Residues Flanking the COOH-terminal C-region of a Model Eukaryotic Signal Peptide Influence the Site of Its Cleavage by Signal Peptidase and the Extent of Coupling of Its Co-translational Translocation and Proteolytic Processing in Vitro*

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The polar, COOH-terminal c-region of signal peptides has been considered to be most important for influencing the efficiency and fidelity of signal peptidase cleavage while the hydrophobic core or h-region appears indispensable for initiating translocation. To identify structural features of residues flanking the c-region that influence the fidelity and efficiency of signal peptidase cleavage as well as co-translational translocation, we introduced six amino acid substitutions into the COOH terminus of the hydrophobic core and seven substitutions at the NH2 terminus of the mature region (the +1 position) of a model eukaryotic preprotein-human pre(Δpro)apoA-I. This preprotein contains several potential sites for signal peptidase cleavage. The functional consequences of these mutations were assayed using an in vitro co-translational translocation processing system and by post-translational cleavage with purified, detergent-solubilized, hen oviduct signal peptidase. The efficiency of translocation could be correlated with the hydrophobic character of the residue introduced at the COOH terminus of the h-region. Some h/c boundary mutants underwent co-translational translocation across the microsomal membrane with only minimal cleavage yet they were cleaved post-translationally by hen oviduct signal peptidase more efficiently than other mutants which exhibited a high degree of coupling of co-translational translocation and cleavage. These data suggest that features at the COOH terminus of the h-domain can influence "presentation" of the cleavage site to signal peptidase. The +1 residue substitutions had minor effects on the extent of co-translational translocation and processing. However, these +1, as well as h/c boundary mutations, had dramatic effects on the site of cleavage chosen by signal peptidase, indicating that residues flanking the c-region of this prototypic eukaryotic signal peptide can affect the fidelity of its proteolytic processing. The site(s) selected by canine microsomal and purified hen oviduct signal peptidase were very similar, suggesting that "intrinsic" structural features of this prepeptide can influence the selectivity of eukaryotic signal peptidase cleavage, independent of the microsomal membrane and associated translocation apparatus.

Several functional roles have been assigned to the NH2-terminal signal peptides during the co-translational translocation of eukaryotic secretory proteins across the endoplasmic reticular (ER) membrane. Initially these 15-30 residue peptides serve as markers for direct recognition by the 54-kDa subunit of signal recognition particle (SRP); Refs. 2-4), thereby distinguishing nascent secretory proteins from proteins destined for other locations. Interaction of SRP with the signal peptide is subsequently released from SRP and translocation begins. A 34-kDa integral-membrane glycoprotein termed the signal sequence receptor is then thought to interact with the signal peptide (7). The signal peptide is subsequently cleaved by signal peptidase during co-translational translocation, releasing the NH2 terminus of the mature secretory protein into the ER lumen. The structural features of signal peptides that are necessary for carrying out these functions have not yet been completely defined. A tripartite domain structure has been identified as a common feature of eukaryotic signal sequences. These three domains include a positively charged NH2 terminus, a hydrophobic core, and a polar COOH-terminal region (known as the n-, h-, and c-regions, respectively; Refs. 8, 9). In view of the enormous diversity in the primary structures of signal peptides, common secondary or tertiary structures are thought to be critical for the productive interactions of these domains with the translocation/processing apparatus of the ER. Correlations between the ability of mutant signals to adopt α-helical structures and their targeting behavior have been noted although under some conditions signal peptides also exhibit β structure (10-13). Functional studies of mutant signal peptides show that the presence of an "intact" h-region is crucial for translocation of the passenger domain. Many lines of evidence suggest that the residues comprising the c-region are very important for recognition and cleavage by signal peptidase. Small, neutral amino acids are usually found...
at the -1 and -3 positions (9, 14). Systematic site-directed mutagenesis of both eukaryotic (15, 16) and prokaryotic pre-proteins (17, 18) have confirmed the importance of the physical-chemical properties of the -1 residue in establishing a proper "context" for signal peptidase cleavage.

The c-region is relatively fixed in length compared with the n- and h-regions (8). Previous work from our laboratory has shown that at least two sites of cleavage in a model preprotein, human pre(Δpro)apolipoprotein(apo)A-II, can "compete" for recognition by signal peptidase (15). This feature allowed us to use site-directed mutagenesis to manipulate the location of the h/c boundary and determine that in addition to the importance of features at the -1 and -3 positions, the processing machinery tends to conserve a distance of 4-5 residues from its h/c junction to the site of cleavage (19).

