Converting Parathyroid Hormone-related Peptide (PTHrP) into a Potent PTH-2 Receptor Agonist*

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Thomas J. Gardeliat, Michael D. Luck, Geoff S. Jensen, Ted B. Usdin§, and Harald J. Öppner

From the Endocrine Unit, Department of Medicine and Children’s Service, Massachusetts General Hospital and Harvard Medical School Boston, Massachusetts 02114 and the §Laboratory for Cell Biology, National Institute of Mental Health, Bethesda, Maryland 20892

Most of the bone and kidney-related functions of parathyroid hormone (PTH) and parathyroid hormone-related peptide (PTHrP) are thought to be mediated by the PTH/PTHrP receptor. Recently, a homologous receptor, the PTH-2 receptor, was obtained from rat and human brain cDNA libraries. This receptor displayed the remarkable property of responding potently to PTH, but not to PTHrP. To begin to define residues involved in the ligand specificity of the PTH-2 receptor, we studied the interaction of several PTH/PTHrP hybrid ligands and other related peptide analogs with the human PTH-2 receptor. The results showed that two sites in PTH and PTHrP fully account for the different potencies that the two ligands exhibited with PTH-2 receptors; residue 5 (His in PTHrP and Ile in PTH) determined signaling capability, while residue 23 (Phe in PTHrP and Trp in PTH) determined binding affinity. By changing these two residues of PTHrP to the corresponding residues of PTH, we were able to convert PTHrP into a ligand that avidly bound to the PTH-2 receptor and fully and potently stimulated cAMP formation. Changing residue 23 alone yielded [Trp23]hPTHrP-(1–36), which was an antagonist for the PTH-2 receptor, but a full agonist for the PTH/PTHrP receptor. Residues 5 and 23 in PTH and PTHrP thus play key roles in signaling and binding interactions, respectively, with the PTH-2 receptor. Receptor-selective agonists and antagonists derived from these studies could help to identify the biological role of the PTH-2 receptor and to map specific sites of ligand-receptor interaction.

The pharmacological profiles of PTH and PTHrP are nearly identical in most in vitro assay systems; and elevated blood levels of PTH (i.e. primary hyperparathyroidism) or PTHrP (i.e. humoral hypercalcemia of malignancy syndrome) have comparable effects on mineral ion homeostasis (1, 2). The similarities in the biological activities of the two ligands can be explained by their interaction with a common receptor, the PTH/PTHrP receptor, which is expressed abundantly in bone and the kidney (3). The binding of either radiolabeled PTH-(1–34) or PTHrP-(1–36) to the PTH/PTHrP receptor is competitively inhibited by either unlabeled ligand (4, 5); thus, the recognition sites for the two ligands in the PTH/PTHrP receptor probably overlap. In both PTH and PTHrP the 15–34 region contains the principal determinants of binding to the PTH/PTHrP receptor, and although these regions show only minimal sequence homology with only three amino acid identities, each 15–34 peptide can block the binding of either PTH-(1–34) or PTHrP-(1–34) (6–8). Furthermore, the amino-terminal portion of each ligand is required for bioactivity, and these probably interact with the PTH/PTHrP receptor in similar ways, since 8 of the first 13 residues are identical in PTH and PTHrP.

The PTH/PTHrP receptor is a member of a distinct family of G protein-coupled receptors (9, 10) that includes receptors for other peptide hormones such as secretin (11), calcitonin (12), and glucagon (13). Using degenerate oligonucleotides corresponding to conserved regions of the PTH/secretin/calcitonin receptor family, Usdin et al. (14) identified a new receptor cDNA derived from rat and human brain that was most closely related to the PTH/PTHrP receptor (51% overall amino acid sequence identity). This new receptor, the PTH-2 receptor, responded efficiently and specifically to PTH-(1–34), but most strikingly, the PTH-2 receptor did not respond at all to PTHrP (14). This observation implied that structural differences in the PTH and PTHrP ligands determined selectivity for the PTH-2 receptor.

Previously, we had prepared a series of PTH/PTHrP hybrid ligands for the purpose of investigating whether homologous domains of PTH and PTHrP could be interchanged (15). In the present study, we have used these hybrid ligands and derivatized peptide analogs to map regions of PTH and PTHrP that contribute to the ligand selectivity of the PTH-2 receptor. These efforts have led to the identification of two residues in the ligands that play major roles in determining the efficiency of binding and signaling interactions with the PTH-2 receptor.

