Parathyroid Hormone-Receptor Interactions Identified Directly by Photocross-linking and Molecular Modeling Studies*

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Parathyroid hormone (PTH) is the major regulator of calcium levels in blood and plays a role in the regulation of bone remodeling (1). Given intermittently, PTH displays anabolic activity in bone and, therefore, has considerable therapeutic potential (2). PTH and PTH-related protein exert their actions via a seven-transmembrane (TM) domain-containing receptor (PTH1-Rc) (3) belonging to a subfamily of related G protein-coupled receptors (4–11). The PTH1-Rc is coupled to both adenyl cyclase/cyclic AMP and phospholipase C/inositol 1,4,5-trisphosphate/cytosolic calcium intracellular signaling pathways (12–15).

Understanding the molecular mechanism of ligand recognition and signal transduction by the PTH1-Rc may identify new directions for the design of novel hormone analogs for the treatment of diseases such as osteoporosis, hypercalcemia of malignancy and hyperparathyroidism (16). In order to directly identify the structural elements involved in PTH–PTH1-Rc interactions, we employed a photoaffinity scanning approach (17). The generation of covalently linked ligand-receptor conjugates and the identification of the cross-linked domains allows mapping of the interface between hormone and receptor. Photoaffinity cross-linking has been successfully applied in defining interactions between small peptides, such as substance P (18–20), cholecystokinin (21), and vasopressin (22), and their receptors. Recently, we used this general approach to identify directly the interaction between position 13 of PTH and a 17-amino acid domain (residues 173–189) of the hPTH1-Rc (17).

We now report the evaluation of a series of photoreactive analogs obtained by a “p-benzoylphenylalanine (Bpa) scan” of the principal receptor activation domain (residues 1–6) of PTH–(1–34). A radiolabeled analog containing a photoreactive moiety at the N terminus, 125I-[Bpa1,Nle8,18,Arg13,26,27,L-2-Nal23,Tyr34]bPTH–(1–34)NH2, maintained full potency and led to the identification of a second “contact domain” between PTH and hPTH1-Rc. This information allows us to create, for the first time, a model describing interactions of the receptor-ligand complex.

Direct mapping of the interface between parathyroid hormone (PTH) and its receptor (hPTH1-Rc) was carried out by photoaffinity scanning studies. Photoreactive analogs of PTH singularly substituted with a p-benzoylphenylalanine (Bpa) at each of the first six N-terminal positions have been prepared. Among these, the analog [Bpa1,Nle8,18,Arg13,26,27,L-2-Nal23,Tyr34]bPTH–(1–34)NH2 (Bpa1-PTH–(1–34)) displayed in vitro activity with potency similar to that of PTH–(1–34). The radioiodinated analog 125I-Bpa1-PTH–(1–34) cross-linked specifically to the hPTH1-Rc stably expressed in human embryonic kidney cells. A series of chemical and enzymatic digests of the hPTH1-Rc–125I-Bpa1-PTH–(1–34) conjugate suggested that a methionine residue (either Met414 or Met425) is involved in this interaction. Site-directed mutagenesis (M414L or M425L) confirmed 125I-Bpa1-PTH–(1–34) cross-linking to the hPTH1-Rc. Molecular modeling studies of the hPTH1-Rc together with the NMR-derived high resolution structure of hPTH–(1–34), guided by the cross-linking data, strongly supports Met425, at the extracellular end of transmembrane helix 6, as the residue interacting with the N-terminal residue of the hPTH–(1–34). The photocross-linking and molecular modeling studies provide insight into the topologic arrangement of the receptor-ligand complex.

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Understanding the molecular mechanism of ligand recognition and signal transduction by the PTH1-Rc may identify new directions for the design of novel hormone analogs for the treatment of diseases such as osteoporosis, hypercalcemia of malignancy and hyperparathyroidism (16). In order to directly identify the structural elements involved in PTH–PTH1-Rc interactions, we employed a photoaffinity scanning approach (17). The generation of covalently linked ligand-receptor conjugates and the identification of the cross-linked domains allows mapping of the interface between hormone and receptor. Photoaffinity cross-linking has been successfully applied in defining interactions between small peptides, such as substance P (18–20), cholecystokinin (21), and vasopressin (22), and their receptors. Recently, we used this general approach to identify directly the interaction between position 13 of PTH and a 17-amino acid domain (residues 173–189) of the hPTH1-Rc (17).

