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Purification of a Novel Mid-region Fragment of Parathyroid Hormone-related Protein
and Evidence of its Secretion by Three Different Cell Types

A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of
the Requirements for the Degree of Doctor of Medicine

by
Katherine Elizabeth Dee
1994

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Abstract

PURIFICATION OF A NOVEL MID-REGION FRAGMENT OF PARATHYROID HORMONE-RELATED PROTEIN AND EVIDENCE OF ITS SECRETION BY THREE DIFFERENT CELL TYPES

Despite presumptive evidence for extensive posttranslational processing, little is known of the native secretory forms of Parathyroid Hormone-related Protein (PTHrP). To identify these forms, PTHrP species were purified from conditioned medium and cell extracts from three cell types: human renal carcinoma (SKRC-1) cells, human keratinocytes, and rat insulinoma cells transfected with the cDNA for PTHrP (1-141) (RIN-141 cells). Amino-terminal and mid-region species were immunoaffinitypurified using anti-PTHrP (1-36) and anti-PTHrP(37-74) columns respectively. Immunopurified species were further resolved by reversed-phase high performance liquid chromatography (RP-HPLC) and identified using region-specific immunoassays. Purified species were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), amino acid sequencing and immunofluorescence studies.

RIN-141 and SKRC-1 cells secreted at least three amino-terminal PTHrP species as well as a novel mid-region fragment. Analysis by SDS-PAGE and amino acid sequencing indicated that the RIN-141 mid-region fragment begins at residue 38 of the cDNA-predicted sequence and is approximately 70 amino acids in length. Comparison of the RP-HPLC elution patterns suggests that SKRC-1 cells and keratinocytes secrete a similar if not identical mid-region fragment. Immunohistochemical studies revealed a secretory granular pattern for the mid-region species and a Golgi pattern for amino-terminal species.

It follows from these studies that 1) three cell lines secrete multiple secretory forms of PTHrP, including a novel mid-region fragment; 2) Arg³⁷ serves as a monobasic endoproteolytic processing site in the PTHrP biosynthetic precursor; 3) PTHrP (1-36) is an authentic secretory form of PTHrP; and 4) the mid-region fragment appears to be packaged into secretory granules.

I Introduction

1. Discovery

The discovery of parathyroid hormone-related protein (PTHrP) as the cause of humoral hypercalcemia of malignancy (HHM) occurred some 60 years after serum calcium concentrations were first discovered to be elevated in many patients with cancer. Early studies in the 1930's and 40's first indicated that the high serum calcium concentrations were almost always associated with skeletal metastases and thus were inferred to result from active bone resorption by tumor cells.²⁸ In 1941, Fuller Albright adumbrated that a humoral mechanism might explain the occurrence of hypercalcemia in a patient with renal cell carcinoma with a solitary skeletal metastasis.¹³ This notion remained unsubstantiated for 15 years until two series of patients in the 1950's were published. In these studies, a *humoral* form of hypercalcemia was demonstrated in all subjects as defined using two major criteria: 1) hypercalcemia occurred in the absence of radiologically evident skeletal metastases, and/or 2) hypercalcemia was corrected by the removal of the primary tumor mass.^{17,71} A 1966 series reviewed 50 patients with malignancy-associated hypercalcemia who met these criteria for HHM. Those patients tended to have squamous, renal, urothelial and gynecologic tumors. In contrast, patients with local osteolytic hypercalcemia (LOH) due to skeletal invasion by their tumors typically suffer from multiple myeloma or metastatic breast carcinoma. It was also observed that the syndrome of HHM was far more common than previously had been thought.⁴⁷

Increasing interest in the search for the mediator(s) of HHM in the 1960's and 70's lead to the consideration of vitamin D-like sterols, prostaglandin E (PGE), and parathyroid hormone (PTH) and its precursors. The earliest studies focused on plant-derived vitamin D analogs (phytosterols) found in the circulation of several patients with breast carcinoma and hypercalcemia.²⁷ However, further investigation proved that these sterols lacked the

potency to cause hypercalcemic effects in the concentrations measured in plasma.²⁹ In 1970, PGE₂ was implicated as a cause of hypercalcemia when studied in animal tumor models.⁴² Studies which followed in humans in the following decade, however, proved that PGE as a source of hypercalcemia is extremely rare.⁵⁶

Albright had originally suggested PTH as the most likely mediator of HHM. The idea of 'ectopic hyperparathyroidism' gained some weight when the use of a first generation PTH radioimmunoassay (RIA) demonstrated elevated PTH levels in patients with certain tumors.⁴ In the 1970's, wide use of the PTH RIA documented detectable (but not elevated) concentrations of PTH in serum and PTH immunoreactivity in tumors.^{3,70} However, controversy grew as to whether PTH was actually the cause of hypercalcemia in these patients. The concentration of PTH measured in these patients varied widely and, in most cases, though detectable by sensitive assays, were disproportionately low, as would be expected as the natural physiologic reaction to high serum calcium produced by some other cause. Over the ensuing decade, it became clear that PTH is not the usual cause of HHM. Recently, a modern two-site immunoradiometric assay (IRMA) has clearly demonstrated that circulating PTH is suppressed or unmeasurably low in patients with HHM.^{7,62} There are several examples in the literature of ectopic PTH production by tumors, but these are exceedingly rare and tend to result from unusual tumor types.^{62,94,114}

In 1980 and 1981, several independent groups published studies of renal calcium, phosphate and most importantly, nephrogenous cyclic adenosine monophosphate (NcAMP) excretion in large series of patients with malignancy-associated hypercalcemia.^{25,45,79,89} Two groups were defined. One group comprised 80% of the patients and displayed characteristics which came to define HHM. These include increased excretion of NcAMP, increased calcium excretion, decreased circulating 1,25(OH)₂D levels and normal to decreased PTH levels. These patients suffered primarily from squamous, renal and urothelial tumors and had a low incidence of bony metastases. The remaining 20% had decreased NcAMP and a high incidence of skeletal metastases.

These patients were hypercalcemic due to their skeletal metastases, primarily from breast and hematologic tumors.

Next, Goltzman²⁶ in 1981 found PTH-like bioactivity in the circulation of patients with HHM, not present in patients with LOH, using a sensitive cytochemical bioassay for PTH. However, the circulating bioactive material was larger than PTH on gel filtration. In addition, these patients demonstrated an uncoupling of bone turnover with increased osteoclastic bone resorption and decreased osteoblastic activity, unlike the coupled bone turnover which is stimulated by high levels of native PTH. Thus, patients with HHM were found to have a circulating factor in their serum with both “PTH-like” and “PTH-unlike” characteristics.

This PTH-like protein was eventually purified simultaneously by three groups, who each managed to overcome the protein's low abundance by employing a PTH-sensitive adenylate cyclase assay for the detection of the factor in conditioned medium from a squamous cell carcinoma,⁵⁸ a renal cell carcinoma,⁹⁵ and from squamous cell and breast tumor extracts.⁹³ The isolated protein, parathyroid hormone-related protein (PTHrP), was found by N-terminal sequencing to possess 13 N-terminal amino acids highly similar to those of PTH. The amino acid sequence distal to this region revealed a unique sequence unlike any other known peptide. The amino acid sequence of this new peptide revealed an explanation for its “PTH-like” and “PTH-unlike” qualities. As will be discussed, the amino-terminal PTH-like sequence corresponds to the receptor-activating region of PTH. The remaining unique sequence explains the PTH-unlike characteristics.

Using an oligonucleotide probe designed using the N-terminal sequence data from the isolated protein, the cDNA for PTHrP was isolated and the gene found to be located on chromosome 12.^{54,96} The gene encoding PTH itself is located on chromosome 11. This pair of chromosomes is thought to have arisen long ago from an ancestral duplication. The location of the the PTH and PTHrP genes on chromosomes 11 and 12,

together with their shared intron-exon structure, raises the likelihood that these genes form two members of a small gene family.

It is now widely acknowledged that PTHrP is the principal cause of HHM. First, using PTHrP RIAs and a two-site PTHrP IRMA, elevated levels of PTHrP have been found circulating in the serum of patients with HHM.^{9,10} Second, synthetic and recombinant PTHrP peptides have been shown to reproduce all of the features of the syndrome, as shown in animals as well as tissues, organs or cells *in vitro*.^{19,32,33,40,58,68,69,88,91,107,112} Third, passive immunization with PTHrP antiserum reverses HHM in animals.^{31,46} These and other studies have established that PTHrP is both necessary and sufficient to cause HHM, with rare exceptions already noted.

2. The PTHrP Gene and mRNA Species

In contrast to the relatively simple organization of the PTH gene, the gene for PTHrP (fig. 1) reveals a high degree of complexity, with at least three promoters, seven exons, and three alternative 3' splicing patterns. The ancestral similarity of the two genes is apparent in the shared structure and organization of the middle exons of the PTHrP gene when compared to the three exons of PTH gene. While the PTH gene is expressed exclusively in the parathyroid gland, the PTHrP gene has been found to be expressed at

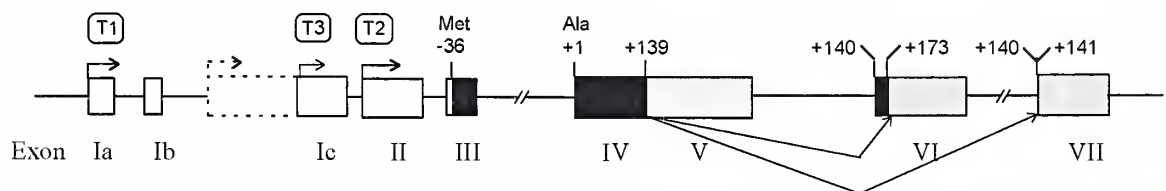


Fig. 1. The organization of the human PTHrP gene. Exons are indicated by boxes designated by a Roman numeral; white--5' noncoding; black--coding; grey--3' noncoding. T1, T2 and T3 indicate upstream and downstream transcription initiation sites respectively; consensus TATA boxes are found upstream of both sites. Other transcription initiation sites lying between T1 and T3 may also be used (dashed line exonic box and arrow). Positive numbers denote the position of nucleotides encoding amino acids in the mature peptide and negative numbers the position of nucleotides encoding amino acids in the prepro region of the peptide. Alternate mRNA splicing options are indicated by arrows connecting coding exons. Modified from Halloran.³⁰

low levels in many tissues throughout the body. In fact, growing evidence is pointing to an almost purely paracrine/autocrine role for PTHrP. Systemic humoral or endocrine effects of PTHrP appear to be found only in the pathologic condition seen in cases of HHM.

