Preparathyroid Hormone-related Protein, a Secreted Peptide, Is a Substrate for the Ubiquitin Proteolytic System*

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Karen Meerovitch‡§, Simon Wing§, and David Goltzman‡§¶

From the ‡Calcium Research Laboratory and *Polypeptide Laboratory, Royal Victoria Hospital, and §Department of Medicine, McGill University, Montreal H3A 1A1, Canada

Parathyroid hormone-related protein (PTHrP) is an important causal factor of hypercalcemia associated with malignancy. PTHrP also modulates cell growth and differentiation of normal cells through mechanisms that include binding to cell surface-specific receptors as well as by possible intracellular routes. To understand the regulation of intracellular PTHrP expression, post-translational processing of PTHrP was investigated. Using cell-free translations it was shown that PTHrP can be ligated efficiently to multiple ubiquitin moieties. Both conjugation to ubiquitin and degradation of prepro-PTHrP synthesized in vitro were ATP-dependent. Translation in vitro in the presence of the proteasome inhibitor MG-132 abolished the degradation of PTHrP. Treatment of cells, cotransfected with hemagglutinin-tagged ubiquitin and histidine-tagged prepro-PTHrP, with MG-132, led to the accumulation of ubiquitinated prepro-PTHrP. Deletion mutagenesis experiments indicated that both the prepro secretory domain and a PEST (amino acid residues Pro (P), Glu (E), and/or Asp (D), Ser (S), and Thr (T)) motif in the COOH-terminal region of the protein were not required as cis-acting determinants for ubiquitination. This is the first report of a wild-type secretory polypeptide serving as a substrate of the ubiquitin proteolytic pathway. These results suggest that the ubiquitin-dependent proteolytic pathway is involved in regulating the metabolic stability of intracellular PTHrP, and this regulation may be an important mechanism for modulating its effects on cell growth and differentiation.

Parathyroid hormone-related protein (PTHrP) is a secreted peptide that is a major pathogenic factor in hypercalcemia associated with malignancy (1, 2). PTHrP shares limited sequence homology with parathyroid hormone (PTH), with 8 of the first 13 amino acids in PTHrP being identical to the corresponding NH2-terminal residues of PTH (for review, see Ref. 3). This limited homology is sufficient for PTHrP to bind to a common PTH/PTHrP receptor and share many of the biological properties of PTH (4, 5). Unlike PTH, PTHrP also contains a bipartite nuclear localization signal in the midregion of the protein (6), which has recently been demonstrated to be capable of targeting PTHrP to the nucleus, and nucleolar localization of PTHrP may be required for enhancing the survival of chondrocytes in culture under conditions that promote apoptotic cell death (7). Although PTHrP was first demonstrated as a tumor product, it is now known to be expressed in a wide array of normal fetal and adult tissues (8). Unlike PTH, PTHrP is not normally present in the circulation, suggesting that it acts locally in an autocrine or paracrine fashion. Studies in several cell types in vitro (6, 9–12), in vivo experiments in transgenic mice that overexpress PTHrP (13), and in vivo studies of PTHrP gene inactivation in mice via homologous recombination (14–16) have all demonstrated that PTHrP plays a role in normal cell proliferation and differentiation including a critical role in skeletogenesis (14–16). In addition, it may also influence tumor cell growth (17, 18).

The regulation by a variety of agents of the expression and secretion of PTHrP has been examined in many different cells. An increase in PTHrP mRNA transcripts is observed rapidly and transiently following exposure of cells to serum, growth factors, and phorbol esters through mechanisms including increased gene transcription and mRNA stability (for review, see Ref. 8). In addition, an increase in constitutive expression and secretion was noted in the progression from the established to the malignant phenotype, suggesting that the protein is dysregulated in tumor cells (12, 19).

A common feature of many regulatory proteins, including oncoproteins, is their short half-life. As increased levels of PTHrP are associated with the transformed phenotype (12, 19), it might be expected that PTHrP instability, and therefore, low steady-state levels of the protein, could be important for properly controlled cell proliferation. To date, no study on the intracellular degradation of PTHrP has been documented. Here we present evidence that PTHrP is a substrate of the ubiquitin-dependent proteolytic system.

