Multiple Sites of Contact between the Carboxyl-terminal Binding Domain of PTHrP-(1–36) Analogs and the Amino-terminal Extracellular Domain of the PTH/PTHrP Receptor Identified by Photoaffinity Cross-linking*

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The carboxyl-terminal portions of parathyroid hormone (PTH)-(1–34) and PTH-related peptide (PTHrP)-(1–36) are critical for high affinity binding to the PTH/PTHrP receptor (P1R), but the mechanism of receptor interaction for this domain is largely unknown. To identify interaction sites between the carboxyl-terminal region of PTHrP-(1–36) and the P1R, we prepared analogs of [I\(^5\), W\(^{23}\), Y\(^{36}\)]PTHrP-(1–36)-amide with individual p-benzoyl-l-phenylalanine (Bpa) substitutions at positions 23, 27, 28, and 33 of native PTHrP. Cross-linking experiments with these Bpa-substituted compounds affinity-labeled the Bpa24-substituted analog, similar to that of the parathyroid hormone-related peptide; Bpa, to residues 96–102; [I\(^5\), W\(^{23}\), Bpa\(^{27}\), Y\(^{36}\)]PTHrP-(1–36)-amide cross-linked to residues 64–95, and [I\(^5\), W\(^{23}\), Bpa\(^{27}\), Y\(^{36}\)]PTHrP-(1–36)-amide cross-linked to residues 151–172. These data thus predict that residues 23, 27, 28, and 33 of native PTHrP are each near to different regions of the amino-terminal extracellular receptor domain of the P1R. This information helps define sites of proximity between several ligand residues and this large receptor domain, which so far has been largely excluded from models of the hormone-receptor complex.

PTH and PTHrP\(^1\) mediate many of their biological effects through the same receptor (1, 2). Peptides containing the first 34 amino acids of PTH and PTHrP are capable of fully activating the PTH/PTHrP receptor (P1R) (1, 2). Studies with PTH and PTHrP ligand analogs and receptor chimeras have suggested that the ligands have two distinct functional domains: the amino-terminal residues, which are important for receptor activation; and the carboxyl-terminal residues, which are important for high affinity binding (3). These data furthermore indicate that the ligand’s amino-terminal portion interacts with the extracellular loops and the membrane-spanning helices of the receptor, and the ligand’s carboxyl-terminal portion interacts with the receptor’s amino-terminal extracellular domain (3). A similar pattern of ligand-receptor interaction has been suggested for other members of this class II family of peptide hormone G-protein-coupled receptors, including the secretin receptor (4) and the PTH-2 receptor (5–7).

Some specific sites of interaction between the amino-terminal portions of PTH-(1–34)/PTHrP-(1–36) and the P1R have been identified by site-directed mutagenesis and photoaffinity cross-linking studies. For example, mutational analyses have shown that residues in extracellular loop 3 and the adjacent sixth membrane-spanning helix (TM6) are critical for mediating ligand-induced receptor activation (2, 8). Consistent with these mutational data, Bpa introduced at position 1 of PTH-(1–34) or position 2 of either PTH-(1–34) or PTHrP-(1–36) was found to cross-link to methionine 425, at the extracellular end of TM6 in the P1R (9, 10). Interactions between the amino-terminal portion of the ligand and the transmembrane domains/extracellular loops of the receptor are also suggested by a study showing that PTH-(1–14) can stimulate cAMP accumulation in a mutant P1R missing most of the amino-terminal extracellular domain as efficiently as it does in the wild-type P1R (11). Furthermore, if a PTH fragment comprising the first 9 amino acids is covalently attached to the amino-terminal end of such a truncated receptor, the resulting ligand-receptor chimera displays constitutive activity, indicative of an intramolecular stimulation of the receptor’s activation domain by the tethered ligand fragment (12).

In contrast to the amino-terminal portion of the ligand, less is known about the interaction of the mid- and carboxyl-terminal region of PTH-(1–34) and PTHrP-(1–36) with the P1R. Initial studies with different P1R chimeras indicated that the carboxyl-terminal portion of PTH-(1–34) interacts with the receptor’s amino-terminal extracellular domain (13). This domain in the P1R is quite large (~190 amino acids, including the 22-amino acid signal sequence) and can be further subdivided based on the exons that encode it: S-(1–25), E1-(26–60), E2-(61–105), E3-(106–141), and G-(142–181) (14, 15) (see Fig. 1 below). Ligand residue 23 (Phe in PTHrP and Trp in PTH) has

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The abbreviations used are: PTH, parathyroid hormone; PTHrP, parathyroid hormone-related peptide; Bpa, p-benzoyl-l-phenylalanine; Bpa\(^{23}\), benzoyl group attached to epsilon-amino group of lysine; Endoglycosidase-F, N-glycosidase F; PAGE, polyacrylamide gel electrophoresis; P1R, PTH/PTHrP receptor; hP1R, human P1R; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
been found to be important for high affinity binding to the P1R and to the PTH-2 receptor (16, 17). Replacement of this residue with a Bpa photoactive group results in affinity cross-linking to residues 23–40 of the rat P1R, at the extreme end of the amino-terminal extracellular domain. Two residues within this region, Thr23 and Gin27, were shown to be important for binding of PTHrP-(7–34) (18). Residues 24–34 of PTHrP-(1–34) (19) and PTH-(1–34) (20) also contribute to high affinity binding to the P1R, and three hydrophobic amino acids within this region of PTH-(1–34), residues Leu24, Leu28, and Val31, have been found to be insufficient to substitution with polar residues (17). A PTH analog with a benzoyl group attached to the epsilon-amino group of lysine 27 has been shown to cross-link to extracellular loop 1 of the human P1R (21), a result that would not have been predicted by the previous receptor mutagenesis and chimera studies (3, 8, 13). Affinity cross-linking studies using a PTH-(1–34) analog with a photoactive benzoyl group attached to Lys13, in the center of the ligand, demonstrated an interaction to P1R residue Arg186 located near the first transmembrane helix (22).