Features NH₂-terminal to the c-region also appear to influence signal peptidase processing. We noted that when the -1 residue (Ala⁹¹) of pre(Δpro)apoA-II was replaced with Gly, roughly equal cleavage between the Gly⁹¹ and Gly⁹² sites occurred. Alterations at the boundary between its n- and h-regions affected the site selected for signal peptidase processing; lengthening of the h-region at its NH₂-terminal end increased the distance between the n/h boundary and the Gly₂₀ residue producing a shift in the site of cleavage to the more NH₂-terminal position (Gly²¹). Ref. 20; Cioffi et al. (21) have studied the effects of dipeptide deletions throughout the h-region of the parathyroid hormone signal peptide. They found that both in vitro co-translational and post-translational cleavage by hen ovioduct signal peptidase (HOSP) were affected by deletions in the NH₂ terminus of the hydrophobic core while deletions in the COOH terminus of the h-region had no deleterious effects.

In this present study we have analyzed the role(s) that amino acids flanking both sides of the c-region have on signal peptidase cleavage and its coupling to translocation. The pre(Δpro)apoA-II Gly²₀ mutant was selected as a "parental" preprotein to begin to assess how systematic amino acid substitutions at the COOH terminus of the h-region of preproapoA-II, can "compete" for recognition by signal peptidase (15). This feature allowed us to use site-directed mutagenesis to manipulate the location of the h/c boundary and determine that in addition to the importance of features at the -1 and -3 positions, the processing machinery tends to conserve a distance of 4-5 residues from its h/c junction to the site of cleavage (19).

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**EXPERIMENTAL PROCEDURES**

**RESULTS AND DISCUSSION**

**Construction of Site Saturation Mutations Flanking the C-region**—By substituting proline residues at one of three positions (Ile¹³, Cys¹, or Ser¹⁸) in pre(Δpro)apoA-II Gly²₀, we previously found that the site and efficiency of cleavage could be correlated with the predicted position of the h/c boundary (19). The Pro²²Gly²₀ mutant had very low translocation and cleavage efficiencies that could be "rescued" by inserting an Ile residue between the Thr¹² and Pro¹³ residues. This result suggested, but did not prove, that (a lack of) hydrophobic character at the COOH terminus of the h-domain of pre(Δpro)apoA-II Pro³Gly²₀ was affecting (limiting) its productive interactions with components of the translocation apparatus. To further examine the role of residues flanking the c-region on co-translational translocation/process, the Ile¹³ residue of the Ile²²Pro²³Gly²₀ mutant was replaced with 6 other residues having varying physical-chemical properties, Ala, Ser, Pro, Gly, Asp, and Lys (Figs. 1 and 2A). As shown in Fig. 1, these mutations progressively decrease the predicted hydrophobicity at the COOH terminus of the h-region.

Another series of mutants was also constructed to systematically investigate the role played by residues COOH-terminal to the h-region of pre(Δpro)apoA-II in regulating the site and efficiency of cleavage by signal peptidase. The Lys¹⁻¹ residue of the Gly²₀ mutant (the +1 position relative to cleavage after Gly²₁) was replaced with Leu, Gly, Ser, Asp, Pro, or Phe (Fig. 2B).

**Efficiency of In Vitro Co-translational Translocation and Cleavage**—A reticulocyte lysate cell-free translation system supplemented with canine microsomal membranes and programmed with wild-type preproc apoA-II or mutant pre(Δpro)apoA-II mRNA was used to determine the effects of the Xaa¹³ and Xaa¹¹ mutations on the efficiency of signal peptidase cleavage. The extent of their translocation was assessed by post-translational treatment of the microsomes with protease K in the presence or absence of the detergent Triton X-100.

