A Constitutively Active Mutant PTH-PTHrP Receptor in Jansen-Type Metaphyseal Chondrodysplasia

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A single heterozygous nucleotide exchange in exon M2 of the gene encoding the parathyroid hormone–parathyroid hormone–related peptide (PTH-PTHrP) receptor was identified in a patient with Jansen-type metaphyseal chondrodysplasia, which changes a strictly conserved histidine residue at position 223 in the receptor's first intracellular loop to arginine. Constitutive, ligand-independent adenosine 3′,5′-monophosphate accumulation was observed in COS-7 cells expressing the mutant PTH-PTHrP receptor but not in cells expressing the wild-type receptor. This finding explains the severe ligand-independent hypercalcemia and hypophosphatemia, and most likely the abnormal formation of endochondral bone, in this rare form of short-limbed dwarfism.

Jansen-type metaphyseal chondrodysplasia (JMC) (*1*), a rare form of short-limbed dwarfism, is associated with asymptomatic but often profound hypercalcemia and hypophosphatemia (*2*, *3*), despite the lack of parathyroid gland abnormalities (*2*, *4*, *5*) and the presence of normal or undetectable amounts of PTH and PTHrP (*6*, *7*, *8*). Although these laboratory findings suggest the presence of an unknown calcium-regulating factor (*6*, *7*, *8*), we considered the possibility of a PTH-PTHrP receptor mutation that results in constitutive receptor activation. Genomic DNA of a patient with JMC was therefore screened for mutations in all coding exons of the PTH-PTHrP receptor gene. A heterozygous His to Arg mutation at position 223 (H223R) was found at the junction between the PTH-PTHrP receptor's first intracellular loop and second membrane-spanning helix (Fig. 1A). Both unaffected parents were homozygous for the normal allele (Fig. 1B). This result and the phenotypic evidence of widespread abnormalities in many tissues suggested that the patient inherited a new germline mutation or developed a new somatic mutation early in development.

To test the consequences of the H223R missense mutation in vitro, we introduced the respective nucleotide exchange into the complementary DNA encoding the wild-type human PTH-PTHrP receptor (HKrk) to generate HKrk-H223R. Ligand-independent constitutive accumulation of adenosine 3′,5′-monophosphate (cAMP) occurred only in COS-7 cells expressing HKrk-H223R but not in cells expressing HKrk (Fig. 2A, inset). Transfection with increasing amounts of HKrk-H223R led to a corresponding increase in basal cAMP accumulation that reached a plateau at about 100 ng per well. In contrast, cells that expressed increasing amounts of HKrk showed no change in basal cAMP accumulation, which was furthermore indistinguishable from that of cells transfected with vector alone (*9*). When challenged with [Nle^{8,18}, Tyr^{34}]bovine PTH(1–34)amide or [Tyr^{36}]human PTHrP(1–36)amide, cAMP accumulation increased only approximately twofold in cells expressing HKrk-H223R, yet more than 20-fold in cells expressing HKrk (Fig. 2A).

Because the PTH-PTHrP receptor activates adenylate cyclase and phospholipase C (*10*), we tested whether the H223R missense mutation led to ligand-independent inositol phosphate (IP) accumulation. Cells that were transfected with saturating amounts of HKrk-H223R showed, in comparison with cells expressing HKrk, no increase in basal IP accumulation. However, challenge with either [Nle^{8,18}, Tyr^{34}]bovine PTH(1–34)amide or [Tyr^{36}]human PTHrP (1–36)amide failed to stimulate the accumulation of IP in cells transfected with HKrk-H223R (Fig. 2B). Because PTH-stimulated IP generation depends on the number of expressed receptors (*11*), we assessed cell-surface expression of wild-type and mutant receptors in which a *Haemophilus influenza* hemagglutinin (HA) epitope had been introduced into the NH_2-terminal extracellular domain by oligonucleotide-directed site-specific mutagenesis (*12*). When cells were transfected with the same increasing amounts of either plasmid, maximal expression of HA-HKrk-H223R was approximately 50% lower than that of HA-HKrk (Fig. 2C). However, when HA-HKrk expression was reduced to match that of HA-HKrk-H223R, ligand-stimulated IP accumulation was readily detectable (Fig. 2D). This indicated that the H223R mutation, and not the lower amount of cell-surface expression, caused the impaired activation of phospholipase C. The transiently expressed mutant HKrk-H223R bound [Nle^{8,18}, Tyr^{34}]bovine PTH(1–34)amide and [Tyr^{36}]human PTHrP (1–36)amide with an apparent binding affinity of 4.1 ± 1.0 and 4.1 ± 0.5 nM, respectively, compared with 10 ± 3.1 and 21 ± 8.0 nM for the wild-type PTH-PTHrP receptor (*13*), and indistinguishable binding data were obtained with HA-HKrk and HA-HKrk-H223R, respectively (*9*). The increased apparent binding affinity observed with cells expressing PTH-PTHrP receptors that contained the H223R mutation appeared to be directly related to a reduced cell-surface expression (Fig. 2C) and is con-

