Birth Defects Research (Part C) 69:352–362 (2003)

REVIEW

PTHrP, PTH, and the PTH/PTHrP Receptor in Endochondral Bone Development

Ernestina Schipani and Sylvain Provot

Endochondral bone development is a fascinating story of proliferation, maturation, and death. An understanding of this process at the molecular level is emerging. In particular, significant advances have been made in understanding the role of parathyroid-hormone-related peptide (PTHrP), parathyroid hormone (PTH), and the PTH/PTHrP receptor in endochondral bone development. Mutations of the PTH/PTHrP receptor have been identified in Jansen metaphyseal chondrodysplasia, Blomstrand's lethal chondrodysplasia, and enchondromatosis. Furthermore, genetic manipulations of the PTHrP, PTH, and the PTH/PTHrP receptor genes, respectively, have demonstrated the critical role of these proteins in regulating both the switch between proliferation and differentiation of chondrocytes, and their replacement by bone cells. A future area of investigation will be the identification of downstream effectors of PTH, PTHrP, and PTH/PTHrP receptor activities. Furthermore , it will be of critical importance to study how these proteins cooperate and integrate with other molecules that are essential for growth plate development.
Birth Defects Research (Part C) 69:352–362, 2003.

INTRODUCTION

Skeletal development proceeds via two mechanisms, intramembranous and endochondral bone formation (Erlebacher et al., 1995). The former, in which mesenchymal cells develop directly into osteoblasts, is involved in the formation of the flat skull bones. The latter, accounting for the development of most other bones, involves a two-stage mechanism, whereby chondrocytes form a matrix template, the growth plate, in which osteoblasts differentiate and initiate the ossification process (Fig. 1). An understanding of this process at the molecular level is emerging (Kronenberg, 2003).

During endochondral bone development, growth plate chondrocytes undergo well-ordered and controlled phases of cell proliferation, differentiation, and apoptosis (Ballock and O'Keefe, 2003; Eames et al., 2003; Kronenberg, 2003; Shum et al., 2003). Round proliferative chondrocytes synthesize type II collagen and form a columnar layer, then stop proliferating and become prehypertrophic chondrocytes that mature into post-mitotic, hypertrophic cells. Hypertrophic chondrocytes express predominantly type X collagen and mineralize the surrounding matrix. This unique maturation/differentiation process is followed by the death of hypertrophic chondrocytes, blood vessel invasion, and finally replacement of the cartilaginous matrix with trabecular bone. Calcified cartilage is resorbed by osteoclasts, and then replaced by bone (the primary spongiosa). With continuing resorption of the primary spongiosa, the primary center splits into two opposite growth plates; in each of these, the maturation of cartilage and subsequent remodeling into bone continues as long as new chondrocytes are generated in the growth plates. As chondrocyte proliferation fuels longitudinal bone growth during postnatal life, the physes are separated by an increasing amount of space that becomes filled with bone marrow. Hypertrophic chondrocytes play a pivotal role in coordinating chondrogenesis and osteogenesis, as hypertrophic chondrocytes provide a scaffold for subsequent formation of trabecular bone. In addition, hypertrophic chondrocytes modulate the formation of the bone collar, the precursor of cortical bone, in the adjacent perichondrium.

This review will summarize the critical role of parathyroid hormone-related peptide (PTHrP), parathyroid hormone (PTH), and the PTH/PTHrP receptor in endochondral bone development.

PTHRP, PTH AND THE PTH/PTHRP RECEPTOR

PTH, an 84–amino acid polypeptide, is a major regulator of mammalian mineral ion homeostasis in postnatal life (Fig. 2A and C). The two major target organs of PTH action are bone and kidney (Kronenberg et al., 1993). In the kidney, PTH acts at two sites: in the proximal tubule, PTH activates 1-alpha hydroxylase, the enzyme respon-

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Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/bdrc.10028

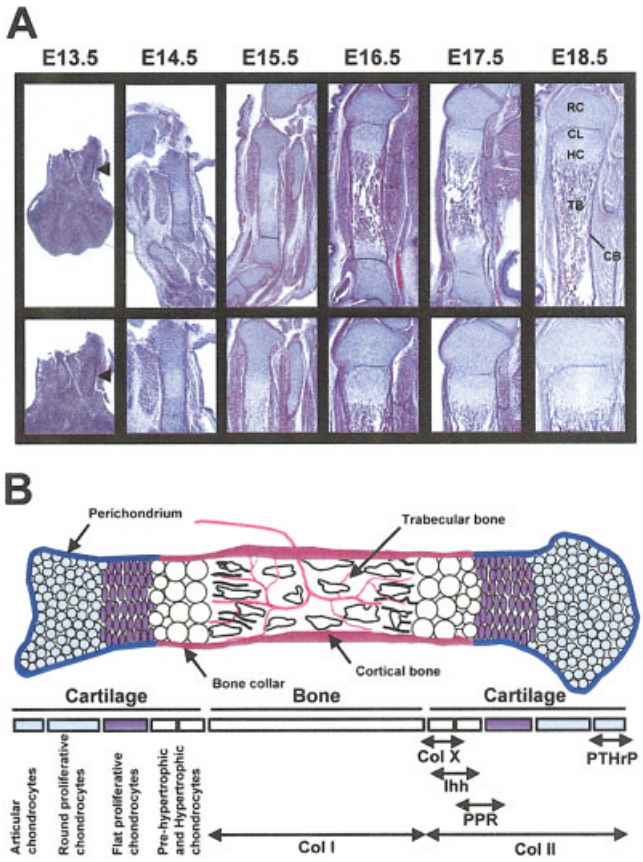

Figure 1. Endochondral bone formation. **A:** Representative pictures of tibia, at different stages of embryonic mouse development, are shown. The lower panels correspond to a higher magnification view of the upper panels. See text for more details. RC: round proliferative chondrocytes; CL: columnar layer; HC: hypertrophic chondrocytes; CB: cortical bone; TB: trabecular bone. Arrow heads on the far left panels indicate the tibia. **B:** Schematic representation of a mouse tibia at late stage of fetal development. Characteristic markers for bone, periarticular, flat, prehypertrophic, and hypertrophic chondrocytes are noted.

