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# Release of Signal Peptide Fragments into the Cytosol Requires Cleavage in the Transmembrane Region by a Protease Activity That Is Specifically Blocked by a Novel Cysteine Protease Inhibitor*

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**Signal peptides of secretory and membrane proteins are generated by proteolytic processing of precursor proteins after insertion into the endoplasmic reticulum membrane. Liberated signal peptides can be further processed, and the resulting N-terminal fragments are released toward the cytosol, where they may interact with target proteins like calmodulin. We show here that the processing of signal peptides requires a protease activity distinct from signal peptidase. This activity is inhibited specifically with a newly developed cysteine protease inhibitor, 1,3-di-(*N*-carboxybenzoyl-L-leucyl-L-leucyl)amino acetone ((Z-LL)$_2$ ketone). Inhibitor studies revealed that the final, (Z-LL)$_2$ ketone-sensitive cleavage event occurs within the hydrophobic transmembrane region of the signal peptide, thus promoting the release of an N-terminal fragment into the cytosol.**

Secretory proteins and most membrane proteins of eukaryotic cells are expressed as a pre-protein with an N-terminal signal sequence that is essential for protein targeting to the endoplasmic reticulum (ER)[1] membrane and entry into the translocon (1). Signal sequences are usually released from the precursor protein by signal peptidase during passage of the growing polypeptide chain through the ER membrane. Little is known about the fate of the liberated signal peptides. However, they have been postulated to have important biological functions both in the lumen of the ER as well as in the cytosol (2).

Signal peptides, liberated from the precursor protein, can be processed further, resulting in fragments that are released from the membrane (3, 4). In the case of the hormone preprolactin (p-Prl) and the human immunodeficiency virus-1 gp160, the N-terminal portion of the respective signal peptide is released into the cytosol and binds to $Ca^{2+}$/calmodulin *in vitro* (5). These findings imply that processing of liberated signal peptides releases functional peptides that may influence signal transduction pathways in the cell.

More recently, a distinct peptide derived from an N-terminal portion of a signal sequence was found to play a crucial role in immune surveillance of healthy cells. The signal peptides of polymorphic major histocompatibility complex class I molecules contain a highly conserved sequence that is capable of binding to so-called non-polymorphic major histocompatibility complex class I molecules (HLA-E in human) (6). At the cell surface, this peptide-HLA-E complex specifically interacts with an inhibitory receptor on natural killer (NK) cells, thereby monitoring indirectly the level of class I molecule expression (7, 8). Presentation of the signal peptide-derived epitope is dependent on the transporter associated with antigen presentation (TAP) and is independent of the proteasome (9). These results suggest that the signal peptide of class I molecules is processed in a manner analogous to the p-Prl signal sequence, leading to release of the epitope-containing portion toward the cytosol.

In eukaryotes, proteases involved in the processing of signal peptides have yet to be characterized or identified. Protease IV and oligopeptidase A process signal peptides in *Escherichia coli*, but homologous proteases have not been found in eukaryotes (10). A new class of metalloproteases that catalyze so-called intramembrane proteolysis has recently been discovered (11). These proteases cleave their substrate proteins within transmembrane regions and promote release of protein domains toward the cytoplasmic or exoplasmic side of the membrane. Signal peptides may be processed by a similar type of protease that cleaves the peptide within the transmembrane region and facilitates release of peptide fragments from the membrane.

In the present study, we characterize the processing of the p-Prl signal peptide. By using different types of protease inhibitors, we ascribe signal sequence cleavage and signal peptide processing to distinct proteolytic activities. A new inhibitor is introduced that prevents signal peptide processing efficiently without affecting signal peptidase and other proteases such as the lysosomal cathepsins and the proteasome. We show that processing promotes release of the $Ca^{2+}$/calmodulin binding signal peptide portion from the membrane into the cytosol. Furthermore, we have determined the cleavage site of the final processing reaction to lie in the center of transmembrane region of the signal peptide.

## EXPERIMENTAL PROCEDURES

*Synthesis of (Z-LL)$_2$ Ketone*—The ketone inhibitor (Z-LL)$_2$ ketone was synthesized in a single step by coupling an excess of commercially available Z-LL-OH with diamino acetone using the coupling reagent

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[1] The abbreviations used are: ER, endoplasmic reticulum; APP, β-amyloid precursor protein; p-Prl, preprolactin; RM, rough microsomes; SREBP, sterol regulatory element-binding protein; (Z-LL)$_2$ ketone, 1,3-di-(*N*-carboxybenzoyl-L-leucyl-L-leucyl)amino acetone. BOC, di-*t*-butoxycarbonyl-; Bio-, biotin; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; HATU, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate.