MATERIALS AND METHODS

Peptides and Reagents—The preparation and initial characterization of PTH/PTHrP hybrid ligands was described previously (15). [Nle8,21,Tyr34]rat (r)PTH-(1–34)NH2 was purchased from Bachem Fine Chemicals (Torrance, CA). The antagonist, [Leu11,D-Trp12]hPTHrP-(7–34)NH2, was purchased from Peninsula Laboratories Belmont, CA. All other peptides were prepared by the biopolymer synthesis facility at Massachusetts General Hospital (Boston, MA) using solid-phase chemistry and Fmoc (N-(9-fluorenyl)methoxycarbonyl) protecting groups. [125I]-[Nle8,21,Tyr34]rPTH-(1–34)NH2 was prepared by chloramine-T oxidation and was high performance liquid chromatography-purified (16). Na125I (2,000 Ci/mmol) was purchased from DuPont NEN. Dulbecco’s modified Eagle’s medium, EGTA/Tris, and 100 × antibiotic mixture (10,000 units/ml penicillin G and 10 mg/ml streptomycin) was from Life Technologies, Inc.; fetal bovine serum was from Hyclone (Logan, UT).

DNA Transfection of COS-7 Cells—The cDNAs encoding the human PTH/PTHrP receptor (17) and the human PTH-2 receptor (18) were carried on the expression vectors pcDNA-1 and pcDNAI/Amp (InVitrogen, San Diego, CA), respectively. COS-7 cells were cultured and transfected as described previously (18). Cells were transfected in 24-well plates using plasmid DNA (200 ng/well) purified by cesium chloride/
ethidium bromide gradient centrifugation. Ligand binding and cAMP accumulation assays were performed 3 days after transfection, by which time the cell density reached 500,000 ± 100,000 cells/well.

Ligand-Receptor Binding—Binding reactions were performed as described previously (18). Each well (final volume = 300 μl) contained 26 fmol of [125I]-[Nle8,21,Tyr34]rPTH-(1–34)NH2 (100,000 cpm) and various amounts (0.4–300 pmol) of an unlabeled competitor ligand, both diluted in binding buffer (50 mM Tris-HCl, pH 7.7, 100 mM NaCl, 5 mM KCl, 2 mM CaCl2, 5% heat-inactivated horse serum, 0.5% heat-inactivated fetal bovine serum). Incubations were at room temperature for 2 h, except for experiments involving Scatchard analysis, which were performed at 4°C for 6 h. At the end of the binding reaction the cells were rinsed three times with 0.5 ml of binding buffer, lysed with 0.5 ml of 5 mM NaOH, and the entire lysate was counted. Nonspecific binding (Bn) was calculated as the total radioactivity bound to cells in the absence of unlabeled PTH ligand, minus nonspecific binding of tracer. IC50 values (dose of competing ligand which resulted in 50% inhibition of [125I]-[Nle8,21,Tyr34]rPTH-(1–34)NH2 binding) were determined from plots of log(B/Bn – B) versus log(competitor).

For Scatchard analyses, homologous competition binding studies were performed that used [125I]-[Nle8,21,Tyr34]rPTH-(1–34)NH2 (26 fmol/well) and varying amounts (1.2–300 pmol) of the same unlabeled ligand. Estimates of the number of receptors/cell derived from these studies assumed a single class of binding sites and a transfection efficiency of 20% (18).

Intracellular Cyclic AMP—Transfected COS-7 cells were rinsed with 500 μl of binding buffer and 200 μl of IBMX buffer (Dubelco’s modified Eagle’s medium containing 2 mM IBMX, 1 mg/ml bovine serum albumin, 35 mM Hepes-NaOH, pH 7.4) and 100 μl of binding buffer or binding buffer containing various amounts of a PTH or PTHrP analog were added. The plates were incubated for 60 min at room temperature. The buffer was then withdrawn, and the cells were lysed by placing the plates on powdered dry ice and adding 0.5 ml of 50 mM HCl. The acid lysate was diluted 1/30 in distilled H2O, and an aliquot (5–50 μl) was analyzed for cAMP content by radioimmunoassay. Emax values, doses of ligand that resulted in 50% of the maximum response (Emax), obtained for that ligand with the relevant receptor, were determined from plots of log(E/Emax – E) versus log(1/[L]) or log(1/[L] + IC50).