The human gene sequence encodes three mRNA species which correspond to resultant peptides with different carboxy termini (1-139), (1-141), and (1-173) (see fig. 3). Little is known about the splicing patterns employed for these three messages among different tissues. Recent work has put forth the possibility of tissue-specificity of PTHrP transcripts and processing of their respective protein products.¹¹⁰ Interestingly, the 3' untranslated regions are rich in mRNA instability motifs comprising multiple repeated patterns of the sequence 'AUUUA.' Such motifs lead to the rapid degradation of those mRNA species which contain them. These motifs are commonly present in genes controlling cellular proliferation and differentiation. These motifs encoded in the 3' untranslated region of PTHrP mRNA species are highly conserved from mouse to man and are the only downstream elements conserved in the PTHrP gene among species. Since the transcription rates of the PTHrP gene are in general comparable to those of more abundant mRNA species, and since the half-lives of PTHrP mRNAs are short, these sequences may appear to account for the low levels of PTHrP found in most tissues. Furthermore, the existence of these sequences implies an autocrine/paracrine role for the protein product.^{29,39,53,54,82,96,111} A thorough discussion of the structure and regulation of the PTHrP gene is beyond the scope of this paper. However, a picture is emerging of a widely expressed, locally-acting regulatory peptide. The marked conservation among species suggests that its actions are crucial to normal development. This has been borne out by the findings of Karapalis et. al.,³⁹ who have shown that the homozygous disruption of the PTHrP gene in mice has lethal consequences.

3. Gene Expression and Regulation

Use of molecular biologic and sensitive immunochemical techniques has demonstrated the expression of the PTHrP gene in a wide array of adult tissues (skin, bone marrow, stomach, brain, kidney, heart, vascular smooth muscle, skeletal muscle, lung, pancreatic islets, parathyroid, adrenal, testis, ovary, uterus, lactating mammary gland and placenta) as well as fetal tissues (brain, liver, heart, lung, intestine, skeletal muscle, chorioallantoic membrane, and yolk sac).³⁰ The study of PTHrP gene regulation has illuminated a large number of factors which affect its transcriptional activity. The following is an overview of some of these areas of investigation.

Several hormones have been implicated in this regulation. For instance, studies *in vitro* have shown that transcriptional activity is reduced by increasing levels of glucocorticoids and $1,25(\text{OH})_2\text{D}_3$ in a human thyroid C-cell line.^{35,50} Activity in an osteoblast-like cell line is stimulated, however, by epidermal growth factor (EGF) and phorbol esters. Since EGF is mitogenic in these cells, it has been proposed that PTHrP may act as a paracrine mediator of the mitogenic response in these cells.^{22,44,75}

Studies *in vivo* have also demonstrated hormonal modulation of PTHrP expression. Studies with rats have shown that post-partum suckling in the first two weeks stimulates the lactating mammary gland to increase PTHrP production abruptly, and that when suckling ceases, PTHrP drops back to low levels.^{8,108} This response was shown to result from changes in prolactin levels.⁹⁷ Studies with pregnant rats have also produced evidence that estrogens stimulate the production of PTHrP message in the rat uterus,¹⁰⁰ as do estrogen analogs such as tamoxifen.⁹⁹

The syndrome of HHM has been seen as a complication not only of solid tumors but also of adult T-cell leukemia caused by the human T-cell leukemia virus type 1 (HTLV-1).⁴¹ Studies have shown a correlation between infection with the HTLV-1 virus and expression of the PTHrP gene, and suggest that viral factors may direct PTHrP production in infected cells.^{59,60}

The expression of PTHrP has also been shown to be responsive to stretch. One group examined rats with unilateral pregnancy, where the developing fetus is located in only one horn of a bifid uterus. This can occur naturally or by surgical manipulation. Investigators detected dramatic elevations of PTHrP mRNA only in the gravid horn.⁹⁸ This group's work has shown that a biomechanical stimulus causes the synthesis of PTHrP. Further research has shown that PTHrP mRNA in the rat urinary bladder rises with increasing bladder volume.¹⁰⁹ As will be discussed later, PTHrP has been shown to relax smooth muscle in various systems.^{57,83,84,101,104} It may be, therefore, that PTHrP plays an autocrine/paracrine modulatory role in accommodation of intraluminal volume in hollow viscera and vascular tissue.

4. The Peptide and its Regulation

Multiple forms of PTHrP are synthesized and secreted by cells. As described earlier, the gene encodes three different precursor proteins which are translated from three alternatively spliced mRNA transcripts (fig. 3). Each of these three mRNA species encodes a putative 36 amino acid 'prepro' sequence, followed by three mature protein isoforms, each of which is identical in the initial 139 amino acids, but differs in its carboxyl terminus (1-139, 1-141, 1-173). PTH, by comparison, is synthesized as one isoform comprising 84 amino acids.

The primary structure of PTHrP may be divided into several regions (fig.2). The N-terminal sequence from amino acids 1-13, with 70% homology to the corresponding amino acids in PTH, is the signal transduction activating region and confers PTH-like biologic activity. The sequence from amino acids 14-34 is divergent from PTH yet, surprisingly, is necessary for binding to the PTH receptor. The region spanning 35-111 is highly conserved among species: the 70 amino acid stretch between residues 38-108 in the human sequence differs by only two amino acids from its counterparts in the rat and mouse, and by 11 amino acids from that in the chicken.^{30,87} Such a degree of conservation

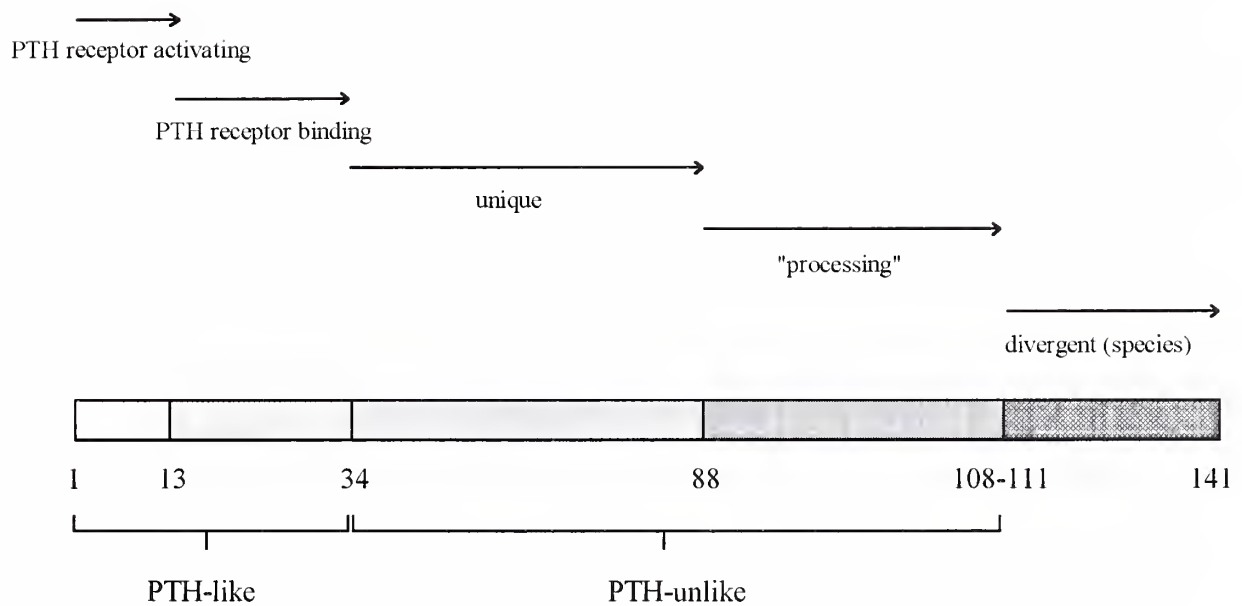


Fig. 2. Structural and functional domains of PTHrP (1-141). See text for details. Modified from Stewart and Broadus.⁸⁷

surpasses that of such important peptides as insulin and growth hormone, suggesting a crucial, yet undetermined physiological role. This region is rich in potential post-translational processing sites as is discussed below in section I.8. Finally, the C-terminal sequences diverge greatly among species. Though it has been speculated that these regions may confer tissue specificity, this is yet to be determined.⁶⁵

The secondary and tertiary structure of PTHrP (1-34) has been modeled using the circular dichroism model and compared to PTH (1-34). This model predicts similar structures for N-terminal PTHrP and PTH with two α -helices (the binding core) and a connecting hydrophilic bridge (activating region).^{16,30}

There are several regulatory mechanisms controlling the availability of the peptide. In addition to transcriptional controls mentioned above, steady state mRNA levels appear to be regulated by mRNA stability. In addition, post-translational processing steps such as proteolytic cleavage, glycosylation and amidation, may play a significant role in PTHrP regulation and function, and is discussed further in section I.8.

The existence of multiple regulatory mechanisms at the gene, mRNA and protein levels support the building impression of a diverse set of local control mechanisms over the availability of PTHrP. This adds more weight to the idea of PTHrP as a locally-secreted and locally-acting autocrine/paracrine hormone, which is regulated close to its sites of action.

5. PTHrP Receptors

The mechanism of action of PTHrP, at least as it acts in HHM, has been shown to be through PTH receptors in bone and kidney. Indirect evidence of this was inferred in early studies in which patients were found to have increased nephrogenous cAMP excretion despite low PTH levels.⁸⁹ In fact, as already discussed, this was the basis of early renal cortical adenylate cyclase bioassays for PTHrP. Direct support was gained when radioligand binding experiments demonstrated that synthetic PTHrP (1-34) could compete equally effectively with PTH (1-34) for binding to PTH receptors in bone and kidney.⁹⁵ In fact, once the PTH receptor was cloned, both PTH and PTHrP were shown to bind with equal affinity to this single receptor.¹

The fact that PTHrP and PTH can act via the same receptor in bone and kidney does not rule out the possibility that PTHrP might have its own unique receptors in other tissues. The hypothesis has been put forth that such receptors may reside in nonclassical PTH target tissues. Pharmacologic amounts of PTH have been shown to cause relaxation in vascular smooth muscle,^{18,102} uterus,⁸⁴ gastric smooth muscle,⁵⁷ and tracheal smooth muscle,¹¹³ yet there is no evidence that PTH acts on these tissues at physiologic levels. These studies, however, have shown equal potencies of PTH and PTHrP on these tissues. The search for a unique PTH receptor has only begun. Preliminary findings strongly indicate that unique PTHrP receptors do exist, for example, in keratinocytes and pancreatic islet cells.^{24,64} The study of the secretory forms of the protein has demonstrated that receptors for non-amino-terminal secretory forms of PTHrP are likely to exist as well.

This paper will present evidence of a secreted fragment which does not include the N-terminal PTH-like sequence; any physiologic effect of this novel peptide would likely result from action on a novel receptor.

