Molecular Dissection of the NH2-Terminal Signal/Anchor Sequence of Rat Dipeptidyl Peptidase IV

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Abstract. Dipeptidyl peptidase IV (DPPIV) is a membrane glycoprotein with a type II orientation in the plasma membrane. As shown in a cell-free translation system, the amino-terminal 34 amino acids of rat DPPIV are involved in translocating nascent polypeptide across the membrane of microsomes and in anchoring the translocated polypeptide in the microsomal membrane. The amino-terminal sequence performing this dual function is composed of: a central hydrophobic core of 22 amino acid residues; 6 amino-terminal residues preceding the hydrophobic core (MKTPWK); and 6 residues following the hydrophobic core. The six residues preceding the hydrophobic core are exposed on the outside (cytoplasmic side) of the microsomal membrane. Site-directed mutagenesis studies show that deletion of this cytoplasmic domain, excluding the amino-terminal initiating methionine, does not affect translocation of nascent DPPIV polypeptide, but does affect significantly anchoring of the translocated polypeptide in the microsomal membrane. In contrast, changing the two cytoplasmic Lys to Glu residues or shortening of the hydrophobic core from 22 to 15 residues or converting the last Ile of the shortened hydrophobic core into Ala affects neither translocation across nor anchoring of the DPPIV polypeptide in the microsomal membrane. These and other structural features of the DPPIV amino-terminal signal-anchor sequences are discussed along with other types of sequences for their role in targeting nascent polypeptides to the RER.

SECRETORY, lysosomal, and membrane proteins are synthesized on ribosomes bound to the RER and are co-translationally translocated across the membrane of the RER (Blobel, 1980; Sabatini et al., 1982; Wickner and Lodish, 1985). These proteins are initially targeted to the RER by short amino acid sequences present in the nascent polypeptide chain. Four types of RER targeting sequences have been identified. Secretory, lysosomal, and type I membrane proteins have amino-terminal signal amino acid sequences which translocate downstream sequences of the nascent growing polypeptide across the membrane of the RER with subsequent removal of the amino-terminal signal from the polypeptide by signal peptidase (von Heijne, 1983). Certain type II membrane proteins such as influenza neuraminidase (Bos et al., 1984; Sivasubramanian and Nayak, 1987), dipeptidyl peptidase IV (DPPIV)[1] (Hong and Doyle, 1987, 1988; Hong et al., 1989), aminopeptidase N (Olsen et al., 1988; Watt and Yip, 1989), sucrase-isomaltase (Hunziker et al., 1986), γ-glutamyl transpeptidase (Laperche et al., 1986), and SPC 22/23 (Shelness et al., 1988) have amino-terminal sequences that both translocate downstream polypeptide sequences and anchor the polypeptide in the membrane of the RER. These signals have short hydrophyllic sequences preceding the hydrophobic membrane-spanning core and there is no proteolytic cleavage of the signal. Other type II membrane proteins such as the asialoglycoprotein receptor (Spiess and Lodish, 1986; Holland and Drickamer, 1986), transferrin receptor (Zerial et al., 1986), and HLA-Dr invariant chains (Lipp and Dobberstein, 1986) have signal sequences that function in both translocation and anchoring; these proteins, however, have a longer hydrophyllic sequence (20 or more amino acids) preceding the hydrophobic membrane-spanning core and since there is no signal peptidase cleavage of these proteins they have longer cytoplasmic domains and more internally localized membrane-spanning domains. Finally, sequences have been identified in proteins such as cytochrome P-450 (Monier et al., 1988), the first transmembrane domain of rhodopsin (Friedlander and Blobel, 1985), and the modified signal sequence of multilineage colony–stimulating factor (Haeuptle et al., 1989) that anchor the polypeptide in the membrane of the RER without further translocation of additional downstream sequences. Again no cleavage by signal peptidase occurs with these proteins. The only feature common among these various types of RER targeting sequences in the presence of a hydrophobic domain. The mechanism(s) by which these various targeting sequences promote insertion into the RER seems to be related since interaction with signal recognition particles is involved (Gilmore et al., 1982; Meyer et al., 1982; Walter and Blobel, 1982). Therefore, the different anchoring functions of these various types of RER targeting sequences most likely reflect events occurring after initial

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1. *Abbreviation used in this paper*: DPPIV, dipeptidyl peptidase IV.

targeting to the membrane. The structural features that distinguish these RER targeting and anchoring sequences are not yet known. In this paper we have used site-directed mutagenesis to analyze the relative importance of various structural features in the amino-terminal domain of DPPIV for its targeting to and anchoring in the membrane of the RER.