Based upon the available physical data, two computer models have been constructed for the complex formed between PTH-(1–34) and the P1R, and these models include interactions between residues in the carboxyl-terminal portion of PTH-(1–34) or PTHrP-(1–36) and residues in the amino-terminal extracellular domain of the P1R (23, 24). The first model, by Röls et al. (23), utilized a solution-phase NMR structure of the ligand, which indicates two α-helices separated by a flexible linker region. Using the previously described cross-link between residue 13 in PTH-(1–34) and Arg186 of the P1R as a constraint (22), the model aligns the carboxyl-terminal helix of PTH-(1–34) with a putative α-helix comprising residues 180–189 of the receptor, at the proximal portion of the amino-terminal extracellular domain (23). This model was later modified to include the cross-link sites of ligand positions 23 and 27, but the revised model was not assessed by molecular dynamic simulations (25). A second model was reported with the recent x-ray crystallographic structure of PTH-(1–34) (24). The crystal structure showed the peptide to contain an extended α-helix spanning nearly the entire chain length (residues 3–32), with a 15° bend in the center (24). Using this structure of the ligand, along with the previously determined cross-linking sites of ligand positions 1 (9) and 13 (22), a detailed alignment was suggested for the carboxyl-terminal region of PTH-(1–34) and the amino-terminal extracellular domain of the P1R. This alignment predicted a hydrophobic interaction between residues Trp23, Leu24, and Leu28 of PTH-(1–34) and Phe173, Leu174 of the P1R; and a polar interaction between Lys27 of PTH-(1–34) and Glu169 of the P1R. The accompanying computer-based model of PTHrP-(1–34) complexed with the P1R predicts the same ligand-receptor interactions, with the exception that Leu27 of PTHrP-(1–34) also participated in the hydrophobic interactions with residues Phe173 and Leu174 of the P1R (24).

To help further elucidate the interactions that occur between the carboxyl-terminal region of the ligand and the P1R, we undertook a photoaffinity cross-linking study utilizing PTHrP-(1–36) analogs with individual Bpa substitutions of residues 22–35. Although not all of the analogs were informative, the overall results establish that multiple regions of the amino-terminal extracellular domain of the P1R are in close proximity to the carboxyl-terminal portion of PTHrP-(1–36).

**EXPERIMENTAL PROCEDURES**

**Materials**—[155,159,163]PTTHrP-(1–36)-amide and analogs of this peptide with Bpa substitutions placed in positions 22–35, along with rat [Nε6,21,25]PTTH-(1–34)-amide (rPTH-(1–34)), rat [Nε6,21,Bpa27,29] PTH-(1–34)-amide, and [Bpa27,29]PTTHrP-(1–36)-amide were synthesized by the Protein and Peptide Core Facility at Massachusetts General Hospital (Boston, MA) by the solid-phase method on PerkinElmer Life sciences model 430A and 431A synthesizers. Peptides were purified by reverse-phase chromatography, and the primary structure was confirmed by amino acid analysis and mass spectrometry.

The [125I]-labeled Bpa-containing peptides (3 million cpm/well in 6-well plates or 800 ng of plasmid/well in 24-well plates) were used in the radioligand-binding assay. Receptor autoradiography and cross-linking experiments, cells were seeded in either B-27 medium (for COS-7 cells transfection) or 15-mm plates (HKrK-B7 cells). For transfection, once the monolayer of COS-7 cells reached ~80% confluency, cells were transfected by the DEAE-dextran method as described (29) using 200 ng of plasmid DNA/well in 24-well plates or 800 ng of plasmid/well in 6-well plates. After 3 days, cells were used for the experiments. HkrK-B7 cells were used in experiments once the monolayer reached confluence.

**Radiolabeling of Peptides**—Radiolabeled peptides were prepared by chloramine-T iodination followed by high pressure liquid chromatography purification using a 30–50% acetonitrile gradient in 0.1% trifluoroacetic acid over 30 min as previously described (29). In brief, [125I]-labeled radioligands, rPTH-(1–34) or [155,159,Y23,Y36]PTTH-(1–36)-amide, were incubated with cells expressing P1R in the presence of varying concentrations (0–10 μM) of unlabeled peptide. After 4-h incubation at 15 °C, the binding mixture was removed and cells were rinsed twice with cold buffer. Cells were lysed, and the entire lysate was counted for gamma irradiation. Intracellular cAMP accumulation was measured by radioligand assay as previously described (5).

**Photoaffinity Labeling of PTH/PTHrP Receptors**—Cells either transiently or stably expressing P1R or P1R mutants were incubated with [125I]-labeled Bpa-containing peptides (3 million cpm/well in 6-well plates, 10–20 million cpm/well in 15-mm plates) for 6 h at 4 °C. Cells were rinsed twice and covered with cold buffer. The dishes were placed on ice under a UV light source (Black Ray long-wave lamp, 366 nm, 7000 microwatts/cm2; UV Products, San Gabriel, CA) at a distance of ~5 cm for 15 min. Cells were rinsed twice with cold acidic buffer (50 mM glycine, 0.15 μM NaCl, pH 2.5) to remove noncovalently bound ligand. Cells were lysed using Triton buffer (1% Triton X-100, 50 mM Tris-HCl (pH 8), 0.15 μM NaCl) and centrifuged at 1500 × g. The supernatant was centrifuged at 80 °C. For partial purification of cross-linked ligand-receptor complexes, wet gels were cut into strips and counted for gamma irradiation. Gel strips with peak radioactivity were electrophoresed in a dialysis bag at 100 V for 2 h. Eluted samples were concentrated using Centricon-10 filters (Millipore Co., Bedford, MA).

