PARATHYROID HORMONE-RELATED PROTEIN, ITS REGULATION OF CARTILAGE AND BONE DEVELOPMENT, AND ROLE IN Treating Bone Diseases

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Martin, TJ. Parathyroid Hormone-Related Protein, Its Regulation of Cartilage and Bone Development, and Role in Treating Bone Diseases. Physiol Rev 96: 831–871, 2016. Published May 3, 2016; doi:10.1152/physrev.00031.2015.—Although parathyroid hormone-related protein (PTHrP) was discovered as a cancer-derived hormone, it has been revealed as an important paracrine/autocrine regulator in many tissues, where its effects are context dependent. Thus its location and action in the vasculature explained decades-long observations that injection of PTH into animals rapidly lowered blood pressure by producing vasodilatation. Its roles have been specified in development and maturity in cartilage and bone as a crucial regulator of endochondral bone formation and bone remodeling, respectively. Although it shares actions with parathyroid hormone (PTH) through the use of their common receptor, PTHR1, PTHrP has other actions mediated by regions within the molecule beyond the amino-terminal sequence that resembles PTH, including the ability to promote placental transfer of calcium from mother to fetus. A striking feature of the physiology of PTHrP is that it possesses structural features that equip it to be transported in and out of the nucleus, and makes use of a specific nuclear import mechanism to do so. Evidence from mouse genetic experiments shows that PTHrP generated locally in bone is essential for normal bone remodeling. Whereas the main physiological function of PTH is the hormonal regulation of calcium metabolism, locally generated PTHrP is the important physiological mediator of bone remodeling postnatally. Thus the use of intermittent injection of PTH as an anabolic therapy for bone appears to be a pharmacological application of the physiological function of PTHrP. There is much current interest in the possibility of developing PTHrP analogs that might enhance the therapeutic anabolic effects.

I. INTRODUCTION: HOW THE PTHrP CONCEPT DEVELOPED

In reviewing the physiological roles of parathyroid hormone-related protein (PTHrP), we move from its discovery as a hormone produced by certain cancers to its functions in many tissues in development and postnatally. The effects of PTHrP excess in cancer that led to its discovery are dramatic, but the subsequent realization that PTHrP functions physiologically as a multifunctional cytokine, surprising as it was at first, has revealed it as an intriguing local regulator of organ function. This review focuses on its role in bone and cartilage physiology and how it relates as a local factor to the circulating hormone, parathyroid hormone (PTH), with which it shares certain crucial structural features, and to which it is related in evolution. That will be introduced by discussing the properties of the protein and gene, as well as important aspects of PTHrP biology, including its local functions in various tissues other than bone, and its capacity to be transported specifically into the nucleus. It is

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hoped that this will provide essential background to consideration of the roles of PTHrP in bone.

As is the case with many aspects of our understanding of the biology of bone and its disorders, the story that develops of PTHrP can be traced back to Fuller Albright at the Massachusetts General Hospital in 1941. In discussing a patient with high plasma calcium and low phosphorus accompanying a non-parathyroid cancer, he suggested that the hypercalcemia might be due to production by the cancer of PTH or something very like it (4). Primary hyperparathyroidism due to excess circulating PTH was known to result in a high plasma calcium and low phosphorus. Albright knew from his own work and that of others that removal of the parathyroid glands as a source of PTH resulted in low calcium and elevated phosphorus, and furthermore, that PTH administration to animals increased the blood calcium, promoted phosphorus excretion, and lowered the plasma phosphorus level.

II. HYPERCALCEMIA IN CANCER

This suggestion of Albright took on a life of its own some few years later, when the concept was developed of the “ectopic” production by cancers of peptide hormones, beginning with adrenocorticotropic hormone (ACTH) (196, 233). The idea became prevalent in the literature that ectopic PTH production (“ectopic PTH syndrome”) could explain the hypercalcemia and hypophosphatemia that was noted with certain cancers in the absence of bone metastases, particularly with squamous cell cancers of lung, and with kidney and certain neuroendocrine cancers (38, 182, 308). Before specific radioimmunoassay for PTH was developed, when they examined extracts of several cancers of hypercalcemic subjects, Tashjian et al. (338) identified immunochemical cross-reactivity with bovine PTH. In this work they recognized the difficulty they confronted, of lack of a sensitive assay for PTH biological activity. They concluded that the tumor antigen was either PTH or a very closely related protein. Their proviso that their finding did not imply identity with PTH proved to be a judicious one in light of later events.

A number of studies were supportive of the idea of tumor production of PTH. Immunohistology was used to show PTH-like immunoreactivity in several cancers associated with nonmetastatic hypercalcemia (262). Importantly, the development of the PTH radioimmunoassay by Berson and Yalow (28) resulted in demonstration of significantly elevated levels of PTH in plasma, tumor extracts, and culture media from affected patients (38, 113, 229, 234). Nevertheless, doubts began to appear concerning the nature of the cancer activity. A breast cancer extract from a hypercalcemic patient yielded PTH immunoreactivity that failed to dilute in parallel with pure PTH standard (234). Importantly, the PTH radioimmunoassay improved, as new antisera were developed that were better defined for specificity. These showed that levels of circulating PTH in the cancer patients, although elevated in absolute terms, were consistently lower than those in primary hyperparathyroidism with similar degrees of hypercalcemia. Moreover, they differed immunochemically, again by diluting nonparallel to standards (25, 285, 290). Most compelling evidence came from the findings that immunoreactive PTH could not be detected in either plasma or tumor extracts of a series of hypercalcemic patients, although the same extracts promoted bone resorption in vitro (16, 274). The use in one of the latter studies (274) of several antisera directed against different sequences within PTH added to the increasing doubt that authentic PTH was being produced by these cancers.

There was little doubt that a tumor product was the cause of the hypercalcemia in these patients without bone metastases, since there were several well-documented examples of reversion of plasma calcium levels to normal after removal of the cancer by surgery (38). The conclusion by the end of the 1970s though was that the tumor-derived activity was not PTH itself, but was likely something that resembled PTH sufficiently to be recognized to some extent by some antisera and not by others, and that was capable of influencing calcium and phosphorus metabolism in a manner that resembled PTH action. Thus, by that time the concept had gone full cycle, back to what Albright had proposed in 1941, “something very like PTH.” Since “ectopic PTH syndrome” appeared to be no longer appropriate, the term humoral hypercalcemia of malignancy (HHM) was coined (216).

III. THE CANCER PRODUCT IS PTH RELATED

Real advances in identifying the cause of HHM began to be made in the early 1980s, by which time more specific radioimmunoassays for PTH had been developed. Three excellent clinical studies showed that HHM patients had suppressed levels of PTH but increased phosphate excretion and nephrogenous cAMP (180, 293, 323), all of which indicated that the responsible tumor-derived factor was not authentic PTH, but that it exerted physiological actions very similar to those of PTH.

There was an obvious need for sensitive biological assays of PTH activity. The difficulties reported by Tashjian et al. (338) in attempting to relate PTH immunologic and biologic activity in cancer extracts with the crude in vivo assay available in the 1960s had illustrated this. Using an ultra-sensitive cytochemical assay for PTH that measured the activation of glucose-6-dehydrogenase in guinea pig kidney tubule cells, Goltzman et al. (109) showed that culture medium from a tumor derived from a HHM patient released readily assayable activity that could be blocked by peptide
receptor antagonists of PTH, but not by PTH antibody (324). Sensitive and specific though this bioassay was, it was too cumbersome and time-consuming to support any effort at purification.

What made the identification and ultimately the purification of PTHrP possible was the availability of a specific biological assay for PTH activity that was highly sensitive, reproducible, capable of high throughput, and with a rapid turn-around time. The assay was based on the recently developed rat osteogenic sarcomas that exhibited PTH-responsive adenyl cyclase (208, 217). The cells of these tumors possessed many features in common with osteoblastic cells (264), and cultured cells and cell lines derived from them responded specifically to PTH with dose-dependent increases in cAMP (15, 264). In view of the new concept that HHM pathogenesis was based on some factor that closely resembled PTH in action, it was logical to use this response as a bioassay.

IV. PURIFICATION AND AMINO ACID SEQUENCE OF PTHrP

The biological criteria applied in the search for the unidentified PTH-related activity from tumors or culture media derived from patients with HHM were that 1) it should promote adenyl cyclase activity strictly in cells known to be PTH targets; 2) it should be inhibited by peptide receptor antagonists of PTH-receptor interaction, but not by anti-PTH neutralizing antisera; and 3) PTH should be undetectable in patients’ plasma by radioimmunoassay. These stringent requirements were demonstrated in early studies of PTH-like activity identified in tumor extracts from patients and animals with HHM (109, 287, 324) and in culture media from one such tumor line (327). Transplantation of tumors from hypercalcemic patients into immune-deficient mice resulted in development of hypercalcemia in the absence of bone metastases (327).

The evidence from all of this work was overwhelming in indicating that a non-PTH activity was produced by these cancers, that it acted upon the PTH receptor to activate adenyl cyclase, and that the available biological assay provided an amenable path to purification. That nevertheless took some years to achieve.

With use of a cell line from a squamous cell carcinoma of the lung from a patient who had HHM, a novel active 17- to 18-kDa protein was purified and sequenced through the first 16 amino acid residues, revealing that 8 of the first 13 residues were identical to those in PTH, but no PTH was expressed by the cells, and anti-peptide antibodies against this protein did not cross-react with synthetic PTH (1-34) (324). The sequence of the first 50 residues was obtained with further purified protein (332). In view of the structural and functional similarity to PTH, the protein was called PTH-related protein (PTHrP) for operational purposes (244), pending the choice of a definitive name (222). Perhaps regrettably in light of the many roles of PTHrP distinct from those of PTH, the name has not been changed. Biologically active PTHrP was secreted when the tumor cell cDNA was introduced into monkey kidney cells (332). Studies with synthetic peptides showed that the biological action of PTHrP at the PTH receptor was contained within the first 34 residues, as was known to be the case with PTH (166), and the structural requirements for activity within that domain of the protein were the same as those that had been known for many years with PTH (346). PTH-like activity was also purified from a breast cancer of a patient with humoral hypercalcemia (43), and an amino-terminal amino acid sequence was obtained from that material (325), as well as from a renal cortical carcinoma cell line (326).

V. CLONING OF PTHrP

Cloning of PTHrP from was achieved using cDNA libraries from the same cell line as above (BEN cells) (85), a cell line established from a HHM patient, allowing prediction of a prepropeptide of 36 amino acids and a mature protein of 141 amino acids, confirming the significant homology with PTH about the NH₂-terminal region that had been evident from protein sequencing (332) [FIGURE 1]. Regions of identity with PTH are boxed and illustrate that the high degree of NH₂-terminal homology is not maintained throughout the remainder of the molecule, with the divergent sequence of PTHrP beyond residue 13 not resembling any other known protein sequence. The pre-pro sequences of PTH and PTHrP are also distinct, but the chemical properties of the residues in pre-pro PTHrP are similar to those in pre-pro PTH (332). Thus cleavage of the pre-sequence was predicted to generate a basic pro-sequence that would be cleaved to liberate the mature protein. The homology and structural overlap between PTHrP and PTH suggested that this might have resulted from gene duplication of a common ancestor of the PTH gene (332). Subsequent work provided support for this (63, 65, 75, 348). The protein has remained known as PTHrP and the human gene as PTHLH, with the mouse gene designated Pthlh (http://www.ncbi.nlm.nih.gov/gene/?term=PTHrP). The same protein was identified in two animal models of HHM, the Leydig cell tumor in Fisher rats and apocrine cell adenocarcinoma of the anal sac in dogs (374). Cloning and amino acid sequence prediction of PTHrP from many species came in subsequent years, revealing the very high degree of conservation of sequence among species (FIGURE 1).

When further cloning identified the same cDNA product from a renal cancer cell line (213, 214), the multiple RNA transcripts noted on Northern analysis suggested that alternative splice products were likely. This was established with data showing two further cDNA species, predicting PTHrP...
variants of 139 and 173 amino acids in length (214, 342, 393), thus explaining the observation in initial cloning experiments of a clone containing a divergent 5' untranslated region (332). Of all the PTHrP sequences predicted to date, only human PTHrP undergoes alternate mRNA splicing that yields three variants (not shown in Figure 1). Mature human PTHrP could consist of either 141 amino acids, or 139- and 173-residue molecules as a result of alternate transcripts (213, 342). The fact that human PTHrP is the only species with more than one protein product is of much interest, posing questions yet to be answered, of the significance of these COOH-terminal differences in the human gene product.

VI. GENE STRUCTURE AND REGULATION

The human PTHLH gene was characterized (212, 330, 393) including its three promoter regions (two TATA boxes and a GC-rich region) (Figure 2) and splicing patterns, showing PTHrP to be encoded by a single copy PTHHL gene located on chromosome 12p11.2-p12.1, distinct from the PTH gene, which is located on chromosome 11p15.4 (35). Despite the greater complexity of PTHHL than the PTH gene, the two are similar in their overall genomic organizations, having similar exon-intron boundaries (Figure 2). Chromosomes 11 and 12 are thought to have arisen through duplication events from a single common ancestral chromosome (211). Together with the fact that there are several examples of other pairs of related genes located on chromosomes 11 and 12 (Table 1) (35, 347), this reinforced the view that PTHrP and PTH are related through a gene duplication event in evolution. Both have a very long evolutionary history, but it is still unknown when the duplication took place, although the greater complexity of the PTHLH gene developed relatively recently in evolution (62). A more comprehensive review of the PTHLH gene is provided in Suva et al. (329), including information from more recent extensive databases.

The human PTHLH gene is composed of nine exons spanning ~15 kb, transcribed by three functionally distinct promoters, and with primary mRNA transcripts alternatively spliced to yield protein products of 139, 141, and 173 residues (Figure 2) (78, 79, 161, 252). 5' to exons I and IV are canonical TATA promoters (P1 and P3), whilst 5' to exon III is a GC-rich promoter region (P2) (44, 170, 212, 330, [44x339]variants of 139 and 173 amino acids in length (214, 342, 393), thus explaining the observation in initial cloning experiments of a clone containing a divergent 5' untranslated region (332). Of all the PTHrP sequences predicted to date, only human PTHrP undergoes alternate mRNA splicing that yields three variants (not shown in Figure 1). Mature human PTHrP could consist of either 141 amino acids, or 139- and 173-residue molecules as a result of alternate transcripts (213, 342). The fact that human PTHrP is the only species with more than one protein product is of much interest, posing questions yet to be answered, of the significance of these COOH-terminal differences in the human gene product.