6. HHM and the Mechanism of Action of PTHrP

HHM is the most common paraneoplastic syndrome, most often occurring in squamous cell and renal cell carcinomas.^{9,10,25,47,89} In addition, about 30% of breast cancer-associated hypercalcemia is caused by PTHrP production rather than by skeletal metastases.³⁷ Lymphomas associated with HTLV-1 infection also produce PTHrP.^{59,60} Finally, tumors which traditionally produce hypercalcemia by direct skeletal invasion, such as myeloma, may also, on occasion, meet the criteria for HHM with increased serum PTHrP and urinary NcAMP levels.^{9,10,25,89}

In patients with HHM, the high PTHrP concentrations have been shown to increase serum calcium by action on bone and kidney. Yet there remain three unexplained differences between hyperparathyroidism and HHM. First, in the bone of patients with HHM, unlike the increased coupled bone turnover seen in hyperparathyroidism, there is decreased osteoblastic activity uncoupled from highly increased osteoclastic bone resorption. It is known that osteoclasts lack PTH receptors, and that PTH acts on osteoblasts, which in turn stimulate osteoclastic activity. Though PTHrP may indeed act in this way, it is yet to be discovered how the differential between the effects of PTH and PTHrP are created. There may be PTHrP receptors on other cells present in the marrow which may in turn stimulate osteoclastic activity.⁷⁸ On the other hand, osteoblasts, though few in number, may still be the target of action and have the potency to effect such a response from osteoclastic cells. There is little data to support such theories and they remain speculative.

Second, in the kidney, PTHrP appears to act, as does PTH, on PTH receptors causing an increase in NcAMP and a decrease in the renal phosphate threshold.³⁰ Animal studies using short synthetic peptides have shown that PTHrP increases calcium

reabsorption in the kidney.^{20,74,81,85} However, patients with HHM have been shown to be relatively hypercalciuric compared to those with primary hyperparathyroidism.²⁵

Third, there is also a discrepancy in vitamin D production. $1,25(\text{OH})_2\text{D}$ levels in patients with HHM are reduced compared to those found in patients with hyperparathyroidism.^{25,89} If all of the actions of PTHrP are exclusively mediated via the PTH receptor, the differences between hyperparathyroidism and HHM remain difficult to explain. In addition, the nature of the actual secreted forms of the peptide and the mechanism whereby they act have yet to be elucidated. Investigation of the structure of these native forms and their physiology may reveal specific mechanisms of action which account for such discrepancies.

7. The Roles of PTHrP in Normal Physiology

Since PTHrP is nearly ubiquitous in human tissues, the protein has been implicated, logically, in a multitude of roles in normal physiology. Those highlighted here are elaborated in much greater detail elsewhere.³⁰ PTHrP, found in abundance in keratinocytes, has been found to stimulate an increase in intracellular calcium without stimulating adenylate cyclase, and investigations continue as to a possible role as a mitogen and/or differentiation factor.⁶⁴ As already mentioned, PTHrP has been found to act as a smooth muscle relaxant in the vasculature,^{18,102} myometrium,⁸⁴ stomach,⁵⁷ bladder,¹⁰⁹ and trachea.¹¹³ In the kidney, PTHrP has been shown to display growth-factor-like effects on renal fibroblasts, and stimulates the proliferation of renal carcinoma cells.^{30,87} In contrast, among lymphocytes PTHrP inhibits proliferation, and is manufactured only by transformed or activated cells.⁵⁵ In the pancreas, PTHrP binds to islet cells and stimulates an increase in intracellular calcium.²⁴

There has been a tremendous amount of research on PTHrP and its role in fetal and neonatal physiology. Fetal calcium levels are maintained at a higher level than found in maternal serum. While fetal PTH is very low, PTHrP is produced in a variety of fetal

tissues and the placenta.³⁰ Studies have accumulated indicating an important role for PTHrP in fetal-maternal calcium regulation. A sheep placental model experiment showed that the fetal parathyroid makes PTHrP which stimulates calcium transport across the placenta.⁷⁶ Similar studies showed that PTHrP promotes magnesium placental transport as well as calcium.^{51,76} Studies with thyroparathyroidectomized lambs showed that PTHrP's effects on the fetus include stimulating fetal bone remodeling, and increasing serum calcium, renal calcium reabsorption, urinary pH, and circulating $1,25(\text{OH})_2\text{D}$ concentrations.¹⁴

Another example of interest is the role of PTHrP in lactation, since there are particularly high levels of the peptide in breast milk. Suckling produces a rise in prolactin levels, followed by a sharp rise in PTHrP and its secretion into milk. One study showed increasing levels of calcium in the breast milk corresponded with increasing levels of secreted PTHrP.⁴⁸ It may be acting as a local paracrine factor within the breast to stimulate calcium transport into milk. It could also play a role in calcium homeostasis in the neonate. Once ingested, the protein could be taken up intact by pinocytosis to act until the infant's PTH axis is fully active. One study showed that neonates who were formula-fed had a steeper decline in calcium levels than those who were breast-fed.⁸ The diverse areas of investigation are proving that the role of PTHrP in normal physiology is truly far-reaching.

8. Posttranslational Protein Processing

From the cDNA-predicted PTHrP amino acid sequences, it is known that PTHrP contains several classic endoproteolytic processing sites. The peptide contains many clusters of basic residues (arginine (R), and lysine (K)) from singlets to quintuplets (fig. 3). Endoproteolytic processing of prohormone precursors has been studied extensively, including in mammalian systems.^{23,49} The processing signals best known are dibasic and tetrabasic sites, which are cleaved in mammals by a family of proteins which act as

PTHrP amino acid sequence. The enzyme peptidyl alpha amidating monooxygenase (PAM) is known to be active in the processing of many prohormones including α -melanocyte stimulating hormone (α -MSH), oxytocin, antidiuretic hormone (ADH), corticotrophin releasing hormone (CRH), and thyroid releasing hormone (TRH).^{5,21,23,49,52} The consensus sequence consists of [X-gly-dibasic], where X denotes the residue which is ultimately amidated, and the adjacent glycine donates the amino group. The protein is first cleaved by a furin-like enzyme which cuts at the carboxyl end of the dibasic residues. Then a carboxypeptidase cleaves off the basic residues leaving X-gly, which is then amidated by PAM. The two amidation consensus sites within PTHrP are at prolines at position 86 or 94 in the sequence (fig. 3), which would yield a proline amide at the carboxyl terminus. PAM is known to have a widespread tissue distribution which overlaps in most cases with that of PTHrP. It is notable that many of the PAM substrates are made more active by amidation, leaving open the possibility that the amidation of PTHrP may significantly alter its biologic activity, strengthening the importance of identifying the actual secreted forms.

Once it was observed that the cDNA-predicted amino acid sequence contained multiple potential processing sites, it was suspected that PTHrP would be posttranslationally processed in some way. It is known that for PTHrP, as is the case with many other secreted peptides, the 36 amino acid 'prepro' sequence which allows passage into the endoplasmic reticulum is cleaved cotranslationally by signal peptidase.⁶⁵ In addition, the 'pro' sequence ending with KR at the -2 and -1 positions is known to be cleaved, since the mature peptide starts with an alanine at position +1. By analogy to other secreted peptides, this sequence is most likely cleaved in the Golgi apparatus or secretory granule. The purpose of this 'pro' region in PTHrP, and in other peptides, has been speculated to relate to intracellular targeting or stability, but may also serve some unknown function.^{23,49,105}

In addition, there is evidence that PTHrP is glycosylated. In keratinocytes, sugar residues have been found on an N-terminal secretory form of the peptide.¹⁰⁶ Since there are no N-glycosylation consensus sites (Asn-X-Ser/Thr), it is assumed to be O-glycosylated. This O-glycosylation may play a role in intracellular trafficking and stability, or may influence the plasma half-life or biologic potency as is the case for erythropoietin, leutinizing hormone (LH), human chorionic gonadotropin (hCG), and thyroid stimulating hormone (TSH).^{38,61,73,80}

The findings presented in this study provide the first evidence that PTHrP is endoproteolytically cleaved within the cell and secreted in distinct fragments. Moreover, proof is offered that a monobasic processing site utilizing the arginine at position 37 of the amino acid sequence signals cleavage of the protein. The novel peptide isolated from three different cell lines begins at amino acid 38 and is approximately 60-70 amino acids in length. This highly conserved sequence promises to play a crucial role in cellular physiology throughout human tissues.

II Materials and Methods

1. Cell Lines

The human renal carcinoma line SKRC-1 was provided by Dr. Neil Bander at the Memorial-Sloan Kettering Cancer Center. These cells were selected for study as an example of a naturally-occurring cell line which produces PTHrP and which causes HHM *in vivo*. These cells were grown in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 200 IU/ml penicillin, and 1% L-glutamine, as described in detail previously.¹⁰³ Rat insulinoma (RIN 1046-38) cells were provided by Dr. Michael Appel at the University of Massachusetts. These cells were selected for transfection and overexpression of PTHrP since they faithfully process a variety of other neuroendocrine preprohormones. A PTHrP cDNA fragment encoding amino acids 1-141 of the human sequence⁵⁴ was ligated into the retroviral vector pLJ which contains a neomycin selectable marker.⁴² RIN cells were then transfected using the lipfection method, and stable transfectants were selected using the antibiotic G148. These cells, henceforth referred to as "RIN-141" cells, were grown in RPMI containing 10% fetal bovine serum, 200 IU/ml penicillin, and 1% L-glutamine. Each cell line was maintained at 37°C in a humidified atmosphere of 5% CO₂, 95% air. Human neonatal keratinocytes were grown in primary culture, and medium was harvested from these cells as previously described.¹⁰⁶

2. Stability of ¹²⁵I-PTHrP(1-141) in the Presence of SKRC-1 and RIN-141 Cells

Prior studies had shown¹⁰⁶ that addition of protease inhibitors to growth medium in concentration sufficient to prevent degradation of PTHrP following secretion was deleterious to the health of the cells and their fidelity in processing. Accordingly, the stability of PTHrP exposed to confluent cells in culture without protease inhibition was examined using ¹²⁵I-PTHrP(1-141), as described previously.¹⁰⁶ Briefly, recombinant PTHrP(1-141) (provided by Dr. Glenn Hammonds, Genentech, S. San Francisco, CA)

was iodinated with Na ¹²⁵I (Amersham Corp.) using the Enzymobead method (Bio-Rad). ¹²⁵I-PTHrP(1-141) was added to both SKRC-1 and RIN-141 cells grown to confluence in 24-well plates. At the time points described in fig. 4, medium containing ¹²⁵I-PTHrP(1-141) and its degradation products was removed from the wells, combined with protease inhibitors as described below, and analyzed by SDS-PAGE and autoradiography. These studies demonstrated that the majority of PTHrP(1-141) remained undegraded at 90 minutes; this 90-min. time point was therefore selected for harvesting "protease-protected" medium as described below.