Ubiquitin-dependent protein degradation is a nonlysosomal, ATP-dependent proteolytic pathway (for review, see Ref. 20). The biochemical mechanism involves covalent ligation of the 76-amino acid polypeptide, ubiquitin, to e-amino groups of lysine residues in target proteins by the action of ubiquitin-activating enzyme, ubiquitin-conjugating enzymes and ubiquitin-protein ligases. The resulting mult ubiquitinated protein is either deubiquitinated by isopeptidases or degraded by a multicatalytic protease complex, the 26 S proteasome. Mult ubiquitination has been implicated, for example, in the rapid degradation of a number of cytosolic regulatory proteins such as transcriptional regulators, oncoproteins, and regulators of cell cycle progression (for review, see Ref. 21). In addition, degradation of the cystic fibrosis transmembrane conductance regulator (CFTR) involves the binding of ubiquitin to specific cytosolic domains of the protein, and ubiquitination has been shown to mediate endocytosis of CFTR (22). Ubiquitination also plays a role in the proteolytic processing of certain signaling proteins, including cytokines and their receptors, and adhesion molecules (23). In these cases, ubiquitination appears to act as a latency factor, delaying signal transduction until after ubiquitination and subsequent proteolytic processing has occurred (24). Ubiquitination of PTHrP may function in a similar manner, delaying proteolytic processing of the protein until it reaches the appropriate subcellular compartment.
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regulator was reported to involve the ubiquitin-proteolytic pathway, suggesting that ubiquitin and the proteasome play a role in the maturation of ER-targeted proteins (22, 23). In this study, we show that prepro-PTHrP is ubiquitinated in vitro and in vivo in cultured cells. In addition, we also show that in transfected cells treated with an inhibitor of the proteasome, ubiquitinated PTHrP accumulated. To determine whether we could identify a cis-acting sequence that confers ubiquitination, we deleted the pre pro domain of PTHrP and a COOH-terminal PEST motif and have observed that these features are not essential for the destabilization of PTHrP. This is a novel report of a secreted polypeptide hormone that is a substrate for the ubiquitin proteolytic pathway. We propose that the proteasome proteolytic pathway is involved in regulating the intracellular level of PTHrP, which in turn would have consequences for its role as a modulator of cell growth and differentiation.

EXPERIMENTAL PROCEDURES

Materials—ATP, S, hexokinase, calpain inhibitor II, E64, and RNase A were all purchased from Boehringer Mannheim. 2-Deoxyglucose was obtained from Sigma. The proteasome inhibitor MG-132 (50 M) was gift from Myogenes. Ubiquitin aldehyde was a gift from A. Ciechanover. An expression plasmid encoding ubiquitin fused at the NH2 terminus to hemagglutinin (HA) sequences was from D. Bohmann (24). The cDNAs encoding prepro-PTHrP and pre pro-PTH were kindly supplied by G. Hendy. Polyclonal antisera to PTHrP (1–34) was raised in a rabbit purchased from Oncogene Science. The mouse monoclonal anti-HA antibody (clone 12CA5) was purchased from Boehringer Mannheim.

Plasmid Construction—All PTHrP constructs were derived from the cDNA encoding rat prepro-PTHrP (PLPm10) (25). For transfection studies, prepro-PTHrP was expressed with a histidine tag at the COOH terminus using the mammalian expression vector pRC/CMV (Invitrogen). Tagged prepro-PTHrP cDNA was generated by PCR using the universal primer as the forward primer and oligonucleotide PTHrPHistag (5′-GCTCTAGATCGTGTATGGTGATGGT-3′) as the reverse primer on PLPm10 as template. The PTHrP Histag primer encodes the last five codons of mature PTHrP except the last two codons T and H are replaced by E and F (italic). The primer also contains 5′ bovine serum albumin (bold type), a termination codon (underlined), and ends with an Xbal restriction site. The amplified product was digested with HindIII and XbaI and ligated into the HindIII and XbaI sites of pRC/CMV, generating plasmid CMV PTHrP-His6 for use in transient transfections.