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Note that although there is no intron between exons 6 and 7 in the human PTHLH gene, we have applied this nomenclature because this junction provides a splice donor site for acceptors at the beginning of exons 8 and 9. Promoter 1 (P1) and P2 transcripts contain exon III sequences in common, but neither contains the P3-specific 5' untranslated sequence of exon IV (FIGURE 2). Although the activity of each of these promoters has not been established for all PTHLH-expressing cells and tissues, it appears that the TATA promoter 5' to exon IV (P3) is the most active promoter in all PTHrP-producing cells examined to date (44, 316, 317). In their structural organizations, the chicken, mouse, and rat Pthlh genes share substantial homology with the human PTHLH gene (FIGURE 2). The conservation of exons equivalent to human exons IV, V, VI, and IX among the rat, mouse, and chicken PTHLH genes suggests that these exons constitute the minimal PTHLH gene structure. To date, only one promoter region has been defined for the mouse and rat genes, equivalent to the P3 promoter of the human gene. Nucleotide sequence comparison of the mouse and rat genes to the human gene demonstrated substantial homology over the GC-rich promoter region (P2) and exon III of the human gene, suggesting that this region is also transcribed in rodents. Confirmation of this idea was achieved when transcripts corresponding to human exon III were detected in a clonal rat line (PT-r) (394) and a HHM-associated rat Leydig cell tumor H500 (138). However, there is no evidence to suggest that the
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mouse or rat has exons equivalent to exons I or II of the human PTHLH gene, although they are considerably simpler than the human gene (FIGURE 2).

A. Promoters and Alternative Splicing

The multiple promoters in the human PTHLH gene could have implications for alternate promoter use in tissue-specific and/or developmentally regulated expression of PTHrP. Since the PTHLH genes from all species described to date have a functional promoter region equivalent to the human P3, but not P1 or P2 promoters, it is likely that the dominant transcriptional regulation of PTHrP occurs through the P3 promoter. Apart from specifying different PTHrP isoforms, the alternative transcripts confer different properties to the corresponding mRNA species. The three isoforms of human PTHrP have different 3' UTRs (FIGURE 2), but all contain multiple copies of an AUUUA instability motif (213, 332, 342). This motif has been associated with the rapid turnover of mRNA from cytokines and oncogenes (307). PTHrP mRNA has a short half-life of ~90–120 min, with a report of a somewhat longer half-life of ~4 h for the PTHrP(1-173) isofrom (304). Comparisons among the 3’ UTR sequences reveal differences in the number and length of instability motifs, consistent with the individual mRNA species having distinct half-lives. The nonamer sequence UUAUUUAUU has been found to have a greater effect on mRNA stability than the minimal sequence AUUUA, but the differential effects of motifs of varying lengths have not been extensively explored for the PTHLH gene. The instability of PTHrP mRNA is a notable feature in all species, which it shares with many cytokines. It is one of the properties of the molecule that equips it well to function as a paracrine or autocrine regulator, together with the susceptibility of the protein itself to proteolysis (see below).

B. Transcriptional Regulation

The transcriptional regulation of the human PTHLH gene may also be the target of noncoding RNAs (ncRNAs). MicroRNAs (miRNAs), small ncRNAs, are able to influence gene expression and posttranslational processing by binding complimentary DNA and mRNAs, as well as complexing with proteins with diverse functions including splicing and epigenetic modification (29, 86, 228). There are at least 28 putative miRNA binding sites in PTHLH (29). One confirmed species is miR-33a, which has been proposed to repress PTHLH expression in human A549 cells (181), and may suggest an important role in the progression of malignancy. In addition, in human MDA-MB-231 breast cancer cells, miR-520/373 overexpression resulted in the suppression of the PTHLH gene, as well as plasminogen activator inhibitor-1 and angiotensin-like 4 in vitro and in vivo (164).

In addition to direct transcriptional regulation, the PTHLH gene is also subject to regulation by a multitude of growth factors, cytokines, and hormones, some acting by transcriptional, and others by posttranscriptional mechanisms. Those that act transcriptionally do so through promoter regions or by binding to positive or negative response elements located in the upstream promoter regions of the PTHLH gene (330). Factors such as CREB, vitamin D3, Ku antigen, Ets1, Tax1, and SP-1 have been shown to bind to DNA-response elements located within PTHLH promoter regions, and either up- or downregulate PTHrP expression (53, 78, 79, 96, 161, 252). The induction of PTHrP expression in response to many of these factors is rapid, indicating that in some circumstances, PTHLH is an immediate early gene, a classification that it shares with transcription factors and oncogenes (6), and implies that PTHrP expression is also under strict temporal regulation.

Both transforming growth factor (TGF)-β and epidermal growth factor (EGF) potentiate modulation of PTHrP expression pretranslationally by influencing promoter usage, and posttranslationally by stabilizing mRNA transcripts (125, 170, 230, 235, 377), both of these growth factors stimulating transcription from P2 and P3 and excluding exon IX from spliced forms (125, 170). A matter of interest is whether alternative splicing products of the human PTHLH gene can be differentially regulated. Heath et al. (125) found that the stability of mRNA for the (1-139) isoform, but not for the (1-141) isoform, was increased by EGF treatment in human A549 cells (181), and implies that PTHrP expression is also under strict temporal regulation.

A comprehensive analysis of tissue specific or development-
studies have been possible. The question cannot be addressed in murine studies because simpler structures of the rat and mouse genes (FIGURE 2), which undergo no alternative 3' splicing, preclude any studies of the tissue specificity of generation of alternate transcripts.

VII. PROTEIN STRUCTURE AND FUNCTIONAL DOMAINS

On the basis of the primary amino acid sequence compared with PTH, PTHrP can be divided into distinct regions. These will be discussed as protein domains (FIGURE 3), with the question of posttranslational processing considered in the next section.

The intracellular “prepro” and “pro” precursors (−36 to −1) of the mature peptide are necessary for intracellular trafficking and secretion of polypeptide. The next functional region is critical for almost all of the agonist effects of PTHrP on the PTH1 receptor expressed on classic PTH target tissues (bone, kidney) that regulate calcium metabolism. This encompasses the first 13 residues of the mature protein, which is the region showing highest degree of primary sequence homology with PTH (FIGURE 1); in fact, 8 of the first 13 residues are identical. The earliest data with synthetic peptides showed that the structural requirements for activation of the PTH receptor (PTHR1) by PTHrP were contained within the first 34 residues and were very similar to those known at the time for PTH (166, 346). A homologous receptor, PTHR2, was discovered subsequently in the brain (354), whose physiological ligand is tuberoinfundibular peptide 39 (TIP39). PTH(1–34) binds to and activates PTHR2, but PTHrP(1–34) does not (104). Sequence within PTH(1–14) and PTHrP(1–14), particularly histidine at position 5 of PTHrP, was considered to contribute largely to the ability of PTHR2 to discriminate between PTH and PTHrP (23).

The 14–36 region of PTHrP, which has almost no homology with the primary amino acid sequence of PTH (FIGURE 1), nevertheless appears to be critical for the binding of PTHrP to the classical PTHR1 and for its subsequent activation. Numerous investigators have used competitive binding assays to show that PTHrP(1–36) bind the receptor with approximately equal affinity, while shorter NH2-terminal fragments of either PTH or PTHrP do not (260). This activity reflects the strong similarity of the secondary/tertiary structure of PTH and PTHrP, despite the differences in primary amino acid sequence in this region.

There are a number of other activities within the highly conserved region of PTHrP from amino acids 36 to 139 (FIGURE 3) whose importance is in some cases less well established. This region is encoded by all three isoforms of human PTHrP mRNA, and of the functions ascribed to domains within it, few have been definitively and repeatedly demonstrated. Apart from the nuclear localizing capability, which we will consider further below, the most established of these is that the “mid-molecule” portion, between residues 35 and 84, is responsible for promoting calcium transport across the placenta, making calcium available for fetal skeletal development. These findings have been established convincingly through pharmacological experiments in sheep (289) and genetic studies in mice (175). Further evidence was provided (47) that the mid-molecule region producing the placental effect is within PTHrP(67–86). This was ascribed to PTHrP(38–94)amide, a product generated by secretory processing in the neuroendocrine RIN cells (382). It is interesting to note that the same peptide was found to be a potent inducer of cytosolic calcium and promote phospholipase C activation, without any effect on cAMP (258). No receptor has been identified for this action, which is independent of PTHR1.

Other actions of PTHrP independent of PTHR1 have been documented in pharmacological experiments only and are less well established. In the isolated perfused rat kidney, both PTHrP(1–141) and (1–108) briefly promoted bicarbonate excretion, followed by a prolonged and substantial

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**FIGURE 3.** Functional domains of PTHrP. The 3 isoforms resulting from alternative splicing terminate at 139, 141, and 173 amino acids (aa). The prepro region (blue) includes the signal sequence (−36 to 1 aa). The PTH-like PTH1R region (green) binds to the PTH1R receptor (1–34 aa). The region responsible for placental calcium transport is stippled (67–86 aa). The nuclear localizing sequence (NLS) (yellow) is (67–94 aa), and the nuclear export sequence (NES) (pink) is (116–136 aa). The osteostatin region (orange) is (107–111 aa). The region that is mitogenic in osteoblast and vascular smooth muscle cells (striped) is (108–139 aa).
The COOH terminus of PTHrP has received quite some attention. The first indication of some activity was the finding that PTHrP(107–139) inhibits osteoclast activity and bone resorption by isolated rat osteoclasts in vitro, an effect that was ascribed to PTHrP(107–111), which was called “osteostatin” (88–90). The osteostatin peptide was also found to inhibit resorption in vivo when injected over calves in mice (59), and furthermore, to promote osteoblast proliferation in experiments in which PTHrP(107–139), (107–111), and (107–120) were effective, but PTHrP (122–139) was not (58). The antiresorptive effect of osteostatin in organ culture has been controversial, with some investigators not finding this effect in vitro (153, 245, 315).

A number of other effects of COOH-terminal PTHrP have been consistently reported. Although PTHrP(107–139) and osteostatin were found to be anti-proliferative in UMR 106 rat osteosarcoma cells (356), transient exposure to PTHrP(107–139) in vitro enhanced human osteoblastic cell differentiation through interaction with the VEGF receptor-2 (67) and, through activation of that same receptor, enhanced human osteoblast cell survival (7). Both stimulation of intracellular calcium and activation of protein kinase C through a receptor of unknown identity were proposed as signals in these actions (356, 357), but to the present time, no receptor has been identified. Some in vivo experiments have suggested biological activity of administered osteostatin and PTHrP(107–139). When neonatal mouse bones were labeled with [3H]tetracycline, release of skeletal radioactivity promoted by PTHrP(1–34) administration was inhibited by osteostatin (286). In mice rendered diabetic with streptozotocin, treatment with PTHrP(107–139) promoted bone healing after marrow ablation (203), and PTHrP(1–34) and PTHrP(107–139) at equal treatment doses of 80 μg·kg⁻¹·day⁻¹ were equally effective at partially restoring the bone lost after ovariectomy in mice (66).

Although there remains some controversy about these actions of the COOH-terminal region of PTHrP with regard to their reproducibility and their relevance to physiology, their possible functions should continue to be pursued, because there are a number of reasons to suspect that there might be important activities in that domain. These various pharmacological effects of COOH-terminal PTHrP are reminiscent of similar studies with COOH-terminal fragments of PTH, where biological effects were identified (80, 83, 334), but no receptors identified or mechanism(s) ascertained. Potential phosphorylation sites have been pointed out within the PTHrP(107–139) sequence (69), and several potential O-linked glycosylation sites. Furthermore, in using the two-hybrid approach to search for binding partners for PTHrP, Conlan et al. (56) found specific binding to β-arrestin 1. This binding was localized to the PTHrP(122–141) region of the molecule (56), providing the opportunity of interaction of PTHrP with other components of the MAPK scaffold and thus retention of PTHrP in the cytoplasm. The presence of putative phosphorylation sites within the COOH terminus of PTHrP could be potential targets for regulation by such β-arrestin interactors.

An aspect of the pharmacology of COOH-terminal PTHrP that should be noted is that this is the domain of PTHrP that is the least conserved among all species. Within PTHrP(107–139), human and mouse have 7 residues in common and rat and human 13 in common (FIGURE 1), but in vitro and in vivo studies in rat and mouse invariably used human PTHrP(107–139), apart from those studies carried out with PTHrP(107–111), the common sequence to all of these species. Later in this review (see sect. X), we discuss the work in which COOH-terminal PTHrP(107–139) was needed in addition to the NLS to stimulate mitogenesis in vascular smooth muscle cells (69, 226). Even in that work (69) it was human PTHrP that was used to transfect rat smooth muscle cells.

If there are indeed important functions within the COOH terminus, why is its sequence so divergent among species, unlike the sequence (107–111), which is so very highly conserved? One possibility is that this sequence, TRSAM, is the most important contributor to the reported effects of COOH-terminal PTHrP. That could only be resolved with further work, including identifying receptor and signaling mechanisms. Alternatively, conformation of the COOH-terminal region might be such that allows it to act across these species. The same might be said for the PTHR1 receptor-binding domains of PTH and PTHrP, located between residues 15 and 34, where the primary sequences differ markedly (103).

B. Alternative Splice Variants and Proteolytic Products

Of the three alternate splice variants of human PTHrP, there is no evidence allowing distinction of functions. The mRNAs for each of these are commonly expressed in various tissues and cancers. Even less is known of the region comprising amino acids 142–173. Its tissue distribution, processing, or function remain unknown, although PTHrP(141–173) immunoreactivity has been identified.
in plasma (42) and in human amnion (37). It might be presumed that the actions ascribed to regions of the molecule beyond the NH2-terminal 34 amino acids are mediated by unique receptors, although these have yet to be discovered. It is relevant to ask, why are there three splice variants of human PTHrP, and what differences exist among actions and significance of the 139, 141 and 173 variants?