3. Preparation of Conditioned Medium

SKRC-1 cells, RIN-141 cells and human epidermal keratinocytes were each grown to confluence in 40 150-cm² flasks. Six ml of medium was added to each flask of confluent cells for exactly 90 min. Medium was then harvested and fresh medium was added to each of the cultures. This procedure was repeated up to five times in a 24 hr. period until 6 liters of SKRC-1, 5.5 liters of RIN-141 and 1 liter of keratinocyte 90 min. conditioned-medium were collected. This medium was immediately chilled and supplemented with protease inhibitors, which included (final concentrations) aprotinin (20µg/ml) (Boehringer-Mannheim), bacitracin (100µg/ml) (Sigma), chymostatin (50µg/ml) (Boehringer-Mannheim), EDTA (3mM) (Sigma), leupeptin (15µg/ml) (Boehringer-Mannheim), pepstatin A (45µg/ml) (Bachem Bioscience, Philadelphia, PA), and phenylmethanesulfonyl fluoride (45µg/ml) (Sigma). This protease-protected medium was stored at -20°C until further use.

4. Immunoaffinity Purification of PTHrP Fragments

Six liters of SKRC-1, 5.5 liters of RIN-141 and 1 liter of keratinocyte 90-min. conditioned medium were applied in 250-ml aliquots to a 300-µl anti-PTHrP(1-36) immunoaffinity column at 4°C, as described in detail previously.¹⁰⁶ To ensure complete

removal of amino-terminal PTHrP species, the medium that flowed through the column was applied to the anti-PTHrP(1-36) affinity column a second time. This second "flow through" medium, determined by immunoassay to be free of amino-terminal PTHrP, was then applied in 100-ml aliquots to a 300 μ l anti-PTHrP(37-74) immunoaffinity column. The PTHrP species adherent to the affinity columns were eluted with 5 ml of 200 mM glycine HCL, pH 2.5. The acid eluates from each column were further purified using RP-HPLC on Vydac (Separations group, Hesperia, CA) 218TP104 analytical C-18 columns using gradients of 28-38% and 15-33% acetonitrile in water containing 0.1% trifluoroacetic acid as described in figs. 3-5 and 8 respectively.

5. PTHrP Immunoassays

PTHrP immunoreactivity in medium and in column fractions was detected using three previously described PTHrP immunoassays (see fig. 1, *lower panel*). The PTHrP(1-36) RIA employs a polyclonal anti-PTHrP(1-36) antibody at a titer of 1:1500 and human ³⁶Tyr-PTHrP(1-36) amide as standard and radioligand.¹⁰ It recognizes both the (1-36) fragment and the full-length PTHrP species with a sensitivity of 50 pM. The PTHrP(37-74) RIA employs a polyclonal sheep anti-PTHrP(37-74) antibody at a titer of 1:2,000, and a synthetic human PTHrP(37-74) as standard and radioligand. It recognizes both the (37-74) fragment and the full-length PTHrP species with a sensitivity of 10 pM.¹¹ Lastly, the PTHrP immunoradiometric assay (IRMA) recognizes only those species of PTHrP which include both the (1-36) and (37-74) PTHrP epitopes; it uses an affinity-purified rabbit anti-PTHrP(37-74) as capture antibody, a second iodinated affinity-purified rabbit anti-PTHrP(1-36) as signal antibody, and a synthetic human PTHrP(1-74) as standard. It has a sensitivity of 1 pM.¹⁰

6. Extraction of SKRC-1 and RIN-141 Cells

SKRC-1 and RIN-141 cells were grown to confluence in 10 150-cm² flasks each. The cells were first rinsed with phosphate-buffered saline, and then 3 ml of 4M guanidine isothiocyanate, 8% (v/v) 2-mercaptoethanol, containing 50 mM Tris-HCl, pH 7.5, and 25 mM EDTA, were added to each flask for 5 min. on ice. The cells were then scraped into a centrifuge tube, ice-cold ethanol was added to final concentration of 30% ethanol, and this ethanol-guanidinium extract was chilled at 4°C for 10 min. Following centrifugation at 16,000 x g for 10 min. at 4°C, the supernatant was removed and dialyzed using a Spectrapor-3 membrane (3,000 molecular weight cut-off) against 16 liters of water at 4°C over 48 hrs. The dialyzed extract was centrifuged at 16,000 x g for 10 min. at 4°C and the supernatant collected and resolved using RP-HPLC as described in fig. 5.

7. Immunofluorescence

Cells were plated onto coverslips (Fisher), grown until 75% confluent, and then fixed with 2% paraformaldehyde at room temperature for 45 min. The cells were rinsed with 10 mM phosphate-buffered saline, 0.2% bovine serum albumin, and then permeabilized for 15 min. with 0.05% saponin in 10mM phosphate-buffered saline, 0.2% bovine serum albumin. Affinity-purified rabbit antibodies to PTHrP regions (1-36) and (37-74)^{10,108} were then added to the permeabilized cells for 2 hours at room temperature. After rinsing, anti-rabbit fluorescein isothiocyanate-conjugated goat F(ab')₂ fragments (Tago Immunologicals, Burlingame, CA) were added to the cells at a 1:200 dilution in a darkened moisture chamber for 1 hr. For insulin immunohistochemistry, anti-insulin guinea pig antibodies (ICN Biomedicals, Inc., Costa Mesa, CA) were added as primary antibody and were detected using an anti-guinea pig fluorescein isothiocyanate-conjugated antibody (Organon Teknika, Inc., Durham, NC) at a 1:200 dilution. Golgi staining was performed with the mouse monoclonal antibody 53FC3² (provided by Dr. A. Danoff and Dr. R. Angeletti at the Albert Einstein College of Medicine) and then detected using anti-

rabbit, rhodamine-B-conjugated goat F(ab)₂ fragments (Tago). Cells were then preserved in *p*-phenylenediamine and photographed using a Zeiss Axiophot fluorescent microscope.

8. Analysis of Purified Species

Tricine-SDS-PAGE and western analysis were performed using 10-18% gradient gels. Transfer to immobilon nylon membranes (Millipore, Bedford, MA) was performed using standard methods, 5% bovine serum albumin as blocker, and polyclonal rabbit anti-PTHrP(1-74) antibody at a titer of 1:200 as primary antibody, and a silver-enhanced, gold-labeled second antibody method for detection (Janssen Products, Olen, Belgium).

Dot blots of synthetic peptides PTHrP(1-36), (37-74), and (1-74) were performed by placing 10 x 1µl drops directly onto nylon membranes, allowing the paper to dry between drops, to final amounts of 1, 10, 30 and 100 ng for each peptide. Blotting was then performed in an identical manner as described for western blots.

Size-exclusion HPLC was performed using SW 300 and I 125 protein pak columns connected in series. The mobile phase was 30% acetonitrile, 70% H₂O and 0.1% trifluoroacetic acid. The flow rate was 0.7 ml/minute.

9. Amino Acid Sequencing

Amino Acid sequencing was performed in the William Keck Protein and Nucleic Acid Chemistry Facility at Yale University using an Applied Biosystems model 407A gas phase sequencer.

10. Procedures Performed by This Author

This author performed the harvesting of conditioned media from both RIN-141 and SKRC-1 cells, immunoaffinity purification of mid-region species, RP-HPLC and size exclusion HPLC, radioimmunoassay, tricine-SDS-PAGE, western blot, and dot blot of the various PTHrP species. Harvesting of medium and immunopurification of the amino-

terminal species, their analysis by RIA and RP-HPLC, and immunohistochemistry was performed by Dr. Neil Soifer. Degradation studies and immunopurification of PTHrP species from cell extracts performed by Mr. Terrence Wu.

III Results

1. Stability of PTHrP in Conditioned Medium

The purified species of PTHrP from conditioned media must be a valid representative of the secreted form of the peptide. Previous studies have shown the instability of secreted PTHrP in plasma⁷² and in culture.¹⁰⁸ To demonstrate the stability of PTHrP species in SKRC-1 and RIN-141 cell culture conditions, exogenous iodinated PTHrP(1-141) was incubated with these cell lines for the times indicated in figure 4. Panel A shows the degradation of PTHrP in the presence of cultured keratinocytes described previously,¹⁰⁶ where the initial single 23-25 kDa band is broken down to smaller components over time. SKRC-1 cells are much less active in degrading PTHrP (*panel B*), and RIN-141 cells show little or no degradation over time (*panel C*). From this data it was determined that the secreted species of PTHrP from these cell lines would not suffer significant degradation by 90 minutes of incubation; thus purification of secreted species was performed from serial collections of 90 minute medium.

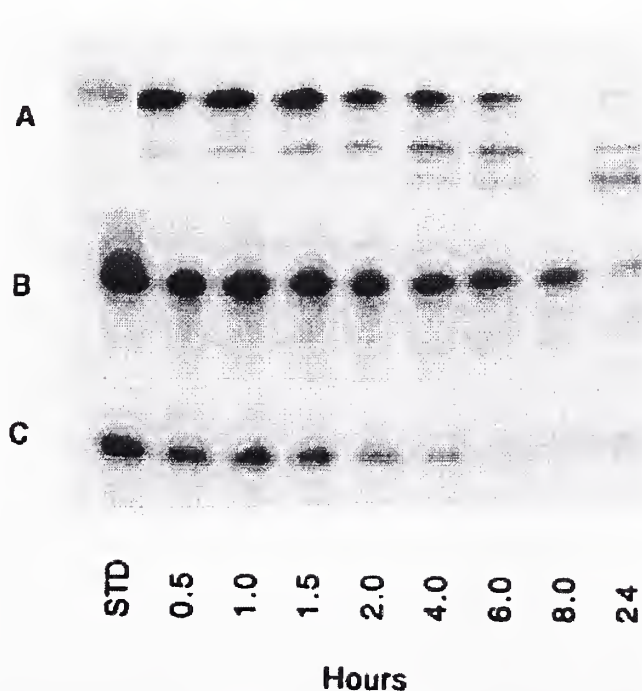


Fig. 4. Degradation of iodinated, recombinant PTHrP(1-141) after exposure to confluent cells under non-protease-protected conditions. Radiolabeled PTHrP was analyzed by SDS-PAGE and autoradiography after collection at the time points indicated. *STD* indicates intact peptide prior to cell exposure. *A*, cultured human epidermal keratinocytes described previously.¹⁰⁶ *B*, cultured SKRC-1 cells. *C*, cultured RIN-141 cells.

