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Research Article

We report the absence of functional parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptors (PTH/PTHrP receptor) in Blomstrand chondrodysplasia, a genetic disorder characterized by advanced endochondral bone maturation. Analysis of PTH/PTHrP receptor genomic DNA from a patient with Blomstrand chondrodysplasia demonstrated that the patient was heterozygous for a point mutation (G--> A substitution at nucleotide 1176) inherited from the mother. Analysis of PTH/PTHrP receptor cDNA demonstrated that: (a) this point mutation caused the deletion of the first 11 amino acids of exon M5 (encoding the fifth transmembrane domain of the receptor), resulting from the use of a novel splice site created by the base substitution; (b) the mutant receptor was well expressed in COS-7 cells, but did not bind PTH or PTHrP, and failed to induce detectable stimulation of either cAMP or inositol phosphate production in response to these ligands; and (c) the paternal allele was not expressed. Thus, only the abnormal and nonfunctional PTH/PTHrP receptors encoded by the maternal allele were expressed by chondrocytes from this patient. In view of the known role played by the PTH/PTHrP receptor in bone and cartilage development, these results strongly support the conclusion that the absence of functional PTH/ PTHrP receptors is responsible for the skeletal abnormalities seen in Blomstrand chondrodysplasia, abnormalities that are the mirror image of those observed in Jansen's chondrodysplasia. These findings […]

Absence of Functional Receptors for Parathyroid Hormone and Parathyroid Hormone–related Peptide in Blomstrand Chondrodysplasia

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Abstract

We report the absence of functional parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptors (PTH/PTHrP receptor) in Blomstrand chondrodysplasia, a genetic disorder characterized by advanced endochondral bone maturation. Analysis of PTH/PTHrP receptor genomic DNA from a patient with Blomstrand chondrodysplasia demonstrated that the patient was heterozygous for a point mutation (G→A substitution at nucleotide 1176) inherited from the mother. Analysis of PTH/PTHrP receptor cDNA demonstrated that: (*a*) this point mutation caused the deletion of the first 11 amino acids of exon M5 (encoding the fifth transmembrane domain of the receptor), resulting from the use of a novel splice site created by the base substitution; (*b*) the mutant receptor was well expressed in COS-7 cells, but did not bind PTH or PTHrP, and failed to induce detectable stimulation of either cAMP or inositol phosphate production in response to these ligands; and (*c*) the paternal allele was not expressed. Thus, only the abnormal and nonfunctional PTH/PTHrP receptors encoded by the maternal allele were expressed by chondrocytes from this patient. In view of the known role played by the PTH/PTHrP receptor in bone and cartilage development, these results strongly support the conclusion that the absence of functional PTH/PTHrP receptors is responsible for the skeletal abnormalities seen in Blomstrand chondrodysplasia, abnormalities that are the mirror image of those observed in Jansen's chondrodysplasia. These findings emphasize the importance of signaling through this receptor in human fetal skeletal development. (*J. Clin. Invest.* 1998. 102:34–40.) Key words: PTH/PTHrP receptor • point mutation • alternate splicing • null allele • Blomstrand chondrodysplasia

Introduction

The parathyroid hormone (PTH)/PTH-related peptide (PTHrP)[1] receptor, a G protein–coupled receptor, plays a key role in the regulation of calcium and phosphorus metabolism in fetal and adult life by mediating the actions of PTH on its target organs (1). Recent studies have demonstrated that signaling through the PTH/PTHrP receptor, in addition to its role in regulating mineral metabolism, also plays an essential role in fetal development due to the important regulatory effects of PTHrP on the development of cartilage and bone (2–4). In this regard, mice with disruptions of either the PTH/PTHrP receptor or PTHrP genes exhibit multiple, severe skeletal defects characterized by an advanced endochondral bone formation that prove lethal in utero or shortly after birth (2, 4). This suggests that genetic abnormalities of the PTH/PTHrP receptor gene might also lead to severe developmental abnormalities in humans. Consistent with this idea, the constitutive activation of the PTH/PTHrP receptor has been demonstrated recently to be responsible for Jansen's chondrodysplasia, a disease characterized by delayed endochondral bone formation (5, 6). Heretofore, the effects of inhibitory mutations of the human PTH/PTHrP receptor have not been described. In particular, defects in this receptor are not responsible for pseudohypoparathyroidism type Ib (7–9), a condition characterized by hypocalcemia and hyperphosphoremia due to selective renal resistance to PTH, in which such abnormalities had been suspected previously (10–12). Here, we report that Blomstrand chondrodysplasia, a genetic disorder characterized by advanced endochondral bone maturation (13, 14), a defect that is the mirror image of that observed in Jansen's chondrodysplasia, results from the absence of functional PTH/PTHrP receptors.