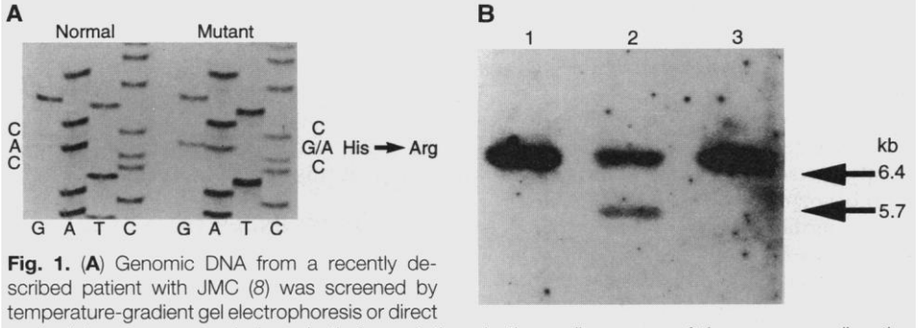

Fig. 1. (**A**) Genomic DNA from a recently described patient with JMC (*8*) was screened by temperature-gradient gel electrophoresis or direct nucleotide sequence analysis or both for mutations in the coding exons of the gene encoding the PTH-PTHrP receptor (*28*). A heterozygous A to G change, which introduces a restriction site for Sph I, was identified in exon M2, which encodes portions of the first intracellular loop and of the second membrane-spanning domain. This nucleotide exchange causes a His to Arg mutation at position 223 of the PTH-PTHrP receptor. Genomic DNA from both healthy parents revealed no abnormality in exon M2 (*9*). (**B**) To confirm these polymerase chain reaction findings, ≈5 μg of genomic DNA from the patient (lane 2) and her healthy mother (lane 3) and father (lane 1) was digested with Sph I before analysis by Southern (DNA) blot. The blot was probed with an ≈1.5-kb Xho I fragment from the most 3′ portion of the human genomic DNA clone HG-2 (*28*). The patient's DNA revealed two large hybridizing DNA species of ≈5.7 and ≈6.5 kb, respectively, and a small fragment of ≈0.8 kb (*9*). DNA from both parents showed only a single ≈6.5-kb DNA fragment that hybridized approximately twice as intensely as did the same DNA fragment from the patient. Position and size of the DNA markers are indicated by arrows.

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sistent with previous findings made with the use of wild-type and mutant receptors (*11*, *12*).

Actions of PTH and PTHrP on kidney and bone explain the abnormalities in mineral ion metabolism found in diseases of excess hormone production such as primary hyperparathyroidism (*14*) and the syndrome of humoral hypercalcemia of malignancy (HHM) (*15*), respectively. The striking laboratory similarities between these diseases and JMC are undoubtedly caused by the dominant expression of the mutant, constitutively active, PTH-PTHrP receptor in the two most important organs of calcium and phosphate homeostasis. These findings thus provide evidence that the recently isolated PTH-PTHrP receptor is the major mediator of the endocrine functions of PTH and PTHrP.

However, PTHrP and the PTH-PTHrP receptor are also expressed in adjacent cells within the metaphyseal growth plate, which suggests a paracrine-autocrine role for PTHrP (*16*, *17*). Furthermore, the targeted ablation of the gene encoding PTHrP in mice through homologous recombination results in a perinatally lethal phenotype with severe chondrodysplasia (*18*), and a similar, but more severe, phenotype that leads to death in utero is observed in mice that lack the PTH-PTHrP receptor (*19*). At least in the animals lacking PTHrP, the osseous malformations are most likely caused by the abnormal proliferation and differentiation of growth-plate chondrocytes into hypertrophic cells (*17*). PTHrP, through its interaction with the PTH-PTHrP receptor, thus appears to play a prominent role in bone elongation and ossification. This hypothesis is supported by our findings in JMC, which indicate that an activating receptor mutation causes not only the abnormalities in mineral ion homeostasis but also the abnormal formation of endochondral bone. The latter findings appear to be reciprocal to those in mice lacking PTHrP or the PTH-PTHrP receptor (*5*).

