The Hydrophobic Residues Phenylalanine 184 and Leucine 187 in the Type-1 Parathyroid Hormone (PTH) Receptor Functionally Interact with the Amino-terminal Portion of PTH-(1–34)*

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Recent mutagenesis and cross-linking studies suggest that three regions of the PTH-1 receptor play important roles in ligand interaction: (i) the extreme NH2-terminal region, (ii) the juxtamembrane base of the amino-terminal extracellular domain, and (iii) the third extracellular loop. In this report, we analyzed the second of these segments in the rat PTH-1 receptor (residues 182–190) and its role in functional interaction with short PTH fragment analogs. Twenty-eight singly substituted PTH-1 receptors were transiently transfected into COS-7 cells and shown to be fully expressed by surface antibody binding analysis. Alanine-scanning analysis identified Phe184, Arg186, Leu187, and Ile190 as important determinants of maximum binding of 125I-labeled bovine PTH-(1–34) and 125I-labeled bovine PTH-(3–34) and determinants of responsiveness to the NH2-terminal analog, PTH-(1–14) in cAMP stimulation assays. Alanine mutations at these four sites augmented the ability of the COOH-terminal peptide [Glu22,Trp23]PTHrP-(15–36) to inhibit the cAMP response induced by PTH-(1–34). At Phe184 and Leu187, hydrophobic substitutions (e.g. Ile, Met, or Leu) preserved PTH-(1–34)-mediated cAMP signaling potency, whereas hydrophilic substitutions (e.g. Asp, Glu, Lys, or Arg) weakened this response by 20-fold or more, as compared with the unsubstituted receptor's response. The results suggest that hydrophobicity at positions occupied by Phe184 and Leu187 in the PTH-1 receptor plays an important role in determining functional interaction with the 3–14 portion of PTH.

The PTH-1 receptor is a class II G protein-coupled receptor (1–3) that plays an important role in two distinct biological processes: the control of calcium ion concentrations in the blood and pattern formation in the developing skeleton (4). Most of these processes: the control of calcium ion concentrations in the blood (1) that plays an important role in determining ligand binding affinity (5–10), and that the portions of these receptors containing the extracellular loops and transmembrane domains have also been shown to contribute to ligand binding (11–14).

Several groups are utilizing a variety of approaches to identify specific points of contact between PTH-(1–34) and the PTH-1 receptor. Both mutational and photochemical cross-linking studies on the PTH-1 receptor have suggested that portions of the NH2-terminal extracellular receptor domain interact with the COOH-terminal (positions 15–34) binding domain of PTH (8, 15); similar observations have been made for the other class II receptors (16, 17). Separate mutational and cross-linking studies have indicated that portions of the extracellular loops of the PTH-1 receptor contact residues within the amino-terminal (positions 1–14) portion of the ligand (17–21) and that this interaction is sufficient for stimulating the cellular cAMP response (22).

The receptor region at the COOH-terminal base of the extracellular domain (residues 182–190; cf. Fig. 1) was initially identified as a candidate ligand binding site by a homolog-scanning mutagenesis strategy. Replacement of this region of the rat PTH-1 receptor with the corresponding region of the secretin receptor abolished binding of PTH-(1–34) without affecting surface expression (23). Independent studies have demonstrated that a [Lys13(e-p-Bz2)]PTH-(1–34) analog cross-linked to this same region of the human PTH-1 receptor (24) and suggested that Arg186 was the reactive site (25). In the current study, we explore further the role of nine amino acids in this juxtamembrane segment of the NH2-terminal domain of the rat PTH-1 receptor in determining the functional interaction with PTH-(1–34). The results reveal four receptor residues that modulate interaction with the 3–14 portion of PTH and suggest that hydrophobicity is required for optimal ligand-binding and cAMP-signaling potency at Phe184 and Leu187.

MATERIALS AND METHODS

Peptides—[Nle8,18,Tyr34]bPTH(3–34)NH2 (PTH-(3–34)) was purchased from Bachem (Torrance, CA). [Nle8,18,Tyr34]bPTH(1–34)NH2 (PTH-(1–34)), and [Glu22,Trp23,Tyr34]human PTHrP(15–36)NH2 (Glu22,Trp23)PTHrP(15–36) were prepared on an Applied Biosystems model 431A peptide synthesizer using Fmoc (N-(9-fluorenyl)methoxy-carbonyl) protecting group chemistry and trifluoroacetic acid-mediated cleavage/deprotection (MGH Biopolymer Synthesis Facility, Boston, MA); these peptide were then purified by high performance liquid chromatography and lyophilized. The peptide rPTH-(1–14)NH2 (rPTH-(1–14)) was synthesized on a multiple peptide synthesizer (Advanced Chemtech model 396 MBS) and desalted by adsorption on a C18-containing cartridge (Sep-Pak). All peptides were reconstituted in 10 mM acetic acid and stored at ~80 °C. The purity, identity, and stock concentration of each compound was secured by analytical high per-
PHT-1 Receptor Binding Determinants