The PTHrP sequence is so rich in mono- and multibasic cleavage sites (FIGURE 4) that it is not surprising that many peptide segments of the molecule have been detected in cell culture and in the sera of patients, concordant with the view that there are a number of different biologically active domains within the molecule. Together with the unstable mRNAs of PTHrP, this marked susceptibility to proteolytic degradation can be viewed as a property that is supportive of PTHrP functioning physiologically as a paracrine/autocrine regulator in many tissues. Together with the unstable mRNAs of PTHrP, this marked susceptibility to proteolytic degradation can be viewed as a property that is supportive of PTHrP functioning physiologically as a paracrine/autocrine regulator in many tissues. This lability of the protein could ensure its rapid disappearance after local actions. In accordance with this, NH2-terminal PTHrP cannot be detected convincingly in the circulation of normal subjects using NH2-terminally directed assays (115), but multiple PTHrP species have been isolated from plasma (41, 42) and from the urine of patients with HHM (140). In patients with renal failure, COOH-terminal epitopes can be identified in the absence of detectable NH2-terminal epitopes, reflecting the fact that a COOH-terminal fragment is disposed of by the kidney (140). Defining the circulating forms of PTHrP has proven to be extremely difficult because of the reliance on multiple sequence-specific antibodies for detection, the low amount of circulating PTHrP, and the need for assays with greater sensitivity and specificity. Such is the reliance upon antibodies for detection and assay that absolute precision about circulating sequences has not been possible.

FIGURE 4. Known and putative proteolytic sites within human PTHrP. Amino acid numbering of human PTHrP is (1–36) to (173). Specific residue numbers and amino acids are indicated associated with potential cleavage sites. Potential proteolytic enzymes are listed in italics.
The use of a mass spectrometry-based assay identified the fragment at concentrations in the 50 ng/ml range, confirming the lack of cross-reactivity with existing PTHrP immunooassays, which may be expected to detect the fragment at this concentration. Why this should circulate at such an apparently high level, and what is its function, remain to be established.

A. Processing in Cell of Origin?

Quite apart from the functional domains within PTHrP and its susceptibility to proteolytic cleavage as discussed above, there is evidence that in some cells PTHrP can be processed in ways that result in secretion of constituent peptides.

Central issues concerning posttranslational proteolytic processing of PTHrP are to what extent it takes place within the cell of origin before secretion, and whether such processing takes place in an isoform- and tissue-specific manner. The processing of the COOH-terminal fragment (107–139) could be detected in cells transfected with PTHrP(1–139), with little detection of the fragment when cells were transfected with the (1–141) and (1–173) isoforms, suggesting isoform specific processing, at least under these circumstances (77, 391). The difficulty in generalizing from observations such as this is illustrated by the differential processing observed between cell lines, where the mid-region (38–74) was detected in rat insulinoma (RIN) cells, but not in Chinese hamster ovary (CHO) cells (391). The secretion of these peptides also differs, with the mid-region of PTHrP secreted by a regulated secretory pathway (RIN cells), and NH₂- and COOH-terminal forms detected when the constitutive pathway was used (CHO cells). The constitutive secretory pathway is common to all cells, whereas the regulated secretory pathway is used by specific cell types such as neuroendocrine cells (reviewed in Ref. 40). Regulated secretion is used for specialized secretory products such as neurotransmitters or hormones. These proteins are packaged at high concentrations into secretory vesicles that are sequestered in the cytosol until an extracellular signal stimulates their secretion.

Such cell-specific use of the regulatory and constitutive pathways for PTHrP has been observed. In neuroendocrine cells, evidence was obtained for the processing and secretion of daughter peptides [i.e., (1–36), (38–84), either free or amidated COOH terminus, and (107–138)] via the regulatory pathway (259, 314, 391). In nonneuroendocrine cells that use the constitutive pathway of secretion, each of these regions could be detected in the culture media, but these experiments using immunoassay could not exclude the possibility that the measured immunoreactivity was contained within parent protein. The authors recognized this (272) but favored the idea that peptide portions were secreted because they had shown these to be present in protease-protected media (280, 314, 391).

While these in vitro studies of secretion are of interest, they do not allow any conclusions about the form of PTHrP released in a particular tissue, e.g., bone or smooth muscle, where the constitutive secretion pathway is used, and the released PTHrP acts on the same or in an adjacent cell in an autocrine or paracrine manner, respectively. This question becomes particularly important when considering the local role of PTHrP in bone and any attempt to mimic this action therapeutically. This will be discussed in section XIX.

The physiological implications of posttranslational processing of PTHrP are still not well understood (231, 259, 260), nor can we exclude the possibility that many of the peptide products of cleavage are just that, simply breakdown products of a paracrine/autocrine factor whose work is completed in that tissue. Nevertheless, it is abundantly clear that PTHrP has a number of bioactivities contained within domains of the protein, as discussed above. This might support the idea of PTHrP as a polyhormone, as is the case with many neuroendocrine proteins that give rise to families of mature secretory peptides that can also access the nucleus (144). These forms may have their own receptors to elicit signaling pathways that are in part regulated by isoform- and cell-specific processing and secretion. It may also be that the great susceptibility of PTHrP to endoproteolytic cleavage is the result of evolutionary pressure to fit it so well as a paracrine and autocrine regulator with a very short tissue half-life, and at the same time be a source of peptide products that serve other purposes, either as circulating hormones or as local agents.

B. Glycosylation and Phosphorylation of PTHrP

Recombinant PTHrP purified after expression in bacteria was consistently found to behave at a molecular weight on gels that was somewhat greater than the predicted 16 kDa (121, 343), likely as a result of its very basic nature. When the PTHrP sequence was first determined, it was pointed out that the molecule contains no N-glycosylation sites. Nevertheless, when PTHrP was purified from large volumes of medium from keratinocyte cultures (381), it was found to be glycosylated, appearing as a diffuse band on gel electrophoresis and with a molecular weight of 18,000 that was reduced to 10,000 following treatment with deglycosylating enzyme. This suggested that the predominant PTHrP form in the culture supernatants was less than full-length molecule, but that it was glycosylated. The deglycosylation did not influence the molecular weight of PTHrP that had been expressed by bacteria (381). PTHrP produced by rat coronary artery endothelial cells was also found to be glycosylated (301), with an apparent molecular weight of ~40,000. Glycosylated PTHrP immunoprecipitated from media was found to be ~10 times more potent than PTHrP(1–34) in promoting contraction of ventricular cardiomyocytes. It seems likely from these observations that
PTHRP is indeed glycosylated, most likely through O-glycosylation sites. How this might affect the function of PTHrP is still not known. The significance of glycosylation is a matter requiring further study.

Phosphorylation is a potent posttranslational modifying process for many proteins. The importance of phosphorylation of Thr-85 in excluding PTHrP from the nucleus will be discussed later in relation to nuclear transport of PTHrP. A number of consensus phosphorylation sites have been pointed out in the human PTHrP(107–139) domain, with serine and threonine residues which are not identically located between species, but are broadly conserved (69). This was introduced in discussion of the possibility of the COOH terminus having a transactivating role in PTHrP action (see sect. VII).

**IX. RECEPTOR AND SIGNALING**

The type 1 parathyroid hormone receptor (PTHR1), which mediates the biological actions of both PTH and PTHrP (152, 166), is a seven-transmembrane G protein-coupled receptor member of a family that includes receptors for calcitonin, secretin, and glucagon. Within the PTHrP sequence, amino acids 1–34 are sufficient to mediate PTHR1 binding of comparable efficacy to PTH(1–34) (152). The receptor is expressed in many tissues that also express PTHrP, often in the same cells or in those immediately adjacent. This close juxtaposition of cells expressing PTHrP and PTHR1 is consistent with its function as a paracrine/autocrine factor in many tissues, including bone (219). It should be noted though that interrogation of genome databases revealed that two PTH receptors are present in the invertebrate *Ciona intestinalis* (154), as well as in insects (193). The ligands are not known, but the finding suggests roles for this signaling pathway before bone and cartilage evolved.

Structural studies of PTH and PTHrP peptides using NMR have indicated consistently that there is in each peptide a relatively stable α-helical portion within the sequence PTHrP(15–34) (19, 20, 52, 224, 225). This appears to be amphipathic in nature, with opposing hydrophobic and hydrophilic faces, and peptide mutational analyses revealed the importance of residues on the hydrophobic face for binding to the receptor (105, 256). With the use of mutant receptors and altered ligands, a “two-site” model of interaction of ligand with PTHR1 has been developed, in which the COOH-terminal portion of the PTH(1–34) fragment binds to receptor extracellular domain, and then the NH2-terminal portion, which is responsible for signaling, interacts with the transmembrane domain to induce the conformational change required for signaling (103).

Activation by either PTH or PTHrP ligand couples PTHR1 to the Gαq/adenyl cyclase/cAMP/protein kinase A (PKA) signaling pathway predominantly, but it is capable of coupling also to several other pathways, including the Gα12/13 phospholipase C/ITP/protein kinase C (PKC) and Gα12/13 phospholipase D (PLD)/RhoA pathways (311) and the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK1/2) signaling cascade (106, 313, 333) (FIGURE 5). The two ligands show very similar agonist response profiles in activating the various signaling pathways. These comparisons have usually been made between PTHrP and PTH peptides consisting of the NH2-terminal activating domain. In studies of a number of analogs, selective activation of ERK1/2 through the Gs/PKA pathway was achieved by using Trp12,-PTHrP(1–36) (106). Furthermore, an inverse agonist for Gs coupling, (d-Trp12,-Tyr38)-PTH(7–34) (PTH-βarr), was found to act as an inverse agonist in signaling by a G protein-independent β-arrestin-mediated signaling pathway (102, 106, 107).

A comparison of activities among PTHrP(1–141), (1–108), and (1–34) and PTH(1–34) showed that the preparations were equipotent on a molar basis in promoting cAMP production in osteoblast-like osteosarcoma cells (121). The dominant signaling pathway mediating responses to PTHrP and PTH is the cAMP/PKA pathway, even if the other signaling pathways might help regulate responses in certain tissues (119, 120, 392). With the use of a number of substituted peptides, it has been possible to conclude that cAMP signaling plays the major role in mediating the bone anabolic and renal phosphaturic responses to responses to the PTH/PTHrP ligands (248, 390). Downstream effectors include the cAMP-response element binding protein CREB and AP-1 transcription factors.

**A. A Comparison of PTH(1–34) and PTHrP(1–36) Signaling**

A new idea of the nature of signaling through the PTHR1 that is relevant to the action of PTHrP comes from work showing the PTHR1 is able to form a unique high-affinity conformation even in the absence of G protein coupling (70, 71). G protein-coupled receptors had been thought to exist in a low-affinity state and move to high affinity upon G protein coupling (68). PTH(1–34) was found to form a stable high-affinity complex in the absence of G protein coupling, whereas PTHrP(1–36) did so only to the G protein-coupled receptor. The result of this was that the cAMP response to PTH(1–34) was somewhat more prolonged than that to PTHrP(1–36).

In HEK293 cells transfected to overexpress PTHR1, PTHrP(1–36) bound to receptor more rapidly and dissociated more rapidly than PTH(1–34). Furthermore, elevated cAMP levels in target cells declined more rapidly with PTHrP(1–36), leading to the overall conclusion that differences in binding domains determined that binding and signaling are more prolonged with PTH(1–34) than with
PThrP(1–36) (71, 91, 103, 364). The results were consistent with analysis of crystal structures of PTH(1–34) and of PThrP(1–36) bound to a specific domain in the PTHR1. PTH(1–34) and PThrP(1–36) had similar amphipathic helical structure in their PTHR1-binding domains, despite the sequence differences, but the binding of PTH(1–34) was tighter and of longer duration than that of PThrP(1–36) (269).

The overall conclusion from the new experiments was that PTH(1–34) and PThrP(1–36) bind to receptor in a similar way, but dissociate by distinct mechanisms, with PThrP(1–36) dissociating fully from the receptor, but PTH(1–34) forming a persistent complex with the receptor, undergoing internalization and a persistent active state at an endosomal location (91). These findings illustrate intriguing differences in details of early cellular responses between these two very closely related peptides. It will be important to understand in due course what significance this has for later events; for example, the extent and duration of CREB phosphorylation, and of expression of genes responsive to this pathway, such as c-fos, TNFS11 (RANKL) and TNFRSF11b (osteoprotegerin), and others.

Interesting though these findings are in specifying initial cell response mechanisms to PTH(1–34) and PThrP(1–36), their relevance to physiology remains to be established, given that PThrP functions physiologically as a paracrine autocrine effector in interacting with PTHR1. Thus the molecular form of PThrP available for paracrine and autocrine action on receptors in bone (or in blood vessels) has not yet been specified. Evidence has been obtained from in vitro studies (discussed in sect. VIII) that PThrP is processed and secreted by neuroendocrine cells to forms that include an NH₂-terminal peptide of PThrP(1–36) (259, 280, 314, 391). Such evidence does not exist for cells that use the constitutive secretion pathway, and that lack the ability to package proteins into secretory granules. This includes mesenchymal cells of the osteoblast lineage and smooth muscle cells. Until established otherwise, the molecular species released by osteoblast lineage cells in bone, or smooth muscle cells in blood vessels, for autocrine/paracrine action is assumed to be the full-length PThrP, which is very susceptible to proteolysis, as discussed above. It cannot be assumed that PThrP(1–36) is generated as a form of the protein at those sites. Following an early indication that PThrP(1–141) and PTH(1–34) exhibited superimposable dose responses in treatment of osteoblast-like cells (121), there have been no detailed published analyses of interaction of full-length (or near full-length) PThrP with receptor and resulting cell activation. This question of the differences in early actions

**FIGURE 5.** Signal transduction pathways in ligand-induced activation of PTHR1. Ligand binding leads to association with Gs α subunit and adenylyl cyclase activation, or with Gq α that activates phospholipase C-β (PLC-β). MAPK can be involved through interaction of PTHR1 with the MAPK scaffolding protein β-arrestin 2. (Figure drawn by L. Conlan.)
of PTH(1–34) and PTHrP(1–36) will be taken up again in section XVIII discussing PTHrP in therapy.