HATU and collidine (Fig. 1*B*). The resulting product was then isolated by column chromatography and identified by NMR and mass spectrometry. The biotin derivative, $(Bio\text{-}LL)_2$ ketone, was synthesized as the corresponding di-*t*-butoxycarbonyl-capped derivative $(BOC\text{-}LL)_2$ ketone as described for $(Z\text{-}LL)_2$ ketone, except that BOC-LL-OH was used in place of Z-LL-OH. The resulting BOC-capped derivative was de-protected by brief trifluoroacetic acid treatment followed by precipitation in ether. The activated, nitrophenyl ester of biotin was used to alkylate both free N termini, resulting in $(Bio\text{-}LL)_2$ ketone. The identity of the compound was confirmed by NMR and mass spectrometry.

*Competition Experiments*—Extracts were prepared from the dendritic cell line DC2.4 and the fibroblast cell line NIH-3T3 by mechanical disruption using glass beads in buffer A (50 mM Tris, pH 5.5, 1 mM dithiothreitol, 5 mM $MgCl_2$, 250 mM sucrose) at pH 5.5 (DC2.4 cells) or pH 7.4 (NIH-3T3 cells). Protein concentrations were determined using BCA reagents. Samples of DC2.4 lysates (100 $\mu$g of total protein) were incubated with increasing concentrations (as indicated) of the two ketone inhibitors $(Bio\text{-}LL)_2$ ketone and $(Z\text{-}LL)_2$ ketone and the peptide aldehyde leupeptin for 30 min at room temperature. Cysteine proteases were then labeled by the addition of $^{125}$I-JPM-565 ($\sim 10^6$ cpm/sample) followed by separation by SDS-PAGE and analysis by autoradiography. The same protocol was used for analysis of proteasome activity in NIH-3T3 cells except the peptide aldehyde MG-132 was used instead of leupeptin, and samples were labeled with $^{125}$I-NLVS (12) (data not shown).

*Plasmids and Transcription*—The *Hin*dIII/*Eco*RI fragment of pGEM4/p-Prl (13) coding for p-Prl was transferred into pGEM3Z (Promega) under the control of the SP6 promotor to give pGEM3Z/p-Prl. To prepare mRNA coding for full-length p-Prl, plasmid pGEM3Z/p-Prl was linearized with *Eco*RI and transcribed *in vitro* with SP6 RNA polymerase at 42 °C in the presence of 500 $\mu$M $m^7G(5')ppp(5')G$ CAP analogue (New England Biolabs) (14). To prepare mRNA coding for p-Prl/86, the respective coding region was amplified with polymerase chain reaction using *Pfu* DNA polymerase (Stratagene), SP6 primer, and a reverse primer starting at the 5′-end with the triplet that corresponds to the 86th amino acid residue. When translation was supposed to terminate by proper termination of translation at a given residue (*e.g.* for synthesis of reference peptides), a TAG stop codon was introduced at the relevant position, and a reverse primer starting with 5′-NNNNNNNNNCTA- was used for polymerase chain reaction. Polymerase chain reaction-amplified DNA fragments were transcribed *in vitro* with SP6 RNA polymerase as described above (15).

*In Vitro Translation and Translocation*—Translations of mRNA coding for p-Prl were performed in 25 $\mu$l of reticulocyte lysate (Promega) containing [$^{35}$S]methionine (Amersham Pharmacia Biotech) and, where indicated, 1.5 eq of nuclease-treated rough microsomes prepared from dog pancreas (16), $(Z\text{-}LL)_2$ ketone (0.25 $\mu$l of a 500 $\mu$M stock solution in $Me_2SO$), or $Me_2SO$ (0.25 $\mu$l). Samples were incubated for 30 min at 30 °C. Samples containing microsomes were next diluted with 25 $\mu$l of RM buffer (50 mM HEPES-KOH, pH 7.6, 50 mM potassium acetate, 2 mM magnesium acetate, 1 mM dithiothreitol, 250 mM sucrose), and the salt concentration was raised to 500 mM potassium acetate. After incubation for 5 min on ice, membranes were separated by a 3-min centrifugation through a 100-$\mu$l sucrose cushion (RM buffer with 500 mM potassium acetate and 500 mM sucrose) at 48,000 rpm and 4 °C in a Beckman TLA100 rotor (4). Samples without microsomes were supplemented with 50 $\mu$l of saturated $(NH_4)_2SO_4$ to precipitate the bulk of proteins. The precipitate was recovered by centrifugation and washed once with 500 $\mu$l of 5% trichloroacetic acid and twice with 150 $\mu$l of acetone. Membrane and protein pellets were prepared for SDS-PAGE as described below.

