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The amino-terminal structures that determine topological orientation of cytochrome P-450 in microsomal membrane

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We previously showed that the amino-terminal region of P-450 is responsible not only for targeting to endoplasmic reticulum (ER) membrane but also for stable anchoring to the membrane. In the present study, we introduced several mutations or deletions into the signal-anchor region of the chimeric proteins in which the amino-terminal regions of two forms of cytochrome P-450 were fused to the mature portion of interleukin 2. The amino-terminal acidic amino acid residues were replaced with basic amino acid residues or the hydrophobic core sequences were partially deleted, and these mutant proteins were assayed *in vitro* for their capacity to be inserted into or translocated across the ER membrane. The proteins that received the former manipulations were processed and the IL-2 portion was translocated across the membrane. In one case, the processing did not occur, thereby enabling the chimeric protein to anchor on the luminal side of the ER. Those that received the latter manipulation were also processed and the IL-2 portion translocated across the ER. These results strongly suggest that the signal-anchor function is determined both by the amino-terminal charged amino acid residues and by the length of the hydrophobic stretch.

Key words: cytochrome P-450/membrane insertion/membrane biogenesis/microsomes/signal-anchor sequence

Introduction

Proteins of the endocytotic–exocytotic pathways are first translocated across or inserted into endoplasmic reticulum (ER) membrane by a mechanism that involves the signal sequences of the proteins, signal recognition particle (SRP), and docking protein (Walter *et al.*, 1981; Gilmore *et al.*, 1982; Meyer *et al.*, 1982). The membrane proteins that are targeted to ER and span the membrane once are categorized into those with cleavable signal sequences and those with uncleavable signal sequences. The former have a stop-transfer sequence, separately from the signal sequence, in the molecule. Such proteins are inserted into the membrane with type I orientation (Garoff, 1985) exposing their amino-terminal portion in the lumen, and carboxy-terminal portion in the cytoplasm. VSV-G protein (Lingappa *et al.*, 1978), UDP-gluculonyl transferase (McKenzie, 1986), insulin receptor (Ebina *et al.*, 1985) and M6P receptors (Morgan *et al.*, 1987) are the examples.

On the other hand, the protein categorized into the latter have combined ER-targeting and stop-transfer sequences, signal-anchor sequences (Markoff *et al.*, 1984; Lipp and Dobberstein, 1986a; Schmid and Spiess, 1988) usually at or near the N-terminal portion, and inserted into the membrane with their C-termini exposed either to the cytoplasm (type I) or to the lumen of the ER (type II) (Garoff, 1985). P-450 (Sakaguchi *et al.*, 1987; Nelson and Strobel, 1988; Monier *et al.*, 1988) and probably NADPH-cytochrome P-450 reductase (Black and Coon, 1982) and epoxide hydrolase (Dubois *et al.*, 1979) belong to the former. The invariant chain of class II histocompatibility antigens (Lipp and Dobberstein, 1986b), the transferrin receptor (Schneider *et al.*, 1984), neuraminidase (Bos *et al.*, 1984) and the asialoglycoprotein receptor (Spiess and Lodish, 1986) belong to the latter.

How, then, is the membrane topology of those proteins with signal-anchor sequences determined? Proteins with uncleavable signal sequences are translocated across the membrane but would be released to the lumen of the ER if the hydrophobicity of the signal sequences are low. If the hydrophobicity of the signal sequence is sufficiently high on the other hand, the translocated segments would remain bound to the luminal side of the membrane, thus assuming type II topology.

It has been shown that the function of type II signal-anchor sequence is influenced by the N-terminal flanking sequence adjacent to the signal-anchor sequence and hence, upon the deletion of the cytoplasmic flanking region, the type II signal-anchor sequence is converted to a signal sequence (Lipp and Dobberstein, 1986; Schmid and Spiess, 1988). It has also been reported that the type I signal-anchor sequence of P-450 IIC2 is converted to the secretory signal sequence after the replacment of negatively charged amino acid residues at the N-terminal portion with positively charged ones (Szczesna-Skorupa *et al.*, 1988; Szczesna-Skorupa and Kemper, 1989).

Haeuptle *et al.* (1989) have shown that otherwise secretory cleavable signal sequence of the colony stimulating factor (CSF) is influenced by the regions NH_2- and COOH-terminally flanking the signal and converted to a type II signal-anchor sequence when the flanking regions are modified.

To learn more about the structural characteristics that determine the orientation of the type I or type II proteins with signal-anchor sequences, we chose, in the present study, two microsomal P-450 species, rabbit P-450 LM6, a methylcholanthrene-inducible form (Kagawa *et al.*, 1987) and rat P-450(M-1), a constitutively expressing male specific form (Matsumoto *et al.*, 1986). Chimeric proteins, in which the signal-anchor sequences of the P-450s (29 amino acid residues for the former and 21 amino acid residues for the latter) were fused to mature interleukin 2, were introduced with various mutations and deletions in the signal-anchor segments by the manipulation of their cDNAs. The cDNAs were expressed in the transcription–translation system and

the products were analyzed for integration or translocation into dog pancreas microsomes.

