Parathyroid Hormone-Related Protein: Evidence for Isoform- and Tissue-Specific Posttranslational Processing†

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ABSTRACT: Parathyroid hormone-related protein (PTHrP) is expressed by malignant tumors and leads to the syndrome of humoral hypercalcemia of malignancy. It is also expressed by a wide variety of nonmalignant tissues, in which it appears to play distinct paracrine and/or autocrine roles. The human PTHrP gene encodes three cDNA-predicted initial translational products of 139, 141, and 173 amino acids. Most human cell lines contain mRNAs encoding all three PTHrP isoforms. The physiological rationale for the existence of these three highly similar transcripts is unknown. In order to determine whether the protein products derived from these three transcripts differ, we transfected Chinese hamster ovary (CHO) cells and rat insulinoma (RIN) cells individually with cDNAs encoding human PTHrP(1–139), PTHrP(1–141), and PTHrP(1–173). Cell extracts and conditioned medium were then chromatographed using reversed-phase HPLC and analyzed using region-specific PTHrP immunoassays. As we had previously observed in SKRC-1 (renal cell carcinoma) and RIN(1–141) cells, multiple amino-terminal PTHrP species as well as a separate midregion PTHrP species were identified in all six cell lines. In addition, both CHO and RIN cell lines transfected with the PTHrP(1–139) construct contained a previously unrecognized carboxy-terminal fragment that reacted with a PTHrP(109–138) antiserum. This carboxy-terminal fragment was physically distinct from the midregion fragment discovered earlier and was also present in conditioned medium, indicating that it is a secretory form, rather than a biosynthetic intermediate or a degradation product. Surprisingly, RIN and CHO cells transfected with PTHrP(1–141) or -(1–173) contained little of this carboxy-terminal fragment, suggesting that isoform-specific protein processing exists for PTHrP. In addition, while RIN cells produced a single predominant amino-terminal species, CHO cells contained two, approximately equimolar, amino-terminal species, indicating the existence of cell-specific protein processing. These studies indicate that the posttranslational processing of PTHrP is highly complex. Specifically, (a) multiple amino-terminal PTHrP secretory forms, as well as a midregion form, are generated by cell lines containing each of the three PTHrP transcripts; (b) the Arg^{37} cleavage that generates the midregion fragment occurs in the Golgi apparatus, as both constitutive and regulated secretory cell types are capable of performing this cleavage; (c) a previously unrecognized carboxy-terminal fragment of PTHrP is secreted; and (d) processing of PTHrP appears to be both isoform- and cell-specific. Complete structural determination of each of these fragments is critical to understanding PTHrP physiology and pathophysiology.

Parathyroid hormone-related protein (PTHrP) was originally isolated from human cancers associated with the syndrome of humoral hypercalcemia of malignancy (HHM) (Bilezikian, 1990; Broadus & Stewart, 1994; Halloran & Nissenson, 1992; Stewart & Broadus, 1990a,b; Strewler & Nissenson, 1990). Since then, the presence of either PTHrP protein, mRNA, or both has been documented in a multitude of normal, non-malignant human and animal tissues (Bilezikian, 1990; Broadus & Stewart, 1994; Halloran & Nissenson, 1992; Stewart & Broadus, 1990a,b; Strewler & Nissenson, 1990). These include sites as diverse as lactating breast, pituitary, placenta, bone, uterus, CNS, and epidermis, as well as fetal liver, kidney, and parathyroid (Bilezikian, 1990; Broadus & Stewart, 1994; Halloran & Nissenson, 1992; Stewart & Broadus, 1990a,b; Strewler & Nissenson, 1990). An emerging literature suggests that PTHrP may play a paracrine or autocrine role in (a) fetal and adult growth and development, (b) transepithelial calcium transport in several tissues, and (c) smooth muscle relaxation (Bilezikian, 1990; Broadus & Stewart, 1994; Halloran & Nissenson, 1992; Stewart & Broadus, 1990a,b; Strewler & Nissenson, 1990).

The human gene for PTHrP resides on chromosome 12, in a position analogous to that of the parathyroid hormone (PTH) gene on chromosome 11 (Bilezikian, 1990; Broadus & Stewart, 1994; Halloran & Nissenson, 1992; Stewart & Broadus, 1990a,b; Strewler & Nissenson, 1990). The PTHrP gene consists of multiple exons, which give rise, through alternative splicing, to three mRNA transcripts, encoding mature products of 139, 141, and 173 amino acids (Figures 1A and 2). These sequences share a common "prepro" sequence (amino acids −36 to −1) and are identical through amino acid 139. Thereafter, the three PTHrP mRNAs diverge, encoding three initial translation products with three distinct carboxy termini. Human tissues and tumors which express PTHrP may contain

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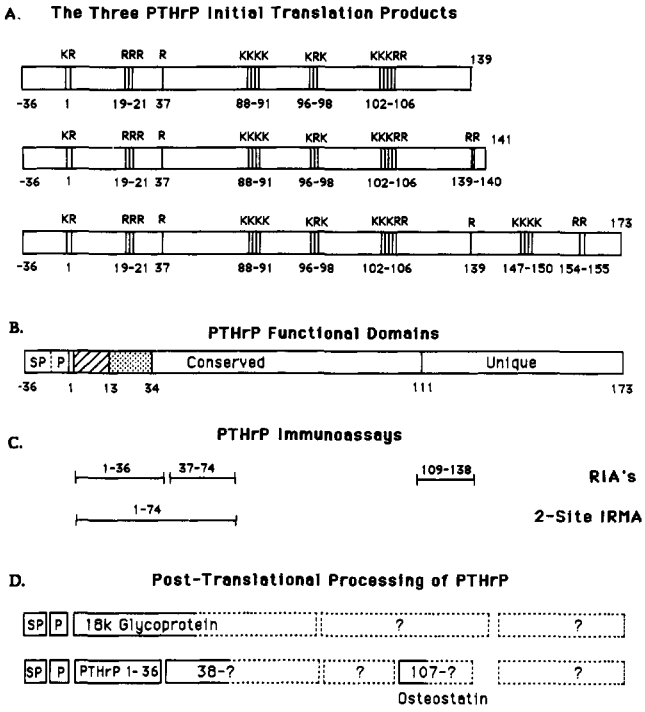

FIGURE 1: Posttranslational processing of PTHrP. Panel A shows the three PTHrP initial translation products predicted by the three cDNAs. Each has a "prepro" sequence (or signal peptide, SP) followed by 139 identical amino acids. Each has a unique carboxy terminus at or after amino acid 139, as shown in greater detail in Figure 2. K = lysine. R = arginine. Panel B shows the functional domains of PTHrP which include (a) the prepro region, or signal peptide (amino acids −36 to −1), (b) a 13 amino acid region which is 70% homologous to the corresponding region of parathyroid hormone (amino acids 1–13), shown in hatches, (c) a region with steric or three-dimensional but no primary amino acid sequence homology to PTH (amino acids 14–34), shown in dots, (d) a region which is highly conserved among species but is distinct from PTH (amino acids 35–111), and (e) a unique region which is less highly conserved among species. Panel C indicates the regions against which the immunoassays are directed. RIA indicates radioimmunoassay, and IRMA, immunoradiometric assay. Panel D indicates PTHrP species which are known to exist (solid boxes) or postulated to exist as described in the text (dashed boxes). [Adapted from Broadus and Stewart (1994).]

	135	136	137	138	139	140	141	142	143	144 173
PTHrP(1-139)	Glu	Leu	Asp	Ser	Arg					
PTHrP(1-141)	Glu	Leu	Asp	Ser	Arg	Arg	His			
PTHrP(1-173)	Glu	Leu	Asp	Ser	Arg	Thr	Ala	Leu	Leu	Trp .... Leu

FIGURE 2: Amino acid sequences of the three carboxy termini of the three PTHrP initial translation products.

all three transcripts, but some tissues appear to express one of them preferentially (Brandt et al., 1992; Mangin et al., 1989; Thiede et al., 1988). While the existence of these three transcripts implies unique biological roles for each, distinct secretory forms of PTHrP which arise from these three transcripts have not yet been identified.