We now report the evaluation of a series of photoreactive analogs obtained by a “p-benzoylphenylalanine (Bpa) scan” of the principal receptor activation domain (residues 1–6) of PTH–(1–34). A radiolabeled analog containing a photoreactive moiety at the N terminus, 125I-[Bpa1,Nle8,18,Arg13,26,27,L-2-Nal23,Tyr34]bPTH–(1–34)NH2, maintained full potency and led to the identification of a second “contact domain” between PTH and hPTH1-Rc. This information allows us to create, for the first time, a model describing interactions of the receptor-ligand complex.

**EXPERIMENTAL PROCEDURES**

Materials—Boc-protected amino acids, N-hydroxybenzotriazole, N,N′-dicyclohexylcarbodiimide, and p-methylbenzylidene resin were purchased from Applied Biosystems (Foster City, CA). Boc-(3-iodo)tyrosine[O-(4-BrBz)] was from Peninsula Laboratories (Belmont, CA). B&J brand dichloromethane, N-methylpyrrolidone, and acetoni-benzylamine resin were purchased from Pierce. Cyanogen bromide was from Aldrich. Na235I was obtained from Amersham Pharmacia Biotech. Endoglycosidase F/N-glycosidase F; Fura-2, fura-2/acetomethyl ester; G protein, guanylate cyclase/binding protein, human; HEK, human embryonic kidney; Lys-C, lysyl endopeptidase; MD, molecular dynamics; Nal, naphthylalanine; Nle, norleucine; PTH (1–34), [Nle8,18,Tyr34]bPTH–(1–34)NH2; 125I-[Nle8,18,Tyr34]bPTH–(1–34)NH2; PTH (7–34), [Nle8,18,p-Trp12,Tyr34]bPTH–(7–34)NH2; Rc, receptor; RP-HPLC, reverse phase-high performance liquid chromatography; TM, transmembrane; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine;
**Parathyroid Hormone-Receptor Bimolecular Interactions**

**TABLE I**

| Analog | Bpa1 | Bpa2 | Bpa3 | Bpa4 | Bpa5 | Bpa6 |
|--------|------|------|------|------|------|------|
| M (a)  | 4362 | 4334 | 4346 | 4304 | 4320 | 4305 |
| Calculated |       |      |      |      |      |      |
| Found  | 4302 | 4335 | 4347 | 4305 | 4321 | 4306 |
| HPLC |      |      |      |      |      |      |
| t<sub>1/2</sub> (min) | 21.9 | 21.5 | 22.3 | 24.7 | 21.0 | 20.0 |
| k<sub>b</sub> | 6.3 | 6.2 | 6.4 | 7.2 | 7.0 | 6.7 |
| a Molecular weights were measured by electron spray-mass spectrometry.
| b Reverse-phase HPLC analyses were carried out on a Vydac C-18, 300-Å column (4.6 × 150 mm, 5 μm) at a flow rate of 1.0 ml/min and monitored at 220 nm. Linear gradient from 20 to 50% (v/v) eluant B in A over 30 min, where eluant A is 0.1% (v/v) trifluoroacetic acid in water and eluant B is 0.1% (v/v) trifluoroacetic acid in acetonitrile.
| c k = (t<sub>1/2</sub> - t<sub>1/2</sub>), where t<sub>1/2</sub> and t<sub>1/2</sub> are the retention times of the peptide and the front, respectively, in the conditions described in footnote b.

**Photoaffinity cross-linking of 125I-Bpa1, adenyl cyclase activity 72 h after transfection.** Activation of adenyl cyclase at pH 8.5, containing Triton X-100 (0.1% w/v) and SDS (0.01% w/v). Small scale photoaffinity cross-linking of transiently transfected COS-7 cells, grown to overconfluence, was carried out in 24-well tissue culture plates. Cells were washed with D-MEM and were treated with 200 μl of DMSO and either 25 μl of 125I-Bpa1-PTH(1–34) in vehicle (PBS, 0.1% BSA), or vehicle alone added. Reactions were incubated at room temperature, 1–2 × 10<sup>5</sup> cpm of 125I-Bpa1-PTH(1–34) (total volume 25 μl) added to each well, and incubated an additional 15 min at room temperature. Plates were cross-linked in a Stratalinker for 30 min as described earlier. Each well was washed once with PBS, cells lysed with 0.5 ml of Laemmli sample buffer, shaken in dish for 10–30 min, and homogenized. Tubes were incubated on a rocking platform at room temperature for 2–3 h, and analyzed by SDS-PAGE.