Activating receptor mutations were recently implicated in some human diseases. In rhodopsin, such mutations cause either rare forms of retinitis pigmentosa (*20*) or congenital stationary night blindness (*21*); in the thyroid-stimulating hormone receptor, they cause either thyroid adenomas (*22*) or congenital nonautoimmune hyperthyroidism (*23*); in the luteinizing hormorne receptor, they cause gonadotropin-independent male precocious puberty (*24*); and in the calcium-sensing receptor, they cause hypoparathyroidism (*25*). Similarly, murine melanocyte-stimulating hormone receptor mutations result in either constitutively active or hypersensitive receptors that determine the fur coat color (*26*). Our findings concerning the PTH-PTHrP receptor extend this list of activating mutations to yet another family of heterotrimeric

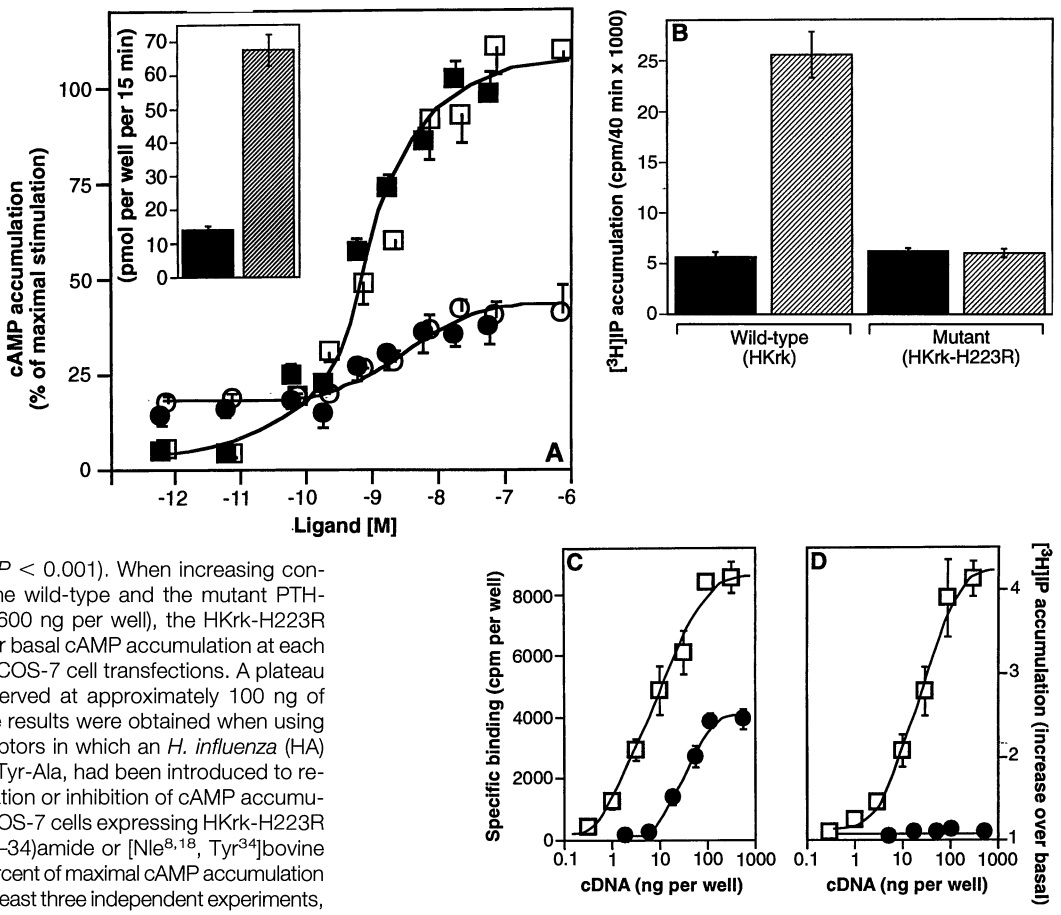

Fig. 2. Functional evaluation of mutant and wild-type PTH-PTHrP receptors (*29*). (**A**) Cyclic AMP accumulation. Measurement of intracellular cAMP accumulation in COS-7 cells transiently expressing HKrk (squares) or HKrk-H223R (circles) (100 ng of plasmid DNA per well) after stimulation with increasing concentrations of either [Nle^{8,18}, Tyr^{34}]bovine PTH(1–34)amide (filled squares and circles) or [Tyr^{36}]human PTHrP(1–36)amide (open squares and circles). Maximal cAMP accumulation was 338 ± 59 pmol per well per 15 min for PTH and 395 ± 95 pmol per well per 15 min for PTHrP when COS-7 cells expressing Hkrk were tested. Basal cAMP accumulation of COS-7 cells expressing either HKrk (filled bar) or HKrk-H223R (shaded bar) is shown in the inset (14.1 ± 1.1 versus 67.5 ± 4.8 pmol per well per 15 min; $P < 0.001$). When increasing concentrations of the plasmid encoding the wild-type and the mutant PTH-PTHrP receptor were compared (3 to 600 ng per well), the HKrk-H223R mutant revealed three- to fivefold greater basal cAMP accumulation at each of the plasmid concentrations used for COS-7 cell transfections. A plateau of basal cAMP accumulation was observed at approximately 100 ng of plasmid DNA per well. Indistinguishable results were obtained when using wild-type and mutant PTH-PTHrP receptors in which an *H. influenza* (HA) epitope, Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala, had been introduced to replace residues 93 to 101 (*9*). No stimulation or inhibition of cAMP accumulation was observed after treatment of COS-7 cells expressing HKrk-H223R