A prepro-PTHrP construct lacking the pre pro sequence was derived from PLPm10 using PCR-based mutagenesis. To create this deletion construct, two overlapping primers were synthesized which both contain the same deletion of nucleotides encoding amino acids 36 to 1. These “inside” primers were used in separate PCRs together with “outside” universal primers in the left PCR and with outside prepro-PTHrP Histag primer in the right PCR. The amplified products were gel purified. Then a subsequent reamplification of these purified overlap fragments with only the outside primers resulted in production of the full-length product containing the deletion. This PCR product was ligated into pRC/CMV as above generating CMV PTHrPΔP-His6. A plasmid in which the PEST region was deleted was generated by PCR using the universal primer as the forward primer and PESThis6 primers as reverse primer. PESThis6 is 5′-GCTCTAGATCAGTGTATGGTGATGGT-3′, which lacks nucleotides encoding amino acids 126–139 of the mature PTHrP, leaving intact distally the sequence encoding EFHHHHHH and a termination codon. The PCR-amplified product was ligated into pRC/CMV as described above generating CMV PTHrPΔP-Es6. A construct containing the double deletion ΔP and ΔPΔEST was generated by PCR using the universal and PESThis6 primers on the purified PCR product obtained from PTHrPΔP-His6. To express human prepro-PTHrP, cDNA encoding this peptide was excised from plasmid pMTU125 (26) using BamHI and HindIII and then ligated into the expression vector pKS (Stratagene) which had been cut with the same enzymes. All constructions were verified by sequencing.

Cells, Media, and Transfections—COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium (plus 4.5 g/liter glucose; Life Technologies, Inc.) supplemented with 10% fetal calf serum and antibiotic-antimycotic (Life Technologies, Inc.) in a humidified atmosphere at 37 °C with 5% CO2. COS-7 cells were transiently transfected with Lipofectin (Life Technologies, Inc.) according to the instructions of the manufacturer. The Lipofectin concentration was 12 μg/ml, and 4 μg of CMV-based expression plasmid and 2 μg of ubiquitin expression plasmid were added per 60-mm dish.

Stable expression of prepro-PTHrP was created in a Chinese hamster lung fibroblast cell line, E36 (kindly provided by William Dunn). The cells were maintained at 30 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. The cells were transfected with Lipofectin reagent and CMV PTHrP-His6. Following lipofection, cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 4148 (1 mg/ml) at 30 °C. Colonial clones were isolated, expanded, and assayed for PTHrP expression by Northern blotting.

Conditioned media from transfected cells were collected at various times and kept frozen at −80 °C. Levels of PTHrP in the media were determined by an amino-terminal radioimmunoassay kit from Immuno. Treatment with Protease Inhibitors—48 h after transfection, cells were incubated in fresh Dulbecco’s modified Eagle’s medium plus 10% fetal calf serum containing either 50 μM MG-132 or dimethyl sulfoxide (MeSO) vehicle alone (final concentration 0.2%) for up to 24 h. In control experiments, 50 μM E64 or 50 μM calpain inhibitor II was used in place of MG-132.

Cell Lysis and Nickle Affinity Chromatography—Cell monolayers were washed once in saline. Cell monolayers were washed once in saline, then lysed in lysis buffer (500 μl of 8 x urea, 0.1 M sodium phosphate buffer (pH 8.0), 0.01 M Tris-HCl (pH 8.0)). Lysates were incubated on ice for 10 min, centrifuged at 16,000 g for 10 min at 4 °C, and the supernatants were stored at −80 °C. Protein concentrations in the lysates were determined by the Bradford assay (Bio-Rad).

To isolate histidine-tagged proteins, equal amounts of total protein from each sample (up to 300 μg of total protein) were loaded on Ni2+–NTA-agarose spin columns (Qiagen) pre-equilibrated with lysis buffer. Columns were spun at 2,000 rpm for 2 min in a microcentrifuge. Three washes were performed with 600 μl of 8 x urea, 0.1 M sodium phosphate buffer, 0.01 M Tris-HCl (pH 6.3). Proteins were eluted with 200 μl of 8 x urea, 0.2 M HCl.