The three PTHrP initial translation products are punctuated by basic amino acid motifs which are likely to serve as endoproteolytic posttranslational processing sites in PTHrP, as they do in a host of other endocrine or neuroendocrine peptides, including proopiomelanocortin (POMC), somatostatin (SRIF), atrial natriuretic peptide (ANP), the glucagons, chromogranin A, gastrin, cholecystokinin (CCK), molluscan egg-laying hormone, and many others (Fricker, 1991; Loh, 1993; Steiner et al., 1992). For example, the arginine–lysine doublet at positions −2 and −1 in the putative prepro peptide is a dibasic cleavage site in PTHrP, as it is in POMC, proinsulin, preproparathyroid hormone, and other peptides (Fricker, 1991; Loh, 1993; Steiner et al., 1992). In addition, the monobasic arginine at position 37 (Figure 1A) has been shown by Soifer et al. to be a posttranslational processing site in PTHrP (Soifer et al., 1992), analogous to the single arginine processing sites in SRIF, ANP, CCK, and many other neuropeptides (Fricker, 1991; Loh, 1993). The Arg^{37} cleavage leads to the generation of PTHrP(1–36) and to a midregion PTHrP that begins at residue 38 and appears to end in the 88–106 region (Figure 1D) (Soifer et al., 1992). Rabbani et al. (1993) have shown that multiple amino-terminal PTHrP species are produced and secreted by rat Leydig cells.

The 88–106 region (Figure 1A) is rich in multibasic endoproteolytic sites that may be recognized by processing enzymes such as the recently identified furin, Kex-2, PC-1/3, and PC-2 family of subtilisin-like endoproteolytic processing enzymes (Fricker, 1991; Loh, 1993; Steiner et al., 1992). As a result of posttranslational cleavages at these processing sites as well as other types of posttranslational modifications [e.g., *O*-glycosylation (Wu et al., 1991) and, potentially, carboxy-terminal amidation (Eipper & Mains, 1988)], PTHrP may be viewed as a "prohormone" or "polyhormone" which gives rise to a family of related but structurally and functionally distinct peptides.

Thus, the 36 amino acid PTH-like amino terminus of the mature peptide (Figure 1B,D) mimics the effects of parathyroid hormone and leads to the syndrome of humoral hypercalcemia of malignancy through which PTHrP was identified (Bilezikian, 1990; Broadus & Stewart, 1994; Halloran & Nissenson, 1992; Stewart & Broadus, 1990a,b; Strewler & Nissenson, 1990; Rabbani et al., 1993). The midregion of the molecule, comprising amino acids 38–111, is highly conserved among species (Bilezikian, 1990; Broadus & Stewart, 1994; Halloran & Nissenson, 1992; Stewart & Broadus, 1990a,b; Strewler & Nissenson, 1990). Synthetic peptides derived from this region have been shown to stimulate calcium transport across the placenta from mother to fetus (Care et al., 1990). The carboxy terminus contains a highly conserved stretch of five amino acids in positions 107–111. This region begins immediately after a presumed endoproteolytic processing site at 102–106 (Figure 1A). Synthetic PTHrP(107–111) has been demonstrated to be a potent inhibitor of osteoclastic bone resorption, leading to its designation as "osteostatin" (Fenton et al., 1991), although these observations have been challenged by Sone et al. (Sone et al., 1992). Peptides containing this region have been shown to be present in the circulation of patients with chronic renal failure on hemodialysis (Burtis et al., 1990) and in patients with HHM. Finally, the extreme carboxy terminus of the peptide, which includes residues 141–173, is encoded by exon 5 of the human gene (Bilezikian, 1990; Broadus & Stewart, 1994; Halloran & Nissenson, 1992; Stewart & Broadus, 1990a,b; Strewler & Nissenson, 1990). This sequence is "uniquely human" in the sense that it is not encoded by the rat, mouse, or chicken genes. The physiologic function of this peptide is entirely speculative at present. The family of PTHrPs which have been documented or theorized to exist at the time of this writing are summarized in Figure 1D.

In the current study, we sought to explore three questions. First, what is the rationale for the existence of the three closely related but carboxy-terminally distinct PTHrP mRNAs and their initial translation products? Does the translation of these three similar but distinct mRNAs lead to production of a distinct pattern of protein products? Since naturally occurring human PTHrP-producing cell lines express multiple PTHrP transcripts, and since the natural level of PTHrP expression

is low, we explored this question of "isoform-specific post-translational processing" by stably transfecting nonexpressing cell lines with cDNAs encoding each of the three possible PTHrP translation products and analyzing the processing intermediates and secretory forms of PTHrP using a panel of region-specific immunoassays (Figure 1C).

Second, since PTHrP is naturally expressed by cells which represent the "regulated" secretory pathway (Kelly, 1985) (e.g., pituitary, islet, parathyroid, and adrenal medulla) as well as by cells which are generally considered to employ the "constitutive" or "bulk flow" secretory pathway (Kelly, 1985) (e.g., liver, kidney, osteoblast, epidermal keratinocyte, amnion, and smooth muscle), we wondered whether differences in PTHrP processing might occur in rat insulinoma (RIN) cells —generally regarded as a paradigm for the regulated secretory pathway—as opposed to Chinese hamster ovary (CHO) cells, which are generally considered to process and secrete peptides via the constitutive secretory pathway.

Third, studies on the posttranslational processing of PTHrP have, until this point, focused on the 1–36 and 37–74 regions of the peptide (Rabbani et al., 1993; Soifer et al., 1992; Wu et al., 1991). With the availability of antisera directed against 109–138 epitopes in the peptide (Burtis et al., 1990; Orloff et al., 1993), with the evidence, noted above, that a peptide related to this region circulates in humans with renal failure and with HHM (Burtis et al., 1990), and with evidence that this region contains osteostatin bioactivity (Fenton et al., 1991), we sought to identify and initially characterize a putative carboxy-terminal PTHrP secretory form.

We report here that (a) PTHrP posttranslational processing is indeed isoform-specific, (b) PTHrP processing is also tissue-specific, and (c) a novel carboxy-terminal form of PTHrP is processed and secreted.

MATERIALS AND METHODS

Cell Lines. RIN cells (strain 1046-38) were obtained from Dr. Michael Appel at the University of Massachusetts, Worcester, MA, and were stably transfected using the lipofectin method with three cDNAs encoding the three PTHrP isoforms [(1–139), (1–141), and (1–173)] ligated into the pLJ vector (Korman et al., 1987) as described previously (Figure 3A) (Soifer et al., 1992). RIN cells containing the PTHrP(1–139) construct are hereafter referred to as RIN-(1–139); those containing PTHrP(1–141), as RIN(1–141); and those containing PTHrP(1–173), as RIN(1–173). The nucleotide sequences of the constructs were confirmed by DNA sequencing. Stable transfectants were selected using G418. RIN cells were then subcloned and grown to confluence in RPMI medium containing 10% FBS, 200 IU/mL penicillin and streptomycin, and 1% L-glutamine.

Methotrexate-sensitive, dihydrofolate reductase deficient (DHFR-deficient) CHO lines (line CHO K1 DUX-B11) were also transfected with three analogous PTHrP(1–139), -(1–141), and -(1–173) constructs. These expression constructs were truncated at their 3′ ends and ligated into the DHFR-containing, methotrexate-amplifiable expression vector pFR-SRα (Figure 3 B). The constructs were confirmed by sequencing. PTHrP-expressing CHO cells were selected using methotrexate, subcloned, and grown in DMEM deficient in nucleotides and containing methotrexate. This medium was supplemented with nucleotide-free 5% FBS, 200 IU/mL penicillin, and 1% L-glutamate. RNase protection analysis of all six transfected CHO and RIN lines confirmed the expression of the appropriate mRNA species.