with either [Nle^{8,18}, Tyr^{34}]bovine PTH(3–34)amide or [Nle^{8,18}, Tyr^{34}]bovine PTH(7–34)amide (*9*). Data are given as percent of maximal cAMP accumulation with HKrk and represent mean ± SE of at least three independent experiments, each done in duplicate or triplicate. (**B**) IP accumulation. Hydrolysis of total IPs (IP_1, IP_2, and IP_3) was assessed as described (*10*) in the absence (filled bars) or presence (shaded bars) of [Tyr^{36}]human PTHrP(1–36)amide (10^{-6} M) with the use of COS-7 cells transfected with either HKrk or HKrk-H223R (100 ng of plasmid DNA per 24 wells). Indistinguishable results were obtained when the experiments were done with HA-HKrk and HA-HKrk-H223R or with [Nle^{8,18}, Tyr^{34}]bovine PTH(1– 34)amide (*9*). Data are the mean ± SE of at least two independent experiments each done in triplicate. (**C**) Assessment of cell-surface expression. COS-7 cells were transfected with increasing concentrations of the plasmid DNA encoding HA-HKrk (open squares) or with HA-HKrk-H223R (filled circles). Cell-surface expression of PTH-PTHrP receptors was determined as described with the use of the monoclonal antibody 12CA5 and a second radiolabeled antibody to mouse immunoglobulin (*12*). Data are the mean ± SE of at least two independent experiments each done in triplicate. (**D**) IP accumulation. COS-7 cells were transfected with increasing concentrations of the plasmid DNA encoding HA-HKrk (open squares) or with HA-HKrk-H223R (filled circles). Hydrolysis of IPs was assessed as described (*10*) in the absence or presence of [Tyr^{36}]human PTHrP(1–36)amide (10^{-6} M). Data are the mean ± SE of at least two independent experiments each done in triplicate.

guanosine triphosphate–binding protein (G protein)–coupled receptors. Because the equivalent of the histidine residue investigated in the present study is strictly conserved in all members of this receptor family (*27*), it may be of general functional importance in these G protein–coupled receptors.

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29. The H223R mutation was introduced into the plasmid encoding HKrk by oligonucleotide-directed site-specific mutagenesis (*12*) to give HKrk-H223R. The mutation was confirmed by enzymatic digestion with the restriction enzyme Sph I and by nucleotide sequence analysis. Plasmid DNAs encoding the wild-type and the mutant PTH-PTHrP receptor were then transiently expressed in COS-7 cells (*10, 13*).

