild-type protein. However, the net charge of the first hydrophilic residues taken together is positive in all our constructs. The negative charges at the other end of the signal may be dispensable as well, since in many signal/anchor sequences the flanking carboxy-terminal charges are positive.

*Signal–SRP interaction*

The wheat germ system offers the possibility to analyse the first step in membrane insertion, the interaction of the signal with SRP, separated from the subsequent processes. Binding of SRP to the signal sequence in the absence of membranes causes translation of the nascent chain to arrest (Walter and Blobel, 1981). We find, as expected, that all signal mutants that can be inserted into microsomes are also arrested in translation by SRP. Of the mutants that are not inserted, at least two, pSA32 and pSA11/30,

interact strongly with SRP and are efficiently blocked in translation. It cannot be excluded that for initiation of translocation a second less redundant signal is necessary, which possibly overlaps with the SRP recognition sequence. But it seems more likely that the deletions in pSA32 and pSA11/30 created structures that, while still recognized by SRP, are incompatible with insertion. This could be caused by the new sequence at the point of deletion (and, in some cases, the insertion of linker sequences) or by bringing a normally more distant segment of the polypeptide (e.g. the proline-rich region) close to the functional domain of the signal. In any case, our results indicate that signal recognition by SRP alone is not sufficient to initiate membrane translocation.

From the available literature and our results, it becomes clear that the information content of ER signal sequences is very low. (A similar situation pertains to mitochondrial targeting sequences; Baker and Schatz, 1987.) This suggests that the high specificity of protein sorting to the ER and other organelles is not brought about only by these signals themselves. Accessability of the signal for interaction with SRP is also essential and depends on the structure of the rest of the polypeptide. Amino-terminal signals may often be accessible only early in translation and become hidden as the protein folds. The conformation of the amino-terminal domain preceding an internal signal is required to keep this sequence exposed. Furthermore, as is indicated by our insertion-incompetent mutants that nevertheless bind SRP, there are additional requirements beyond a recognition site for SRP that have to be fulfilled for successful membrane integration. Further experiments will be needed to elucidate the structural prerequisites for these processes.

## Materials and methods

*Materials*

SP6 RNA polymerase, staphylococcal nuclease, soybean trypsin inhibitor, exonuclease *Bal*31, endo-β-N-acetylglucosaminidase H (Endo H), and DNA sequencing reagents were from Boehringer Mannheim. Other modifying and restriction enzymes and linkers were purchased from Stehelin AG (Basel) and New England Biolabs. L-[$^{35}$S]methionine was from New England Nuclear. Wheat germ was provided by General Mills. TPCK-treated trypsin was purchased from Worthington Biochemicals, RNase inhibitor from Promega Biotech.

*Signal deletion constructs*

The techniques and conditions used for DNA manipulations were essentially as described by Maniatis *et al.* (1982).

To prepare deletions of the sequence encoding the membrane-spanning domain of the receptor H1, the cDNA was digested with exonuclease *Bal*31 either from the *Bam*HI site (at position 178) immediately downstream or from the *Pst*I site (at position 94) upstream of this segment. To this end, pSA1 was linearized with *Bam*HI, and pSA1-C (the 5′ portion of the cDNA up to nucleotide 195, containing a single *Pst*I site, cloned in the vector pSP64) was linearized with *Pst*I. Linear DNA (2 μg) was incubated with 1 U of nuclease *Bal*31 in 40 μl of 20 mM Tris-HCl, pH 7.2, 0.6 M NaCl, 12.5 mM $MgCl_2$, 1 mM EDTA, for 1–16 min at 30°C. The reaction was stopped by adding 25 mM EGTA and by ethanol precipitation. *Cla*I linkers (CATCGATG) were ligated to the DNA ends and digested with *Cla*I. The linear DNA was re-ligated to the circular form for transformation. By isolating and sequencing a number of these transformants, a collection of plasmids with varying deletions on either side of the segment encoding the membrane-spanning domain, all containing a *Cla*I site, was generated. The 3′ region of the cDNA (*Bam*HI to *Eco*RI) was re-inserted into the clones derived from pSA1-C.

By recombining the 5′ and 3′ portions (with respect to the *Cla*I site) of the deletion plasmids, new hybrid plasmids were created containing progressive deletions in the region of interest and an uninterrupted reading frame. These plasmids were named pSA # / #, with the numbers referring to the numbers of the original deletion clones from which the 5′ and the 3′ portions were derived, respectively. In some cases (pSA11/5, pSA11/19, pSA11/29) the *Cla*I site had to be cut with *Cla*I), filled in with the Klenow fragment of DNA polymerase I and re-ligated in order to obtain a continuous reading frame. For pSA11/30, the *Cla*I site was cut with *Cla*I and filled in with Klenow. Then, another *Cla*I linker (CATCGATG) was inserted. In pSA24 and pSA25, both the 5′ and the 3′ portions of the cDNA were derived from the same deletion clones which fortuitously had continuous reading frames. For pSA25B, the 5′ portion of pSA25 up to the *Cla*I site was ligated to the 3′ portion of pSA1 starting from the *Bam*HI site, after the unpaired ends of both had been filled in with Klenow. pSA32 and pSA34 are 'cloning artefacts' discovered by sequencing. pSA33 is another accidental clone generated when pSA23/30 was prepared: a nucleotide had been lost and three *Cla*I linkers had been inserted.