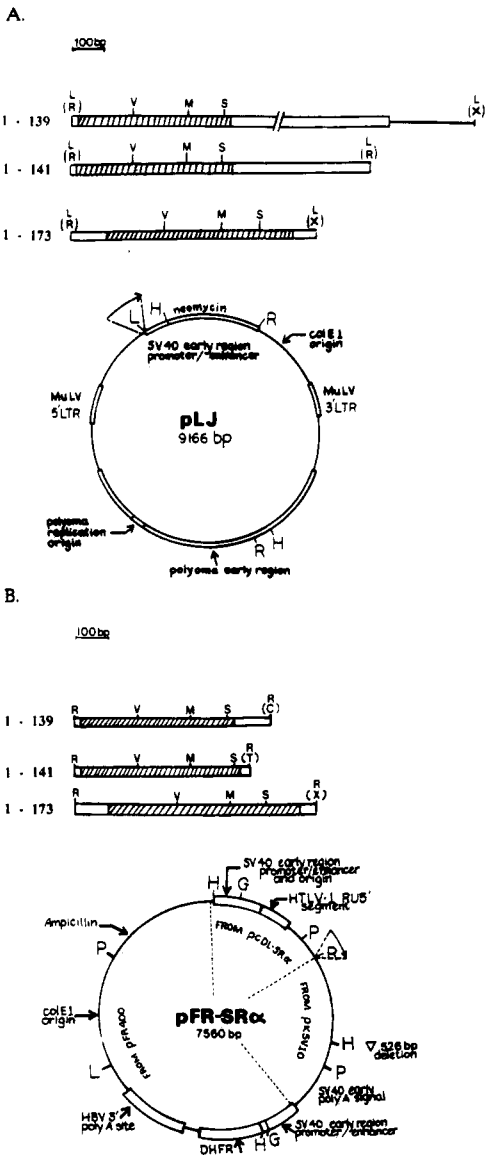

FIGURE 3: PTHrP expression vectors. (A) Human PTHrP cDNAs of 1600, 1025, and 825 bp, which encode the mature peptides 1–139, 1–141, and 1–173, respectively (Thiede et al., 1988; Mangin et al., 1988, 1989), were inserted into the *Sal*I site of the eukaryotic expression vector pLJ (Korman et al., 1987) with linkers. Inserts in this vector are transcribed from the upstream murine leukemia virus LTR and are terminated at a poly(A) addition site within the downstream LTR. A neomycin selectable marker driven by an SV40 promoter/enhancer is contained within the primary transcriptional unit. (B) Human PTHrP cDNAs of 665, 560, and 825 bp, which encode the mature peptides 1–139, 1–141, and 1–173, respectively, were inserted into the *Eco*RI site of the expression vector pFR-SRα. This vector was assembled from the shuttle vector pKSV10 (Pharmacia, Piscataway, NJ) and the DHFR expression vector pFR400 (Horwich et al., 1986) by Arthur Horwich (Yale University) and further modified by the addition of the SRα promoter, which is derived from the HTLV-1 LTR (Takebe et al., 1988). In both panels, striped boxes in the cDNA inserts signify the coding regions and clear boxes signify 5′ or 3′ untranslated regions. Enzyme abbreviations are as follows: C, *Cla*I; G, *Bgl*I; H, *Hin*dIII; L, *Sal*I; M, *Sma*I; P, *Pst*I; R, *Eco*RI; S, *Sac*I; V, *Pvu*II, T, *Sty*I; X, *Xmn*I.

PC-3 cells, a human prostate carcinoma line which expresses PTHrP (Rodan et al., 1983), were obtained from American Type Culture Collection, Rockville, MD, and were grown in F12 medium. SKRC-1 cells, a human renal carcinoma line which expresses PTHrP, were grown in DMEM medium. These cells had previously been used to construct a cDNA library from which PTHrP was cloned (Mangin et al., 1988), and they cause hypercalcemia when passaged in athymic mice

Table 1: PTHrP Immunoassay Sensitivity and Specificity

		specificity: cross-reactivity with PTHrP and PTH (%)					
PTHrP immunoassay	sensitivity (pM)	PTHrP(1–36)	PTHrP(1–74)	PTHrP(37–74)	PTHrP(109–138)	PTHrP(1–141)	PTH(1–84)
1–36 RIA	50	100	100	0	0	100	0
37–74 RIA	10	0	100	100	0	100	0
109–138 RIA	10	0	0	0	100	1	0
1–74 IRMA	1	0	100	0	0	100	0

(Weir et al., 1988). YCC-SQ-1 cells are a human squamous carcinoma line which expresses both PTHrP and its receptor, and they have been described previously (Orloff et al., 1992). A253 cells, a squamous carcinoma line which also expresses PTHrP and its receptor (Orloff et al., 1992), were obtained from American Type Culture Collection. YCC-SQ-1 and A253 cells were grown in calcium-free DMEM (Merendino et al., 1986). Finally, primary cultures of human neonatal foreskin keratinocytes were obtained and cultured as described previously (Merendino et al., 1986). Medium was harvested from each of the six stably transfected cell lines (RIN and CHO) and from each of the five natural PTHrP-expressing cell lines following 24–48 h of exposure to cells.

Cellular Protein Extraction. Each of the six RIN and CHO PTHrP-expressing cell lines was grown to confluence in ten 150-cm^2 flasks. The medium was discarded at confluence. Cellular proteins were extracted in 3 mL of ice-cold guanidinium isothiocyanate as described previously (Soifer et al., 1992). Ice-cold ethanol was added to a final volume of 30%, and nucleic acids were precipitated by chilling to 20 °C for 15 min, followed by centrifugation at 16000*g* for 10 minutes. The supernatant, containing cellular guanidinium isothiocyanate-soluble proteins, was dialyzed at 4 °C using a 3500M_r cutoff dialysis membrane (Spectrapor-3, Los Angeles, CA) against 16 L of deionized water. These cellular protein extracts were centrifuged at 16000*g* for 10 min and frozen at –20 °C until fractionation by RP-HPLC.

RP-HPLC. Cell extracts and conditioned medium were analyzed by reversed-phase HPLC, using a Vydac 218TP104 C_{18} column (Separations Group, Hysperia, CA) as described previously (Burtis et al., 1987; Soifer et al., 1992; Wu et al., 1991) and in the captions of Figures 4–7. A gradient of acetonitrile and water containing 0.1% trifluoroacetic acid (TFA) was employed. One-milliliter fractions were collected in siliconized tubes, lyophilized, and resuspended in immunoassay buffer.

PTHrP Immunoassays. Four region-specific PTHrP immunoassays were employed. The regions recognized by these assays are represented diagrammatically in Figure 1C. The PTHrP(1–36) RIA is a nonequilibrium assay and employs a polyclonal sheep antiserum (S_2) raised against PTHrP(1–74). S_2 was used at a titer of 1:1500, and Tyr^{36}PTHrP(1–36) amide was used both as radioligand and as standard. The assay was performed in 300 uL of PBT (10 mM phosphate-buffered saline, pH 7.4, 10.0% bovine serum albumin, and 0.1% Triton X-100) buffer and involved a 24-h preincubation of antiserum at 4 °C with sample or standard followed by an addition of radioligand and a second 24-h incubation. Phase separation was accomplished using charcoal–dextran as described previously (Burtis et al., 1990). The detection limit of this assay is 50 pM. The PTHrP(37–74) and PTHrP-(109–138) RIAs and the PTHrP(1–74) IRMA have been described in detail previously (Burtis et al., 1990, 1994). The detection limits and specificities of these asssays are summarized in Table 1. Each chromatogram shown in Figures 4–7 is representative of two to five immunoassays for each of these assays. Each immunoreactive peak was dosed out in the appropriate RIA and shown to parallel the assay standard.