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Ras-Dependent Induction of Cellular Responses by Constitutively Active Phosphatidylinositol-3 Kinase

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Phosphatidylinositol (PI)-3 kinase is one of many enzymes stimulated by growth factors. A constitutively activated mutant, p110*, that functions independently of growth factor stimulation was constructed to determine the specific responses regulated by PI-3 kinase. The p110* protein exhibited high specific activity as a PI-3 kinase and as a protein kinase. Expression of p110* in NIH 3T3 cells induced transcription from the *fos* promoter. Co-expression of dominant negative Ras blocked this response. When expressed in *Xenopus laevis* oocytes, p110* increased the amount of guanosine 5′-triphosphate–bound Ras, caused activation of the Ras effector Raf-1, and induced Ras-dependent oocyte maturation. These findings show that PI-3 kinase can stimulate diverse Ras-dependent cellular processes, including oocyte maturation and *fos* transcription.

PI-3 kinase activity has been correlated with the actions of growth factors and oncogenes (*1*). Studies with mutants of platelet-derived growth factor (PDGF) receptor have shown that PI-3 kinase is required for PDGF-mediated mitogenic signaling (*2*). In fibroblast cell lines, a mutant PDGF receptor that no longer associated with PI-3 kinase did not activate c-Ras (*3*). Although this observation implies that PI-3 kinase acts upstream of Ras in PDGF-stimulated signaling, the interpretation of this type of experiment is complicated by the possibility that several signaling molecules share the same binding site. To investigate directly whether PI-3 kinase can transmit signals through a Ras-dependent pathway, we generated a form of PI-3 kinase that is constitutively active and does not require receptor stimulation.

The catalytic subunit of PI-3 kinase, p110, exhibits enzymatic activity in mammalian cells only when bound, through its NH_2-terminal region, to the p85 subunit or to a 102–amino acid fragment of the inter–Src homology region 2 (iSH2) region of p85 (*4–6*). We postulated that a molecule composed only of elements of p85 and p110 that are essential for enzymatic activity might be constitutively active. To generate an activated p110 mutant, p110*, we covalently attached the iSH2 region of p85 to the NH_2-terminus of p110. A hinge region composed of a "glycine-kinker" was inserted between iSH2 and p110 to allow the iSH2 domain to more easily interact with the p110 NH_2-terminal domain (Fig. 1). The p110* protein exhib-

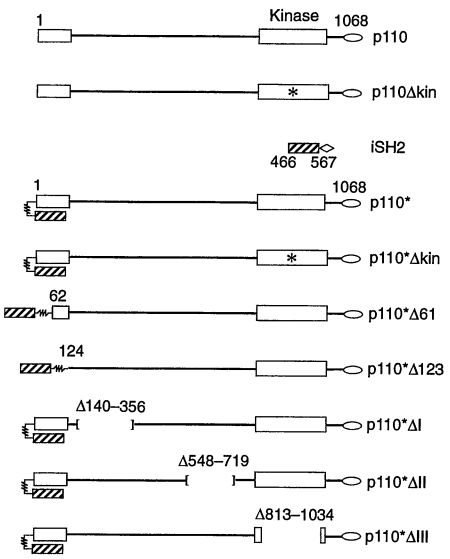

Fig. 1. Schematic representation of the p85 and p110 derivatives used in this study (*16*). The p110 constructs were tagged at the COOH-terminus with the Myc epitope (oval); the iSH2 fragment of p85 contained a COOH-terminal influenza virus hemagglutinin (HA) epitope tag (diamond). The box labeled Kinase represents the p110 region with similarity to the catalytic domain of protein kinases. The domain responsible for the interaction with the iSH2 domain of the p85 subunit is shown as a small box at the p110 NH_2-terminus. The p110Δkin protein is a kinase-deficient version of p110 in which the ATP-binding site was mutated (*16*), as indicated by an asterisk within the catalytic domain. The iSH2 domain of p85 that is required for catalytic activity is represented by a striped bar. The first and last amino acids of fragments are numbered with respect to their position in the wild-type p85 or p110 sequence. The p110* protein is a constitutively active chimera that contains the iSH2 domain of p85 fused to the NH_2-terminus of p110 by means of a flexible glycine-kinker (*16*). Plasmid p110*Δkin is the kinase-deficient version of p110*. Plasmids p110*Δ61 and p110*Δ123 lack 61 or 123 amino acids, respectively, from the p110 NH_2-terminus and can no longer associate with iSH2 (*5*). Plasmids p110*ΔI, p110*ΔII, and p110*ΔIII are mutants that have internal deletions within the p110 structure as indicated (*16*).