X. NUCLEAR IMPORT OF PTHrP

Although the actions of PTHrP in the nucleus are not fully defined, current evidence suggests that a nuclear action plays important roles in both normal and malignant cells, and thus are likely to be pertinent to PTHrP action in cells of the osteoblast lineage. The first evidence that PTHrP might participate in intracellular signaling pathways was the nuclear and nucleolar localization of PTHrP in chondrocytes, where its translocation was dependent on a highly basic region of PTHrP(87–107) homologous to nuclear localization sequences within human retroviral regulatory proteins (349). The nuclear localization was essential for the ability of PTHrP to confer enhanced survival on the chondrocytes following serum starvation (126). In the study of how PTHrP gains access to the nucleus, it was found that when PTHrP(1–141) was transiently expressed in COS-1 cells, it could be detected in cytoplasm and nucleus as well as at the cell membrane (3). This required preservation of the integrity of the nucleolar targeting sequence, and particularly, residues 87–91 within that sequence (3). With the use of a combination of PTHrP peptides, exogenously applied PTHrP was found to target to the nucleus without participation of the PTHR1, but requiring an intact nucleolar targeting signal (NTS). These important studies introduced an entirely new concept to the biology of PTHrP. Having been discovered as a hormone in cancer, it was first realized to be an autocrine/paracrine factor in normal physiology, and then followed an extension to the latter function by virtue of a likely nuclear role that could be either autocrine or intracrine.

Nuclear localization of PTHrP was reported to increase vascular smooth muscle cell proliferation (226), whereas PTHrP acting through PTHR1 in the same cells decreased proliferation and enhanced muscle relaxation. This is a striking dichotomy of outcome that depends on the mode of delivery of PTHrP within the cell. It needs to be kept in mind in any organ in which PTHrP is acting in an autocrine/paracrine, and possibly also an intracrine manner. Perhaps even more remarkably, the increased mitogenesis in vascular smooth muscle cells resulting from PTHrP transfection was found to require not only the NLS, but also the COOH-terminal (108–139) domain of the molecule (69). In smooth muscle cells (255) and keratinocytes (186), PTHrP expression was found to be cell cycle-dependent. In keratinocytes, the highest levels of PTHrP mRNA appeared to be a response to mitogenic factors only at the G1 phase of the cell cycle during which PTHrP localized to the nucleolus (186), whereas in PTHrP-overexpressing A10 vascular smooth muscle cells, PTHrP localized in cells that were dividing or completing cell division (226).

A. Nuclear Localizing Sequence and Importin β

The NLS of PTHrP was defined by Lam and colleagues as between residues (67–94) (183, 187); however, there is debate over the precise region required. Others have proposed that PTHrP has a bipartite NLS (227) and that residues (86–107) are required for nuclear localization and include a nucleolar localization sequence (126). The region (67–94) includes the residues that are necessary for efficient binding to the nuclear transport factor importin β1 (183, 187), rather than to the conventional NLS-binding importin α subunit (FIGURE 6). In support of this sequence, importin β and the monomeric GTP-binding protein Ran were shown to mediate the nuclear import of PTHrP in vitro via the nuclear pore complex in the absence of importin α (183). Furthermore, deletion of the basic residues of the NLS resulted in complete cytoplasmic localization of PTHrP (183). Studies using a series of alanine-mutated PTHrP constructs and truncated importin β derivatives showed that PTHrP residues (83–93) are absolutely essential for importin β1 recognition, with residues (71–82) additionally required.

B. PTHrP import mechanism

FIGURE 6. Conventional and PTHrP nuclear import mechanisms. The conventional nuclear import mechanism involves the nuclear localizing sequence (NLS) of the protein forming a complex with importin α, importin β, nuclear transport factor 2 (NTF2), and RanGDP. This complex is transported through the nuclear pore complex (NPC) into the nucleus. The PTHrP import mechanism involves the NLS of PTHrP forming a complex with just importin β and RanGDP, which is then transported into the nucleus through the nuclear import complex. Inclusion of NTF2 and importin α inhibits the nuclear import of PTHrP.
for high-affinity binding (185). The region of (67–94) also includes a candidate nucleus (CcN) and nucleolus localizing motif, and in particular, a threonine residue at 85 amino acids, as a p34cdc2 kinase phosphorylation site (183). The CcN motif is similar to that described for the archetypal CcN-containing protein, SV40 T-antigen (145, 146), comprising also consensus protein kinase CK2 and confirmed cyclin-dependent kinase phosphorylation sites, phosphorylation of which results in exclusion of PTHrP from the nucleus.

This led to a focus of interest upon importin β and to studies showing that the phosphorylation status of the threonine residue at 85 amino acids is crucial in determining the nuclear localization of PTHrP (184). Most importantly though, support for this region of PTHrP as the NLS comes from the crystal structure of importin β bound to PTHrP (67–94), and identification of the specific binding sequence within importin β (53). It remains possible that there may be more than one contributing segment to nuclear translocation, including the triple arginine motif at position 19–21, that is highly conserved across species (FIGURE 1), and which could mediate nuclear trafficking of the recently identified circulating PTHrP (12–48) isoform (371).

B. Intracrine Actions of PTHrP

The idea that intracrine action is a feasible prospect came from studies showing altered translation initiated from sites downstream of the initiator methionine gave rise to forms of PTHrP that could not be secreted but were available for nuclear transport (251). Such a mechanism was proposed for a second eukaryotic protein, FGF-3, which initiates translation at a classic AUG codon and possibly an upstream CUG site. In either case, if the former is used, the protein may follow the secretory pathway; if the latter codon is used, the protein may be translocated first to the cytosol and then the nucleus (8, 226, 251). FIGURE 7 represents these aspects of PTHrP action.

Evidence was obtained for two possible mechanisms by which PTHrP could gain entry to the nucleus from outside the cell. Internalization and nuclear localization of fluorescein isothiocyanate-labeled PTHrP(1–108) required cells expressing the PTHR1 (183). In a study using PTHrP mutant constructs in an osteoblastic cell line, MC3T3-E1, it was concluded that PTHrP needs to be secreted and internalized in an autocrine/paracrine manner via the PTHR1 for NLS-mediated shuttling to the nucleus (101). In other work though (see sect. X), PTHrP gained entry to the cell independently of the PTHR1, with the transport of PTHrP(1–141) to the nucleus after binding to the cell surface requiring an intact NLS (3). The latter effect was abrogated by pretreatment with PTHrP(74–113) alone, indicating the involvement of a cell surface receptor other than the PTHR1 in endocytosis-dependent nucleolar translocation. Of course it is possible that there could be more than one means of PTHrP gaining entry to the target cell in an autocrine/paracrine manner. If PTHR1-dependent nuclear translocation of PTHrP is used, similar to mechanisms op-

![FIGURE 7. Trafficking pathways of PTHrP. After synthesis as a preprohormone, PTHrP is targeted to the ER before secretion (1), or can be subject to proteasomal degradation (7). Secreted PTHrP acts in a paracrine or autocrine manner by binding to PTHR1 (2), activating signaling, and can be internalized (6) and escape degradation to localize in the nucleus (4). PTHrP can remain intracellular to act in an intracrine manner; sometimes as a result of translation from an alternative start codon (8), and then transported to the nucleus by importin β (3, 5). See text for details.](http://physrev.physiology.org/10.220.33.2/7/27/2017)
erating with interleukin (IL)-5, growth hormone (147), and vascular endothelial growth factor (200), the PTHR1 should be found in the nucleus. There is some evidence for that, with the PTHR1 being found by immunostaining in the nucleus of some cells of all tissues examined in the rat (kidney, liver, small intestine, uterus and ovary) with PTHrP mRNA protein and mRNA localized in the same or adjacent cells (372, 373). Nuclear localization of PTHR1 was also observed in UMR 106, ROS 17.2/8, MC3T3-E1, and SaOS-2 cells (372, 373). Furthermore, the cytoplasmic tail of the PTHR1 at residues (471–488) has a putative NLS similar to that of PTHrP, although whether this is required for nuclear transport has not been shown (266).

PTHRP appears so far to be the only protein classified at least in some circumstances as a hormone that possesses a CcN motif and displays differential cellular localization (nuclear/nucleolar versus cytoplasm). Most proteins that possess a CcN motif are of large molecular weight, often greater than 45 kDa (146). Several lines of evidence converge to suggest that PTHrP is likely to exert many presumably important functions from within the cell: 1) the finding of the CcN motif; 2) the redistribution within the cell as a result of phosphorylation at Trypt; 3) the striking cell cycle dependence of PTHrP location; 4) identification of nuclear localization of PTHrP from the extracellular site; 5) the participation of PTHrP with importin β in a specific, regulated nuclear import process; and 6) the uncovering of other non-canonical intracellular trafficking sequences within PTHrP. In the many tissues in which PTHrP plays a potent local role, including bone and cartilage, these observations need to be studied further.

Although clearly implicated in delaying apoptosis and promoting proliferation in certain cell types, the precise role of PTHrP in the nucleus/nucleolus remains unclear. RNA has been shown to bind PTHrP (2) through a distinct motif in the nucleolar targeting sequence (261), and PTHrP inhibits rRNA synthesis (1). These findings together suggest a role for PTHrP, perhaps in conjunction with other proteins, as a nuclear export factor for RNA. This would be consistent with its ability to shuttle between nuclear/nucleolar and cytoplasmic compartments. Among the most important tasks will be to identify nuclear binding partners for PTHrP, including the possibility that it could bind DNA directly, as a transcription factor.

The fact that PTHrP nuclear localization is integral to its function in any cell studied so far implies that strategies to block PTHrP nuclear localization could have important effects on target cell function.

XII. PTHrP AND PLACENTAL CALCIUM TRANSPORT

Fetal plasma calcium levels had long been known to be significantly higher than maternal, indicating independent control of fetal calcium homeostasis. Use of an ultrasensitive cytochemical bioassay for PTH activity revealed that human fetal plasma contained readily assayable PTH activity despite PTH levels being suppressed to unmeasurable levels (5), and the same cytochemical assay had revealed PTH-independent activity in extracts of cancers from patients with humoral hypercalcemia (110). With the discovery of PTHrP, the obvious question to be raised was whether what was being assayed in the fetal circulation was the same as the cancer-derived PTHrP, and that PTH was suppressed in the fetus in the face of relative hypercalcemia. These findings were the stimulus for experiments in pregnant sheep, in which the fetal lambs were thyroparathyroidectomized at day 110 of pregnancy and allowed to proceed to term. Evidence was obtained that PTHrP promoted the transport of calcium across the placenta, making it available for mineralization of the growing fetal skeleton (289), with subsequent experiments using this experimental model showing that a mid-molecular region of PTHrP was responsible for this placental effect. It could be achieved by PTHrP(67–86) (47) or by PTHrP(38–94) (382). In the studies in sheep it was concluded that PTHrP was the predominant product of the fetal parathyroid glands, where PTHrP was identified by immunostaining. This appears not to be the case in all species though, since PTHrP could not be detected in fetal rat parathyroids (352), and fetal mouse parathyroids were found to be not necessary for placental calcium transport (176). There is no information on human fetal parathyroid content, but PTHrP mRNA and protein have been detected in adult human parathyroids that are normal, hyperplastic, or adenomatous (14, 64, 139).

The approach used by Kovacs and colleagues was an ingenious one, where they used genetically manipulated mice and administered $^{45}$Ca and $^{51}$Cr-EDTA to pregnant mice. Placental calcium transfer was then estimated from the ap-
pearance of the isotopes in the fetal circulation, where it was 25% lower in \textit{Pthlh} null fetuses after 5 min, and 40% lower after 40 min (175). Their conclusion that PTHrP stimulates placental calcium transport was supported also by their finding that isotope accumulation was 50% higher in \textit{Pthr1} null mice, that have high circulating levels of PTHrP, than in their controls. Since \textit{Pthr1} null mice are unable to respond to the NH$_2$-terminal PTHrP (or PTH), this is consistent with the placental effect of PTHrP being due to action of another domain of the molecule that makes use of a yet uncharacterized receptor. A comprehensive review of the role of PTHrP in placental calcium transfer is provided by Kovacs (173).

**XIII. PTHrP ACTIONS ON VASCULAR AND OTHER SMOOTH MUSCLE**

The location and actions of PTHrP as a paracrine regulator of smooth muscle function, particularly in the vasculature, are such that they provide information that is useful in considering PTHrP in bone. For that reason this is being discussed briefly. It is striking to note how the discovery of PTHrP suddenly provided an explanation for the long-standing observations of PTH actions that were difficult to fit into a coherent physiological concept. The same might be said of bone, as will be discussed in a subsequent section.

PTH was known for many years to relax smooth muscle from a variety of tissues (review in Ref. 240). After the discovery of PTHrP, substantial evidence was produced that localized PTHrP mRNA and protein to the smooth muscle layer in many tissues, together with experiments demonstrating relaxation in response to PTHrP. The common receptor PTHR1 was localized to smooth muscle cells in those same organs (57, 353). Consistent with the idea that the PTH/PTHrP effects on smooth muscle were through actions upon PTHR1 was the finding that cultured vascular smooth muscle cells respond to PTHrP(1-34) with generation of cAMP (254), and in the aorta, PTHrP-induced cAMP production was correlated with decreased tension (142).

Most important of all, though, it had been known since the 1950s that injection of parathyroid extract in animals resulted in dose-dependent increases in blood flow through a range of vascular beds, accompanied by decreases in blood pressure (48, 49). The new discoveries made it clear that this was not the normal function of PTH, but instead, reflected a local physiological role of PTHrP produced in smooth muscle beds of the stomach and intestine, uterus, urinary bladder, and arterial vessels, acting in all those tissues as a muscle relaxant through PTHR1 (219, 268, 277). The paracrine production and action of PTHrP in local...
vascular beds comes into action as required physiologically, for example, to relax vessels constricted by the action of angiotensin II (270). When PTH was administered systemically, with simultaneous activation in many sites throughout the vasculature, the response of general vasodilatation and decline in blood pressure noted by Charbon and others some decades ago should not be surprising (48, 49).