**Enzymatic and Chemical Digestions of the 125I-Bpa1-PTH(1–34)-Receptor Conjugate—**Batches of SDS-PAGE-purified radiolabeled hormone-receptor conjugates and fragments were prepared in small volumes (typically 10–20 μl) of 25 mM Tris-HCl, pH 7.4, Triton X-100 (0.1% w/v), SDS (0.01% w/v). Endo-F digestions were carried out at 37 °C for 24 h, according to the manufacturer’s procedure. Lys-C digestions were performed by two 24-h treatments with 0.15 units (in 10 μl) of water at 37 °C. BNPS-skatol digests were carried out with 2 mg/ml BNPS-skatole in 70% acetic acid at 37 °C for 24–48 h in the dark. CNBr digestions were performed with 50 mg/ml CNBr in 70% formic acid at 37 °C for 24 h in the dark. Samples were dried on Speed-Vac and dissolved in 2 μl of H<sub>2</sub>O prior to analysis.

**Electrophoresis and Autoradiography—**Electrophoretic analyses were performed with 7.5% SDS-PAGE for the hormone-receptor conjugates and 16.5% Tricine/SDS-PAGE for the cleavage products. Appropriate molecular weight markers (Amersham Pharmacia Biotech and Bio-Rad) were included in each gel. Gels were dried and exposed to x-ray films (X-Omat, Eastman Kodak Co.) with intensifying screens (Kodak). Purity and structure of the peptides were confirmed by reverse-phase HPLC analyses. The retention times of the peptide and the front, respectively, in the conditions described in footnote b.

**Intracellular Calcium Determinations—**The stimulation of increases in intracellular calcium levels following treatment with PTH(1–34) and the Bpa-containing analogs was assessed spectroscopically in Fura-2-loaded HEK-293/C-21 cells as described (26).

**Photoaffinity Cross-linking, Membrane Protein Preparation, and SDS-PAGE Purification—**Photoaffinity cross-linking of 125I-Bpa1, Nle<sup>3</sup>-Arg<sup>13</sup>,20-Tyr<sup>23</sup>,27-bPTH(1–34)NH2 was carried out as described (17). Briefly, confluent HEK-293/C-21 cells were harvested with 0.5 mM EDTA, washed twice with PBS, and resuspended in D-MEM at a density of 2 × 10<sup>5</sup> cells/ml. This suspension was incubated at room temperature for 30 min in the presence of 0.3 μCi (~0.5 nmol) of 125I-Bpa1-PTH(1–34), after which cells were placed on ice in a Stratalinker (Stratagene) at a distance of 10 cm from six 15-watt 365 nm UV lamps and irradiated for 1 h. Cells were then washed five times with PBS, resuspended in 50 mM Tris, pH 8.5, and lysed by five cycles of freezing and thawing. Membranes were obtained by centrifugation at 45,000 rpm for 2 h at 4 °C. Membranes were solubilized in 25 mM Tris, pH 8.5, containing Triton X-100 (2% v/v) at room temperature for 2 h. Proteins were precipitated by adding five volumes of cold acetone and redissolved in 25 mM Tris, pH 8.5, containing SDS (2% w/v). Proteins were reduced with 100 mM dithiothreitol for 2 h at 37 °C and alkylated with 200 mM iodoacetamide for 15 min at room temperature. Reduction was divided and concentrated on Centricon 50 (Amicon), diluted with reducing Laemmli sample buffer, and loaded on a 7.5% (v/v) SDS-PAGE. After autoradiography, the radioactive 125I-Bpa1-PTH(1–34)–Receptor conjugate was excised from the gel, passively eluted in 100 mM NH<sub>4</sub>HCO<sub>3</sub>/SDS (0.01% w/v), pH 7.5, and submitted to concentration and buffer exchange on Centricon 50 (Amicon) to 25 mM Tris, pH 8.5, containing Triton X-100 (0.1% v/v) and SDS (0.01% v/v). Small scale photoaffinity cross-linking of transiently transfected COS-7 cells, grown to overconfluence, was carried out in 24-well tissue culture plates. Cells were washed with D-MEM and were treated with 200 μl of DMSO and either 25 μl of 125I-Bpa1-PTH(1–34) in vehicle (PBS, 0.1% BSA), or vehicle alone added. Reactions were incubated at room temperature, 1–2 × 10<sup>5</sup> cpm of 125I-Bpa1-PTH(1–34) (total volume 25 μl) added to each well, and incubated an additional 15 min at room temperature. Plates were cross-linked in a Stratalinker for 30 min as described earlier. Each well was washed once with PBS, cells lysed with 0.5 ml of Laemmli sample buffer, shaken in dish for 10–30 min, and homogenized. Tubes were incubated on a rocking platform at room temperature for 2–3 h, and analyzed by SDS-PAGE.