**Chemical/Enzymatic Cleavage**—For cleavage at methionine residues, the gel-purified, radiolabeled ligand-receptor complexes were incubated with CNBr (100 mM) in 70% formic acid at 20 °C for 24 h. After
TABLE I

| PTHrP-(1–36) analog | Binding IC₅₀ | cAMP EC₅₀ | E₅₀ max
|----------------------|-------------|----------|--------|
| Parent               | 17 ± 4      | 12 ± 1   | 126 ± 11 |
| Bpa²²                | 20 ± 9      | 10 ± 1   | 157 ± 49 |
| Bpa²³                | 25 ± 11     | 4.4 ± 0.1| 129 ± 6 |
| Bpa²⁴                | 175 ± 25    | 18 ± 1   | 131 ± 16 |
| Bpa²⁵                | 39 ± 14     | 10 ± 1   | 141 ± 8 |
| Bpa²⁶                | 20 ± 9      | 7.0 ± 0.3| 140 ± 26 |
| Bpa²⁷                | 21 ± 2      | 6.6 ± 0.2| 138 ± 13 |
| Bpa²⁸                | 8.1 ± 2.4   | 7.8 ± 0.1| 152 ± 8 |
| Bpa²⁹                | 36 ± 16     | 11 ± 1   | 132 ± 10 |
| Bpa³⁰                | 53 ± 11     | 10 ± 1   | 118 ± 35 |
| Bpa³¹                | 6.9 ± 1.4   | 4.7 ± 0.1| 159 ± 11 |
| Bpa³²                | 57 ± 14     | 12 ± 1   | 191 ± 10 |
| Bpa³³                | 74 ± 17     | 31 ± 1   | 171 ± 7 |
| Bpa³⁴                | 42 ± 6      | 30 ± 1   | 165 ± 12 |
| Bpa³⁵                | 47 ± 4      | 48 ± 2   | 173 ± 25 |

The remaining ligands did not provide sufficient yields of gel-purified photoaffinity-labeled material to permit subsequent digestive mapping.

The cross-linked complex formed between ¹²⁵I-[⁵⁻Bpa²³,Y³⁶]PTHrP-(1–36)-amide and the hP₁R in HKrk-B₇ cells was gel-purified and digested at methionine residues with CNBr. The size of the resulting radioactive conjugate fragment observed by Tricine gel electrophoresis and subsequent autoradiography was ~7 kDa (Fig. 2, lane 2). Based on the previously identified cross-link interval for [⁵⁻Bpa²³,Y³⁶]PTHrP-(1–36)-amide and the rat P₁R (residues 23–40) (18), the observed fragment would be most consistent with cross-linking to either residues 23–32 or 33–63 of the hP₁R (see Fig. 1). To identify which of these two receptor fragments represented the site of cross-linking, [⁵⁻Bpa²³,Y³⁶]PTHrP-(1–36)-amide was cross-linked to COS-7 cells expressing a mutant hP₁R (delta-E2) in which residues 61–105 (encoded by exon E2), including Met³⁶, are deleted. This receptor mutant has been previously shown to have PTH binding and activation properties similar to those of the wild-type receptor (8, 13). In contrast to the radiolabeled band seen with COS-7 cells expressing the wild-type hP₁R, which was indistinguishable from the band observed with HKrk-B₇ cells (data not shown), the CNBr-digested complex obtained with the delta-E2 mutant showed a broad, broad band at ~50 kDa (Fig. 2, right panel). This size was consistent with a cross-link to the glycosylated receptor fragment that comprises residues 33–60/106–189. The smaller CNBr-generated band observed with the wild-type hP₁R affinity labeled by ¹²⁵I-[⁵⁻Bpa²³,Y³⁶]PTHrP-(1–36)-amide thus corresponds to the receptor fragment comprising residues Thr³³ through Met⁶³.

Different results were obtained when complexes of hP₁R and
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Fig. 2. CNBr digest of [I5,Bpa27,Y36]PThrP-(1–36)-amide cross-linked to hP1R. 125I-[I5,Bpa27,Y36]PThrP-(1–36)-amide was bound to LLC-PK1 cells stably transfected with hP1R (lanes 1 and 2) or COS-7 cells transiently transfected with the hP1R-delta E2 mutant (lane 3). Bound ligand was photofinity cross-linked to the receptor as described under “Experimental Procedures.” Cells were lysed, the ligand-receptor complex was gel-purified, and the resulting sample placed in formic acid for 24 h in the absence (lane 1) or presence (lanes 2 and 3) of CNBr. Samples were analyzed on Tricine/SDS-PAGE followed by autoradiography at −80 °C. The positions of size markers are indicated in kDa. [I5,Bpa23,Y36]PThrP-(1–36)-amide cross-linking to COS-7 cells transfected with wild-type hP1R showed the same banding pattern as was seen in LLC-PK1 cells stably expressing hP1R (data not shown). Below the gel is shown a linear representation of the amino-terminal, extracellular domain of the hP1R with the location of three methionine residues (●) and the four sites for potential N-linked glycosylation (●). The delta E2 mutant of the hP1R lacks residues 61–105 (including methionine 63); the shaded region indicates the region cross-linked by [I5,Bpa23,Y36]PThrP-(1–36)-amide in the wild-type hP1R.

either radiolabeled [I5,W23,Bpa27,Y36]PThrP-(1–36)-amide, [I5,W23,Bpa28,Y36]PThrP-(1–36)-amide, or [I5,W23,Bpa23, Y36]PThrP-(1–36)-amide were digested with CNBr. Each of these digested ligand-receptor complexes showed a broad band of ∼50 kDa upon Tricine gel analysis, indicating that the CNBr-generated ligand/receptor conjugate fragment was glycosylated (Fig. 3). These findings were consistent with cross-linking of each of the three Bpa groups to the region of the amino-terminal extracellular domain of the receptor delimited by Glu64 and Met105, a region that is carboxy to the [I5,Bpa23,Y36]PThrP-(1–36)-amide cross-linking site (see Fig. 1).

