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Inefficient Membrane Targeting, Translocation, and Proteolytic Processing by Signal Peptidase of a Mutant Preproparathyroid Hormone Protein*

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A preproparathyroid hormone allele from a patient with familial isolated hypoparathyroidism was shown to have a single point mutation in the hydrophobic core of the signal sequence. This mutation, changing a cysteine to an arginine codon at the −8 position of the signal peptide, was associated with deleterious effects on the processing of preproparathyroid hormone to proparathyroid hormone *in vitro*. To examine the biochemical consequence(s) of this mutation, proteins produced by cell-free translation of wild-type and mutant cRNAs were used in assays that reconstitute the early steps of the secretory pathway. We find that the mutation impairs interaction of the nascent protein with signal recognition particle and the translocation machinery. Moreover, cleavage of the mutant signal sequence by solubilized signal peptidase is ineffective. The consequence of this mutation on processing and secretion of parathyroid hormone is confirmed in intact cells by pulse-chase experiments following transient expression of the mutant protein in COS-7 cells. The inability of the mutant signal sequence, however, to interfere with the targeting and processing of other secreted proteins does not support obstruction of the translocation apparatus as the mechanism underlying the dominant mode of inheritance of hypoparathyroidism in this family.

Proteins destined for residence within membranes or for secretion contain hydrophobic amino-terminal sequences referred to as signal sequences (1). These sequences direct the nascent polypeptides bound to ribosomes to form a functional junction with rough endoplasmic reticulum (RER)[1] membranes, thereby assuring the translocation of the growing polypeptide chain into the lumen of the endoplasmic reticulum and its subsequent cleavage by the luminal-localized signal peptidase enzyme (for review, see Ref. 2).

The nascent secretory protein is localized to the endoplasmic reticulum via a targeting apparatus consisting of the signal recognition particle (SRP) and its membrane-bound receptor on RER. The SRP binds to the signal sequence as it emerges from the large ribosomal subunit. This results in a transient delay or even arrest of translation (3) aimed in preventing premature folding of the precursor protein. When the SRP-ribosome complex encounters the SRP receptor or "docking protein," a series of reactions take place that result in the insertion of the nascent chain into the translocation site, release of SRP, resumption of translation, and initiation of translocation. GTP binding and its hydrolysis are required for these events to take place (4–6). As the nascent polypeptide transverses the translocation channel (7, 8) and emerges into the lumen of the endoplasmic reticulum, it is modified further by the signal peptidase enzyme complex that catalyzes the endoproteolytic cleavage of the signal sequence.

Only a small number of natural mutations in human signal sequences have been reported to have direct correlation with defective secretion and associated pathological states (9–11). We have described such a mutation in the signal peptide of one allele of preproparathyroid hormone (prepro-PTH) gene from a kindred with a form of familial isolated hypoparathyroidism (9). This is an inherited metabolic disorder characterized by hypocalcemia and hyperphosphatemia resulting from lack of biologically active circulating PTH, the major calcium-regulating peptide. In this family, the disorder was inherited as an autosomal dominant trait (12). The single point (T to C) mutation changed the codon at position −8 (signal peptide residues are numbered negatively starting from the site of cleavage toward the amino terminus) of the signal peptide of prepro-PTH from cysteine to arginine, thereby disrupting the hydrophobic core of the signal sequence (Fig. 1). Associated with this change was a dramatic impairment in the processing of the *in vitro* translated mutant prepro-PTH protein to pro-PTH by microsomal membranes (9).

Which step(s) of the early secretory process is affected by this mutation is not readily evident. Conceivably it could preferentially affect one or all of the steps involved, such as binding to SRPs, targeting to the RER, translocation through the membrane, and proteolytic processing by signal peptidase. In this report, we have systematically examined each of these steps using mutant and wild-type forms of *in vitro* translated prepro-PTH proteins by assaying their interaction with components of these various processes. Moreover, the consequence of this mutation on processing and secretion of PTH was examined in intact cells by transient expression of the mutant protein in COS-7 cells. Finally, we have used a co-transfection assay to

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[1] The abbreviations used are: RER, rough endoplasmic reticulum; SRP, signal recognition particle; PTH, parathyroid hormone; PAGE, polyacrylamide gel electrophoresis.

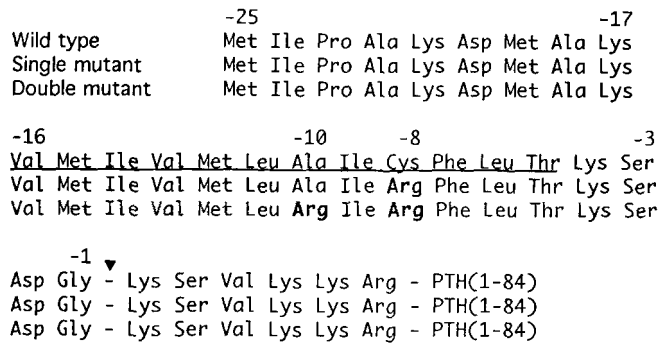

FIG. 1. **Signal peptide sequence of human prepro-PTH.** Amino acids −25 to −1 constitute the signal peptide of wild-type and mutant (single and double) forms of human prepro-PTH. The 6 residues following the signal peptidase cleavage site (*arrowhead*) make up the pro sequence, and the 84 amino acids of mature PTH follow. Residues −16 to −5 (*underlined*) comprise the hydrophobic core of the signal peptide. The described patient's mutation (Cys → Arg at the −8 position; single mutant) and the additional substitution at the −10 position of the signal peptide (Ala → Arg; double mutant), are indicated by boldface. *Numbers above* the amino acids indicate their position relative to the signal peptidase cleavage site.