Both the extent of cleavage and translocation were dramatically altered depending on the nature of the residue at position 13. Fig. 2A tabulates the efficiency of translocation (of all species) and cleavage for each preprotein. As previously observed for the pre(Δpro)apoA-II Gly²₀ and Pro³Gly²₀ mutants (19), uncoupling of translocation from cleavage was evident for some of the substitutions (e.g. Ile¹³, Pro¹³, Asp¹³). For example, both processed and unprocessed Ile³³Pro³⁴Gly²₁ polypeptides were protected from protease digestion but were

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2 The "Experimental Procedures" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
performed once again for this study. The data presented for the
functional signal peptidase cleavage and translocation of the
completely coupled to cleavage (e.g. Ala13, Lys13). When these
Other substitutions resulted in translocation that is almost
undetectable or too low to accurately quantify (<15%).

The percent signal peptide cleavage at the sites indicated is based on
Edman degradation of co-translationally processed polypeptides labeled with [35S]Succinyl (see "Experimental Procedures"). Cleavage data for the Gly20, Pro14 Gly20, and Ile13 Pro14 Gly21 mutants was reported
previously (19). Cleavage site analysis for the Ile13 Pro14 Gly21 mutant was
performed once again for this study. The data presented for the
remainder of the Xaa'3 Pro'4 Gly21 mutants represents the results of
one experiment. The results shown for each of the Gly20 Xaa21 mutants
is the average of two independent experiments each done in duplicate
(the duplicates varied by less than 13%). The percent cleavage and
translocation data were calculated after taking into account the loss
of the initiator Met (see "Experimental Procedures"). Within each
experiment, the data was normalized to that obtained for wild-type
preproapoA-II (its cleavage and translocation were set at 100%). The
bars shown for the percent translocation of the Gly20 Xaa21 mutants
represent the average of two experiments. The remainder of the
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completedly digested in the presence of detergent (see below).
Other substitutions resulted in translocation that is almost
completely coupled to cleavage (e.g. Ala13, Lys13). When these
data are normalized to the value for wild-type preproapoA-II,
the order of translocation efficiency for the Xaa13 Pro14 Gly21
mutants was Ile > Ala > Ser > Gly > Pro > Lys > Asp.
while the order of cleavage efficiency was Ile > Ala > Ser > Lys > Gly > Pro, Asp. The ability of the residue at position 13 to
rescue translocation appears related to its hydrophobicity
(Fig. 1, inset). Specifically, the data suggests that there is a
minimum threshold of hydrophobicity that must be achieved
for efficient translocation to occur (greater than +2 on the
Kyte-Doolittle scale; Ref. 36).

The translocation and cleavage efficiency of the Gly20 Xaa21
were not dramatically altered when Lys20 was replaced with 7
other residues (Fig. 2B). All mutants were cleaved and translocated with an efficiency close to that of wild-type human
preproapoA-II. In addition, most of the amino acid substitu-
tions at position 21 (Leu, Ser, Asn, Pro, Phe) result in
increased coupling of translocation and cleavage relative to
the parental pre(Apro)apoA-II Gly20 mutant (data not shown).

Membrane Sedimentation Assay—The compartmentaliza-
tion assays suggested that for some mutants, translocation
can occur without cleavage. To further verify these observa-
tions, wild-type preproapoA-II, pre(Apro)apoA-II Gly20, and
pre(Apro)apoA-II Ile13 Pro14 Gly21 were analyzed using a mem-
brane sedimentation assay (Fig. 3). mRNAs were translated
in the presence of microsomes. Protease K was then added
with or without detergent. Samples treated with protease K
in the absence of detergent (lane 3) were adjusted to 10 mM
PMSF to inactivate the protease. All samples were subse-
sequently spun through a 0.5 M sucrose cushion in the presence
of 0.5 M KOAc. ApoA-II polypeptides were immunoprecip-
itated from the resulting supernant and pellet fractions and
subjected to SDS-PAGE. Most of the processed wild-type
polypeptides sediment with the microsomes (compare lanes 4
and 5). The slight amount of processed wild-type protein in
the supernant may indicate a lack of complete sedimentation
of the microsomes or a lack of microsome integrity during
fractionation. Both the protease-protected, processed, and
unprocessed Gly20 and Ile13 Pro14 Gly21 mutant polypeptides
sediment with the microsomes demonstrating that unproc-
essed chains are indeed translocated.