sible for hydroxylating 25-hydroxyvitamin D, and inhibits phosphate reabsorption by blocking sodium-dependent phosphate cotransport; in the distal tubule, it stimulates calcium absorption against an electrochemical gradient. In bone, PTH-mediated activation of osteoblasts leads to increased osteoclast number and resorptive activity. The primary physiological consequences of increasing serum PTH are, therefore, an increase in serum calcium, a decrease in serum phosphate, and an increase in circulating 1, 25-dihydroxyvitamin D_3.

PTHrP was first discovered as the cause of humoral hypercalcemia of malignancy (HMM) syndrome (Broadus and Stewart, 1994). This syndrome is characterized by serum levels of calcium and phosphate that are essentially indistinguishable from those observed in patients with hyperparathyroidism (i.e., hypercalcemia and hypophosphatemia), but with low or normal PTH levels. PTHrP and PTH share a limited sequence homology, with only eight identical residues in the first 34 amino acids (Fig. 2A). Unlike PTH, PTHrP synthesis is not regulated by serum calcium, and circulating levels of PTHrP are very low in healthy adults. PTHrP is produced in a large variety of normal adult and fetal tissues, including cartilage, heart, kidney, hair follicles, placenta, breast, lungs, and many epithelial surfaces. This tissue distribution pattern suggests that PTHrP serves biological functions other than those linked to the regulation of mineral ion metabolism (Fig. 2C). Indeed, the hypothesis that PTHrP plays a broad role as an autocrine/paracrine factor has now been confirmed by studies that have shown dramatic developmental abnormalities in mice genetically modified to either overexpress or not express PTHrP (see below).

Despite their limited sequence homology, PTH and PTHrP bind and activate the same PTH/PTHrP receptor with almost indistinguishable high affinity (Fig. 2B) (Jüppner et al., 1991; Abou-Samra et al., 1992; Schipani et al., 1993). As discussed in detail below, the PTH/PTHrP receptor mediates both the endocrine actions of PTH and the autocrine/paracrine actions of PTHrP (Kronenberg et al., 1998). This places the PTH/PTHrP receptor as a central regulator of both mineral ion homeostasis and bone development. Consistent with the ability of the PTH/PTHrP receptor to recognize PTH and PTHrP, mRNA transcripts that encode the PTH/PTHrP receptor are found in a wide variety of fetal and adult tissues, with the highest level of expression seen in kidney, bone, and cartilage (Urena et al., 1993; Lee et al., 1994, 1995, 1996).

Together with the receptors for calcitonin (Lin et al., 1991) and secretin (Ishihara et al., 1991), the PTH/PTHrP receptor belongs to a distinct group of G protein–coupled receptors termed Family B (Gardella and Juppner, 2001). All members of this secretin/calcitonin/PTH receptor family have seven membrane-spanning domains, and a relatively long amino-terminal extracellular domain (approximately 160 amino acids) that contains six conserved, functionally important cysteine residues, and up to four potential N-linked glycosylation sites. Approximately 45 amino acid residues, which are dispersed throughout the transmembrane domains and in the amino-terminal extracellular portion, are strictly conserved in all members of this receptor family, and are likely to have important functions in ligand binding, signal transduction, or both.

Like all members of the Family B receptors, the PTH/PTHrP receptor is coupled to signal effector molecules by heterotrimeric ($\alpha\beta\gamma$) guanine nucleotide binding proteins (G

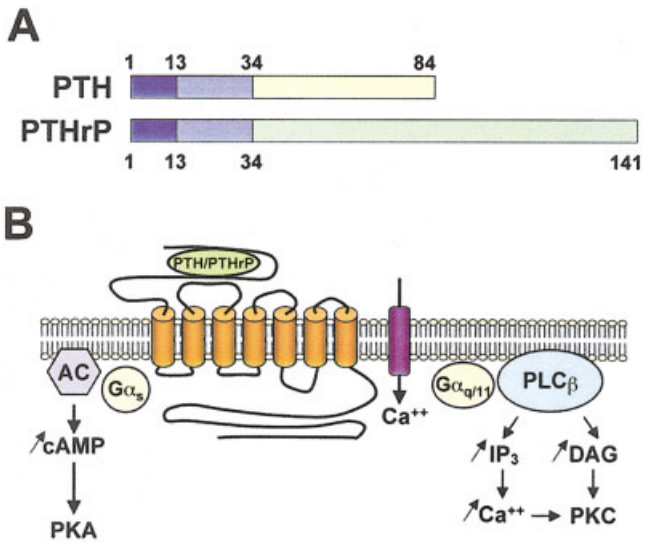

Figure 2. PTH, PTHrP, and the PTH/PTHrP-receptor. **A:** Schematic representation of the parathyroid hormone (PTH) and PTH related peptide (PTHrP). The three domains, defined on the basis of degree of homology between the two ligands, are indicated. The purple rectangles represent the regions of greater homology (dark purple) and lesser homology (light purple). The C-terminal part of these two ligands diverged. **B:** Downstream effectors of the PTH/PTHrP receptor. PTH and PTHrP share the same, unique seven-transmembrane G-protein–coupled receptor, and the PTH/PTHrP receptor. Upon binding of its ligand, the PTH/PTHrP receptor can activate adenylate cyclase (AC) through Gαs, and phospholipase Cβ (PLCβ) through Gαq/11. Activated AC then stimulates the formation of 3′, 5′-adenosine monophosphate (cAMP), which in turn activates protein kinase A (PKA). Activated PLCβ stimulates the formation of diacylglycerol (DAG) and 1,4,5-inositol triphosphate (IP3). In turn, DAG activates protein kinase C (PKC), and the production of IP3 leads to an increase of intracellular free Ca^{++}. The PTH/PTHrP receptor can also stimulate extracellular influx of Ca^{++} through regulation of calcium channels. **C:** Target tissues and function for PTH and PTHrP. See text for more details.