define the mechanism by which this specific mutation could cause clinical hypoparathyroidism in the presence of a second, apparently normal (9), PTH allele.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases, Klenow fragment of *Escherichia coli* DNA polymerase I, and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). Endoglycosidase H (endo-β-*N*-acetylglucosaminidase H) was from Boehringer Mannheim. Rabbit reticulocyte lysate, wheat germ extract, and canine pancreatic rough microsomes were purchased from Promega Corp. SRPs prepared from freshly excised canine pancreas and eluted from an aminopentyl-agarose column (13) were a generous gift from Reid Gilmore, University of Massachusetts, Worcester, MA.

Construction of Plasmids—Plasmid pWT84 was prepared by inserting a *Dde*I-*Xba*I fragment of human prepro-PTH cDNA encoding prepro-PTH(1–84) within the *Hin*dIII-*Xba*I cloning sites of the mammalian expression vector pCDM8 (9). The single point mutation (T to C) was introduced at the −8 position of the signal peptide of prepro-PTH cDNA using oligonucleotide-directed mutagenesis (14), resulting in plasmid pSM84 (single mutant; see Fig. 1). Plasmid pSM84 was then further modified by two additional point mutations introduced into the −10 position of the signal peptide (GCA to CGA; Ala to Arg), resulting in plasmid pDM84 (double mutant; Fig. 1).

Plasmids pWT83, pSM83, and pDM83, all containing prepro-PTH(1–83) sequences without the termination codon, were constructed by subcloning a *Hin*dIII-*Pst*I fragment containing the prepro sequences from plasmids pWT84, pSM84, and pDM84, respectively, into plasmid SP-PTH(*Xba*I/*Bal*31/*Cla*I/#6) comprising mature PTH(1–83) sequences without the termination codon (15).

In order to construct a plasmid encoding a protein of the same size as prepro-PTH but lacking a functional signal sequence, *Nco*I linkers were introduced into the *Sma*I site in the polylinker of SP-PTH(*Xba*I/*Bal*31/*Cla*I/#7) (15), and a 225-base pair *Hae*III-*Hin*dIII fragment derived from the same plasmid was ligated into the *Nco*I site. This introduced an ATG initiation codon followed by PTH sequences (51-82)-Ala in place of the signal sequence. This plasmid was then restricted with *Cla*I and *Hin*dIII and ligated to a *Dpn*I-*Hin*dIII fragment isolated from plasmid SP-PTH (15), containing sequences encoding PTH(1–84). The resulting plasmid (pRM84, for random mutant signal sequence), encoded Met-PTH(51–82)-Ala-Ser-PTH(1–84).

To introduce an *N*-linked glycosylation site into the PTH-coding sequence of plasmids pWT84 and pSM84, synthetic oligonucleotides, encoding Ser-Asn-Gly-Ser-Gly-Glu-Gly-Val-Glu-Ser, were ligated into the unique *Taq*I site of the prepro-PTH coding sequence (the underlined sequence indicates the consensus sequence for *N*-glycosylation). The resulting plasmids, pWT84(G) and pSM84(G), differed from pWT84 and pSM84, respectively, only by the insertion of 9 amino acids between residues Ser-17 and Met-18 of the mature PTH protein.

In Vitro Transcription, Translation, and Analysis of Products—Plasmids were linearized and sense RNA strands were transcribed using either T7 or SP6 RNA polymerase (15). Translation reactions in rabbit reticulocyte lysate and in wheat germ extract were performed according to the manufacturer's (Promega) procedure. Translation products were immunoprecipitated using affinity-purified goat anti-human PTH(1–34) antiserum and subjected to 15–20% continuous gradient SDS-polyacrylamide gel electrophoresis (PAGE). Autoradiography was performed after treating the gel with EN^{3}HANCE (DuPont NEN) as described previously (15). The identity of PTH-related peptides was established by amino-terminal radiosequence analysis (16).

Posttranslational Membrane Binding—Truncated cRNAs missing the termination codon were transcribed from plasmids pWT83, pSM83, and pDM83 and translated in the rabbit reticulocyte lysate cell-free system for 15 min at 24 °C. Aurintricarboxylic acid was added to 0.1 mM to inhibit translation initiation, and after another 15-min incubation, emetine was added (1 mM final concentration) to block peptide elongation. Incubation was continued for another 15 min, 4 eq (1 eq refers to 1 μl of the original rough microsome preparation that has been adjusted to a concentration of 50 A_{280} units/ml; see Ref. 17) of microsomal membranes/25 μl of translation mixture were added, and incubation continued for 15 min at 24 °C. Translation products were then incubated at 0 °C for 10 min. Insertion into the membranes was assessed by centrifugation of the membranes through either a physiological salt- or an EDTA-sucrose step cushion (15, 18). Supernatants containing membrane-free peptides and pellets were immunoprecipitated and subjected to SDS-PAGE analysis, as described (15).