[I5,W23,Bpa28,Y36]PThrP-(1–36)-amide contained the most carboxy-terminal Bpa-substitution analyzed to date for PTh or PThrP. The complex between radiolabeled [I5,W23,Bpa28, Y36]PThrP-(1–36)-amide and the hP1R was evaluated by enzymatic digestion with LysC or ArgC. Each enzyme digest revealed cross-linking to a large, glycosylated fragment, along with a smaller band migrating with the 3.5/2.35-kDa size markers (Fig. 4, lanes 1 and 3). The mobility of the smaller band was indistinguishable from that of the free ligand (not shown), suggesting that some cross-linked radioligand was released from the gel-purified complex during the digestion process. After Endoglycosidase-F treatment, the larger band in the LysC-digested sample was reduced to ∼5 kDa (Fig. 4, lane 2). Only two glycosylated fragments are predicted after complete

Fig. 3. CNBr digest of [I5,W23,Bpa27,Y36]PThrP-(1–36)-amide, [I5,W23,Bpa28,Y36]PThrP-(1–36)-amide, and [I5,W23,Bpa23, Y36]PThrP-(1–36)-amide cross-linked to hP1R. 125I-Labeled PThrP-(1–36) analogs containing a Bpa substitution at either position 27 (lanes 1 and 2), position 28 (lanes 3 and 4), or position 33 (lanes 5 and 6) were bound to LLC-PK1 cells stably transfected with hP1R. Bound ligand was photofinity cross-linked to the receptor as described under “Experimental Procedures.” Cells were lysed, the ligand-receptor complex was gel-purified, and the resulting sample was placed in formic acid for 24 h in the absence (−; lanes 1, 4, 5) or presence (+; lanes 2, 3, 6) of CNBr. Samples were analyzed on Tricine/SDS-PAGE followed by autoradiography at −80 °C. The positions of size markers are indicated in kDa. Below the gel is shown a schematic representation of the amino-terminal, extracellular domain of the hP1R with the location of three methionine residues (●) and the four sites for potential N-linked glycosylation (●). The shaded region indicates the cross-linked region in the wild-type hP1R for each of the three ligands.

LysC digestion of the hP1R; these fragments consist of residues 142–172 (LysC-1) and residues 173–240 (LysC-2). Deglycosylated LysC-2, when cross-linked to [I5,W23,Bpa23, Y36]PThrP-(1–36)-amide, has a calculated mass of 11 kDa, considerably larger than the observed radiolabeled ∼5 kDa band. The calculated mass of deglycosylated LysC-1 cross-linked to the receptor is 6.5 kDa, much closer to that of the observed band. The LysC digest thus narrowed the [I5,W23,Bpa23,Y36]PThrP-(1–36)-amide cross-link site to the hP1R region between residues 142–172. Endoglycosidase-F treatment of the ArgC-digested sample eliminated the larger, more diffuse band but a prominent new band could not be detected (Fig. 4, lane 4). The faint band at ∼16 kDa, which appeared after deglycosylation, presumably derived from a partial ArgC or Endoglycosidase-F digestion, because complete ArgC digestion of the P1R is predicted to generate two glycosylated fragments, residues 151–162 or residues 163–179, and the calculated sizes of these receptor fragments (after deglycosylation) when cross-linked to [I5,W23,Bpa23,Y36]PThrP-(1–36)-amide are 3.2 and 3.9 kDa, respectively. Furthermore, based on the comigration of the 2.35- and 3.5-kDa markers, these smaller fragments would not be distinguishable from the free radioligand fragment (residues 22–36 of 125I-[I5,W23,Bpa23,Y36]PThrP-(1–36)-amide generated by ArgC cleavage; molecular mass = 2.1 kDa) on the Tricine gel (Fig. 4, lane 4). The overlap between LysC-1 (residues 142–172) and the combined ArgC fragments (residues 151–179) indicates that cross-linking of [I5,W23,Bpa23,Y36]PThrP-(1–36)-amide occurred between receptor residues Asn151 and Lys172. This
Fig. 4. LysC and ArgC digest of [I\textsuperscript{5},W\textsuperscript{23},Bpa\textsuperscript{27},Y\textsuperscript{36}]PTHrP-(1–36)-amide, cross-linked to hP1R. [I\textsuperscript{5},W\textsuperscript{23},Bpa\textsuperscript{27},Y\textsuperscript{36}]PTHrP-(1–36)-amide was bound to LLC-PK\textsubscript{1} cells stably transfected with hP1R. Bound ligand was photoaffinity cross-linked to the receptor as described under “Experimental Procedures.” Cells were lysed, the ligand-receptor complex was gel-purified, and the resulting sample was placed in LysC (lanes 1 and 2) or ArgC (lanes 3 and 4) for 24 h. Samples in lanes 2 and 4 were subsequently treated with Endoglycosidase-F to remove N-linked sugars. Samples were analyzed on Tricine/SDS-PAGE followed by autoradiography at 80 °C. The positions of size markers are indicated in kDa. Free [I\textsuperscript{5},W\textsuperscript{23},Bpa\textsuperscript{27},Y\textsuperscript{36}]PTHrP-(1–36)-amide ligand, which is released after cross-linking during the digestion process, is visible in all lanes at the 3.5/2.35-kDa size markers. Below the gel is shown a linear representation of the amino-terminal, extracellular domain of the hP1R with the location of five lysine (K) and arginine (R) residues (●) and the four sites for potential N-linked glycosylation (○). The shaded region indicates the cross-linked region in the wild-type hP1R.