Materials and Methods

Materials

Restriction enzymes were from Promega Biotec (Madison, WI), Bethesda Research Laboratories (Gaithersburg, MD), Pharmacia P-L Biochemicals (Uppsala, Sweden), and New England Biolabs (Beverly, MA). Sp6 RNA polymerase, pGEM-4Z plasmid, rabbit reticulocyte lysate, QRI DNase, and RNAsein were from Promega Biotec. Dog pancreas microsomal membranes (RM) were from Amersham Corp. (Arlington Heights, IL). The cap analogue m7 G(5′)ppp(5′)G was from Pharmacia P-L Biochemicals. [^{35}S]Methionine (800 Ci/mmole) was from Dupont-New England Nuclear (Boston, MA). The Muta-Gene M13 in vitro Mutagenesis kit was from Bio-Rad Laboratories (Richmond, CA). The DNA sequencing kit with Sequenase (version 2.0) was from United States Biochemical Corp. (Cleveland, OH).

Site-directed Mutagenesis of DPPIV cDNA

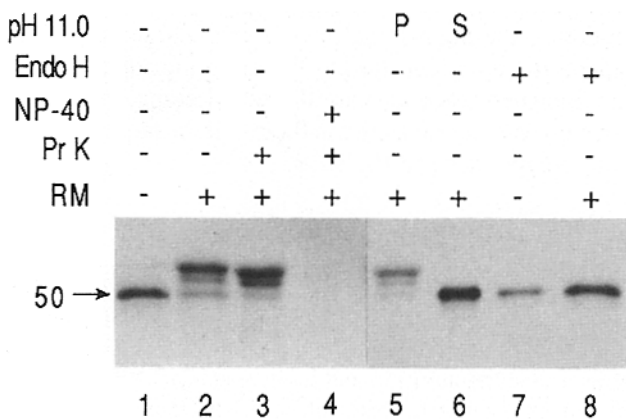The full-length cDNA for DPPIV was subcloned into the Eco RI site of

Figure 1. SDS-PAGE analyses of mutated DPPIV made in a cell-free system. The DNA for the NH$_2$-terminal 34 amino acid sequence of DPPIV was linked to that for the COOH-terminal region (residue 356 to COOH terminus) of DPPIV which contains neither signal nor anchor sequence (see Materials and Methods for details of the pDPPIV/Δ35-356 construct). The RNA derived was translated in vitro with rabbit reticulocyte lysates supplemented with (lanes 2-6 and 8) and without (lanes 1 and 7) microsomal membranes. The 50-kD polypeptide is not translocated and is accessible to proteinase K digestion (not shown, refer to lane 3). The mobility of this untranslocated polypeptide in SDS-PAGE is not affected by endo H treatment (lane 7). The two upper forms (lane 2) are translocated and glycosylated at one or two sites, respectively. The majority of the translocated polypeptides are protected from proteinase K digestion except for a few residues (suggested by a slight but reproducible increase in gel mobility) (lane 3). This protection was abolished when the microsomal membrane was permeabilized by detergent NP-40 (lane 4). Endo H converted these two translocated polypeptides into forms with identical sizes as the untranslocated polypeptide (lane 8). The translocated polypeptides are anchored to the membrane (lane 5), whereas the untranslocated polypeptide is extractable in sodium carbonate solution (lane 6). All reactions in this and subsequent figures represent immunoprecipitated products resolved by 8% SDS-PAGE.

M13mp19. Mutagenesis of this cDNA was performed essentially as described in the protocols supplied by the company except that the M13 DNA was propagated in bacterial strain CJ236 twice before the preparation of U-containing single-strand DNA for the synthesis of the mutagenic strand. Under these conditions the efficiency of mutagenesis is routinely 75-100%. Mutations in the DNA were confirmed by sequencing. Once a mutation was identified, double-strand replicating form DNA was prepared by standard protocols. The mutated DPPIV cDNA was excised out of the double-strand M13 DNA and was cloned into the Eco RI site of the in vitro expression vector pGEM-4Z. The following oligonucleotides were used to produce mutations in the DPPIV insertion signal: GCCGCCCAACCATGGTTCT-TCTGGGACTGCTTGG (denoted M1); GCCCAACCATGGAGACACCGT-GGGAGGTTCTTCTGGG (M2); GCGCTTGTCACCATCATCAACAAA-GATGAAGCGGCC (M3); and GCGCTTGTCACCATCGCCAACAAA-GATGAAGCGGCC (M4). M1 was used to delete the amino acids between the initiating NH$_2$-terminal methionine and the hydrophobic core; M2 was used to change the two lys residues in the amino-terminal cytoplasmic domain into glu residues; M3 was used to delete the last seven residues from the hydrophobic core; and M4 was used to change ile at position 15 of the truncated hydrophobic core produced by M3 into ala. All oligonucleotides were purified by gel electrophoresis and were 5′ phosphorylated using T4 polynucleotide kinase.

