Importance of the Amino Terminus in Secretin Family G Protein-coupled Receptors

INTRINSIC PHOTOOFFINITY LABELING ESTABLISHES INITIAL DOCKING CONSTRAINTS FOR THE CALCITONIN RECEPTOR*

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The calcitonin receptor is a member of the class B family of G protein-coupled receptors, closely related to secretin and parathyroid hormone receptors. Although mechanisms of ligand binding have been directly explored for these receptors, current knowledge of the molecular basis of calcitonin binding to its receptor is based on receptor mutagenesis. In this work we have utilized the more direct approach of photoaffinity labeling to explore spatial approximations between distinct residues within calcitonin and its receptor. For this we have developed two human calcitonin analogues incorporating a photolabile p-benzoyl-L-phenylalanine residue in the mid-region and carboxyl-terminal half of the peptide in positions 16 and 26, respectively. Both probes specifically bound to the human calcitonin receptor with high affinity and were potent stimulants of cAMP accumulation in calcitonin receptor-bearing human embryonic kidney 293 cells. They covalently labeled the calcitonin receptor in a saturable and specific manner. Further purification, deglycosylation, specific chemical and enzymatic cleavage, and sequencing of labeled wild type and mutant calcitonin receptors identified the sites of labeling for the position 16 and 26 probes as receptor residues Phe137 and Thr30, respectively. Both were within the extracellular amino terminus of the calcitonin receptor, with the former adjacent to the first transmembrane segment and the latter within the distal amino-terminal tail of the receptor. These data are consistent with affinity labeling of other members of the class B G protein-coupled receptors using analogous probes and may suggest a common ligand binding mechanism for this family.

Calcitonin, a hypocalcemic peptide hormone, is secreted from the thyroid gland in response to elevations in serum calcium levels. Its hypocalcemic effect is mediated by inhibition of bone resorption by osteoclasts and enhancement of renal calcium excretion. These actions are important for its widespread clinical use for treatment of bone disorders, including Paget’s disease, osteoporosis, and hypercalcemia of malignancy (1, 2).

Knowledge of the molecular basis of ligand binding is important for structure-based drug design. At the present time, our understanding of the mechanism of calcitonin binding to its receptor is based predominantly on limited chimeric receptor studies (8–10). However, currently there is no working model to predict how the two molecules might interact. In this work, we attempt to establish initial constraints that will contribute to the development of a model for the interaction of calcitonin with its receptor. With our success with the secretin receptor amino terminus.

The calcitonin receptor is closely related to the secretin and parathyroid hormone (PTH) receptors in the class B family of the G protein-coupled receptor superfamily, also having a long structurally unique amino-terminal domain that contains six conserved Cys residues. It shares ~30% identity with the secretin receptor and 32% with the PTH 1 receptor. The human calcitonin receptor has three isoforms resulting from alternative mRNA splicing. Isoform I has 490 amino acids, including a 22-residue signal sequence and a 16-residue insert in the predicted first intracellular loop domain (residue 175–190) that is absent in isoforms II and III. Apart from this 16-amino acid insert, isoform III also has the first 47 residues missing at the receptor amino terminus.

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(11–17), we use the more direct and powerful approach of photoaffinity labeling. For this we have developed two photolabile radioiodinatable agonist probes by incorporating a photolabile residue, p-benzoyl-L-phenylalanine (Bpa), into the midregion of the human calcitonin peptide in position 16 and into the carboxyl-terminal half of the ligand in position 26. Both probes bound to the human calcitonin receptor specifically and with high affinity and efficiently covalently labeled the receptor. By sequential targeted enzymatic and chemical fragmentation reactions, the ligand binding region for the position 16 probe was localized to a domain within the amino terminus of its receptor adjacent to the first transmembrane domain, whereas that for the position 26 probe was localized within the distal amino terminus of the receptor. Using radiochemical Edman degradation sequencing, the specific residues labeled by these probes were identified as Phe137 and Thr30, respectively. These represent the first experimentally derived residue-residue approximations between calcitonin-like agonists and this receptor and should be very helpful for docking this ligand in a molecular model.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human calcitonin was purchased from Bachem (Torrance, CA). Cyanogen bromide (CNBr) and solid phase oxidant N-chlorobenzenesulfonamide (IODO-BEAD) were purchased from Pierce. Endoproteinase Lys-C was from Calbiochem. Endoproteinase F (Endo F) was produced in our laboratory (18). All other reagents were analytical grade.

