Importance of the Propeptide Sequence of Human Preproparathyroid Hormone for Signal Sequence Function*

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The function of amino-terminal pro-specific peptides (propeptides), sequences often found on intermediate precursor forms of secreted proteins, is poorly understood. Human proparathyroid hormone (prepro-PTH), a precursor protein containing such a propeptide, is initially synthesized as a precursor containing a 25-amino acid signal sequence, a 6-amino acid propeptide, and the 84-amino acid mature secreted peptide. Cloned cDNA encoding prepro-PTH and synthetic oligonucleotides were used to generate a mutant missing precisely the pro-specific sequences. The effects of this deletion on signal sequence function and on secretion per se were assessed after expression of the mutant cDNA in intact cells and in a cell-free translation system using synthetic mRNA in the presence of microsomal membranes. The mutant precursor protein was inefficiently translocated and cleaved, and cleavage occurred both at the normal site and within the signal sequence. Thus, for the eukaryotic protein prepro-PTH, sequences immediately downstream and separate from the classically defined signal sequence facilitate accurate and efficient signal function.

Secretory proteins are generally translated with amino-terminal sequences, termed signal sequences, that mediate interaction with the membrane of the rough endoplasmic reticulum (Blobel and Dobberstein, 1975a, 1975b; Hortsch and Meyer, 1986; Walter et al., 1984). In some cases, after signal cleavage the resultant protein is identical to the mature secreted form. Often, however, intermediate precursors, called pro-proteins, missing the signal sequence but containing peptide residues not found in the mature secreted protein, are found intracellularly. The extra, pro-protein-specific regions, termed propeptides, are proteolytically processed from the intermediate precursor only at the distal aspect of the secretory pathway, in the trans-Golgi network (Githiffs and Simons, 1986) and developing secretory granule (Orci et al., 1986). Cleavage occurs after dibasic residues, by enzymes still being characterized (Steiner et al., 1984).

Unlike signal sequences, pro-specific sequences are unlikely to have one uniform function from protein to protein, because their lengths, locations in the precursor, and structures vary so greatly (Docherty and Steiner, 1982). Possible functions of propeptides include: 1) ensuring correct folding of the protein (Steiner and Clark, 1968); 2) providing a minimum length needed for transport across the endoplasmic reticulum; 3) providing linkage for coordinate translation of related secreted peptides, such as the cleavage products of proopiomelanocortin (Herbert and Uhler, 1982); 4) conferring a diverse spectrum of actions to a family of alternatively cleaved precursors, such as the precursor of somatostatins SRIF-14 and SRIF-28 (Noe and Spiess, 1983); 5) acting as sorting sequences (Guan and Rose, 1984) to guide precursors through the secretory pathway from the rough endoplasmic reticulum to the Golgi, where cleavage of the propeptide ensues; 6) directing conformation of protein, as suggested by the observation that the propeptide plays a role in post-translational γ-carboxylation of Factor IX (Jorgensen et al., 1987); or 7) forming a functional unit with the signal peptide, to assure optimal signal function.

The site of cleavage of the signal from any precursor protein can be predicted fairly reliably, with rules based on the observed frequency of certain amino acid residues, relative to the cleavage site, in already characterized signals (Perlman and Halvorson, 1983; von Heijne, 1983, 1984, 1986a, 1986b). From such analyses, a pattern of acceptable and forbidden sequences bordering the cleavage site emerged. Acceptable cleavage domains conform to the following rules: the residue at position −1 from the cleavage site must be small (i.e. Ala, Ser, Gly, Cys, Thr, or Gin); and the residue at position −3 must not be aromatic, charged, large or polar, or a proline. This (−3, −1) rule, combined with the expected distribution of other amino acids within the cleavage domain (−13 to +2), has been used to construct a weight matrix to calculate the probability of cleavage at a specific site within the cleavage domain of eukaryotic proteins. The analysis has shown further that signal cleavage usually occurs within a window of 4–10 residues from the end of the hydrophobic core and that sequences immediately distal to the cleavage site tend to minimize choices for alternate cleavage sites (von Heijne, 1986b).