SDS-PAGE and Immunoblotting—Equilibrated amounts of protein from total cell lysates or equal volumes of material eluted from the Ni2+-NTA-agarose columns were denatured in SDS sample buffer and separated on 12.5% polyacrylamide–sodium dodecyl sulfate (SDS) gels. Proteins were transferred in 25 mM Tris, 192 mM glycine, 20% Methanol onto polyvinylidene difluoride (Trans-Blot, Bio-Rad). Blots were blocked with TBS (20 mM Tris-HCl (pH 7.5), 137 mM NaCl) plus 0.1% Tween 20 and also contains 5% bovine serum albumin. Polyclonal antibody against amino acids 1–34 of human PTHrP was used at a 1:400 dilution in TBS, 3.5% bovine serum albumin. The monoclonal antibody 12CA5, specific for a nine-amino acid HA peptide sequence (YPYDVPDYA) from influenza HA, was used at 2.5 μg/ml. The antigen-antibody complexes were visualized using appropriate secondary antibodies (Bio-Rad) and the ECL detection system as recommended by the manufacturer (Amersham Corp.).

In Vitro Transcriptions and Translations—pSK (Bluescript, Stratagene) containing rat pre pro-PTHrP cDNA (PLPm10) was linearized with BglII, pks-hPTH was linearized with HindIII. A 5-μg portion was transcribed in vitro using T7 RNA polymerase (Promega Biotec) under conditions recommended by the supplier. A 0.5-μg portion of the resulting RNA was translated in 12.5 μl of reticulocyte lysate (Promega Biotec) translation mix in the presence of 100 μCi of [3H]leucine at 30 °C for the times indicated in the figure legends. Aliquots were removed and fractionated by electrophoresis in 12.5% polyacrylamide–SDS gels. Gels were fixed and processed for fluorography with En’Hance (DuPont NEN), dried, and exposed to X-Omat AR (Kodak) film.

Ubiquitination Conjugation Assay—Bacterial expression plasmids for glutathione-tagged yeast ubiquitin were from J. Huibregtse (27). Glutathione-S-transferase (GST) fusion constructs were expressed in Escherichia coli DH5α and affinity purified on glutathione-Sepharose (Pharmacia Biotech Inc.) under conditions recommended by the manufacturer. Bound proteins were eluted with 10 μM glutathione (Boehringer Mannheim). For analysis of PTHrP-His6 elution from GST-ubiquitin, 12.5 μl of in vitro translated H-labeled PTHrP was incubated at 37 °C for up to 3 h in a total volume of 30 μl in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 2 mM MgCl2, 1 mM dithiothreitol, 2 mM ATPβS, and 5 μg of GST or GST-ubiquitin. Aliquots of the reaction were terminated with SDS sample buffer and subjected to SDS-PAGE (12.5%), fluorography, and autoradiography.
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Degradation Assay—Degradation assays were performed in rabbit reticulocyte lysate translation reactions programmed with prepro-PTHrP mRNA and [3H]leucine. After 30 min of translation, 25-μl aliquots were diluted to a final reaction volume of 30 μl containing 0.3 μg/ml RNase A, 15 mM MgCl₂, and either 1 mM ATP or 1 mM ATP-S. In assays involving ATP depletion of the lysate, 20 mM 2-deoxyglucose and 1.5 units hexokinase were added instead of ATP. Reactions were incubated at 30 °C for indicated times. Aliquots of the reactions were terminated by the addition of SDSPAGE buffer and analyzed on SDS-PAGE as described above.

When used, the protease inhibitors MG-132 (50 μM) or calpain inhibitor II (50 μM) or vehicle (Me₂SO) were added at the onset of reticulocyte lysate translations and incubated at 30 °C for the indicated times. Ubiquitin aldehyde was added at 30 μM.