Protease Protection Assay—To assay for co-translational translocation, wild-type and single-mutant cRNAs were translated in rabbit reticulocyte lysate cell-free system in the presence of canine pancreas microsomal membranes. After a 15-min incubation at 24 °C, proteinase K (20 μg/ml final concentration) was added to the translation reaction mixtures either alone or in combination with Triton X-100 (1%), and incubation was continued for a further 60 min on ice. After inactivation of the protease with phenylmethylsulfonyl fluoride (final concentration, 2 mM), radiolabeled proteins were subjected to immunoprecipitation and SDS-PAGE analysis.

Endoglycosidase H Digestion—Transcribed products of pWT84(G) and pSM84(G) were translated in the absence or presence of microsomal membranes, immunoprecipitated, and treated with endoglycosidase H (10 milliunits) as described previously (19). Following overnight digestion, bovine serum albumin was added as a carrier (final 0.25%), and the samples were precipitated by ice-cold trichloroacetic acid (15% final concentration). Following centrifugation, the precipitates were resuspended in 0.1 M Tris-HCl, pH 7.4, and prepared for SDS-PAGE analysis as usual.

Signal Peptidase Assay—EDTA-stripped, nuclease-treated rough microsomes were prepared from canine pancreas as described (17). Aliquots of rough microsomes were resuspended by homogenization in ice-cold buffer (20 mM HEPES, pH 7.6, 50 mM NaCl) to a final concentration of 50 A_{280} units/ml. Solubilization of signal peptidase with sodium deoxycholate was performed as described previously (20). Briefly, one volume of 10% (w/w) sodium deoxycholate was mixed with 19 volumes of membrane suspension. The resultant clear solution was centrifuged at 100,000 × *g* for 4 h, and the supernatant was frozen in liquid nitrogen. Wild-type and single-mutant prepro-PTH and prepro-PTH(G) cRNAs were translated in a wheat germ cell-free system, and translation products were used as substrates for detergent-solubilized signal peptidase (20). A typical 50-μl posttranslational cleavage assay contained 20 μl of the detergent extract, 20 μl of wheat germ translation mixture, and 10 μl of water. Posttranslational cleavage by signal peptidase was allowed to proceed at 25 °C for 90 min.

Pulse-Chase Analysis—COS-7 cells cultured in 12-well (22-mm diameter) plates in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics were transfected with plasmids pWT84, pSM84, and pDM84 using the DEAE-dextran protocol followed by 10% dimethyl sulfoxide shock (21). Five days later, cells were rinsed twice with Dulbecco's modified Eagle's medium without methionine and supplemented with 2% dialyzed fetal calf serum and then incubated for 15 min at 37 °C in the same medium containing [^{35}S]methionine (40 μCi/well). The cells were then washed once with 2 ml of Dulbecco's modified Eagle's medium (with methionine, supplemented with 10% fetal bovine serum), and then incubated further in the same medium for the times indicated. Conditioned media were saved for immunoprecipitation, and cells were lysed with 0.5 ml of lysis buffer (0.01 M Tris-HCl, pH 7.2, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 5 mM EDTA, 0.02% sodium azide, 0.15 M NaCl) with 50 μl of phenylmethylsulfonyl fluoride at a final concentration of 500 μg/ml. DNAs of the cell lysates were sheared with a 22-gauge needle, and the extracts were briefly centrifuged prior to immunoprecipitation.

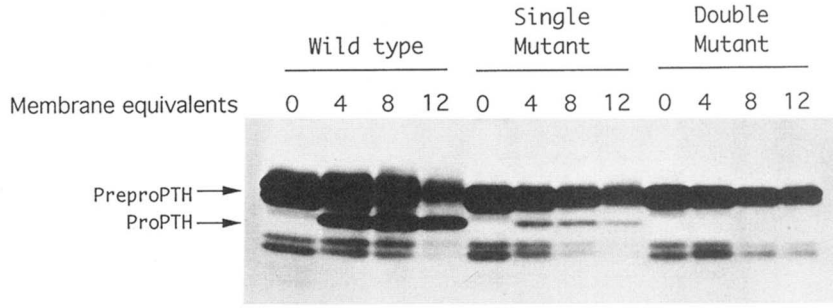

FIG. 2. **Processing of normal and mutant prepro-PTH.** Autoradiogram of [^{3}H]leucine-labeled proteins derived from the translation of cRNAs transcribed from plasmids containing wild-type (pWT84), single mutant (pSM84), and double mutant (pDM84) prepro-PTH cDNA. Translation was performed in the rabbit reticulocyte lysate cell-free system in the absence (0 eq) or presence of increasing amounts (4, 8, and 12 eq) of canine pancreatic microsomal membranes.

Competition Studies—COS-7 cells were transfected with pWT84 or pSM84 either alone or in combination with pWT52 encoding for human prepro-PTH(1–52) (22). After the indicated times post-transfection, cells were labeled with [^{35}S]methionine for 15 min at 37 °C, as described above. Cells were then lysed and processed for immunoprecipitation and SDS-PAGE analysis.