Methods

Case report. The case evaluated in these studies has been described recently (15). The fetus, stillborn at 25 wk, was the result of the second pregnancy of a nonconsanguineous couple. The diagnosis of Blomstrand chondrodysplasia was made during pregnancy by sonographic examination, and was confirmed after delivery by the demonstration of characteristic radiologic and histologic findings, including a striking increase in bone density and extremely advanced skeletal maturation (15). 1 yr later, the mother gave birth to a healthy boy. However, her fourth pregnancy terminated spontaneously at 33 wk of gestation with the delivery of a dead girl with the same syndrome.

Southern blot analysis of genomic DNA. Genomic DNA from the proband and the mother was extracted from blood samples by standard methods. Southern blot analysis was performed according to standard procedures using probes prepared from plasmids containing either PTH/PTHrP receptor cDNA (16) or the genomic sequence corresponding to the 5′ promoter regions as described by Schipani et al. (16). Genomic DNAs were cut by HindIII or XbaI (PTH/PTHrP receptor cDNA probes) and by SstI (5′ promoter and PTH/PTHrP receptor cDNA probes) before Southern blotting. In all experiments, genomic DNA from the fetus, his mother, control fibroblasts, and normal human kidney were analyzed in parallel.

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1. *Abbreviation used in this paper:* PTH/PTHrP receptor, PTH and PTH-related peptide receptor.

Table I. Sequences of Primers Used to Amplify Exons S, G, M3, and EL2, Their Adjacent Intron/Exon Borders, and the 3′ Untranslated Region of the PTH/PTHrP Receptor

PCR product	Primer sequence	Size
		bp
Exon S	5′-GCAGCTCTGCACCCCCTACC-3′	232
	5′-*GACTGCGTGCCTTAGACCTACTCC*-3′	
Exon G	5′-TGCTGGAAGGGGTGGGGATTAC-3′	274
	5′-*CGTGTGGGTGGGAGTGAATTTATCT*-3′	
Exon M3	5′-CCCCCAGCCCAGCCCTGACT-3′	328
	5′-*GGGGCGGGATGTGCTGTGTG*-3′	
Exon EL2	5′-CTGGGTCTCTGTGGGCAGTCTT-3′	187
	5′-*CGCACATCCCACCCACTCTC*-3′	
3′ untranslated	5′-TCCTCAACGGCTCCTGCTCA-3′	322
	5′-*CCCTCCGCCACAGCTTTCC*-3′	

*Primers in italics are reverse primers.

Sequencing of genomic DNA. All coding exons of the PTH/PTHrP receptor gene were amplified by PCR using a mixture of 1 U *Taq* (GIBCO BRL, Gaithersburg, MD) and 0.06 U Pfu (Stratagene, La Jolla, CA) DNA polymerases in a final volume of 50 μl. Primers used to amplify exons S, EL2, G, and M3, and the 3′ untranslated region are shown in Table I. Other primers were as described by Schipani et al. (7). Amplification products were sequenced in both directions by the dideoxy chain-termination method.

Evaluation of PTH/PTHrP receptor mRNA. Chondrocyte cell cultures were established from tissue obtained from the proband at the time of delivery. A fibroblast cell line from a normal subject, human kidney tissue, and SaOS-2 osteosarcoma cells were used as controls. Cells were maintained in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum as described (10). Total RNA was extracted from cells and tissue using the method of Chomczynski and Sacchi (17), and cDNA was synthesized as described previously (18). Amplification of the entire PTH/PTHrP receptor cDNA in four overlapping fragments was performed using the primers described in Table II. Sequencing of cDNA was performed as described above.