The buffer was then withdrawn, and the cells were lysed by placing the plates on powdered dry ice and adding 0.5 ml of 50 mM HCl. The acid lysate was diluted 1/30 in distilled H2O, and an aliquot (5–50 μl) was analyzed for cAMP content by radioimmunoassay. Emax values, doses of ligand that resulted in 50% of the maximum response (Emax), obtained for that ligand with the relevant receptor, were determined from plots of log(1/[L]) versus log(1/[L] + IC50).

Results

The binding and cAMP signaling responses obtained for PTH-(1–34) ([Tyr34]hPTH-(1–34)NH2) and PTHrP-(1–36) ([Tyr36]hPTHrP-(1–36)NH2) in COS-7 cells expressing the human PTHrP/PTH and human PTH-2 receptors are shown in Fig. 1. With the PTHrP/PTH receptor, both PTH-(1–34) and PTHrP-(1–36) fully inhibited binding of the radioligand, [125I]-[Nle8,21,Tyr34]rPTH-(1–34)NH2, although PTH-(1–34) was 4.8-fold more potent than PTHrP-(1–36) (IC50 values were 39 and 285 nM, respectively, p < 0.001; Fig. 1A and Table I).

With cells expressing the PTH-2 receptor, PTH-(1–34) bound with high apparent affinity, while PTHrP-(1–36) bound poorly with a potency that was more than 100-fold weaker than that of PTH-(1–34) (Fig. 1B and Table I).

Both PTH-(1–34) and PTHrP-(1–36) were full and potent agonists of cAMP production in COS-7 cells expressing the PTHrP/PTH receptor (Fig. 1C and Table I). In contrast, only PTH-(1–34) was a potent agonist with the PTH-2 receptor, while PTHrP-(1–36) was nearly inactive (Fig. 1D). These results confirm the markedly different cAMP-stimulating activities of PTH and PTHrP with the PTH-2 receptor (14) and demonstrate that at least part of the signaling defect that PTHrP has with this receptor can be attributed to weak binding interactions.

Scatchard analyses of competition binding data indicated that the PTH-2 receptor was expressed on the surface of COS-7 cells to levels that were 5-fold lower than the level of expression attained by the PTH/PTHrP receptor (Fig. 2, A and B). This difference in expression levels is likely to explain the lower cAMP responses compared with the PTH/PTHrP receptor (Fig. 1). Although, the reason for the lower expression of the PTH-2 receptor is unclear at present, expression was fully adequate for comparing the interactions of different PTH and PTHrP analogs with this receptor.

FIG. 1. Ligand-binding and cAMP signaling profiles of PTH/PTHrP and PTH-2 receptors. COS-7 cells transfected with human PTH/PTHrP receptors (A and C) or human PTH-2 receptors (B and D) were evaluated for competitive inhibition of radi iodinated binding (A and B) or ligand-induced cAMP accumulation (C and D). Binding studies were performed at room temperature for 2 h and used [125I]-[Nle8,21,Tyr34]rPTH-(1–34)NH2 (100,000 cpm/well) as radioligand and varying amounts of unlabeled [Tyr36]hPTHrP-(1–34)NH2 (●) or [Tyr36]hPTHrP-(1–36)NH2 (▲) as competitor ligands. The maximum amount of tracer that specifically bound to PTH/PTHrP and PTH-2 receptors in the absence of competitor (Bn) was ~25 and ~5% of total radioactivity added, respectively. For cAMP stimulation assays, cells were treated with ligand for 1 h at room temperature in the presence of IBMX. Symbols in C and D are the same as in A and B. Data are the mean (± S.E.) of 3–10 experiments, each performed in duplicate.