proteins; Fig. 2B). Upon binding of its ligand, the PTH/PTHrP receptor can activate adenylate cyclase (AC) through Gαs, and phospholipase Cβ (PLCβ) through Gαq/11 (Bringhurst et al., 1993; Iida-Klein et al., 1997). Activated AC then stimulates the formation of 3′,5′-adenosine monophosphate (cAMP), which in turn activates protein kinase A (PKA). Activated PLCβ stimulates the formation of diacylglycerol (DAG) and 1,4,5-inositol triphosphate (IP3). In turn, DAG activates protein kinase C (PKC), and the production of IP3 leads to an increase in the intracellular free Ca^{++}. The PTH/PTHrP receptor can also stimulate the extracellular influx of Ca^{++} through regulation of calcium channels (Swarthout et al., 2002). Furthermore, recent studies have also indicated that the PTH/PTHrP receptor can activate protein kinase C through a PLC-independent pathway (Whitfield et al., 2001). While the best-characterized second messenger of the PTH/PTHrP receptor is undeniably cAMP, activation of the phospholipase C and protein kinase C pathways by PTH is likely to play a significant role in renal phosphate transport (Iida-Klein et al., 1997), chondrocyte differentiation (Guo et al., 2002), and osteoblast proliferation (Carpio et al., 2001).

THE PTH/PTHRP RECEPTOR IN HUMAN CHONDRODYSPLASIAS

The critical role of the PTH/PTHrP receptor in endochondral bone development is highlighted by the discovery that two devastating chondrodysplasias, Blomstrand's lethal chondrodysplasia and Jansen's metaphyseal chondrodysplasia, are caused by mutations of this protein. More recently, mutant PTH/PTHrP receptors have been also identified in some cases of human enchondromatosis.

Blomstrand's Lethal Chondrodysplasia (BLC)

Blomstrand et al. (1985) reported the first case of BLC, and several other cases then followed (Oostra et al., 2000). The disease is characterized by prenatal lethality, premature and abnormal bone mineralization and ossification, and shortened limbs (Fig. 3B). Endochondral bone formation is markedly advanced in BLC fetuses, and the columnar proliferative layer in the mutant growth plate is virtually absent. In two recent cases of BLC, defects in tooth and mammary gland development were noted (Wysolmerski et al., 2001). The disease appears to have an autosomal recessive pattern of inheritance, as most BLC cases are derived from consanguineous parents.

Genetic studies of BLC patients led to the determination that BLC is caused by inactivating mutations in the PTH/PTHrP receptor (Fig. 3A) (Jobert et al., 1998; Karaplis et al., 1998; Zhang et al., 1998; Karperien et al., 1999). Thus far, three types of mutant PTH/PTHrP receptors have been identified, and all three have been studied in vitro using recombinant expression systems. The first mutant, Δ373-383-PPR, lacks a region of the fifth transmembrane domain of the PTH/PTHrP receptor due to a single nucleotide change that affects mRNA splicing (Jobert et al., 1998). Despite having normal cell-surface expression, Δ373-383-PPR does not bind or respond to PTH or PTHrP. The second identified muta-

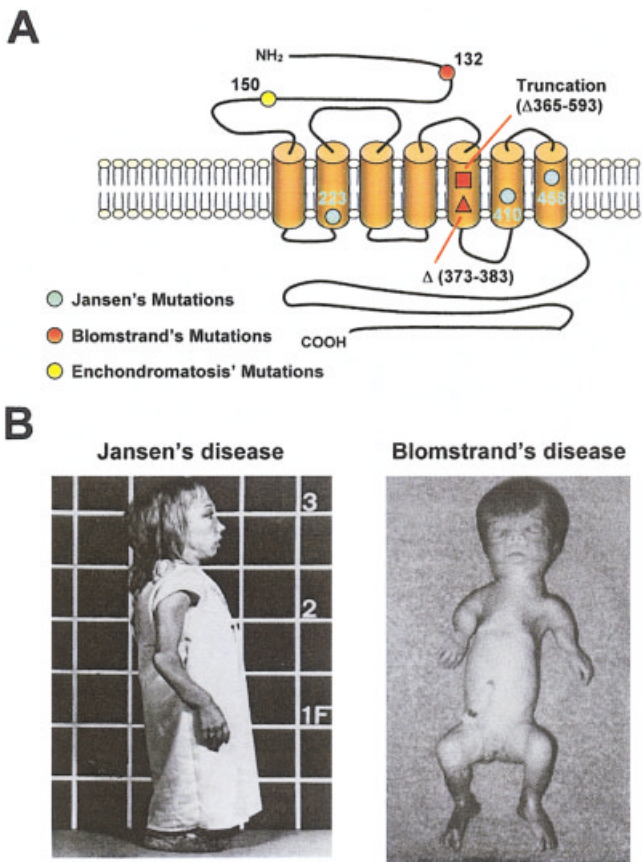

Figure 3. The PTH/PTHrP receptor in human diseases. **A:** Schematic representation of the PTH/PTHrP receptor showing the mutations identified in patients with Jansen's metaphyseal chondrodysplasia (in green), Blomstrand's lethal chondrodysplasia (in red), and enchondromatosis (in yellow). Mutations are missense point mutations unless specified. See text for more details. **B:** View of a patient with Jansen's metaphyseal chondrodysplasia at the age of 22 years (left panel), and a postmortem view of a baby with Blomstrand's lethal chondrodysplasia (generous gift of Dr. Caroline Silve, right panel).