We found that the type I signal-anchor sequences of the P-450s were converted to secretory signal sequences or type II signal-anchor sequences either upon replacement of the amino-terminal acidic amino acid residues with basic amino acid residues or upon partial deletions of the hydrophobic core of the signal-anchor sequence. We therefore conclude that the hydrophobicity of the hydrophobic core sequence as well as the charge at the amino-terminus of type I signal-anchor sequences are the major factors that determine the function of the sequence.

Results

Effect of positive charge introduction to P-450 LM6 signal-anchor sequence

As we have already shown, the amino-terminal region consisting of <29 amino acid residues of P-450 LM6 functions both as the signal sequence and as the stop-transfer sequence (Sakaguchi *et al.*, 1987). By the presence of this signal-anchor sequence at the amino-terminus, P-450 LM6 should assume the type I topology in the microsomal membrane (type I signal-anchor sequence). The N-terminal regions of two other species of cytochrome P-450 are also reported to have similar functions (Monier *et al.*, 1988; Szczesna-Skorupa *et al.*, 1988).

When the signal-anchor regions of microsomal P-450s are compared with typical eukaryotic secretory signal sequences, two prominent differences should be noted. One is the lack of a positively charged amino acid residue preceding the hydrophobic core sequence. Almost all hydrophobic core sequences of eukaryotic signal peptides, on the other hand, are preceded usually by a positively charged amino acid residue (von Heijne, 1984). The other is that the hydrophobic core sequences of the signal-anchor regions are longer than those of secretory signal sequences (see Table I).

We therefore addressed a question of whether these signal-anchor sequences are converted to translocation signal sequences upon changing these parameters. As shown in Table I, the signal-anchor sequence of P-450 LM6 has two repeated stretches of uncharged amino acid residues and they are both preceded by a negatively charged amino acid residue, Asp4 and Glu15, respectively. We first converted these negatively charged amino acids to positively charged ones (Figure 1A) and examined the insertion of modified chimeric proteins into ER membrane. The results are shown in Figure 2. When the mRNA coding the original chimeric protein, LM6[N29]/IL2, was translated in the presence of RM, no processing was observed (compare lanes 1 and 2 in Figure 2) and the protein was digested by externally added trypsin both in the absence (lane 3) and presence (lane 4) of Triton X-100. This result confirms our previous experiment (Sakaguchi *et al.*, 1987). LM6[N29]/IL2 translated in the presence of RM was tightly associated with the membrane and not extractable with 50 mM Na_2CO_3 (pH 11) (Sakaguchi *et al.*, 1987).

When the mRNA for LM6[N29](++/−)/IL2 in which Arg and Lys were introduced in place of Asp4 and Phe5 respectively, was translated in the presence of RM processing of the mutated fusion protein occurred (lane 6, arrow head). The processed form was resistant to externally added trypsin (lane 7, arrow head), but not in the presence of 1% Triton X-100. It should be noted that the apparent mobility of the processed band corresponded to that of mature IL2 (lane 18), suggesting that the newly formed secretory signal sequence of the fusion protein was cleaved by signal peptidase at the same point as that of pre-IL2.

It is thus clear that upon replacement of a negatively charged amino acid residue by two positively charged amino acid residues, the signal-anchor sequence was converted to a secretion signal. Moreover, when only one positively charged amino acid residue was introduced by replacing Asp4 with Lys, the modified chimeric protein, LM6[N29](+/−)/IL2, was also processed by RM (lane 10, arrow head). The processed band was resistant to trypsin digestion (lane 11, arrow head), although some portion of the unprocessed chimeric protein is visible in lane 11. Apparently, the replacement of the negatively charged amino acid by a positively charged amino acid is sufficient for changing the function of the signal-anchor sequence of P-450 LM6.

The structural feature of the signal-anchor region of P-450 LM6 is somewhat different from those of other microsomal P-450 species in that the long hydrophobic core sequence is interrupted by Glu15. We therefore, constructed LM6[N29](−/++)/IL2 where Thr14 and Glu15 were both replaced by Arg, and its co-translational interaction with RM was examined as above. As shown in lanes 12−14 in Figure 2, LM6[N29](−/++)/IL2 was also processed and