formance liquid chromatography, matrix-assisted laser desorption/ionization mass spectrometry, and indirect acid analysis. Radiolabeling of PTH-(1–34) and PTH-(3–34) was performed using 125I-Na (2200 Ci/mmole, NEN Life Science Products) and chloramine-T; the resultant 125I-labeled ligand was purified by high performance liquid chromatography.

**PTH Receptor Mutagenesis**—The construction and initial characterization of the pCDNA-1-based plasmid encoding the epitope-tagged amino-terminally truncated rat PTH-1 receptor (rNt-HA) has also been described previously (22). In this receptor, residues 23–181 have been removed, and a nine-amino acid HA tag joined to a tetraglycine linker (YPYDVPDYAGGG-) has been inserted between Ala23 and Glu182. Signal peptide cleavage is predicted to occur between Ala18 and the tyrosine of the HA tag (27). The Phe184 point mutation was incorporated into rNt-HA as described above.

**Cell Culture and DNA Transfection**—Stock solutions of EGTa/trypsin and antibiotics were from Life Technologies, Inc.; fetal bovine serum was from Hyclone Laboratories (Logan, UT). COS-7 cells were cultured at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with fetal bovine serum (10%), penicillin G (20 units/ml), streptomycin sulfate (20 μg/ml), and amphotericin B (0.05 μg/ml) in a humidified atmosphere containing 5% CO2.

**Transfection of COS-7 cells** were performed using DEAE-dextran as described previously (17). COS-7 cells were transfected in 24-well plates when the cells were 85–95% of confluency using 200 ng of plasmid DNA that was purified by cesium chloride/ethidium bromide gradient centrifugation for each well. Twenty-four to sixteen hours prior to assay, the cells were treated with fresh media and shifted to a humidified incubator containing 5% CO2 that was set at 33 °C (17, 28). Assays were conducted 72–96 h after transfection, at which point ~20% of the cells were transfected and expressed surface wild type PTH receptors at a density of about 5 × 106/17 (17).

**Ligand-binding Assays**—Binding reactions were performed with transiently transfected COS-7 cells in 24-well plates. Cells were rinsed with 0.5 ml of binding buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM KCl, 2 mM CaCl2, 5% heat-inactivated horse serum, 0.5% fetal bovine serum, adjusted to pH 7.7 with HCl) and treated successively with 200 μl of binding buffer and 100 μl of binding buffer containing ~100,000 cpm of 125I-labeled rPTH-(1–34) (~26 fmol; final volume ~300 μl). The cells were incubated at 15 °C for 4 h and placed on ice; the binding medium was removed, and the cells were rinsed three times with 0.5 ml of cold binding buffer and lysed with 0.5 ml of 5 N NaOH. The entire lysate was counted for γ-irradiation. Nonspecific binding was determined in cells transfected with the pCDNA-1 vector and was typically less than 1.5% of the total radioactivity added.

**PHT-1 Receptor Expression**—Measurements of surface expression for the HA epitope-tagged COS-7 cells were performed with intact transfected COS-7 cells in 24-well plates. Cells were washed with 0.5 ml of binding buffer and then incubated with 0.25 ml of binding buffer containing the mouse monoclonal antibody 12CA5 (Roche Molecular Biochemicals) at 1 μg/ml for 2 h at 15 °C. The buffer was removed, and the cells were then washed three times with 0.5 ml of binding buffer and incubated for an additional 2 h at 15 °C with 0.25 ml of binding buffer containing ~400,000 cpm of 125I-labeled goat anti-mouse IgG antibody (NEN Life Science Products). The buffer was withdrawn, and the cells were then washed three times with 0.5 ml of binding buffer and lysed with 5 N NaOH. The entire lysate was counted for γ-irradiation. Nonspecific binding was determined in cells transfected with the pCDNA-1 vector and was typically less than 0.5% of the radioactivity added.