In the gastrointestinal tract, PTHrP mRNA was detected in rat stomach, duodenum, and colon (57), positive immunoreactivity for PTHrP was found in the muscle layer of the stomach, and rat fundic strips were relaxed by PTHrP(1-34) (240). Precontracted smooth muscle cells isolated from guinea pig ileum also relax when exposed to PTHrP(1-34), a response attenuated by PTHR1 antagonist (34). Mechanical stretch increases PTHrP expression in vivo in bladder (389), rat uterus (61), and aortic smooth muscle (271). In vitro, the mechanical stimulus of rocking increased PTHrP expression in primary cultures of vascular smooth muscle cells, in a manner dependent on oscillation rate (253, 271). Thus increased PTHrP production following vasoconstriction or stretch may provide a mechanism to limit or reverse these actions through the relaxant action of PTHrP on smooth muscle. The examples of the vascular dilatation effect of PTHrP and its relaxation effect in an organ such as the bladder illustrate powerfully the concept that locally produced PTHrP is responsible for actions previously ascribed to PTH through pharmacological studies of the latter.

As several growth factors and cytokines have been shown to modulate PTHrP production, and these substances are often produced in the same tissues as PTHrP, it is likely that unique paracrine loops exist in each tissue to control PTHrP expression. For example, in bone, PTHrP is produced by osteoblasts, and its secretion is enhanced by TGF-β-expression. For example, in bone, PTHrP is produced by unique paracrine loops exist in each tissue to control PTHrP production in the same tissues as PTHrP, it is likely that actions of the hormone PTH, will be discussed further in relation to PTHrP actions in bone.

### XIV. PTHrP in Fetal Development

As early as the eight-cell stage in murine embryogenesis, PTHrP was detected in developing trophocytoderm cells and in cells lining the blastocoele cavity that are likely primitive endoderm (358). In the rat, mRNA for PTHrP could be detected in polar and trophoblast cells at 7.5 days of gestation, with strong hybridization apparent by day 18.5 in ectoderm-derived tissues such as epithelial cells of the dental lamina and hair follicles, as well as in endoderm- and mesoderm-derived tissues such as lung (at day 15.5) and perichondrium (days 16.5–19) (306). In the skeletal system, PTHrP mRNA and protein were detected in the early mesenchyme and immature cartilage of the vertebral column, tail, and long bones. The intensity of PTHrP expression was noted to decrease gradually until the onset of ossification, when PTHrP-positive chondrocytes and osteoblasts were observed. The spatial and temporal distribution of PTHrP correlated closely with that of PTHr1 in the mouse and rat (162, 191), with the coupling of expression so striking that it suggests precise coregulation of the two genes during fetal development.

When PTHrP and its receptor were sought by in situ hybridization during fetal development of rats (15–20 days gestation), both PTHrP and PTHr1 mRNAs were detected not only in the skeleton, but also in many extraskeletal tissues, where PTHrP mRNA was expressed mainly in surface-lining cells, whereas receptor mRNA was expressed mainly in adjacent mesenchymal cells. The expression patterns of PTHrP and its receptor mRNAs during fetal development suggested PTHrP’s roles as a paracrine factor and its involvement in epithelial-mesenchymal (-like) interactions (191). Further functional evidence for the roles of PTHrP and its receptor in mammalian fetal development comes from studies with genetically manipulated mice, which will be discussed further. Homozygous deletion of the Ptrlh gene is neonatal lethal, with mice dying shortly after birth with multiple skeletal defects (see sect. XV) (157). The Pthr1 deletion was even more lethal, with death occurring in utero (188).

Analogous profiles of PTHrP expression were generated in the human fetus (82, 242, 243). PTHrP mRNA was expressed in both the syncytiotrophoblastic and cytotrophoblastic layers in first-trimester chorionic villi, and both peptide and mRNA in the ectodermal cells of the avascular amnion (82). Ectodermal tissues strongly positive for PTHrP at 7–8 wk include the epidermis, otic placode, and tooth bud; endodermal tissues include lung, liver, pancreas, stomach, intestine, and hindgut; while those of mesodermal origin include perichondrium and developing kidney (82). Later in development (18–20 wk gestation), PTHrP expres-
sion is evident in cardiac and skeletal muscle, vascular smooth muscle, neural tissues, and areas of both endochondral and intramembranous bone in the limb buds and calvaria, respectively (82, 242, 243).

**XV. PTHrP IN CARTILAGE AND ENDOCHONDRAL BONE FORMATION**

With its identification in tumors associated with the syndrome of HHM, PTHrP was recognized as a potent bone-resorbing agent in vivo and in vitro. The pathogenesis of hypercalcemia in HHM in large part reflects the bone-resorbing properties of PTHrP (222, 321), and there is ample evidence that cancer-derived PTHrP contributes to bone metastasis establishment and growth by promoting the formation of osteoclasts in the host bone. This is the subject of a number of reviews (118, 218, 320) and will not be considered here. The findings that PTHrP exerted a number of additional actions on bone and bone-derived cells suggested a physiological role of PTHrP in bone, but the most prominent early findings came with respect to its role in cartilage and endochondral bone formation.

**A. Endochondral Bone Formation**

The first evidence of a physiological role for PTHrP in chondrocyte biology was provided when mice were generated null for the Pthlh gene using homologous recombination (10, 156, 157, 192). Neonatal mice homozygous for Pthlh gene ablation exhibited severe skeletal abnormalities at birth and died within 24 h, most likely from respiratory failure as a consequence of widespread abnormalities of endochondral (intracartilaginous) bone development. Pthlh homozygous mutant mice were characterized by a domed skull, short snout and mandible, protruding tongue, and disproportionately short limbs, whereas their nonskeletal organs and tissues appeared normal. These abnormalities were evident throughout the endochondral skeleton (axial as well as appendicular), whereas in contrast, no abnormality was noted at that stage in skeletal structures that develop predominantly by intramembranous ossification.

The process of endochondral skeletal development requires strict coordination of temporally and spatially specific differentiation of chondrocytes to replace the cartilage template through ossification (367). Most of the long bones of the body develop by endochondral bone formation. First, a miniature hyaline cartilage model is formed by condensation of mesenchymal cells, and second, the cartilage model continues to grow and serves as a structural scaffold which is then resorbed and replaced by bone (FIGURE 9; Refs. 177, 207).

The epiphyseal growth plate separates the diaphysis from the epiphysis and is responsible for the longitudinal growth of bones. During development its orderly progression is essential for normal skeletal growth and structure. Histologically the layer is divided into five zones: 1) resting (hyaline) cartilage. These cells contain the precursors for the next zone of actively dividing cells. 2) Proliferating zone chondrocytes undergo successive mitotic divisions to form rows of cells that parallel the direction of growth, synthesize collagen type II and aggrecan, and are ultimately responsible for longitudinal bone growth. 3) Zones of maturation and hypertrophy are where cell division ceases and chondrocytes mature and become enlarged and vacuolated, synthesizing matrix rich in collagen type X. They begin producing vascular endothelial growth factor and other factors, that attract blood vessels and osteoclasts to digest calcified cartilage matrix in the next zone. They also direct adjacent perichondrial cells to become osteoblasts and form bone collar. 4) In the zone of calcification and cartilage degeneration, hypertrophied chondrocytes die and cartilage matrix becomes calcified and invaded by osteoclasts and capillaries from marrow cavity. 5) In the zone of ossification/primary spongiosa, osteoprogenitors invade and differentiate to osteoblasts that gather on spicules of calcified cartilage matrix and begin bone formation. This is followed by resorption of calcified cartilage/bone complex and re-

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**FIGURE 9.** Growth plate interactions of PTHrP and Ihh. PTHrP is produced by chondrocytes at the end of long bones. It stimulates proliferation of adjacent chondrocytes and delays them from further differentiating into prehypertrophic and then hypertrophic chondrocytes. Synthesis of Ihh by hypertrophic chondrocytes begins when the signal of PTHrP is no longer able to reach those cells. Ihh increases proliferation and accelerates differentiation into prehypertrophic chondrocytes, promotes the formation of osteoblasts from adjacent perichondrial cell, and completes a feedback control system by promoting PTHrP production at the articular end (see text for further details). [Modified from Maes and Kronenberg (207), with permission from Elsevier.]
modelling of the bone to form the mature trabecular network.

A number of factors contribute to the perinatal death of \textit{Pthlh} null mice, including abnormal ribcage development that does not allow adequate respiration, respiratory distress accentuated by reduced surfactant production, and impaired type II alveolar cell development. They have a severe chondrodystrophic phenotype that also includes short-limbed dwarfism, characterized by premature chondrocyte differentiation and accelerated bone formation (10, 157). Microscopic examination of the growth plates of bones in the \textit{Pthlh} (−/−) mutant mice revealed a marked reduction in the height of the resting and proliferating chondrocyte zones as a result of the decreased number of cell divisions, disorganization of the cartilage columns in the hypertrophic zone, and reduced deposition of matrix molecules such as type II collagen (156). PTHrP thus proved to be necessary for normal proliferation of chondrocytes. The untimely maturation of the skeleton was presumably a consequence of the reduced proliferation and accelerated differentiation/premature hypertrophy and apoptosis of chondrocytes within the growth plate.

\section*{B. Indian Hedgehog and PTHrP Relationship}

The molecular pathway of PTHrP action during endochondral bone formation was discovered to involve Indian hedgehog (Ihh) (365). Ihh is one of the mammalian homologs of the \textit{Drosophila} segment known as hedgehog, that includes also Sonic and Desert (232). This gene in \textit{Drosophila} regulates segment polarity, and in many organisms hedgehog genes regulate the correct embryonic patterning during development (110, 143). Hedgehog signaling results in the activation of Smoothened (Smo), a 7-transmembrane protein homologous to G protein-coupled receptors. Smo is kept inactive by binding to the membrane protein patched (ptc). Hedgehog binding to ptc releases Smo, which triggers gene activation through participation of the Gli transcription factors (232).

\textit{Ihh} was known to be expressed in prehypertrophic chondrocytes, and overexpression of its gene in developing chick limbs blocked chondrocyte differentiation in a manner similar to that seen with overexpression of PTHrP in chondrocytes. It was noted (188, 365) that, just as PTHrP and the PTHR1 localize to the growth plate region of long bones, so too does Ihh. The studies by Lanske et al. (188) and Vortkamp et al. (365) showed that PTHrP and the PTHR1 are downstream effectors of the Ihh pathway that regulate the correct spatial and temporal progression of chondrocyte differentiation that determines the rate and extent of long bone formation (FIGURE 9).

In the developing skeleton Ihh was noted to be coexpressed with the PTHR1 in prehypertrophic chondrocytes (365). PTHrP is undetectable in fetal bones of mice deficient in Ihh, and their chondrocytes show accelerated differentiation (319). This phenotype is rescued by transgenically expressing constitutively active PTHR1 using the Collagen IIα(1) promoter (159). Ihh is required for the synthesis of PTHrP and, once secreted, initiates the signaling events that result in secretion of PTHrP in the peri-articular perichondrium.

The chondrocytes of the articular cartilage secrete PTHrP that acts upon PTHR1 in proliferating chondrocytes that have relatively low numbers of receptors. PTHrP maintains chondrocyte proliferation, ensuring they divide sufficiently before differentiating to the hypertrophic state. The more distant prehypertrophic chondrocytes are also PTHrP targets, with more abundant PTHR1 receptors, where PTHrP action is thought to delay further differentiation. When chondrocytes are sufficiently far from the PTHrP source, their differentiation progresses and they produce Ihh, which then cycles back to promote formation of more PTHrP. When the prehypertrophic cells differentiate into hypertrophic chondrocytes, Ihh is no longer expressed, therefore shutting off this negative feedback system; thus new cells are allowed to proceed down the differentiation pathway (365). These processes are followed by vascular invasion that brings in cells that differentiate to osteoblasts, forming the primary spongiosa. Importantly also, Ihh acts independently of PTHrP to promote conversion of proliferating to columnar chondrocytes (172) and the formation of osteoblasts from adjacent perichondrial cells (201, 202).

The negative paracrine feedback loop in which Ihh regulates PTHrP to modulate Ihh levels provides a mechanism whereby the rate and number of cells committed to hypertrophic differentiation is modulated in a spatially specific manner, thus allowing regulated elongation of endochondral bone (160, 178, 207) (FIGURE 9).

\section*{C. PTHR1 and the Cartilage Phenotype}

Genetic ablation of \textit{Pthr1} resulted in mice with skeletal abnormalities similar to, but far more severe than, those of \textit{Pthlh} null mice, with lethality at day E9.5 of gestation (188). The cartilage phenotypes in \textit{Pthlh} and \textit{Pthr1} null mice are so similar that the PTHR1 must play at least a large role in mediating PTHrP action. That view is supported by studies in genetically manipulated mice establishing that PTHrP uses Gs activation of adenylyl cyclase/cAMP/protein kinase A pathway to keep chondrocytes proliferating (21, 54). This pathway also provides phosphorylation of the chondrocyte transcription factor SOX9 (137). Phosphorylated SOX9 slows the differentiation of chondrocytes and therefore could contribute to the action of PTHrP in restraining chondrocyte differentiation. An explanation for the difference in severity of phenotype might be related to the fact that some PTHrP intracrine actions might not re-
quire the PTHR1 (126, 183, 186, 226). The fact that PTHrP and PTHR1 both localize to the growth plate in long bones makes it likely that a major signaling mechanism is through that receptor, and thus requires the NH2-terminal domain of PTHrP, but there are enough unanswered questions remaining about molecular mechanisms that the possibility of other pathways of PTHrP action should be kept in mind. This applies particularly to its likely nuclear actions; nuclear localization of PTHrP was essential for PTHrP to confer enhanced survival on the chondrocytes following serum starvation (126). Indeed, that demonstration of nuclear and nucleolar localization of PTHrP in chondrocytes was the first evidence that intracellular PTHrP might contribute to signaling pathways.

Thus, whether the deficiency be of ligand or receptor, chondrocytes become hypertrophic closer to the ends of long bones. The appearance of hypertrophic chondrocytes could be delayed by overexpression of PTHrP in chondrocytes (375) or by expression of constitutively active PTHR1 (299). The latter mice showed a severe delay in chondrocyte differentiation and decreased mineralization in the endochondral skeleton at birth, thus a mirror image of mice lacking the Ptibh or Pthr1 genes, which were characterized by accelerated chondrocyte differentiation (157, 188).