Truncated mRNA coding for p-Prl/86 was translated for 10 min at 30 °C in 50 $\mu$l of reticulocyte lysate containing [$^{35}$S]methionine and nuclease-treated rough microsomes (3 eq). After translation, samples were diluted with 50 $\mu$l of RM buffer, and microsomes were treated with 500 mM potassium acetate and recovered by centrifugation through a sucrose cushion as described above. Microsomes were resuspended in 120 $\mu$l of RM buffer, and samples were split in 20-$\mu$l aliquots. To one aliquot, 0.5 $\mu$l of $Me_2SO$ was added, and the sample was placed on ice; to other aliquots, 0.5 $\mu$l of 40 $\mu$M $(Z\text{-}LL)_2$ ketone, 400 $\mu$M $(Bio\text{-}LL)_2$ ketone, 20 mM calpain inhibitor I, or 20 mM dichloroisocoumarin (all in $Me_2SO$) was added, and the samples were pre-incubated for 3 min at 22 °C. Nascent chains were released by adding 1 $\mu$l of 100 mM puromycin and further incubation at 22 °C for 15 min. Proteins were next either precipitated by adding trichloroacetic acid to 10% and prepared for SDS-PAGE (see below) or membranes were separated by a 10-min centrifugation at 100,000 rpm and 4 °C in a Beckman TLA100 rotor, and the membrane pellet and supernatant were prepared for SDS-PAGE as described below. Translations of mRNAs coding for reference peptides (p-Prl/18, p-Prl/20, p-Prl/25, p-Prl/27, and p-Prl/30) were performed in 25 $\mu$l of wheat germ extract containing [$^{35}$S]methionine (17).

*Signal Peptide Processing with Solubilized ER Membrane Proteins*—To obtain solubilized ER membrane proteins, rough microsomes were first prepared from dog pancreas (17). Associated ribosomes and peripheral membrane proteins were removed by treatment with puromycin/high salt (18), and lumenal proteins were depleted by exposure of microsomes to alkaline pH (19). Membranes were next resuspended (1–2 eq/$\mu$l) by using a Dounce homogenizer in solubilization buffer containing 50 mM Tris-HCl, pH 7.8, 50 mM potassium acetate, 2 mM magnesium acetate, 125 mM sucrose, 1 mM dithiothreitol, and 2% CHAPS. The sample was incubated for 30 min on ice, and non-solubilized proteins were subsequently removed by centrifugation in a TLA100.4 rotor (Beckman Instruments) for 30 min at 75,000 rpm and 4 °C.

For the processing assay, 2 $\mu$l of p-Prl/30 translation mixture (see above) were added to 35 $\mu$l of assay buffer (25 mM HEPES-KOH, pH 7.6, 100 mM potassium acetate, 2 mM magnesium acetate, 1 mM dithiothreitol). Where indicated, 1 $\mu$l of 40 $\mu$M $(Z\text{-}LL)_2$ ketone was added; 1 $\mu$l $Me_2SO$ was added to all the other samples. The reaction was started by the addition of 2 $\mu$l (2 eq) of rough microsomes, puromycin-treated, and content-depleted microsomes or detergent-solubilized microsomes. After incubation at 30 °C for 1 h, proteins were precipitated by adding trichloroacetic acid to 10%, and samples were prepared for SDS-PAGE (see below).

*Electrophoresis*—Proteins and peptides were analyzed by SDS-PAGE using Tris-Bicine gels (20). Membrane pellets were solubilized in 12 $\mu$l of sample buffer containing 360 mM BisTris, 160 mM bicine, 1% SDS, 50 mM dithiothreitol, 15% sucrose, 0.01% bromphenol blue, and 0.004% Serva blue. Supernatants (20 $\mu$l) obtained after treatment with puromycin were supplemented with 1 $\mu$l of wheat germ extract, and proteins were precipitated by adding trichloroacetic acid to 10%. The precipitate was recovered by centrifugation, washed twice with 150 $\mu$l of acetone, and solubilized in 12 $\mu$l of sample buffer. All samples were incubated for 20 min at 65 °C. Proteins were finally separated on 14.25% acrylamide, 0.75% bis-acrylamide, 8 M urea gels (70 × 80 × 1 mm). Labeled proteins were visualized by a STORM PhosphorImager (Molecular Dynamics).

## RESULTS

*Synthesis of Peptide Ketone Inhibitors*—Analysis of the amino acid sequence surrounding the putative intra-membrane cut site of the signal peptide from preprolactin and human immunodeficiency virus-1 gp160 indicated that both contain a cluster of hydrophobic amino acids on either side of the scissile amide bond (Fig. 1*A*). Based on this information, we designed protease inhibitors that contain a central electrophile surrounded by aliphatic amino acid residues. Previously, Veber and co-workers (21, 22) found that symmetrical ketone derivatives served as potent inhibitors of the cysteine protease cathepsin K. These compounds bind to the enzyme such that the peptide portions of the molecules on either side of the ketone electrophile occupy both the prime and non-prime binding sites. We reasoned that analogs of these compounds might be well suited for mimicking the hydrophobic core of the signal peptide and, therefore, might serve as potent inhibitors of the putative signal peptide peptidase activity.

We first synthesized the simple symmetrical di-leucine-containing derivative in which both N termini were capped with the hydrophobic carboxybenzoyl group ($(Z\text{-}LL)_2$ ketone; Fig. 1*B*). The related compound in which the Z capping group was replaced with a *t*-butoxycarbonyl group was also synthesized. This derivative was de-protected with acid, resulting in a di-free amino derivative that was then alkylated by biotin ($(Bio\text{-}LL)_2$ ketone; Fig. 1*B*).