**Peptide Synthesis**—The probes, human (Ile8, Bpa16, Arg18)calcitonin (Bpa16 analogue or probe) and (Ile8, Arg18, Bpa26)calcitonin (Bpa26 analogue or probe), were designed to contain a photolabile Bpa in position 16 or 26, respectively, for covalent labeling of the calcitonin receptor. Both probes contained a naturally occurring Tyr residue in position 12 or 26, respectively, for covalent labeling of the calcitonin receptor. By sequential targeted enzymatic and chemical fragmentation reactions, the ligand binding region for the position 16 probe was localized to a domain within the amino terminus of its receptor adjacent to the first transmembrane domain, whereas that for the position 26 probe was localized within the distal amino terminus of the receptor. Using radiochemical Edman degradation sequencing, the specific residues labeled by these probes were identified as Phe137 and Thr30, respectively. These represent the first experimentally derived residue-residue approximations between calcitonin-like agonists and this receptor and should be very helpful for docking this ligand in a molecular model.

**Ligand Binding**—Receptor binding of calcitonin, the Bpa16 analogue, and the Bpa26 analogue was characterized in a standard assay using membranes from the HEK293-CTR cell line. Membranes (−10 μg) were incubated with a constant amount of radioligand, [125I]calcitonin (3–5 pM), in the presence of increasing concentrations of non-radioiodinated calcitonin or the Bpa16 analogue or the Bpa26 analogue (0–1 μM) for 1 h at room temperature in Krebs-Ringer-HEPES medium (25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 1 mM KH2PO4, 1.2 mM MgSO4, 2 mM CaCl2, 1 mM phenylmethylsulfonyl fluoride, 0.01% soybean trypsin inhibitor) containing 0.2% bovine serum albumin. Bound and free radioligand were separated using a Skatron cell harvester (Molecular Devices, Sunnyvale, CA) with glass fiber filters that had been soaked in 0.3% Polybrene for 1 h, and bound radioactivity was quantified in a γ-spectrometer. Nonspecific binding was determined in the presence of 1 μM calcitonin and represented <20% of total binding.

**Biological Activity Assay**—The agonist activities of the Bpa16 and Bpa26 analogues were studied for stimulation of cAMP in HEK293-CTR cells using a competition binding assay (Diagnostic Products Corp., Los Angeles, CA). Cells were stimulated with increasing concentrations of calcitonin or the Bpa16 or Bpa26 analogue at 37 °C for 30 min, and the reactions were stopped by adding ice-cold perchloric acid. After adjusting the pH to 6 with KHCO3, cell lysates were cleared by centrifugation at 9000 rpm for 10 min, and the supernatants were used in the assay as previously described (24). Radioactivity was quantified by scintillation counting in a Beckman LS6000.

**Photoaffinity Labeling Studies**—For covalent labeling studies, plasma membranes from receptor-bearing HEK293-CTR cells containing ~50 μg of protein were incubated with ~0.1 μM [125I]calcitonin or Bpa16 or Bpa26 analogue at 37 °C for 1 h at room temperature before photoactivation for 30 min at 4 °C in a Rayonet photochemical reactor (Southern New England Ultraviolet, Hamden, CT) equipped with 3500-A lamps. To scale up receptor purification, a larger amount of membranes (~150–200 μg) was incubated with each radiolabeled probe (~0.5 μM) in the absence of competing calcitonin. After photoactivation, membranes were washed, pelleted, solubilized in SDS sample buffer, and applied to a 10% SDS-polyacrylamide gel for electrophoresis (25). Radiolabeled bands were detected by autoradiography.

**Peptide Mapping**—Radioactive receptor bands were cut out from the gel and homogenized in a Dounce homogenizer in water followed by lyophilization and ethanol precipitation. Purified materials were used for chemical or enzymatic cleavage experiments. CNBr and endoproteinase Lys-C were used to separately or sequentially cleave the labeled receptor using procedures previously described (16). The products of cleavage were resolved on 10% NuPAGE gels using MES running buffer (Invitrogen). After electrophoresis, labeled bands were identified by...
exposure to x-ray film with intensifying screens at \(-80^\circ\) C. Aliquots of affinity-labeled receptor and relevant receptor fragments were deglycosylated with endoglycosidase F, as described previously (23).

