Residues in the Membrane-spanning and Extracellular Loop Regions of the Parathyroid Hormone (PTH)-2 Receptor Determine Signaling Selectivity for PTH and PTH-related Peptide*

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The parathyroid hormone (PTH)-2 receptor displays strong ligand selectivity in that it responds fully to PTH but not at all to PTH-related peptide (PTHrP). In contrast, the PTH-1 receptor (PTH/PTHrP receptor) responds fully to both ligands. Previously it was shown that two divergent residues in PTH and PTHrP account for PTH-2 receptor selectivity; position 23 (Trp in PTH and Phe in PTHrP) determines binding selectivity and position 5 (Ile in PTH and His in PTHrP) determines signaling selectivity. To identify sites in the PTH-2 receptor involved in discriminating between His5 and Ile5, we constructed PTH-2 receptor/PTH-1 receptor chimeras, expressed them in COS-7 cells, and tested for cAMP responsiveness to [Trp23]PTHrP-(1–36), and to the nondiscriminating peptide [Ile5,Trp23]PTHrP-(1–36) (the Phe23 → Trp modification enabled high affinity binding of each ligand to the PTH-2 receptor). The chimeras revealed that the membrane-spanning/loop region of the receptor determined His5/Ile5 signaling selectivity. Subsequent analysis of smaller cassette substitutions and then individual point mutations led to the identification of two single residues that function as major determinants of residue 5 signaling selectivity. These residues, Ile241 at the extracellular end of transmembrane helix 3, and Tyr318 at the COOH-terminal portion of extracellular loop 2, are replaced by Leu and Ile in the PTH-1 receptor, respectively. The results thus indicate a functional interaction between two residues in the core region of the PTH-2 receptor and residue 5 of the ligand.

EXPERIMENTAL PROCEDURES

Peptides and Reagents—The preparation and initial characterization of [Trp33,Tyr36]PTHrP-(1–36)amide and [Ile6,Trp23,Tyr36]PTHrP-(1–

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cassette and point mutations were introduced by oligonucleotide-directed mutagenesis (18). Panels A-D show cAMP responses; one experiment performed in duplicate and representative of five others, is shown. Panels E-H show results from competition binding studies which used $^{125}$I-[Nle$^{8,21}$,Tyr$^{34}$]rPTH-(1–34)amide as a radioligand; data (mean ± S.E.) from six separate experiments, each performed in duplicate, were combined.

36amide was described previously (14). Herein, these two peptides are referred to as [Trp$^{23}$]PTHrP-(1–36) and [Ile$^5$,Trp$^{23}$]PTHrP-(1–36), respectively. These PTHrP analogs, and other peptides used in the study, were prepared by the biopolymer synthesis facility at Massachusetts General Hospital (Boston, MA), as were the DNA oligonucleotides used in receptor mutagenesis experiments. The PTH analog [Nle$^{8,21}$,Tyr$^{34}$]rPTH-(1–34)amide was radioiodinated by the chloramine-T procedure, and the product was purified by reverse phase high performance liquid chromatography (16). $^{125}$I-Na (2,000 Ci/mmol) was used. This miniprep DNA was quantified by ethidium bromide staining of agarose gels, and was transfected at a concentration of 100 units/ml. Cells were transfected in 24-well plates using plasmid DNA (200 ng/well) that was purified by cesium chloride/ethidium bromide gradient centrifugation, except for the initial screening of the cassette mutants, in which phenol-extracted miniprep DNA was used. COS-7 cells were cultured at 37 °C as has been found for other G protein-coupled receptors (19). The cells were then used for binding and cAMP stimulation assays.