Immunohistochemistry. Immunohistochemistry was performed as described in detail previously (Soifer et al., 1992). Briefly, RIN(1–139) cells were grown to approximately 50% confluence and fixed in 2% paraformaldehyde at 25 °C for 45 min. The cells were then washed with phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin and permeabilized for 15 min with 0.05% saponin in PBS. Immunoaffinity-purified rabbit polyclonal antisera directed against the PTHrP 1–36 and 37–74 regions were employed as detailed previously (Soifer et al., 1992). Polyclonal and immunoaffinity-purified rabbit antisera (R32) (Burtis et al., 1990) directed against the 109–138 region were employed at titers of 1:200 and 1:30, respectively. Fixed and permeabilized cells were exposed to antiserum diluted in PBS for 2 hours at 27 °C. After rinsing, a fluorescein-labeled anti-rabbit antiserum (Tago Immunologicals, Burlingame, CA) was added at a titer of 1:200 for 2 h in PBS containing 0.2% BSA and 0.05% saponin. The coverslips were then rinsed with PBS and mounted in 70% glycerol and 30% PBS containing *p*-phenylenediamine (1 mg/mL). Controls included identical studies performed (a) without primary antiserum, (b) with nonimmune antiserum in place of the primary antiserum, and (c) with immune serum previously exposed to 10^{-6} M PTHrP-(1–36), -(37–74), or -(109–138) as appropriate. These control studies showed no staining. The staining patterns of secretory granules (i.e., insulin-containing granules) and Golgi apparatus have been described previously (Soifer et al., 1992).

RESULTS

Processing of PTHrP(1–139), -(1–141), and -(1–173) in RIN Cells. In order to determine whether the three PTHrP mRNA species lead to the generation of three different profiles of PTHrP peptides, we stably transfected cDNAs encoding each of the three PTHrP isoforms into RIN cells and examined the cell extracts for PTHrP species following RP-HPLC using a panel of region-specific immunoassays (Figure 1C). The results of these studies are shown in Figure 4. Each of the three RIN lines contained a peak of midregion [i.e., PTHrP-(37–74)] immunoreactivity (peak I), which we have previously identified as a secretory form of the peptide that is devoid of 1–36 immunoreactivity and which begins at amino acid 38 of the PTHrP cDNA sequence (Figure 1D) (Soifer et al., 1992). Each of the RIN lines also contained a second minor peak of 37–74 immunoreactivity (peak II) of uncertain composition.

Each of the three PTHrP-expressing RIN cell lines also contained a large peak of PTHrP(1–36) immunoreactivity (peak III). This amino-terminal peak has been identified previously (Soifer et al., 1992), both in RIN cell extracts and as a secretory form of the peptide in RIN(1–141) conditioned medium (Figure 1). In the three RIN lines, peak III contains 1–36, 37–74, and IRMA immunoreactivity, suggesting that this peptide contains both N-terminal and midregion epitopes. RIN(1–139) and RIN(1–173) cells also contain additional minor peaks of PTHrP(1–36) activity (peak IV). These

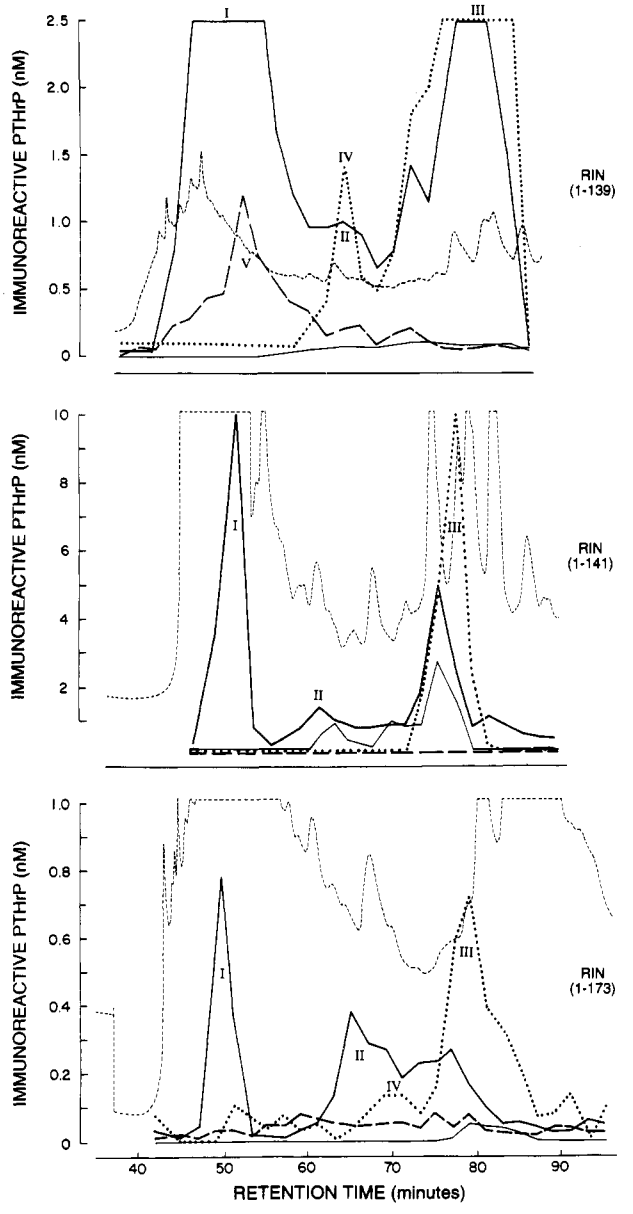

FIGURE 4: Reversed-phase HPLC of the three RIN cell extracts. The top panel shows RIN(1–139) extracts; the middle panel, RIN-(1–141); and the bottom panel, RIN(1–173). The flow rate was 0.5 mL/min. The gradient is a 70-minute 26–38% water/acetonitrile/0.1% TFA gradient; 1-mL fractions were collected at 2-min intervals. In each panel, the fine dashed line indicates protein absorbance at OD_{210}; the thick solid line, PTHrP(37–74) immunoreactivity; the dotted line, 1–36 immunoreactivity; the heavy dashed line, 109–138 immunoreactivity; and the thin solid line, 1–74 immunoreactivity. Each time point is the mean of a minimum of two radioimmunoassays. Immunoreactive peaks are labeled I–V as discussed in the text.

amino-terminal peaks have previously been demonstrated to be present in protease-protected conditioned medium from PTHrP(1–141)-expressing RIN cells (Soifer et al., 1992) and are thus secretory forms of the peptide.

The most striking finding in Figure 4 is that the RIN(1–139) cells contain a previously unrecognized peak of PTHrP-(109–138) immunoreactivity (peak V). In contrast to the findings in RIN(1–139) cells, peak V is undetectable in RIN-(1–141) and RIN(1–173) cells, despite the fact that the concentration of PTHrP(109–138) in this peak (1 nM) is approximately 50–100 times the detection limit of the PTHrP-(109–138) RIA, and despite the fact that extreme tail region PTHrP measured using a PTHrP(141–173) RIA is easily measurable (1000 pM) in RIN cells expressing the PTHrP-

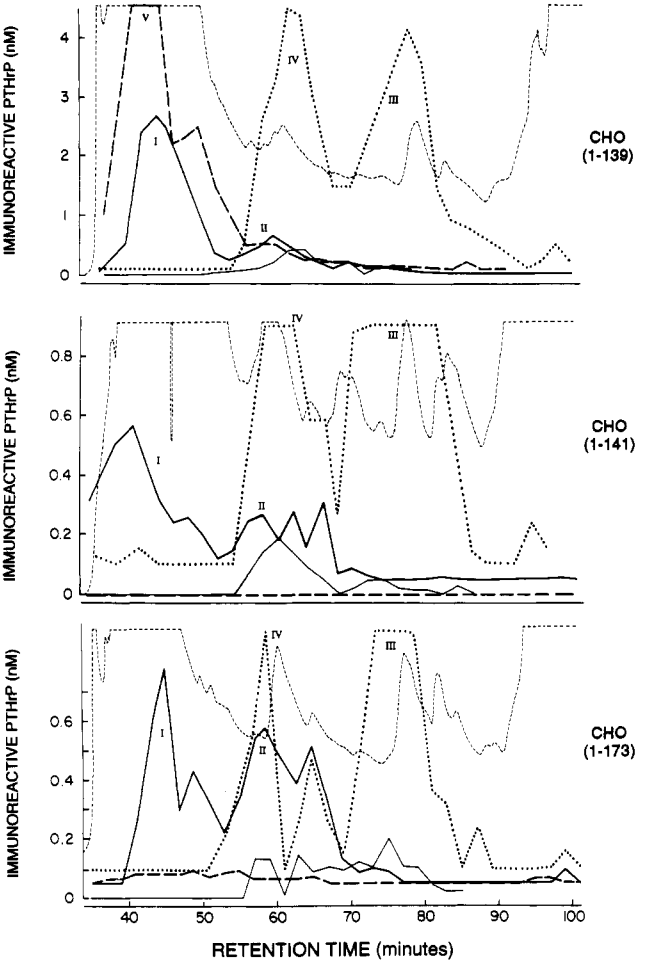

FIGURE 5: Reversed-phase HPLC of the three CHO cell extracts, arranged to correspond to the RIN cell extracts in Figure 4: the upper panel is CHO(1–139); the middle panel, CHO(1–141); and the lower panel, CHO(1–173). The gradient employed and the code for the dotted, dashed, and solid lines are the same as in Figure 4.