An uncleaved signal peptide might be expected to anchor
the translocated mature domain to the luminal side of
the membrane (37). Therefore, the nature of the interaction of
the protease-protected species with the microsomal mem-
brane was characterized by incubating material treated as
shown in lane 3 with sodium carbonate, pH 11.5. This treat-
ment has been shown to extract luminal and peripheral mem-

FIG. 2. Summary of the site and efficiency of co-transla-
tional signal peptidase cleavage and translocation of the
pre(Apro)apoA-II mutants. Panel A shows the amino acid sequence
around the cleavage site of the Gly20, Pro14 Gly20, and Xaa13 Pro14 Gly21
mutants while the Gly20 Xaa21 mutants are shown in panel B. A lack of
sequence divergence from the Gly20 mutant is indicated by a dashed.
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were not dramatically altered when Lys20 was replaced with 7

FIG. 3. Co-sedimentation assay of translation products with
the membrane fraction. The treatments indicated in lanes 1, 2, 3,
and 5 were performed as described under "Experimental Procedures".
Equal aliquots of translation samples treated as indicated in lane 3
were subsequently processed as follows prior to immunoprecipitation
and analysis by SDS-PAGE. Lanes 4 and 5, the aliquot was centri-
fuged through a 0.5 M sucrose cushion in the presence of 0.5 M KOAc,
and separated into supernatant and pellet fractions; lanes 6 and 7,
the sample was diluted 29-fold into 100 mM Na2CO3, pH 11.5, and
was separated into supernatant and pellet fractions by centrifugation.
The supernatant fractions from each treatment were loaded in lanes
4 and 6 while the membrane pellet fractions were loaded in lanes 5
and 7. For further details see "Experimental Procedures."
brane proteins leaving only integral membrane proteins associated with membranes (29). As shown for preproapoA-II in lanes 6 and 7 of Fig. 3, a majority of the processed, protease-protected form is present in the supernatant and is partially degraded while a small, but detectable, amount remains with the membrane pellet. Similar results are obtained for the Gly\(^{20}\) and Ile\(^{13}\)Pro\(^{14}\)Gly\(^{21}\) mutants. An examination of the polypeptides which sediment with the microsomes under these conditions (lane 7) reveals unprocessed Ile\(^{13}\)Pro\(^{14}\)Gly\(^{21}\) protein in the sodium carbonate-treated membranes. While no processed chains are apparent in the sodium carbonate supernatant (lane 6), we cannot exclude the possibility that they were preternaturally degraded during preparation of this fraction. There is an enrichment of unprocessed Ile\(^{13}\)Pro\(^{14}\)Gly\(^{21}\) protein in the pellet (lane 7) compared with the starting material (lane 3), however, the amount of unprocessed protein is a fraction of that present in the starting material. While the data suggest that retention of the signal peptidase enhances its interaction with the bilayer, a large fraction of unprocessed, mutant preprotein does not appear to be associated with the membrane in a fashion typical of integral membrane polypeptides.

**Determination of the Site of Co-translational Signal Peptidase Cleavage**—To assess the effect of these mutations on the fidelity of signal peptidase cleavage, automated sequential Edman degradation was performed on \(^{14}C\)lysyldeuterated, processed polypeptides purified from *in vitro* translation reactions carried out in the presence of canine microsomes. Since pre(pre\(^{\Delta}\)apoA-II has a cysteine residue at position 24, the site of signal peptidase cleavage could be determined by noting the cycle(s) which yield deuterated amino acid derivatives. Fig. 2A shows the sites of cleavage for the Gly\(^{20}\), Pro\(^{13}\)Gly\(^{20}\), and Ile\(^{13}\)Pro\(^{14}\)Gly\(^{21}\) mutants as well as for the other Xaa\(^{1}\)Pro\(^{2}\)Gly\(^{3}\) mutants. When the Ile\(^{13}\) residue of pre(pre\(^{\Delta}\)apoA-II Gly\(^{20}\) is changed to a Pro, cleavage shifts so that the major site of processing occurs after Gly\(^{18}\) with minor cleavage after Gly\(^{21}\) (19). Cleavage of the pre(pre\(^{\Delta}\)apoA-II Ile\(^{13}\)Pro\(^{14}\)Gly\(^{21}\) mutant occurs exclusively after its Glu\(^{15}\) residue. When Ile\(^{13}\) is replaced by Ala, Ser, Gly, or Lys all of the cleavage occurs after Gly\(^{21}\), a more frequently observed -1 residue than Glu (8, 9, 15). We were not able to recover enough processed Pro\(^{13}\)Pro\(^{14}\)Gly\(^{21}\) and Asp\(^{13}\)Pro\(^{14}\)Gly\(^{21}\) protein to sequence since they are very poorly cleaved. These results demonstrate that certain structural features at the h/c boundary can induce signal peptide cleavage to occur after very uncommon -1 residue (i.e. Glu) and that the Ile\(^{13}\)Pro\(^{14}\)Gly\(^{21}\) mutant may assume a unique conformation that restricts signal peptidase cleavage to the Glu\(^{18}\) position.