To investigate the function of propeptides, we have chosen as a model the secretory protein preproparathyroid hormone (prepro-PTH). Biosynthetic studies indicate that, in the parathyroid gland, parathyroid hormone (PTH) is formed first as the larger peptide prepro-PTH. This precursor undergoes two successive proteolytic cleavages to yield PTH; the transitory amino-terminal signal peptide is cleaved off in the rough endoplasmic reticulum co-translationally to yield the intermediate precursor pro-PTH (Cohn and Elting, 1983; Habener et al., 1979) which is processed later in the Golgi to produce PTH (Habener et al., 1979). Secreted mature PTH is...
an unglycosylated 84-residue protein whose classical actions are to raise blood calcium and lower blood phosphorus by acting on bone, kidney, and, indirectly, on intestine (Habener et al., 1984). The primary translation product, pre-pro-PTH, has a 25-amino acid signal, followed by the highly basic 6-residue propeptide sequence.

The small size of pre-pro-PTH's propeptide makes unlikely most of the hypotheses about function outlined above. The human propeptide (Lys-Ser-Val-Lys-Arg), is cleaved from pre-PTH just before secretion, presumably in the trans-Golgi tubular network. Processing occurs after the dibasic residues Lys-Arg. Pre-PTH is not secreted from cells (Habener, 1979), and neither the pro-specific fragment nor any of the its possible degradation products accumulates in the cell (Habener, 1979); thus, any role for the propeptide must be an intracellular one.

The propeptide of pro-PTH should serve as an appropriate model for analyzing the function of other short amino-terminal propeptide sequences. To test for potential functions, we have used a human pre-pro-PTH cDNA clone to generate a mutant gene (called pre(Apro)-PTH) lacking precisely and only the pro-specific sequences. The propeptide deletion was created by oligonucleotide-directed mutagenesis. Two independent but parallel approaches were used in the analysis: the mutant pre-PTH cDNA was integrated stably into eukaryotic secretory cells using a retroviral vector; furthermore, the cDNA was expressed by translation of synthetic mRNA in a cell-free extract containing microsomal membranes. The studies reported here define a role for the propeptide in signal function, since the pre(Apro)-PTH mutant precursor is 1) inefficiently translocated, 2) inefficiently processed to PTH-related peptides, and 3) cleaved at both the normal site and at an alternate site located two amino acids proximal to the normal site within the signal sequence. These results indicate that, in the eukaryotic protein pre-pro-PTH, sequences immediately downstream and separate from the classically defined signal facilitate several aspects of signal sequence function.

**EXPERIMENTAL PROCEDURES**

**Materials**

Restriction enzymes, T4 DNA ligase, and Escherichia coli RNA polymerase were from New England Biolabs. Formalin-fixed Staphylococcus aureus (IgG-sorb) was from the Enzyme Center, Inc. Polymerase were from New England BioLabs. Formalin-fixed Staphylococci secretory cells using a retroviral vector; furthermore, the polymerase were from New England BioLabs. Formalin-fixed Staphylococci secretory cells using a retroviral vector. The cells were replated every 5-7 days, using 0.05% trypsin and 0.02% EDTA and EDTA, and subjected to automated Edman degradation using a Beckman 1215C protein sequenator and a 0.1 M quadrupole program as described previously (Kronenberg et al., 1983).

**Plasmid Constructions—**The methods for preparation of plasmid DNA, cleavage with restriction enzymes, purification of fragments, and gel electrophoresis procedures were performed as described (Hindley et al., 1981). A plasmid encoding pre(Apro)-PTH was constructed using oligonucleotide-directed mutagenesis (see “Results”). The synthetic 82-base oligonucleotide fragments were synthesized on a Model 380A DNA synthesizer from Applied Biosystems, by the solid-phase phosphoramidite method. Sequencing of the mutant cDNA, to confirm the sequence (after cloning), was by the chemical method of Maxam and Gilbert (Maxam and Gilbert, 1980). The pZIP + -gpt was a gift of R. Mulligan.

**DNA Transfections and Viruses—**DNA transfections were performed with 10 μg of DNA per ml by the protamine sulfate method of Shields and Blobel (1978), or purchased from Promega Biotec.

**RESULTS**

Construction of pre(Apro)-PTH Deletion Mutant cDNA—Oligonucleotide-directed mutagenesis was used to delete the 18 nucleotides that encode the 6-amino acid propeptide from pre-pro-PTH. Synthetic HindIII and BamHI cleavage sites had previously been inserted at the ends of human pro-pre-PTH cDNA (Born et al., 1986). Employing a BLII site fortuitously located precisely at the end of the prepro-sequence,
the entire "prepro" coding sequence was excised by digestion with HindIII and with BglII. Two 82-base oligonucleotides were synthesized and provided the complementary strands encoding the entire 25-amino acid prepeptide with appropriate cohesive ends. The oligonucleotides were annealed by slow cooling from 68 °C to room temperature, in low salt buffer (10 mM Tris, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol) at 100 ng/μl for each strand. This double-stranded sequence was then ligated just upstream from the coding sequences for mature PTH. This construction thus deleted precisely and only the sequences encoding the prepeptide, generating pre(Apro)-PTH cDNA. The sequence, orientation and reading frame of the deletion plasmid were confirmed by DNA sequencing using the chemical method (Maxam and Gilbert, 1980).