Fig. 5. LysC and ArgC digest of [I\textsuperscript{5},W\textsuperscript{23},Bpa\textsuperscript{27},Y\textsuperscript{36}]PTHrP-(1–36)-amide, cross-linked to hP1R. [I\textsuperscript{5},W\textsuperscript{23},Bpa\textsuperscript{27},Y\textsuperscript{36}]PTHrP-(1–36)-amide was bound to LLC-PK\textsubscript{1} cells stably transfected with hP1R (lanes 1 and 2) or COS-7 cells transiently transfected with the wild-type hP1R (lane 3) or hP1R-K141R mutant (lane 4). Bound ligand was photoaffinity cross-linked to the receptor as described under “Experimental Procedures.” Cells were lysed, the ligand-receptor complex was gel-purified, and the resulting sample placed in LysC (lanes 1, 3, 4) or ArgC (lane 2) for 24 h. Samples were analyzed on Tricine/SDS-PAGE followed by autoradiography at 80 °C. The positions of size markers are indicated in kDa. Below the gel is shown a linear representation of the amino-terminal, extracellular domain of the hP1R with the location of six lysine (K) and arginine (R) residues (●) and the four sites for potential N-linked glycosylation (○). The shaded region indicates the cross-linked region in the wild-type hP1R.

Cross-link site lies in a region of the amino-terminal extracellular domain that is encoded by exon G (see Fig. 1).

Mapping of the [I\textsuperscript{5},W\textsuperscript{23},Bpa\textsuperscript{27},Y\textsuperscript{36}]PTHrP-(1–36)-amide cross-link site was next undertaken. Digestion of the covalent ligand-receptor complex with either LysC or ArgC revealed nonglycosylated fragments of ~6.5 and ~4 kDa, respectively (Fig. 5, lanes 1 and 2). The best candidate segment for the LysC fragment comprised residues Glu\textsuperscript{96}–Lys\textsuperscript{141}, even though the predicted size of this ligand-receptor complex was 8.1 kDa, i.e., ~2 kDa larger than that estimated from the Tricine gel. To assess this plausible assignment of the LysC fragment, a mutant receptor with Lys\textsuperscript{141} mutated to arginine (K141R) was prepared. When transiently expressed in COS-7 cells, this mutant receptor showed binding and cAMP activation responses to rPTH-(1–34) that were indistinguishable from those of the wild-type receptor (data not shown). After cross-linking of [I\textsuperscript{5},W\textsuperscript{23},Bpa\textsuperscript{27},Y\textsuperscript{36}]PTHrP-(1–36)-amide and LysC digestion of the covalent ligand-receptor complex, COS-7 cells expressing the wild-type P1R showed a radiolabeled band similar to that observed with HKrk-B7 cells (Fig. 5, lane 3). COS-7 cells expressing the K141R mutant receptor yielded a radiolabeled fragment that was much larger (~50 kDa) than that observed with the wild-type receptor. In addition, this fragment was likely to be glycosylated, as indicated by its mobility and the broad banding pattern (Fig. 5, lane 4). This experiment confirmed that cross-linking of Bpa\textsuperscript{27} occurred within the LysC fragment comprising residues Glu\textsuperscript{96}–Lys\textsuperscript{141}, even though the epsilon-amino group of lysine 27 (Bpz) was found to cross-link to the first extracellular loop of the P1R. The difference between these data and our current findings could be due to the use of different scaffold peptides (a PTH analog in Greenberg’s experiments versus a PTHrP analog in our studies), or to the use of structurally different benzophenone adducts (Bpz in Greenberg’s experiments versus Bpa in our studies). To investigate these possibilities, we synthesized two additional Bpa\textsuperscript{27} analogs: [Bpa\textsuperscript{27,Y\textsuperscript{36}}]PTHrP-(1–36)-amide (lacking the I\textsuperscript{5} and W\textsuperscript{23} modifications) and [Nle\textsuperscript{10,21},Bpa\textsuperscript{27,Y\textsuperscript{34}}]rat PTH-(1–34)-amide. The two analogs bound to hP1R and stimulated cAMP

Our findings with [I\textsuperscript{5},W\textsuperscript{23},Bpa\textsuperscript{27},Y\textsuperscript{36}]PTHrP-(1–36)-amide were quite different from those obtained in the previously published cross-linking studies by Greenberg et al. (21), in which a PTH-(1–34) analog with a benzoyle group attached to the epsilon-amino group of lysine 27 (Bpz) was found to cross-link to the first extracellular loop of the P1R. The difference between these data and our current findings could be due to the use of different scaffold peptides (a PTH analog in Greenberg’s experiments versus a PTHrP analog in our studies), or to the use of structurally different benzophenone adducts (Bpz in Greenberg’s experiments versus Bpa in our studies). To investigate these possibilities, we synthesized two additional Bpa\textsuperscript{27} analogs: [Bpa\textsuperscript{27,Y\textsuperscript{36}}]PTHrP-(1–36)-amide (lacking the I\textsuperscript{5} and W\textsuperscript{23} modifications) and [Nle\textsuperscript{10,21},Bpa\textsuperscript{27,Y\textsuperscript{34}}]rat PTH-(1–34)-amide. The two analogs bound to hP1R and stimulated cAMP
Cross-linking of PTHrP to the PTH/PTHrP Receptor