RESULTS

Processing of Wild-type and Mutant prepro-PTH—To assess the processing of wild-type and mutant forms of prepro-PTH to pro-PTH, transcribed sense RNA strands were translated in a rabbit reticulocyte lysate cell-free system in the absence or presence of increasing concentrations of canine pancreatic microsomal membranes. In this co-translational assay, substitution of Cys → Arg at the −8 position of the prepro-PTH signal peptide (single mutant) resulted in impaired processing to pro-PTH as compared with the wild-type form (Fig. 2). The addition of a second charged residue within the hydrophobic core of the signal peptide (Ala → Arg at the −10 position, double mutant) completely abolished its proteolytic processing by rough microsomes. Maximal processing of wild-type and single-mutant precursors occurred when microsomal membranes were added to a final concentration of 4 eq/25 μl of translation mixture.

Interaction with SRP—The impaired processing of the mutant prepro-PTH proteins by microsomal membranes may result from the inability of SRP to recognize efficiently the altered signal peptides. Binding of SRP to the signal sequence of nascent secretory proteins induces a site-specific elongation arrest of translation (3, 23). We, therefore, assessed the interaction of the mutant prepro-PTH signal sequences with SRP by examining the effect of exogenous SRP on inhibition of translation in a wheat germ cell-free system (Fig. 3). The mRNAs for pWT84, pSM84, pDM84, pRM84, and rabbit globin were translated in the presence (final concentration 0.02 and 0.04 A_{280} units/ml) or absence of exogenous SRP. The translation of the wild-type protein was inhibited significantly more than the translation of the mutant peptides by exogenous SRP. Moreover, translation of the single-mutant form was impaired to a greater extend than that of the double mutant. The addition of SRP did not affect the synthesis of globin (cytoplasmic protein with no signal peptide) and PTH-like protein with a random mutant signal peptide. These results suggested that the observed inhibition in processing of the mutant precursors is, at least, partly due to the inability of their signal sequences to interact effectively with SRP.

Translocation-competent Binding—To assess binding of the mutant prepro-PTH proteins to microsomal membrane components, truncated cRNAs for wild-type and mutant forms of prepro-PTH missing the termination codon were transcribed from plasmids pWT83, pSM83, and pDM83 and translated in the rabbit reticulocyte lysate cell-free system, thereby "freezing" the nascent proteins on ribosomes. After translation was complete, rough microsomes were added, and insertion into membranes was assessed following centrifugation of the reaction mixture through either a physiological salt- or an EDTA-sucrose step gradient. Only nascent chains tightly bound to the translocation apparatus pellet with the membranes in the presence of high concentrations of EDTA (18). When membranes were added to the reaction mixture post-translationally, the propeptide (wild-type and single mutant) as well as the prepropeptide bands were seen in the precipitates (Fig. 4*A*). Moreover, peptides pelleting with the rough microsomes were resistant to extraction with EDTA, suggesting that translocation-competent binding of the ribosome-nascent chain complex to the membranes had taken place. This post-translational assay suggested that the mutant proteins can be targeted to microsomal membranes and bind in a translocation-competent manner. Although the mutant forms were able to engage the translocation apparatus, they did so less efficiently than the wild-type form. Equal loading of the various reaction mixtures was verified by examining corresponding supernatant fractions using SDS-PAGE analysis (Fig. 4*B*). Thus, the amount of nascent protein that sedimented with rough microsomes paralleled the affinity of SRP for the respective peptides (wild-type > single mutant > double mutant) likely reflecting their degree of interaction with SRP. As shown in Fig. 4*A*, processing of the mutant forms to pro-PTH was again markedly impaired when compared with the wild-type protein, even though translocation-competent binding had been achieved. These results suggested that additional processes in the early steps of the secretory pathway, *i.e.* translocation and/or interaction with signal peptidase, may be impaired by the mutant signal sequences.

Protease Sensitivity of Membrane-bound Nascent Chains—To determine the location of the nascent chains within the rough microsomes, we examined their sensitivity to digestion with proteinase K either in the absence or presence of Triton X-100. Protein products that are translocated to the lumen of the microsomal vesicles would be protected from digestion by proteolytic enzymes that are unable to enter these vesicles. As shown in Fig. 5, the processed peptides (pro-PTH) were protected by the membranes from proteolysis by proteinase K. The protection of these forms must have resulted from their sequestration into the lumen of the microsomal vesicles. This was confirmed by the addition of Triton X-100 to the reaction mixture, thereby, permeabilizing the membrane bilayer and allowing the protease to gain access to all protected polypeptides. The minor unprocessed single-mutant product that is protected from proteolysis may represent unprocessed nascent protein that has not fully translocated, yet is protected from proteolysis by the ribosomes and the tight ribosome-membrane junction required for translocation.

N-Glycosylation of a Modified PTH Sequence—Since the mutant signal sequence might be a poor substrate for signal peptidase, cleavage alone is an insufficient criterion for the localization of PTH peptides. The fact that a fraction of the mutant prepro-PTH was protected from proteolysis raises the question of how far into the translocation process the uncleaved precursor has progressed. Because glycosylation is restricted to the lumen of the endoplasmic reticulum, the addition of carbohy-

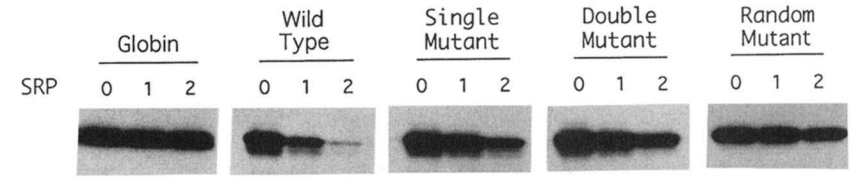

FIG. 3. **Effect of SRP on translation.** The cRNAs for wild-type (pWT84), single mutant (pSM84), double mutant (pDM84), random mutant (pRM84), and rabbit globin were translated in a wheat germ cell-free system in the absence (*0*) or presence of exogenous SRP (final concentration 0.02 (*1*) and 0.04 (*2*) A280 units/ml).