Table II. Primers Used to Amplify the Entire cDNA of the PTH/PTHrP Receptor in Four Overlapping Fragments

Primers	Sequence	Size
		bp
Pair 1: S_{10}*	5′-GCGGCCCTAGGCGGTGG	451
G_{460}	5′-*GGCATGGCCTTTGTGATTGAA*	
Pair 2: G_{455}	5′-CATGCCTACCGACGCTGTGACC	595
$M4_{1049}$	5′-*TGACCCACACAGCCACGAAGAC*	
Pair 3: $M3_{952}$	5′-CCTCATCTTCATGGCCTTCTTCTC	510
T_{1462}	5′-*GTCCAGCGGCTCCAAGATTTC*	
Pair 4: $M7_{1409}$	5′-TTCTGCAATGGCGAGGTACAA	478
T_{1885}	5′-*GGAAATCATTCAACCACCCATCTT*	

Primers in italics are reverse primers. *The letter refers to the name of the exon on which the 5′ end of the primer is located, and the subscript number indicates the position of the 5′ end. The expected PCR product sizes (bp) are also indicated.

Construction of expression plasmids. A vector permitting expression of the wild-type PTH/PTHrP receptor with an epitope Tag (19) added at the COOH-terminal end (PTH-R/wt) was obtained by ligating the dodecapeptide sequence coding for the Tag marker to the COOH-terminal end of the full-length PTH/PTHrP receptor cDNA, and subcloning the fragment into pcDNA1-Amp (Invitrogen, La Jolla, CA). The functional properties of the tagged and native receptors were indistinguishable (data not shown). To obtain a vector permitting the expression of the abnormal PTH/PTHrP receptor identified in this study (PTH-R/Δ373-383), a fragment encompassing the deletion was produced by PCR (bases 676–1316), digested with NspI and EagI restriction enzymes, and ligated to the tagged wild-type PTH/PTHrP receptor cDNA plasmid that had been digested previously with the same restriction enzymes. The construction was verified by DNA sequencing.

RNase protection analysis. A riboprobe specific for a fragment of the abnormal PTH/PTHrP receptor mRNA encompassing the 33-bp deletion in exon M5 was prepared by amplifying a 477-bp fragment of the deleted PTH/PTHrP receptor cDNA using oligonucleotide pair 3 described in Table II. The PCR fragment was cloned in the pTAg plasmid using the LigATor cloning system (R & D Systems, Abingdon, UK). The plasmid was linearized by digestion with XbaI, and the riboprobe was synthesized using T7 RNA polymerase. RNase protection analysis was performed as described (10, 18). The unprotected riboprobe migrated at 589 bp. When the riboprobe hybridized to the deleted receptor mRNA, a protected fragment of 477 bp was observed. When the riboprobe hybridized to the wild-type receptor, two fragments, one at 191 bp, the second at 286 bp, were obtained.

Evaluation of receptor expression and function by transient expression. The techniques used for transient expression of wild-type and mutated PTH/PTHrP receptors in COS-7 cells, and the evaluation of PTH-induced cAMP production, PTH-induced inositol phosphate accumulation, and binding of [^{125}I]hPTHrP(1-34) by transfected cells have been described previously (18, 20). Expression of the Tag epitope was detected by immunofluorescence on permeabilized cells as described (19, 21). No staining was observed when untransfected COS-7 cells were incubated with the anti-Tag antibody.

Results

Evaluation of genomic DNA coding for the PTH/PTHrP receptor. To evaluate the possibility that defects in the PTH/PTHrP receptor gene were responsible for the abnormalities in the stillborn infant described above, each of the 14 coding exons and the 3′ untranslated region of this gene were amplified by PCR and sequenced. A single heterozygous base change (G→A, nucleotide 1176) was detected in exon M5, which encodes the fifth transmembrane domain of the receptor (Fig. 1, *A* and *B*). No other base changes were identified. Amplification and sequence analysis of the mother's genomic DNA revealed the same base substitution, indicating maternal inheritance of the trait. The base substitution removed an NciI restriction site from the normal sequence. Amplification of genomic DNA and subsequent digestion with NciI confirmed the presence of a heterozygous base change in the affected fetus and in his mother.

These studies evaluating genomic DNA raised two important issues. First, two different abnormalities could be produced by the G→A base substitution identified. This mutation could result in a Arg to Gln substitution at amino acid 383 of the resulting PTH/PTHrP receptor. Alternatively, this substitution creates a novel splice acceptor site (TCC**A**G↓GT) 33 nucleotides downstream from the normal splice acceptor site for exon M5 (Fig. 1, *C–E*). If this splice site were used, it would result in a mutant receptor in which the first 11 amino acids