The sequences of all constructs were confirmed by sequencing the affected regions after subcloning into M13 by the Sanger 'dideoxy' procedure (Sanger *et al.*, 1977). Figure 6 shows the nucleotide sequence of all constructs in the region where they differ from the wild-type cDNA.

*In vitro transcription and translation*

For *in vitro* transcription, 2 μg of plasmid DNA linearized with *Eco*RI were incubated in 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 0.5 mM each of ATP, CTP, GTP and UTP, 10 mM dithiothreitol (DTT), 50 U RNasin, and 20 U of SP6 RNA polymerase, in a total volume of 50 μl for 1 h at 40°C. Nucleic acids (containing ~2–3 μg of RNA) were then precipitated with ethanol and re-dissolved in 25 μl of water.

Wheat germ extract for *in vitro* translation was prepared following the procedure of C.W.Anderson *et al.* (1983). Dog pancreas microsomes were isolated and column-washed according to Walter and Blobel (1983a). SRP and SRP-free (salt-washed) microsomes were prepared as described by Walter and Blobel (1983b) without additional purification on a sucrose gradient. Both the wheat germ extract and the microsomes were treated with staphylococcal nuclease (0.6 U/μl in 2 mM $CaCl_2$, reaction stopped by adding 4 mM EGTA) to remove endogenous RNA.

The translation reactions were performed as described by C.W.Anderson *et al.* (1983) for 90 min at 30°C, except that a potassium acetate concentration of only 40 mM was used, which improved the translation efficiency. Approximately 100 ng of RNA (1 μl of the preparation described above) and ~5 μCi [$^{35}$S]methionine were used in a standard 12.5 μl reaction. Samples were incubated for 90 min at 30°C. While the presence of normal microsome preparations inhibited the *in vitro* translation efficiency by at least a factor of 3, salt-washed microsomes did not inhibit. For translation experiments with membranes, therefore, salt-washed microsomes were used and supplemented with SRP in order to restore the insertion competence of the system. The concentrations of both components were titrated to yield maximal insertion efficiency for the wild-type asialoglycoprotein receptor derived from the plasmid pSA1 (0.25 Eq. of salt-washed microsomes, as defined by Walter and Blobel, 1983a,b, and 0.25 μl of SRP, as eluted from ω-aminopentyl agarose, per 12.5 μl reaction). For protease protection experiments, the reaction solutions were chilled on ice and incubated with 50 μg/ml of TPCK-treated trypsin for 1 h at 4°C. To permeabilize the microsomes in control samples, Nonidet P-40 was added to a final concentration of 0.5% together with the protease. Trypsin was inactivated by addition of soybean trypsin inhibitor to a final concentration of 200 μg/ml. Samples to be digested with Endo H were mixed with an equal volume of 0.3 M sodium-citrate, pH 6, 1% SDS, boiled for 2 min, incubated with 1 mU of Endo H per 25 μl for 1 h at 37°C.

To study the interaction of SRP with the nascent polypeptides, translations were performed as above but with 0.5 μl SRP per 12.5 μl reaction (i.e. twice as much as for an insertion experiment) and without microsomes.

To assess the strength of the interaction of proteins inserted into microsomes with the hydrophobic core of the membrane, the microsomes were extracted at alkaline pH as described by Gilmore and Blobel (1985) translation reactions were adjusted to pH 11 with 0.1 N NaOH, incubated for 10 min on ice, loaded onto a 100 μl sucrose cushion (0.2 M sucrose, 60 mM Hepes, pH 11, 150 mM potassium acetate, 2.5 mM magnesium acetate, 1 mM DTT) and centrifuged in a Beckman Airfuge using the A-100/30 rotor for 10 min at 30 p.s.i. After centrifugation the entire supernatant including the cushion was removed and the pellet resuspended in water.

For analysis by SDS–PAGE, all samples were precipitated with 10% trichloroacetic acid, re-dissolved in SDS-sample buffer and loaded onto 12.5% polyacrylamide gels (Laemmli, 1970). After electrophoresis, the gels were fixed, soaked in 1 M sodium salicylate, 1% glycerol, dried and fluorographed on pre-flashed Kodak X-Omat SB-282 film for 1–2 days at −70°C. For quantitation, fluorographs were scanned with a Camac TLC Scanner II equipped with an integrator.

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