The site(s) of cleavage of the Gly\(^{20}\)Xaa\(^{1}\) mutants are shown in Fig. 2B. When the Lys\(^{31}\) residue of the Gly\(^{20}\) mutant is replaced with 7 other residues, the relative proportion of cleavage between the Gly\(^{18}\) and Gly\(^{21}\) sites shifts so that in every case more cleavage occurs at Gly\(^{18}\). The extent of shifting to the Gly\(^{18}\) site depends on the residue at position 21 thus allowing us to conclude that the relative suitability for these amino acids as +1 residues in pre(pre\(^{\Delta}\)apoA-II is as follows: Lys > Ser > Gly > Phe > Leu > Asp > Asn > Pro. These data suggest that the +1 position of eukaryotic prepeptides is under selective pressure to define the site of signal peptidase cleavage.

Using the PARA-SITE algorithm (38), we were not able to correlate any physical chemical property of the residues at position 21 (e.g. charge, polarity, side chain size, hydrophobicity) with the extent of shifting of cleavage to the Gly\(^{18}\) site. Also, no significant correlation was found between predicted secondary structures (e.g. Chou Fasman rules; Ref. 39) and the site of cleavage. The most dramatic alteration in the site of cleavage occurred as a result of the Lys\(^{31}\) to Pro\(^{31}\) substitution which caused all of the cleavage to be redirected back to the Gly\(^{18}\) site. Only three examples of Pro at the +1 position of secretory proteins were found within a database of 583-eukaryotic signal peptides (40). Therefore, although signal peptidase cleavage of a Xaa-Pro bond is possible, the data, taken together, suggest that this type of bond is relatively difficult for the enzyme to break. Signal peptidase has been shown to cleave on the COOH-terminal side of a Pro residue (i.e. serves as a -1 rather than a +1 amino acid) in pheasant prelysozyme c (41) and in our pre(pre\(^{\Delta}\)apoA-II Pro\(^{31}\) mutant (15). We speculate that steric constraints introduced by the Pro side chain at the +1 position or the nature of the Xaa-Pro bond, which involves a tertiary rather than a secondary amine, may limit cleavage by signal peptidase.

**Post-translational Cleavage by Purified HOSP**—These results indicated that *in vitro* signal peptidase processing can be modulated by the introduction of point mutations at the COOH-terminus of the hydrophobic core and at the +1 position. In addition, some mutations enhanced or reduced the extent of uncoupling of translocation with cleavage. The experiments were performed using closed microsomal membrane bilayers which contain signal peptidase activity as well as other components of the translocation machinery. Therefore, the observed modulation in cleavage efficiency could be due to the substrate requirements of signal peptidase and/or to changes in the interactions of the mutant preproteins with components responsible for their presentation to signal peptidase. To address this issue, we determined the sensitivity of the mutants to post-translational cleavage by solubilized, purified HOSP assayed in the presence of detergent-phospholipid micelles.