**Molecular Modeling—**The molecular model of the hPTH1-Rc was developed using the topological arrangement of the TM helices of rhodopsin (29). To identify the location of the TM portions of the hPTH1-Rc, assumed to be α-helices, a hydrophobicity profile (30) was calculated for a region of the protein data bank, especially for a region of high homology in the protein data bank, following the substitution-table confined, requiring minor adjustments, following the substitution-table.
ectopic N-terminal tail of the hPTH1-Rc contiguous to the TM1 helix. The homologous regions of each of these protein structures were analyzed for secondary structural features and then incorporated into the molecular model.

To refine the molecular model, molecular dynamics (MD) simulations and energy minimization were carried out with the CVFF91 force field within the Discover program (Biosym/MSI). To mimic the environment of the membrane, a two-phase simulation cell consisting of H2O and CCl4 was utilized. The explicit solvent simulations were carried out following previously published procedures (33). All molecular modeling was carried out with the Insight II program (Biosym/MSI).

RESULTS

Characterization of Bpa-containing PTH-(1–34) Analogs—

Binding affinities for the hPTH1-Rc stably expressed in HEK-293 cells (clone C-21) were measured by competition with [125I][Nle8,18,Tyr34]bPTH-(1–34)NH2 (125I-PTh-(1–34)) (Fig. 1A). Agonist activity (stimulation of adenylyl cyclase and increase in intracellular calcium levels) was determined in HEK-293/C-21 cells (25) (Fig. 1, A and C). The substitution of Ala1 with Bpa in PTH-(1–34) generated Bpa1-PTH-(1–34), which displays a pharmacological profile similar to that of the parent peptide PTH-(1–34) (IC50 = 4.5 nM; EC50 = 2 nM and [Ca2+]i = 130 nM at 10−7 M ligand and IC50 = 25 nM, EC50 = 0.8 nM and [Ca2+]i = 100 nM at 10−7 M ligand for PTH-(1–34) and Bpa1-PTH-(1–34), respectively). Bpa substitution of Val2 caused a 17-fold reduction in binding affinity accompanied by a 10-fold reduction in adenylyl cyclase activity and only 50% mobilization of intracellular calcium relative to PTH-(1–34). Despite a 60-fold reduction in binding affinity, Bpa2-PTH-(1–34) displayed full potencies for the stimulation of both adenylyl cyclase and intracellular calcium transients (Fig. 1). Substitution at positions 3, 4, and 5 led to analogs with very weak binding affinity, weak stimulation of adenylyl cyclase, and no effect on [Ca2+]i.

Characterization of Bpa-containing PTH-(1–34) Analogs—

Bpa 3-PTH-(1–34), 5, Bpa 4-PTH-(1–34), 6, Bpa 5-PTH-(1–34), and 7 yielded a single diffuse band migrating at 7 kDa (Fig. 2A). The substitution of Ala1 with BNPS-skatole. Exhaustive Lys-C treatment produced a single band with apparent mass of 7 kDa (Fig. 3A, lane 2). Moreover, the formation of the ligand–receptor conjugate was completely inhibited in the presence of excess (17 μM) unlabeled agonist PTH-(1–34) or antagonist PTH-(1–34) (Fig. 2A, lanes 3 and 4, respectively). The apparent molecular mass of the cross-linked band was similar to that observed for the conjugate obtained through photocross-linking of a position 13 benzophenone-containing PTH-(1–34) analog, [Nle8,18,Lys13(ε-pBz)2,2-i-Nal23,Tyr34]bPTH-(1–34)NH2 to the same hPTH1-Rc-expressing cells (28). Endo-F-mediated deglycosylation of the 87-kDa band shifted the complex to 70-kDa band (Fig. 2B, lane 2) as described previously (17, 34–36).

Identification of the Ligand Binding Domain—The 87-kDa 125I-Bpa1-PTH-(1–34)–Rc conjugate was purified from 7.5% SDS-PAGE (Fig. 1, lane 2). The apparent molecular mass was determined by analytical RP-HPLC (data not shown). The apparent molecular mass of the cross-linked band was similar to that observed for the conjugate obtained in Fura-2-loaded HEK-293/C-21 cells obtained in a single experiment. The numbers of the bars refer to the following compounds: 1, PTH-(1–34), 2, Bpa1-PTH-(1–34) (○), Bpa2-PTH-(1–34) (×), Bpa3-PTH-(1–34) (■), Bpa4-PTH-(1–34) (□), Bpa5-PTH-(1–34) (△), and Bpa6-PTH-(1–34) (△) are shown. Experiments were carried out in triplicate. Curves in panels A and B show the mean ± S.E. of three independent experiments. C, stimulation of intracellular calcium release by 10−7 M PTH-(1–34) and Bpa-containing PTH-(1–34) analogs in Fura-2-loaded HEK-293/C-21 cells obtained in a single experiment. The numbers of the bars refer to the following compounds: 1, PTH-(1–34), 2, Bpa1-PTH-(1–34), 3, Bpa2-PTH-(1–34), 4, Bpa3-PTH-(1–34), 5, Bpa4-PTH-(1–34), 6, Bpa5-PTH-(1–34), and 7 Bpa6-PTH-(1–34). Similar results were obtained in two additional experiments.