*Analysis of Specificity of Ketone Inhibitors*—To initially determine the specificity of both the Z and biotin ketone derivatives, we performed competition experiments in crude cellular extracts. Lysates from the dendritic cell line DC2.4 were incubated with increasing concentrations of both $(Z\text{-}LL)_2$ ketone and $(Bio\text{-}LL)_2$ ketone for 30 min at room temperature (Fig. 2). After pre-incubation, a radiolabeled general cysteine protease

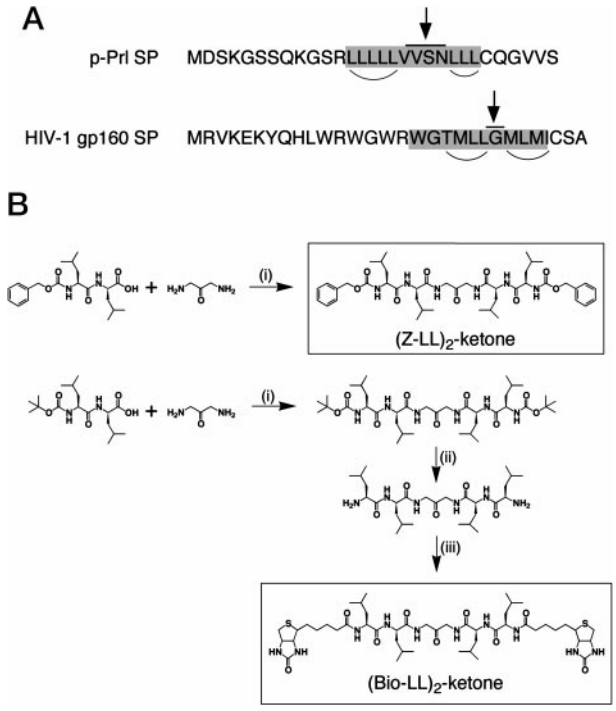


FIG. 1. **Synthesis of symmetrical di-leucine-containing ketones.** *A*, sequence of the signal peptide of preprolactin and human immunodeficiency virus-1 gp160. The transmembrane regions are shown *shaded*, and *brackets* are used to indicate the clusters of long hydrophobic amino acid residues on either side of the predicted signal peptide peptidase cleavage region (*arrow*). *B*, synthesis of di-leucine ketone derivatives from di-amino acetone. (i) HATU, collidine in *N*,*N*-dimethylformamide; (ii) 50% trifluoroacetic acid in $CH_2Cl_2$; (iii) biotin-*p*-nitrophenyl-ester, *N*,*N*-diisopropylethylamine in *N*,*N*-dimethylformamide.

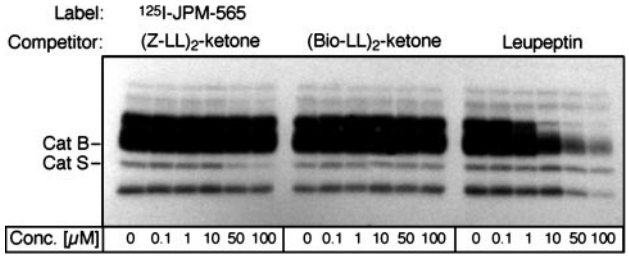


FIG. 2. **Di-leucine-containing ketones do not inhibit the lysosomal cysteine proteases.** The ketone derivatives $(Z\text{-}LL)_2$ ketone, $(Bio\text{-}LL)_2$ ketone, and the peptide aldehyde leupeptin were added to extracts from the dendritic cell line DC2.4 at the concentrations indicated. Lysates were incubated for 30 min at room temperature, and then the cysteine protease affinity label $^{125}$I-JPM-565 was added. *Cat*, cathepsin.

inhibitor $^{125}$I-JPM-565 was added to the extracts. This compound covalently modifies the active site of most of the papain family of cysteine proteases. Intensity of labeling of protease targets serves as readout of enzymatic activity. Thus, prior modification of the active site of the enzyme by either $(Z\text{-}LL)_2$ ketone or $(Bio\text{-}LL)_2$ ketone is observed as a loss of labeling by $^{125}$I-JPM-565. The results from DC2.4 extracts indicated that at low concentrations (0.1–10 $\mu$M) neither the Z nor biotin-capped derivatives are reactive toward any of the multiple lysosomal cysteine proteases targeted by the general cysteine protease label $^{125}$I-JPM-565. The Z compound at high concentrations specifically blocks labeling of a single 25-kDa polypeptide. The general cysteine protease inhibitor leupeptin blocked labeling of most of the JPM-565-reactive proteases, indicating that the assay was an effective readout of lysosomal cysteine protease activity. Therefore, biological effects observed from treatment of samples with low concentrations of the inhibitors $(Z\text{-}LL)_2$ ketone and $(Bio\text{-}LL)_2$ ketone are not likely due to inhibition of lysosomal proteases.