In this study, we prepared fifteen p-benzoyl-L-phenylalanine (Bpa)-substituted analogs of PTHrP-(1–36) for use in photoaffinity cross-linking experiments with the human P1R. The Bpa moieties replaced the ligand’s natural residues at positions 22–35, thus enabling the study of sites of interaction between the PTHrP-(1–36) binding domain and the P1R. Receptor contacts were mapped for ligand positions 23, 27, 28, and 33, and accumulation indistinguishably from [I\(^5\),W\(^{23}\),Bpa\(^{36}\),Y\(^{36}\)]PTHrP-(1–36)-amide (data not shown). The radioiodinated analogs were cross-linked to the hP1R and digested with CNBr. Both compounds showed a cross-linking pattern similar to that of [I\(^5\),W\(^{23}\),Bpa\(^{27}\),Y\(^{36}\)]PTHrP-(1–36)-amide, i.e. CNBr digestion resulted in a 50-kDa glycosylated fragment (Fig. 6). Glycosylation was confirmed by sequential CNBr/Endoglycosidase-F treatment, which resulted in a smaller fragment that is different from that containing the site of cross-linking for [I\(^5\),W\(^{23}\),Bpa\(^{36}\),Y\(^{36}\)]PTHrP-(1–36)-amide. In combination with the data obtained after CNBr digestion (see Fig. 3, lanes 3 and 4), the cross-link interval for [I\(^5\),W\(^{23}\),Bpa\(^{28}\),Y\(^{36}\)]PTHrP-(1–36)-amide can be reduced to the region between Glu\(^{64}\) and Lys\(^{86}\). Within this region, complete LysC digestion of the P1R is predicted to generate five fragments; three of which, after cross-linking to [I\(^5\),W\(^{23}\),Bpa\(^{36}\),Y\(^{36}\)]PTHrP-(1–36)-amide, have a predicted size of 4–5 kDa. The two bands observed experimentally could thus be due to cross-linking to two separate sites in the Glu\(^{64}\)-Lys\(^{86}\) interval (although partial enzymatic digestion of this region cannot be excluded). Although we were unable to resolve this mapping further, the results clearly indicate that, similar to the site of [I\(^5\),W\(^{23}\),Bpa\(^{27}\),Y\(^{36}\)]PTHrP-(1–36)-amide cross-linking, [I\(^5\),W\(^{23}\),Bpa\(^{28}\),Y\(^{36}\)]PTHrP-(1–36)-amide cross-links to residues within the nonessential E2 region of the amino-terminal extracellular domain of the P1R (8, 13).

**DISCUSSION**

In this study, we prepared fifteen p-benzoyl-L-phenylalanine (Bpa)-substituted analogs of PTHrP-(1–36) for use in photoaffinity cross-linking experiments with the human P1R. The Bpa moieties replaced the ligand’s natural residues at positions 22–35, thus enabling the study of sites of interaction between the PTHrP-(1–36) binding domain and the P1R. Receptor contacts were mapped for ligand positions 23, 27, 28, and 33, and accumulation indistinguishably from [I\(^5\),W\(^{23}\),Bpa\(^{36}\),Y\(^{36}\)]PTHrP-(1–36)-amide (data not shown). The radioiodinated analogs were cross-linked to the hP1R and digested with CNBr. Both compounds showed a cross-linking pattern similar to that of [I\(^5\),W\(^{23}\),Bpa\(^{27}\),Y\(^{36}\)]PTHrP-(1–36)-amide, i.e. CNBr digestion resulted in an 50-kDa glycosylated fragment (Fig. 6). Glycosylation was confirmed by sequential CNBr/Endoglycosidase-F treatment, which resulted in a smaller fragment that is different from that containing the site of cross-linking for [I\(^5\),W\(^{23}\),Bpa\(^{36}\),Y\(^{36}\)]PTHrP-(1–36)-amide. In combination with the data obtained after CNBr digestion (see Fig. 3, lanes 3 and 4), the cross-link interval for [I\(^5\),W\(^{23}\),Bpa\(^{28}\),Y\(^{36}\)]PTHrP-(1–36)-amide can be reduced to the region between Glu\(^{64}\) and Lys\(^{86}\). Within this region, complete LysC digestion of the P1R is predicted to generate five fragments; three of which, after cross-linking to [I\(^5\),W\(^{23}\),Bpa\(^{36}\),Y\(^{36}\)]PTHrP-(1–36)-amide, have a predicted size of 4–5 kDa. The two bands observed experimentally could thus be due to cross-linking to two separate sites in the Glu\(^{64}\)-Lys\(^{86}\) interval (although partial enzymatic digestion of this region cannot be excluded). Although we were unable to resolve this mapping further, the results clearly indicate that, similar to the site of [I\(^5\),W\(^{23}\),Bpa\(^{27}\),Y\(^{36}\)]PTHrP-(1–36)-amide cross-linking, [I\(^5\),W\(^{23}\),Bpa\(^{28}\),Y\(^{36}\)]PTHrP-(1–36)-amide cross-links to residues within the nonessential E2 region of the amino-terminal extracellular domain of the P1R (8, 13).
all of these contacts were found to occur in the receptor’s amino-terminal extracellular domain. All of the Bpa-substituted analogs activated the P1R with potencies similar to that of \([I^5,W^{23},Y^{36}]\text{PTHrP-(1–36)}-\text{amide}\) (Table I). Thus, although we cannot determine the actual state of the receptor at the time of photolabeling, it is at least possible that ligand cross-linking occurred to the P1R in its activated, G-protein-coupled conformation. Binding to the P1R was only minimally affected by most of the Bpa substitutions, with the exception of \([I^5,W^{23},Bpa^{24},Y^{36}]\text{PTHrP-(1–36)}-\text{amide}\), which showed an ~10-fold reduced ability to compete with \(^{125}\)I-labeled rPTH-(1–34) at the hP1R (Table I). The conserved Leu at position 24 in PTH was previously shown to be intolerant to substitution with polar residues (~1000-fold reduced binding affinity) (17). In contrast, other mutationally intolerant ligand residues nearby (e.g. at positions 23, 28, and 31 (16, 17)) tolerated the Bpa substitution with no apparent reduction in binding affinity. At position 23, Bpa replaced a tryptophan, and the structural similarity between these two residues may explain why the substitution was tolerated. At positions 28 and 31, Bpa replaced similarly hydrophobic isoleucine residues; the tolerance of these positions to substitutions with Bpa and intolerance to substitutions with polar residues (17) suggests that these two residues are involved in important hydrophobic interactions.