tion arose from a single nucleotide exchange that led to the P132L mutation in the N-terminal extracellular domain (P132L-PPR) (Karaplis et al., 1998; Zhang et al., 1998). When studied in COS cells, this receptor shows slightly decreased expression, decreased specific ligand binding, and decreased cAMP formation upon exposure to agonist ligands. A homozygous deletion in exon EL2 was identified in a third BLC patient (Karperien et al., 1999). The resultant protein, Δ365-593-PPR, lacks transmembrane domains 5, 6, and 7, the connecting loops, and the cytoplasmic "tail," and does not display any measurable response to PTH. Notably, the degree of skeletal abnormalities observed in BLC patients can be correlated with the severity of the PTH/PTHrP receptor mutation that they carried: P132L-PPR (the least deleterious mutation) resulted in less severe defects than did either Δ365-593-PPR or Δ373-383-PPR.

Jansen's Metaphyseal Chondrodysplasia (JMC)

Jansen's metaphyseal chondrodysplasia (JMC) is a rare autosomal dominant disorder characterized by short-limbed dwarfism secondary to severe abnormalities of the growth plate, and hypercalcemia (Fig. 3B) (Jansen, 1934; Jüppner and Schipani, 1997). Clinical findings in JMC patients include severe short stature, disproportionately short limbs, and micrognathia (Frame and Poznanski, 1980). The laboratory findings in JMC patients are also reminiscent of primary hyperparathyroidism: severe and asymptomatic hypercalcemia, hypophosphatemia, decreased tubular reabsorption of phosphate, increased urinary excretion of cAMP, and elevated circulating levels of $1,25\text{-}(OH)_2$VitD (Rao et al., 1979; Kruse and Schütz, 1993; Parfitt et al., 1996).

The pathogenesis of JMC has been obscure for many years. Although JMC and hyperparathyroidism share many symptoms, parathyroid gland abnormalities were not detected in JMC patients, and circulating levels of PTH and PTHrP were either normal or undetectable. Although early investigators postulated the existence of an unknown calcium-regulating agent, it was later considered that the same symptomatology—hypercalcemia in the absence of elevated PTH—might be observed if the patients had a mutation in the PTH/PTHrP receptor that led to ligand-independent (constitutive) activation of the receptor (Schipani et al., 1995; Yasuda et al., 1996). Accordingly, genomic DNA from a JMC patient was screened for mutations in the coding exons for the PTH/PTHrP receptor, and a heterozygous mutation that changed residue 223 from histidine to arginine was found (Fig. 3A). Since the original report in 1995, the genomic DNA from nine other JMC patients has been examined: seven patients had the H223R heterozygous nucleotide exchange, and two patients had distinct heterozygous mutations in the PTH/PTHrP receptor (T410P and I458R; as shown in Fig. 3A) (Schipani et al., 1996, 1999). None of these mutations has been detected in the genomic DNA taken from a large number of healthy individuals, and there is only one familial case in which the affected mother of a JMC patient also had the mutation (Schipani et al., 1996). Thus, while it appears that at least the H223R mutation has a dominant mode of inheritance, the dataset suggests that the three JMC mutations normally arise as new germline mutations or as spontaneous somatic mutations that appear early in life.

When the corresponding mutant receptors (H223R-, T410P-, and I458R-PPR) were examined in vitro, it was found that cells expressing these receptors demon-

strated increases in basal cAMP signaling that correlated with the amount of DNA transfected, indicating that the mutant receptors were indeed constitutively active (Schipani et al., 1995, 1996, 1999). Relative to those that expressed the wild-type PTH/PTHrP receptor, cells expressing either the H223R-PPR or T410P-PPR mutation mounted submaximal responses to either PTH or PTHrP (approximately two-fold above basal). In contrast, cells expressing the I458R-PPR mutation showed the same maximal cAMP accumulation in response to PTH or PTHrP as did the wild-type receptor. While none of the three JMC receptors activated the phospholipase C pathway in the absence of exogenous PTH, both T410P-PPR and I458R-PPR were able to stimulate a normal phosphoinositol response to agonist ligands. In contrast, PTH did not elicit increases in phosphoinositol production from cells containing the H223R-PPR mutation. In competition assays using radiolabeled PTH analogs, it is possible to demonstrate that all three of the JMC constitutively active receptors bind PTH with either normal or modestly enhanced affinity. Despite the subtle differences in the interactions of each of the three receptors with PTH, the corresponding JMC patients did not show any obvious differences in their clinical or biochemical presentations. It thus appears that the PTH-independent activation of the cAMP pathway, evidenced by these mutant receptors, is the origin of the pathology of JMC (Schipani et al., 1995, 1996, 1999).