2 It is of interest to note that hybrid 1 and hybrid 5, as well as (H7)[Tyr34]rPTH-(1–34), competed very poorly for binding to the PTH/PTHrP receptor, yet efficiently stimulated cAMP formation (Table I). We have found that radiiodinated hybrid 5 peptide binds efficiently to the PTH/PTHrP receptor in COS-7 cells (specific binding ~8% of total radioactivity added, T. Gardella and H. Ju¨ppner, unpublished data). It thus appears that these altered ligands bind to the PTH/PTHrP receptor, but at a site that does not fully overlap with that used by [125I]-[Nle8,21,Tyr34]rPTH-(1–34)NH2.
Table 1

Binding to PTH/PTHrP or PTH-2 receptors. COS-7 cells transfected with plasmid DNA (200 ng/well) encoding either the human PTH/PTHrP receptor (A) or human PTH-2 receptor (B) were evaluated in competition binding studies performed for 6 h at 4 °C, as described under “Materials and Methods.” The radioligand was [125I]Nle-21, Tyr-34]PTH-(1–34)amide (100,000 cpm/well), and the competitor ligand was the same unlabeled peptide. Data are the mean (±S.E.) of data from the number of experiments indicated (n), each performed in duplicate. ND, not determined.

| Ligand | Binding, IC<sub>50</sub> (nM) | cAMP | PTH/PTHrP receptor | Binding, IC<sub>50</sub> (nM) | cAMP | PTH-2 receptor |
|--------|-----------------|------|-------------------|-----------------|------|----------------|
| n | pmol/well | n | pmol/well | n | pmol/well | n | pmol/well | n | pmol/well |
| PTH-(1–34) | 39 ± 4 | 7.06 ± 0.18 | 113 ± 13 | 5 | 16 ± 4 | 7.06 ± 0.18 | 81 ± 5 | 5 | 2,140 ± 250 | 10 | >1,000 | 11 | 1 ± 5 | 5 |
| PTHrP-(1–36) | 285 ± 47 | 10.32 ± 0.04 | 103 ± 10 | 5 | 706 ± 96 | 4 | >1,000 | 11 | 5.3 ± 0.6 | 3 |
| Hybrid-1 | 7,900 ± 370 | 4 | 0.23 ± 0.04 | 140 ± 17 | 3 | 500 ± 62 | 4 | >1,000 | 11 | 6.2 ± 0.4 | 3 |
| Hybrid-2 | 4,970 ± 1,560 | 4 | 0.43 ± 0.16 | 133 ± 14 | 3 | 11 ± 3 | 4 | >1,000 | 11 | 6.7 ± 0.6 | 4 |
| Hybrid-3 | 19 ± 6 | 4 | 0.27 ± 0.06 | 107 ± 17 | 4 | 6,050 ± 990 | 4 | >1,000 | 11 | 1 ± 4 | 4 |
| Hybrid-4 | 1,000 ± 471 | 4 | 0.42 ± 0.13 | 95 ± 9 | 4 | 210 ± 4 | 2.1 ± 0.5 | 58 ± 12 | 3 |
| [Ile<sup>6</sup>]PTHrP-(1–36) | 41 ± 11 | 6 | 0.26 ± 0.08 | 112 ± 9 | 3 | 695 ± 171 | 6 | 7.0 ± 2.1 | 80 ± 5 | 3 |
| [Trp<sup>6</sup>]PTHrP-(1–36) | 47 ± 8 | 5 | 0.26 ± 0.05 | 123 ± 14 | 4 | 30 ± 7 | 5 | >1,000 | 12 ± 1 | 4 |
| [Ile<sup>6</sup>, Trp<sup>6</sup>]PTHrP-(1–36) | 16 ± 3 | 5 | 0.21 ± 0.06 | 109 ± 10 | 4 | 10 ± 1 | 5 | 0.5 ± 0.3 | 65 ± 7 | 4 |
| [His<sup>6</sup>]PTH-(1–34) | 5,100 ± 1,000 | 4 | 0.93 ± 0.37 | 116 ± 19 | 3 | 249 ± 18 | 4 | >1,000 | 10 ± 1 | 3 |
| [Phe<sup>6</sup>]PTH-(1–34) | 95 ± 11 | 4 | 0.23 ± 0.02 | 104 ± 16 | 4 | 453 ± 53 | 4 | 7.6 ± 2.2 | 50 ± 8 | 4 |
| [His<sup>6</sup>, Phe<sup>6</sup>]PTH-(1–34) | >10,000 | 3 | 1.18 ± 0.29 | 110 ± 19 | 3 | >10,000 | 3 | >1,000 | 7.1 ± 0.9 | 3 |
| PTH-(15–36) | >10,000 | 4 | ND | 4 | 9,970 ± 970 | 4 | ND |
| [Trp<sup>6</sup>]PTHrP-(15–36) | >10,000 | 4 | ND | 4 | 370 ± 100 | 4 | ND |
| [Glu<sup>6</sup>, Trp<sup>6</sup>]PTHrP-(15–36) | >10,000 | 4 | ND | 4 | 137 ± 36 | 4 | ND |
| PTH-(15–34) | >10,000 | 3 | ND | 3 | 624 ± 116 | 3 | ND |
| [Phe<sup>6</sup>]PTH-(15–34) | >10,000 | 4 | ND | 4 | >10,000 | 4 | ND |