Table I. Comparison of the amino-terminal sequences of microsomal P-450s

	Microsomal cytochrome P-450s	References
	− + + +	
a	M L D T G L L L V V I L A S L S V M L L V S L W Q Q K I R G R L P P G P T P L P	Nagata *et al.* (1987)
	− + + +	
b	M E P S I L L L L A L L V G F L L L L V R G H P K S R G N F P P G P R P L P L L	Nelson and Strobel (1988)
	− + + +	
c	M P S V Y G F P A F T S A T E L L L A V T T F C L G F W V V R V T R T W V P K G	Nelson and Strobel (1988)
	− − + + +	
LM6	M V S D F G L P T F I S A T E L L L A S A V F C L V F W V A G A S K P R V P K G	Kagawa *et al.* (1987)
	− + + +	
M-1	M D P V L V L V L T L S S L L L L S L W R N S P G R G K L P P G P T P L P I I G	Yoshioka *et al.* (1987)
	1 10 20 30 40	

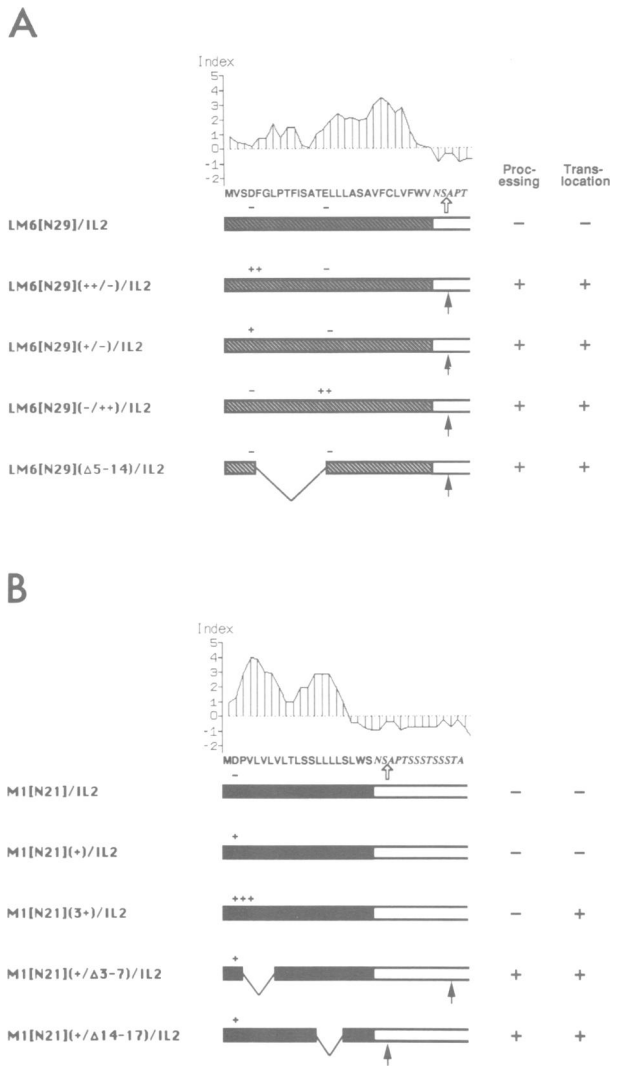

Fig. 1. (**A**) Schematic representation of LM6[N29]/IL2 and its mutant proteins. LM6[N29]/IL2 is a chimeric protein in which the signal peptide portion of pre-IL2 was replaced by the N-terminal 29 amino acid residues of P-450 LM6 leaving authentic signal peptidase cleavage site of pre-IL2. The mutant proteins were constructed by manipulation of LM6[N29]/IL2 cDNA as described in Materials and methods. + and − above the boxes represent positively and negatively charged amino acid residues, respectively. Hatched boxes and open boxes represent regions originated from P-450 LM6 and the mature portion of IL2, respectively. Putative signal peptidase cleavage sites are shown by arrows and the authentic signal peptidase cleavage site of pre-IL2 is shown by an open arrow. (**B**) Schematic representation of M1[N21]/IL2 and its mutant proteins. M1[N21]/IL2 was constructed by replacing the signal peptide portion of pre-IL2 by the N-terminal 20 amino acid residues of P-450(M-1) plus one amino acid insertion and authentic signal peptidase cleavage site is retained. The mutant proteins were constructed from M1[N21]/IL2 cDNA. Symbols in the figure are the same as in (A) except that filled boxes represent regions originated from P-450(M-1). The hydropathy plots of LM6[N29]/IL2 and M1[N21]/IL2 are shown above their amino acid sequences, respectively.

the mature IL2 translocated the membrane. Though the efficiency of processing as well as that of translocation of LM6[N29](−/++)/IL2 was rather low in this particular experiment, it was possibly due to the variances among experiments. Almost the same translocation efficiency as other variants was observed in a repeated experiment. We therefore conclude that LM6[N29](−/++)/IL2 was