D. Rescue of Pthlh Null Mice

The above findings provided useful direction to experiments in which the primary skeletal defects were corrected in Ptibh knockout mice by crossing Ptibh(+/−) heterozygous mice with a type II collagen promoter-targeted transgenic line, thus generating a Ptibh null mouse that expressed moderate amounts of PTHrP only in chondrocytes (267, 384). This resulted in rescue of the Ptibh null mice, which survived for at least 4 mo. The mice displayed a number of abnormalities, including dwarfing, craniofacial chondrodystrophy, failed tooth eruption, and epidermal differentiation, as well as a defect in early branching morphogenesis of the mammary epithelium and abnormal epidermal differentiation (95, 267, 384). Such mice died of unknown causes. Collectively, quite apart from what their features taught about the skeleton, they provided an approach to dissecting the postnatal actions of PTHrP in organs other than the skeleton (385).

E. Related Human Chondrodysplastic Syndromes

Some rare human genetic abnormalities were found to recapitulate these abnormalities recognized in Pthr1 and Ptibh null mice that were characterized at about the same time. Blomstrand’s chondrodysplasia (OMIM no. 215045), first described in 1985, is an autosomal recessive syndrome characterized by shortened long bones and increased bone density due to marked reduction of proliferating and resting chondrocyte numbers at the growth plates of long bones (32). Such advanced skeletal maturation is invariably fatal, with death occurring shortly after birth. This autosomal recessive form of dwarfism was noted to resemble the skeletal abnormalities of Ptibh and Pthr1 null mice (158). Examination of the PTHR1 gene in patients with Blomstrand’s chondrodysplasia revealed a single point mutation in the codon encoding the highly conserved proline 132, located in the NH2-terminal tail of PTHR1. This mutation encoded a leucine and led to loss of PTHrP binding and, consequently, lack of activation in target cells (155, 395). Consistent with observations from null mice that PTHrP and PTHR1 have roles in other tissues, infants with Blomstrand’s chondrodysplasia have distorted tooth development, lack mammary buds, and suffer cardiovascular abnormalities (277, 383).

The reverse mechanism was found to operate in Jansen-type metaphyseal chondrodysplasia (OMIM no. 156400), an autosomal dominant condition arising from constitutive activation of the PTH1R (298–300) and also in transgenic mice in which expression of a constitutively active PTH1R was targeted to the growth plate (300). The sequences of the chondrocyte maturation program in the genetically manipulated mice and in Jansen-type chondrodysplasia subjects are similar. In the mouse study, targeted expression of constitutively active PTH1R corrected at birth the growth plate abnormalities of Ptibh null mice and allowed prolonged survival. These “rescued” animals lacked tooth eruption and showed premature epiphyseal closure indicating the requirement of PTHrP for both processes. Therefore, overexpression of PTHrP or constitutive activation of PTH1R in the growth plate ultimately results in a similar pattern of abnormalities in endochondral bone formation. The human and mouse genetic studies were reassuring in showing that these local functions, investigated extensively in the mouse, are reflected in human physiology and disease.

A further cartilage connection with a human genetic disorder involving PTHrP is that in brachdactyly type E (BDE) (OMIM no. 613382), in which a microdeletion of 900 kb was discovered in the PTHLH gene, and four other loss-of-function mutations in others with this abnormality (171). BDE is an autosomal dominant congenital skeletal malformation in which there is shortening of the bones of hands and feet, sometimes in the setting of short stature. A family was reported with a balanced translocation on chromosome 12 that harbored dysregulated expression of PTHLH (206). Subsequently, the same group found in a further family a conserved long noncoding RNA (lncRNA) that interacted with the PTHLH and SOX9 loci (205). This was proposed to result in loss of the coordination between PTHLH and SOX9 needed for normal chondrogenic transcriptional control. The brachdactyly phenotype is expressed also in acrodysostosis (ADO), where a more severe
form of brachdactyly is associated with facial dysostosis and nasal hypoplasia, as well as resistance to signaling through PTHR1 (reviewed in Ref. 309). In ADO the defects occur in genes expressing either the type 1 regulatory subunit of PKA (PRKAR1A) (309) or phosphodiesterase 4 (PDE4D) (199), both of which are involved directly in signaling from PTHrP/PTH. These defects result in impaired activation of PKA, in contrast to the PRKAR1A mutations resulting in activated PKA, that have been associated with osteosarcoma (241) (discussed in sect. XVIII). In view of the finding that histone deacetylase 4 (HDAC4) is involved in PTHR1 signaling (176a), it is noted that an HDAC4 mutation has been associated with a brachydactyly with a range of severe mental defects (378). It should be mentioned that at the present time no human genetic mutations in PTHHLH have been recognized that could involve nuclear actions of the protein.

A connection between PTHR1 and height has been noted (239). In identifying a polymorphism in a tetranucleotide repeat region of the PTHR1 gene P3 promoter that functions as a repressor sequence, a genotype was identified with significantly greater adult height than others, and significantly lower levels of bone resorption markers (239). This was consistent with the idea that PTHR1 is the critical mediator of PTHrP effect in the growth plate and that the PTHR1 gene is central to these three disorders featuring reduced bone length: Blomstrand’s chondrodysplasia, Jansen’s chondrodysplasia, and BDE.

**XVI. PTHrP IN BONE**

**A. PTHrP in Osteoblast Lineage Cells**

This important developmental role for paracrine/autocrine PTHrP in endochondral bone formation (see above) was demonstrated relatively soon after the discovery of the cancer hormone (157). It took some further years before conclusions could be reached about involvement in bone formation postnataally.

PTHrP mRNA and protein were shown to be produced in osteoblast-rich cultures of rat, mouse, and human origin (117, 243, 328, 351). These findings were supported by in vivo evidence showing production by both early osteoblast precursors (191) and actively synthesizing osteoblasts (328) (FIGURE 10). In long bones undergoing intramembranous bone formation in an induced defect model in the rabbit, both PTHrP mRNA and protein were expressed throughout the osteoblast lineage (163).

In a Pthlh-lacZ reporter mouse PTHrP promoter activity was identified in the fibrous layer of the periosteum and in ligament and tendon insertions (50). This led to generation of mice in which Pthlh was deleted in periosteum and tendon insertion sites by targeting with Cre-recombinase directed by the scleraxis promoter gene (Scx) (370). This resulted in failure of modeling in long bones associated with decreased production of RANKL and hence osteoclast formation, with long bone deformities that were reproduced in Scx-Cre-Tnfsf11 mice (i.e., deficient in RANKL). There is such a role for PTHrP in long bone growth, distinct from its effects through endochondral bone formation. That work was extended to show in Scx-Cre-Pthlh mice that periosteum-derived PTHrP induces osteoblast activity and bone formation and regulates fracture healing in cortical bone (369).

**B. Less Bone in PTHrP-Deficient Mice**

Instructive as the Pthlh null mice were in leading to discovery of the central role of PTHrP in endochondral bone formation in development, these mice ultimately were the instrument that revealed the importance of PTHrP postnatally in bone remodeling, where it provides a crucial stimulus to osteoblast differentiation and bone formation.

First, in the Pthlh null mice, osteoblastic progenitor cells (and chondrocytes) were observed to contain inappropriate accumulations of glycogen, which indicated a defect, meta-
Both physiologic or otherwise, in cells of the osteogenic lineage arising as a consequence of PTHrP deficiency (10). Second, although mice lacking Pthlh die immediately after birth (10), haploinsufficient Pthlh mice were born phenotypically normal, but had low bone mass by 3 mo of age (9). This was characterized by a marked decrease in trabecular thickness and connectivity, and an abnormally high number of adipocytes in the bone marrow compared with wild-type littermates (9). The low bone mass was due to decreased bone formation as a result of lesser recruitment of bone marrow precursors coupled with increased osteoblast apoptosis compared with wild-type. Osteoclast formation was also reduced. There may be a net imbalance at each bone multicellular unit (BMU), but likely also a decreased number of active BMUs, resulting in overall decrease in bone mass.

Such was the accumulated evidence for an important function of PTHrP in bone that mice were generated with osteoblast-specific deletion of PTHrP, giving rise to a skeletal phenotype (236) (FIGURE 11) that faithfully reproduced that recognized a decade earlier in the Pthlh+/− mice after ~3 mo of age. Thus the lack of PTHrP in osteoblasts was reflected in reduced bone formation, but also fewer osteoclasts, giving rise to a state in which both formation and resorption were reduced, but in which there was a net loss of bone. The mice with PTHrP-deficient osteoblasts were normocalcemic, emphasizing the fact that PTHrP action in bone is not required for the maintenance of calcium homeostasis.

The finding of low bone mass in Pthlh haploinsufficient mice that is reproduced in osteoblast-specific Pthlh-null mice constituted a striking and influential discovery that yielded physiological insights into bone remodeling and into PTHrP function that relate to PTH action. If PTHrP plays such a part at remodeling sites, prolonged exposure could theoretically result in excessive osteoclast generation and hypercalcemia (215), such as occurs with prolongation of PTH1R expression also raised the intriguing possibility that some PTHrP local effects might result from actions other than those using the common receptor PTH1R (396).

The question of the relative roles of PTH and PTHrP in the regulation of bone remodeling is an intriguing one, asking to what extent does the hormone PTH contribute to bone remodeling, either directly or indirectly? Some light has been shed on this question, with implications for our understanding of bone modeling and remodeling. When bone marrow ablation, in which formation precedes resorption during recovery (331), was performed in Pth-null mice, osteogenesis and osteoclast formation were delayed and impaired, and Pthlh expression in osteoblasts was increased (396). When a similar experiment was carried out in Pth-null mice crossed with Pthlh haploinsufficient mice, response to bone marrow ablation generated fewer osteoblasts, less bone formation, and fewer osteoclasts than Pth null mice (396). These data suggest a role for PTH in recruiting osteoprogenitor cells, with locally generated PTHrP being necessary both for their further differentiation and for the generation of osteoclasts, and PTH required for promotion of osteoclastic resorption to make calcium available for mineralization (386, 396). Observations made in these experiments regarding the timing of the changes in PTH1R expression also raised the intriguing possibility that some PTHrP local effects might result from actions other than those using the common receptor PTH1R (396).

Other mouse genetic studies were also helpful in discerning differences in PTH and PTHrP physiology. Global knockout of Pth in the mouse resulted in hypocalcemia and hyperphosphatemia, and a bone phenotype of increased tra-

![FIGURE 11. PTHrP haploinsufficient mice (top panel) have decreased bone (BV/TV), increased trabecular separation (Tb.Sp), decreased mineral apposition rate (MAR), and increased osteoblast apoptosis. In mice null for PTHrP in osteoblasts (bottom panel), cortical and trabecular MAR are decreased, and osteoblast apoptosis is increased. [From Miao et al. (236).]
becular and cortical bone volume, the opposite of that with \textit{Pthlh} null mice of the same age (237). The genetic experiments taken together indicate that PTH does not function physiologically to promote bone formation; rather, it acts as a regulator of extracellular fluid calcium homeostasis postnatally and in the fetus, and in the latter case is essential in providing calcium for mineralization of bone. The fact that \textit{Pth} null mice remained hypocalcemic and hyperphosphatemic when crossed with \textit{Pthlh}/H11001/H11002 mice illustrates the lack of a calcium regulatory role for PTHrP. Its physiological role after development is that of a local regulator of bone remodeling (formation and resorption) that does not contribute significantly to the maintenance of extracellular fluid calcium. A hypothesis embracing the relative roles of PTH and PTHrP is that the hormone PTH governs modeling effects through PTHR1 in the fetus and newborn, and the paracrine/autocrine PTHrP in its action upon bone becomes important when remodeling supervenes some time after birth.

D. Lessons From Mice Lacking Mid- and COOH-Terminal Domains

Genetic experiments in mice support the likelihood that biological activities exerted by domains within PTHrP that do not require interaction with PTHR1 may be distinct. Knock-in of PTHrP(1–84) lacking both the NLS and COOH-terminal region while retaining the bioactive NH2-terminal region, resulted in multiple abnormalities and early lethality in mice at 2–3 wk of age (238). Homozygous mice exhibited skeletal growth retardation and decreased bone mass associated with reduced proliferation and increased apoptosis of osteoblasts. Together with fewer osteoblasts there was less trabecular and cortical bone and fewer osteoclasts. Impaired growth plate chondrocyte proliferation in these mice resulted in narrower proliferative zones, with markedly shorter long bones. This differed quite clearly from the severe chondrodystrophy of \textit{Pthlh} null mice, which resulted from premature differentiation of chondrocytes (10). The \textit{Pthlh}(1–84) knock-in mice also exhibited early senescence with altered expression patterns and subcellular distribution of proliferative- and senescence-related genes in multiple tissues. Many organs failed. The brain was assessed specifically in these mice, where decreased neural cell proliferation and increased apoptosis, with abnormal brain shape and structures, identified the need for PTHrP in maintenance of normal synaptic transmission and plasticity (116).

In a second model, knock-in of PTHrP(1–66), which excludes a significant portion of the mid-region, resulted in a similar, but even more severe phenotype, with similar abnormal skeletal development including chondrodysplasia, impaired hemopoiesis and mammary development, dysregulated energy metabolism, and death of most mice by 5 days of age (344). These mouse studies convincingly demonstrate that many actions of PTHrP are not mediated solely by the NH2-terminal region, but that the absence of the mid-region, NLS or COOH-terminal region of PTHrP, result in greatly impaired commitment and survival of both osteogenic and hemopoietic precursors, as well as defects in other organ systems that remain to be fully defined.
Circulating PTHrP could not be detected in normal human subjects using the most sensitive and validated assays available (41, 115), reinforcing the view that postnatally in mammals, PTHrP functioned as a locally generated regulator in the many tissues in which it is produced. In lactating mothers though, PTHrP was readily detected in the circulation, with readily measurable plasma PTHrP being detected in 63% of lactating mothers but in none of the non-lactating mother controls (114). Such measurable PTHrP levels in lactation were shown subsequently in the mouse (174, 362) and in other human subjects (318).