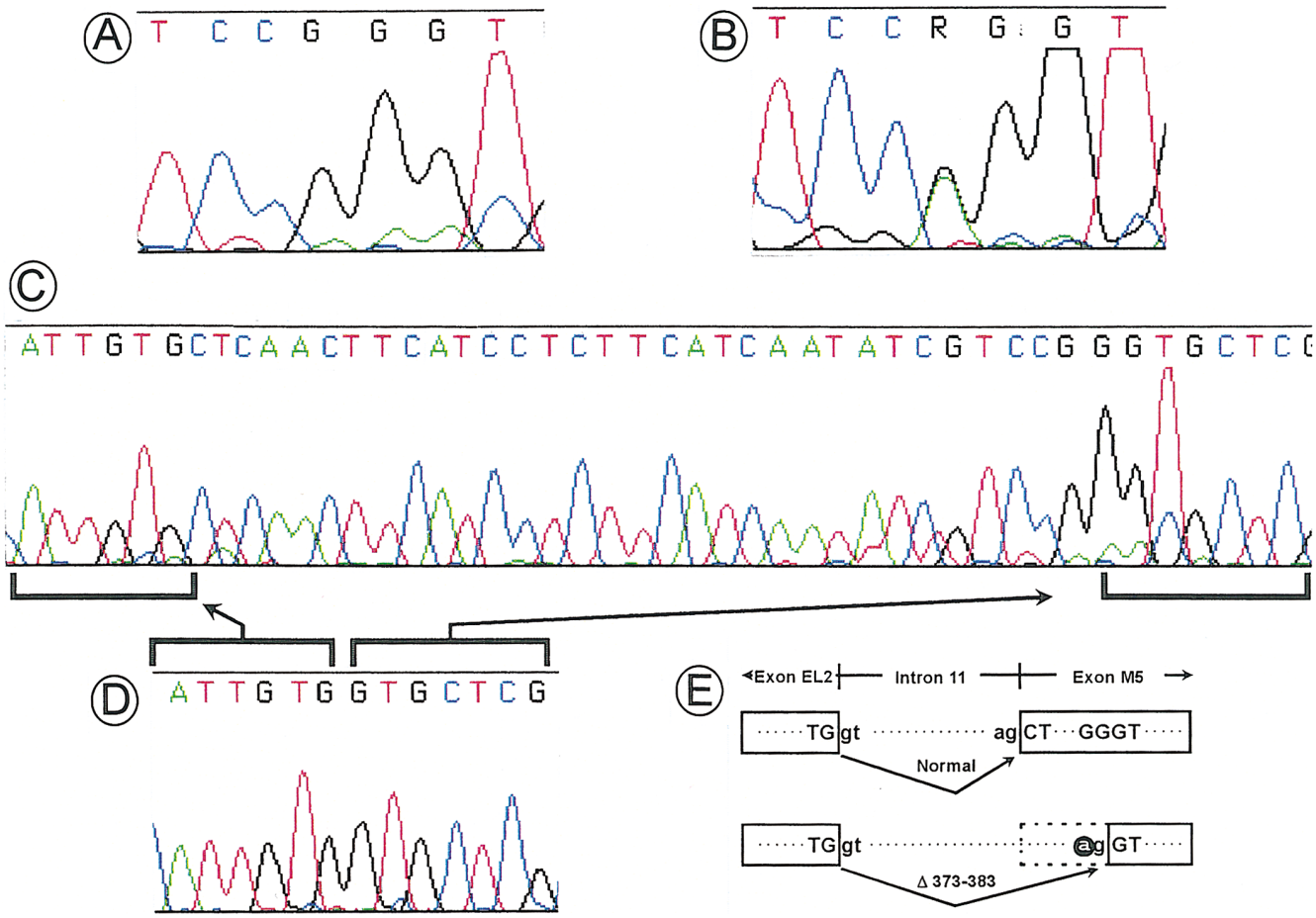

Figure 1. Nucleotide sequences of genomic and complementary DNA encoding portions of the PTH/PTHrP receptor from the stillborn infant with Blomstrand chondrodysplasia and a normal subject. Comparison of genomic DNA sequences coding for a portion of exon M5 from the normal subject (*A*) and the infant with Blomstrand chondrodysplasia (*B*) revealed a single heterozygous G→A mutation at position 1176 (indicated by R in the sequence). The same mutation was detected in the mother. The sequence of the cDNA at the boundary of exons EL2 and M5 from the normal subject (*C*) contains 33 nucleotides not present in the sequence obtained for cDNA from the infant with Blomstrand chondrodysplasia (*D*). As shown in *E*, this results from the use of the novel splice site created by the G→A mutation (*dark circle*) during processing of mRNA in the infant, and results in the deletion of amino acids 373–383 in the corresponding receptor protein.

coded by exon M5 are deleted (Fig. 1 *E*). Secondly, only a single heterozygous defect was identified in the fetus. It is known that mice heterozygous for the knockout of the PTH/PTHrP receptor gene are phenotypically normal (4). Thus, if abnormal PTH/PTHrP receptor activity were responsible for the fetus' abnormalities, another defect, presumably affecting receptor expression by the paternal allele, would have to be postulated. The availability of chondrocyte-like cells from this fetus, which express PTH/PTHrP receptor mRNA, allowed these issues to be resolved.