Construction of pDPPIV/Δ35-356

To generate a mutant of DPPIV lacking residues 35-356 the DNA was first cut between codons 34 and 35 with Not I (followed by conversion of the resulting sticky ends into blunt ends). The DNA was next cut between codons 355 and 356 with Stu I (creating blunt ends). The blunt-ended DNA was ligated and transformants were screened for the deletion.

In Vitro Transcription

The cDNAs for normal DPPIV, M1-M4 mutated DPPIV, and pDPPIV/Δ35-356 were all placed under the control of the Sp6 promotor in the pGEM-4Z vector. Plasmid DNAs were linearized using restriction enzyme Hind III except where indicated otherwise in the text. Linearized DNAs were transcribed in 50-μl reactions with sP6 as described previously (Hong and Doyle, 1988). In vitro translation of these transcribed RNAs, translocation, and other methods were performed as described by us previously (Hong and Doyle, 1988, and references therein).

Results

The Amino-Terminal 34 Amino Acid Sequence of DPPIV Is Sufficient for the Signal/Anchor Function

In previous studies (Hong and Doyle, 1988) we have shown that a truncated form of DPPIV having residues 1-356 is translocated, glycosylated, and anchored in microsomal membranes after translation of its mRNA in a cell-free system. The only candidate region in this polypeptide for providing the translocation/anchor function is the amino-terminal hydrophobic domain. Furthermore, a form of DPPIV containing residues 297 to the carboxy terminus is neither translocated nor membrane anchored in the cell-free system. Finally, we determined that the amino-terminal hydrophobic domain is not proteolytically cleaved as a consequence of membrane insertion. We concluded, then, that the amino-terminal hydrophobic region acts as both a translocation signal and a membrane anchor. To test this conclusion a construct was made in which codons 35-356 were deleted (denoted pDPPIV/Δ35-356). This deletion links the amino-terminal 34 residues to the carboxy terminal domain containing amino acid residues 357 to the carboxy terminus of DPPIV. When mRNA derived from this construct was translated in a cell-free system in the absence of dog pancreas microsomal membranes one polypeptide with M_r of ~50,000 (Fig. 1, lane 1) and insensitive to digestion with endoglycosidase H

(Fig. 1, lane 7) was produced. In the presence of microsomes two additional larger polypeptides were produced (Fig. 1, lane 2); these most likely represent translocated polypeptides that have acquired one or two asn-linked glycans, since there are two sites for asn-linked glycosylation in the M_r 50,000 polypeptide. This was confirmed by endoglycosidase H treatment of the immunoprecipitated translation products, which converted the two higher M_r polypeptides into the one with M_r of 50,000 (Fig. 1, lane 8). As expected for the translocated polypeptides the two glycosylated products were for the most part resistant to proteinase K digestion. Proteinase K caused a slight increase in the mobility of the two polypeptides (Fig. 1, lane 3) suggesting that most of the polypeptide was protected from protease digestion by the microsomal membrane. Such protection is consistent with a cytoplasmic localization of the six most amino-terminal amino acids in DPPIV. To determine if the translocated polypeptides were indeed anchored in the membrane, the translation reaction was diluted in carbonate buffer, pH 11, and membrane fragments were collected by centrifugation. The glycosylated polypeptides were in the membrane pellet (Fig. 1, lane 5) while the nonglycosylated M_r 50,000 polypeptide precursor was only in the supernatant fraction resulting from the alkaline permeabilization of the microsomes (Fig. 1, lane 6). Together with our previous results, these studies show that the amino-terminal 34 residues are necessary and sufficient for both translocating across and anchoring DPPIV in the microsomal membrane. In fact, the amino-terminal 35 residues of DPPIV can translocate and anchor in the membrane the carboxy terminal 212 residues of rat hepatic lectin 1 (Holland and Drickamer, 1986) which forms part of the asialoglycoprotein recognition system and which does not contain signal or anchor sequence (data not shown).