Among the compounds generated for our studies, the Bpa\(^{23,27,28}\), Bpa\(^{27}\), Bpa\(^{28}\), and Bpa\(^{33}\)-substituted analogs yielded sufficient gel-purified material to permit digestive mapping of the cross-link site in the P1R. Our previous studies with \([I^5,Bpa^{23},Y^{36}]\text{PTHrP-(1–36)}-\text{amide}\) and the rat P1R (18) demonstrated that cross-linking occurred to a site between the amino-terminal residues 23 and 40. We now extended these studies to include the human P1R, for which we determined that cross-linking occurred to a site within residues 33–63, an extreme amino-terminal receptor segment encoded mostly by exon E1 (see Fig. 1). Taken together, the results with rat and human receptors indicate that residue 23 of PTHrP-(1–36) likely contacts the receptor between residues 33–40; a conclusion that is consistent with alanine-scan studies, which identified Thr\(^{33}\) and Gln\(^{37}\) as important determinants for PTHrP-(7–36) binding (18).

The analog \([I^5,W^{23},Bpa^{27},Y^{36}]\text{PTHrP-(1–36)}-\text{amide}\) cross-linked to a site between receptor residues 96–102, within the region of the amino-terminal extracellular domain encoded by exon E2. The E2-encoded region is not present in the P1R of lower vertebrate species (32, 33) and can be removed without affecting receptor function (8, 13). In addition, residue Lys\(^{37}\) of PTH has been shown to be moderately tolerant to substitution (17, 34). Thus the combined data do not support a critical functional role for the E2-encoded P1R domain or the residue at position 27 of PTH-(1–34) or PTHrP-(1–36). The cross-linking site recently identified by Greenberg et al. (21) for a PTH analog benzoyl-tagged at Lys\(^{27}\) in the first extracellular loop was not identified with our Bpa\(^{27}\)-containing PTH-(1–34) or PTHrP-(1–36) analogs, each of which cross-linked to the amino-terminal extracellular domain of the receptor. The divergence of these results most likely reflects the different spatial properties of the photoreactive benzoyl group in the ligands used by the two groups, because it is attached distally to the lysine side chain (Bpz) in the ligand used by Greenberg et al. (21), whereas it is incorporated into the peptide chain as \(p\)-benzoyl-L-phenylalanine (Bpa) in each of our analogs. Similar to the lack of functional effect that occurs with the deletion of the E2-encoded region, removal of a large portion of extracellular loop 1 (including the Bpz\(^{27}\) cross-link site) from the P1R has little or no effect on receptor function (28). Thus, the photoreactive moiety at position 27, in either the Bpa or Bpz configuration, cross-links to one of two receptor segments that have been found to be nonessential. Although these data do not identify a critical ligand-receptor contact, they do lead to the important conclusion that the first extracellular loop and portions of the amino-terminal extracellular domain of the P1R are close to each other, at least when the receptor is occupied by agonist ligand.

The analog \([I^5,W^{23},Bpa^{28},Y^{36}]\text{PTHrP-(1–36)}-\text{amide}\) cross-linked to a site between receptor residues 64–95, and thus in a different portion of the exon E2-encoded region from that which reacted with \([I^5,W^{23},Bpa^{28},Y^{36}]\text{PTHrP-(1–36)}-\text{amide}\). However, unlike PTH residue Lys\(^{27}\), Leu\(^{28}\) is intolerant to many amino acid substitutions (17). The reason why Bpa located at an apparently critical position in the ligand (e.g. position 28) cross-linked to a nonessential domain of the receptor is not clear. One possibility is that residue 28 (Leu in PTH, Ile in PTHrP) contacts a critical hydrophobic pocket that is located close to the...
E2-encoded domain such that the Bpa-substituted analog can cross-link to the E2 region. Another possibility is that residue 28 initially contacts a critical hydrophobic pocket in the P1R, but this contact is not maintained during the time of photolabeling, perhaps because of a conformational change in the receptor. A third possibility relates to the hypothesis that some amphipathic peptide ligands, including PTH and PTHrP (35), associate initially with the cell membrane and then diffuse two-dimensionally to their cell surface receptors, where a more specific high affinity interaction occurs (36). Residue 28 is located on the hydrophobic face of the carboxyl-terminal amphipathic helix and thus, according to this hypothesis, may participate in a nonspecific but important interaction with the lipid component of the cell membrane. If such an interaction with the cell membrane is the primary role of residue 28, then [I\(^8\),W\(^{22}\),Bpa\(^{28}\),Y\(^{36}\)]PTHrP-(1–36)-amide would not necessarily be expected to cross-link to an essential P1R region. Our current data cannot distinguish between these possible explanations. In any case, it seems unlikely that the proximity between the Bpa moiety in [I\(^8\),W\(^{22}\),Bpa\(^{28}\),Y\(^{36}\)]PTHrP-(1–36)-amide and receptor residues 64–95 indicates a role of this receptor region in determining high affinity ligand binding. Nevertheless, the apparent proximity between these specific sites in the ligand and the receptor does provide important topographical information about the receptor’s amino-terminal extracellular domain when occupied by agonist ligand.