**Intracellular Cyclic AMP**—Stimulation of transiently transfected COS-7 cells was performed in 24-well plates. Cells were rinsed with 0.5 ml of binding buffer and treated with 200 μl of cAMP assay buffer (Dulbecco’s modified Eagle’s medium containing 2 mM 3-isobutyl-1-methylnitane, 1 mg/ml bovine serum albumin, 35 mM Hepes-NaOH, pH 7.4), and 100 μl of binding buffer containing varying amounts of peptide analog (final volume ~300 μl). The medium was removed after incubation for 30 min at 37 °C, and the cells were frozen (~80 °C), lysed with 0.5 ml of 50 mM HCl, and refrozen (~80 °C). The cAMP content of the diluted lysate was determined by radioimmunoassay using unlabeled cAMP as a standard. The cAMP EC50 values were determined using nonlinear regression (see below).

**Inhibition Studies**—The cAMP stimulation protocol described above was utilized for inhibition studies with some minor modifications. Cells were rinsed with 0.5 ml of binding buffer and treated successively with 100 μl of cAMP assay buffer, 50 μl of binding buffer containing varying doses of [Glu23,Trp28]PThP (15–36), and 100 μl of cAMP assay buffer containing 1 nM dose of rPTH-(1–34) (final volume ~250 μl). Cells were incubated for 30 min at room temperature and processed as above. The dose of [Glu23,Trp28]PThP (15–36) that inhibited the PTH-(1–34)-mediated cAMP response by 50% (IC50) was calculated using nonlinear regression analysis (see below).

**Data Calculation**—All calculations were performed using Microsoft® Excel. Nonlinear regression analysis of binding and cAMP stimulation data was performed using four parameters, defined as the Minimum (Min), Maximum (Max), midpoint (IC50), and slope of the response curve. The predicted response (ya) for a given dose (x) of peptide was calculated using the following equation: $y_a = Min + \frac{(Max - Min)x}{1 + (IC50/x)^p}$, where I represent the primary data, and the Excel “Solver function” was then used to vary the parameters in order to minimize the differences between the predicted and observed responses (least-squares method) (29). The statistical significance between two data sets was determined using a one-tailed Student’s t test, assuming unequal variances for the two sets.

**RESULTS**

We introduced individual alanine substitutions at each position in the 182–190 region of the rat PTH-1 receptor (Fig. 1) and analyzed the effects on receptor function in transiently transfected COS-7 cells (Table I). The surface expression of the alanine-substituted mutants ranged from 85 to 113% of the wild type receptor, as judged by antibody-binding analysis (Table I). Four of the mutations (Phe184 → Ala, Arg186 → Ala, Leu187 → Ala, and Ile190 → Ala) reduced the capacity of the receptor to bind the agonist tracer 125I-PTH-(1–34) by 4-fold or more (Fig. 2). The strongest effect occurred with the Phe184 → Ala mutation, which reduced binding to 4 ± 0.4% of the binding seen with the wild type receptor. A similar pattern was observed when the alanine-substituted mutant receptors were tested for their capacity to bind the partial agonist tracer 125I-PTH-(3–34) (Table I). Each of the nine alanine-substituted mutant receptors mediated a comparable maximal (40-fold) increase in intracellular cAMP in response to high doses of PTH-(1–34), as was observed with the wild type receptor (Table I). The cAMP-stimulating potency of PTH-(1–34) with most of the alanine-substituted mutants was similar to the potency seen with the wild type receptor (EC50 1.4 ± 0.3 nM, Table I), but the Phe184 → Ala mutation resulted in an 8-fold decrease in potency of PTH-(1–34) agonist peptide relative to rWT-HA (p = 0.02, Fig. 3).

In order to analyze the effect of the alanine mutations on the NH2-terminal signaling domain of PTH-(1–34), we utilized the COOH-terminally truncated rPTH-(1–14)NH2. As reported previously (22), stimulation of rWT-HA with a 100 μM dose of PTH-(1–14) induced a 14-fold increase in cAMP formation, relative to the basal response. Stimulation of the alanine-substituted mutants with the same dose of PTH-(1–14) revealed that Phe184 → Ala, Arg186 → Ala, Leu187 → Ala, and Ile190 → Ala each showed a 7–20-fold reduced responsiveness to this peptide (Fig. 4). CAMP formation was relative to rWT-HA (p < 0.05). To analyze the effect of the alanine mutations on cAMP responses involving the COOH-terminal signaling domain of PTH-(1–34), we utilized the fragments [Glu22,Trp28]PThP (15–36)NH2 (30) and tested its ability to block the CAMP response induced by a 1 nM dose of PTH-(1–34) with each mutant receptor. With rWT-HA, a 10 μM dose of [Glu22,Trp28]PThP (15–36)NH2 did not significantly inhibit the cAMP response induced by PTH-(1–34) (Fig. 5A), a result that is con-
subtracted in the calculation.