Enchondromatosis

Enchondromas are common benign cartilage tumors of bone that can occur as solitary lesions or, in enchondromatosis, as multiple lesions. Recently, the heterozygous missense mutation R150C has been identified in the PTH/PTHrP receptor (R150C-PPR) of two patients with enchondromatosis (Fig. 3A) (Hopyan et al., 2002). One patient had inherited the mutation from the father, who had only a very mild chondrodysplasia; in the second case the mutation was just limited to the tumor tissue. When tested in vitro, the mutant R150C-PPR appeared to be very poorly expressed, and displayed a severe impairment of both binding to PTH and cAMP production upon challenge with the agonist, very likely a result of impaired cell surface expression. However, cells transfected with the mutant R150C-PPR appeared to have higher basal cAMP levels than controls, when basal cAMP values were corrected for the level of receptors on the cell surface. The mutant R150C-PPR is thus constitutively active. Interestingly, the patient heterozygous for the R150C mutation, unlike the patients with Jansen's disease, did not display any obvious sign of chondrodysplasia besides the enchondromas, and was not hypercalcemic. Of course, a variety of considerations could explain the obvious differences. The R150C mutation could affect receptor cell surface expression in a more severe manner than the Jansen mutations, or the Jansen mutations so far identified could be intrinsically more potent in terms of constitutive activity. Alternatively, it is possible that constitutive activity is not the only critical feature of the R150C-PPR, and that other signaling properties, as yet not explored, could also be affected by the R150C mutation, in addition to the increase of basal cAMP levels. The mechanism that leads to formation of enchondroma in the absence of any obvious sign of chondrodysplasia in patients carrying the R150C mutation is still largely unknown. In order to address this question, the effect of the R150C mutation on Indian Hedgehog (Ihh) activity has been investigated in vitro (Hopyan et al., 2002). Ihh is a morphogen that has a critical role in endochondral bone formation (see below). Transcriptional assays in HeLa cells co-transfected with a Hedgehog-responsive reporter gene and either R150C-PPR or the wild-type PTH/PTHrP receptor showed that only the mutant receptor, and not the wild-type receptor, resulted in a constitutive activation of the reporter. The finding suggests that the R150C mutation overactivates the Ihh signaling pathway. This result is somehow surprising, especially in light of in vitro and in vivo data showing a negative regulation of Ihh by PTHrP (see below). More studies will be necessary in order to better understand how the R150C-PPR mutant leads to activation of the Ihh pathway and to formation of enchondromas.

The discovery of mutant PTH/PTHrP receptors as causes of these human diseases clearly underscores the critical developmental role of the PTH/PTHrP receptor in endochondral bone formation. Numerous genetic models have been generated, which have been instrumental in understanding the role of PTHrP, PTH, and their receptor during development. Some of these models are described in the next paragraph.

GENETIC MANIPULATIONS OF PTHRP, PTH, AND THE PTH/PTHRP RECEPTOR IN ENDOCHONDRAL BONE

Genetically modified animals have dramatically demonstrated the critical developmental role of PTHrP. In the growth plate, PTHrP mRNA is expressed by perichondrial cells and proliferating chondrocytes in the periarticular region, whereas the PTH/PTHrP receptor mRNA is expressed at low levels by proliferating chondrocytes in columns and at higher levels by prehypertrophic chondrocytes (Lanske et al., 1996; Vortkamp et al., 1996). Homologous ablation of the PTHrP gene in mice (*PTHrP*$^{-/-}$) results in animals that die during the perinatal period (Karaplis et al., 1994). These mice show severe abnormalities in the bones that form through the endochondral process; in particular, they display a dramatic shortening of the snout, mandible, and extremities, and a reduced diameter of the ribcage. The contracted and hypoplastic character of the ribcage in these mice is the one feature that is mostly responsible for their inability to survive after birth. *PTHrP*$^{-/-}$ mice also show severe abnormalities in mammary gland epi-

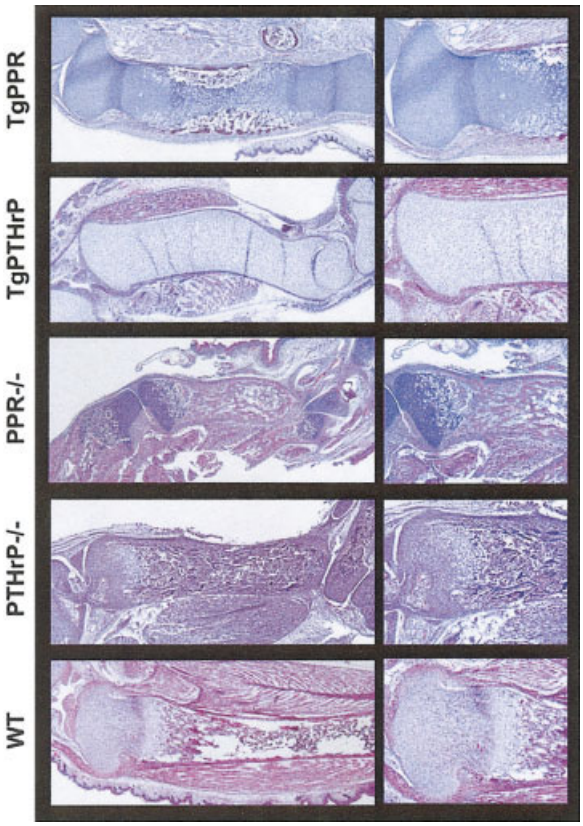

Figure 4. Role of PTHrP and the PTH/PTHrP receptor in endochondral bone development. **A:** Histology of tibia obtained from wild-type (WT), PTHrP$^{-/-}$, PPR$^{-/-}$ mice, or transgenic mice overexpressing in cartilage PTHrP (TgPTHrP) and a constitutively active form of the PTH/PTHrP receptor (TgPPR). Representative sections of E18.5 (PPR$^{-/-}$) or newborn tibia (WT, PTH$^{-/-}$;PTHrP$^{-/-}$, TgPTHrP, and TgPPR) stained with hematoxylin and eosin (H&E) are shown. **B:** Higher magnification view of the panels presented in (A).

thelial development, suggesting that PTHrP plays a crucial role in epithelial–mesenchyme interactions (Wysolmerski et al., 1995, 1998).