2. Immunoaffinity Purification of Secreted PTHrP Species

a. SKRC-1 Cell Line, amino-terminal species

Six liters of 90 minute conditioned medium was harvested under protease-protected conditions. This initial media contained approximately equimolar amounts of PTHrP (1-36) and PTHrP(37-74) species by radioimmunoassay (Table 1). After extraction with the anti-PTHrP(1-36) affinity column, virtually all of the 1-36 activity was stripped from the media, as measured by 1-36 RIA. The acid eluates from the PTHrP (1-36) affinity column were further purified by RP-HPLC and examined by 1-36 RIA and 1-74 IRMA (fig.5, *top panel*). SKRC-1 media contained three major amino-terminal species. Peak I contains both 1-36 and 1-74 immunoreactivity, and migrated in a position analogous to that of the glycosylated amino-terminal peptide isolated previously from keratinocytes.¹⁰⁶ Peak II contains only 1-36 activity and thus represents a smaller amino-terminal fragment. Peak III contains both 1-36 and 1-74 activity, yet elutes with a very different retention time than does peak I, and thus may represent a larger amino-terminal fragment distinct from previously isolated species.

b. SKRC-1 Cell Line, mid-region species

The SKRC-1 conditioned medium, after being stripped of PTHrP(1-36) immunoactivity, was found to retain the same concentration of PTHrP(37-74) immunoactivity as had the initial medium, indicating the presence of a mid-region, non-amino-terminal PTHrP species in the medium. This medium, already stripped of PTHrP(1-36) immunoactivity was then affinity-purified using an anti-PTHrP(37-74) antibody column, and resolved by RP-HPLC in the same fashion as were the amino-terminal species (fig. 5, *bottom panel*). The SKRC-1 medium contained a previously unrecognized, mid-region PTHrP peptide which eluted as a single peak with a unique retention time, earlier than that of any other species identified to date. In

Table 1. Immunoreactive PTHrP species in conditioned medium. Quantities of PTHrP present in SKRC-1- and RIN-141-conditioned medium and cell extracts. “90-min conditioned medium” and “guanidinium extract” refer to immunoreactive quantities available prior to purification. The PTHrP concentration was significantly higher in conditioned medium than in cells. Numbers in parentheses indicate the recovery of immunoreactivity given as a percent of the original immunoreactivity. PTHrP recovery after purification was less than 40%.

	<u>SKRC-1</u> (pmol)	<u>RIN-141</u> (pmol)	
90-min conditioned medium			
1-36	1,158 (100) ^a	1,788 (100) ^b	^a pmol/6 liters
37-74	870 (100)	2,822 (100)	^b pmol/5.5 liters
1-74	324 (100)	468 (100)	
N-terminal HPLC peak III			
1-36	107 (9.2) ^c	28 (2) ^d	^{c,d} pmol/peak
1-74	25 (7.7)	2.2 (0.5)	
Mid-region fragment			
37-74	330 (37.9)	750 (26)	
Guanidinium extract			
1-36	2 ^e	1.7 ^f	^{e,f} pmol/flask
37-74	1.3	0.85	
1-74	0.85	0.7	

addition, this peptide was present in larger amounts than were any of the amino-terminal fragments (fig. 5; table 1).

c. Transfected RIN-141 cell line, amino-terminal species

RIN-141 conditioned medium (5.5 liters) was harvested and immunoaffinity purified using an anti-PTHrP(1-36) antibody column in the same manner as was the SKRC-1 medium. The immunoactivity in the RP-HPLC purified fractions is shown in fig. 6, *top panel*. As in the SKRC-1 purified medium, the RIN-141 medium contained three amino-terminal species. However, all three peaks demonstrated both 1-36 and 1-74 immunoreactivity.

d. Transfected RIN-141 cell line, mid-region species

As had been the case with SKRC-1 media, the RIN-141 conditioned media, stripped of amino-terminal immunoactivity, was found to retain full mid-region immunoactivity. This amino-terminally stripped media was further purified using an anti-

PTHrP(37-74) antibody column as had been performed with the SKRC-1 media, and the acid-eluates resolved by RP-HPLC. The resultant peak in (37-74) RIA activity contained neither (1-36) RIA or (1-74) IRMA immunoactivities (fig. 6, *bottom panel*), demonstrating, as was found in the mid-region SKRC-1 medium, that RIN-141 conditioned medium contains a single, non-amino-terminal fragment which elutes at an identical retention time as does the SKRC-1 fragment. This mid-region PTHrP species is present in much larger quantities in RIN cell medium than in SKRC-1 medium, reflecting the overexpression of PTHrP in RIN cells.

3. PTHrP Species in Cell Extracts

Despite the demonstration of low levels of degradation of PTHrP by media proteases, it remained of some concern that the purified species from 90 minute media represented artifactually degraded products from a larger secreted peptide. To disprove this possibility, cell extracts of both SKRC-1 and RIN-141 were analyzed for the presence of intracellular PTHrP species prior to secretion. Guanidine isothiocyanate/ 6-mercaptoethanol extraction was employed for its immediate protease-inactivating property, simplicity of use and yield comparable to other methods used previously.⁹⁰ Extracts were applied directly to RP-HPLC without preliminary immunoaffinity purification. Fig. 7 shows the elution patterns for (1-36) RIA, (37-74) RIA and (1-74) IRMA immunoactivities in SKRC-1 cells (*top panel*), and RIN-141 cells (*bottom panel*). Multiple amino-terminal fragments are present in both SKRC-1 and RIN-141 cells prior to secretion, most of which contain both 1-36 and 37-74 epitopes with similar elution patterns as were demonstrated in conditioned medium.

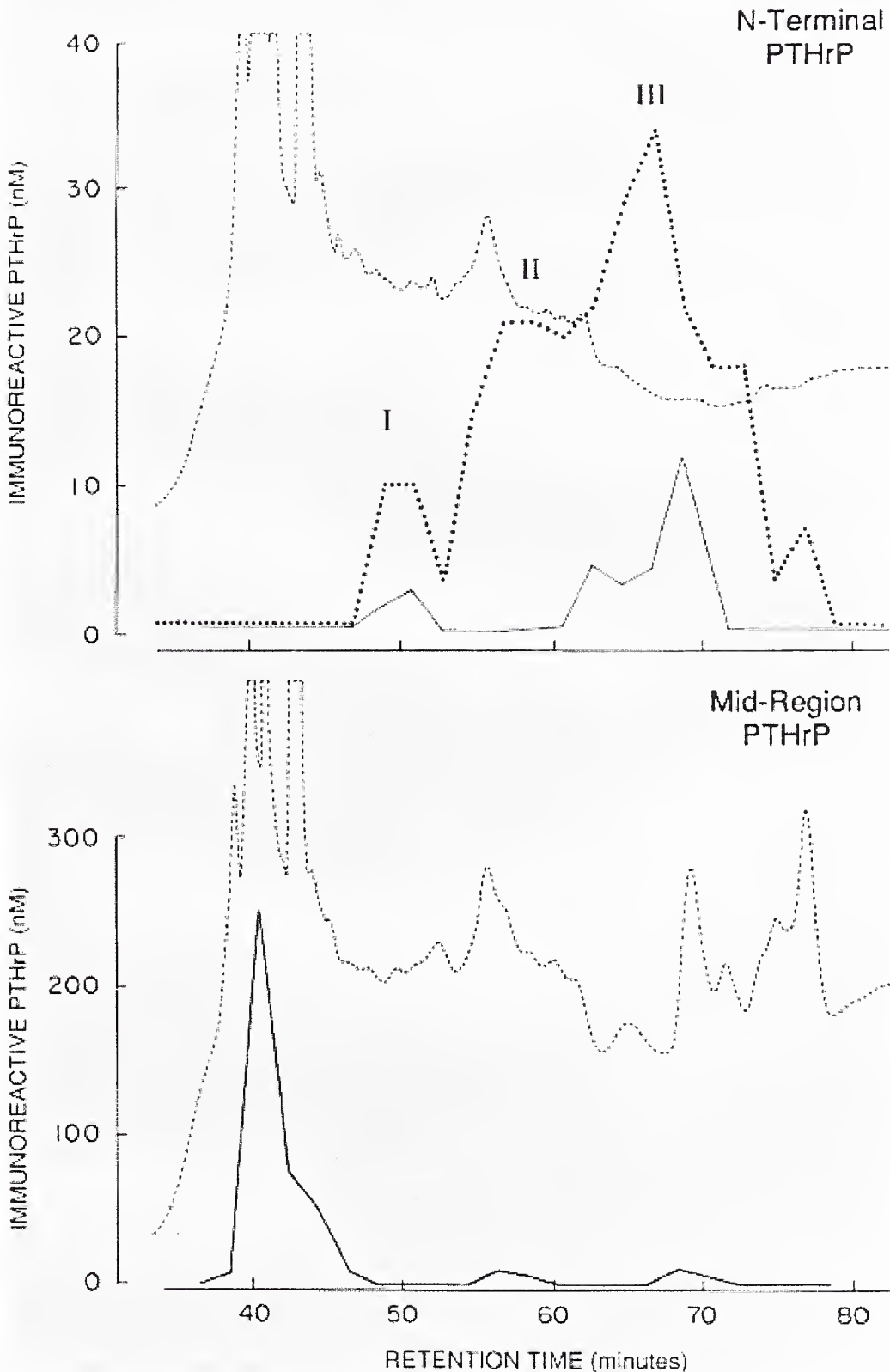


Fig. 5. Immunoreactive PTHrP species from SKRC-1-conditioned medium. RP-HPLC chromatogram (*fine dotted line*) with retention time and elution patterns are shown at A_{210} . The gradient is 28-38% acetonitrile in water containing 0.1% trifluoroacetic acid. *Upper panel*, amino-terminal PTHrPs. The *heavy dotted line* indicates (1-36) RIA activity. The *thin solid line* indicates (1-74) IRMA activity. Multiple species are present, with three major peaks (I, II, and III). *Lower panel*, non-amino-terminal PTHrP. A single peak is present, consisting only of (37-74) RIA (*heavy dotted line*) activity.