RESULTS

Characterization of Prepro-PTHrP Translated in Vitro—Prepro-PTHrP mRNA was synthesized in vitro and translated in a rabbit reticulocyte lysate in the presence of [3H]leucine. Following SDS-PAGE and autoradiography a principal band of protein was observed migrating at 27 kDa (Fig. 1, lane 2). The molecular mass of prepro-PTHrP based on its deduced amino acid sequence is 14 kDa. This aberrant migration of prepro-PTHrP has been noted previously and results from its highly basic net charge (28). Faster migrating bands were also observed and are presumably due to premature termination of translation or nonspecific degradation in the lysate. In addition to the full-length protein, translation in reticulocyte lysate generated higher molecular mass bands which were not visualized when the same RNA preparation was translated in a wheat germ lysate (Fig. 1, lanes 2 and 4). These higher molecular mass bands migrated approximately 15 and 8 kDa slower than prepro-PTHrP. On longer exposure, several additional bands with regular 8-kDa spacing were apparent near the top of the gel (data not shown). This ladder of higher molecular mass bands is reminiscent of the post-translational covalent attachment of ubiquitin to target proteins in reticulocyte lysate, which is an established source of ubiquitinating enzymes and proteasome complexes (29). We therefore explored the possibility that prepro-PTHrP is indeed a substrate for ubiquitin conjugation and degradation.

Conjugation of GST-Ubiquitin to Prepro-PTHrP in Vitro—To determine if the higher molecular mass bands obtained in these reactions were the result of prepro-PTHrP ubiquitination, we added ubiquitin to the reticulocyte translation extract, as a fusion protein linked to GST. If ubiquitin conjugation to prepro-PTHrP were to occur, then the addition of the 33-kDa GST-ubiquitin protein should increase the molecular mass of prepro-PTHrP from 27 to 60 kDa. The addition of GST-ubiquitin but not GST alone to the translation reaction resulted in the appearance of a novel band that migrated with the predicted mobility of a prepro-PTHrP-GST-ubiquitin conjugate (Fig. 2, lanes 2 and 3).

To test the specificity of this ubiquitination of prepro-PTHrP, we performed the conjugation assay on prepro-PTH, which has limited homology to prepro-PTHrP. Prepro-PTH translated in reticulocyte lysate was observed not to be a substrate for GST-ubiquitin conjugation (Fig. 2, lanes 4–6). This experiment suggests that ubiquitin is conjugated specifically to prepro-PTHrP in vitro.