The premature mineralization and ossification seen with *PTHrP*$^{-/-}$ mice appears to be related to the premature transition of proliferative chondrocytes to hypertrophic chondrocytes in the fetal growth plate (Fig. 4). Consistent with these findings, the study in which PTHrP overexpression was targeted to tissues expressing the type II collagen promoter (*TgPTHrP*) shows that the transgenic animals are born with shortened limbs (Fig. 4). This is apparently due to delayed mineralization and decelerated chondrocyte maturation in the fetal growth plate (Weir et al., 1996).

As previously discussed, the well-ordered and controlled proliferation, differentiation, and apoptotic death of the growth plate chondrocytes is crucial for proper control of bone elongation, since it sets the stage for the timing of the replacement of the cartilage matrix with a trabecular bone matrix. In particular, the switch from a proliferative to a postproliferative state determines the number of chondrocytes in the proliferative versus the hypertrophic pool. The animal models described above demonstrate that PTHrP is critically involved in this switch.

Later studies with the *PTHrP*$^{-/-}$ mice have shown that PTHrP is one of the mediators of Ihh activity in the growth plate. As previously mentioned, Ihh is a member of a family of proteins important for embryonic patterning that are highly expressed in the transition zone between proliferating and hypertrophic cells and in hypertrophic chondrocytes. It appears that Ihh inhibits chondrocyte differentiation by increasing PTHrP synthesis by the periarticular chondrocytes (Lanske et al., 1996; Vortkamp et al., 1996), and thereby delays the mineralization of the cartilage matrix. Overexpression of Ihh protein by the injection of a recombinant retrovirus into embryonic chick limbs delays hypertrophy of growth plate chondrocytes, as does addition of an active NH_2-terminal fragment of Sonic Hedgehog (Shh, a relative of Ihh known to mimic Ihh actions in chondrocytes) to embryonic mouse limbs in vitro. These gain-of-function phenotypes are associated with an increase in PTHrP mRNA expression in the perichondrial region at the end of long bones. Consistent with these findings, the Shh fragment has no effect on *PTHrP*$^{-/-}$ mouse limbs. Furthermore, mice homozygous for a null mutation in the Ihh gene (*Ihh*$^{-/-}$) have no detectable PTHrP mRNA in their growth plate, and hypertrophic chondrocytes predominate in the *Ihh*$^{-/-}$ growth plate late in fetal development, in association with a dramatic inhibition of chondrocyte proliferation (St-Jacques et al., 1999). Taken together, these data suggest that Ihh delays the switch from proliferation to hypertrophy of chondrocytes by stimulating PTHrP production in the periarticular region of the growth plate. In addition, Ihh is also a potent stimulator of chondrocyte proliferation (St-Jacques et al., 1999; Long et al., 2001b). Because Ihh stimulates PTHrP expression, which in turn keeps the chondrocytes in the proliferative pool, and thereby delays Ihh production, a negative Ihh/PTHrP feedback loop is then established in the growth plate (Fig. 6). Consistent with this model, expression of a constitutively active PTH/PTHrP receptor in chondrocytes of *Ihh*$^{-/-}$ mice prevented premature chondrocyte hypertrophy (Karp et al., 2000). Interestingly, it did not rescue the decreased chondrocyte proliferation. These experiments demonstrate that the molecular mechanism preventing chondrocyte hypertrophy is distinct from that which drives proliferation. Ihh positively regulates PTHrP, which is sufficient to prevent chondrocyte hypertrophy and maintain a normal domain of cells competent to undergo proliferation. In contrast, Ihh

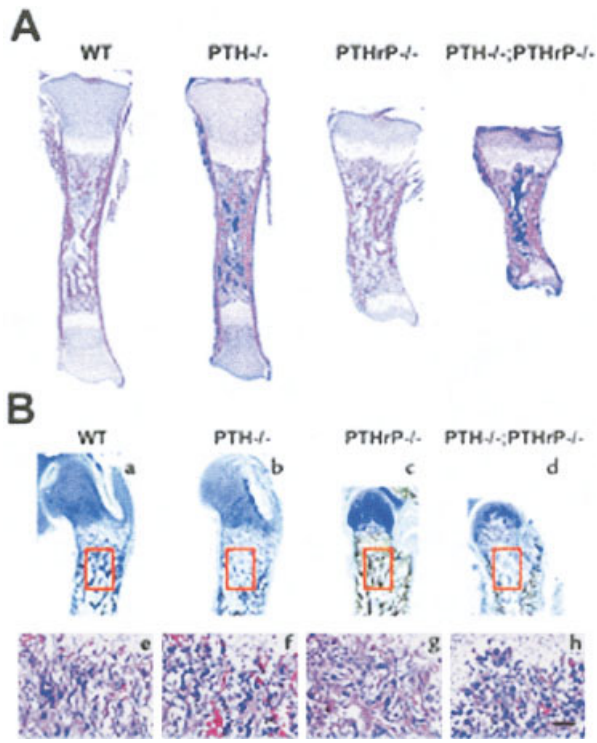

Figure 5. Role of PTH and PTHrP in transition from cartilage to bone and in primary spongiosa formation. **A:** Sections of newborn tibias from wild-type(WT), $PTH^{-/-}$, $PTHrP^{-/-}$, $PTH^{-/-}$/$PTHrP^{-/-}$ mice stained with H&E. **B:** (a–d) Undecalcified sections of femur stained with von Kossa stain, obtained from newborns $PTH^{-/-}$ or $PTHrP^{-/-}$ animals or PTH;PTHrP double-null mouse ($PTH^{-/-}$;$PTHrP^{-/-}$); (e–h) enlargement of the primary spongiosa stained with H&E of the null animals (with permission from JCI)

is necessary for normal chondrocyte proliferation in a pathway that cannot be rescued by PTHrP signaling.