AFFINITY-PURIFIED CONDITIONED MEDIUM - RIN CELLS

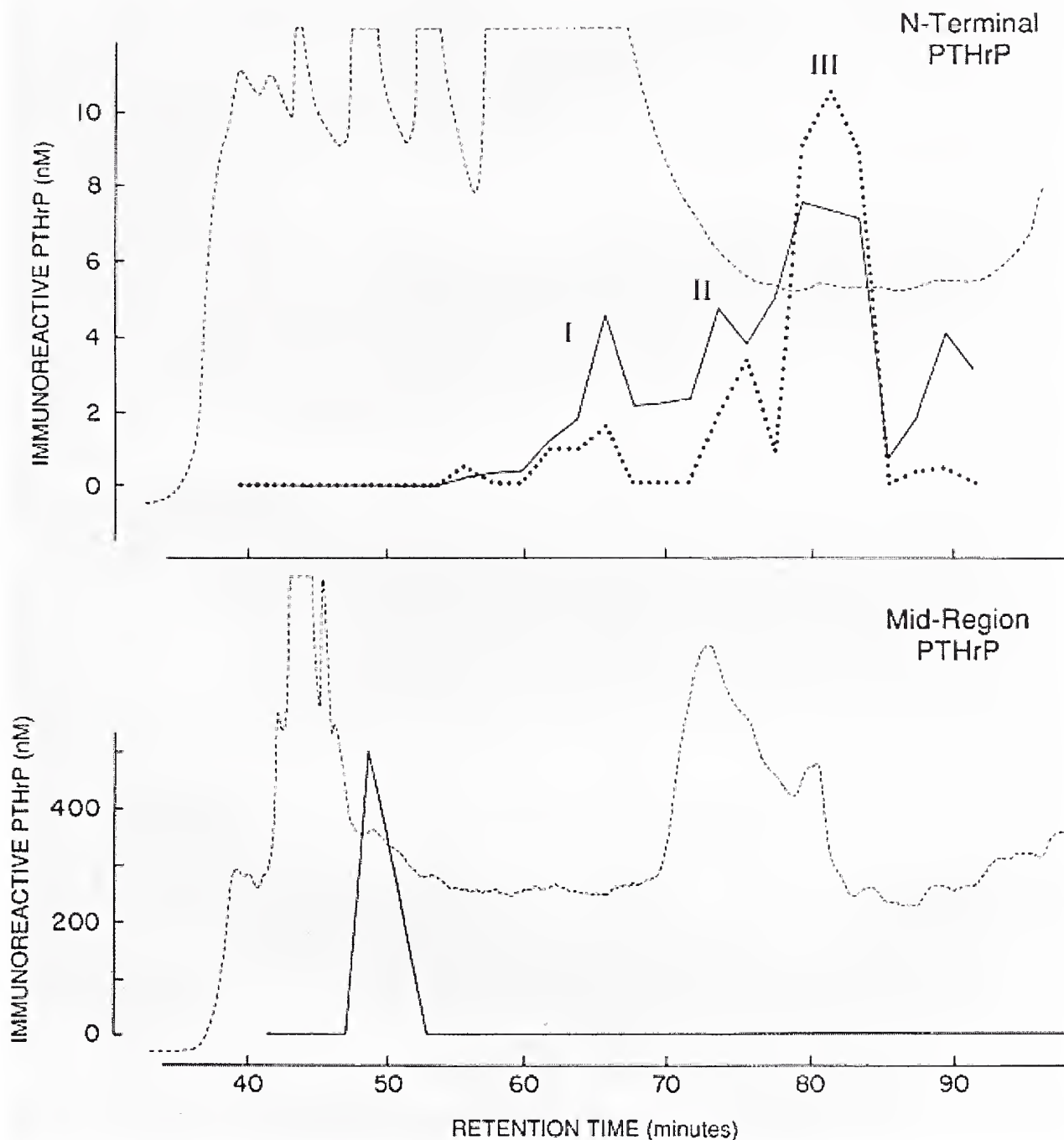


Fig. 6. Immunoreactive PTHrP species from RIN-141-conditioned medium. RP-HPLC chromatogram (*fine dotted line*) with retention time and elution patterns are shown at A_{210} . *Upper panel*, amino-terminal PTHrPs. Multiple species are present, with three major peaks (I, II, and III). (1-74) IRMA concentrations (*thin solid line*) are represented at 50 X actual concentrations to allow better visualization. *Lower panel*, non-amino-terminal PTHrP. A single peak is present, consisting only of (37-74) RIA activity (*heavy solid line*). The *heavy dotted line* indicates (1-36) RIA activity. The gradient is 28-38% acetonitrile in water containing 0.1% trifluoroacetic acid.

GUANIDINIUM CELL EXTRACTS

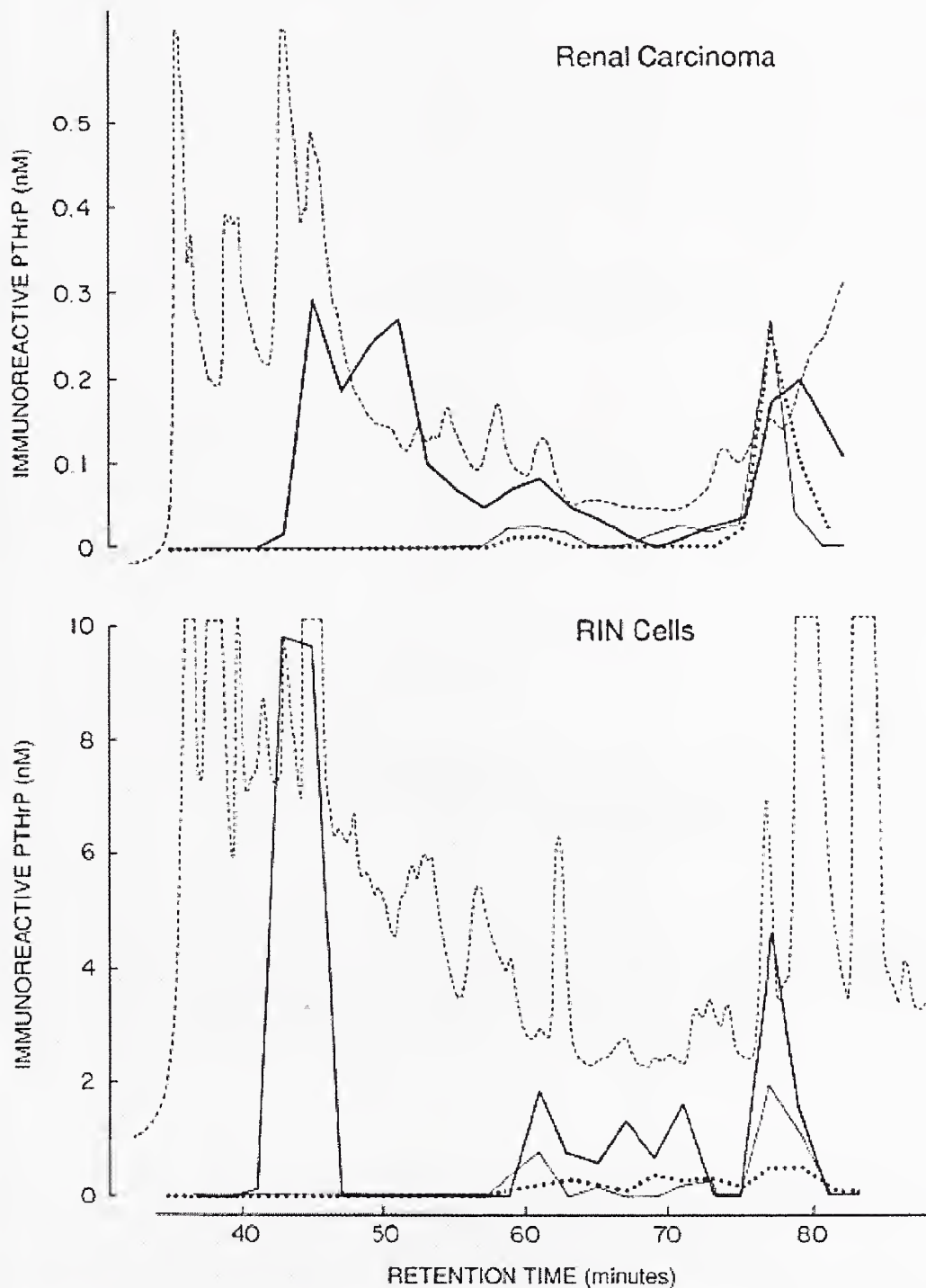


Fig. 7. Immunoreactive PTHrP species from cell extracts. RP-HPLC chromatogram (*fine dotted line*) with retention time and elution patterns shown. *Upper panel*, SKRC-1 cell extract. Multiple amino-terminal species are present by (1-36) RIA (*heavy dotted line*) and (1-74) IRMA (*thin solid line*). Multiple (37-74) RIA (*heavy solid line*) immunoreactive fragments are also present, including an early eluting peak that corresponds to the non-amino-terminal peak found in conditioned medium. *Lower panel*, RIN-141 cell extract. Multiple amino-terminal and non-amino-terminal species are present. An early eluting fragment contains only (37-74) RIA immunoreactivity (*heavy solid line*). The gradient is 28-38% acetonitrile in water containing 0.1% trifluoroacetic acid.

More importantly, a non-amino-terminal (37-74) immunoreactive fragment was present within both SKRC-1 and RIN-141 cells prior to secretion. This fragment eluted in the same position as did the corresponding peptide derived from conditioned medium (fig.s 5-7). This evidence confirms that the mid-region PTHrP is processed to its mature form within the cell prior to secretion. As had been observed in conditioned medium, the mid-region peptide was present at a much higher concentration in cell extracts than were the amino-terminal fragments. The overexpression of PTHrP in RIN cells produced levels of the peptide that were 30 times higher in RIN-141 cells than in SKRC-1 cells (Table 1). Finally, the SKRC-1 cells contained a second non-amino terminal (37-74) immunoreactive peak which is not present in RIN-141 cells, nor in either of the conditioned medium preparations.

4. Analysis of the Mid-region PTHrP Fragment

a. Tricine SDS-PAGE

The mid-region fragment purified from conditioned media from the two cell lines shown in the bottom panels of fig.s 3 and 4 were analyzed by Tricine SDS-PAGE. One hundred ng of the RIN-141 fragment and 30 ng of the SKRC-1 fragment were shown to migrate as single homogeneous bands of approximately 7kDa each, slightly below synthetic PTHrP(1-74) (fig. 8).

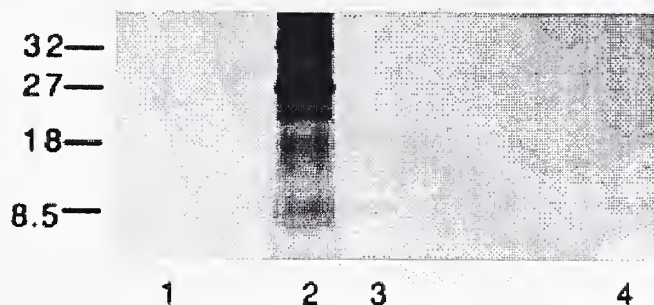


Fig. 8. Tricine SDS-PAGE and silver stain of the (37-74) immunoreactive, non-amino-terminal fragment. Lane 1, 100µg of the (37-74) PTHrP fragment from RIN-141-conditioned medium. Lane 2, molecular weight markers. Lane 3, 100ng of synthetic PTHrP (1-74), molecular mass ~8.5 kDa. Lane 4, 30µg of the (37-74) PTHrP fragment from SKRC-1-conditioned medium. Each (37-74) species appears as a homogeneous narrow band with a molecular mass ~7kDa.

b. Western Blot

In order to confirm that the band visualized on SDS-PAGE was identical to the mid-region PTHrP detectable by RIA, western blotting was employed. It was found that the synthetic PTHrP(37-74) peptide itself migrates anomalously making comparison of purified fragments to a standard (37-74) fragment impossible. In addition, the (37-74) region, when applied to nitrocellulose paper, stains poorly with the same affinity-purified anti-PTHrP(37-74) antibodies used in the affinity purification of these peptides, preventing its detection by western blot of PTHrP fragments in this region. To confirm this, varying concentrations of synthetic PTHrP(37-74) were compared in a dot blot to the same concentrations of synthetic PTHrP(1-36) and PTHrP(1-74). The staining of the 37-74 peptide is sparse compared to the other two synthetic peptides. It was concluded that western blotting using the available mid-region PTHrP antibody would not be useful in the study of PTHrP processing.

c. Size-exclusion HPLC

Attempts to analyze the purified fragments by size-exclusion HPLC failed to add any additional information as to their nature or size. Acid-eluates from the anti-PTHrP(37-74) immunoaffinity column were resolved using two size-exclusion affinity columns in series. The resultant peaks were too broad to accurately determine size of the various fragments and were too widely overlapping to properly resolve the 37-74 RIA curve from the 1-36 RIA curve.

d. Amino Acid Sequencing

Amino Acid sequencing of 30 pmol of the RIN-141 mid-region fragment purified from conditioned media yielded 34 cycles of amino acid sequence identical to amino acids 38-71 of the cDNA predicted PTHrP sequence (fig. 9). Thus the amino terminus of the

mid-region fragment secreted by RIN-141 cells begins at amino acid 38 of the cDNA-predicted peptide.