There was other existing and developing evidence suggesting involvement of PTHrP. Calcium and vitamin D requirements in hypoparathyroid patients had been noted to decline during lactation (46, 292). In lactating rats, suckling-associated increases in urinary cAMP and phosphate were reported (388), and a venous-arterial gradient in plasma PTHrP was found across the mammary gland in the goat (282). All of these findings were consistent with the view that PTHrP produced in mammary tissue is released systemically during suckling and is available to produce an endocrine effect. Hypercalcemia associated with benign breast hypertrophy, and resolving following bilateral mastectomy, was documented (223, 359). PTHrP was demonstrated in breast tissue in one such case (168).

Although the question was inevitably posed of the biological purpose of such high concentrations of PTHrP in milk, the answer remains unknown. An early study showed that treatment of neonatal mice with neutralizing anti-PTHrP (179) did not affect serum calcium or whole body calcium content, and more recently no correlation was found between milk PTHrP levels and infant PTHrP, calcium, or phosphate levels (345). On the other hand, when milk from mothers with breast-targeted ablation of PTHLH was provided to sucking mice, low PTHrP in milk was associated with high skeletal ash calcium content (210), indicating that PTHrP might actually reduce gut absorption of calcium. One idea that has been considered was that PTHrP might contribute to neonatal intestinal epithelium maturation through the known PTHR1 in those cells, but no evidence has been obtained for this.

In seeking the source of the large amount of calcium required for milk production, diet can contribute, and renal losses of calcium are reduced in lactation, but the major source is considered to be the skeleton, with bone resorption appreciably exceeding bone formation throughout lactation. Lactating women lose 5–19% of bone mineral over 6 mo, whereas rats and mice lose 30% of their skeletal mass over 3 wk (362). In studies in genetically manipulated mice, it was shown that mobilization of skeletal calcium by resorption during lactation was not dependent on either PTH or 1,25(OH)2 vitamin D3 (174). Circulating PTHrP levels in lactating women were found to correlate with bone mineral...
density (318). Furthermore, bone loss correlated with the duration of amenorrhea in lactating women, and bone resorption rates correlated inversely with circulating estradiol levels in lactating mice (312).

The conclusion from mouse and human studies is that in lactation, PTHrP secreted by the breast into the systemic circulation acts together with estrogen deficiency to promote bone resorption and cause bone loss during lactation (385). There has been much recent interest in the mechanisms of lactational bone loss, predominantly through studies in mice. Although it might have been expected that osteoclastic resorption would explain the bone loss in lactation, some evidence has been obtained that bone loss in lacunae surrounding osteocytes might also contribute. It had been noted that inactivation of osteoclasts with bisphosphonate treatment blocked the loss of bone mineral density in lactating mice by only 60%, suggesting additional mechanisms (361). On the other hand, in more recent work the same group found that treatment of lactating mice with OPG-Fc completely inhibited bone resorption and the rapid lactational bone loss (12). Thus, whatever the contribution of osteocytes, osteoclastic resorption surely has an important part to play, likely under the influence of PTHrP.

The possible contribution of osteocytes to lactational bone loss resurrects the concept of “osteocytic osteolysis,” according to which osteocytes influence their perilacunar space by dissolution of mineral (339, 340). Using backscatter scanning electron microscopy to measure lacunar area, Qing et al. (278) found that in lactating mouse bone, osteocytic lacunar area was significantly enlarged and that these returned to levels of virgin mice within a week of forced weaning. Significant increases were found in osteocyte expression of a number of genes associated in osteoclasts with bone resorption, including tartrate-resistant acid phosphatase (TRAP), cathepsin K, and matrix metalloproteinase 13. Importantly, in these experiments, mice lacking PTHR1 in osteocytes (275) underwent significantly less perilacunar bone loss, which would imply loss of a PTHrP response. Furthermore, infusion of PTHrP(1–36) (278) for several days significantly increased numbers of TRAP-positive osteocytes in cortical and trabecular bone. This latter finding is reminiscent of earlier data showing that PTH infusion resulted in changes in cortical bone consistent with osteocytic osteolysis (339). The combined data suggest that perilacunar loss of bone, or at least of bone mineral, could contribute to the lacunar enlargement (and bone loss) of lactation. It has been reported in abstract form that PTHrP is produced by osteocytes, and mice with osteocyte specific knockout of PTHrP results have significantly less bone (11). Together with the data obtained in PTHR1-deficient mice, this suggests that osteocyte-derived PTHrP could mediate any contribution made by osteocytes to lactational bone loss.

There is much current interest in the osteocyte, the most abundant cell in bone by far (33, 39), and one that communicates with its fellows and with surface osteoblasts and osteoclasts by a complex canalicular system that provides amply for signals to be transmitted. It will be something of a surprise if the osteocyte is eventually proven to be able to remove both mineral and matrix, just as an osteoclast does, but that is the direction of these current experiments. An alternative possibility is that what is being removed is mineral only. Either way, we are being taken back to the idea of osteocytes playing an important role in calcium homeostasis (24, 340), and PTHrP might be at the center of this.

XVIII. PTHrP IN OSTEOSARCOMA

When PTHrP soon after its discovery was predicted to be a local paracrine agent in so many tissues in the body, including bone, its possible involvement in primary bone cancer was thought of, but little was done to investigate that. Some decades earlier, when osteosarcoma (OS) was induced in rats by radiophosphorus injection, tumors developed whose cells were rich in alkaline phosphatase, made a bone-like ground substance and mineralized it, and were markedly responsive to PTH with dose-dependent increases in cAMP accumulation (217). These tumors were maintained through transplantation in the rat, and developed into the UMR series of clonal cell lines (264) that have been used for many experimental purposes since that time. An early interest in the induced OS was to establish the significance of its PTH regulation, but in that study, removing the source of PTH by parathyroidectomy had no influence on any aspect of OS (141). That work was carried out many years before the existence of PTHrP was appreciated, but it has become clear now that the ligand for the PTHr1 in OS is PTHrP rather than circulating PTH.

Studies in OS cell lines from several species found PTH responsiveness to be a common, if not universal, feature of OS (108). Consistent with a role for PTHr1 in OS, higher expression of PTHr1 mRNA was detected in a human OS series of metastatic or relapsed samples than those in primary sites, and overexpression of PTHr1 in an OS cell line increased proliferation and invasion (392). In the same work, overexpression of PTHr1 in a human OS cell line conferred increased proliferation, motility, and invasion without adding PTHrP or PTH, implying an autocrine PTHrP role. In vitro evidence was obtained for autocrine production of PTHrP in human OS (288) and in UMR 106 rat OS cells (265).

OS is the most common primary tumor of bone, occurring mainly in the second decade of life. It arises from malignant osteoblastic mesenchymal cells that produce an osteoid matrix that mineralizes in the case of the osteoblastic subtype of OS (60%) (18). The less mineralized fibroblastic and the chondroblastic subtypes comprise ~30%, with rarer sub-
types the remainder (376). There is evidence in support of either a mesenchymal stem cell origin of OS, or origin from mutations occurring within a population of cells that are committing or committed osteoblast progenitors. Origin from a mesenchymal stem cell can result not only in OS but also in other sarcoma types as well, including poorly differentiated soft tissue sarcoma (387). Based on evidence from several genetically induced OS models that mimic the human disease (27, 197, 246, 294, 366) (FIGURE 13), the cell of origin for OS has been proposed to come from within the population of cells undergoing osteoblast differentiation (247). This view is strongly supported by the finding that when p53 is knocked out in late osteoblasts using osteocalcin-Cre, as many as 44% of mice developed OS (279), even though only <2% of the null cells were actively synthesizing DNA, confirming the late, nonproliferating state of these cells.

These models of OS have made it possible now to study aspects of the hormonal control of osteoblast malignancy. Osteoblast-restricted Cre:lox deletion of Trp53 (p53) and Rb1 (Rb) in the mouse results in a phenotype resembling fibroblastic osteosarcoma in humans (366). This approach was extended by carrying out shRNA-based knockdown of p53, resulting in generation of the osteoblastic subtype of OS, the most common clinical subtype (246). In each of these subtypes of OS, all tumors produce PTHrP that acts through the PTHR1 in the same cells to activate adenyl cyclase, protein kinase A, and the transcription factor CREB (131). The abundance of PTHrP, of receptor-mediated response, and CREB activation are all greater in the osteoblastic type of OS compared with the fibroblastic (131) (FIGURE 13), a reflection of the greater differentiation state of the osteoblastic than the fibroblastic tumor. Together these OS models recapitulate all aspects of the disease process, from initiation and establishment to invasion and dissemination to distant sites with a ≥70% rate of spontaneous metastasis possible in both models, thus reproducing three-quarters of the clinically managed disease with the osteoblastic and fibroblastic subtype models (both familial and sporadic).

A striking feature of these tumors was that most of the PTHrP is identified intracellularly, with very little secretion,
leaving open the question whether there is an intracrine action of PTHrP in these tumors also. Knockdown of PTHR1 in either OS resulted in impaired cell growth and invasion in vitro, increased apoptosis, and profound growth inhibition of transplanted tumors in vivo (131). Knockdown of PTHR1 had a very similar effect (131). Knockdown of Creb had an even greater inhibitory effect on OS apoptosis and growth, more so in the osteoblastic tumors, so much so that it was not possible to develop stable shCreb cell lines. Normal murine osteoblasts, on the other hand, survived depletion of either PTHrP or CREB. Thus an autocrine circuit was identified, of the PTHrP-PTHRI-PKA-CREB axis as a critical proliferation and survival pathway in the maintenance of OS. This extends the significance of PTHrP in cancer to this primary tumor of bone. It was discovered by virtue of its production by cancer cells and circulation as a hormone that acted generally upon the skeleton to promote excessive bone resorption and was found also to act locally to promote resorption and facilitate bone metastasis formation and expansion (320). In view of the production and roles of PTHrP in the osteoblast lineage, perhaps it is not surprising that it is involved in primary bone tumors also.

In breast cancer, the possible involvement of PTHrP in primary cancer, in addition to its role in skeletal complications, has also been raised. In a long-term, prospective study of unselected patients, tumors positive for PTHrP at surgery were independently predictive of improved patient survival, with reduced metastases at all sites, including bone (127, 128). Such a mechanism is distinct from the bone resorbing action later in disease. On the other hand, of two independent studies of genetically induced breast cancer in mice, one concluded that loss of PTHrP expression resulted in poorer outcomes in breast cancer (94), and the other concluded the opposite, that PTHrP promotes the initiation and progression of primary tumors (194). Since PTHrP has an important role in early mammary gland development (383), it might not be surprising if it were to play a part in early stages of breast cancer development. In the case of the breast involvement, no link has been sought with the osteoblastic lineages, perhaps it is not surprising that it is involved in primary bone tumors also.

A role of cAMP-dependent PKA-CREB activation in OS has been proposed. In mice in which osteocalcin promoter-driven SV40T/t antigen was used, OS were identified that were low in the α regulatory subunit of PKA type I (PRKAR1A) and were highly invasive, leading to the conclusion that Prkar1a was an OS tumor suppressor (241). The functional consequence of reduced PRKAR1A is enhanced PKA activity. Consistent with elevated PKA activity, an OS was also identified with amplification of Prkaca, that codes for the catalytic component of PKA, and Prkaca RNA was overexpressed in that tumor. The findings point to activation of the PKA/CREB cascade as a contributor to OS invasion and growth. It is relevant to note that mammary-specific deletion of Prkar1a in mice using MMTV-Cre deletion resulted in multiple breast cancers, identifying the PKA-CREB pathway in this cancer also (26). No link with breast-derived PTHrP was explored.

These studies point to osteosarcoma being associated with prolonged stimulation of the PKA-CREB axis. There is no evidence to allow any link between these observations and the known outcome of toxicology studies which resulted in an extremely high incidence of osteosarcoma in rats treated from a few weeks old for 2 years with PTH (296, 337, 355). Of course, no comparable treatment regime has been used in human studies or is likely to be. Although there is a Food and Drug Administration (FDA) therapeutic warning concerning the matter, there is no clinical evidence linking osteosarcoma to prolonged increase in PTH, for example, in chronic renal failure or primary hyperparathyroidism (336).

XIX. PTHrP IN TREATMENT OF OSTEOPOROSIS?

By the 1980s, greatly renewed interest had developed in the findings of the 1930s, that intermittent (daily) injections of PTH in animals resulted in more bone (22, 305). These early efforts (283, 284) were rewarded ultimately by investment in a major clinical trial that established the anti-fracture efficacy of PTH(1–34) (249). Since any consideration of the use of PTHrP as a treatment for osteoporosis arises from its likely physiological role as the locally generated agent in bone, acting through the PTHR1, it is appropriate to discuss briefly how the anabolic action of PTH through PTHR1 is thought to be achieved.

A. Bone Remodeling

The skeleton undergoes a continual process of renewal throughout life, replacing ~5–10% of existing bone each year. Its functions are to remove old or damaged bone and to respond to mechanical loading. The process is nonuniform, differing from one bone to another, between cortical and trabecular bone, and from one trabecular site to another. It takes place in focal packets throughout the skeleton, known as BMUs. The cellular sequence within each BMU begins with osteoclastic bone resorption and is followed by new bone formation by osteoblasts. BMUs are initiated asynchronously throughout the skeleton, geographically and chronologically separated from each other.

The concept of the BMU was initiated by Frost and coworkers (100, 124) and has been much reviewed (220, 250, 263). Activation of BMUs consists of the attraction of osteoclasts to the site to be remodeled, followed by the phase of bone resorption that lasts in human bone 2–3 wk. During
the next, reversal, phase, lasting 2–3 wk, the resorbed bone surface is cleared of collagen fragments by the action of mononuclear cells of the mesenchymal lineage that lay down a cement line. The final, formation stage is that in which recruited osteoblast precursors begin to differentiate at the site, ultimately replacing exactly the amount that had been removed by resorption, in a process that takes 3–4 mo (310).

To maintain bone mass, bone resorption and formation need to be balanced at the cellular level in the BMU, with the volumes of bone resorbed by the osteoclasts of a BMU and formed by the osteoblasts of that BMU being equal, so that no permanent bone loss occurs. This implies regulation of the number of BMUs, regulation of the numbers of osteoclasts and osteoblasts in each BMU, regulation of the activity of the participating cells, and regulation of their life-spans.