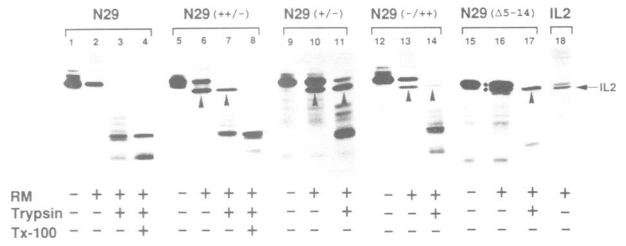

Fig. 2. Translocation of LM6[N29]/IL2 and its mutant proteins synthesized *in vitro* across microsomal membranes. cDNAs coding for the chimeric proteins and pre-IL2 were transcribed by SP6 RNA polymerase, and the mRNAs were translated in the wheat-germ cell-free system in the absence (−) (lanes 1, 5, 9, 12 and 15) or presence (+) (lanes 2, 6, 10, 13, 16 and 18) of dog pancreas microsomes (RM). Aliquots of the translation mixtures were digested with trypsin in the absence (lanes 3, 7, 11, 14 and 17) or presence (lanes 4 and 8) of 1% Triton X-100. Total reaction mixtures were directly subjected to SDS−PAGE and the protein bands were visualized by fluorography. N29, N29(++/−), N29(+/−), N29(−/++), and N29(Δ5−14) indicate LM6[N29]/IL2, LM6[N29](++/−)/IL2, LM6[N29](+/−)/IL2, LM6[N29](−/++)/IL2, and LM6[N29](Δ5−14)/IL2, respectively.

processed and translocated as efficiently as other mutant proteins.

Effect of the length of hydrophobic core sequence on the function of P-450 LM6 signal-anchor sequence

As already described, microsomal P-450s have longer hydrophobic amino acid stretches at their amino-terminal regions (see Table I and also Nelson and Strobel, 1988). We addressed a question if these longer hydrophobic stretches are responsible for the signal-anchor function. To test this possibility, we constructed LM6[N29](Δ5−14)/IL2, a deletion mutant of LM6[N29]/IL2, in which amino acid residues 5−14 were deleted. It should be notified that this manipulation resulted in putting Asp4 and Glu15 together and placing them just in front of the shortened hydrophobic stretch.

Unexpectedly, this deletion mutant was processed by RM (lane 16 in Figure 2) and the mature portion, which showed the same mobility as mature IL2, was translocated across the membrane as revealed by trypsin resistancy (lane 17, arrow head). The observation indicates that a hydrophobic core sequence preceded by negatively charged amino acid residues can function as a translocation signal if the length of the hydrophobic core sequence is shorter than some threshold.

Similar experiments were performed with LM6[N20]/IL2, a chimeric protein consisting of the amino-terminal 20 amino acid residues of P-450 LM6 and mature IL2. Although this chimeric protein had almost the same length of the amino-terminal portion of P-450 LM6 as LM6[N29](Δ5−14)/IL2, it could not bind to the membrane of RM as described in the previous paper (Sakaguchi *et al.*, 1987).

Although LM6[N29](Δ5−14)/IL2 and LM6[N20](++/−)/IL2 contain almost the same length of the amino-terminal portion of P-450 LM6, the hydropathic indices (Kyte and Doolittle, 1982) are clearly different. The index of the former is 1.0 and that of the latter is 0.2, indicating that the hydrophobicity, in addition to the length of the hydrophobic core sequence, is an important factor for the function of their amino-terminal portions as secretory signal. As it is interesting to know if the signal and signal-anchor

Table II. Effect of SRP on the translation of LM6[N20]/IL2, LM6[N29]/IL2 and its mutant proteins

Proteins	Inhibition of translation (%)
LM6[N20]/IL2	50
LM6[N29]/IL2	74
LM6[N29](++/−)/IL2	87
LM[N29](Δ5−14)	51
Porin	−3

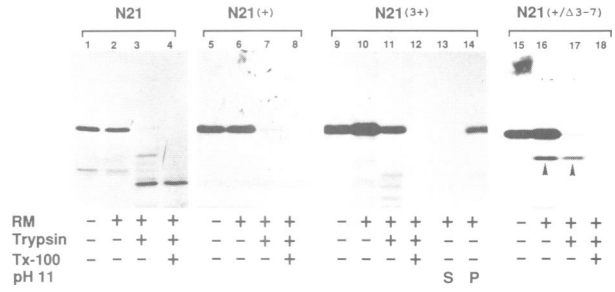

Fig. 3. *In vitro* translocation of M1[N21]/IL2 and the mutant proteins across microsomal membranes. cDNAs coding for the chimeric proteins were transcribed *in vitro* and the mRNAs were translated in the wheat-germ cell-free system in the absence (−) (lanes 1, 5, 9 and 15) or presence (+) (lanes 2, 6, 10, 13, 14 and 16) of dog pancreas microsomes (RM). Aliquots of the translation mixtures were digested with trypsin in the absence (lanes 3, 7, 11 and 17) or presence (lanes 4, 8, 12 and 18) of 1% Triton X-100. In the case of M1[N21](3+)/IL2 chimeric protein, an aliquot of the translation mixture containing the microsomes was treated with 50 mM Na_2CO_3 (pH 11.1) and centrifuged to separate it into supernatant (S) and membrane (P) fractions (lanes 13 and 14). The samples were subjected to SDS−PAGE followed by fluorography. N21, N21(+), N21(3+) and N21(+/Δ3−7) indicate M1[N21]/IL2, M1[N21](+)/IL2, M1[N21](3+)/IL2 and M1[N21](+/Δ3−7)/IL2, respectively. Other conditions are the same as in Figure 2.