Radioligand-receptor Binding—Binding reactions were performed as described previously (18). Each well (final volume = 300 μl) contained 26 fmol of $^{125}$I-[Nle$^{8,21}$,Tyr$^{34}$]rPTH-(1–34)NH$_2$ (100,000 cpm) and various amounts (0.4–300 pmol) of unlabeled competitor ligand; peptides were diluted in binding buffer (50 mM Tris-HCl, pH 7.7, 100 mM NaCl, 5 mM KCl, 2 mM CaCl$_2$, 5% heat-inactivated horse serum, 0.5% heat-inactivated fetal bovine serum). Incubations were at room temperature for 2 h, except for experiments performed for Scatchard analysis, which were performed at 4 °C for 6 h. At the end of the binding reactions the cells were rinsed 3 times with 0.5 ml of binding buffer, lysed with 0.5 ml of 9 M NaOH, and the entire lysate was counted. Non-specific binding of tracer (NSB), determined in wells containing 1 μM [Nle$^{8,21}$,Tyr$^{34}$]rPTH-(1–34)NH$_2$, was 1–1.5% of total counts added. Maximum specific binding (B0) was calculated as the total radioactivity bound to cells in the absence of unlabeled ligand minus NSB. IC$_{50}$ values (dose of competing
ligand that resulted in 50% inhibition of $^{125}$I-[Nle$^5$,Trp$^{23}$]rPTH-(1–34)NH$_2$ binding) were determined from plots of log($B/B_0 - B$) versus log$[ligand]$, where $B_0$ is the maximum dose (1 M) of the ligand that resulted in 50% inhibition of $^{125}$I-[Nle$^5$,Trp$^{23}$]rPTH-(1–34)NH$_2$ binding. Cell surface receptor numbers were estimated from Scatchard analyses of homologous competition binding studies that were performed with $^{125}$I-[Nle$^5$,Trp$^{23}$]rPTH-(1–34)NH$_2$ (26 fmol/well) and varying amounts (12–300 pmol) of the same unlabeled ligand. Calculations of the number of receptors per cell assumed a single class of binding sites and a transfection efficiency of 20% (6, 20).

Intracellular Cyclic AMP—Transfected COS-7 cells were rinsed with 500 µl of binding buffer, and 200 µl of IBMX buffer (Dulbecco’s modified Eagle’s medium containing 2 mM 3-isobutyl-1-methylxanthine, 1 mg/ml bovine serum albumin, 35 mM Hepes-NaOH, pH 7.4) and 100 µl of binding buffer or binding buffer with various amounts of peptide added. The plates were incubated for 60 min at room temperature; the buffer was then withdrawn and the cells were lysed by adding 0.5 ml of 50 mM HCl and freezing. The diluted lysate (1:30 in distilled H$_2$O) was analyzed for cyclic AMP content by radioimmunoassay. For the initial screening of the mutants, we compared the cAMP response of each receptor to the corresponding dose of ligand (6).

RESULTS

The difference in the ligand selectivities of the PTH-1 receptor and the PTH-2 receptor can be seen in the cAMP response profiles shown in Fig. 1, panels A and B. The PTH-1 receptor responded fully and equally to both [Ile$^5$,Trp$^{23}$]rPTH-(1–36) and [Ile$^5$,Trp$^{23}$,Tyr$^{36}$]rPTH-(1–36)amide and [Trp$^{23}$]rPTH-(1–36) but not at all to [Trp$^{23}$,Tyr$^{36}$]rPTH-(1–36), which has histidine at position 5. In contrast, the PTH-2 receptor responded fully to [Ile$^5$,Trp$^{23}$]rPTH-(1–36) but not at all to [Trp$^{23}$]rPTH-(1–36). The ligand selectivity of the PTH-2 receptor is not due to a difference in binding affinities because the two analogs exhibited comparable potencies in their ability to inhibit the binding of $^{125}$I-[Nle$^5$,Trp$^{23}$]rPTH-(1–34)NH$_2$ to its response to the PTH-2 receptor in these experiments is due primarily to the Phe$^{23}$ → Trp modification; this substitution of a PTHrP residue by the corresponding PTH residue markedly enhances binding potency at the PTH-2 receptor without affecting cAMP signaling (14). A small improvement in binding potency was also seen for the His$^5$ → Ile modification, however, this improvement was not receptor specific (Fig. 1, E and F) and was much smaller in magnitude than the effect of the substitution on PTH-2 receptor signaling.

To localize the region of the PTH-2 receptor involved in His$^5$-Ile$^5$ signaling selectivity, we constructed a pair of chimeras in which the amino-terminal extracellular domains of the human PTH-1 and PTH-2 receptors were reciprocally interchanged and tested the chimeras in cAMP stimulation assays for responsiveness to [Trp$^{23}$]rPTH-(1–36) and to the nondiscriminating control peptide [Ile$^5$,Trp$^{23}$]rPTH-(1–36). The 1E2 receptor chimera, which has the amino-terminal extracellular...
domain of the PTH-1 receptor connected (via an EcoRI site) to the mid- and carboxyl-terminal region of the PTH-2 receptor, discriminated between the two ligands (Fig. 1C), whereas the reciprocal chimera 2E1 responded fully to each ligand (Fig. 1D). These results indicated that the membrane-spanning and loop portion of the receptor determines His5/Ile6 signaling selectivity.