Evaluation of PTH/PTHrP receptor mRNA structure and expression. The entire sequence of the PTH/PTHrP receptor cDNA obtained from the fetus with Blomstrand chondrodysplasia was amplified in four overlapping fragments, one of which included the portion coding for exon M5. These amplification products migrated as a single band of the expected size, except for the product containing the portion of the PTH/PTHrP cDNA coding for exon M5 (pair 3). Amplification of this portion of the PTH/PTHrP cDNA generated a single band that was ~ 30 bp shorter than the expected 510-bp size observed with control cDNA (Fig. 2). Sequencing this abnormal band revealed that it resulted from amplification of PTH/PTHrP receptor mRNA formed using the novel splice site produced by the G→A substitution identified in the maternal allele of the genomic DNA (Fig. 1, *D* and *E*). Consequently, this mRNA codes for a receptor in which the first 11 amino acids in exon M5 (amino acids 373–383) have been deleted.

Despite that the mutation resulting in the 33-bp deletion of the PTH/PTHrP receptor mRNA affected only the maternal allele of the infant's DNA, an amplification product corresponding in size to normal PTH/PTHrP receptor was not observed (Fig. 2, lane *1*), suggesting that expression from the paternal allele was abnormal. To confirm this finding, RNA was extracted from the patient's cultured chondrocyte-like cells and control fibroblasts, and expression of mRNAs corresponding to normal and partially deleted PTH/PTHrP receptor was evaluated by RNase protection assays. When RNA from the fetal chondrocytes was studied, a protected fragment at 477 bp corresponding to the deleted receptor was seen (Fig. 3). In contrast, bands at 191 and 286 bp corresponding to the wild-

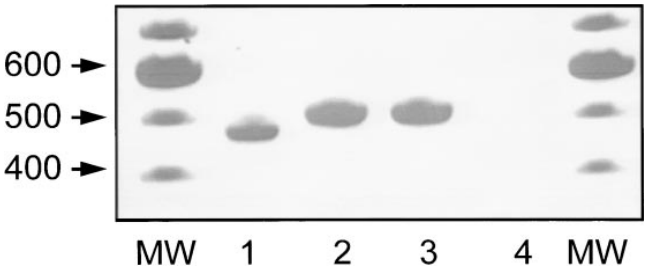

Figure 2. Amplification of PTH/PTHrP receptor cDNA prepared from chondrocytes from the infant with Blomstrand chondrodysplasia (lane *1*), from human SaOS-2 osteosarcoma cells (lane *2*), and from human kidney (lane *3*). PCR was performed using the primer pair 3 described in Table II, which amplifies a fragment which includes exon M5, and whose expected size is 510 bp. The migration of the 100-bp ladder molecular weight standard (*MW* lanes) and the corresponding molecular weights (*arrows*) are also shown. Lane *4*, Amplification performed in the absence of template.

type PTH/PTHrP receptor mRNA were not present. When RNA from the control fibroblasts was studied, the two expected fragments at 191 and 286 bp were observed (Fig. 3). These studies confirm the presence of deleted, but not wild-type specific mRNA in cultured chondrocytes obtained from the infant, whereas only normal PTH/PTHrP receptor mRNA was detected in control samples. Thus, expression of the shortened PTH/PTHrP receptor mRNA encoded by the maternal allele accounted for all detectable transcripts in this patient, indicating that little or no mRNA from the paternal allele was present in the patient's chondrocytes.