sequences are recognized by SRP to different extents, we examined the SRP-dependent translation arrest (Walter *et al.*, 1981) with LM6−IL2 chimeric proteins. As shown in Table II, all the chimeric proteins examined were more or less recognized by SRP. However, porin, the mitochondrial major outer membrane protein (Mihara and Sato, 1985), was not recognized by SRP at all. It should be noted that the translation of LM6[N20]/IL2, which do not bind to RM, was arrested by SRP to a similar extent to LM6[N29](Δ5−14)/IL2 which is translocation competent.

Therefore, there is no significant correlation between the extent of translation arrest and the function of the signals.

Examination of the signal-anchor sequence of P-450(M-1)

Compared with other forms of microsomal P-450, the signal-anchor sequence of P-450 LM6 is rather anomalous in that the amino-terminal hydrophobic region is longer and interrupted with a negatively charged amino acid residue. We therefore addressed questions of whether the amino-terminal regions of other forms of cytochrome P-450 work in the same way as P-450 LM6 in anchoring the passenger molecule to the outer surface of RM, and we examined the signal-anchor sequence of P-450(M-1) (Matsumoto *et al.*, 1986) which is constitutively expressed in male rat liver.

As shown in Table I, the amino-terminal portion of P-450(M-1) contains a hydrophobic stretch of amino acid extending from the 3rd residue to the 20th, which could be the signal-anchor sequence of the protein. The 20 amino acid residues of P-450(M-1) were connected to mature IL2 (Figure 1B) and the chimeric protein was expressed in the *in vitro* transcription−translation−translocation system. The chimeric protein was not processed by dog RM (lanes 1 and 2 in Figure 3), and was digested by externally added trypsin (lane 3). When the reaction mixture was treated with 50 mM Na_2CO_3 (pH 11.1) followed by centrifugation to separate supernatant and membrane fractions, the chimeric protein was recovered exclusively in the membrane fraction (data not shown).

These experiments indicate that the chimeric protein M1[N21[/IL2 was co-translationally inserted into the RM membrane, but its IL2 portion did not translocate into the luminal side, remaining bound to the outer surface of the membrane. It is clear that the amino-terminal 21 amino acid residues of P-450(M-1) function as the type I signal-anchor sequence.

Replacement of a negatively charged amino acid residue, Asp2 at the amino-terminal end of the signal-anchor sequence, by a positively charged amino acid residue, Lys, did not alter the membrane topology of the chimeric protein (lanes 5−8 in Figure 3). We further constructed another chimeric protein, M1[N21](3+)/IL2, in which Asp2, Pro3, and Val4 were replaced by Lys, Arg, and Lys, respectively. The chimeric protein translated in the presence of RM was not processed as shown in Figure 3 (compare lanes 9 and 10). However, it was resistant to externally added trypsin (lane 11) although it was digested by the protease in the presence of 1% Triton X-100 (lane 12).

The results thus obtained strongly suggest that M1[N21](3+)/IL2 did translocate across the RM membrane without being processed. However, another possibility still remained that the activity of trypsin was somehow inhibited by the presence of RM. To rule out this possibility, we took advantage of the fact that protein translocation across the membrane is strongly inhibited after alkylation of RM with N-ethylmaleimide (NEM) (Walter and Blobel, 1980).

If the resistance to trypsin depended upon the ability of RM to translocate the peptide, then the protected band of M1[N21](3+)/IL2 would not appear when NEM-treated RM was used for the translocation assay. As shown in Figure 4, lanes 1−3, the chimeric protein synthesized in the presence of NEM-RM was almost completely digested by added trypsin. The control experiment was performed with preprolactin (lanes 4 and 5). These results strongly indicate that M1[N21](3+)/IL2 translocated across the RM membrane without being processed.

As shown in lanes 13 and 14 in Figure 3, the translocated chimeric protein was not extracted with 50 mM Na_2CO_3 (pH 11) from the RM membrane. These results clearly indicate that the translocated chimeric protein was firmly attached to the inner surface of the membrane, that is, inserted into the membrane with the type II orientation.