From the parent construct, two other plasmids were made; one based upon an E. coli expression plasmid pGL101 (Guarner et al., 1980) for studies employing cell-free translation assays using synthetic mRNA, and one based upon the plasmid pZIP-v-gpt (analogous to pZIP-neoSV(X)l (Cepko et al., 1984) but containing the gpt gene) used to generate recombinant retrovirus that, in turn, was used to infect GH4Cl cell lines. In the pGL101-based construct, transcription of pre(Apro)-PTH mRNA was driven from the lac promoter in vitro using E. coli RNA polymerase. Messenger RNA produced in this reaction was then used to program cell-free translation of pre(Apro)-PTH protein. Clonal GH4Cl cell lines stably expressing mutant human pre(Apro)-PTH cDNA were obtained by infection of cultures with recombinant retrovirus by calcium-phosphate precipitation. These cultures package infective viral particles that contain only the pre(Apro)-PTH RNA. Medium harvested from these cultures 20 h later contained the recombinant retrovirus that was used to infect GH4Cl cells. Infectected cultures were grown under guanine-xanthine phosphoribosyl transferase (gpt) selection in the presence of mycophenolic acid. Clones expressing gpt activity, and presumably pre(Apro)-PTH, were isolated.

Processing of Pre(Apro)-PTH in Cell-free Extracts—Translation reactions using synthetic mRNA, carried out in reticu-locyte lysate in the presence of canine pancreatic microsomal membranes, were used to assay in vitro the early events in the processing of pre(Apro)-PTH. In this assay, proteins are cotranslationally translocated across the membrane, and signal sequences are cleaved by signal peptidase, located on the inner face of the membrane (Jackson and Blobel, 1977; Kreil, 1981). The mRNA was transcribed in vitro from normal and mutant linearized plasmids (20 μg), using E. coli RNA polymerase. The template DNA was digested with RQ1 DNase (20 units), and the mRNA was extracted with phenolchloroform and ethanol-precipitated. Messenger RNA concentration was measured spectrophotometrically after resuspending the pellets in H₂O. Both normal and mutant constructs yielded identical amounts of message (data not shown). Synthesis of prepro-PTH was performed with undiluted mRNA while synthesis of pre(Apro)-PTH was performed with mRNA diluted 1:5 and 1:10 (see below).

Translation reactions were performed in the absence or in the presence of increasing concentrations of rough micro- somes from canine pancreas. To assay for proteolytic pro-cessing by signal peptidase, proteins were translated in the presence of [³H]methionine, immunoprecipitated with affinity-purified anti-PTH antisera, separated on 15–20% SDS-polyacrylamide gradient slab gels, and processed for fluorography.

The results of translation reactions directed by pPTHlac3 (Born et al., 1986), a pGL101-based plasmid encoding normal human prepro-PTH, are shown in the upper panel of Fig. 1. The primary translation product in the absence of membranes (0 equivalents) was prepro-PTH. The more rapidly migrating immunoprecipitatable proteins are PTH-related peptides that are generated by internal initiation at the methionines at positions 7, 39, and 49 in the prepro-PTH molecule. The identity of all PTH-related peptides was established by amino-terminal radiosequence analysis (data not shown). Translation in the presence of increasing concentration of rough microsomes from canine pancreas (1, 2, and 4 equivalents) resulted in cleavage of the signal, producing pro-PTH. Increasing the concentration of membranes generated more processed pre-PTH and less prepro-PTH, with almost complete conversion at the highest dose (4 equivalents) of membranes employed.

When message concentrations were equal, more pre(Apro)-PTH peptide was translated than normal prepro-PTH (data not shown). This variable efficiency of translation presumably results from the differences in the mRNA structures: the prosequence deletion and differences in the 5' - or 3'-untranslated regions.