Fig. 2. Scatchard analysis of PTH-(1–34) binding to PTH/PTHrP or PTH-2 receptors. COS-7 cells transfected with plasmid DNA (200 ng/well) encoding either the human PTH/PTHrP receptor (A) or human PTH-2 receptor (B) were evaluated in competition binding studies performed for 6 h at 4 °C, as described under “Materials and Methods.” The radioligand was [125I]Nle-21, Tyr-34]PTH-(1–34)amide (100,000 cpm/well), and the competitor ligand was the same unlabeled peptide. Data are the mean (±S.E.) of data from the number of experiments indicated (n), each performed in duplicate. ND, not determined.

The most informative hybrid analogs were hybrid 3 and hybrid 4. Hybrid 4, PTHrP-(1–21)/PTH-(22–34), bound to the PTH-2 receptor with high potency (IC<sub>50</sub> = 11 nM), similar to PTH-(1–34) (Table I). Extending the PTHrP sequence by two residues yielded hybrid 3, PTHrP-(1–23)/PTH-(24–34), which competed poorly for binding to the PTHrP receptor (IC<sub>50</sub> = 1,000 nM) and bound with even weaker potency to the PTH-2 receptor (IC<sub>50</sub> = 6,000 nM). In this regard, the binding profile of hybrid 3 resembled that of PTHrP-(1–36).

Substitutions at positions 22 and 23—comparing the structures and binding properties of hybrids 3 and 4 led us to the hypothesis that the divergent residues at positions 22 and/or 23 contribute to PTH-2 receptor binding selectivity. To initially test the role of these residues in receptor interaction, we prepared shorter length PTHrP-(15–36) fragments in which Phe<sup>22</sup> and Trp<sup>23</sup> were replaced by the corresponding Glu and Trp residues of PTH. The unsubstituted parent peptide PTHrP-(15–36) competed weakly for binding to the PTH-2 receptor (Fig. 4B). The binding potency improved considerably with the combined Glu<sup>22</sup> and Trp<sup>23</sup> substitution. Most of this enhancement could be accounted for by the single substitution of Trp for Phe (Table I). Binding of either unsubstituted or substituted PTHrP-(15–36) fragments to the PTHrP receptor could not be detected in these competition assays (Fig. 4A).

The Phe<sup>23</sup> to Trp modification was introduced into full-length PTHrP-(1–36), where it was found to enhance binding potency for the PTH-2 receptor by 71-fold, as compared with the binding of PTHrP-(1–36) itself (Fig. 4B, Table I). The reciprocal change of Trp<sup>23</sup> to Phe in PTH-(1–34) led to a 28-fold reduction in binding potency for the PTH-2 receptor, as compared with unsubstituted PTH-(1–34) (Fig. 5D and Table I). Interestingly, the divergent residues at position 23 could also account for some of the difference in the apparent binding affinities that PTH and PTHrP exhibited for the PTHrP receptor. Thus [Trp<sup>23</sup>]PTH-(1–36) bound to the PTHrP receptor with 6-fold stronger affinity than did PTHrP-(1–36) (Fig. 5A; Table I), and [Phe<sup>23</sup>]PTH-(1–34) bound to this receptor with 2–3-fold weaker affinity than did PTH-(1–34) (Fig. 5C; Table I). The effect of these position 23 changes on binding to
the PTH/PTHrP receptor could be predicted from the binding properties that hybrid 3 and hybrid 4 displayed with this receptor (Table I).