The results indicate that the introduction of three positively charged amino acid residues in front of the hydrophobic core of the amino-terminal signal-anchor region of P-450(M-1), converted the region into a translocation signal. Incidentally, the absence of processing resulted in type II membrane

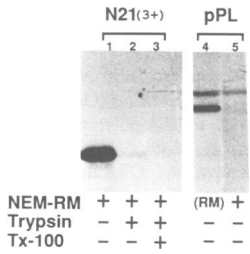

Fig. 4. Effect of NEM treatment on the translocation of M1[N21](3+)/IL2 and preprolactin across microsomal membranes. cDNAs for M1[N21](3+)/IL2 and preprolactin were transcribed *in vitro* and the mRNAs were translated in the wheat-germ cell-free system in the presence of dog pancreas microsomes (RM) (lane 4) and NEM-treated microsomes (NEM-RM) (lanes 1, 2, 3 and 5). Aliquots were treated with trypsin in the absence (lane 2) or the presence (lane 3) of Triton X-100. The reaction mixtures were subjected to SDS−PAGE followed by fluorography. Other conditions are the same as described in Figure 2.

protein. However, the above results leave the possibility that the deletion of Pro3 and Val4, rather than the introduction of two additional basic amino acid residues, could account for the functional conversion of the signal-anchor sequence to the translocation signal. We therefore constructed another chimeric protein in which Asp2 was replaced by a stretch of three basic amino acids, Lys-Arg-Lys, and found that it also translocated the ER membrane without being processed, and thus ruled out the possibility (data not shown).

As has already been shown for P-450 LM6, the signal-anchor sequence of P-450(M-1) was also converted to a secretory signal sequence on partial deletion of the hydrophobic stretch either from the N- or C-terminal. Typical results are shown in Figure 3, lanes 15−18 for M1[N21](+/Δ3−7)/IL2. This deletion mutant was processed and the processed band was protected against trypsin. The same result was obtained for M1[N21](+/Δ14−17)/IL2 (data not shown). However, the mobility of the processed band of M1[N21](+/Δ3−7)/IL2 was higher than that of mature IL2 indicating that the processing occurred downstream of the authentic processing point. Taking into account both the '−3, −1 rule' of von Heijne (von Heijne, 1983) and the molecular weight decrease caused by processing, the processing occurred possibly 10 amino acids downstream of the authentic processing point.

These results confirm the observation with LM6[N29](Δ5−14)/IL2 that a partial deletion of the hydrophobic core of the signal-anchor sequence can convert its function to a translocation signal. Thus the membrane topology of microsomal P-450s seems to be determined primarily by the length of the hydrophobic stretch of its signal-anchor sequence at the amino-terminal portion.

Discussion

We have demonstrated in this paper that two structural features of the signal-anchor sequence, which consists of a long hydrophobic stretch usually preceded by a negatively charged amino acid, are important for correct insertion of newly synthesized P-450 molecules into the membrane of endoplasmic reticulum. First, in confirming the results of Szczesna-Skorupa *et al.* (1988) for P-450 IIC2, the charged amino acid residue in front of the long hydrophobic stretch is important in the function of the signal-anchor sequences of two microsomal P-450s examined, P-450 LM6 and P-450(M-1). When the N-terminal acidic amino acid residue of the signal-anchor sequences was replaced by one or multiple basic amino acid residues, the otherwise signal-anchor sequences were converted to translocation signals. The number of basic amino acid residue required varied with the P-450 species. In view of a clear difference between P-450 LM6 and P-450(M-1) in the hydrophobicity of their signal-anchor sequences (see Figure 1), these results suggest that the number of basic amino acid residues required for the functional conversion depends on the hydrophobicity of the signal-anchor sequences.

Second, the particular significance, is the finding that the length of the hydrophobic stretch also affect the signal-anchor function, that is, partial deletion of the hydrophobic stretch from either N- or C-terminus converted the signal-anchor sequences to secretory signals. M1[N21](+)/IL2 was inserted into the membrane, but M1[N21](+/Δ3−7)/IL2 and M1[N21](+/Δ14−17)/IL2 were translocated across the membrane.

It should also be noted in this respect that LM6[N29](Δ5−14)/IL2, which has a partially shortened hydrophobic stretch preceded by two acidic amino acid residues, was translocated across RM membrane and processed, indicating that the negatively charged amino acids did not interfere with the function of the translocation signal sequence.

The data described above clearly indicate that the type I signal-anchor fucntion is determined not only by the charged amino acid residues preceding the hydrophobic stretch but also by the length of the hydrophobic stretch itself.