Relatively equivalent amounts of protein were synthesized when the mutant mRNA was diluted 1:5 relative to the normal mRNA (Fig. 1). Even under conditions in which less mutant

![Fig. 1. Cleavage of PTH-related precursors by increasing doses of microsomal membranes.](image-url)
peptide was synthesized (1:10 dilution), the processing was somewhat inefficient. Fig. 1 compares quantitatively the cleavage of normal prepro-PTH to the cleavage of pre(Δpro)-PTH. The relevant bands were scanned, and the fraction of synthesized protein cleaved at each membrane concentration was calculated. Even at low protein concentrations, pre(Δpro)-PTH peptide was inefficiently processed when compared to normal prepro-PTH. The difference between the processing of pre(Δpro)-PTH and prepro-PTH was statistically significant at the p = 0.037 level using the nonparametric triangle test (Hollander and Wolfe, 1973).

Deletion of the propeptide from the prepro-PTH molecule therefore resulted in inefficient cleavage of the signal in a cell-free system, even though the actual signal sequence was unaltered in the mutant precursor. As will be discussed in more detail later, amino-terminal sequence analysis was carried out on the processed PTH product to determine the site of cleavage. The predominant processed protein produced in the reticulocyte lysate was authentic PTH, generated by cleavage of the signal sequence at the normal position (data not shown).

Translocation of Pre(Δpro)-PTH through the Membrane— A proteolytic protection protocol was used to determine the location of precursor and product in the cell-free system, relative to the phospholipid bilayer. Since signal peptidase is located on the inner face of the membrane, any processed product must have been translocated through the membrane in order to have been a substrate for signal peptidase activity. However, translocation can occur without signal cleavage (Hortin and Boime, 1981), and consequently must be measured by an assay independent of cleavage. Limited trypsin/chymotrypsin digestion in the presence of membranes therefore more directly assesses translocation through the membrane. Trypsin and chymotrypsin were added after the completion of translation in the presence of rough microsomes from canine pancreas and [3H]leucine, and samples were incubated for 90 min on ice. Radiolabeled proteins were immunoprecipitated after inactivating the proteases with aprotinin and subjected to SDS-polyacrylamide gel electrophoresis as described above.

Radiolabeled protein resistant to degradation is presumed to be protected within a membrane-bound space, isolated from protease digestion. In the presence of rough microsomes from canine pancreas, pro-PTH cleaved from prepro-PTH was completely protected from limited digestion (Fig. 2, lanes 3 and 6), while precursor prepro-PTH was sensitive to digestion and disappeared. Similarly, processed PTH from pre(Δpro)-PTH was also protected (lanes 11 and 14), while the precursor was not. The first two lanes in each set again demonstrate the inefficient processing of pre(Δpro)-PTH seen in Fig. 1 (compare lane 2 to lane 10 in Fig. 2). The translocation of pre(Δpro)-PTH was therefore less efficient than the translocation of normal prepro-PTH. The PTH-peptides were inherently digestible, since disruption of membrane integrity with detergent led to digestion of both the precursor and the product (lanes 4, 7, 12, and 15) radiolabeled proteins at two different protease concentrations. The peptides were not protected from digestion by a nonspecific hydrophobic interaction with membranes, since addition of microsomes after translation did not protect them from digestion (lanes 5, 8, 13, and 16).

Processing and Secretion of Pre(Δpro)-PTH by Cells Infected with Recombinant Retrovirus—The cell-free studies outlined above indicate that processing of pre(Δpro)-PTH is inefficient. Cell-free systems best assay the early steps in secretion. In order to examine the later stages of secretion, and to confirm the cell-free data, clonal secretory cell lines expressing pre(Δpro)-PTH were established using recombinant retrovirus. The rat pituitary GH4C1 tumor cell line was chosen as the recipient cell line for the transfection studies, because it is a well characterized prolactin-secreting line (Tashjian et al., 1978). These cells do contain some vesicles and secretory granules at the cell periphery, and respond in a physiological manner to thyrotropin-releasing hormone by increased release of prolactin (Tashjian et al., 1978). PTH-derived peptides use the normal secretory pathway in these cells, since clones expressing prepro-PTH respond to a thyrotropin-releasing hormone challenge by increased release of PTH into the media (Hellerman et al., 1984). The pre(Δpro)-PTH recipient GH4C1 clones derived by gpt selection were characterized by immunoprecipitation using affinity-purified goat anti-human PTH antisera. Four independent clones were established and shown to secrete identical products; one was used for the detailed characterization of pre(Δpro)-PTH processing.