To further establish the reactivity of these ketone derivatives, we performed a similar set of competition experiments in NIH-3T3 extracts using the proteasome label $^{125}$I-NLVS as readout. These experiments provided complementary information about the reactivity of the two compounds toward the proteasome. Both the $(Z\text{-}LL)_2$ ketone and the $(Bio\text{-}LL)_2$ ketone showed no modification of the proteasomal active site at concentrations as high as 100 $\mu$M (data not shown). Thus, these compounds do not block the action of the multicatalytic proteasome complex.

*Distinct Proteases Catalyze Signal Sequence Cleavage and Signal Peptide Processing*—To investigate processing of the p-Prl signal peptide, we used a previously established *in vitro* assay that includes synchronized entry of short p-Prl chains into ER-derived rough microsomes (5). Truncated mRNA coding for the 86 N-terminal residues of p-Prl was translated in the presence of rough microsomes. The resulting p-Prl/86 chains were bound to the ribosomes at their C terminus and inserted into the translocons via their N-terminal signal sequence. Signal sequence cleavage did not occur because the p-Prl/86 chains were too short (Fig. 3*A*, *lane 1*). Microsomes were isolated and resuspended in buffer, and p-Prl/86 chains were released from the ribosome by the addition of puromycin. p-Prl/86 chains were translocated, and the signal sequence was cleaved and processed. The liberated, 30-residue signal peptide was seen after a short incubation with puromycin (Fig. 3*A*, *lane 2*), whereas the processed peptide was obtained after longer incubation (Fig. 3*A*, *lane 3*).

To distinguish the individual proteolytic steps of signal sequence cleavage and signal peptide processing, we tested several different classes of protease inhibitors. The newly developed cysteine protease inhibitors $(Z\text{-}LL)_2$ ketone and $(Bio\text{-}LL)_2$ ketone inhibited signal peptide processing without affecting signal peptidase activity (Fig. 3*B*, *lanes 2* and *3*). The apparent $IC_{50}$ value determined by our assay was ~50 nM for $(Z\text{-}LL)_2$ ketone (Fig. 3*C*) and 1–2 $\mu$M for $(Bio\text{-}LL)_2$ ketone (not shown). Similarly, signal peptide processing was inhibited by calpain inhibitor I, another cysteine protease inhibitor, albeit at much higher concentration (Fig. 3*B*, *lane 4*). In contrast, the serine protease inhibitor dichloroisocoumarin inhibited signal peptidase, thereby blocking release of the signal sequence from the precursor protein (Fig. 3*B*, *lane 5*). These results indicate that signal peptide processing requires at least one protease that is distinct from signal peptidase.

*Processing Promotes Release of the N-terminal Signal Peptide Fragment toward the Cytosol*—We have reported that the N-terminal portion of the p-Prl signal peptide is released into the cytosol *in vitro*, where it binds to $Ca^{2+}$/calmodulin upon cleavage and processing (5). To determine whether processing is required for release of the N-terminal peptide into the cytosol, we treated microsomes with $(Z\text{-}LL)_2$ ketone before release of p-Prl/86 chains with puromycin. After treatment with puromycin, we separated microsomes from the release buffer by centrifugation and analyzed the microsomes and buffer fraction separately. In the presence of $(Z\text{-}LL)_2$ ketone, the unprocessed signal peptide is found exclusively in the membrane fraction (Fig. 4*A*, *lane 5*). In the control reaction where the inhibitor is omitted, the signal peptide is processed, and the N-terminal fragment is released from the membrane toward the cytosolic side and, hence, found in the supernatant fraction (Fig. 4*A*, *lane 4*).

Cytosol facilitates the release of the processed p-Prl signal peptide from the membrane, most likely due to the presence of