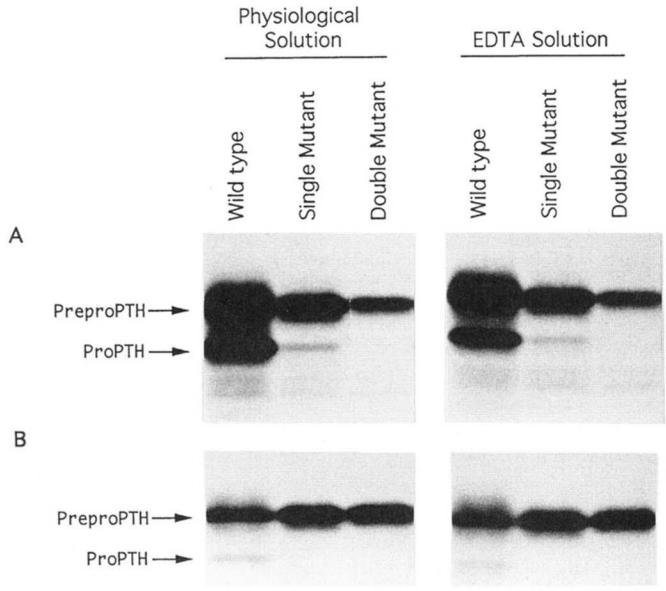

FIG. 4. **Translocation-competent binding to microsomal membranes.** Truncated cRNAs missing the termination codon were transcribed from plasmids encoding wild-type, single mutant, and double mutant cDNAs and translated in rabbit reticulocyte lysate system. Translocation-competent binding to microsomal membranes was assessed by centrifugation of the membranes through either a physiological salt- or an EDTA-sucrose step cushion. Radiolabeled proteins in the pellets (*A*) and corresponding supernatants (*B*) are displayed.

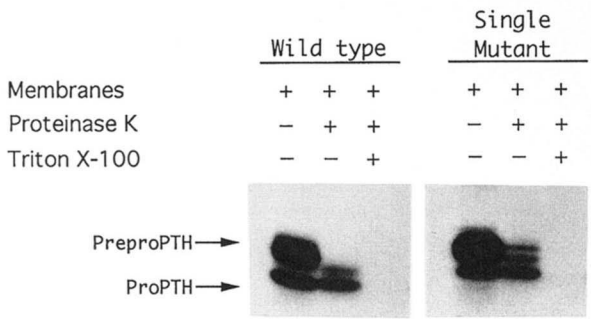

FIG. 5. **Protease sensitivity of membrane-bound nascent chains.** Wild type (pWT84) and single mutant (pSM84) cRNAs were translated in the rabbit reticulocyte lysate system in the presence (+) of dog pancreas microsomal membranes. After translation was completed, reactions were treated with proteinase K (20 μg/ml) with (+) or without (−) the addition of 1% Triton X-100. Radiolabeled translation products were immunoprecipitated and analyzed by SDS-PAGE.

drate to the PTH sequence by microsomal membranes would constitute further evidence of translocation and would not directly require the presence of a suitable substrate for signal peptidase (19). Because the mature PTH protein does not have an *N*-linked glycosylation site, we engineered such a site 23 amino acid residues distal to the signal peptidase cleavage site.

Fig. 6 shows that upon translation of pWT84(G)-transcribed cRNA in reactions supplemented with microsomal membranes, two translational products with PTH immunoreactivity were seen that were not present in the absence of membranes. The smaller product migrated with an apparent molecular weight slightly greater than that of authentic pro-PTH and was therefore felt to be pro-PTH(G). The second product, which migrated more slowly than prepro-PTH(G), was believed to be the glycosylated form of pro-PTH(G). This was confirmed by treatment with endoglycosidase H which, by removing carbohydrate on this peptide, shifted its position on an SDS-PAGE gel to that of pro-PTH(G). Interestingly, prepro-PTH(G) appeared to be processed much more efficiently by canine microsomal membranes than the unmodified form of the protein (compare Figs. 2 and 6), and this may simply reflect the influence of the extended length or the specific structure of the nascent chain.

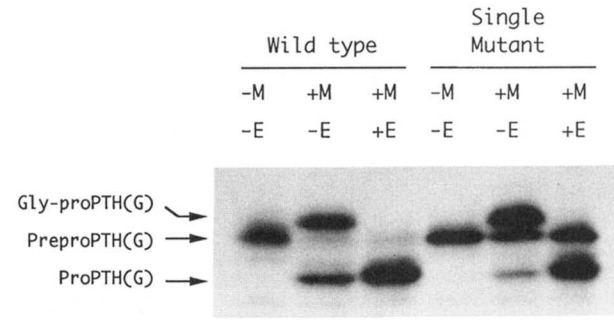

FIG. 6. **Glycosylation of wild-type and mutant prepro-PTH(G).** Plasmids pWT84(G) and pSM84(G) were transcribed *in vitro* and translated in the rabbit reticulocyte lysate cell-free system in the absence (−*M*) or presence (+*M*) of canine microsomal membranes. Translation products were immunoprecipitated and treated with (+*E*) or without (−*E*) endoglycosidase H.