Cells were pulse-labeled for 15 min with [35S]methionine and then chased for the times indicated (Fig. 3a) with medium containing excess nonradioactive methionine. Media and cell lysate samples were immunoprecipitated and separated on

![Fig. 2. Translocation of PTH-related peptides.](image)

![Fig. 3. Pulse-chase analysis of control GH4C1 cells and GH4C1 cells synthesizing prepro-PTH pre(Δpro)-PTH.](image)
15-20% SDS-polyacrylamide gradient slab gels and then processed for fluorography. The results of a pulse-chase analysis of a clone expressing normal prepro-PTH are shown in Fig. 3a. As in normal parathyroid cells, no prepro-PTH precursor was detected after a 15-min pulse labeling of such cells (lane 1), because cleavage and removal of the signal sequence is presumably cotranslational. The predominant products immunoprecipitated at the earliest time point were pro-PTH and PTH (lane 1). With time, pro-PTH was converted to PTH (lanes 2-5), and PTH was found secreted into the medium at 15-30 min post-chase (Fig. 3b). Neither precursor prepro-PTH nor pro-PTH were secreted from the media from these cultures.

In cells that had integrated the pre(Apro)-PTH recombinant retrovirus, the protein that co-migrated with the uncleaved precursor pre(Apro)-PTH marker protein produced in a cell-free extract without membranes (marker not shown) was the predominant PTH-related protein at the end of the 15-min pulse (Fig. 3a). The mutant clonal cell line contains less peptide than the clonal line expressing normal prepro-PTH, presumably because the site of integration affects the level of gene expression. The inefficient processing of the pre(Apro)-PTH precursor (lane 6) was in striking contrast to the efficient processing of normal prepro-PTH (which is therefore not visible in lane 1). The precursor pre(Apro)-PTH, although a predominant band at the earliest time point, rapidly disappeared from the cells. Since the precursor was not secreted into the media (Fig. 3b), it was either rapidly degraded or cleaved to the PTH-sized product in Fig. 3a.

In the medium from cultures expressing pre(Apro)-PTH, PTH-sized peptide was secreted with kinetics similar to that of native PTH, appearing around 30 min (Fig. 3b, lane 8). No bands that co-migrate with PTH-peptides were immunoprecipitated from untransfected GHCl (lanes 11-15).

Sequence Analysis of Products Secreted into the Medium—The sites of cleavage of normal prepro-PTH are accurate in GHCl cells (Hellerman et al., 1984). To characterize the site of cleavage of the precursor pre(Apro)-PTH that generates the PTH-sized protein in GHCl cells, amino-terminal radiasequence analysis was performed. [35S]Methionine was used to label the protein, since mature human PTH contains methionine residues at positions 8 and 18. The cleavage products secreted into the medium during a 2-h labeling of cells were eluted from a gel slice after preparative high resolution polyacrylamide gel electrophoresis and subjected to repetitive automated Edman degradation. The longer gel allowed the resolution of two closely spaced bands of nearly equal intensity. As can be seen in Fig. 4b, sequence analysis of the lower band from the medium revealed [35S]methionine peaks at positions 10 and 18, indicating cleavage at the normal end of the signal sequence, after a glycine residue. This finding confirms the result found with in vitro processing (data not shown). However, sequence analysis of the upper band showed [35S]methionine peaks at positions 10 and 20 (Fig. 4a). Cleavage to produce this peptide therefore occurred after serine 23 of the pre(Apro)-PTH precursor, 2 amino acids back into the signal sequence, yielding Asp-Gly-PTH as the processed product (Fig. 4c). Cleavage at this site conforms to the (-3, -1) rule, although this site is only three amino acid residues downstream from the end of the hydrophobic core of the signal. In the pulse-chase study (Fig. 3a) these two peptides were found both in the media and in the cell lysates. Both normal PTH and Asp-Gly-PTH appeared in both the cell lysate and media samples with the same kinetics. Removal of the propeptide therefore led to ambiguity in the signal sequence at this site identified by signal peptidase for cleavage of the signal peptide, but did not dramatically alter the kinetics of secretion. A second cleaved product was not detected in the cell-free translation reactions, in contrast (Figs. 1 and 2). However, sequence analysis of the product synthesized in vitro suggested that a small amount of Asp-Gly-PTH might be synthesized in the cell-free system (data not shown).