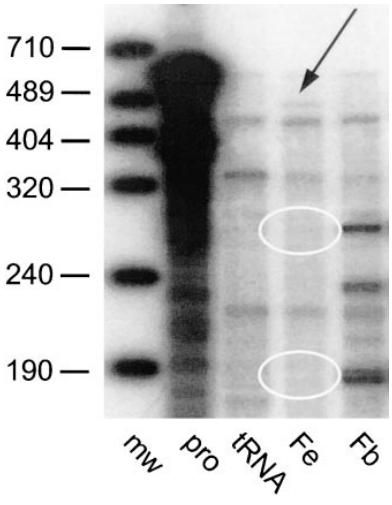

Figure 3. Ribonuclease protection analysis of PTH/PTHrP receptor mRNA expressed in chondrocytes from the fetus with Blomstrand chondrodysplasia (*Fe*) and control fibroblasts (*Fb*). RNase protection assay was performed as described in Methods. 50 μg of total RNA from chondrocytes from the fetus with Blomstrand chondrodysplasia and control fibroblasts were hybridized with a ^{32}P-labeled antisense hPTH/PTHrP receptor riboprobe, as described in Methods. The sequence of the riboprobe corresponds to that of the deleted PTH/PTHrP receptor mRNA. As expected, when RNA from the fetal chondrocytes was studied, bands at 191 and 286 bp corresponding to the wild-type PTH/PTHrP receptor mRNA were not observed (*ellipses*). In contrast, a protected fragment at 477 bp corresponding to the deleted receptor mRNA was seen (*arrow*). When PTH/PTHrP receptor mRNA from control fibroblasts was studied, the two expected fragments at 191 and 286 bp were observed. *mw*, Molecular weight standards; *pro*, unprotected riboprobe; *tRNA*, hybridization of the riboprobe with transfer RNA.

As indicated above, abnormalities in the 3′ untranslated region affecting mRNA stability are unlikely to explain these findings, because analysis of the fetus' genomic DNA from this region was normal. Evidence for genomic rearrangement that might influence expression of the paternal allele was sought, but no rearrangements of the receptor gene were detected by Southern blot analysis of genomic DNA using probes spanning the coding exons and the 5′ flanking promoter regions (data not shown).

Functional properties and expression of the mutant receptor. To investigate the functional consequences of the mutation, a sequence encoding a 12–amino acid epitope tag was added to the COOH-terminal end of the mutant and the wild-type cDNAs, and these sequences were cloned into an expression vector and transiently expressed in COS-7 cells. Transfection of COS-7 cells with increasing amounts of wild-type cDNA led to a dose-dependent increase in cAMP and inositol phosphate production induced by human (h) PTH(1-34) (maximum response, 25–50 ng/well) (Fig. 4, *A* and *B*). By contrast, transfecting the mutant construction (up to 100 ng/well) failed to result in detectable stimulation of cAMP and inositol phosphate production in response to hPTH(1-34) (Fig. 4, *A* and *B*). Similar results were obtained using other PTH/PTHrP receptor ligands [hPTH(1-84) and hPTHrP(1-34)] (data not shown). To investigate the mechanisms responsible for the absence of receptor function, ligand binding by the mutated receptor was evaluated. No [^{125}I]-labeled hPTHrP(1-34) ligand binding to COS-7 cells transfected with the mutated construction was noted, whereas specific binding to cells transfected with the wild-type cDNA was readily detectable (Fig. 4 *C*). To determine whether the mutated receptor was adequately addressed to the plasma membrane, monoclonal antibodies directed against the 12–amino acid tag of the fusion protein were used to detect receptor expression. Interestingly, similar signal intensity was obtained for cells transfected with plasmids containing either wild-type or mutated PTH/PTHrP receptor cDNA, indicating that the two receptors were expressed to an equivalent extent and were both addressed to the plasma membrane (Fig. 4 *D*).

As would be expected for cells that did not express functional PTH/PTHrP receptors, PTH-induced cAMP production was undetectable in chondrocyte-like cells from the infant with Blomstrand chondrodysplasia. In contrast, the patient's chondrocytes adequately responded to forskolin (50 μM) (82-fold increase of cAMP production in response to 50 μM forskolin). Thus, the absence of PTH-induced stimulation of cAMP production in the patient's cells could not be explained by a deficiency in the adenylate cyclase system. Cells from a control subject responded to both PTH and forskolin (6- and 50-fold increases in cAMP, respectively, data not shown).