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sistent with the weak ability of this fragment to inhibit the

maximum specific binding (expressed as percentage of total counts added) to rWT-HA was 2.3 ± 0.1% (antibody), 20 ± 0.5% (1251-PTH-(1–34)), and

26 ± 1% (1251-PTH-(3–34)). The maximum stimulation observed with rWT-HA was 450 ± 19 pmol/well with 0.1 μM PTH-(1–34) and 149 ± 11 pmol/well with 100 μM PTH-(1–14). The basal values observed in the cAMP stimulation assays ranged from 6 to 20 pmol/well and were not subtracted in the calculation.

Maximum specific binding data (n = 3) and cAMP stimulation data (n = 3) were obtained as described under "Materials and Methods." The maximum specific binding (expressed as percentage of total counts added) to rWT-HA was 2.3 ± 0.1% (antibody), 20 ± 0.5% (1251-PTH-(1–34)), and

26 ± 1% (1251-PTH-(3–34)). The maximum stimulation observed with rWT-HA was 450 ± 19 pmol/well with 0.1 μM PTH-(1–34) and 149 ± 11 pmol/well with 100 μM PTH-(1–14). The basal values observed in the cAMP stimulation assays ranged from 6 to 20 pmol/well and were not subtracted in the calculation.

Table I

| Receptor | Maximum specific binding (Bmax) | cAMP stimulation data |
|----------|---------------------------------|-----------------------|
|          | 1251-PTH-(1–34) | 1251-PTH-(3–34) | nM | EC50 | Emax | Maxobs |
| rWT-HA   | 100 ± 2  | 100 ± 2  | 100 ± 2 | 1.4 ± 0.3 | 115 ± 7 | 113 ± 5 |
| EA-182   | 107 ± 3  | 105 ± 2  | 95 ± 3  | 1.3 ± 0.6 | 115 ± 7 | 113 ± 5 |
| VA-183   | 101 ± 2  | 91 ± 2   | 64 ± 2  | 0.6 ± 0.3 | 97 ± 15 | 65 ± 4  |
| FA-184   | 99 ± 1   | 4 ± 0.4  | 6 ± 3   | 11.0 ± 3.5 | 94 ± 8 | 14 ± 2  |
| DA-185   | 106 ± 2  | 116 ± 4  | 137 ± 4 | 0.7 ± 0.3 | 93 ± 12 | 133 ± 5 |
| RA-186   | 113 ± 3  | 125 ± 1  | 7 ± 1   | 1.1 ± 0.1 | 100 ± 9 | 9 ± 1   |
| IA-187   | 94 ± 2   | 10 ± 1   | 23 ± 1  | 2.2 ± 0.8 | 93 ± 7  | 5 ± 1   |
| GA-188   | 93 ± 3   | 120 ± 4  | 123 ± 3 | 1.0 ± 0.6 | 102 ± 8 | 146 ± 5 |
| MA-189   | 102 ± 2  | 94 ± 2   | 84 ± 3  | 0.6 ± 0.2 | 106 ± 11 | 93 ± 6 |
| IA-190   | 85 ± 2   | 24 ± 2   | 16 ± 1  | 1.3 ± 0.5 | 86 ± 8  | 9 ± 1   |
| PD-184   | 115 ± 2  | 2 ± 0.3  | 0.3 ± 0.1 | 111.0 ± 80.1 | 78 ± 2 | 4 ± 1   |
| FE-184   | 115 ± 2  | 2 ± 0.3  | 0.3 ± 0.1 | 111.0 ± 80.1 | 78 ± 2 | 4 ± 1   |
| FG-184   | 113 ± 3  | 3 ± 1    | 1 ± 0.2  | 62.9 ± 18.6 | 70 ± 2 | 9 ± 2   |
| FH-184   | 116 ± 2  | 15 ± 2   | 8 ± 1   | 10.8 ± 0.9 | 87 ± 2 | 14 ± 2  |
| FI-184   | 120 ± 5  | 37 ± 2   | 36 ± 1  | 0.6 ± 0.2 | 112 ± 4 | 55 ± 4  |
| FK-184   | 110 ± 4  | 3 ± 1    | 1 ± 0.2  | 48.5 ± 10.8 | 77 ± 5 | 5 ± 1   |
| FL-184   | 98 ± 2   | 61 ± 2   | 31 ± 2  | 2.3 ± 0.4 | 105 ± 4 | 46 ± 4  |
| FM-184   | 103 ± 3  | 80 ± 3   | 40 ± 3  | 1.8 ± 0.5 | 90 ± 5 | 47 ± 4  |
| FR-184   | 119 ± 4  | 5 ± 1    | 5 ± 1   | 33.2 ± 3.6 | 95 ± 7 | 8 ± 1   |
| FP-184   | 111 ± 3  | 9 ± 2    | 9 ± 2   | 8.7 ± 1.2 | 84 ± 3 | 17 ± 2  |
| DI-185   | 97 ± 2   | 108 ± 1  | 122 ± 2 | 1.5 ± 0.5 | 96 ± 6 | 161 ± 7 |
| DK-185   | 103 ± 1  | 107 ± 1  | 126 ± 2 | 1.4 ± 0.3 | 108 ± 4 | 181 ± 12 |
| RI-186   | 104 ± 2  | 52 ± 2   | 13 ± 1  | 1.1 ± 0.3 | 94 ± 6 | 25 ± 2  |
| RK-186   | 101 ± 2  | 102 ± 2  | 106 ± 1 | 1.5 ± 0.2 | 104 ± 7 | 123 ± 6 |
| LE-187   | 78 ± 3   | 0.8 ± 0  | 1 ± 1   | 493 ± 19.3 | 19 ± 4 | 9 ± 1   |
| LH-187   | 98 ± 2   | 4 ± 1    | 4 ± 0.4 | 30.4 ± 14.6 | 74 ± 4 | 11 ± 2  |
| LI-187   | 104 ± 22 | 83 ± 1   | 87 ± 1  | 1.7 ± 0.4 | 91 ± 2 | 41 ± 3  |
| LR-187   | 99 ± 1   | 2 ± 1    | 7 ± 1   | 406.9 ± 0.8 | 27 ± 1 | 10 ± 1  |
| LV-187   | 99 ± 1   | 69 ± 2   | 61 ± 1  | 4.3 ± 0.4 | 92 ± 1 | 28 ± 3  |