(1–173) isoform. These results were observed in multiple extracts and indicate that among the three RIN cell lines peak V is a unique carboxy-terminal product of the PTHrP-(1–139) isoform.

Processing of PTHrP(1–139), -(1–141), and -(1–173) in CHO Cells. In order to determine whether PTHrP processing differed in a prototypically constitutive secretory cell type as compared to a prototypically regulated secretory cell type, and to determine whether the inability of RIN(1–141) and RIN(1–173) cells to produce the carboxy-terminal PTHrP peak V was tissue- or isoform-specific, the three PTHrP constructs were stably transfected into CHO cells. The three CHO lines were extracted and analyzed by RP-HPLC and region-specific immunoassay as described above for the RIN cells. The results are shown in Figure 5 and reveal important similarities to and differences from those obtained in RIN cells.

First, each of the three CHO lines (Figure 5) contained the previously identified midregion PTHrP(37–74) peak I identified in RIN cells (Figure 4) (Soifer et al., 1992). As with the three RIN cell lines, the three CHO lines also contained a second smaller peak of midregion immunoreactivity (peak II).

Second, as with the three RIN lines (Figure 4), each of the three CHO lines also contained a major amino-terminal PTHrP species (peak III), identified using the PTHrP(1–36) RIA (Figure 5). Unlike the RIN lines, however, this amino-

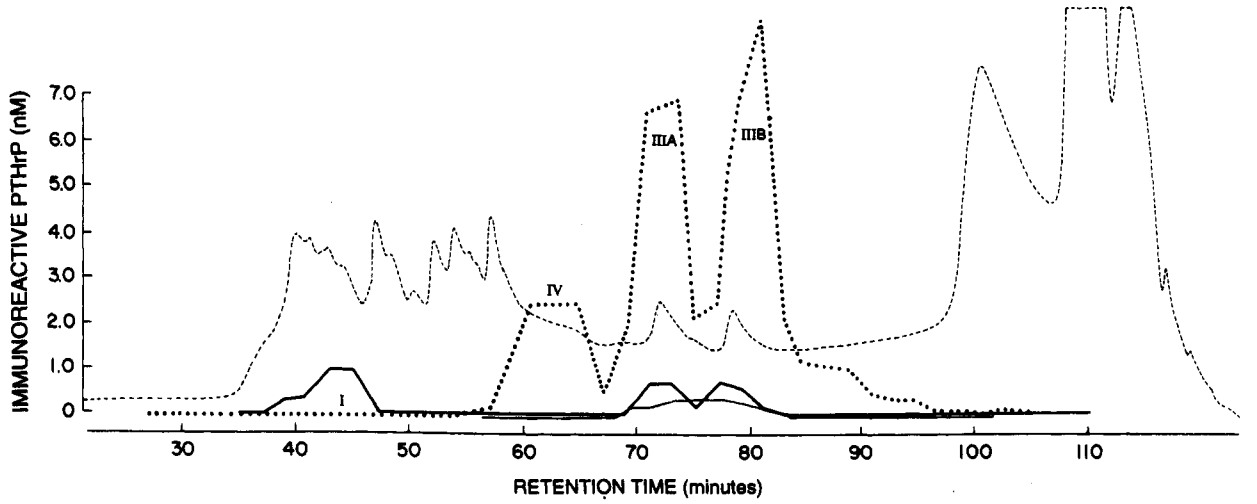

FIGURE 6: Amino-terminal secretory forms of PTHrP in protease-protected conditioned medium from CHO(1–141) cells. Compare to the middle panel of Figure 5. Medium (2 L) was affinity purified using an anti-PTHrP(1–36) immunoaffinity column, resolved using the same reversed-phase HPLC gradient shown in Figures 4 and 5, and immunoassayed using the PTHrP(1–36) RIA. Note that the amino-terminal immunoreactivity in the medium migrates in two distinct peaks, labeled IIIa and IIIb, which comigrate with peak III in the CHO(1–141) extract. Note also that relatively modest amounts of peak IV are present in conditioned medium, whereas it is a major amino-terminal component within the three CHO lines (Figure 5) prior to secretion.

terminal peak had little or no 37–74 immunoreactivity, suggesting that the processing of PTHrP is different in RIN as compared to CHO cells and is therefore cell-specific. Interestingly, and in contrast to results observed in the RIN cells, all three CHO cell lines contained a second major peak of amino-terminal immunoreactivity (peak IV) which had been minor or absent in the three RIN lines (Figure 4). This peak also comigrated with peak II, a midregion peak, suggesting that this could be a single PTHrP species with amino-terminal and midregion epitopes. The abundant expression of this peak in CHO cells contrasts with results seen in RIN cells and represents additional evidence for cell-specific processing of PTHrP.

The most important finding in the three CHO lines, however, was that the carboxy-terminal peak (peak V) identified in the RIN(1–139) cell line using the PTHrP(109–138) immunoassay was also present only in CHO cells which contained the PTHrP(1–139) construct. It was undetectable in the CHO(1–141) and -(1–173) cells, as it was in RIN(1–141) and RIN(1–173) cells. This peak is quantitatively larger in CHO cells than in RIN cells. The expression of this peak appears therefore to be isoform-specific, but not cell-specific.

Amino-Terminal PTHrP Species in CHO(1–141) Conditioned Medium. The studies described thus far relate to intracellular forms of PTHrP. We had previously observed that conditioned medium harvested from RIN(1–141) cells contained multiple amino-terminal PTHrP species corresponding to peak III in Figure 4 (Soifer et al., 1992). We had not previously observed the prominent amino-terminal peak IV expressed by the three CHO cell lines and wondered whether either or both of the two amino-terminal PTHrP peaks (III and IV) were actually secreted by CHO cells. With this goal in mind, we collected 2 L of CHO conditioned medium under conditions of protease protection (Soifer et al., 1992), concentrated the N-terminal PTHrP species using an anti-PTHrP(1–36) immunoaffinity column (Stewart et al., 1991), and resolved the N-terminal PTHrP secretory forms on the same RP-HPLC column and gradient employed in Figures 4 and 5. As shown in Figure 6, CHO(1–141) conditioned medium contained two peaks, labeled IIIa and IIIb, which migrate in the same position as had peak III in the CHO-(1–141) cell extract. In contrast, whereas peak IV is prominent in CHO cell extracts (Figure 5), it appears to be a relatively minor secretory form of PTHrP (Figure 6). Thus, peak IV may serve primarily as a processing intermediate, from which more mature amino-terminal and midregion PTHrP species are generated.

Midregion and Carboxy-Terminal PTHrP Species Are Chromatographically Distinct. In the RIN(1–139) and CHO(1–139) lines (top panels of Figures 4 and 5), peaks I and V comigrate, raising the question of whether these represent a single PTHrP species with both 37–74 and 109–138 epitopes or whether these are distinct peptides. Accordingly, CHO(1–139) cells were extracted and analyzed using a different RP-HPLC gradient (Figure 7, upper panel). As shown in Figure 7, these two peaks can be chromatographically separated, indicating that they represent separate peptides.