Translation of pSM84(G)-transcribed cRNA in the presence of membranes resulted in the appearance of three PTH-immunoreactive products, two of which co-migrated with pro-PTH(G) and its glycosylated form, while the third was consistent with the unprocessed prepro-PTH(G) form. Again, the addition of 9 amino acids to the mature PTH molecule resulted in a more efficient cleavage of the signal sequence by microsomal membranes as compared with the unmodified form (see Figs. 2 and 6). Yet, the mutant signal sequence was once again processed less efficiently than the wild-type sequence, as indicated by the persistence of the unprocessed mutant prepro-PTH(G) form in the immunoprecipitated products. Once cleaved, however, pro-PTH(G) was glycosylated appropriately as confirmed by treatment of the reaction products with endoglycosidase H. The addition of carbohydrate to this moiety provides direct and independent evidence for its translocation, although significantly impaired, across the endoplasmic reticulum membranes. Furthermore, no larger glycosylated product was found that might have represented a protein that was translocated, glycosylated, and yet not cleaved by signal peptidase. Therefore, the uncleaved mutant prepro-PTH(G) was not delivered to the glycosylation machinery on the inner surface of the microsomal membranes.

Processing by Solubilized Signal Peptidase—To determine whether the single-mutant form of prepro-PTH is an unsuitable substrate for signal peptidase, we assessed proteolytic processing of the nascent protein in a translocation-independent assay. Following translation of wild-type and single-mutant prepro-PTH cRNAs in the wheat germ cell-free system, signal peptidase assays were performed by mixing aliquots of

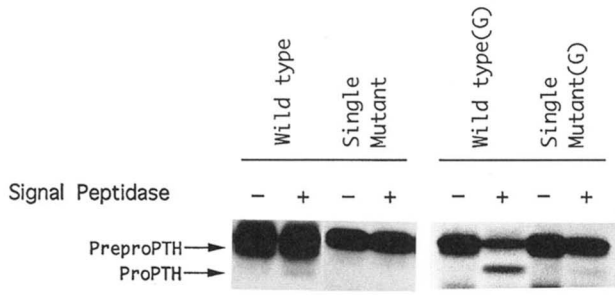

FIG. 7. **Signal peptidase assay.** Following translation of either pWT84- and pSM84- or pWT84(G)- and pSM84(G)-transcribed cRNAs in a wheat germ extract, signal peptidase assays were performed by mixing aliquots of translation mixture and signal peptidase prepared by directly solubilizing canine pancreatic rough microsomes.

translation mixture and signal peptidase prepared by directly solubilizing canine pancreatic rough microsomes. In this post-translational assay, no cleavage was detected for the mutant precursor, while a minor amount of processing was observed for the wild-type form of prepro-PTH (Fig. 7). Since the wild-type prepro-PTH is also an inefficient substrate for solubilized signal peptidase, an attempt was made to increase the sensitivity of the assay using as substrate the modified form of the prepro-PTH molecule containing an engineered glycosylation target site. RNAs encoding the wild-type and single-mutant form of prepro-PTH(G) were translated, and solubilized signal peptidase was added posttranslationally (Fig. 7). As found with intact membranes, wild-type and mutant forms of this modified PTH protein were processed more efficiently than their normal counterparts. Indeed, the mutant prepro-PTH(G) was processed by solubilized signal peptidase unlike its unmodified form, but the extent of cleavage was significantly less than that of the wild-type prepro-PTH(G). These results suggested that the mutation in the signal peptide makes the protein a less suitable substrate for signal peptidase.

Processing and Secretion of Prepro-PTH in COS-7 Cells—To confirm the cell-free data outlined above in intact cells, we examined the processing and secretion of mutant and wild-type forms of prepro-PTH from COS-7 cells transfected transiently with the corresponding expression plasmids. Five days following transfection, cells were pulse-labeled for 15 min with [^{35}S]methionine and then chased for the times indicated in Fig. 8 with medium containing cold methionine. The PTH species in cell extracts and conditioned media were immunoprecipitated and then resolved by SDS-PAGE. At the earliest time point examined (0 min), the predominant product immunoprecipitated in wild-type PTH-transfected cells was pro-PTH, while only barely detectable levels of prepro-PTH precursor were observed (Fig. 8*A*). Moreover, immunoreactive PTH was seen in the culture medium of these cells after 30 min of chase (Fig. 8*B*). The rate of processing seen with the wild-type sequence was in striking contrast to that observed with the mutant forms. In lysates from cells transfected with either mutant, the predominant band at the earlier time point corresponded with the prepro-PTH precursor with proteolytic cleavage to pro-PTH being dramatically reduced in efficiency. With the single mutant, only a small amount of pro-PTH was detected; no pro-PTH was found in cells expressing the double mutant. With the single-mutant form, a band corresponding to prepro-PTH was detectable even after 120 min of chase, consistent with the *in vitro* observed inefficient cleavage of this precursor to pro-PTH by microsomal membranes. Immunoreactive PTH was detectable in culture media of these cells after 30 min of chase but at substantially reduced levels as compared with the wild-type form. The double mutant form of prepro-PTH, although efficiently translated, was not processed to pro-PTH, nor was immunoreactive PTH detectable in the culture media of cells transfected with this plasmid (data not shown). These results demonstrate in intact cells the inefficient processing of the mutant prepro-PTH molecules as compared with the wild-type form. The single-mutant prepro-PTH allows a small amount of normal processing, consistent with the results in the cell-free studies that showed that the single mutant molecule can engage the translocation apparatus, although less efficiently than normal. The double mutant prepro-PTH almost totally fails to engage the translocation apparatus in cell-free extracts and is not processed at all in intact cells.