Degradation of Prepro-PTHrP in Vitro Is ATP-dependent—In time course experiments, the overall intensity of the translated PTHrP bands in the reticulocyte lysate decreased for up to 6 h (Fig. 3, A and B, lanes 1–4). A key feature of ubiquitin-dependent proteolysis is its dependence on ATP, which is required for conjugation of ubiquitin to the substrate and for degradation of the ubiquitinated proteins. To determine whether degradation was ATP-dependent, we depleted ATP from the reticulocyte lysate with 2-deoxyglucose and hexokinase. This resulted in inhibition of PTHrP proteolysis and prevented the appearance of the higher molecular mass bands (Fig. 3A, lanes 5–8). To test the requirement for ATP hydrolysis in the degradation assays we added ATP₆S, a nonhydrolyzable ATP analog, to the reticulocyte lysate. This led to a slight reduction in PTHrP proteolysis (Fig. 3B, lanes 5–8) and stabilization of the higher molec-
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PThrP in Vitro—To characterize the ubiquitination of PThrP in intact cells, an expression plasmid containing the prepro-PThrP cDNA encoding six histidines at the carboxyl terminus was constructed and transiently transfected into COS-7 cells. In addition, the cells were transfected with a vector that expresses high levels of a HA-tagged human ubiquitin (24). 48 h after transfection, cells were cultured for 6 or 24 h in medium containing either the vehicle Me$_2$SO (control), MG-132, calpain inhibitor II, or a cysteine proteinase inhibitor E64. A viability test was performed on control and MG-132-treated cells to assess for potential toxicity. Trypan blue exclusion demonstrated that ~90% of the cells remain viable in the presence of Me$_2$SO- or MG-132-treated cultures. Total cell lysates were subjected to SDS-PAGE and immunoblotted with polyclonal antibody against PThrP (1–34) or monoclonal antibody against PThrP (38–64) (Fig. 5, A and B, respectively). Addition of MG-132, but not Me$_2$SO, calpain inhibitor II, or E64, induced an accumulation of higher molecular mass bands (Fig. 5A). The antibodies against PThrP recognized two main products corresponding to full-length prepro-PThrP and the processed form responding to full-length prepro-PThrP and the processed form. This is more obvious in Fig. 5B since immunoblotting with the monoclonal antibody does not detect an unrelated but similar sized protein that cross-reacts with the polyclonal antibody (Fig. 5A, lanes 1 and 2, mock transfected). A faster migrating band accumulating in the presence of MG-132 may have been due to enzymatic removal of a COOH-terminal fragment. Indeed, several post-translational cleavages resulting in NH$_2$-terminal fragments have been reported for PThrP (8, 33). Interestingly, when the monoclonal antibody against PThrP (38–64) was used (Fig. 5B), the higher molecular mass bands that accumulate in the presence of MG-132 were not detected. This suggests that ubiquitin may be conjugated to PThrP in this region thus masking the ability of the antibody to detect it. This result...
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Fig. 5. Ubiquitination of prepro-PTHrP in vivo. Panel A, COS-7 cells transiently transfected with pBCCMV vector (lanes 1 and 2) or with prepro-PTHrP-His6 (lanes 3–10) together with CMV-HA-ubiquitin (lanes 1–10) were treated for the indicated times with MeSO (DMSO, lanes 1–4), 50 μM MG-132 (lanes 5 and 6), 50 μM calpain inhibitor II (lanes 7 and 8), or 50 μM E64 (lanes 9 and 10). Cell lysates (50 μg of total protein) were immunoblotted with PTHrP (1–34) polyclonal antibody. The closed arrow points to the migration of full-length prepro-PTHrP, and the open arrow points to a putative signal peptide cleavage product, pro-PTHrP. Note that prepro-PTHrP migrates just below a cellular protein that cross-reacts nonspecifically with the PTHrP (1–34) antibody (see Fig. 9A, lanes 1 and 2). Treatment of these cells with MG-132 and analysis of total protein by Western blotting resulted in an accumulation of PTHrP, suggesting that MG-132 inhibits the degradation of PTHrP in vivo (Fig. 7).

To determine whether the higher molecular mass immuno-reactive bands that accumulated in the presence of MG-132 represented ubiquitinated PTHrP, cell lysates from MeSO- or MG-132-treated cells were purified by Ni2+ -NTA affinity chromatography. The material that bound to and was eluted from the column was then examined by Western blotting using a PTHrP (1–34) antibody (Fig. 6). Eluted protein from MG-132- but not MeSO-treated cells retained the same high molecular mass bands that were initially recognized by the PTHrP (1–34) antibody (see Fig. 5A). The bands in Fig. 6 are specific for PTHrP because Ni2+ -NTA affinity columns purify and immunoblotting of non-His6-(mock-) transfected cell extracts did not react with PTHrP (1–34) antibody (see Fig. 9A, lanes 1 and 2). In addition, the higher molecular mass bands, purified on the Ni2+-NTA affinity column, reacted with an antibody against the HA epitope, suggesting that these bands contain ubiquitin (see Fig. 9B). Furthermore, the pattern of higher molecular mass bands detected in transfected cells was identical to that observed from in vitro translations. Taken together, these data strongly suggest that PTHrP is multiubiquitinated in vivo.

To address the question of whether the stability of PTHrP is altered by treatment with MG-132, we created stably transfected Chinese hamster lung fibroblasts that express prepro-PTHrP. PTHrP expression levels in these cells were significantly lower than in transiently transfected COS-7 cells (Fig. 7, compare lanes 3 and 4 with lanes 1 and 2). Treatment of these cells with MG-132 and analysis of total protein by Western blotting resulted in an accumulation of PTHrP, suggesting that MG-132 inhibits the degradation of PTHrP in vivo (Fig. 7).