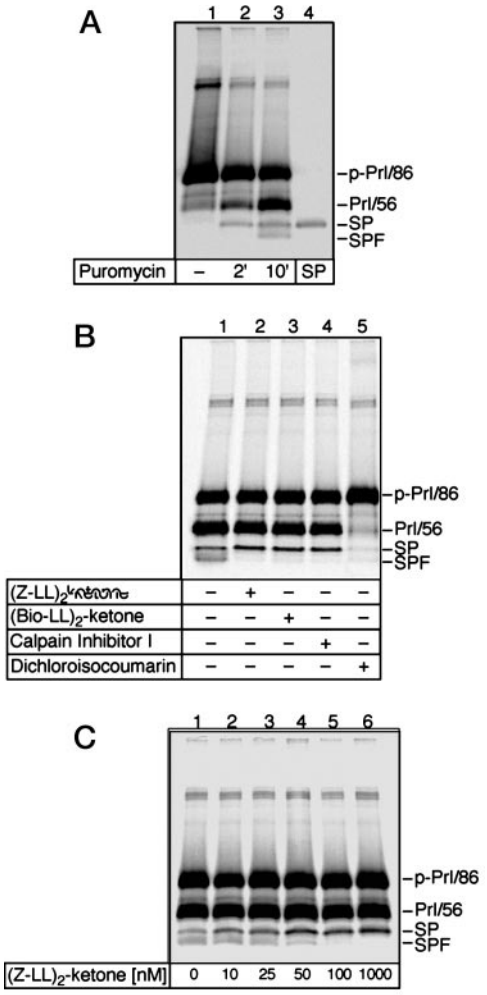


FIG. 3. **(Z-LL)$_2$ ketone inhibits processing of the p-Prl signal peptide.** *A*, cleavage and processing of the p-Prl signal sequence. ER-derived rough microsomes were loaded with ribosome-bound p-Prl/86 chains. Microsomes were next isolated and resuspended in buffer (*lane 1*). To induce synchronized signal peptide cleavage and processing, the p-Prl/86 chains were released from the ribosome by the addition of puromycin. Samples were incubated for 2 min and 10 min, respectively, and subsequently analyzed by SDS-PAGE (*lanes 2* and *3*). *In vitro* translated reference signal peptide is shown in *lane 4*. *SP*, 30-residue-long signal peptide; *SPF*, N-terminal signal peptide fragment. *B*, inhibition of signal peptide processing. p-Prl/86 chains were released from the ribosome as in *A* upon the addition of 1 $\mu$M (Z-LL)$_2$ ketone (*lane 2*), 10 $\mu$M (Bio-LL)$_2$ ketone (*lane 3*), 500 $\mu$M calpain inhibitor I (*lane 4*), and 500 $\mu$M dichloroisocoumarin (*lane 5*). *C*, the apparent $IC_{50}$ value for (Z-LL)$_2$ ketone was determined by releasing p-Prl86 chains upon the addition of increasing amounts of (Z-LL)$_2$ ketone. Half-maximal activation is observed at ~50 nM inhibitor.

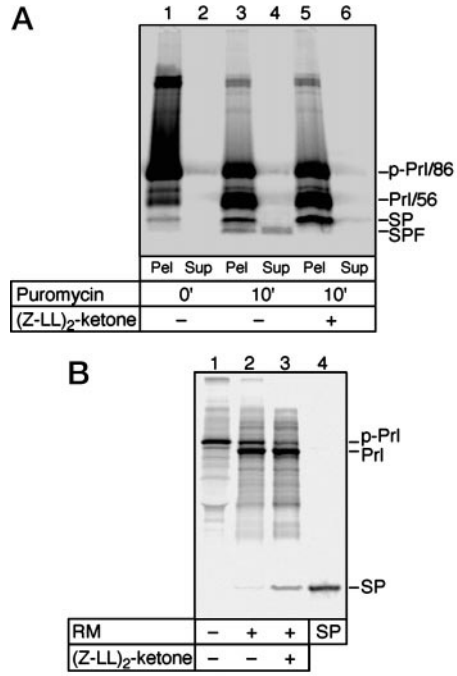


FIG. 4. **Inhibition of processing prevents release of the signal peptide from the membrane.** *A*, microsomes loaded with ribosome-bound p-Prl/86 chains were isolated and resuspended in buffer, and where indicated, 1 $\mu$M (Z-LL)$_2$ ketone was added (*lanes 5* and *6*). p-Prl/86 chains were released from the ribosome by the addition of puromycin (*lanes 3–6*). Samples were incubated for 10 min, and microsomes were subsequently separated from the buffer by centrifugation. Membrane pellets (*Pel*) and buffer fractions (*Sup*) were analyzed separately. The N-terminal portion of the processed signal peptide was released from the membrane (*lane 4*), whereas inhibition of processing retained the signal peptide in the membrane fraction (*lane 5*). *B*, full-length p-Prl chains were synthesized in reticulocyte lysate in the presence of rough microsomes (*lanes 2* and *3*) and (Z-LL)$_2$ ketone (*lane 3*). Microsomes were extracted with 500 mM salt and recovered by centrifugation. The unprocessed signal peptide (*SP*) was retained in the membrane fraction, even in the presence of cytosol (*lane 3*). *Lane 4* shows *in vitro* translated reference signal peptide.

its target, calmodulin (5). To test whether signal peptide processing is required for efficient release of the N-terminal portion, we translated full-length p-Prl in reticulocyte lysate in the presence of microsomes and (Z-LL)$_2$ ketone. Microsomes were extracted with 500 mM salt and sedimented through a sucrose cushion to remove excess cytosolic proteins that interfere with the analysis of small peptides. When (Z-LL)$_2$ ketone was added to the translation mixture, the unprocessed signal peptide was found in the membrane fraction (Fig. 4*B*, *lane 3*). In contrast, no corresponding peptide was obtained in the control reaction without the inhibitor (Fig. 4*B*, *lane 2*). These results show that processing of the p-Prl signal peptide is required for efficient release of the N-terminal peptide portion, even in the presence of cytosol.

*The Signal Peptide Is Processed in the Center of the Transmembrane Region*—The p-Prl signal peptide is thought to be processed in the region between the two leucine clusters of its hydrophobic region (4). To determine the cleavage site, we compared the electrophoretic mobility of the cleavage product with reference peptides. The cleavage product detected must represent the N-terminal fragment, because the p-Prl signal peptide is radioactively labeled at a single methionine residue at its N terminus. Reference peptides were thus easily obtained by *in vitro* translating mRNAs coding for the N-terminal 18-, 20-, 25-, and 30-amino acid residues of the p-Prl signal sequence (Fig. 5*A*).