To further localize the sites involved in residue 5 selectivity, we replaced most of the divergent residues in the membrane-spanning helices and extracellular connecting loops of the PTH-2 receptor with the corresponding residues of the PTH-1 receptor. As shown in Fig. 2, these residues were replaced either by cassette substitution or, for two of the sites (Ala325 and Gly327), by single residue point mutation. All but 3 of the 21 mutant receptors were functional and adequately expressed on COS-7 cells, as judged by the cAMP response to [Ile5,Trp23]PTH-P-(1–34) and the binding of radioiodinated [Nle8,21,Tyr24]rPTH-(1–34)amide (Table 1). The three cassette mutants showing poor cAMP responsiveness and little or no PTH binding, P2R-Cast#2, P2R-Cast#7 and P2R-Cast#18, were possibly not expressed on the cell surface and were considered uninformative. Of the functional mutant receptors, three displayed increased responsiveness to [Trp25]PTHrP-(1–36). These three cassettes are predicted to be located in the amino-terminal portion of extracellular loop 1 (P2R-Cast#8), the extracellular end of helix 3 (P2R-Cast#8), and at the COOH-terminal end of extracellular loop 2 (P2R-Cast#13) (Fig. 2). For each of these three receptor mutants the maximum binding of [Nle8,21,Tyr24]rPTH-(1–34)amide was elevated by a factor of 1.8–3.2, in comparison to the binding observed for the WT PTH-2 receptor (Table 1). This increase in maximum binding of radiolabeled PTH-(1–34) may indicate increased surface expression, enhanced PTH-(1–34) binding affinity, or both. It is unlikely, however, that such effects on surface expression or PTH-(1–34) binding affinity are the basis for the altered cAMP selectivity.

Table I

| Receptor | Maximum binding | As shown in Fig. 2, these residues were replaced either by cassette substitution or, for two of the sites (Ala325 and Gly327), by single residue point mutation. All but 3 of the 21 mutant receptors were functional and adequately expressed on COS-7 cells, as judged by the cAMP response to [Ile5,Trp23]PTH-P-(1–34) and the binding of radioiodinated [Nle8,21,Tyr24]rPTH-(1–34)amide (Table 1). The three cassette mutants showing poor cAMP responsiveness and little or no PTH binding, P2R-Cast#2, P2R-Cast#7 and P2R-Cast#18, were possibly not expressed on the cell surface and were considered uninformative. Of the functional mutant receptors, three displayed increased responsiveness to [Trp25]PTHrP-(1–36). These three cassettes are predicted to be located in the amino-terminal portion of extracellular loop 1 (P2R-Cast#8), the extracellular end of helix 3 (P2R-Cast#8), and at the COOH-terminal end of extracellular loop 2 (P2R-Cast#13) (Fig. 2). For each of these three receptor mutants the maximum binding of [Nle8,21,Tyr24]rPTH-(1–34)amide was elevated by a factor of 1.8–3.2, in comparison to the binding observed for the WT PTH-2 receptor (Table 1). This increase in maximum binding of radiolabeled PTH-(1–34) may indicate increased surface expression, enhanced PTH-(1–34) binding affinity, or both. It is unlikely, however, that such effects on surface expression or PTH-(1–34) binding affinity are the basis for the altered cAMP selectivity. |
therefore focused on the receptor regions defined by cassette mutations 5, 8, and 13.

Replacement of each divergent residue in cassette region 5 by the corresponding residue of the PTH-1 receptor failed to identify a single residue affecting the cAMP response to [Trp^{23}]PTHrP-(1–36) (Fig. 3B and Table I). It is possible that two or more sites in this region cooperatively contribute to His^{7}Ile^{5} signaling selectivity; however, two of the mutations in this set, Val^{207} → Leu (valine 207 changed to leucine, VL-207) and Lys^{208} → Asp (KD-208) were poorly expressed, as indicated by their very low responses to [Ile^{5},Trp^{23}]PTHrP-(1–36)NH_{2} after subtracting the corresponding basal cAMP value. Panel B shows the cAMP responses of the PTH-2 receptors with single point mutations at each of the divergent sites within cassette regions 5, 8, and 13. The data shown are from two separate experiments (mean ± S.E.) each performed in duplicate.

As part of our efforts to understand the mechanisms of ligand interaction in PTH receptors, we are exploring the molecular basis for the unique ligand selectivity of the PTH-2 receptor, which responds to PTH but not PTHrP. In the first stage of these studies, we examined the ligands for amino acid residue divergences that could explain this selectivity. We identified position 23 as the major determinant of binding selectivity, and position 5 as the major determinant of signaling selectivity (14). In the present study we sought to identify the sites in the PTH-2 receptor that enable it to discriminate, on the basis of cAMP signaling, between His and Ile at position 5. The receptor residues involved in this effect were of particular interest, because it seemed likely that they would be at, or near, sites involved in triggering the signal transduction mechanism.