Attempts to sequence 7 pmol of the mid-region fragment purified from SKRC-1 conditioned medium were unsuccessful. In order to analyze the similarity between the two mid-region fragments, a sample of both the RIN-141 and SKRC-1 peptides was run over a RP-HPLC column on a shallow gradient chosen for its superior resolving power. The resultant elution patterns are shown in fig. 10. The SKRC-1 mid-region fragment (*top panel*) and the RIN-141 mid-region fragment (*middle panel*) eluted at identical retention times.

Keratinocytes are well known to produce amino-terminal PTHrP. Since we had not previously analyzed keratinocyte-conditioned medium for the presence of this novel mid-region peptide, protease-protected conditioned medium from keratinocytes was immunoaffinity-purified using an anti-PTHrP(37-74) antibody column and run on the same shallow-gradient HPLC column as the SKRC-1 and RIN-141 fragments had been resolved in fig. 10. The bottom panel shows that a chromatographically indistinguishable mid-region fragment of PTHrP is indeed produced by keratinocytes. Therefore, it is likely that all three cell types secrete a highly similar if not identical mid-region species.

cDNA position	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56
cDNA amino acid	Ala	Thr	Ser	Glu	Val	Ser	Pro	Asn	Ser	Lys	Pro	Ser	Pro	Asn	Thr	Lys	Asn	His	Pro
mid-region amino acid	x	Thr	Ser	Glu	Val	Ser	Pro	Asn	Ser	Lys	Pro	Ser	Pro	Asn	Thr	Lys	Asn	x	Pro
mid-region position	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
cDNA position	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71				
cDNA amino acid	Val	Arg	Phe	Gly	Ser	Asp	Asp	Glu	Gly	Arg	Tyr	Leu	Thr	Gln	Glu				
mid-region amino acid	Val	x	Phe	Gly	Ser	Asp	Asp	Glu	Gly	x	Tyr	Leu	Thr	x	Glu				
mid-region position	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34				

Fig. 9. Amino Acid sequence of the non-amino-terminal, mid-region immunoreactive fragment from RIN-141-conditioned medium. This sequence is compared with the cDNA-predicted amino acid sequence. x refers to amino acids that could not be identified with complete accuracy.

RP-HPLC FLAT GRADIENT

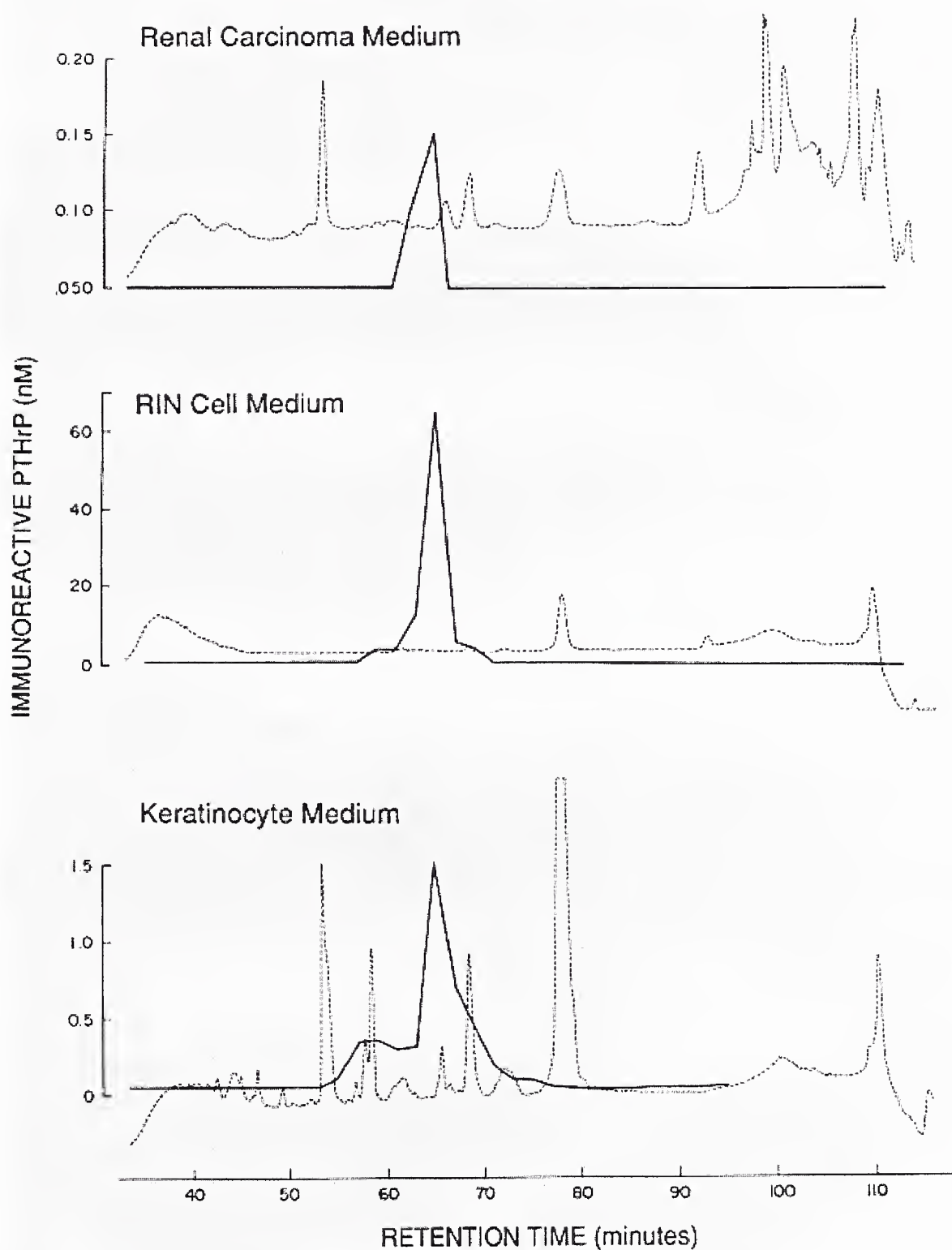


Fig. 10. Elution patterns of the (37-74) immunoreactive, non-amino-terminal fragments in conditioned medium from three different cell lines. RP-HPLC chromatogram (*fine dotted line*) is shown using a 15-33% gradient of acetonitrile in water with 0.1% trifluoroacetic acid. *Upper panel*, SKRC-1 mid-region fragment. *Middle panel*, RIN-141 mid-region fragment. *Lower panel*, normal human keratinocyte mid-region fragment. (37-74) RIA immunoreactivity is indicated by the *solid line*.

5. Immunohistochemical Localization of PTHrP in RIN-141 and SKRC-1 Cells

To determine the intracellular locations of the PTHrP species identified in both cell extracts and conditioned media, SKRC-1 and RIN-141 cells were examined using immunohistochemistry with region-specific PTHrP antibodies. Fig. 11 A and D show a perinuclear pattern when cells are stained with anti-PTHrP(1-36) antibodies, suggesting that the amino-terminal PTHrP species is located primarily in the Golgi apparatus, as seen in fig. 11, G. In contrast, cells stained with anti-PTHrP(37-74) antibodies demonstrate, in addition to perinuclear staining, a prominent peripheral staining pattern typical of proteins contained in secretory granules (fig. 11, B and E). RIN-141 cells stained with anti-insulin antibodies demonstrate a similar peripheral granular staining pattern seen in fig. 11, H. Control studies without the use of primary antibody or with excess concentration of competing peptides display no immunofluorescence (fig. 11, C and F).