Carboxy-Terminal PTHrP (Peak V) Is Present in RIN and CHO Cell Lines and Conditioned Medium. In order to determine whether the carboxy-terminal PTHrP species (peak V) seen in CHO(1–139) and RIN(1–139) cells is exclusively an intracellular form of the peptide or whether it is also a secretory form, medium from each of the six RIN and CHO lines was harvested and assayed for PTHrP(109–138) immunoreactivity. As observed in the six cell extracts and as illustrated in Figure 8A, medium conditioned by RIN and CHO lines transfected with the PTHrP(1-139) constructs contained abundant PTHrP(109–138) immunoreactivity. In contrast, medium conditioned by RIN and CHO lines transfected with the 1–141 and 1–173 constructs contained relatively minor amounts of PTHrP(109–138) immunoreactivity. These findings indicate that peak V is secreted by those cell lines containing the 1–139 isoform and confirm that expression of the carboxy-terminal PTHrP peak V is isoform- but not cell-specific.

Carboxy-Terminal PTHrP Is a Product of Naturally Occurring Human Cell Lines. We have previously shown that amino-terminal and midregion PTHrP peptides are produced by naturally occurring human cell lines such as epidermal keratinocytes and renal carcinomas (Soifer et al., 1992; Merendino et al., 1986; Wu et al., 1991). We have not previously examined naturally occurring human PTHrP cells for carboxy-terminal PTHrP production. As demonstrated in Figure 8B, medium conditioned by naturally occurring

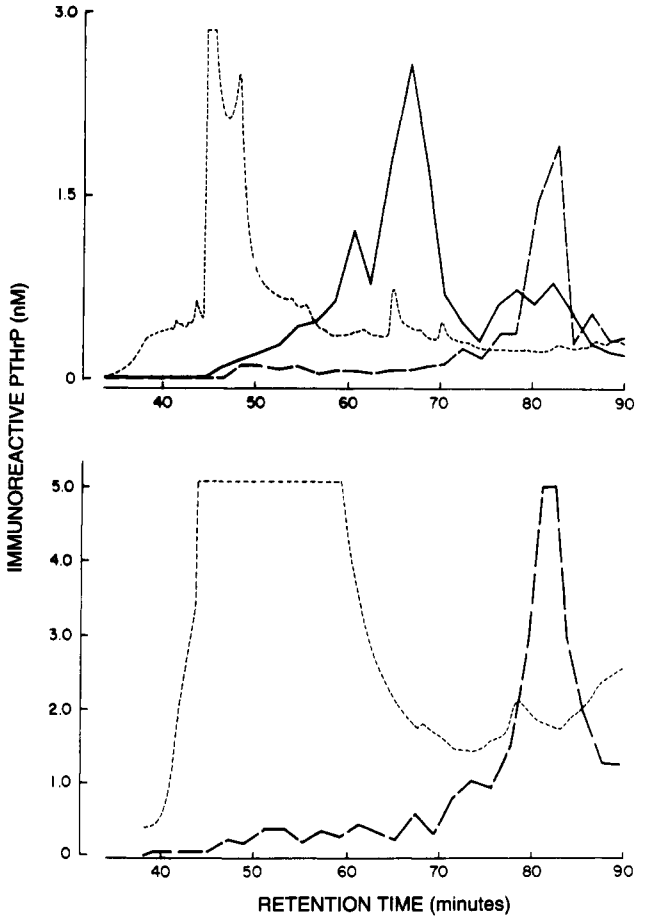

FIGURE 7: (Upper panel) Chromatographic resolution of midregion and carboxy-terminal PTHrP immunoreactivity in CHO(1–139) cell extracts. Extracts were prepared exactly as in Figure 5 but were resolved using a different gradient, in this case a 15–33% 70-min gradient. Peaks I and V, which had comigrated on the initial gradient, are clearly resolved on this gradient, indicating that these two peaks represent distinct peptides. (Lower Panel) Carboxy-terminal PTHrP from conditioned medium comigrates with its counterpart in cell extracts. Five milliliters of CHO(1–139) conditioned medium was resolved by RP-HPLC using the same gradient shown in the upper panel. PTHrP(109–138) immunoreactivity in both CHO(1–139) cell extracts and medium comigrates. Similar results were obtained when RIN(1–139) cell extracts and medium were studied (not shown). These observations indicate that the intracellular and secreted forms of the peptide are similar or identical.

malignant and nonmalignant PTHrP-producing cell lines including squamous, renal, and prostate carcinomas, as well as human epidermal keratinocytes, contains a carboxy-terminal form of the peptide.

The Carboxy-Terminal Form of PTHrP Observed in Conditioned Medium Is Chromatographically Identical to the Carboxy-Terminal Form of PTHrP Observed in Cell Extracts. In order to determine whether the PTHrP(109–138) immunoreactive species identified in conditioned medium in Figure 8A was the same as that found within cells (Figures 4 and 5), medium conditioned by RIN(1–139) and CHO-(1–139) was resolved by RP-HPLC using the same gradient employed in Figure 7, upper panel. As depicted in Figure 7, lower panel, the major carboxy-terminal peak in CHO(1–139) medium eluted in a position identical to that seen in cell extracts. Indistinguishable results were obtained using RIN-(1–139) medium (not shown). This comigration suggests that the carboxy-terminal species found in cell extracts and conditioned medium are highly similar or identical.

Immunohistochemical Intracellular Localization of PTHrP Species. The chromatographic findings described above

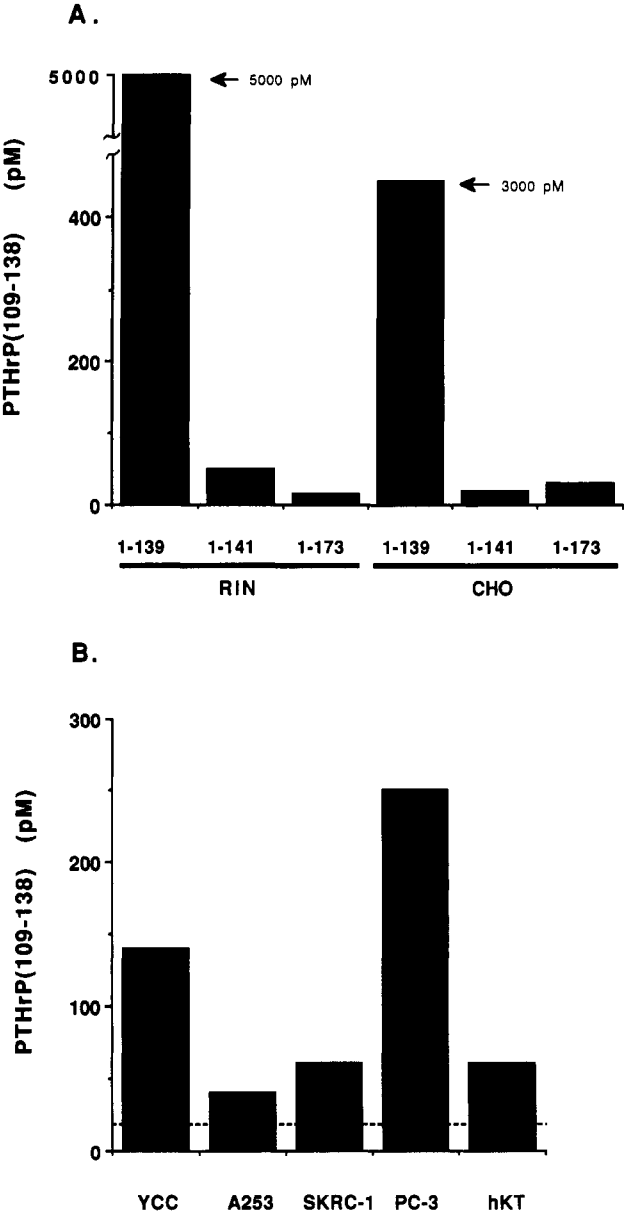

FIGURE 8: Carboxy-terminal PTHrP is present not only in cell extracts but also in conditioned medium. Medium was harvested from each of the cell lines indicated and assayed using the PTHrP(109–138) RIA. The detection limit of the assay is shown as the dotted line in panel B. Panel A shows the results from the stably transfected RIN and CHO lines, and Panel B, those from the five naturally occurring PTHrP-expressing human cell lines.

indicated that endoproteolytic processing of PTHrP occurred intracellularly prior to secretion. To determine the intracellular locations of the processed fragments, immunofluorescent histochemistry was performed on RIN(1–139) cells using antisera directed against PTHrP(1–36), PTHrP(37–74), and PTHrP(109–138). The results of these experiments are shown in Figure 9. PTHrP(1–36) and PTHrP(109–138) localized to the Golgi apparatus exclusively, whereas PTHrP-(37–74) was observed both in the Golgi apparatus and in what appeared to be secretory granules.