Effect of Mutation on Signal Function

We next analyzed the effect of the four mutations (denoted M1-M4) introduced in the amino-terminal signal of DPPIV on translocation across microsomal membranes (see Fig. 2). A major polypeptide with approximate M_r of 80,000 was synthesized using normal mRNA or mRNA transcribed from mutated constructs in the cell-free translation system (Fig. 3, lane 1). When microsomal membranes were added to the translation mixtures an additional polypeptide with approximate M_r of ~100,000 was produced from each of the mRNAs (Fig. 3, lane 2). The M_r 100,000 polypeptide is protected from digestion with proteinase K (Fig. 3, lane 3) in the absence of detergent but is sensitive when the reactions are treated with detergent before proteinase K treatment and immunoprecipitation (Fig. 3, lane 4). The 100,000 M_r polypeptide also is converted to an 80,000 M_r form by endoglycosidase H (Hong and Doyle, 1988). The 100,000 M_r polypeptide, then, is a translocated and glycosylated form of

DPPIV. Each of the mutated amino-terminal sequences promotes translocation of DPPIV polypeptide across the microsomal membrane with efficiencies comparable to the normal nonmutagenized sequence. Therefore, none of the mutations has a significant effect on the ER insertion signal function.

Deletion of the Amino-Terminal Six Amino Acids Abolishes the Amino-Terminal Anchor Function

Cell-free translation reactions containing microsomal membranes were diluted with 0.1 M sodium carbonate, pH 11, and membrane-bound polypeptides were separated from microsomal luminal soluble contents by centrifugation. Under these circumstances the translocated normal nonmutagenized form of DPPIV with M_r of 100,000 was associated mainly with the membrane pellet while the 80,000 M_r polypeptide was mainly in the supernatant fraction (Fig. 4, lanes 1 and 2). Mutations in the M2-M4 constructs showed similar distributions after centrifugation with the translocated polypeptide membrane-associated and the nontranslocated polypeptide in the supernatant fraction. With the M1 construct, however, both translocated and nontranslocated polypeptides made in the cell-free system were present mainly in the supernatant fraction after centrifugation, with only a small amount of the translocated M1 polypeptide associated with the membrane pellet. These results demonstrate that the M2-M4 mutations do not affect significantly the anchor function of the amino-terminal sequence; deletion of the six amino-terminal amino acids in the M1 construct, however, does effectively abolish anchoring of the translocated polypeptide in the microsomal membrane.

The Mutated Amino-Terminal Sequences Are Not Cleaved

To determine whether the amino-terminal sequences resulting from the mutated constructs were cleaved during microsomal translocation in the cell-free system we took advantage of the facts that the second met residue in the DPPIV polypeptide is located at position 283 from the amino-terminal end and that RNA derived from the Pvu II-linearized DNA encodes a truncated polypeptide from the amino-terminus to residue 139. There are two potential sites for asn-linked glycosylation in this truncated polypeptide and the only met is the amino-terminal-initiating met. If any part of the amino-terminal sequence is cleaved during translocation, the translocated and glycosylated polypeptide would not be detected by autoradiography when ^{35}S-met is used as label in the cell-free system. Conversely, if the amino-terminal sequence is not cleaved, radiolabeled and glycosylated polypeptide would be made in the cell-free system. As predicted mRNA derived from Pvu II-linearized DNA constructs produced a polypeptide with M_r of ~15,000, in the absence of
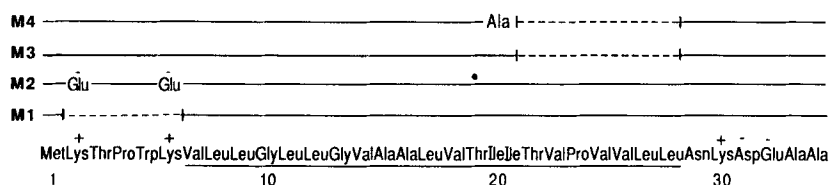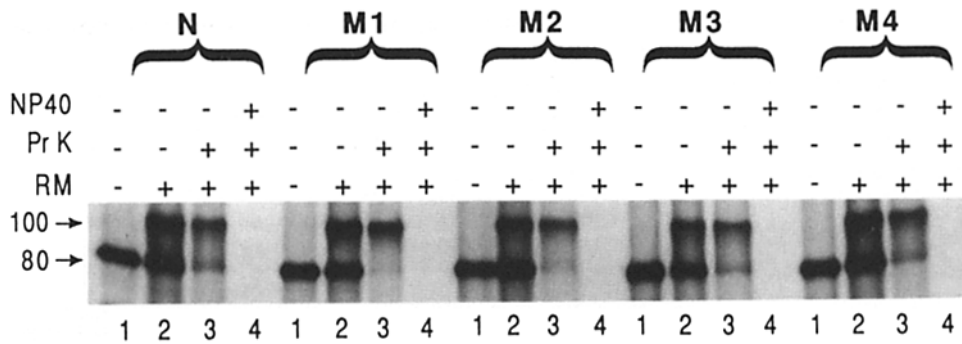