We next evaluated whether the addition of MG-132 altered the amount of PTHrP secreted in the conditioned medium. The levels of secreted PTHrP in conditioned medium of MeSO-treated (control) cells were the same as in MG-132-, calpain inhibitor II-, or E64-treated cells in both transiently transfected COS and stably transfected fibroblast cells (data not shown).

Fig. 6. Specific binding of ubiquitinated PTHrP to Ni2+ -NTA resin. Cell lysates from COS-7 cells coexpressing prepro-PTHrP-His6 and HA-ubiquitin which were treated with 50 μM MG-132 (lanes 1–3) or MeSO (DMSO, lanes 4–6) for 24 h were fractionated on Ni2+ -NTA resin as described under “Experimental Procedures.” Samples of total lysate (load; 21 μg of total protein), flow-through unbound material (FT), and bound, eluted material (eluate), each representing equivalent fractions of the original cell lysate, were electrophoresed and immunoblotted with PTHrP (1–34) antibody. Closed and open arrows point to prepro-PTHrP and pro-PTHrP, respectively. The asterisks mark the position of ubiquitin-PTHrP conjugates formed only in the presence of MG-132.

Sequences in Prepro-PTHrP Required for Ubiquitination—To investigate the cis-acting requirements for PTHrP ubiquitination, we tested two His6-tagged deletion mutants of PTHrP in the transient cotransfection assay described above. Since previous studies have suggested that hydrophobic sequences and amphipathic α-helices may target proteins for ubiquitin-mediated degradation (34), we determined whether the hydrophobic prepro sequence may target PTHrP for degradation. A truncated molecule lacking the NLE-terminal 36-amino acid prepro sequence was therefore created (PTHrPΔP-His6, Fig. 8). PTHrP is also rich in the amino acid residues Pro, Glu, Asp, Ser, and Thr (PEST) in the carboxyl portion of the protein (Fig. 8). PEST-rich sequences have been suggested to function as degradation signals (35) and have recently been implicated to be required for recognition by the ubiquitination process (36–38). To test whether the PEST site in PTHrP serves as a degradation signal, amino acids 126–139 from the mature protein were deleted (PTHrPΔPEST-His6, Fig. 8). In addition, a double mutant was created which lacked both the prepro and the PEST motifs (PTHrPΔPΔPEST-His6). COS-7 cells were cotransfected with full-length PTHrP or the deletion mutants and with HA-tagged ubiquitin. Transfectants were exposed to MeSO or MG-132 for 18 h, and cell lysates were purified on Ni2+-NTA affinity columns. Eluted material was analyzed by immunoblotting with antibody recognizing PTHrP (1–34) (Fig. 9A). As demonstrated by this blot, PTHrP-His6 and
We have demonstrated ubiquitination and proteasome-dependent degradation of a protein, prepro-PTHrP, which is normally a secreted entity. In vitro translation of prepro-PTHrP in reticulocyte lysate revealed the accumulation of higher molecular mass bands. Exogenous addition of ubiquitin in the form of a fusion protein conjugated specifically to prepro-PTHrP and prepro-PTHrP was observed to be degraded in an ATP- and proteasome-dependent fashion in vitro. Expression of prepro-PTHrP and an epitope-tagged ubiquitin in the presence of the proteasome inhibitor MG-132 led to the accumulation in vivo of ubiquitin-PTHrP conjugates. These results indicate that degradation of PTHrP by the ubiquitin-dependent proteolytic pathway may be a mechanism to regulate the intracellular abundance of the protein.

We also examined several putative internal signals that might target PTHrP for ubiquitination. We observed that deletion of the prepro sequence resulted in higher levels of PTHrP within the cell consistent with its impaired secretion, but did not abolish efficient ubiquitination of PTHrP in vivo. Consequently, the prepro region is as a minimum not a sole site of ubiquitination, nor is it essential for directing ubiquitination. We therefore examined an additional putative cis-acting signal for ubiquitination within the mature protein, a 14-amino acid domain of PEST-rich sequences in the COOH-terminal region of the protein. Deletion of only these 14 amino acids did not however abolish ubiquitination. The amino acid sequence extending from residue 109 to residue 126 reveals another domain enriched in PEST amino acids. It is possible that additional deletion of this sequence would have influenced the ubiquitination process, consistent with previous results that multiple PEST-like stretches may be additive in influencing the stability of some proteins (38). Further studies will be required to examine this possibility. Although we were able to detect ubiquitin-PTHrP conjugates using an antibody directed against the 1–34 sequence, we were unable to detect such conjugates using an antibody that recognizes PTHrP (38–64) (Fig. 5B). This lack of reactivity could be due to blockade by ubiquitin moieties of the PTHrP epitope recognized by the antibody and suggests that this region may be a site for ubiquitin ligation.