Effects of Residues 5 and 23 on cAMP Signaling—The above substitutions at position 23 in PTH or PTHrP had little or no effect on cAMP signaling by the PTH-2 receptor; [Trp23]PTHrP-(1–36) was still inactive and [Phe23]PTH-(1–34) was nearly a full agonist (Fig. 6, B and D; Table I). The high potency of hybrid 2, PTH-(1–14)/PTHrP-(15–34), in stimulating cAMP production with the PTH-2 receptor, compared with the weak activity of hybrid 1, PTHrP-(1–14)/PTH-(15–34) (Table I) suggested that residues within the 1–14 sequence of PTH and PTHrP were involved in modulating the signaling properties of the two ligands with this receptor.

Our previous binding studies in ROS 17/2.8 cells revealed that position 5 (Ile in PTH and His in PTHrP) was one divergent site in the 1–14 region that could dramatically affect ligand-receptor interaction (15). We, therefore, tested the effects of reciprocal position 5 substitutions on signaling interactions with the PTH-2 receptor. Strikingly, [Ile5]PTHrP-(1–36) became a full agonist with the PTH-2 receptor, promoting the same maximal cAMP response as that attained by PTH-(1–34) (Fig. 6B). However, the EC50 of the [Ile5]PTHrP-(1–36) response was still 12-fold higher than that of PTH-(1–34) (EC50 values = 7 and 0.6 nm, respectively). We, therefore, combined the Ile5 substitution with the affinity-enhancing Trp23 modification. The resulting analog, [Ile5,Trp23]PTHrP-(1–36), was as potent and efficacious as PTH-(1–34) in both binding and cAMP production with the PTH-2 receptor (Fig. 6B, Table I).

The observation that [Trp23]PTHrP-(1–36) (histidine at position 5) bound to the PTH-2 receptor without stimulating cAMP production suggested that this analog might function as a PTH-2 receptor antagonist. Fig. 7 shows that this analog was indeed at least as potent as [Leu11,D-Trp12]hPTHrP-(7–34)NH2, a highly potent PTH/PTHrP receptor antagonist (19), in inhibiting PTH-(1–34)-induced activation of the PTH-2 receptor. With the PTH/PTHrP receptor, [Trp23]PTHrP-(1–36) was not an antagonist, but instead augmented the agonist response to PTH-(1–34).

**FIG. 3.** Primary structures of PTH, PTHrP, and hybrid ligands. The biologically active regions of PTH, PTHrP, and the PTH/PTHrP hybrid ligands used in this study are shown. Sequences corresponding to PTHrP are shaded. All peptides contained the carboxyl-terminal modification of tyrosineamide and otherwise corresponded to the native human PTH or human PTHrP sequence.

**FIG. 4.** Effects of substitutions at position 23 on the binding properties of PTH-(15–34) and PTHrP-(15–36) carboxyl-terminal fragments. The binding properties of PTH-(15–34) and PTHrP-(15–36) fragment analogs to COS-7 cells expressing either PTH/PTHrP receptors (A) or PTH-2 receptors (B) are shown. Competition binding studies were performed at room temperature for 2 h with [125I]-[Nle8,21,Tyr34]rPTH-(1–34)amide (100,000 cpm/well) as radioligand. The unlabeled competitor ligands used and corresponding symbols are: [Tyr36]hPTHrP-(15–36)NH2 (●); [Glu22,Trp23,Tyr36]hPTHrP-(15–36)NH2 (▲); [Trp23,Tyr36]hPTHrP-(15–36)NH2 (○); and [Trp23]hPTH-(15–34)NH2 (□). Data are the mean (+S.E.) of three or four experiments, each performed in duplicate.