**Radiochemical Sequencing**—For this, the purified fragment from CNBr cleavage of the wild type human calcitonin receptor labeled with the Bpa16 or from cleavage of the S27M/M48I/M49I mutant receptor labeled with the Bpa26 probe was coupled to N-(2-aminoethyl)-1-benzamidinopolypropyl glass beads (Sigma) through the sulfhydryl side chain of Cys residues. Cycles of Edman degradation were repeated manually in a manner that has been previously reported in detail (26), and the radioactivity released in each cycle was quantified in a \(\gamma\)-spectrometer.

**Statistical Analysis**—All observations were repeated at least three times in independent experiments and are expressed as the means \(\pm\) S.E. Binding curves were analyzed and plotted using the nonlinear regression analysis routine for radioligand binding in the Prism software package (GraphPad Software, San Diego, CA). Binding kinetics was determined by analysis with the LIGAND program of Munson and Rodbard (27).

**RESULTS**

**Characterization of Photolabile Calcitonin Probes**—Both the Bpa16 and Bpa26 probes were synthesized by manual solid phase techniques and purified by reversed-phase HPLC, and their identities were verified by mass spectrometry. They both bound saturably and specifically to calcitonin receptor-bearing HEK293-CTR membranes. As shown in Fig. 2, the Bpa16 probe bound to its receptor with similar affinity to that of natural calcitonin (calcitonin, \(K_r = 83 \pm 6\) pm; Bpa16 probe, \(K_r = 168 \pm 18\) pm), whereas the Bpa26 probe had affinity more than an order of magnitude lower (\(K_r = 3.1 \pm 0.3\) nm). They both represented full agonists, simulating cAMP accumulation in HEK293-CTR cells in a concentration-dependent manner, with the Bpa16 probe having higher potency than the Bpa26 probe (calcitonin, \(EC_{50} = 30 \pm 7\) pm; Bpa16 probe, \(EC_{50} = 28 \pm 6\) pm; Bpa26 probe, \(EC_{50} = 97 \pm 14\) pm; Fig. 2).

**Photoaffinity Labeling of the Calcitonin Receptor**—Both the Bpa16 and Bpa26 probes were used to explore their ability to covalently label the calcitonin receptor. As shown in Fig. 3, they labeled the calcitonin receptor specifically and satura
ty. The protein band labeled with each probe migrated on a 10% SDS-PAGE gel at approximate \(M_r = 97,000\) that shifted to \(M_r = 52,000\) after deglycosylation with endoglycosidase F. The differential migration of the labeled glycosylated receptor from earlier studies may relate to species differences and/or to different degrees of receptor glycosylation in distinct cell lines used (19, 28–31). As expected, the labeling was inhibited by increasing concentrations of calcitonin (Bpa16 probe, \(IC_{50} = 81 \pm 9\) nm; Bpa26 probe, \(IC_{50} = 5.0 \pm 1.2\) nm). No radioactive band was present in the affinity-labeled non-receptor bearing HEK293 cell membranes.

**Identification of Domains of Labeling by Peptide Mapping**—We have successfully used CNBr for identification of ligand binding sites of the cholecystokinin receptor (26, 32, 33), the secretin receptor (11, 12, 14–17), and the motilin receptor (34). Here again, we used CNBr as the first indication of domain of labeling for the calcitonin receptor. Theoretically, CNBr cleavage of the calcitonin receptor would yield 16 fragments ranging in molecular mass from 0.1 to 11 kDa, with 2 of the fragments also containing potential sites of N-linked glycosylation (Fig. 4). As shown in Fig. 4, CNBr cleavage of the calcitonin receptor labeled with the Bpa16 probe resulted in a band that migrated on a 10% NuPAGE gel at approximate \(M_r = 9,500\) and did not further shift after deglycosylation with endoglycosidase F. Given the molecular mass of the radiolabeled Bpa16 probe (3657 Da) and the absence of glycosylation, there was only one candidate fragment matching these data. This represents the fragment spanning the amino terminus, the first transmembrane domain, the first intracellular loop, and the second transmembrane domain (Cys134-Met187, molecular mass = 6138 Da). As also shown in Fig. 4, CNBr cleavage of the calcitonin receptor labeled with the Bpa26 probe yielded a band migrating at approximate \(M_r = 20,000\) and shifted to approximate \(M_r = 6,500\) after deglycosylation. Taking into account the molecular mass of the radiolabeled Bpa26 probe (3732 Da) and clear evidence of glycosylation, the first CNBr fragment at the distal amino terminus of the calcitonin receptor is the only candidate that matches these data.