What is, then, the molecular basis for the functional conversion from a signal-anchor to a translocation signal, or *vice versa*? In this respect, the orientation of the hydrophobic region in the membrane during membrane insertion would probably have a tight connection to those functions. Coleman *et al.* (1985) have first suggested the dual functions of signal peptides. When a signal peptide is inserted in a loop configuration (tail-orientation) it works as a translocation signal, whereas it will work as a stop-transfer signal when inserted into the membrane as 'head-orientation' structure. In that connection, Szczesna-Skorupa *et al.* (1988) have recently proposed a model for the topology of the signal-anchor sequence of a microsomal P-450 in which the signal-anchor region is inserted into the membrane in a 'head-orientation' configuration and the introduction of basic amino acids changes the orientation so that the signal-anchor region is inserted in a 'tail-orientation' structure, thus recognized by the translocation machinery of the ER membrane as a translocation signal.

How, then, is the orientation of the signal-anchor sequence in the membrane determined? The hypothesis that an electrical dipole[Δ(C-N)] surrounding the membrane-spanning region is a major determinant for the membrane orientation of proteins has been suggested (Haeuptle *et al.*, 1989; Hartmann *et al.*, 1989; von Heijne, 1989). This hypothesis predicts that proteins with positive electrical dipole in the signal-anchor flanking regions are inserted into the membrane in a type I orientation, while proteins with negative electrical dipole are inserted in a type II orientation.

However, our results indicate another factor which determines the orientation of signal-anchor sequences. The

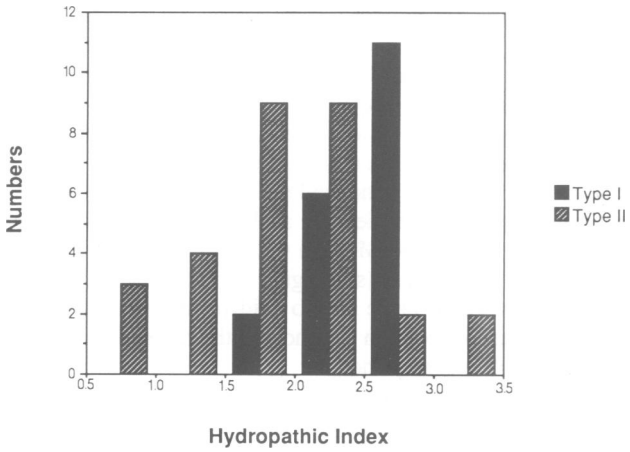

Fig. 5. Distribution of the hydropathic indices of signal-anchor sequences of type I and type II proteins. The hydropathic indices per one amino acid of the signal-anchor sequences, defined by Hartmann *et al.* (1989), of 19 type I and 29 type II proteins were calculated. The type I and type II proteins were separately grouped into six groups according to their hydropathic indices and their numbers are shown in the figure.

amino-terminal portion of P-450 LM6 and P-450(M-1) work as type I signal-anchor sequences, but partial deletion of the hydrophobic core region changes their function from type I to type II without changing electrical dipoles. In order to confirm the importance of the hydrophobicity of the core sequence in the function of signal-anchor sequences, we calculated and compared the hydrophobic indices of 19 type I proteins and 29 type II proteins (Hartmann *et al.*, 1989). As shown in Figure 5, the hydropathic indices of type I proteins are on average higher than those of type II indicating that the orientation of the signal-anchor sequences would be affected by the hydrophobicity of the core sequences. There might be some threshold length of the hydrophobic stretch for the membrane anchor to assume a stable type I orientation. It should be mentioned, however, that this might be true for the type I signal-anchor that locates to the N-terminal end of the molecule. For the proteins with an internally located signal-anchor, the folding state of the N-terminal domain could be another determinant of the orientation (Zerial *et al.*, 1987).

It is well known that the hydrophobic stretches of the signal-anchor sequence of microsomal P-450 are longer and almost double that of the secretory signal peptides. It is tempting to speculate, in this context, that by the existence of the extra hydrophobic portion, the function of the signal peptide is masked and the hydrophobic region is inserted into the membrane as a head-orientation. Upon introduction of basic amino acid residues into the N-terminal portion, the basic amino acid residues now interact with the cytoplasmic surface of the ER membrane and counteract the auxiliary function of the former hydrophobic region, thereby enabling translocation in a loop configuration. Hence the topology of the type I signal-anchor is determined by a balance between the number of N-terminal charged amino acid residues and the hydrophobicity of the signal-anchor sequence.

This hypothesis should be proven by more detailed and systematic experiments. The orientation of the extreme N-terminal portions of the chimeric proteins in the ER membrane should also be determined unambiguously.

Materials and methods

Materials

Restriction enzymes were obtained from Nippon Gene (Tokyo), Takara Shuzo (Kyoto) and Toyobo Biochemicals (Osaka). M13 sequencing kit, T4 polynucleotide kinase, and SP6 RNA polymerase were from Takara Shuzo. pSP64, pSP65, and human placental RNase inhibitor (RNasin) were from Promega Biotech (Madison, USA). Oligonucleotide-directed mutagenesis kit, L-[^{35}S]methionine (1160 Ci/mmol) and [α-^{32}P]dCTP were from Amersham International (Amersham, UK). Oligonucleotides were synthesized by Applied Biosystem, Japan (Tokyo). Wheat germ extract, dog pancreas rough microsomes and the signal recognition particles were prepared as described (Walter *et al.*, 1981).