Discussion

We report that the absence of functional PTH/PTHrP receptors causes Blomstrand chondrodysplasia, a disease characterized by advanced endochondral bone maturation and fetal death. Recent studies in rodents have demonstrated that, in addition to their role in regulating calcium and phosphorus metabolism, PTH/PTHrP receptors play a key role in fetal skeletal development by decelerating the differentiation of growth plate chondrocytes into hypertrophic cells (3, 22). PTH/PTHrP receptors are abundantly expressed by growth

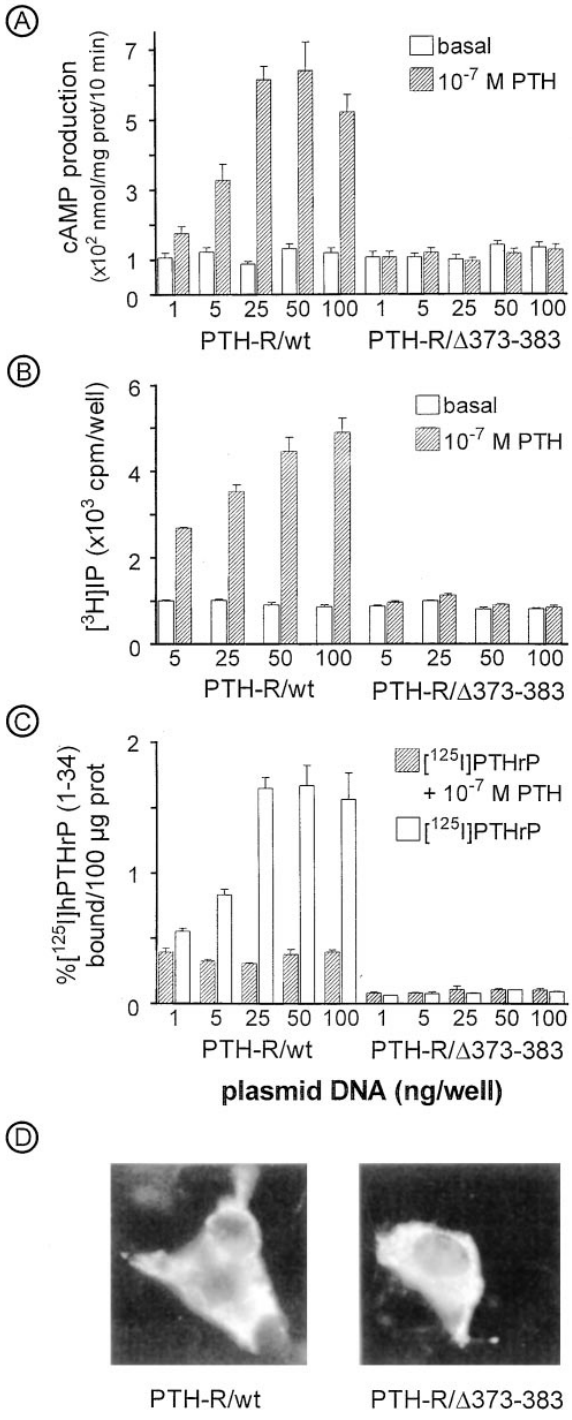

Figure 4. Functional evaluation of wild-type and mutant PTH/PTHrP receptors expressed in COS-7 cells. COS-7 cells were transfected with the indicated amounts of plasmid DNA coding for the wild-type (PTH-R/wt, left) or mutant (PTH-R/Δ373-383, right) PTH/PTHrP receptors, and functional studies were performed 48 h later. (*A*) cAMP production for cells incubated in the absence (basal, *open bars*) or presence of 10^{-7} M hPTH(1-34) (*striped bars*). (*B*) Accumulation of inositol phosphates for cells incubated in the absence (basal, *open bars*) or presence of 10^{-7} M hPTH(1-34) (*striped bars*). No stimulation of cAMP or inositol phosphate production by PTH was detected in untransfected COS-7 cells (data not shown). (*C*) Binding of [^{125}I]hPTHrP(1-34) by cells incubated with radiolabeled ligand only (*open bars*) or radiolabeled ligand in the presence of 10^{-7} M unlabeled hPTH(1-34) (*striped bars*). No binding of [^{125}I]hPTHrP(1-34) by untransfected cells was detectable (data not shown). Data are presented as mean ± SEM for at least two independent experiments, each performed in triplicate. (*D*) Detection of the tagged PTH-R/wt (*left*) and PTH-R/Δ373-383 (*right*) proteins in transfected COS-7 cells by immunofluorescence.