FIG. 1. In vitro characterization of the Bpa-containing PTH-(1–34) analogs. Competition for 125I-PTh-(1–34) binding (A) and dose-response curves for the stimulation of adenylyl cyclase activity (B) in HEK-293/C-21 cells by PTH-(1–34) (○), Bpa1-PTH-(1–34) (■), Bpa2-PTH-(1–34) (×), Bpa3-PTH-(1–34) (□), Bpa4-PTH-(1–34) (△), Bpa5-PTH-(1–34) (△), and Bpa6-PTH-(1–34) (△) are shown. Experiments were carried out in triplicate. Curves in panels A and B show the mean ± S.E. of three independent experiments. C, stimulation of intracellular calcium release by 10−7 M PTH-(1–34) and Bpa-containing PTH-(1–34) analogs in Fura-2-loaded HEK-293/C-21 cells obtained in a single experiment. The numbers of the bars refer to the following compounds: 1, PTH-(1–34), 2, Bpa1-PTH-(1–34), 3, Bpa2-PTH-(1–34), 4, Bpa3-PTH-(1–34), 5, Bpa4-PTH-(1–34), 6, Bpa5-PTH-(1–34), and 7 Bpa6-PTH-(1–34). Similar results were obtained in two additional experiments.

radiolabeled band with apparent molecular mass of ~11 kDa (Ia) (Fig. 3A, lane 2). Similar treatment of the deglycosylated conjugate (~66 kDa) yielded a band with the same apparent molecular mass (data not shown), confirming the absence of glycosylation within the Lys-C-generated fragment Ia. BNPS-skatole treatment of the excised and eluted 11-kDa (Ia) fragment produced a single band with apparent mass of ~7 kDa.

Identification of the Ligand Binding Domain—The 87-kDa 125I-Bpa1-PTH-(1–34)–Rc conjugate was purified from 7.5% SDS-PAGE and subjected to a series of chemical and enzymatic cleavages. The first digestion pathway (I) consisted of enzymatic cleavage at the carboxyl side of lysyl residues with Lys-C, followed by chemical cleavage at the carboxyl side of tryptophanyl residues with BNPS-skatole. Exhaustive Lys-C treatment of the 87-kDa ligand–receptor conjugate yielded a single band shifted the complex to ~70-kDa band (Fig. 2B, lane 2) as described previously (17, 34–36).

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the recombinant hPTH1-Rc.

A receptor's response to PTH-(1–34). However, the mutants differ in their ability to cross-link with 125I-Bpa1-PTH-(1–34) (Fig. 5). The arrow indicates the position of the ~87-kDa cross-linked hPTH1-Re. B, Endo-F-mediated deglycosylation of the 125I-Bpa1-PTH-(1–34)-hPTH1-Re conjugate. The ~87-kDa labeled conjugate was incubated in the absence (lane 1) or presence (lane 2) of endoglycosidase F/N-glycosidase F. The arrow indicates the position of the ~66-kDa deglycosylated labeled receptor. Samples were loaded on 7.5% (w/v) SDS-PAGE. Molecular mass markers are also shown. Similar results were obtained in two additional experiments.

B (Fig. 3A, lane 3).

A second digestion pathway (II), the reciprocal of I, initially yielded a single band migrating at ~14 kDa (IIa) (Fig. 3B, lane 2). Lys-C treatment yielded the final fragment migrating at ~7 kDa (IIb) (Fig. 3B, lane 3), similar to Ib obtained from pathway I.

Treatment with CNBr (III) of the purified intact ligand-receptor conjugate in 70% formic acid solution produced a band of very low apparent molecular mass (IIIa) (~4 kDa, Fig. 3C, lane 2), with electrophoretic mobility similar, if not identical, to that of the free radioligand 125I-Bpa1-PTH-(1–34) (Fig. 3C). In addition, CNBr treatment of the 11-kDa (Ia) fragment obtained after Lys-C digestion produced a similar ~4-kDa band (data not shown).

Fig. 4 summarizes schematically the different fragmentation pathways employed in the analysis of hPTH1-Re–125I-Bpa1-PTH-(1–34) conjugate.