DISCUSSION

The production of PTHrP is complex, and this complexity is multilayered. First, PTHrP is produced by a large variety of neuroectoderm-, mesenchyme-, and endoderm-derived tissues beginning with the preimplantation embryo immediately following fertilization (van de Stolpe et al., 1993)

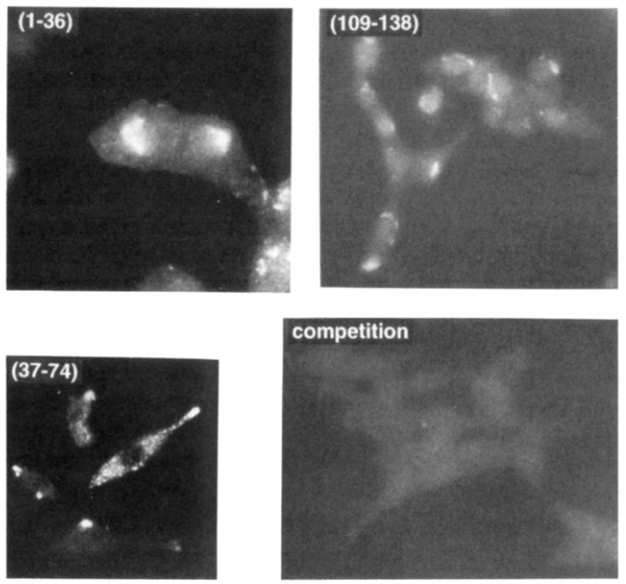

FIGURE 9: Immunohistochemical localization of PTHrP species in RIN(1–139) cells using a PTHrP(1–36) antiserum (panel A), a PTHrP(37–74) antiserum (panel B), or one of three PTHrP(109–138) antisera (panel C). Panel D is a negative control which shows the absence of staining in cells exposed to PTHrP(109–138) antisera which had been preabsorbed by preincubation with 10^{-6} M PTHrP-(109–138). Similar findings were observed in cells incubated without primary antiserum and in experiments in which immunohistochemistry was done in the presence of 10^{-6} M PTHrP(1–36) or PTHrP-(37–74) and the corresponding antiserum. The staining patterns of insulin within secretory granules and an anti-Golgi antibody in these cells have been published previously (Soifer et al., 1992). Note that PTHrP(1–36) and -(109–138) species are found predominantly within the Golgi apparatus, whereas the midregion fragment is found predominantly in what appears to be a secretory granule pattern.

and continuing throughout adult life (Bilezikian, 1990; Broadus & Stewart, 1994; Halloran & Nissenson, 1992; Stewart & Broadus, 1990a,b; Strewler & Nissenson, 1990). Second, transcriptional regulation of the PTHrP gene is complex, involving at least three different promoters, some of which appear to be employed in a tissue-specific fashion (Bilezikian, 1990; Broadus & Stewart, 1994; Halloran & Nissenson, 1992; Stewart & Broadus, 1990a,b; Strewler & Nissenson, 1990). Third, the intron/exon structure of the PTHrP gene is complex, more so, for example, than that of the PTH gene (Bilezikian, 1990; Broadus & Stewart, 1994; Halloran & Nissenson, 1992; Stewart & Broadus, 1990a,b; Strewler & Nissenson, 1990). This complexity gives rise, through alternative splicing, to three distinct mRNA isoforms, illustrated in Figure 1. The 3′ end of each of the three PTHrP mRNA species contains AUUUA-rich motifs, indicating that posttranscriptional control may be important in regulating PTHrP mRNA expression (Bilezikian, 1990; Broadus & Stewart, 1994; Halloran & Nissenson, 1992; Stewart & Broadus, 1990a,b; Strewler & Nissenson, 1990; Ikeda et al., 1989; Zakalik et al., 1992). Fourth, inspection of the three cDNA-predicted PTHrPs indicates that PTHrP, like POMC, ANP, CCK, SRIF, and many other neuroendocrine peptides (Fricker, 1991; Loh, 1993; Steiner et al., 1992), is likely to be subject to extensive posttranslational endoproteolytic processing at mono- and multibasic amino acids (Fricker, 1991; Loh, 1993; Steiner et al., 1992), adding another layer of complexity to an already complicated process of expression. To date, the existence of PTHrP(1–36) (Rabbani et al., 1993; Soifer et al., 1992), an *O*-glycosylated N-terminal PTHrP species (Wu et al., 1991), and a midregion PTHrP which begins at Ala^{38} following a cleavage at Arg^{37} and extends approximately 60 amino acids (Soifer et al., 1992) has been documented. The putative multibasic cleavage sites in the three initial translation products suggest that additional carboxy-terminal PTHrPs, shown as the dotted boxes in Figure 1D, will eventually be identified (Fricker, 1991; Loh, 1993; Steiner et al., 1992). Each of the final secretory forms of PTHrP will presumably prove to have unique physiologic functions, in a fashion analogous to ACTH, the MSHs, γ-lipotrophin, β-lipotrophin, and β-endorphin, which arise from POMC, or the several glucagons, somatostatins, calcitonins, and cholecystokinins, which arise through proteolytic processing from their parent precursor proteins (Loh, 1993; Fricker, 1991; Steiner et al., 1992). Interestingly, the work of Henderson, Kaiser, Goltzman and their co-workers (Henderson et al., 1992; Kaiser et al., 1992) and of Fenton et al. (1991) suggests that in some cases mature amino-terminal and carboxy-terminal secretory species may have opposing actions.

In the current study, we examined PTHrP posttranslational processing by a prototypical regulated secretory cell line (RIN) and a prototypical constitutive secretory cell line (CHO). All six cell lines produced an amino-terminal form of PTHrP (peak III) which we had observed previously both in cell extracts and in medium conditioned by RIN(1–141) cells and by SKRC-1 human renal carcinoma cells (Soifer et al., 1992). This region of the peptide presumably contains the receptor-binding, adenylate cyclase, and cytosolic calcium-stimulating regions of the peptide and appears to account for the similarities between primary hyperparathyroidism and HHM (Bilezikian, 1990; Broadus & Stewart, 1994; Halloran & Nissenson, 1992; Stewart & Broadus, 1990a,b; Strewler & Nissenson, 1990). Complete purification and amino- and carboxy-terminal characterization of this peptide are currently in progress.

In addition, all six of the cell lines studied produced a midregion form of PTHrP (peak I) which we had previously also identified as an intracellular and secretory form of PTHrP in SKRC-1 renal carcinoma cells and in RIN(1–141) cells (Soifer et al., 1992). This region of the peptide is highly conserved among species (Bilezikian, 1990; Broadus & Stewart, 1994; Halloran & Nissenson, 1992; Stewart & Broadus, 1990a,b; Strewler & Nissenson, 1990) and would appear to include the 67–86 region shown by Care and collaborators to mediate calcium transport across the placenta (Care et al., 1990). There was thus no evidence of isoform- or cell-specific processing at the Arg^{37} site. The fact that this cleavage occurs in cells both with and without secretory granules suggests that this cleavage occurs in a site other than secretory granules, such as the Golgi apparatus or the transgolgi network.