plate chondrocytes at the site of transition between proliferation and hypertrophy (23). Delayed endochondral bone formation is observed in transgenic mice in which proliferating chondrocytes overproduce PTHrP (3). Similarly, delayed endochondral bone formation also occurs in Jansen's metaphyseal chondrodysplasia, a disease in which mutations producing constitutive activation of PTH/PTHrP receptors have been identified (5, 6). Conversely, advanced endochondral bone formation is observed in mice that lack either PTHrP or PTH/PTHrP receptors (2, 4). Furthermore, targeted expression of constitutively active PTH/PTHrP receptors delays endochondral bone formation and rescues mice that lack PTHrP (24). Although the abnormalities observed in the PTH/PTHrP receptor and PTHrP knockout models are generally similar, the phenotype observed in mice lacking the PTH/PTHrP receptor is more severe than that of mice lacking PTHrP. Thus, although a similar phenotype might be seen in patients with defects in the PTHrP gene or PTH/PTHrP receptor gene, the severe abnormalities seen in Blomstrand chondrodysplasia are more reminiscent of those occurring in PTH/PTHrP receptor gene knockout mice.

Analysis of genomic DNA and mRNA coding for the PTH/PTHrP receptor in the fetus with Blomstrand chondrodysplasia demonstrated that the absence of functional receptors resulted from the expression of a nonfunctional receptor coded by the maternal allele, associated with the absence of expression of the paternal allele. Thus, the fetus was a compound heterozygote, compatible with the recessive inheritance pattern described for Blomstrand chondrodysplasia (13–15, 25, 26). The abnormality responsible for the absence of expression of the paternal allele was not identified. No other mutations in the coding exons were present, and no abnormality in mRNA splicing other than that detected in the maternal allele was identified. A number of different mechanisms can result in reduced expression, including imprinting of one allele, mutations in the promoter region, or abnormalities reducing the stability of the mRNA. Results in knockout mice indicate that the gene is not imprinted. No mutations in the 3′ noncoding region were identified. Little is known about the factors that control PTH/PTHrP receptor gene expression in fetal and adult life. Two promoters, P1 and P2, have been described in mice and rats (27, 28). The P1 activity is mainly restricted to the adult kidney, while P2 activity is detected in several fetal and adult tissues, including cartilage and bone. The P2 promoter activity is apparently well conserved between humans and mice. In contrast, P1 activity is weak or absent in humans, whereas a third promoter P3, apparently specific to humans, appears to control the PTH/PTHrP receptor expression in a number of tissues including kidney and bone (29). Southern blots exploring these previously described 5′ promoter regions were normal in the patient, indicating that major rearrangements or deletions of these sequences did not explain the absence of expression of the paternal allele. It is noteworthy that although PTH/PTHrP receptor expression is regulated by factors such as cAMP, 1,25(OH)$_2$ vitamin D, and glucocorticoids, no responsive ele-

ments for these factors have been found in the promoter regions identified to date (27–29). These findings suggest the existence of unidentified enhancer or repressor sequences participating in the regulation of the PTH/PTHrP gene expression.

The 33-bp deletion in exon M5 identified in the maternal allele resulted from a point mutation leading to the creation of a new 3′ splice site with conservation of the reading frame. As in our study, the previously reported cases of creation of 3′ splice sites have involved a G→A mutation at position −2 of the newly created splice site. Because their identification requires characterization of cDNA, the detection of mutations resulting in the creation of a novel splice site is relatively uncommon (30) and has not been described for other G protein–coupled receptors (31).

Previous work has suggested that receptor residues in this fifth transmembrane domain are at or near a site that interacts with the NH_2 terminus of PTH (32). Site-directed mutagenesis of nearby amino acids has also suggested the importance of this region for coupling to signal transduction (33). We found that the deletion in this embedded helical region strongly inhibited the binding of ligand, without preventing receptor expression. It is likely that the deletion reported here disrupts the three-dimensional organization of the receptor, thereby preventing normal ligand recognition.

In view of the essential role played by the PTH/PTHrP receptor in bone and cartilage development, this study strongly supports the conclusion that absence of functional PTH/PTHrP receptors is responsible for Blomstrand chondrodysplasia. This study emphasizes the importance of signaling through this receptor in human fetal bone development, and suggests that abnormalities in components of this ligand/receptor system may contribute to the pathogenesis of other osteochondrodysplasias.

Note added in proof: Since the submission of this study, we have identified in another unrelated patient with Blomstrand chondrodysplasia a homozygous missense inactivating mutation in the PTH/PTHrP receptor (Zhang, P., et al., manuscript submitted for publication).

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