The two PTHrP analogs used in our analysis differed by having His or Ile at position 5, and were thus nonfunctional or functional with the WT PTH-2 receptor. Each of the two analogs contained the Phe → Trp modification at position 23, which enabled high affinity binding of either analog to the PTH-2 receptor (14). The role of residue 5 in signaling selectivity was also demonstrated by Behar et al. (15). Importantly, this modification does not influence cAMP signaling, as demonstrated by the ability of [Trp^{23}, Tyr^{36}]PTHrP-(1–36)NH_{2} (na-
COS-7 cells. The peptides tested were [Trp 23, Tyr 36]PTHrP-(1–36)NH₂ and [Ile 5, Trp 23, Tyr 36]PTHrP-(1–36)NH₂. Intracellular cAMP and competition binding assays were performed as described under "Experimental Procedures." Binding experiments used [125I]-[Nle 8, 21, Tyr 34]rPTH-(1–34)amide as a competitor ligand. Values are means ± S.E. of six to twelve experiments performed in duplicate.

### TABLE II

cAMP responses of WT and mutant PTH-1 and PTH-2 receptors

| Receptor | Basal | [Trp 23]PTHrP-(1–36)max | [Ile 5, Trp 23]PTHrP-(1–36)max | [Trp 23]PTHrP-(1–36)EC₅₀ | [Ile 5, Trp 23]PTHrP-(1–36)EC₅₀ |
|----------|-------|-------------------------|-------------------------------|--------------------------|-------------------------------|
| P2R-wt   | 18 ± 2| 135 ± 24                | 134 ± 17                     | 2.5 ± 1.1                | 0.1 ± 0.0                    |
| P2R-IL-244| 25 ± 4| 135 ± 24                | 135 ± 24                     | 2.5 ± 1.1                | 0.1 ± 0.0                    |
| P2R-YI-318| 10 ± 2| 356 ± 55                | 356 ± 55                     | 2.5 ± 1.1                | 0.1 ± 0.0                    |
| P1R-wt   | 18 ± 4| 135 ± 24                | 135 ± 24                     | 2.5 ± 1.1                | 0.1 ± 0.0                    |
| P1R-IL-289| 25 ± 8| 135 ± 24                | 135 ± 24                     | 2.5 ± 1.1                | 0.1 ± 0.0                    |
| P1R-YI-363| 12 ± 3| 135 ± 24                | 135 ± 24                     | 2.5 ± 1.1                | 0.1 ± 0.0                    |

### TABLE III

Scatchard analysis of WT and mutant PTH-1 and PTH-2 receptors

| Receptor | Binding affinity Kᵦ[apparent] | Surface PTH-(1–34)×10⁻⁶ binding sites/cell |
|----------|--------------------------------|-------------------------------------------|
| P2R-WT   | 8.0 ± 1.7                      | 0.5 ± 0.1                                 |
| P2R-IL-244| 3.5 ± 0.2                    | 0.4 ± 0.1                                 |
| P2Rc-YI-318| 3.8 ± 0.8                    | 1.5 ± 0.9                                 |
| P1R-WT   | 10.1 ± 2.6                    | 5.9 ± 2.0                                 |
| P1R-IL-289| 6.0 ± 0.7                     | 3.0 ± 1.0                                 |
| P1R-YI-363| 11.3 ± 1.6                    | 6.8 ± 1.5                                 |

The cAMP responses of the two reciprocal PTH-1/PTH-2 receptor chimeras showed that the major determinants of His ⁵/ Ile ⁶ signaling selectivity mapped to the portion of the receptor containing the membrane-spanning helices and connecting loops. Cassette mutagenesis then revealed three segments that affected this selectivity; these were located in extracellular loop 1, transmembrane helix 3, and extracellular loop 2. Scanning mutagenesis analysis of the extracellular loop 1 segment failed to reveal a single site that led to improved responsiveness to [Trp 23]PTHrP-(1–36), a finding that suggests that multiple residues in this region might be involved in residue 5 recognition.