The cleavage product had mobility identical to that of the 20-residue reference peptide (Fig. 5*A*, *lanes 3* and *4*), indicating that the p-Prl signal peptide is cleaved in the center of its transmembrane region, where the polar residues serine and asparagine interrupt the hydrophobic segment. Interestingly, a common feature of the transmembrane region of signal peptides is the acquisition of a helix-break-helix structure in an apolar environment (23). The break in the helix structure is thought to facilitate membrane entry at the initial phase of protein translocation and may also make the scissile peptide bond accessible to proteolysis during signal peptide processing.

The investigation of signal peptide processing requires ER-derived microsomes that are functional in protein targeting, translocation, and signal sequence cleavage. To analyze processing independently of preceding reactions, we simplified the assay by using the p-Prl signal peptide (p-Prl/30) as the immediate substrate for the cleavage reaction. Because synthetic

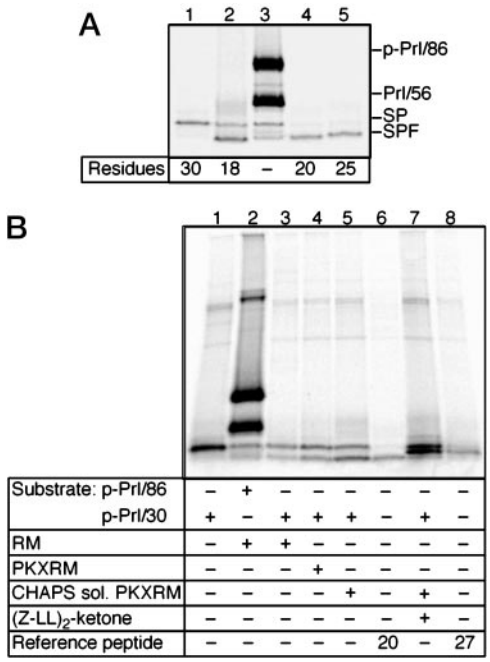


FIG. 5. **The p-Prl signal peptide is processed in the center of the transmembrane region.** *A*, parallel electrophoretic analysis of the signal peptide fragment obtained after puromycin release of p-Prl/86 chains (*lane 3*) compared with *in vitro* translated reference peptides corresponding to the N-terminal 30-, 18-, 20-, and 25-amino acid residues of the p-Prl signal peptide (*lanes 1*, *2*, *4*, and *5*). *B*, signal peptide processing with detergent-solubilized ER membrane proteins. *In vitro* translated signal peptide of p-Prl (p-Prl/30, *lane 1*) was incubated with rough microsomes (*lane 3*), puromycin/high salt and alkali-treated microsomes (PKXRMs, *lane 4*), and CHAPS-solubilized PKXRMs (*lanes 5* and *7*). To one sample, 1 $\mu$M $(Z\text{-}LL)_2$ ketone was added (*lane 7*). The electrophoretic mobility of the resulting cleavage products is compared with the signal peptide fragment obtained after puromycin release of p-Prl/86 chains into rough microsomes (*lane 2*), and 20- and 27-residue-long reference peptides (*lanes 6* and *8*).

signal peptides are known to enter the translocon without the aid of cytosolic components (24), p-Prl/30 is expected to enter the translocon and be processed similarly to the signal peptide that enters the translocon by the conventional protein targeting pathway. Indeed, when *in vitro* translated p-Prl/30 was incubated together with microsomes, we obtained a cleavage identical to the one obtained with the previous assay, where p-Prl/86 chains were inserted into the translocons and cleaved by signal peptidase before the liberated signal peptide could be processed (Fig. 5*B*, *lanes 2* and *3*).

In an initial attempt to characterize the protease that catalyzes processing of the p-Prl signal peptide, we extracted rough microsomes first with puromycin and 600 mM salt to remove the ribosomes and peripherally associated proteins and then with alkali to wash out lumenal proteins (19, 25). The resulting PKXRMs were still active and processed p-Prl/30 to the ~20-residue fragment (Fig. 5*B*, *lane 4*). We then solubilized the residual ER membrane proteins with the detergent CHAPS. When p-Prl/30 was incubated with the detergent-solubilized proteins, it was processed to the ~20-residue fragment, as with intact, untreated microsomes (Fig. 5*B*, *lane 5*). $(Z\text{-}LL)_2$ ketone inhibited the processing reaction, although a ~27-residue fragment was obtained instead (Fig. 5*B*, *lane 7*). The latter cut was most likely performed by signal peptidase that, when solubilized, may have access to a second potential consensus site in the C-terminal extension of the p-Prl signal peptide (26). These results indicate that "intramembrane proteolysis" can be reconstituted using detergent-solubilized membranes.