Fig. 1. Schematic of the PTH-1 receptor. Shown is the predicted domain structure of the rat PTH-1 receptor used in this study and the locations of the eight conserved extracellular cysteines (C) and the nine amino acid HA epitope tag (hatched circles). The expanded view shows the nine-amino acid segment at the juxtamembrane end of the amino-terminal extracellular domain of the receptor (residues 182–190), which was subjected to point mutational analysis, as described under "Materials and Methods" and in succeeding figures. The boundary of the NH2-terminal domain and the first transmembrane domain (TM-1) is shown as predicted by the Proteinpredict algorithm (32).
FIG. 2. Effect of alanine mutations in the PTH-1 receptor on ligand binding. The wild type and mutant rat PTH-1 receptors bearing alanine point substitutions at positions 182–190 were transiently transfected into COS-7 cells and functionally evaluated. The maximum specific binding of \(^{125}\text{I}-\text{bPTH-(1–34)}\) to these receptors is shown and is expressed as the percentage of the specific binding observed for that ligand with the wild type receptor. Shown are data (mean ± S.E.) combined from three individual experiments, each of which was performed in duplicate on a separate day, as described under “Materials and Methods.” Nonspecific binding was subtracted in the calculation.

FIG. 3. Dose-response analysis of PTH-(1–34) stimulation of wild type and mutant PTH-1 receptors. Wild type or alanine-substituted mutant PTH-1 receptors were transiently transfected into COS-7 cells and subsequently stimulated with \(\text{bPTH-(1–34)}\) over the dose range indicated. Shown are data (mean ± S.E.) combined from three individual experiments, each of which was performed in duplicate on a separate day, as described under “Materials and Methods.” The combined rWT-HA curve (\(n = 3\), dashed line) matched for this particular experiment is shown as a control. ●, rWT-HA; ○, FA-184; ▲, RA-186; □, LA-187; ■, IA-190.

To determine if other residues in the NH\(_2\)-terminal extracellular domain of the PTH-1 receptor were required for the functional effects observed for the mutations in the 182–190 region, we utilized a truncated rat PTH-1 receptor (rΔNt-HA) that lacked residues 23–181 and had in their place a nine-amino acid HA epitope tag (Fig. 6A) (22). Introduction of the Phe\(^{184}\) → Ala mutation into rΔNt-HA yielded a truncated mutant receptor that was expressed on the cellular surface to the same level as unsubstituted rΔNt-HA (Fig. 6B). The unsubstituted truncated receptor elicited ~6-fold increases in cAMP levels in response to either 1 \(\mu\text{M}\) PTH-(1–34) or 100 \(\mu\text{M}\) PTH-(1–14); rΔNt/HAFA-184 exhibited little or no response to these peptides (Fig. 6C).