Competition Experiments—A co-transfection assay in COS-7 cells was used to address the issue of dominant transmission of hypoparathyroidism in the family carrying the single-mutant prepro-PTH allele. The objective was to determine whether the inefficient processing of the mutant precursor can result in obstruction of the translocation apparatus and, thereby, to global defects in protein processing. For this study, an expression vector containing sequences encoding a truncated version of prepro-PTH, missing the last 32 residues (WT52), was used to provide a readily distinguishable varient of prepro-PTH with a normal signal sequence.

WT52 was co-transfected into COS-7 cells with either wild-type or mutant prepro-PTH expression vector. Over-expression of the mutant precursor for up to 10 days did not interfere with the processing of prepro-PTH(1–52) (Fig. 9). Although these results may simply reflect lack of sensitivity of the system, they do not support global interference with protein processing at the microsomal membrane level as a consequence of overexpression of the mutant prepro-PTH form.

DISCUSSION

Translocation from the cytoplasm into the endoplasmic reticulum, is a multistep process requiring a functional amino-terminal signal peptide. A signal sequence must perform effectively several distinct functions required for the efficient translocation of secreted proteins. These subfunctions include its recognition and binding to SRP, its interaction with membrane-bound components of the export machinery, opening the protein-conducting channels to initiate translocation, and appropriate presentation to the signal peptidase for cleavage.

Three domains have been identified as a common feature of eukaryotic signal sequences, and considered to be necessary for carrying out these functions: a positively charged NH_2 terminus, a central hydrophobic core of 10–15 amino acid residues, and a polar COOH-terminal region (24, 25). While the COOH-terminal region influences the efficiency and fidelity of signal peptidase cleavage, intactness of the hydrophobic region is indispensable for initiating translocation.

Two reported inherited mutations in the signal sequence of human secreted proteins, namely preprovasopressin (10), and preprofactor X (11), involve the COOH-terminal region of the respective signal peptides. Thus, a point mutation resulting in substitution of Arg for Gly at the −3 position of the factor X signal peptide (Factor $X_{\text{Santo Domingo}}$) blocks cleavage by signal peptidase but does not interfere with targeting and translocation to the RER (11). Similarly, a naturally occurring substitution of Thr for Ala at the −1 position of the signal peptide of preprovasopressin results in central diabetes insipidus (10). This mutant protein, similar to the Factor $X_{\text{Santo Domingo}}$, undergoes inefficient cleavage by signal peptidase, although targeting and translocation to the RER are not measurably affected.

The present study is the first to examine the effect of a naturally occurring substitution at the hydrophobic core of a signal peptide that results in human disease. Prepro-PTH, the precursor of PTH, contains a typical 25-residue amino-terminal

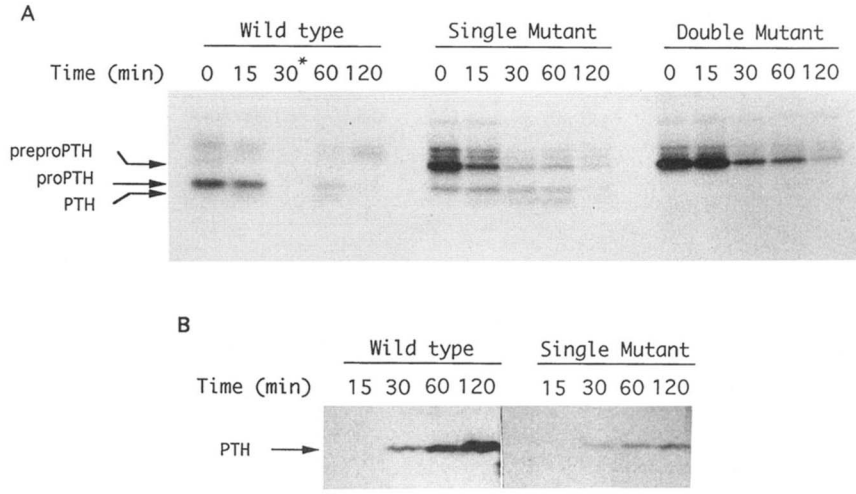

FIG. 8. **Expression of wild-type and mutant forms of prepro-PTH in COS-7 cells.** Plasmids pWT84, pSM84, and pDM84 were transiently transfected into COS-7 cells. Five days following transfection, the cells were pulse-labeled with [^{35}S]methionine for 15 min. At the indicated times following pulse-labeling, the media were removed, and cell extracts were prepared. Both cell extracts (*A*) and media (*B*) were immunoprecipitated using a PTH-specific antibody prior to SDS-PAGE analysis. *, sample not processed.