Characterization of Transiently Transfected COS-7 Cells Expressing Mutated hPTH1-Re—PTH-(1–34) stimulation of adenylyl cyclase in COS-7 cells transiently expressing either the hPTH1[M414L], hPTH1[M425L], or the native hPTH-Rcs resulted in very similar dose-response curves (Fig. 5A). Point mutation of either Met414 or Met425 to Leu does not alter the receptor’s response to PTH-(1–34). However, the mutants differ in their ability to cross-link with 125I-Bpa1-PTH-(1–34) (Fig. 5B). The transiently transfected hPTH1[M414L] and native hPTH Rcs cross-link to 125I-Bpa1-PTH-(1–34) (Fig. 5B, lanes 1 and 3), respectively, generating the anticipated ~87-kDa band corresponding to the 125I-Bpa1-PTH-(1–34)–hPTH1-Re conjugate. This cross-linking can be inhibited competitively by 10−6 M PTH-(1–34) (Fig. 5B, lanes 2 and 4). In contrast, the functional transiently transfected hPTH1[M425L] does not cross-link to 125I-Bpa1-PTH-(1–34) (Fig. 5B, lanes 5 and 6), suggesting that Met425 may be involved in the cross-linking of 125I-Bpa1-PTH-(1–34).

Fig. 3. Chemical and enzymatic digestions of the 125I-Bpa1-PTH-(1–34)-hPTH1-Re conjugate. A, the SDS-PAGE purified ~87-kDa conjugate was incubated in the absence (lane 1) or presence (lane 2) of Lys-C. The excised and eluted Lys-C-derived 11-kDa (Ia) fragment was then treated with BNPS-skatole (IB) (lane 3). Samples were loaded on 16.5% (w/v) Tricine/SDS-PAGE. Molecular mass markers are also shown. B, the SDS-PAGE purified ~87-kDa conjugate was incubated in the absence (lane 1) or presence of BNPS-skatole (lane 2). The excised and eluted ~14-kDa (IIa) band was then treated with Lys-C (IIIb) (lane 3). Samples were loaded on 16.5% (w/v) Tricine/SDS-PAGE. Molecular mass markers are also shown. C, the SDS-PAGE of a mixture of the purified ~87-kDa conjugate in the presence of free ligand (lane 1), and the purified ~87-kDa conjugate incubated in the presence of cyanogen bromide (IIIa) (lane 2). Samples were loaded on 16.5% (w/v) Tricine/SDS-PAGE. Molecular mass markers are also shown. Similar results were obtained in three additional experiments.

Molecular Modeling—Results from the BLAST search of hPTH1-Re(172–189), consisting of the distal end of the ectopic...
The characterization of the bimolecular interaction between the activation domain of PTH-(1–34) and the hPTH1-Rc is of fundamental importance for elucidating the molecular mechanism of signal transduction. To this end, photoaffinity cross-linking of bioactive analogs enables direct identification of “contact domains” and/or “contact points” between ligand and receptor (17–21, 38–42). The goal of this study was to identify fundamental importance for elucidating the molecular mechanism of signal transduction. To this end, photoaffinity cross-linking of bioactive analogs enables direct identification of “contact domains” and/or “contact points” between ligand and receptor (17–21, 38–42). The goal of this study was to identify principal “activation domain” of PTH-(1–34), a conformation of hPTH1-Rc with the ectopic amphipathic helix projecting away from the TM1 helix and this ectopic helix.

Although these data support the presence of an amphipathic α-helix, the orientation of this helix with respect to the bundle of TM helices is not clear. Molecular dynamics simulation of many different starting orientations of the helix were carried out using the two phase simulation cell (33). Throughout the molecular dynamics simulations, the ectopic helix always tended to move away from the bundle of TM helices. Therefore, for the purpose of docking the ligand hPTH-(1–34), a conformation of hPTH1-Rc with the ectopic amphipathic helix projecting away from the TM bundle was utilized.

Molecular modeling of hPTH-(1–34) with the hPTH1-Rc model was then performed, guided by the contact domain identified by previous photoaffinity cross-linking studies (17) (Fig. 6). The conformation of hPTH-(1–34) used in the molecular modeling was obtained from our high resolution NMR studies performed in different environments, including aqueous saline conditions and the presence of dodecylphosphocholine as a membrane mimetic (37).