In order to characterize the chemical basis for the role of the 182–190 region in interacting with PTH-(1–34), we examined the effects of a number of polar and nonpolar mutations in the 184–187 segment of intact rWT-HA (Table I and Fig. 7). All of these point mutations yielded mutant receptors that were well expressed on the cell surface (range = 78–120% of rWT-HA, Table I). Substitution of the polar amino acids Glu, Lys, and

FIG. 4. Effect of alanine mutations in the PTH-1 receptor on stimulation by PTH-(1–14). The wild type and alanine-substituted mutant PTH-1 receptors were transiently transfected into COS-7 cells and subsequently stimulated with a 100 \(\mu\text{M}\) dose of rPTH(1–14). Shown are data (mean ± S.E.) combined from three individual experiments, each of which was performed in duplicate on a separate day, as described under “Materials and Methods.” The basal (unstimulated) level of cAMP in these cells ranged from 10 ± 1 pmol/well (LA-187) to 15 ± 2 pmol/well (MA-189) and was not subtracted in the calculation. [●, untreated; ■, treated with 100 \(\mu\text{M}\) rPTH(1–14).

Arg at position 184 resulted in 10–50-fold reductions in the maximal specific binding of \(^{125}\text{I}-\text{PTH-(1–34)}\), the cAMP-signaling potency of PTH-(1–34), and the cAMP-signaling efficacy of PTH-(1–14) (Table I and Fig. 7). Substitution of the nonpolar amino acids Ile, Met, and Leu at position 184 resulted in less severe (~2-fold) reductions in PTH-(1–34) binding and PTH-(1–14) stimulation and did not significantly impact the stimulation by PTH-(1–34). Mutation of Asp\(^{185}\) to either Ile or Lys did not alter the binding or signaling properties of PTH analogs. In accord with the findings of Adams and co-workers (25), the introduction of lysine at position 186 was well tolerated in both binding and signaling assays. The Arg\(^{186}\) → Ile mutant showed a 2-fold reduction in the maximum specific binding of \(^{125}\text{I}-\text{PTH-(1–34)}\) and a 7.5-fold reduction in the specific binding of \(^{125}\text{I}-\text{PTH-(3–34)}\), as compared with rWT-HA, but responded normally to PTH-(1–34) in cAMP stimulation assays. Interestingly, the Arg\(^{186}\) → Ile mutant receptor was less functionally impaired than the Arg\(^{186}\) → Ala receptor. Substitution of the polar residue Glu, His, or Arg at position 187 with Ala resulted in severe reductions (>15-fold) in PTH-(1–34) binding capacity and the cAMP-stimulation potency of PTH-(1–34), whereas introduction of Ile and Val at this position resulted in small (~2-fold) or no reductions in PTH-(1–34)-binding capacity or cAMP signaling potency of PTH-(1–34).

DISCUSSION

The present study was conducted to explore the functional role(s) of individual residues in the (182–190) juxtamembrane region of the NH\(_2\)-terminal domain of the PTH-1 receptor. The potential importance of this segment of the receptor was recognized previously in a homolog-scanning mutagenesis study (23) and has also been investigated by photochemical cross-linking (24, 25) and spectroscopic methods (31). In the current study, we extended the functional analysis by first performing an alanine-scanning experiment, the results of which suggested that Phe\(^{184}\), Arg\(^{186}\), Leu\(^{187}\), and Ile\(^{189}\) played a role in the optimal binding of \(^{125}\text{I}-\text{PTH-(1–34)}\) (Fig. 2), \(^{125}\text{I}-\text{PTH-(3–34)}\) (Table I), and \(^{125}\text{I}-\text{PTHrP-(1–36)}\) (data not shown). Additional point mutations targeted to this region verified the importance of Phe\(^{184}\) and Leu\(^{187}\) and suggested that side chain hydrophobicity at these positions is a key determinant of ligand/receptor interaction (Table I).
the wild type (f)
tions that reduced the specific binding of 125I-bPTH-(1–34) to weakened binding of PTH-(1–34). In addition, for the mutated into COS-7 cells and subsequently stimulated with a 1 nM dose of wild type and alanine-substituted mutant PTH-1 receptors were transfected into COS-7 cells and subsequently stimulated with a 1 nM dose of [Glu22,Trp23]PTHrP-(15–36) with mutant PTH-1 receptors. cAMP-stimulating potencies of PTH-(1–34) were 8–300-fold less than 6% of that seen for the wild type receptor (Table I).

These results are consistent with the above mutant receptors exhibiting a reduced affinity for PTH-(1–34), although the possibility of additional activation-specific effects caused by the mutations cannot be excluded.