Plasmid constructions

Standard molecular cloning techniques were used as described by Maniatis *et al.* (1982).

Plasmids. pSP-LM6[N20]/IL-2, pSP-LM6[N29]/IL2 and pSP-LM6[N39]/IL2 correspond to pSP-N20/IL2, pSP-N29/Il2 and pSP-N39/IL2 in the previous paper (Sakaguchi *et al.*, 1987), respectively. pSP-IL2 was described (Sakaguchi *et al.*, 1987). pSP-LM6[N20]/IL2 codes for a chimeric protein composed of the N-terminal 20 amino acid residues of P-450 LM6 and mature IL2.

pSP-M1[N21]/IL2 codes for a chimeric protein composed of the N-terminal 20 amino acid residues of P-450(M-1) and mature IL2. For the construction of the plasmid, a restriction enzyme site *Nru*I was introduced in the cDNA coding intact P-450(M-1) and the cDNA was ligated into pSP-65 vector (pSP-M1). pSP-M1 was cut with *Nru*I and *Pvu*I and ligated with pSP-IL2 which had been cut with *Hin*cII and *Pvu*I. In this way, a serine residue was introduced at the connection between P-450(M-1) and mature IL2.

Oligonucleotide-directed mutagenesis

Oligonucleotide-directed mutagenesis kit was used to prepare the following mutant plasmids from pSP-LM6[N29]/IL2. pSP-LM6[N29](++/−): Asp4 and Phe5 were replaced with Arg and Lys, respectively. pSP-LM6[N29](−/++)/IL2: Thr14 and Glu15 were both replaced with Arg. pSP-LM6[N29](+/−): Asp4 was replaced with Lys. pSP-LM6[N29] (Δ5−14): residues from Phe5 through Thr14 were deleted.

The following mutant plasmids were also produced from pPS-M1[N21]/IL2 by oligonucleotide-directed mutagenesis. pSP-M1[N21](+)/IL2: Asp2 was replaced with Lys. pSP-M1[N21](3+): Asp2, Pro3 and Val4 were replaced with Lys, Arg and Lys, respectively. pSP-M1(+/Δ3−7)/IL2: residues from Pro3 through Leu7 were deleted in M1[N21](+)/IL2. pSP-M1(+/Δ14−17)/IL2: residues from Leu14 through Leu17 were deleted in M1[N21](+)/IL2.

The nucleotide sequences of all the constructs described above were determined by the dideoxy method at around the mutated regions to confirm that the expected mutations were introduced.

In vitro transcription and translation

The cDNAs in the above plasmids were transcribed *in vitro* as described by Krieg and Melton (1984). The mRNAs synthesized were translated in a wheat germ S-23 extract in the presence of L-[^{35}S]methionine as described (Sakaguchi *et al.*, 1987). When the cotranslational insertion or translocation of the translation products were to be examined, 4 U/ml of dog pancreas rough microsomes (RM) or NEM-pretreated RM(NEM-RM) were included in 10 μl of the translation mixture. After translation, 1 μl of 2 mg/ml trypsin and 2 μl of 100 mM $CaCl_2$ were added to the reaction mixture and the mixture was further incubated at 23°C for 30 min in the presence or absence of 1% Triton X-100. At the end of the incubation, 1 μl of 4 mg/ml soybean trypsin inhibitor was added and incubated at 0°C for a further 15 min. The final reaction mixture was analyzed on SDS−PAGE followed by fluorography.

Assay for protein insertion into microsomal membrane

The alkali extraction method (Fujiki *et al.*, 1982) was used to assay the insertion of the chimeric proteins into dog pancreas RM. Ice-cold Na_2CO_3 (pH 11.1) was added to 10 μl of the reaction mixture to a final concentration of 50 mM. 10 μl of RM (50 U/ml) were also added as the carrier. After incubation at 0°C for 20 min, the mixture was centrifuged in a Beckman Airfuge for 110 s at 30 p.s.i. to precipitate the membrane. Solubilized proteins in the supernatant fraction were precipitated with 10% TCA. The two fractions thus obtained were analyzed by SDS−PAGE and subsequent fluorography.

Assay for translation arrest by SRP

The translation of the chimeric proteins was conducted in the absence or presence of 500 U/ml of SRP as described (Sakaguchi *et al.*, 1984). The translation products were analyzed by SDS–PAGE followed by fluorography. The percentage of SRP dependent inhibition was calculated after the quantitation of the translation by densitometric scanning of the fluorogram.

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