**FIG. 5.** Effects of substitutions at position 5 and 23 on the binding of PTH-(1–34) and PTHrP-(1–36) to PTH/PTHrP and PTH-2 receptors. The binding of PTHrP-(1–36) analogs (A and B) and PTH analogs (C and D) to COS-7 cells expressing either PTH/PTHrP receptors (A and C) or PTH-2 receptors (B and D) are shown. Competition binding studies (room temperature/2 h) were performed with [125I]-[Nle8,21,Tyr34]rPTH-(1–34)amide (100,000 cpm/well) as radioligand. The unlabeled PTHrP analogs used in A and B and the corresponding symbols are: [Tyr36]hPTHrP-(1–36)NH2 (●); [Ile5,Tyr36]hPTHrP(1–36)NH2 (▲); [Trp23,Tyr36]hPTHrP-(1–36)NH2 (○); and [Ile5,Trp23,Tyr36]hPTHrP-(1–36)NH2 (Ç). The unlabeled PTH analogs used in C and D and the corresponding symbols are: [His5,Tyr34]hPTH-(1–34)NH2 (●); [His5,Tyr34]hPTHrP-(1–34)NH2 (▲); [Phe23,Tyr34]hPTH-(1–34)NH2 (○); and [His5,Phe23,Tyr34]hPTH-(1–34)NH2 (Ç). The binding curves of [Tyr36]hPTHrP-(1–36)NH2 and [Tyr34]hPTH-(1–34)NH2 from Fig. 1, A and B, are shown again for comparison. Data are the mean (+S.E.) of 3–10 experiments, each performed in duplicate.
Within 15–34 portions of the ligand. Evidence for this included the putative intramolecular interactions between the 1–14 and these earlier studies suggested that this residue was involved in the interaction with the PTH/PTHrP receptor (15).

For ROS 17/2.8 cells, we identified residue 5 as an important determinant for interacting with the PTH-2 receptor. In our previous competition binding studies in COS-7 cells expressing either PTH/PTHrP receptors (A and C) or PTH-2 receptors (B and D) are shown. Cells were treated with the indicated amount of peptide for 1 h at room temperature in the presence of IBMX. The PTHrP analogs used in A and B and the corresponding symbols are: [Tyr34]hPTH-(1–34)NH2 (●); [Phe23,Tyr36]hPTHrP-(1–36)NH2 (□); [Ile5,Tyr36]hPTHrP-(1–36)NH2 (▲); and [His5,Phe23,Tyr36]hPTH-(1–36)NH2 (△). The corresponding basalcAMP levels in cells not treated with peptide were 8.4 ± 0.3 and 9.0 ± 1.7 pmol/well, respectively. Data are the mean (±S.E.) of two experiments, each performed in duplicate.

**DISCUSSION**

These studies show that the failure of PTHrP to efficiently bind to and activate the PTH-2 receptor can be attributed to two residues, phenylalanine at position 23 and histidine at position 5. In PTH, which is fully potent with the PTH-2 receptor, these residues are replaced by tryptophan and isoleucine, respectively. By exchanging residues 5 and 23 of PTHrP with the corresponding residues of PTH, we could convert PTHrP into a full and potent PTH-2 receptor agonist.

The histidine at position 5 in PTHrP blocked activation of the PTH-2 receptor. In our previous competition binding studies in ROS 17/2.8 cells, we identified residue 5 as an important determinant for interacting with the PTH/PTHrP receptor (15). These earlier studies suggested that this residue was involved in putative intramolecular interactions between the 1–14 and 15–34 portions of the ligand. Evidence for this included the ability of the His5→Ile substitution in PTHrP to “cure” the deleterious effects that carboxyl-terminal substitutions (i.e. at positions, 19, 21, and 24) had on binding potency. It now appears that the full role of residue 5 in PTH and PTHrP is more complex, since our new data indicate that this residue strongly modulates signaling interactions with the PTH-2 receptor.

The weak binding of PTHrP to the PTH-2 receptor can be attributed to the phenylalanine residue at position 23; replacing this residue by the corresponding tryptophan of PTH improved binding to the PTH-2 receptor by 71-fold. The substitution of Trp for Phe23 could also explain the 6-fold weaker binding that PTHrP-(1–36) exhibited for the PTH/PTHrP receptor, as compared with PTH-(1–34) (Table I). Such weaker binding of PTHrP was observed in our earlier studies with the cloned human PTH/PTHrP receptor expressed in COS-7 cells; although nearly equivalent binding potencies were seen with the d-trp rat PTHrP receptor (17). Earlier studies with the rat PTHrP receptor endogenous to ROS 17/2.8 cells also found comparable binding potencies for the two ligands (4, 5). It may be expected, therefore, that the rat and human PTH/PTHrP receptors, and the human PTH-2 receptor, each differ at a site or sites that recognize residue 23 in the ligand.