Translation of mRNAs encoding human preproapoA-II, pre(pre\(^{\Delta}\)apoA-II Gly\(^{20}\), Asp\(^{13}\)Pro\(^{14}\)Gly\(^{21}\), Ala\(^{13}\)Pro\(^{14}\)Gly\(^{21}\), and Ile\(^{13}\)Pro\(^{14}\)Gly\(^{21}\) was performed in reticulocyte lysates in the absence of microsomal membranes. Aliquots of the translation reactions containing \(^{35}S\)methionine-labeled polypeptides were then diluted into HOSP assay buffer in the presence or absence of HOSP. After incubation for 60 min with HOSP, the reaction products were subsequently immunoprecipitated and subjected to SDS-PAGE. The results of such an experiment are shown in Fig. 4. A majority of the wild-type preproapoA-II polypeptides are cleaved in this assay (lanes 1 and 2), cleavage of pre(pre\(^{\Delta}\)apoA-II Gly\(^{20}\) is detectable but much less efficient. Little or no cleavage is detected for the Pro\(^{13}\)Gly\(^{20}\) and Asp\(^{13}\)Pro\(^{14}\)Gly\(^{21}\) mutants while a minor amount of cleavage is observed for pre(pre\(^{\Delta}\)apoA-II Ala\(^{13}\)Pro\(^{14}\)Gly\(^{21}\). The extent of cleavage of Ile\(^{13}\)Pro\(^{14}\)Gly\(^{21}\) exceeds that of its Gly\(^{20}\) parent (compare lanes 4 and 8) but is still less than that of wild-type preproapoA-II (compare lanes 2 and 8). Therefore, the ability to rescue the inefficient co-translational signal peptide cleavage of Pro\(^{13}\)Gly\(^{20}\) by inserting an Ile NH\(^{\bullet}\) to the Pro\(^{13}\) residue is recapitulated in this post-translational cleavage assay using purified HOSP. The relatively efficient post-translational cleavage of the Ile\(^{13}\)Pro\(^{14}\)Gly\(^{21}\) mutant was surprising in light of the extent of uncoupling of its co-translational translocation and cleavage. Therefore, the Ala\(^{13}\)Pro\(^{14}\)Gly\(^{21}\) mutant appears to require the Ala\(^{13}\) Pro\(^{14}\) Gly\(^{21}\) mutant (15) and the site of cleavage. The most dramatic alteration in the basis for the apparent difference in environment that the Ala\(^{13}\) Pro\(^{14}\) Gly\(^{21}\) mutant was surprising in light of the extensive uncoupling of its co-translational translocation and cleavage. Therefore, the Ala\(^{13}\) Pro\(^{14}\) Gly\(^{21}\) mutant appears to require the Ala\(^{13}\) Pro\(^{14}\) Gly\(^{21}\) mutant (15) and the site of cleavage. The most dramatic alteration in the
incubated with HOSP (±) or buffer only (−). The cleavage products of each mutant was carried out in reticulocyte lysates supplemented with [35S]methionine. Aliquots of the translation mix were subsequently incubated with HOSP (+) or buffer only (−). The cleavage products were then immunopurified, separated by SDS-PAGE, and subjected to autoradiography. The downward and upward pointing arrows indicate the unprocessed and processed apoA-II polypeptides, respectively.

![Diagram of membrane bilayer and signal peptides](image)

**TABLE I**

Comparison of the site of post-translational cleavage by HOSP and co-translational processing by canine microsomes.

| Preprotein   | Post-translational HOSP cleavage site | Co-translational membrane cleavage site |
|--------------|--------------------------------------|----------------------------------------|
| PreproapoA-II | Gly⁹⁰ (63%) Gly³⁰ (45%)              | Gly⁹⁰ (65%) Gly³⁰ (55%)                |
| Pre(Δpro)apoA-II Gly³⁰ | Gly³⁰ (63%) Gly⁹⁰ (45%)              | Gly³⁰ (65%) Gly⁹⁰ (55%)                |
| Pre(Δpro)apoA-II Ile⁵⁰Pro⁴Gly²¹ | Gly⁹⁰ (65%) Gly³⁰ (55%)              | Gly³⁰ (65%) Gly⁹⁰ (55%)                |

Interactions of the nascent polypeptide substrate with the membrane bilayer or with polypeptides that have been postulated to form a "translocation pore." These types of mutations of pre(Δpro)apoA-II thus allow us to operationally distinguish functional interactions with components of the translocation apparatus from functional interactions with the cleavage apparatus.

What is the relationship between the translocation and cleavage apparatus? The translocation apparatus could include a non-catalytic subunit(s) of signal peptidase. Cioffi et al. (21) suggested that one signal peptidase subunit may recognize features in the hydrophobic core region while another subunit carries out cleavage. This model is analogous to that proposed for the processing of mitochondrial preproteins (44) in which a processing enhancing protein (PEP) is thought to bind the presequence and thus enhance interaction of its cleavage site with the catalytic polypeptide, mitochondrial processing peptidase (MPP). Since efficient cleavage of our Ile⁵⁰Pro⁴Gly²¹ mutant is achieved by the two subunits which make up purified HOSP, it would appear that some other component(s) is(are) responsible for "inadequate" presentation of this preproteins to signal peptidase in the microsomal membrane. These components may be associated with the translocation machinery or environment.