![Fig. 4. Schematic summary of the fragmentation pattern observed for the 125I-Bpa1-PTH-(1–34)-hPTH1-Rc following pathways I (—), II ( - - ) and III ( - ). Endo-F, BNPS-skatole, Lys-C, and cyanogen bromide digestions were carried out as detailed under “Experimental Procedures.” • • • indicates data not shown. Molecular masses of the fragments are indicated in kDa and represent the actual size of the digested conjugate fragments including the ligand 125I-Bpa1, PTH-(1–34) (molecular weight 4489).](image)

![Fig. 5. Characterization of COS-7 cells transiently expressing native and point mutant hPTH1-Rc. A, stimulation of adenyl cyclase (cAMP in pmol/well above basal) by PTH-(1–34) in COS-7 cells transiently expressing native hPTH1-Rc (○), and receptor containing mutations M414L (□) and M425L (×). B, SDS-PAGE analysis of 125I-Bpa1-PTH cross-linking to COS-7 cells transiently transfected with native hPTH1-Rc (lanes 1 and 2), and receptor containing mutations M414L (lanes 3 and 4), and M425L (lanes 5 and 6) in the absence (lanes 1, 3, and 5) or presence of 10−6 M PTH-(1–34) (lanes 2, 4, and 6). Size markers (in kDa) are also shown. The arrow to the right of lane 6 indicates the location of the 125I-Bpa1-PTH1-Rc conjugate (~87 kDa).](image)
cally designed for this study. Both Met<sup>8</sup> and Met<sup>18</sup> were replaced by the isosteric Nle residue, rendering the ligand resistant to cyanogen bromide treatment. Replacement of Trp<sub>23</sub> with 2-naphthylalanine (2-Nal) introduces stability toward digestion by Trp-specific reagents. Replacement of all Lys residues with Arg provides resistance to Lys-C-mediated cleavage, and replacement of Phe<sub>34</sub> with Tyr generates a reactive site for incorporation of radioiodine. These modifications were introduced singularly and found to be well-tolerated (23, 43). As demonstrated previously, the combination of all the modifications is, in many cases, also well tolerated (17, 23).

Based on the protein sequence of hPTH1-Rc, an exhaustive Lys-C digestion of the 125I-Bpa<sup>1</sup>-PTH-(1–34)–hPTH1-Rc cross-linked conjugate should generate three fragments with molecular masses of approximately 6.5 kDa: hPTH1-Rc-(487–539), -(409–471), and -(173–240) (5,410.1, 7,291.5, and 8,031.5 Da, respectively). The generation of the Ia fragment (Fig. 4) from the deglycosylated receptor eliminates hPTH1-Rc-(173–240), which contains a documented glycosylation site at Asn<sub>176</sub> (17). The hPTH1-Rc-(487–539) fragment does not contain a tryptophan and therefore will not be cleaved when treated with BNPS-skatole. Therefore, hPTH1-Rc-(409–471) represents the region in the receptor cross-linked to the ligand in conjugate Ia (Figs. 4 and 3A, lane 2).

The smallest and sole overlapping sequence among the BNPS-skatole-generated fragments from both reciprocal digestion pathways (pathway I: hPTH1-Rc-(409–437) and -(438–471), and pathway II: hPTH1-Rc-(1–69), -(362–437), and
The structure of the hPTH1-Rc obtained by homology modeling and MD using a two-phase solvent cell (33) suggests that the segment Arg^{270}–Arg^{283} consists of an amphipathic a-helix whose axis is parallel to the membrane surface and directed away from the helical bundle of the receptor. The structure of hPTH(1–34) used in the molecular modeling was determined by NMR in a zwitterionic, micellar environment (37) as a mimic of the ligand itself (mass of hPTH-(1–34), consisting of 4,487 Da) and distinctly lower than any potential covalent ligand–receptor complex produced by cyanogen bromide cleavage.

Exhaustive cyanogen bromide digestion of the intact ^125^I-Bpa^{1}-PTH-(1–34)–hPTH1-Rc conjugate (87 kDa) (III) (Fig. 4) and of the Lys-C-generated fragment In (11 kDa) yields a similar band IIIa with a low apparent molecular mass (~4 kDa) (Fig. 3C, lane 2). The electrophoretic mobility of this band is similar to that of the ligand itself (mass = 4,487 Da) and distinctly lower than any potential covalent ligand–receptor complex produced by cyanogen bromide cleavage.

Photocross-linking of a benzophenone-containing ligand through insertion into a C-H bond of the S-CH$_3$ group in Met residues will generate upon cyanogen bromide treatment a ligand–CH$_3$SCN adduct, which increases its molecular mass by only 73 Da (19). Electrochemically, the CH$_3$SCN–^125^I-Bpa^{1}-PTH-(1–34) adduct will be indistinguishable from the non-modified photoactive radioligand (Fig. 3C). This adduct could be generated by cross-linking to either one of the two methionine residues present in the minimal contact domain hPTH1-Rc-(409–437) and included in the conjugate fragment (IIb/IIIb) (Figs. 4, 3A, lane 3, and 3B, lane 3). Both methionines, Met^{414} and Met^{425}, are located in TM6 and are therefore potential contact points between the N-terminal residue of PTH and the receptor.