Consistent with the notion that the PTH/PTHrP receptor mediates the action of PTHrP in growth plate development, ablation of the PTH/PTHrP receptor mimics the effect of the PTHrP ablation on chondrocyte differentiation (Lanske et al., 1996). Like *PTHrP*$^{-/-}$ mice, *PPR*$^{-/-}$ mice die around birth, and show dramatic skeletal abnormalities secondary to acceleration of chondrocyte hypertrophy (Fig. 4). These findings are strikingly reminiscent of those reported in Blomstrand's chondrodysplasia. Conversely, consistent with the observations in patients with JMC, transgenic mice, in which the H223R-PPR was targeted to the growth plate by placing its expression under the control of the α1 (II) collagen promoter (*TgPPR*), show delayed mineralization and decelerated chondrocyte maturation in skeletal segments that are formed by endochondral bone development (Fig. 4) (Schipani et al., 1997). This phenotype is very similar to the phenotype of *TgPTHrP* mice with targeted overexpression of PTHrP in the growth plate (Fig. 4) (Weir et al., 1996). The striking similarity between the two animal models indicates that the PTH/PTHrP receptor is the main mediator of PTHrP action in the developing endochondral bone (Chung et al., 2001).

The remarkable parallelism between human and mouse models is further underlined by the phenotype of transgenic mice expressing the R150C-PPR in the chondrocytic growth plate under the control of the collagen type II promoter (Hopyan et al., 2002). Consistent with the findings in humans, long bones in these transgenic mutant mice were not shorter than controls, even though the hypertrophic layer appeared to be reduced in size. More importantly, in adulthood they showed persistence of cartilage islands in the bony diaphyses that were phenotypically similar to the human enchondromas.

As noted above, at first glance the features of the *PPR*$^{-/-}$ mice are similar to those of the *PTHrP*$^{-/-}$ mice, but closer examination reveals that there are indeed differences between these two genetically ablated animals (Lanske et al., 1998). In particular, *PPR*$^{-/-}$ mice show decreased trabecular bone formation in the primary spongiosa, an alteration not seen after PTHrP ablation. These results suggest that another ligand for the PTH/PTHrP receptor plays a role in development. This has been confirmed by the initial report on mice that lack PTH as a result of homologous recombination (*PTH*$^{-/-}$) (Miao et al., 2002): *PTH*$^{-/-}$ mice are viable but dysmorphic, and they uniquely demonstrate a modest expansion in the layer of hypertrophic chondrocytes, and reduced metaphyseal osteoblast and trabecular bone in fetal life and at birth (Fig. 5). The modest expansion in the layer of hypertrophic chondrocytes may be secondary to the abnormal replacement of cartilage by bone, and is therefore consistent with an important role of PTH in primary spongiosa formation during fetal development. Compound *PTH*$^{-/-}$ and *PTHrP*$^{-/-}$ mice mutants display the combined cartilaginous and osseous defects of both single mutants (Fig. 5). These results indicate that coordinated action of both PTH and PTHrP are required to achieve normal fetal skeletal morphogenesis. Furthermore, they clearly demonstrate an essential function of PTH at the cartilage-bone interface, and a unique role for PTH in skeletal development in utero that complements the critical action that has been documented for PTHrP (Miao et al., 2002).

To further explore the role of the PTH/PTHrP receptor in endochondral bone development, an elegant chimeric model has been developed recently that has unveiled another critical role of the Ihh/PTHrP system in endochondral bone development (Chung et al., 1998). Normally, bone collars are formed in the perichondrium abutting prehypertrophic and hypertrophic chondrocytes. In the growth plate of *wt/PPR*$^{-/-}$ chimeric mice, *PPR*$^{-/-}$ chondrocytes hypertrophy ectopically closer to the

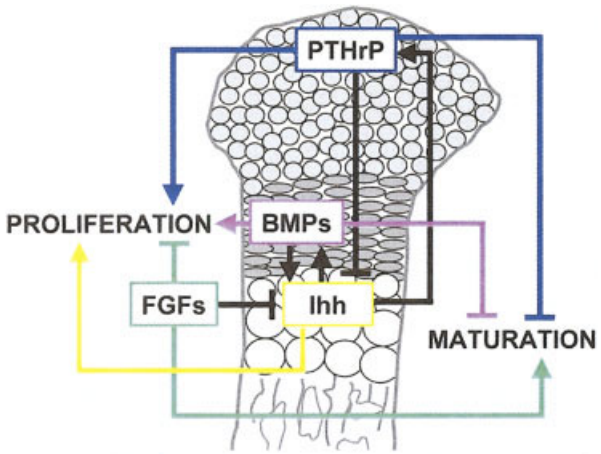

Figure 6. Schematic representation of feedback loops and biological activities of PTHrP, Ihh, BMPs, and FGFs in the fetal growth plate. Ihh induces PTHrP expression at the articular region, and PTHrP in turn represses Ihh expression, generating a negative feedback loop. Ihh also induces the expression of BMPs, which themselves induce Ihh expression in a positive feedback loop. Conversely, FGFs repress Ihh expression. PTHrP, Ihh, and BMPs are positive modulators of proliferation, and negatively affect maturation; the effect of Ihh on maturation is exclusively PTHrP dependent, while Ihh induces proliferation independently of PTHrP; and the effects of BMPs on proliferation and maturation can occur independently of the PTHrP/Ihh axis. FGFs are negative modulators of proliferation, and positively affect terminal maturation; these effects can occur independently of PTHrP/Ihh action.

articular surface, and bone collars are formed in the perichondrium adjacent to the ectopically hypertrophied chondrocytes. A similar chimeric approach has revealed that ectopic hypertrophic chondrocytes lacking both the PTH/PTHrP receptor and Ihh no longer induce ectopic bone collar in the adjacent perichondrium (Chung et al., 2001). These data strongly suggest that Ihh is indeed involved in the formation of the bone collar, and specifies the site at which perichondrium forms bone. Consistent with this notion, mice lacking Ihh do not show osteoblasts in either the primary spongiosa or the bone collar of bones formed by endochondral development (St-Jacques et al., 1999).