Figure 2. Summary of site-directed mutagenesis of the NH₂-terminal sequence of DPPIV.

Figure 3. Effect of mutations on the translocation of DPPIV polypeptide across the microsomal membrane. The RNA produced from normal and mutated DPPIV cDNA was translated without (lane *1*) and with (lanes *2-4*) microsomal membranes. A polypeptide with M_r of ~80 kD was produced in the absence of microsomal membrane (lane *1*). When microsomal membranes were added, a 100-kD polypeptide was produced (lane 2) and this polypeptide is protected from proteinase K digestion (lane 3). Permeabilization of microsomal membranes by detergent abolished this protection (lane 4). All the mutated sequences can promote the production of the 100-kD translocated polypeptide at similar efficiency as the normal signal/anchor sequence. N indicates that RNA is derived from cDNA with normal amino terminus. M1-4 indicates that RNA is derived from cDNAs with the M1, M2, M3, and M4 mutated sequences, shown in Fig. 2, respectively.

microsomal membranes (Fig. 5, lane *1*). With the M1, M3, and M4 constructs polypeptides with increased mobilities (decrease in M_r) after SDS-PAGE were made in the cell-free system. Interestingly, mRNA corresponding to the M2 construct also produced a polypeptide with faster mobility after SDS-PAGE, suggesting that conversion of positive to negative charges affects mobility of polypeptides in the SDS-PAGE system. In the presence of microsomal membranes in the cell-free system, two larger polypeptides were produced from normal truncated and mutagenized truncated constructs. The two larger polypeptides represent glycopeptides that have acquired one or two asn-linked glycans since they can be converted into the 15,000 M_r nonglycosylated polypeptide by treatment with endoglycosidase H (data not shown). The production of labeled glycosylated polypeptides in the cell-free system shows that the amino-terminal sequence promotes translocation of the truncated glycopeptides across microsomal membranes without cleavage of any amino-terminal amino acids. When the cell-free systems were treated with proteinase K after translation in the presence of micro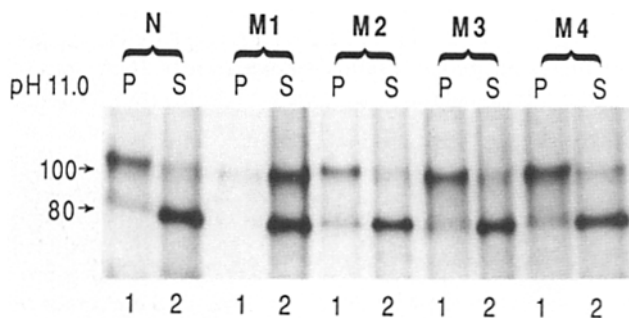somes the glycosylated polypeptides were no longer detected most likely because the amino-terminal met on the outside of the microsomal membrane vesicle was accessible to the protease (data not shown).

Discussion

Cytoplasmic Localization of the Short Sequence Preceding the Hydrophobic Core at the Amino-Terminal Region of DPPIV

The very small decrease in M_r upon proteinase K treatment of polypeptides synthesized and translocated in the cell-free system suggests that the six amino-terminal amino acids preceding the hydrophobic core or membrane spanning domain of DPPIV are exposed on the outside of the microsomal membrane. We (Hong and Doyle, 1988) previously showed by microsequencing techniques that the initiating met residue is present in both the nontranslocated 80,000 M_r and the translocated 100,000 M_r DPPIV polypeptides. When