Using site-directed mutagenesis to produce both M414L and M425L mutated Rcs, the Met residue involved in the cross-linking can be assigned to Met^{425}. The M414L mutant is fully active and like the native PTH1-Rc it photocross-links to the non-modified photoreactive radioligand (Fig. 3C). This adduct could be generated by cross-linking to either one of the two methionine residues present in the minimal contact domain hPTH1-Rc-(409–437) and included in the conjugate fragment (IIb/IIIb) (Figs. 4, 3A, lane 3, and 3B, lane 3). Both methionines, Met^{414} and Met^{425}, are located in TM6 and are therefore potential contact points between the N-terminal residue of PTH and the receptor.

The structure of hPTH1-Rc obtained by homology modeling and MD using a two-phase solvent cell (33) suggests that the segment Arg^{270}–Arg^{283} consists of an amphipathic a-helix whose axis is parallel to the membrane surface and directed away from the helical bundle of the receptor. The structure of hPTH(1–34) used in the molecular modeling was determined by NMR in a zwitterionic, micellar environment (37) as a mimic of the cellular membrane. Throughout the MD simulations, deviations from the experimentally determined structure of hPTH(1–34), consisting of a-helices for residues 4–10 and 20–32, were not allowed. A number of starting structures with Lys^{13} of hPTH placed at different locations along the 17-amino acid cross-linking domain hPTH1-Rc-(173–189) (17) were used for MD simulations. During these simulations, utilizing the biphasic solvent mixture to mimic the membrane environment (33), the interactions between the amphipathic helix of the receptor, segment 179–186, just exterior to TM1, and the C-terminal helix (residues 20–32) of hPTH were optimized. Throughout these simulations, the N-terminal residue of hPTH could be easily placed in close proximity to Met^{425} which is on the surface of the membrane at the C-terminal end of TM6 (Fig. 6). In contrast, all attempts to place position 1 of hPTH in close proximity to Met^{414} while maintaining the experimentally determined conformation of hPTH and Lys^{13} of hPTH close to its cross-linking domain, failed. In our model, Met^{414} is on the intracellular half of TM6, projecting toward the membrane, a full three helical turns removed from Met^{425}. Thus, the bio-chemical analysis of the ^125^I-Bpa^{1}-PTH-(1–34)–hPTH1-Rc conjugate, site-directed mutagenesis of the hPTH1-Rc, and molecular modeling simulation strongly suggest that Met^{425} is the “contact point” of the hPTH1-Rc and the cross-linking site for ^125^I-Bpa^{1}-PTH-(1–34).

Previous mutagenesis studies have implied that TM6 and the third extracellular loop are important for hormone binding and signal transduction. Homologous substitution of these regions in the rat Rc with the corresponding portions of either the opossum PTH1-Rc (45) or the secretin Re (46) identified several residues (i.e. Leu^{247}, Trp^{347}) which affect hormone binding and/or signaling. Interestingly, mutation of Thr^{410} in TM6 generated a constitutively active receptor associated with the clinical skeletal disorder, Jansen’s metaphyseal chondrodysplasia (47). TM6 seems to be directly involved in signaling in other G protein-coupled receptors. Mutations in this region of the m5 muscarinic (48) and a-factor (49) receptors result in constitutive receptor activation. In addition, TM6 is contiguous with the third intracellular loop, which has been implicated in the interaction of G proteins in several seven TM-domain-containing receptors, including the PTH1-Rc (50).

The identification of Met^{425} in the extracellular end of TM6 as the contact point for the N terminus of PTH, together with the emerging model of ligand-receptor interaction, offers new insights into the nature of hormone-receptor interactions and signal transduction in this system. The contact of the principal activation domain of PTH-(1–34) with residues in the extracellular end of TM6, which in turn is connected to the third intracellular loop (considered to contain a G protein contact domain) suggests a possible relay mechanism that communicates an extracellular stimulus (i.e. agonist binding) into an intracellular signaling event (i.e. activation of the G protein).

Our modeling of the bimolecular PTH–hPTH1-Rc interaction potentially can be greatly enhanced by identification of the specific amino acid in hPTH1-Rc involved in cross-linking with residue 13 of hPTH. Narrowing the 17-amino acid contact domain to a smaller fragment, plus identification of additional contact domains in the Rc with other amino acids of the hormone, will refine our experimentally based model and provide greater detail regarding the hPTH–hPTH1-Rc bimolecular interface.

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