## DISCUSSION

The role of signal sequences in protein targeting and membrane insertion is well established (27). The fate of signal peptides beyond cleavage from the pre-protein, however, remains unclear. Degradation may be the immediate destination for most signal peptides that are cleared from the ER membrane by as of yet unidentified proteases. However, in some cases signal peptides have been shown to perform functions downstream of precursor protein processing. Dissecting the pathway followed by signal peptides, as presented here, reveals a possible mechanism for how the ER membrane is cleared from peptides and indicates an approach toward the identification of the distinct components involved in the release process.

*Signal Peptide Processing Includes a Novel Type of Intramembrane Proteolysis*—Cleavage of proteins in transmembrane regions and concomitant release of protein domains or peptides from the membrane, as reported here for the signal peptide of p-Prl, has become increasingly evident and is involved in cellular differentiation, lipid metabolism, and presumably, the unfolded protein response (28, 29). The process was named intramembrane proteolysis, assuming that cleavage occurs in the plain of the lipid bilayer, although the proof of proteolysis within the membrane is not provided so far (28).

In animal cells, at least three proteins undergo intramembrane proteolysis according to the definition given above. These proteins are SREBPs (sterol regulatory element-binding proteins) and Notch, transmembrane proteins of the ER and Golgi/plasma membrane, respectively, whose cytosolic transcription factor domains are liberated upon activation, and APP ($\beta$-amyloid precursor protein), which can be processed to the amyloid peptide A$\beta$ suspected to cause Alzheimer's disease (30, 31).

SREBPs and Notch are cleaved close to the cytosolic end of their respective transmembrane regions of type II topology (N in, C out) and type I topology (N out, C in), respectively. In contrast, APP is cleaved in the center of its transmembrane region, which has type I topology. The latter type of cleavage is analogous to the processing of the preprolactin signal peptide, which is also cleaved in the center of the transmembrane region. However, the membrane orientation of the signal peptide is opposite to that of APP. This finding adds a missing link to the group of proteases that perform intramembrane proteolysis: a protease that cleaves in the center of a type II-oriented transmembrane region (29).

With the exception of S2P, proteases that promote cleavage in transmembrane regions have not been identified so far. This putative metalloprotease catalyzes cleavage within one of the two membrane anchors of SREBPs and was identified by complementation cloning (32). Data base searches revealed a family of S2P-like metalloproteases with the common, unusual feature of a HE*XX*H motive within a transmembrane region, suggesting that intramembrane proteolysis is a process conserved in evolution from bacteria to man (11).

Signal peptide processing is inhibited by cysteine protease inhibitors. Inhibition is particularly efficient with the novel $(Z\text{-}LL)_2$ ketone, designed according to the expected cleavage site within the signal peptide. Metalloprotease inhibitors such as EDTA ($\leq$25 mM) and *o*-phenanthroline ($\leq$5 mM) have no effect (not shown). These findings suggest that the unidentified signal peptide peptidase belongs to another class of proteases that cleave presumably in the plane of the lipid bilayer (29). The molecular identification of signal peptide peptidase remains a challenging task for future research.

Intramembrane proteolysis and concomitant release of a functional peptide also occurs in bacteria. The eubacterium *Enterococcus faecalis* secretes an octapeptide pheromone cAF1

that is derived from a signal sequence (33). The pheromone is generated from a precursor protein by cleavages at two sites. First the signal sequence, which contains the octapeptide, is cleaved off by signal peptidase at the extracellular side of the plasma membrane. Then the signal peptide is processed within the transmembrane region by a designated protease Eep that resembles S2P (28, 33).

The processes of SREBP activation and cAF1 release are strikingly similar to the generation of the p-Prl signal peptide fragment described herein. Signal peptidase cleaves the translocating p-Prl chain in the ER lumen and liberates the signal peptide that becomes anchored in the ER membrane. A subsequent cut within the transmembrane region favors the release of the N-terminal peptide portion toward the cytosol. This signal peptide fragment binds to $Ca^{2+}$/calmodulin *in vitro* upon release from the membrane, which led to the speculation that the released peptide may influence calmodulin-dependent signal transduction pathways in a cell (5).

Signal peptides of other proteins may likewise have functions beyond protein targeting and membrane insertion (2). Signal peptide processing could promote the release of these peptides from the ER membrane, as shown here for the preprolactin signal peptide. Liberated into the cytosol or the exoplasmic space, signal peptide fragments can report on the synthesis of major histocompatibility complex class I molecules (7, 8) or inducing a mating response (33). Specific protease inhibitors such as $(Z\text{-}LL)_2$ ketone are powerful tools to further elucidate the role of signal peptide processing *in vitro* and possibly in living cells. Furthermore, such inhibitors may facilitate the biochemical identification of the elusive signal peptide peptidase, particularly in combination with the assay that allows monitoring of signal peptide processing using detergent-solubilized ER membrane proteins. The approach to address intramembrane proteolysis presented here may be applied similarly to identify related proteases such as γ-secretase- and S2P-like proteases.

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