The effects on activity of a variety of modifications at position 23 in PTH have been investigated previously in studies with PTH/PTHrP receptors. Rosenblatt and co-workers found that the addition of a bulky ortho-nitrophenylsulfonyl group to the indole nitrogen of Trp23 had little or no effect on bioactivity (20), nor did a naphthylalanine substitution at this site (21). However, methylation of the backbone nitrogen at Phe23, or the substitution of d-Trp for Trp23, severely reduced receptor-binding affinity (22). Our own mutational analysis of residues 23–34 of PTH-(1–84) showed that cysteine substitution of Trp23 reduced bioactivity by more than 10-fold, while leucine substitution reduced activity by 50% (23). NMR studies on PTH analogs (24–27) and PTHrP analogs (28, 29) suggest that the aromatic residue at this position in either ligand is involved in intramolecular hydrophobic interactions. Residue 23 may, therefore, be important for maintaining ligand structure, as well as modulating receptor-binding interactions.

Because the PTHrP-(1–36) analog containing the Trp23 modification exhibited high binding affinity for the PTH-2 receptor without having detectable agonist activity, we evaluated it as a potential PTH-2 receptor antagonist. This analog was at least as potent as [Leu11,D-Trp12]hPTHrP-(7–34)NH2 (●), or [Trp23,Tyr36]hPTH-(1–36) (△), plus a near-maximal stimulatory dose of the agonist, [Tyr34]hPTH-(1–34)NH2 (1 nM), and then incubated in the presence of IBMX for 30 min at room temperature. The resulting intracellular cAMP levels are expressed as the percent of the cAMP levels in cells treated with [Tyr34]hPTH-(1–34)NH2 (1 nM), which were 167 ± 17 and 70 ± 8 pmol/well for the PTHrP receptor and the PTH-2 receptor, respectively. The corresponding basal cAMP levels in cells not treated with peptide were 8.4 ± 0.3 and 9.0 ± 1.7 pmol/well, respectively. Data are the mean (±S.E.) of two experiments, each performed in duplicate.

**Antagonism of PTH-(1–34)-induced cAMP formation in COS-7 cells expressing PTH/PTHrP or PTH-2 receptors.** COS-7 cells expressing either PTH/PTHrP receptors or PTH-2 receptors were treated with various doses of [Leu11,d-Trp12]hPTHrP-(7–34)NH2 (●), or [Trp23,Tyr36]hPTH-(1–36) (△), plus a near-maximal stimulatory dose of the agonist, [Tyr34]hPTH-(1–34)NH2 (1 nM), and then incubated in the presence of IBMX for 30 min at room temperature. The resulting intracellular cAMP levels are expressed as the percent of the cAMP levels in cells treated with [Tyr34]hPTH-(1–34)NH2 (1 nM), which were 167 ± 17 and 70 ± 8 pmol/well for the PTHrP receptor and the PTH-2 receptor, respectively. The corresponding basal cAMP levels in cells not treated with peptide were 8.4 ± 0.3 and 9.0 ± 1.7 pmol/well, respectively. Data are the mean (±S.E.) of two experiments, each performed in duplicate.
tophan at this site, as is found in all native PTH ligands, is fully compatible with binding to the PTH/PTHrP receptor. It may be that phenylalanine at position 23 in PTHrP has been selected for its ability to inhibit binding to the PTH-2 receptor. Likewise, the conserved histidine at position 5 in PTHrP may have been selected for, because it blocks signaling interactions with the PTH-2 receptor. Together, these two conserved residues in PTHrP would ensure that productive interactions with the PTH-2 receptor do not occur.

In summary, we have identified two sites in PTH and PTHrP that account for the ligand selectivity of the PTH-2 receptor. We are now constructing chimeras between the PTH-2 and PTH/PTHrP receptors, as one approach toward identifying the cognate receptor sites involved in this ligand selectivity. The functional profiles of such receptor chimeras interacting with modified PTH and PTHrP ligands should help refine and constrain models of the complexes formed between these peptide ligands and their G protein-coupled receptors.

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