To localize the region of the ligand affected by the mutations in the 182–190 region of the receptor, we examined the ability of the mutant receptors to interact with PTH ligands of varying length. The Phe184Ala, Leu187Ala, and Ile190Ala receptors each (i) reduced the specific binding of 125I-bPTH-(1–34), (ii) reduced the cAMP-signaling responsiveness to PTH-(1–14), and (iii) enhanced the ability of [Glu22,Trp23]PTHrP-(15–36)NH2 to inhibit the cAMP response mediated by PTH-(1–34). In addition, every receptor in this study that exhibited impaired capacity to bind 125I-bPTH-(3–34) also demonstrated reduced responsiveness to PTH-(1–14) (Table I). These data suggest that the mutations in the 182–190 region of the PTH-1 receptor alter interactions with the 3–14 portion of PTH-(1–34). These receptor mutations at the COOH-terminal end of the amino-terminal domain thus

All of the mutant receptors in this study were expressed near wild type levels on the surface of transfected COS-7 cells, as judged by antibody binding. The decreases in maximum specific binding of radiolabeled PTH tracers observed for several of these mutants suggest that certain substitutions in the 182–190 region reduce ligand binding affinity. The low specific binding of radiolabeled PTH-(1–34) (less than 3% of total counts added) observed with the wild type receptor is diminished in the 182–190 region of the PTH-1 receptor, which was not subtracted in the calculation. Differences between the antagonized and unantagonized responses that are demarcated with an asterisk are significant (p < 0.0005), while all others are not (p > 0.15).
receptor were to be induced cAMP signaling. If the 182–190 region of the intact receptor interact with the 3–14 region of PTH-(1–34) and that the hydrophobicity of Phe184 and Leu187 and possibly the at positions 184, 186, 187, and 190 would form a contiguous role of Phe184 in r
out affecting receptor expression. These data indicate that the D
full-length PTH-1 receptors and thus suggest that Phe 184 does for its interaction with PTH-(1–34).

Deletion of most of the amino-terminal domain of the PTH-1 receptor (residues 23–181) abolishes detectable binding of [125I]labeled PTH analogs; however, we recently showed that surface expression levels and cAMP-signaling responsiveness to PTH-(1–34) and PTH-(1–14) are partially preserved in this truncation (e.g. Trp317 → Ala, which impaired the binding of PTH-(1–34) but not PTH-(3–34)) (15, 22, 23).

Deletion of most of the amino-terminal domain of the PTH-1 receptor (residues 23–181) abolishes detectable binding of [125I]-labeled PTH analogs; however, we recently showed that surface expression levels and cAMP-signaling responsiveness to PTH-(1–34) and PTH-(1–14) are partially preserved in this truncated r(ΔNt)-HA receptor (22). The insertion of the Phe184 → Ala mutation into r(ΔNt)-HA severely diminished the cAMP-signaling responses to both PTH-(1–34) and PTH-(1–14) without affecting receptor expression. These data indicate that the role of Phe184 in r(ΔNt)-HA is homologous with its role in the full-length PTH-1 receptors and thus suggest that Phe184 does not depend on the major portion of the amino-terminal domain for its interaction with PTH-(1–34).

Recently, Pellegrini, et al. (31) determined the structure of a synthetic peptide containing residues 168–198 of the PTH-1 receptor in a micellar solution of dodecylphosphocholine using NMR spectroscopy and demonstrated that residues 180–189 of this receptor fragment formed an amphipathic α-helix, which was lipid-associated. These authors suggested that the solvent-exposed hydrophobic face of this helix interacts with charged residues in the COOH-terminal portion of PTH-(1–34) (31). Our current functional data are most consistent with the view that residues Phe184, Arg186, Leu187, and Ile190 in the COOH-terminal portion of PTH-(1–34) and PTH-(1–14) are partially preserved in this truncation at the receptor’s extreme amino terminus (24).