Figure 4. Effect of the M1-4 mutations on anchoring the DPPIV polypeptide in the membrane. RNA derived from normal and mutated DPPIV cDNA was translated in vitro with rabbit reticulocyte lysates in the presence of microsomal membranes. After translation, the reaction was diluted 15-fold in volume with 0.1 M sodium carbonate at pH 11.0 and kept on ice for 30 min. Membrane-associated polypeptides (lane *1*) were separated from those not anchored in the membrane (lane 2) by centrifugation. N and M1-4 have the same meaning as in Figs. 2 and 3.
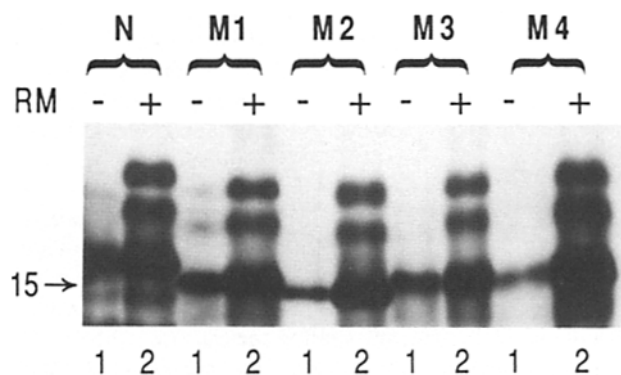

Figure 5. Effect of the M1-4 mutations on cleavage of amino-terminal residues. DNA for normal and mutated DPPIV was linearized by Pvu II restriction and transcribed with Sp6 polymerase. The RNA derived was translated in the presence (lane 2) or absence (lane *1*) of microsomal membranes. The polypeptides with M_r of ~15 kD were not translocated or glycosylated. The two polypeptides with higher M_r represent the translocated and glycosylated polypeptides, the presence of which indicates the initiation Met residue remains in the polypeptide.

the translocated polypeptide in the microsomal membrane, however, is treated with proteinase K the initiating met is accessible to the protease. The inability to detect the translocated polypeptides labeled with ^{35}S met after proteinase K treatment in microsomes in the cell-free system using truncated mRNA transcripts reported here is consistent with our previous observations. We conclude then that the most amino-terminal six amino acids of DPPIV are located on the outside of the microsomal membrane after cell-free translation and translocation which is equivalent to a cytoplasmic localization in situ.

The Six Amino Acids in the Cytoplasmic Domain Play a Role in Anchoring DPPIV in the Plasma Membrane

When the six amino acids in the cytoplasmic domain of DPPIV were removed by deletion in the M1 construct, succeeding or downstream sequences were still translocated at a high efficiency in the cell-free system; similar results to these were obtained with studies on the HLA-Dr invariant chain (Lipp and Dobberstein, 1986) and the H1 subunit of the asialoglycoprotein receptor (Schmid and Spiess, 1988). In the case of DPPIV lacking the cytoplasmic six amino acid domain there is a pronounced effect on anchorage of the translocated polypeptide in the microsomal membrane, implying that the short cytoplasmic domain preceding the membrane spanning domain is essential for locking DPPIV in the lipid bilayer. The fact that a small portion of the translocated polypeptide which lacks the cytoplasmic domain remains associated with the membrane after treatment at pH 11 and centrifugation of the cell-free extract might indicate two populations of translocated polypeptide with somewhat different affinity for the lipid bilayer. In previous work from other laboratories it has been difficult to determine whether sequence preceding the hydrophobic core of an internal signal sequence is important as an anchor sequence because deletion in this region made the internal signal sequence cleavable by signal peptidase (Lipp and Dobberstein, 1986; Schmid and Spiess, 1988).

The Positive Charge Preceding the Hydrophobic Core Is Not the Determinant for the Membrane Orientation of the Amino-Terminal Signal/Anchor Sequence

Changing the negative charges at the amino-terminal end of cytochrome P450 into positively charged residues converted the membrane anchor signal of this membrane protein into a secretory type signal resulting in translocation across the microsomal membrane and glycosylation (Szczesna-Skorupa et al., 1988; Szczesna-Skorupa and Kemper, 1989). Our results with the M2 construct of DPPIV in which two lys residues were converted into two glu residues in the amino-terminal domain had no effect on either translocation or anchoring of the polypeptide in the membrane, suggesting that net negative charge is as compatible as net positive charge with these functions. These results suggest that net charge preceding the hydrophobic domain is not the sole determinant for translocation of succeeding downstream sequences. It appears more likely that net negative charge is necessary but not sufficient for the amino-terminal region of cytochrome P450 acting as a nontranslocating signal/anchor. Further studies using additional mutations and hybrid molecules to-

gether with structural studies should shed more light on these issues.

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