An important finding in the current study is the processing and secretion of a previously unrecognized carboxy-terminal form of PTHrP, which contains PTHrP(109–138) immunoreactivity. This peptide is present both within cells and in conditioned medium, indicating that it is not a biosynthetic intermediate but a secretory form of the peptide. At present, the physiological function of this region of PTHrP remains speculative, but several points are worth emphasizing. First, a carboxy-terminal fragment of PTHrP recognized by the same PTHrP(109–138) antiserum is present in the plasma of patients with humoral hypercalcemia of malignancy (Burtis et al., 1990), as well as in those with acute and chronic renal failure (Burtis et al., 1990; Orloff et al., 1993). Second, this peptide is likely to contain the PTHrP(107–111) region that is highly conserved among species and which appears to have potent osteoclast-inhibiting activity (Fenton et al., 1991). Third, it may be related to the carboxy-terminal fragment of

PTHrP identified by Imamura et al. using a PTHrP(127–141) RIA in the urine of patients with HHM (Imamura et al., 1991). This peptide was produced both by RIN cells and by CHO cells. This observation, together with the fact that it was identified in the Golgi apparatus of RIN cells but, unlike the midregion fragment, was not identified in secretory granules, suggests that the carboxy-terminal form of PTHrP is secreted by the constitutive and not the regulated secretory pathway. Further, this fragment appeared in the medium conditioned by all five different naturally occurring human PTHrP-producing cell lines examined, indicating that it is not simply a product of the bioengineered cell lines. The fact that every cell line examined (RIN, CHO, SKRC-1, A253, YCC SQ-1, PC3, and hKT) produces this product indicates that tissue-specific expression is not a characteristic of this fragment of PTHrP, at least among the seven cell lines examined.

A second important finding in the current study is that this carboxy-terminal form of PTHrP was found only in RIN and CHO cells containing the PTHrP(1–139) construct, indicating that production of peak V in cell extracts and medium is in some sense isoform-specific or isoform-dependent. Since the human gene has the capacity to generate all three PTHrP transcripts, we would expect that each of the three transcripts would encode proteins with distinct structures that might perform physiologically important but distinct functions. In this sense, it is not surprising that the 1–139 and 1–173 peptide isoforms, with such different carboxy termini (Figures 1A and 2), would give rise to daughter peptides in which the carboxy termini are differentially processed. It is surprising, however, that processing of the 1–139 and 1–141 transcripts, which differ in their carboxy termini by only two amino acids (Figure 2), would lead in one case (1–139) to exuberant expression of peak V and in the other (1–141) to negligible amounts of the same peptide. Close inspection of the C-termini of these two isoforms (Figure 2) sheds little light on the mechanisms responsible for this differential processing: in one case, the 1–139 translation product would presumably be trimmed by carboxypeptidase E or a related enzyme to a peptide terminating at Ser^{138}; in the second case, the Arg^{139}-Arg^{140} segment in the 1–141 transcript would likely be cleaved by one of the subtilisin family of enzymes between Arg^{140} and His^{141} (Fricker, 1991; Loh, 1993; Steiner et al., 1992) and then trimmed to the same Ser^{138} by carboxypeptidase (Fricker, 1991; Loh, 1993; Steiner et al., 1992). Thus, one can only speculate as to why these similar precursors might lead to profoundly different carboxy-terminal forms. It may be that the two additional amino acids alter the folding of the carboxy-terminal form of the peptide produced by the 1–141 and 1–173 cell lines in such a way as to render it unrecognizable by the 109–138 antiserum. Clarification of these issues will require further study. Nonetheless, these findings begin to provide a rationale for the apparent redundant existence of exons 4 and 6 in the human gene and for both the 1–139 and 1–141 isoforms of the peptide.

A third important finding in the current study is that PTHrP processing appears to be cell-specific. Two examples were encountered. First, each of the three RIN cell lines contained a quantitatively important peak (peak III) of midregion immunoreactivity, detected using the 37–74 RIA. This midregion peak comigrated with a peak of PTHrP(1–36) immunoreactivity. In contrast, none of the three CHO lines contained a midregion 37–74 peak in the region of peak III. A second, more striking example of tissue-specific processing is demonstrated by peak IV, which was abundant in the three CHO lines but quantitatively scarce or absent in the three RIN lines. Since this peak is present in CHO conditioned medium in only small amounts (Figure 6), it is presumed that it is a biosynthetic intermediate of PTHrP, analogous, for example, to the ACTH/joining peptide precursor in POMC, which is identified within cells but only in small amounts in medium (Mains et al., 1991).

RIN cells secrete insulin via the regulated secretory pathway and store insulin within typical secretory granules (Soifer et al., 1992). We had previously demonstrated, using RIN cells overexpressing PTHrP(1–141), that PTHrP(1–36) immunoreactivity was confined to the Golgi apparatus, while the midregion fragment identified using the PTHrP(37–74) antiserum was present in what appeared to be secretory granules and displayed an intracellular distribution similar to that of insulin in these same RIN cells (Soifer et al., 1992). In order to define the intracellular location of the carboxy-terminal fragment identified with the PTHrP(109–138) antiserum, immunohistochemistry was performed on RIN-(1–139) cells using three PTHrP(109–138) antisera. PTHrP-(109–138) staining was observed with each antiserum only in the Golgi apparatus, in a pattern similar to that seen with the PTHrP(1–36) antiserum. In contrast, the staining obtained with the PTHrP(37–74) antiserum, while present in Golgi, was predominantly scattered throughout the cytoplasm and cell periphery in what appeared to be secretory granules. These findings have two possible explanations. It may be that all three PTHrP secretory forms are packaged into secretory granules in RIN cells and are secreted via the regulated secretory pathway. In order for this possibility to be true, one must postulate that PTHrP species identified with the 1–36 and the three 109–138 antisera are folded, posttranslationally processed, or packaged into secretory granules in a manner which obscures the epitopes which are recognized by the 1–36 and 109–138 antisera which were identified in the cell extracts and in conditioned medium. The other possibility is that the endoproteolytic cleavages which generate the three secretory species occur in the Golgi apparatus (i.e., prior to packaging within secretory granules) and that the three fragments are packaged separately, with the midregion fragment entering the regulated secretory pathway and the amino- and carboxy-terminal secretory forms entering the constitutive secretory pathway. In support of the latter possibility is the observation that the aplysia egg-laying prohormone (pro-ELH), a model neuroendocrine secretory peptide, is cleaved into multiple secretory fragments, which are packaged into different vesicle classes in distinct cellular locations (Fisher et al., 1988). In this regard it is noteworthy that Deftos and his colleagues (Deftos et al., 1993) have identified carboxy-terminal PTHrP in secretory granules at the electron microscopic level in atrial cardiocytes. Whether this apparent packaging of carboxy-terminal PTHrP into dense core secretory granules is due to differences in the carboxy-terminal antibody used in their work compared to those we used, or whether atrial cardiocytes process PTHrP differently than do RIN cells, remains uncertain at present. Clarification of this issue will require formal evaluation of the PTHrP secretory mechanisms in these and other cell types.

These studies indicate that the posttranslational processing of PTHrP is a complex process, which includes both cell-specific and isoform-specific components. In addition, PTHrP posttranslational processing steps may include *O*-glycosylation and carboxy-terminal amidation. This complexity of processing is not limited to PTHrP expressed in transfected cell lines but occurs in naturally occurring human PTHrP-secreting

cells as well. Finally, while CHO and RIN cells endoproteolytically process and glycosylate human peptide hormones with reasonable fidelity, it is conceivable that these rodent cell lines may not process the human form of PTHrP as they would the rat form, or that the human cells process the human isoform of PTHrP in a different manner than that described herein for rodent cells. These considerations, together with the studies presented here, underscore the need to characterize fully the secretory forms of PTHrP so that the physiology of this complex family of peptides can be meaningfully studied.

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