"HOT AREAS" OF FUTURE INVESTIGATIONS

Signaling Pathways Downstream of the PTH/PTHrP Receptor

As mentioned above, the PTH/PTHrP receptor can activate more than one G protein. The in vivo and in vitro characterization of mutant receptors carrying mutations identified in JMC supports the hypothesis that cAMP is a critical mediator of PTH/PTHrP receptor activity in the growth plate. A major role for cAMP/PKA signaling is also highlighted by the recent observation, in a chimeric mouse model, that growth plate chondrocytes lacking Gsα exhibit accelerated differentiation (Drs. Chung and Kronenberg, personal communication). Consistent with this model, a knock-in mutant mouse, in which the wild-type PTH/PTHrP receptor had been substituted with a mutant receptor (DSEL) impaired in its ability to increase intracellular levels of IP3, displays a significant delay in chondrocyte differentiation (Guo et al., 2002). These two downstream effectors of PTH/PTHrP receptor activity, cAMP and IP3, thus seem to have opposite or distinct effects on growth plate development. IP3 signaling via the PTH/PTHrP receptor appears to slow down the proliferation and to hasten the differentiation of chondrocytes, actions that oppose the dominant effects of PTH/PTHrP receptors and involve cAMP-dependent signaling pathways. Interestingly, lack of IP3 signaling also increases PTH/PTHrP receptor levels in chondrocytes (Guo et al., 2002).

Further studies will now be required in order to understand how the different signaling pathways downstream of the PTH/PTHrP receptor are connected and integrated, and to identify the crucial downstream effectors of PTH/PTHrP receptor activity in chondrocytes. In this regard, our knowledge of potential downstream targets of cAMP/PKA signals in chondrocytes is expanding. It has been shown that the master chondrogenic factor Sox9 (Bi et al., 1999, 2001; Akiyama et al., 2002;) is phosphorylated by PKA upon PTHrP treatment (Huang et al., 2001). This phosphorylation results in an increased DNA affinity of Sox9, and thus increases Sox9 transcriptional activity (Huang et al., 2000). Sox9 is required during sequential steps of the chondrocyte differentiation pathway; it is critical for commitment of mesenchymal cells towards chondrocytes, positively regulates chondrocyte proliferation, and delays their terminal differentiation. Sox9 is highly expressed by proliferative chondrocytes in vivo; however, it is currently not clear whether this factor directly or indirectly regulates their proliferation. Another known target of cAMP/PKA signaling is the cAMP response element binding protein (CREB). In primary cultures of chondrocytes, it has been shown that PTHrP induces phosphorylation of CREB, which binds and activates the transcription of the Cyclin D1 promoter, and activates cell division (Beier et al., 2001; Jonescu et al., 2001). Interestingly, however, in vivo, chondrocytes lacking PTHrP or its receptor do not display any decrease in CREB phosphorylation in comparison to normal cells, and transgenic mice expressing a dominant-negative CREB have a phenotype that is very different from that of the *PTHrP*$^{-/-}$ mouse (Long et al., 2001a). This indicates that the PTHrP/cAMP/PKA signal may promote chondrocyte proliferation independently of PKA/CREB signaling, through a yet uncharacterized signaling pathway.

Integration and Cooperation of the PTH/PTHrP Receptor Signaling System with Other Signaling Systems during Endochondral Bone Development

In recent years, it has become progressively clear that endochondral bone development is the result of a complex and integrated network of numerous endocrine and paracrine activities. PTHrP, PTH, and the PTH/PTHrP receptor are part of these critical signaling systems, but a large number of other factors have been extensively investigated both in vitro and in vivo. For example, numerous studies have established that fibroblast growth factor (FGF) signaling, and bone morphogenetic protein (BMP) signaling, respectively, interact with the Ihh/PTHrP pathway (Minina et al., 2001, 2002; Ornitz and Marie, 2002). It is well known that gain-of-function mutations of the FGF receptors are the cause of the most common forms of human chondrodysplasias (Vajo et al., 2000). Consistent with these findings, FGF signaling represses chondrocyte proliferation, and accelerates terminal differentiation of hypertrophic chondrocytes (Ornitz and Marie, 2002). Studies involving organ culture of bone explants in vitro suggest that the effect of FGFs on chondrocyte proliferation is independent of the suppression of Ihh activity (Minina et al., 2002). However, it is still an open question whether the effects of the FGF signaling pathway in chondrocytes in vivo are, at least in part, dependent on the Ihh/PTHrP system. Differently from FGFs and similarly to Ihh/PTHrP, BMPs are positive modulators of chondrocyte proliferation, and they negatively regulate chondrocyte terminal differentiation (Minina et al., 2001, 2002). Consistent with these findings, a positive autoregulatory feedback loop between BMPs and Ihh/PTHrP exists (Minina et al., 2001). Interestingly, a role for BMPs in chondrocyte proliferation and maturation that is independent of Ihh/PTHrP has also been suggested (Minina et al., 2001).

It will be exciting and challenging to dissect how all these different pathways interact and cooperate in order to generate the complex network of actions that determine and tightly regulate endochondral bone development.

ACKNOWLEDGMENTS

We thank Dr. Henry M. Kronenberg for critical review of the manuscript. We also thank Dr. Tatsuya Kobayashi for providing histological slides and for helpful discussion.

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