The analog [I\(^8\),W\(^{22}\),Bpa\(^{28}\),Y\(^{36}\)]PTHrP-(1–36)-amide cross-linked to a site between residues 151–172 of the hP1R, within the N-glycosylated region (37) encoded by exon G (see Fig. 1). Within this cross-link interval, the cassette replacement and deletion-based mutational analyses that have been performed so far are largely uninformative, because the receptor modifications tested eliminated or severely diminished cell surface expression (8, 28). Our current studies suggest that point mutational analysis of this region may ultimately reveal important functional roles for individual residues in the cross-linked interval.

The cross-linking data presented in our study do not provide support for the components of the two previously mentioned computer-based models of the PTH/PTHrP ligand-receptor interaction that concern the amino-terminal extracellular domain of the receptor and the carboxyl-terminal portion of the ligand. The model by Rôls et al. (23) aligns the carboxyl-terminal helix of the ligand with a putative α-helix comprising residues 180–189 of the receptor. However, none of the cross-linking sites identified in our studies were mapped to this receptor domain. The model proposed by Jin et al. (24) predicts specific receptor interactions for ligand residues Trp\(^{23}\), Lys\(^{27}\), and Leu\(^{28}\) of PTH-(1–34), and for residues Phe\(^{23}\), Leu\(^{27}\), and Ile\(^{28}\) of PTHrP-(1–34) to an α-helical region comprising residues 169–177 of the receptor. This receptor region is also distant from the cross-linking sites of our corresponding Bpa-substituted PTHrP-(1–36) analogs. In light of these discrepancies between the reported computer models and the new cross-linking data provided herein, it now seems likely that alternative modes of interaction between the carboxy-terminal helix of PTH-(1–34)/PTHrP-(1–36) and the amino-terminal extracellular domain of the P1R need to be considered.

The cross-linking intervals identified in our studies are well separated from each other in the receptor’s primary structure (Fig. 8A). These separations therefore predict that the amino-terminal region of the P1R forms a folded structure in which the identified ligand-receptor contact points converge. Additionally, there are six highly conserved cysteine residues in the amino-terminal extracellular domain of the hP1R (positions 48, 108, 117, 131, 148, and 170) that are likely to be involved in intramolecular disulfide bonds. A plausible pattern of disulfide bonding has recently been determined using an amino-terminal fragment of the P1R, which, although generated in Escherichia coli, binds PTH-(1–34) with the expected affinity (38). Based on these studies, disulfide bridges are likely to occur between residue pairs Cys\(^{48}\)/Cys\(^{117}\), Cys\(^{108}\)/Cys\(^{148}\), and Cys\(^{131}\)/Cys\(^{170}\) (38). Although these findings have yet to be confirmed in the full-length P1R, or in an hP1R fragment produced in a mammalian expression system, we find that the proposed pattern of disulfide bridging is at least compatible with the current cross-linking data. Reconciliation of these two sets of data can occur with the receptor’s amino-terminal extracellular domain assuming a spiral shape that partially encircles the ligand’s carboxyl-terminal helix, as illustrated in Fig. 8B. However, it is important to note that the cross-linking data could also be compatible with other patterns of disulfide bridging, which would presumably give a different overall topology. The Bpz cross-link between ligand residue 27 and extracellular loop 1 of the P1R (21) helps to orient the ligand's terminal helix with respect to the juxta-membrane region of the receptor. Satisfying this constraint, together with those imposed by the cross-linking sites for ligand positions 1 (9) and 13 (22), appears to require greater flexibility between the amino-terminal and carboxyl-terminal regions of the ligand than that observed in the crystalline structure of PTH-(1–34) (24); indeed the degree of folding of the ligand may be greater than that pictured in Fig. 8B. Note that a considerable degree of flexibility between the amino-terminal and carboxyl-terminal domains of PTH-(1–34) has been observed in solution-phase NMR studies (39). In summary, results obtained in our studies identified four new sites of contact between residues in the carboxyl-terminal portion PTHrP-(1–36) and the amino-terminal extracellular domain of the human P1R. One of these contact sites was also confirmed with a PTH-(1–34) analog. The findings should help constrain 3-dimensional structural models of ligand-P1R interaction, particularly in regards to the receptor’s large amino-terminal extracellular domain, which now appears to contain multiple sites that contribute to ligand interaction. Additional studies, aimed at further refining the topology of the P1R binding pocket, may ultimately assist in understanding the fundamental mechanism(s) by which this, and potentially other related family B receptors, bind and respond to their specific ligands.

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Multiple Sites of Contact between the Carboxyl-terminal Binding Domain of PTHrP-(1–36) Analogs and the Amino-terminal Extracellular Domain of the PTH/PTHrP Receptor Identified by Photoaffinity Cross-linking

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