**DISCUSSION**

The role played by amino-terminal propeptides in the complex process of secretion has not been clarified. In order to characterize potential functions of propeptides, we have used human prepro-PTH as a model secretory protein, and deleted the hexapeptide prosegment by oligonucleotide-directed mutagenesis of cloned cDNA. This precise deletion created a cDNA encoding a peptide consisting of the naturally occurring 25-residue-long signal peptide from prepro-PTH fused in phase with the mature PTH molecule. We have used this cDNA to create vectors that program both in vitro reactions and intact cells for the expression of the pre(Apro)-PTH precursor peptide.

We have demonstrated that precise deletion of the propeptide-specific domain from the prepro-PTH molecule leads to inefficient translocation and processing of the signal sequence from the precursor pre(Apro)-PTH. Furthermore, the deletion of the pro-specific peptide leads to ambiguity in the signal cleavage sites chosen by signal peptidase. These results suggest that sequences immediately downstream from (carboxyl-terminal to) the classically defined signal sequence are im-
Propeptide Sequence Important for Signal Function

When the processing efficiency of the pre-

3 The presence of a prepeptide is essential for proper signal function. The possible generality of this observation is suggested by the exonic organization of the human, bovine, and rat PTH genes (Vasicek et al., 1983; Weaver et al., 1984; Heinrich et al., 1984). The first exon of the PTH genes contain 5'-noncoding sequences. Exon II contains both the entire signal and sequences encoding the first four amino acid residues of the propeptide domain (Vasicek et al., 1983; Weaver et al., 1984, Heinrich et al., 1984). Given the hypothesis that exons are organized into functional domains (Gilbert, 1978), this organization supports the hypothesis that the propeptide segment, necessary for accurate cleavage and efficient translocation, be considered as part of the domain of the signal sequence. Many other signal-encoding exons similarly include portions of pro-encoding sequences.

Deletion of the propeptide portion of prepro-PTH does not dramatically alter the kinetics of secretion of PTH-related peptides in GH4C1 clones expressing pre(Apro)-PTH. Thus, the processed PTH-sized doublet travels the secretory pathway with relatively the same kinetics as does normal PTH. The rapid secretion of PTH peptides from the cells synthesizing pre(Apro)-PTH suggests that the pro-specific signal sequence is not required for efficient transport of the PTH molecule from the endoplasmic reticulum to the Golgi.

Translocation of pre(Apro)-PTH through the membrane was less efficient than the translocation of wild type prepro-PTH in cell-free extracts. This result is particularly interesting and somewhat puzzling because the signal is unaltered in the pre(Apro)-PTH mutant peptide. The explanation for this result is not obvious. Perhaps the efficiency of cleavage by signal peptidase is reduced; in that case, the uncleaved precursor might fail to cross the membrane or even slip back into the cell-free equivalent of the cytoplasm. Alternatively, the propeptide may directly influence translocation by a mechanism unrelated to inefficient signal cleavage.

One of the most striking alterations in phenotype of the pre(Apro)-PTH precursor is the use of alternate cleavage sites by signal peptidase. In an attempt to define the features present in the mutant precursor peptide that might be responsible for the use of the alternate cleavage site, the signal sequence was characterized by von Heijne's probabilistic method (von Heijne, 1986b). This method allows comparison of the mutant precursor to sequences of other characterized precursor proteins to predict appropriate cleavage sites. Since the residues bordering the alternative cleavage site are identical in the mutant and normal peptide, there is no difference in the calculated probability that the alternate site, within the signal sequence, will be used. While the probability of cleavage at the alternate site is high for both peptides (data not shown), the alternate site is, in fact, only used in the context of the pre(Apro)-PTH peptide. This site is used even though it is only 3 residues from the end of the hydrophobic core, outside of the cleavage window. Furthermore, a second site with high probability, after the serine at position 1 plus 1 in mature PTH, is within the window for cleavage, and is used in related mutants missing the last several residues of the signal sequence as well as the propeptide, but not in the pre(Apro)-PTH precursor.

Examination of the primary structure of the pre(Apro)-PTH mutant thus fails to completely predict its phenotype. It has been suggested (Perlman and Halvorson, 1983) that an alteration of β-turn potential and interruption of helical domains may be important structural features in determining the site of signal peptidase cleavage. Secondary structure predictions of the prepro-PTH molecule (Chou and Fasman, 1978) demonstrate that the pro-specific segment has no marked tendency to form secondary structure, either of the helix or β-sheet type (data not shown). This possibility unstruc-
established, however, that deletion of the prosegment of pre-pro-PTH results in reduced efficiency of processing and in ambiguity in the sites selected by signal peptidase for cleavage in vitro and in intact cells. The prosegment therefore contributes to accurate and efficient signal function.

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