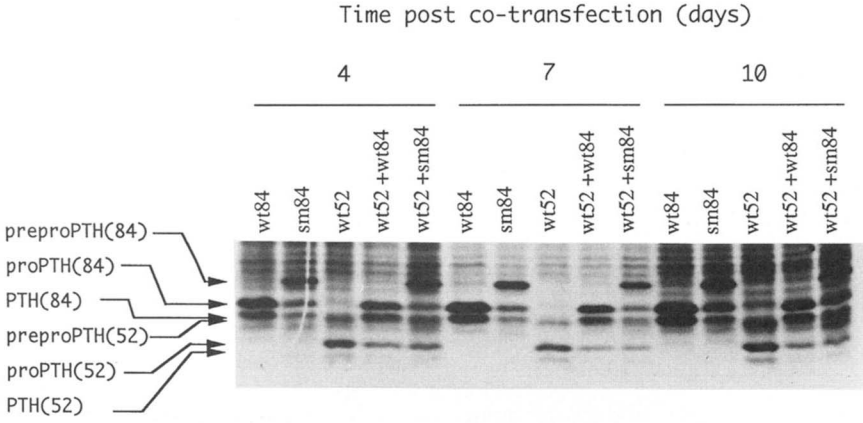

FIG. 9. **Co-transfection of wild-type and mutant prepro-PTH.** Plasmids pWT84 and pSM84 were transfected into COS-7 cells either alone or in combination with pWT52. After the indicated number of days posttransfection, cells were pulse-labeled with [^{35}S]methionine for 15 min. Cell extracts were then prepared and immunoprecipitated prior to SDS-PAGE analysis.

signal sequence followed by a 6-residue prospecific peptide and the mature hormone (residues 1–84; see Fig. 1). The hydrophobic core of the human prepro-PTH signal peptide is composed of 12 contiguous uncharged amino acids (residues −5 to −16 of the signal peptide).

In the present study, we have demonstrated that substitution of a charged amino acid, Arg for Cys, in the signal peptide hydrophobic core of prepro-PTH impairs co-translational translocation as well as posttranslational cleavage by isolated signal peptidase. The impairment was even more evident when two charged residues were introduced in the hydrophobic core of the signal sequence. In contrast to deletion mutants (26), this change interferes not only with translocation and cleavage by signal peptidase but also with binding to SRP. Since substitution of a hydrophobic amino acid, leucine, for cysteine or its deletion was ineffective in modifying translocation and processing (26), the present findings would suggest that a change in the hydrophobicity of the core is responsible for the observed global disruption in the processing of the mutant prepro-PTH. Similarly, single charged amino acids introduced in the hydrophobic core of the *E. coli* maltose binding protein signal peptide impair secretion of the protein into the external periplasmic compartment of the cell (27).

It is rather intriguing that three distinct reports of inherited mutations in the signal sequence of human secreted proteins, namely prepro-PTH (9), preprovasopressin (10), and preprofactor X (11), demonstrate inheritance of the associated disorder in an autosomal dominant fashion. As is the case for the other two reported disorders, however, it remains unclear why individuals with a mutated PTH allele have hypoparathyroidism. From transfection studies in COS-7 cells, it would appear that expression of the mutant allele does lead to secretion of PTH, albeit inefficiently. Moreover, the normal allele would be expected to produce sufficient circulating PTH to maintain calcium homeostasis. The only other reported case of familial isolated hypoparathyroidism segregating with a mutation in the PTH gene, involved a point mutation affecting intron splicing and was associated with autosomal recessive inheritance of the disorder (28). Since heterozygous individuals for this mutant allele were unaffected, it would appear that one normal PTH allele is sufficient for maintaining calcium homeostasis.

Hypopathyroidism in the presence of one normal PTH allele would therefore suggest that the mutant gene product exerts a dominant negative effect *in vivo*. The mutant protein might interfere with the normal targeting and processing of other secreted proteins, including the normal PTH precursor. Such interference might even lead to destruction of parathyroid tissue in affected individuals; unfortunately, this is difficult to evaluate because the tissue is not readily accessible. Export incompatibility, however, has been observed in *E. coli* expressing transport-defective β-galactosidase leading to lethal jamming of the cellular export machinery (29).

The phenotype of the single-mutant prepro-PTH suggests that it might have dominant negative effects under appropriate conditions. The mutation allows a fraction of the prepro-PTH precursor to enter the translocation machinery, but the mutant protein is then cleaved inefficiently by signal peptidase. The inability of the uncleaved protein to reach the glycosylation machinery (assessed using the precursor modified by inclusion of a glycosylation signal) suggests that the precursor is not transported fully across the microsomal membrane. Such a protein that engages the translocation apparatus but fails to

move through the apparatus efficiently might well have dominant negative effects. The competition experiment in Fig. 9 failed to demonstrate such an effect; perhaps higher levels of protein expression or a longer term experiment are needed. Nevertheless, the observation that all three reported human signal sequence mutations involve proteins that partly engage the secretory apparatus and appear to have dominant effects suggests that *in vivo* these mutant proteins cause dominant secretory dysfunction.

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