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REFERENCES
1. Segre, G. V., and Goldring, S. R. (1993) Trends Endocrinol. Metab. 4, 309–314
2. Kolakowski, L. F. (1994) Receptors Channels 2, 1–7
3. Jüppner, H., Abou-Samra, A.-B., Freeman, M., Kong, X.-F., Schipani, E., Richards, J., Kolakowski, L. F., Jr., Hock, J., Potts, J. T., Jr., Kronenberg, H. M., and Segre, G. V. (1991) Science 254, 1024–1029
4. Kronenberg, H., Abou-Samra A, Bringhurst, F., Gardella, T., Jüppner, H., and Segre, G. (1997) in Genetics of Endocrine and Metabolic Disorders (Thakker, R. ed) pp. 389–420. Chapman & Hall, London
5. Lee, C., Gardella, T., Abou-Samra, A.-B., Nussbaum, S., Segre, G., Potts, J. T., Jr., Kronenberg, H., and Jüppner, H. (1994) Endocrinology 135, 1488–1495
6. Nicole, P., Du, K., Couvineau, A., and Laburthe, M. (1998) J. Pharmacol. Exp. Ther. 284, 744–50
7. Stroop, S., Kuestner, R., Serwold, T., Chen, L., and Moore, E. (1995) Biochemistry 34, 1050–1057
8. Jüppner, H., Schipani, E., Bringhurst, F. R., McClure, I., Keutmann, H. T., Potts J. T., Jr., Kronenberg, H. M., Abou-Samra, A.-B., Segre, G. V., and Gardella, T. (1994) Endocrinology 134, 879–884
9. Holzmöller, M. H., Hadac, E. M., Ulrich, C. D., and Miller, L. J. (1996) J. Pharmacol. Exp. Ther. 279, 555–560
10. Cao, Y.-J., Gimpl, G., and Fahrenholz, F. (1995) Biochem. Biophys. Res. Commun. 212, 673–680
11. Couvineau, A., Rouyer-Fessard, C., Maoret, J. J., Gaudin, P., Nicole, P., and Laburthe, M. (1996) J. Biol. Chem. 271, 12785–800
12. Turner, P. R., Mefford, S., Bambino, T., and Nissenson, R. A. (1998) J. Biol. Chem. 273, 3830–3837
13. Dautzenberg, F., Wille, S., Lohmann, R., and Spies, J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 4941–4946
14. DeAlmeida, V., and Mayo, K. (1998) Trends Endocrinol. Metab. 9, 87–91
15. Mannstadt, M., Luck, M., Gardella, T., and Jüppner, H. (1998) J. Biol. Chem. 273, 16890–16896
16. Dong, M., Wang, Y., Pinon, D., Hadiac, E., and Miller, L. (1999) J. Biol. Chem. 274, 903–909
17. Bergwitz, C., Jusseaume, S., Luck, M., Jüppner, H., and Gardella, T. (1997) J. Biol. Chem. 272, 28861–28868
18. Bergwitz, C., Gardella, T., Flannery, M., Potts, J. T., Jr., Kronenberg, H., Goldring, S., and Jüppner, H. (1997) J. Biol. Chem. 272, 26469–26472
19. Gardella, T. J., Jüppner, H., Wilson, A. K., Keutmann, H. T., Abou-Samra, A. B., Segre, G. V., Bringhurst, F. R., Potts, J. T., Jr., Nussbaum, S. R., and Kronenberg, H. M. (1994) Endocrinology 135, 1186–1194
20. Gardella, T., Luck, M., Fan, M.-H., and Lee, C. (1996) J. Biol. Chem. 271, 12620–12625
21. Bisello, A., Adams, A. E., Mierke, D., Pellegrini, M., Rosenblatt, M., Suva, L., and Chorev, M. (1998) J. Biol. Chem. 273, 22498–22505
22. Luck, M. D., Carter, P. H., and Gardella, T. (1999) Mol. Endocrinol. 13, 670–680
23. Lee, C., Luck, M., Jüppner, H., Potts, J. T., Jr., Kronenberg, H., and Gardella, T. (1995) Mol. Endocrinol. 9, 1269–1278
24. Zhou, A., Bisello, R., Bisello, A., Nakamoto, C., Rosenblatt, M., Suva, L. J., and Chorev, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 3644–3649
25. Adams, A., Bisello, A., Chorev, M., Rosenblatt, M., and Suva, L. (1998) Mol. Endocrinol. 12, 1673–1683
26. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 488–492
27. Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997) Protein Eng. 10, 1–6
28. Abou-Samra, A., Liu, X., and Segaloff, D. (1996) J. Biol. Chem. 271, 4518–4527
29. Bowen, W., and Jerman, J. (1995) Trends Pharmacol. Sci. 16, 413–417
30. Gardella, T., Luck, M., Jensen, G., Uusdin, T., and Jüppner, H. (1996) J. Biol. Chem. 271, 19888–19893
31. Pellegrini, M., Bisello, A., Rosenblatt, M., Chorev, M., and Mierke, D. (1998) Biochemistry 37, 12737–12743
32. Rost, B., Casadio, R., Fariselli, P., and Sander, C. (1995) Protein Sci. 4, 521–533