Since these mutants contain multiple potential sites of processing, it was possible that post-translational HOSP cleavage occurred at different sites than those observed during co-translational translocation across canine microsomal membranes. For those examples where a comparative study was done (Table I), the sites of cleavage were the same. (i) The cleavage of Gly³⁰ at two sites is recapitulated by HOSP. (ii) The Pro⁵⁰Gly³⁰ mutant is also cleaved at two sites when incubated post-translationally with HOSP with most of the cleavage shifted to the Gly³⁰ residue, a trend also observed with in vitro co-translational processing (19). (iii) Cleavage of the Ile⁵⁰Pro⁴Gly²¹ mutant occurred only after the Glu⁶⁵ residue in both systems. The cleavage after Glu⁶⁵ raises the question of why this residue is typically excluded from the −1 position of signal peptides (8, 9). We have proposed (20) that the positioning (i.e. topology) of the signal peptide in the membrane bilayer may influence the site of signal peptidase cleavage. This was based on the observation that the position of the n/h and h/c boundaries of pre(Δpro)apoA-II affects the site of its co-translational signal peptidase cleavage (20). It is likely that the selectivity of signal peptidase for a single specific −1 residue is derived in part from limitations (restrictions) in the presentation of alternative sites due to the conformation of the nascent preprotein. The fact that signal peptidase chooses Glu⁶⁵ over Gly³⁰ for the Ile⁵⁰Pro⁴Gly²¹ mutant in both assays suggests that constraints on the presentation of alternative sites are imposed by an intrinsic signal peptide structure that may exist independently of the presence or absence of the membrane bilayer and components of the translocation apparatus. Direct comparison of the cleavage of additional mutants that have alterations in the predicted positions of both the n/h and h/c boundaries by HOSP and canine microsomes should shed more light on these issues.

This study, combined with the results of other recent studies focusing the n/h boundary of pre(Δpro)apoA-II (20) and hydrophobic core of preproparathyroid hormone (21), suggests that the mechanism of eukaryotic signal peptidase cleavage is dependent not only on features present within the c-region but is also influenced by other regions of the signal and by the +1 residue. Many of the alterations outside of the c-region which influenced the site of signal peptide cleavage were not predicted to do so based on available methods for predicting signal cleavage sites. For example, von Heijne’s (45) weight matrix prediction method gave the Glu⁸⁵ site of the Ile⁵⁰Pro⁴Gly²¹ mutant a much lower score than the Gly³⁰ site which was not used by signal peptidase (data not shown). Therefore, it is likely that these predictive methods could be enhanced by the results of this and related studies which employ systematic mutagenesis of eukaryotic prepeptides.

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Features Influencing Cleavage of Eukaryotic Signal Peptides

Purified Signal Peptide Cleavage Assay. Translation of rabbit reticulocyte lysate was carried out in the presence of microsomal membranes as detailed above. Signal peptide-containing cleavage was performed by incubating a total of 200,000 cpm of translation product, 15 ml of confident buffer (10 mM Tris, 200 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM 2-mercaptoethanol, 5 mM MgCl₂, 100 nM phosphatidylcholine, 20 mM DTT, pH 8.0) and 3 ml of purified bovine signal peptidase and incubating the solution at 30°C for 60 min. Aliquots of the reaction were separated by SDS-PAGE (10% polyacrylamide gel electrophoresis) and immunoblotted with antibodies against cleaved or uncleaved signal peptide.

N-terminally Probed Protein Sequence Analysis of Concurrently Processed Mutant Proteins. Translation reactions were carried out in the presence of 125I-labeled tyrosine (NEN Research Products, Boston, MA). The resultant 125I-labeled translation product was subjected to automated protein sequence determination using an on-line (13) automated sequencer (model 132, Applied Biosystems, Bedford, MA). The amount of each protein was determined using a PhosphorImager and quantitation of the sequence data was determined using Genestudio software.
Residues flanking the COOH-terminal C-region of a model eukaryotic signal peptide influence the site of its cleavage by signal peptidase and the extent of coupling of its co-translational translocation and proteolytic processing in vitro.

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