The subcellular compartment in which PTHrP undergoes ubiquitination and degradation as well as the precise role that degradation plays in the function of PTHrP remain unknown. Since PTHrP is a secreted protein, ubiquitination could be occurring in the secretory pathway. Although the ubiquitin proteolytic system has generally been regarded as a cytosolic or nuclear pathway, recent studies have identified the presence of ubiquitin and ubiquitin-activating enzyme associated with a post-ER/pre-Golgi compartment (39). In addition, lactacytin, a specific inhibitor of the proteasome, was shown to block the degradation of a mutant precursor of glycosylphosphatidylinositol-linked protein in a pre-Golgi compartment (40). The luminal degradation of mutated secretory protein α1-antitrypsin is suggested to be proteasome-dependent via association with the transmembrane chaperone protein calnexin (41). Recent studies have provided evidence that ER-targeted aberrant proteins can be degraded by the 26 S proteasome in the cytoplasm. Although ubiquitin-dependent degradation of the integral cytoxic fibrosis transmembrane conductance regulator protein most likely occurs on the cytoplasmic side of the ER membrane (22, 23), the proteasome-dependent degradation of some aberrant luminal polypeptides (42–44) is proposed to be due to retrograde transport of these proteins at least partially out of the ER into the cytosol, where they are subsequently degraded by the proteasome. Taken together, these studies suggest that ER-
associated degradation of secretory proteins can involve the proteasome and are consistent with our observations that prepro-PTHrP can be a substrate for the ubiquitin-proteolytic system.

The ubiquitination and degradation of PTHrP may therefore be occurring at least in part in the cytoplasmic compartment of the cell. It has been suggested previously that the ubiquitin system could be involved in the degradation of secreted proteins that escape the secretory pathway (45). Thus, a possible function of ubiquitination may be to metabolize PTHrP that has entered the cytoplasm. This may simply provide a clearance mechanism for a peptide normally destined for secretion. Although overexpression of PTHrP in transiently transfected COS cells may have facilitated aberrant localization of the peptide in the cytoplasm, we have observed a similar effect of MG-132 on PTHrP degradation in stably transfected Chinese hamster lung fibroblasts, which express at least 100-fold lower levels of PTHrP than do COS cells (Fig. 7).

Although PTHrP is a secreted peptide, currently available data indicate potential intracellular roles for this protein. It has recently been shown that PTHrP is localized in the nucleus where it has been suggested to play a role in preventing cells from undergoing apoptotic cell death (7). The mechanism for nuclear localization of PTHrP is not known but is dependent on a consensus nuclear localization signal in the midregion of the protein (7). Another role for PTHrP is the regulation of functions described above. Furthermore, a recent report has suggested that intracellular PTHrP abundance may be dependent on the stage of the cell cycle. In this study in nontransformed asynchronously growing cells immunoreactive PTHrP was found to accumulate in the G2 + M phase of the cell cycle in the absence of any changes in mRNA expression, suggesting that this accumulation occurs due to inhibition of degradation rather than enhanced synthesis (47). In contrast to the reported finding of cell cycle-dependent PTHrP accumulation in normal cells, immunoreactive PTHrP abundance in the squamous carcinoma cell line SCC and in the Rice 500 Leydig cell line does not appear to display variation during the cell cycle (47). This discrepancy may suggest that post-translational control of PTHrP abundance may be defective in cancer cells. Further work will be necessary to explore a potential link between cell cycle and PTHrP ubiquitination in both normal and transformed cells.

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