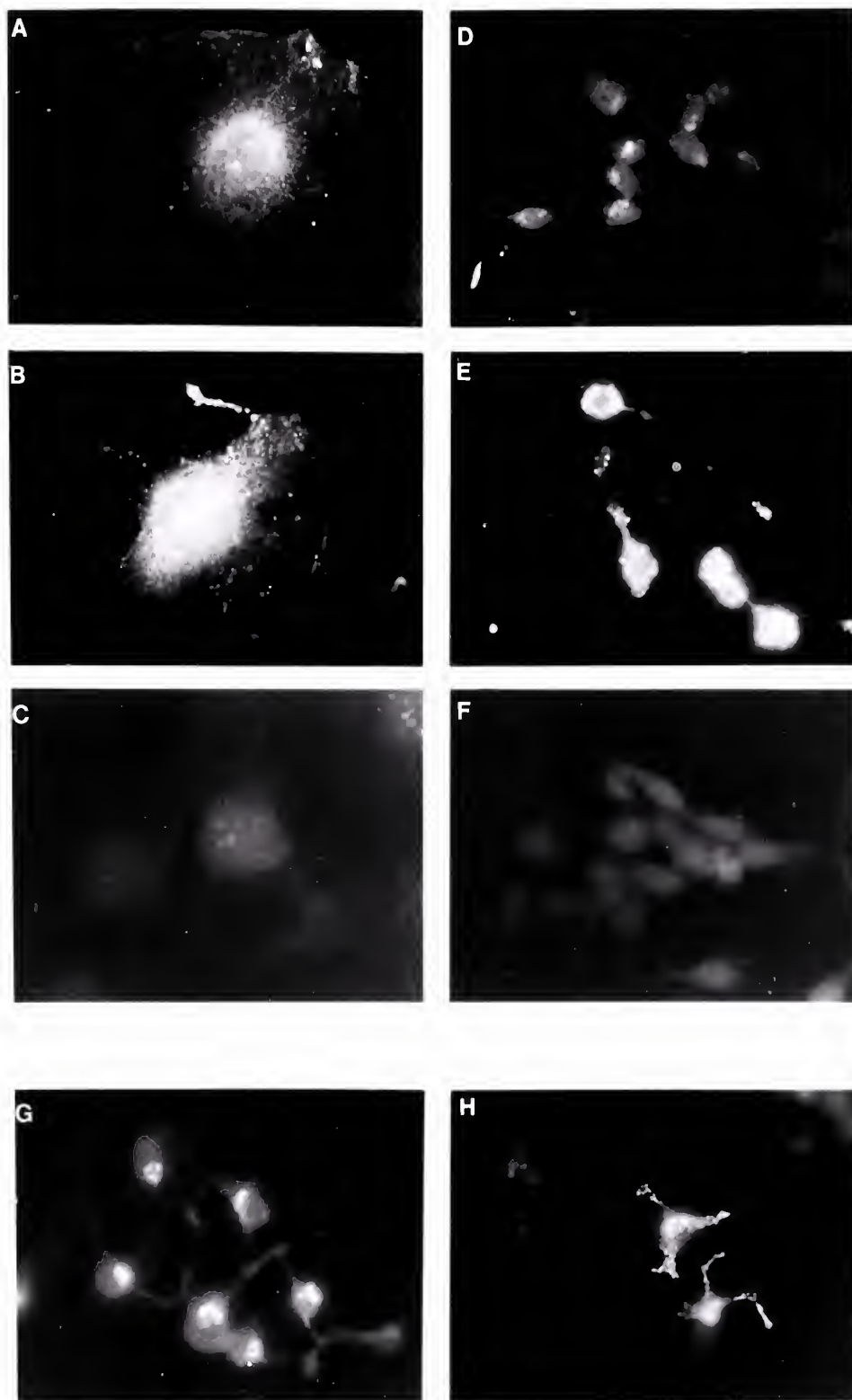


Fig. 11. PTHrP Immunohistochemistry using affinity-purified, region-specific antibodies. A. SKRC-1 cell stained with anti-PTHrP (1-36) antibody. B. SKRC-1 cell stained with anti-PTHrP (37-74) antibody. C. SKRC-1 cell stained without the addition of a primary antibody. Similar results (not shown) were obtained when affinity-purified antibody was added to a non-PTHrP-producing renal carcinoma cell line. D. RIN-141 cell stained with anti-PTHrP (1-36) antibody. E. RIN-141 cell stained with anti-PTHrP (37-74) antibody. F. RIN-141 cell stained without the addition of primary antibody. G. RIN-141 cells stained with anti-Golgi antibody. H. RIN-141 cells stained with anti-insulin antibody.

IV Discussion

Considering the widespread distribution of PTHrP in adult and fetal tissues and the lethality of PTHrP gene “knockout,” one must imagine a significant and essential role it might play in cellular physiology. Borne out in many studies of normal physiology and malignant cells, the theory that PTHrP acts a widespread autocrine/paracrine factor, with growth factor and other actions, has gained general acceptance. The investigation of this role until now has relied on the use of synthetic amino-terminal peptides manufactured based on their homology to PTH. The low abundance of PTHrP has prevented large-scale harvesting of the native forms for further study. Identification of the native secreted and circulating forms would be a helpful advance in the study of the physiologic action of PTHrP.

Study of the molecular biology of PTHrP has produced a multitude of possible regulatory steps in the biosynthesis of the protein, such that one might predict many alternative final peptide forms (fig.s 1,3). In addition to its several mRNA species, PTHrP has in its sequence many putative proteolytic processing sites and potential sites for C-terminal amidation and O-glycosylation. It has previously been shown that one secreted species of PTHrP from human keratinocytes is an 18-kDa O-glycosylated amino-terminal form of the peptide.¹⁰⁶ These cells are useful to study since they represent a normal cell type. However, keratinocyte studies require continuous preparation of primary cultures of human keratinocytes. In addition, the forms secreted from these cells may differ from those secreted by malignant cells in the syndrome of HHM, or from those produced by the various neuroendocrine cell types which produce PTHrP, such as the pancreatic beta cell, the pituitary somatotrope, the adrenal medulla and the CNS neuron.

Two additional cell lines, RIN-141 cells and SKRC-1 human renal carcinoma cells, were included in this study with these considerations in mind. Both secrete PTHrP in

relative abundance, and neither has demonstrated the propensity to degrade exogenously added iodinated PTHrP(1-141) as has been observed for keratinocytes (fig. 4).

Several new secretory forms of PTHrP were discovered during this study. At least three amino-terminal species (immunoreactive with the anti-PTHrP(1-36) immunoaffinity column) were isolated from both RIN-141 and SKRC-1 cells, most of which contained (37-74) immunoreactivity as well. The precise structure of these N-terminal species is currently under study. The earliest eluting of these species from both RIN-141 and SKRC-1 cells comigrates with the glycosylated N-terminal species isolated from human keratinocytes. Whether these forms are indeed glycosylated has yet to be determined.

The most important discovery made in this study was the isolation of a novel mid-region form of PTHrP secreted by all three cell types. The existence of such a mid-region peptide had been suggested by the recognition of cleavage sites surrounding this area in the cDNA predicted amino acid sequence (fig.3).^{30,87} The mid-region fragment, missing the N-terminal sequence of the protein, was not recognized by the PTHrP (1-36) antiserum and does not contain amino-terminal epitopes. It is recognized by a mid-region PTHrP antiserum. This peptide migrates as a single peak both on RP-HPLC and SDS-PAGE, is present both in conditioned medium following secretion and within cells prior to secretion. It has an amino terminus which begins at amino acid 38 of the cDNA-predicted PTHrP sequence.

Several implications follow from these observations. First, the PTHrP biosynthetic precursor must be cleaved at the arginine residue at position 37 in the sequence. Proteolytic cleavage at monobasic sites, most commonly a single arginine, is a common occurrence in protein processing as was discussed in the Introduction. Second, although there are many serine and threonine residues in this sequence which might serve as O-glycosylation sites, all of these residues were identified in the amino-acid sequencing of the isolated peptide from residues 38-71, and thus it is unlikely that the peptide is O-glycosylated prior to amino acid 71. This is further supported by the appearance of the

peptide on SDS-PAGE as a sharp band (fig. 8), in contrast to the broad appearance of the glycosylated species isolated from keratinocytes.¹⁰⁶ Third, the estimated molecular mass is approximately 7 kDa, which implies a length of 60-70 amino acids. This would place the carboxy-terminus of the fragment in one of two intensely basic regions of the peptide, amino acids 96-98 or 102-106 (fig. 3), long hypothesized to be proteolytic processing sites for PTHrP.^{30,87} Current studies are aimed at precisely defining this C-terminus of the fragment. The low abundance of the peptide make this task extremely difficult.

Since the mid-region fragment from SKRC-1 cells was available only in small amounts, N-terminal sequencing of this peptide was not possible. It was possible to more precisely examine its behavior on chromatographic evaluation. The SKRC-1 fragment migrated in an analogous position on SDS-PAGE to the mid-region fragment isolated from RIN-141 cells, and was found to elute at a similar or identical position on RP-HPLC on two different gradients. It is likely then, although not proven, that the mid-region fragment isolated from SKRC-1 cells begins at position 38 of the cDNA-predicted sequence. After simultaneously isolating mid-region fragments from these two cell lines, a similar study of keratinocyte-conditioned medium was performed in search of a comparable peptide secreted from these cells. One liter of protease-protected keratinocyte-conditioned medium was first stripped of N-terminal immunoreactivity and then examined for mid-region immunoreactivity. Human keratinocyte-conditioned medium was shown to contain a mid-region fragment of PTHrP which is very similar if not identical to that secreted by RIN-141 and SKRC-1 cells (fig. 10).

The fact that this mid-region fragment results from cleavage at the arginine at position 37 lends indirect evidence that PTHrP(1-36) is a secreted form of the peptide. Synthetic PTHrP(1-36) has been manufactured in the past with this processing site in mind. However, this represents the first evidence that this sequence may be an actual secreted form of the protein.

Much consideration went into determining whether the species collected were secretory forms of PTHrP or merely degradation products formed after secretion into the media. First, extensive precautions were undertaken to prevent artifactual proteolysis, including the use of protease inhibitors and collection of 90-minute medium. Second, degradation studies were undertaken which proved that both SKRC-1 and RIN-141 cells display little breakdown of exogenously added iodinated PTHrP(1-141). Third, it was shown that cells extracted with guanidinium, which inactivates proteases, contained what appears to be the identical mid-region fragment isolated from conditioned medium. Finally, immunohistochemical studies indicate that the mid-region fragment localizes to peripheral granules which are most likely secretory vesicles, whereas the N-terminal species localizes to the perinuclear Golgi apparatus. The peripheral granular pattern is commonly seen when staining for proteins which undergo regulated secretion, as seen in immunohistochemical staining for insulin. These findings all support the idea that PTHrP is manufactured in the cell as a larger precursor molecule, undergoes proteolytic cleavage intracellularly and then is secreted as at least two separate fragments-- one amino-terminal and one non-amino-terminal species.

Since both these species are secreted from the cell, it is interesting that they localize to different areas according to immunohistochemical staining. One might postulate that the mid-region fragment is routed to the regulated secretory pathway, whereas the amino-terminal fragment is routed to a constitutive secretory pathway. However, it is possible that the different patterns of localization may result from idiosyncratic staining problems. For instance, it may be that the amino-terminal species does indeed reside in secretory vesicles, but the amino-terminal antibody used for staining might not recognize the species due to folding or packaging of the peptide, or some other post-translational modification. Further study is needed to trace the intracellular trafficking of the various PTHrP species.

It was mentioned in the Introduction that the mid-region of PTHrP is a very highly conserved sequence across species. Considering that the region is flanked by proteolytic processing sites, the conserved sequence from 38-108 is speculated to act in some crucial role in physiology. This study has provided additional evidence that this sequence is indeed processed into a separate fragment, and likely targeted into a regulated secretory pathway. Moreover, this fragment is so produced by three different cell lines. In terms of understanding the physiology of this mid-region secretory form of PTHrP, Care, et. al.¹⁵ have recently shown that a fragment of PTHrP which includes amino acids 67-86 is capable of regulating calcium transport across the placenta. It is unknown whether the peptide identified by Care bears the same carboxy-terminus as does that isolated in our study. It will be interesting to determine whether the mid-region fragment we have isolated demonstrates this calcium-regulatory property.

If the physiologic role of this new peptide is related to calcium homeostasis, this finding could generate more insight into the action of PTHrP in HHM. This could explain the mysterious differences in bone coupling, $1,25(\text{OH})_2$ vitamin D metabolism and renal calcium handling which occur in HHM and hyperparathyroidism. In addition to action on PTH receptors by the amino-terminal fragment of PTHrP, the mid-region fragment may simultaneously alter calcium regulation independently and create the different biochemical profile and bone turnover unique to HHM. On the other hand, if one makes any comparisons of PTHrP and its processing to other protein precursors such as POMC and their final end products, one need not invoke related actions of the resulting peptide hormones. They may vary as widely as do ACTH, α -MSH and endorphins. More detailed study of this mid-region fragment and its role in physiology may produce evidence of an important molecular role in the basic function of a broad range of human tissues.

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