The molecular basis by which Ile ⁴⁴₄ and Tyr ⁴₃₈ affect ligand selectivity is not known. The three-dimensional structure of the PTH-2 receptor, or any G protein-coupled receptor, with the exception of rhodopsin (24), has not been determined. The two-dimensional schematic of the core region of the PTH-2 receptor shown in Fig. 2 is based mainly on evolutionary and hydrophathy analyses of the primary structure (25). Although the end points of the seven membrane-spanning helices have not been firmly established, it is predicted that Ile ⁴⁴₄ and Tyr ⁴₃₈ lie at, or close to, the boundary of the extracellular fluid and the lipid membrane. These two sites could thus be in a reasonable position for interacting with the ligand. Peptide-binding sites in another class of peptide hormone receptors, the tachykinin receptors, have been mapped to similar locations (13). Our functional data do not exclude the possibility that the mutations at Ile ⁴⁴₄ and Tyr ⁴₃₈ have allosteric effects on other residues, but global changes in receptor structure seem unlikely, because there was little or no effect of the mutations on
the binding and signaling properties of [Ile\(^5\),Trp\(^{23}\)]PTHrP-(1–36) or rPTH-(1–34), or on receptor expression levels. One question to consider in these studies is whether the PTH-2 receptor and the PTH-1 receptor engage their ligands in a similar fashion. In an effort to address this question we introduced reciprocal mutations, LI-289 and YI-363, into the PTH-1 receptor and tested the mutants for the ability to discriminate between analogs with His or Ile at position 5. No effect on cAMP signaling responsiveness was detected for either receptor mutation; we were thus unable to determine whether equivalent sites in the PTH-1 and PTH-2 receptors are involved in residue 5 recognition. That neither mutation was sufficient for conferring His\(^5\)/Ile\(^5\) signaling selectivity to the PTH-1 receptor indicates that multiple PTH-2 receptor residues are required for this effect, as was suggested by the initial cassette mutagenesis studies in which mutations at three distinct sites led to a loss of selectivity (Fig. 3).

The bioactive regions of PTH and PTHrP differ considerably in primary structure (26), yet most studies indicate that the two ligands bind to the same site in the PTH-1 receptor (21–23). To examine whether the PTH-2 receptor sites that we identified here also influence interactions with position 5 of PTH, we studied the binding and signaling properties of [His\(^5\)]hPTH-(1–34). This analog is inactive in cAMP assays with the PTH-2 receptor, though, it binds with adequate affinity (14). As with PTHrP, both the IL-244 and YI-318 mutations were able to rectify the signaling defect of [His\(^5\)]PTH-(1–34) (Fig. 6). These results suggest that the histidine at position 5 in both [Trp\(^{23}\)]PTHrP-(1–36) and [His\(^5\)]PTH-(1–34) is recognized by the same region of the PTH-2 receptor.

The isoleucine at position 244 is conserved in the rat and human PTH-2 receptor (1, 27), and leucine is preserved at the homologous site in each PTH-1 receptor (Xenopus, rat, mouse, human, porcine, and opossum). The same pattern of evolutionary preservation is seen for the Tyr\(^{318}\) site in extracellular loop 2. Residue 5 in the ligands also shows this trend; the polar histidine is found here in all PTHrP ligands, and a hydrophobic isoleucine or methionine residue is found at the corresponding site in each vertebrate PTH sequence. It may be that the two receptor sites and the cognate residue 5 of the ligands are under the same evolutionary constraints. These constraints would ensure that PTHrP specifically interacts with the PTH-1 receptor to mediate its biological actions, including the regulation of embryonic bone development (28, 29) and not with the PTH-2 receptor, which is expressed in several different tissues, albeit with unknown functional consequences (27). Whether PTH is the actual ligand for the PTH-2 receptor is unknown; recent evidence suggests that the hypothalamus contains a novel peptide that selectively activates the PTH-2 receptor (30).

For the PTH receptors, and other members in this same peptide hormone receptor family, the amino-terminal extracellular domain has been shown to play an important role in ligand-binding affinity and specificity (3, 5, 8–10, 31, 32). Several other studies on these receptors have implicated the extracellular loops or transmembrane helices in ligand binding or signaling interactions (6–8, 10, 11). Our present studies with
Fig. 6. Effect of receptor mutations on signaling by [His]3,Tyr34]hPTH-(1–34). The ability of the analogs [His]3,Tyr34]hPTH-(1–34)NH2 (△) and [Tyr34]hPTH-(1–34)NH2 (●) to stimulate cAMP with wild-type and mutant PTH-1 and PTH-2 receptors expressed in COS-7 cells is shown. For each receptor, the intracellular cAMP levels are expressed as a percent of the maximum response attained by that receptor with [Tyr34]hPTH-(1–34)NH2. Shown are data from a single experiment performed in duplicate; the results are representative of three independent experiments.

the PTH-2 receptor provide additional information on the functional map of the ligand interaction surface of the receptor, as they identify specific residues in helix 3 and extracellular loop 2 that modulate the signaling selectivity determined by residue 5 in the ligand.

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