### B. How PTH Exerts Its Anabolic Effect

The anabolic effect of PTH requires that it be administered intermittently. It is administered by daily subcutaneous injection, with PTH(1–34) approved for the treatment of osteoporosis in a number of countries (249). The efficacy of PTH as a skeletal anabolic therapy was shown to be dependent on intermittent injections, each achieving a sharp peak of PTH in the blood (99). More sustained elevation of PTH levels favors osteoclast formation through increasing RANKL and therefore osteoclast production. This pharmacokinetic requirement is well illustrated by the attempts to develop anabolic therapies by using calcilytic agents to release PTH from the parathyroid gland. These attempts have resulted in sustained increases in endogenous PTH leading to increased osteoclastogenesis and cortical bone loss (97, 112, 151).

Studies of PTH pre- and posttreatment bone biopsies in 55 women used histomorphometry to classify anabolic surfaces as modeling, remodeling, or mixed. The conclusion was that the anabolic response was both modeling and remodeling based, with the predominant effect on remodeling (204). Other work identified remodeling under the influence of PTH accounting for over 70% of the anabolic effect and modeling for the remainder (198). Furthermore, in the latter work, it was estimated that much of the modeling effect might be due to extension of bone formation from the resorbed space of BMUs to adjacent quiescent surfaces, essentially “overfilling” of the BMU. These findings support the view that intermittent PTH treatment increases the number of BMUs but each produces a positive bone balance (13, 74, 148, 150, 302).

Current views of the anabolic action of PTH are that it increases the recruitment and activation of BMUs, that it acts on committed osteoblast precursors to promote their differentiation, inhibits osteoblast and osteocyte apoptosis (149, 150), and inhibits the production of the bone formation inhibitor sclerostin (165). There is also a possibility that activation of the resorption component of remodeling by PTH treatment generates activities that enhance the osteoblast differentiation effect. The latter may be explained in part by production of osteoblast-stimulating activities by the osteoclasts themselves (111, 221), or by release of matrix-bound growth factors [TGF-β, insulin-like growth factor I (IGF-I)] in the resorption process (335, 386) that enhance the availability of mesenchymal stem cells (TGF-β), or their differentiation in the osteoblast lineage (IGF-I). In addition to remodeling, evidence of a modeling effect of intermittent PTH includes the findings that PTH can increase osteoblast formation by converting bone lining cells to mature osteoblasts in vivo (169), and that PTH treatment of 4-day-old mice (solely bone modeling) for 17 days results in increased amount of bone (73).

The PTH anabolic effect has been thought to be blunted when administered with a resorption inhibitor (30, 72, 92). However, PTH treatment combined with Denosumab (190, 350), with responses assessed by BMD measurement or bone microarchitecture, suggested a net benefit even in the presence of such near-complete resorption inhibition. If this is the case, it would seem likely that such an effect results from stimulation of modeling by PTH in those circumstances (303). It is relevant to note that prolonged treatment of OVX monkeys with Denosumab resulted in increased bone through modeling, suggesting that this proceeds unabated despite virtually complete inhibition of remodeling (257).

The resorption component of PTH action continues to be of interest, since an effect at the BMU of as short a duration as possible could minimize stimulation of osteoclast formation and activity that occurs with repeated injection of PTH(1–34) or PTH(1–84). This has been at the center of discussion of data obtained with the trial of daily injections of PTHrP(1–36), which has been suggested to have almost purely anabolic action because increases in resorption markers are minimal.

### C. Rationale of Anabolic Therapy With PThrP

The mouse genetic experiments of Karaplis and colleagues (215, 236) revealed actions of locally generated PThrP that reproduce the known anabolic effects of intermittent PTH, namely, to stimulate bone formation by promoting the differentiation of committed osteoblast precursors and by inhibiting apoptosis of mature osteoblasts and osteocytes (150). An obvious corollary was that when used as a skeletal anabolic therapy, PTH is simply reproducing the local endogenous action of PThrP. Such a shared function between the hormone and its related cytokine was reminiscent
of the relationship between growth hormone and IGF-I, where the relationship is functional in many tissues (81).

With the recognition that PTHrP produced by cells within the osteoblast lineage is the endogenous ligand for the PTH1R in osteoblasts, several truncated forms of the molecule were investigated as anabolic agents. The obvious susceptibility of the full-length PTHrP to proteolytic degradation made it unsuitable for use as a parenteral treatment. The form of PTHrP subject to the most attention has been PTHrP(1–36), which has activity identical to that of PTH(1–34) in activating the cAMP/PKA pathway through the PTHR1. The main rationale for this choice appears to have been that PTHrP(1–36) was identified as a secreted product of neuroendocrine cells that use the secretory pathway, through which proteins are packaged at high concentrations in vesicles before processing and secretion (40, 382) (as discussed in sect. VIII). The nature of PTHrP secreted through the constitutive pathway of secretion, as used by osteoblast lineage cells of mesenchymal origin, has not been determined. Since those cells do not have the features required for the secretory pathway, the local physiological product in bone available for paracrine/autocrine action cannot be assumed to be PTHrP(1–36), but is more likely to be full-length PTHrP, or fragments generated rapidly by extracellular proteolysis (see sect. VIII).

D. Effects of PTHrP Peptides in Human Subjects

The earliest studies were aimed at determining whether parenterally administered PTHrP had effects in human subjects. PTHrP(1–34) was somewhat less potent than PTH(1–34) when infused into normal human subjects (98). Single injections of several doses of PTHrP(1–36) (maximum 3.20 μg/kg) resulted in increased phosphate excretion and nephrogenous cAMP and increased plasma 1,25(OH) vitamin D₃ (130). Similar outcomes were obtained with an infusion study in which the highest PTHrP(1–36) dose even resulted in a decrease in resorption marker excretion (273). No PTH-treated groups were included in either of these for comparison, but in a further study the same group claimed equal potency of PTHrP(1–36) and PTH(1–34) as the outcome of 6-h infusions of two doses of PTHrP(1–36) with one dose of PTH(1–34) (87). Despite the conclusion of equal potency based on these infusion studies, when anabolic studies were undertaken, the doses of PTHrP(1–36) required to increase levels of anabolic markers were manyfold higher than those of PTH(1–34) (134).

E. Anabolic Effects of PTHrP(1–36)

Courses of intermittent injections were then explored, seeking evidence for an anabolic effect of PTHrP(1–36). An earlier comparison in rats showed an anabolic effect of PTHrP in which it was clear that PTHrP(1–34) was less potent than PTH(1–34), probably ~25% as effective (132). A later 6-mo study in ovariectomized rats showed that daily single doses of PTHrP(1–36) of 40 μg·kg⁻¹·day⁻¹ were less effective and less hypercalcemic than the same dose of PTH(1–34) (322). The anabolic effect of intermittent injection of PTHrP(1–36) in human subjects, as assessed by measurement of bone formation markers, was suggested to be relatively free of the resorptive effect of PTH, in that markers of resorption did not increase to the extent that they do with PTH treatment (134, 135). Furthermore, PTHrP(1–36) was not accompanied by hypercalcemia at the highest dose used at that time (400 μg/day).

A 3-mo, randomized study was carried out in three groups of 35 postmenopausal women given daily subcutaneous PTH(1–34) (20 μg) or PTHrP(1–36) (400 or 600 μg) (133). The 600 μg/day PTHrP group was discontinued because of hypercalcemia. Of the two remaining groups, the increases in bone mineral density were similar: the bone formation marker P1NP increased appreciably more in the PTH(1–34) group (171%) than in the PTHrP(1–36) group (46%), and the increase in the CTX resorption marker in the latter group was evident at 3 mo, whereas it occurred at 2 mo with PTH(1–34). The findings are consistent with a view that less of the injected PTHrP(1–36) was reaching target sites in bone. Such an outcome would be due to a difference in pharmacokinetics of the two, with PTHrP(1–36) degraded more rapidly following injection, and thus not so widely distributed to activate BMUs. There is currently no evidence to exclude this.

The possibility of PTHrP(1–36) being purely, or predominantly, anabolic (17, 133, 134) is an interesting one, but difficult to explain. An explanation that has been offered (17, 136) arises from studies carried out in cells overexpressing PTH1R, where PTHrP(1–36) and PTH(1–34) differed in their initial receptor interaction mechanisms (see sect. IX for discussion of this work). The action of PTHrP(1–36) was restricted to the cell surface, while PTH(1–34) was more readily internalized and thus more likely to activate persistently though G protein interactions (91, 103). The authors propose that this differential response might explain why PTH(1–34) exhibits a greater resorption response than PTHrP(1–36), when each of the peptides is used by daily injection (134). If that is indeed the case, it might be expected that differing initial interactions with the target cell should be mirrored by different later events, such as the rapidity and duration of CREB activation and the expression of specific genes. Such evidence will be important to obtain. Furthermore, translating these findings regarding very early cell interactions to the physiological events in remodeling is hampered by the lack of understanding of the nature of the PTHrP protein presented to the osteoblast lineage in bone remodeling.
F. A PTHrP Analog?

An alternative approach to anabolic therapy through the PTHrP1 under investigation currently is the use of a PTHrP analog in which 5 of the 13 residues between 22 and 34 of PTHrP(1–34) have been altered, with the NH2-terminal 21 residues remaining the same. The rationale for these changes in this peptide, known as Abaloparatide, has not been published. There are a number of reports in abstract form only of bone anabolic activity of Abaloparatide (e.g., Ref. 123). In the one published paper at the time of preparing this manuscript (189), treatment of postmenopausal women for 24 wk with 40 or 80 μg/day resulted in increases in BMD similar to those in subjects treated with teriparatide, 20 μg/day. The resorption marker CTX was the same in teriparatide and all groups of abaloparatide after 12 wk, and after 24 wk was increased by 77% with the highest dose of abaloparatide and 86% by teriparatide (P < 0.003).

On this basis, the authors suggest that resorption markers increase with Abaloparatide to a lesser extent than they do with teriparatide (189), and that the mechanism of action of Abaloparatide might be similar to that proposed for PTHrP(1–36), interacting with the PTHR1 with kinetics different from those operating with PTH(1–34) (see above). Evidence in support of that has been obtained from studies of the initial action of Abaloparatide on cAMP formation (122).

Further studies are clearly required to establish whether there are any real differences between the actions of NH2-terminal domains of PTH and PTHrP on target tissues. The data so far pertain entirely to initial action on receptor, and to duration of cAMP formation, without any assessment of later cellular responses, including early events in PKA activation and subsequent stimulation or inhibition of expression of appropriate genes. The differences between the two in initial interaction with receptor that have been observed to the present time are not sufficient to explain any difference in perceived pharmacological responses to the two.

G. Anabolic Action Through the PTHR1 Pathway

Since the anabolic action of PTH is achieved predominantly through increasing the number and activity of BMUs (198, 204), and since both resorption and formation are integral to remodeling, the resorption effect must surely be inevitable. That is so if an anabolic agent is acting through the PTH anabolic pathway mechanisms. When PTH is administered in treatment by daily injection, increased bone formation markers (e.g., P1NP) are significant within days, and a more gradual rise in resorption markers (e.g., CTX) occurs some weeks or even months later (45, 249). These responses can readily be explained. The direct action of PTH on osteoblast lineage cells in existing BMUs or newly initiated BMUs is expected to result in rapid release of cell-derived P1NP, whereas with each activated BMU in response to PTH there would be a gradual accumulation of resorption products of osteoclast activity in an increasing number of BMUs, culminating eventually in increased detectable circulating resorption markers. For these reasons it is argued that a purely, or even a predominantly, anabolic action (17, 31) through the mechanism ascribed to anabolic PTH might be very difficult to achieve, since resorption is an essential feature of the active BMU, and the PTH anabolic mechanism requires the recruitment and activation of new BMUs.

On the other hand, the very fact that in some circumstances PTH might promote modeling leaves open the possibility that these PTHrP-related peptides currently under investigation might promote bone modeling at the high doses being administered, which have lesser effects on remodeling because their pharmacokinetics leaves them less available to act upon the PTHR1 in the appropriate osteoblast lineage cells. It is even possible that the PTHR1 receptor number varies sufficiently among the potentially responsive members of the osteoblast lineage, and that BMU activation is sacrificed for responses of lining cells or osteocytes; there is no such information available. Thus, if a PTH/PTHrP molecule is less effective at increasing the number of BMUs or the volume of bone resorbed by each, either because of reduced potency or less availability to the PTHR1, then a reasonable inference is that any increase in bone formed is by modeling. Establishing this would require quantitative histomorphometry before and after treatment, such as has been obtained with PTH treatment of human subjects (198, 204).

The local role of PTHrP in remodeling that is suggested by the mouse genetic experiments fits well with this, contributing to the activity of BMUs that are active asymmetrically throughout the skeleton at those sites where they are needed for repair or for removal of old bone (FIGURE 12). Pharmacologically administered bolus doses of PTH (or of any agonist acting through the PTHR1) would be expected to activate many more BMUs with treatment. This would be reflected in rapid increases in cell-based formation markers and less rapid increase in the resorption products that result from degradation of matrix.

XX. CONCLUSION

It could not have been predicted that uncovering the cause of the humoral hypercalcemia of malignancy would reveal a protein that has so many functions beyond its cancer roles. The sequence similarity between PTHrP and PTH within the NH2-terminal regions allows them to share actions through the common G protein-coupled receptor PTHR1. PTHrP was found to function as an autocrine/paracrine regulator in several organs. In cartilage it is necessary for the correct spatial and temporal expression of chondrocyte differentiation that determine normal endochondral bone
formation and the rate and extent of long bone formation. Its production by cells of the osteoblast lineage serves its role in bone remodeling, a function also demonstrated through the use of mouse genetics. PTHrP has other properties that are not shared with PTH. The most important of these are its ability to be transported to the nucleus, where its functions are yet to be specified, and the biological activities contained within the remainder of the molecule, including the ability to promote placental calcium transport. Whereas the physiological role of PTH is predominantly that of calcium homeostatic control, PTHrP is essential in development for normal endochondral bone formation and in maturity for bone remodeling. The pharmacological use of intermittent PTH injections in promoting bone formation in osteoporosis reflects the physiological function of PTHrP. Currently there is much interest in capitalizing on this by developing PTHrP analogs for therapeutic trial.

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