Endoproteinase Lys-C, which specifically cleaves at Lys residues, was used either separately or sequentially with CNBr to further refine the labeled receptor domain for the Bpa16 probe. As shown in Fig. 5, endoproteinase Lys-C cleavage of the intact calcitonin receptor labeled with the Bpa16 probe yielded a glycosylated fragment band (\(M_r = 26,000\), top right panel, third lane) that migrated at approximate \(M_r = 5,500\) (top right panel, fourth lane) after deglycosylation. This represents the fragment His121-Lys141. Taken together with the above CNBr data, the labeling domain for the Bpa16 probe was now narrowed to the segment Cys134-Lys141. This conclusion was further supported by endoproteinase Lys-C cleavage of the labeled \(M_r = 9,500\) fragment resulting from the CNBr digestion of the labeled intact calcitonin receptor (Figs. 4 and 5). This sequential digestion yielded a labeled fragment that migrated at approximate \(M_r = 4,500\) (Fig. 5, top right panel, second lane, the labeled segment Cys134-Lys141).

Endoproteinase Lys-C was also used sequentially with CNBr to refine the labeled receptor domain for the Bpa26 probe. As also shown in Fig. 5 (bottom right panel), endoproteinase Lys-C...
cleavage of the labeled Mr/H11005/20,000 fragment resulting from CNBr digestion of the labeled intact calcitonin receptor yielded a radioactive band migrating on a 10% NuPAGE gel at approximate Mr/H11005/19,000 (Fig. 5, bottom right panel, fourth lane). The receptor labeled with each probe migrated at approximate Mr/H11005/97,000 and shifted to approximate Mr/H11005/52,000 after deglycosylation with endoglycosidase (EF). No bands were detected in affinity-labeled non-calcitonin receptor-bearing HEK293 cell membranes.

Moreover, endoproteinase Lys-C cleavage of the deglycosylated Mr/H11005/6,500 CNBr fragment (Fig. 5, bottom right panel, second lane) from the intact calcitonin receptor labeled with the Bpa26 probe yielded a radioactive band shifting to approximate Mr/H11005/5,500 (Fig. 5, bottom right panel, third lane). These data, suggesting that the 22-amino acid signal sequence was cleaved in the mature calcitonin receptor, clearly demonstrate that the first endoproteinase Lys-C fragment Leu23-Met37 at the distal amino terminus of the calcitonin receptor represented the domain of labeling for the Bpa26 probe.

To further refine the region of labeling for the Bpa26 probe, a receptor mutant was developed to introduce an additional site for CNBr cleavage, representing the Ser27 to Met (S27M) calcitonin receptor mutant, and was transiently expressed in COS
FIG. 5. Endoproteinase Lys-C cleavage of the affinity-labeled calcitonin receptor and its CNBr fragment. Shown are typical autoradiographs of 10% NuPAGE gels used to separate the products of endoproteinase Lys-C digestion of both intact calcitonin receptor and its CNBr fragments labeled with the Bpa16 (top panel) and Bpa26 (bottom panel) probes as well as diagrams illustrating the predicted cleavage sites. As shown in the top panels, Lys-C cleavage of the intact receptor labeled with the Bpa16 probe resulted in a band migrating at approximate $M_r = 26,000$ (third lane) that shifted to approximate $M_r = 5,500$ (fourth lane) after deglycosylation with Endo F, representing the fragment His121-Lys141 (gray and black circles in the top left diagram). Sequential Lys-C cleavage of the $M_r = 9,500$ CNBr fragment labeled with the Bpa16 probe (first lane) resulted in a shift of this fragment to approximate $M_r = 4,500$ (second lane, the labeled segment Cys134-Lys141). Taken together, these data identified the 8-residue segment Cys134-Lys141 as the domain of labeling for the Bpa16 probe (black circles in the diagram on the top left). As shown in the bottom panels, Lys-C cleavage of the $M_r = 20,000$ CNBr fragment (bottom right, first lane) from the calcitonin receptor labeled with the Bpa26 probe resulted in a band migrating at approximate $M_r = 19,000$ (bottom right, fourth lane), whereas Lys-C cleavage of the deglycosylated $M_r = 6,500$ CNBr fragment (bottom right, second lane) yielded a band shifting to approximate $M_r = 5,500$ (bottom right, third lane). These data suggested the fragment Leu23-Lys37 at the distal amino terminus of the calcitonin receptor was the domain of labeling for the Bpa26 probe (black circles in the bottom left diagram).
Molecular Basis of Ligand Binding to the Calcitonin Receptor

Photoaffinity labeling has been used for labeling calcitonin receptors (27, 28, 30–35) and has been employed to study the molecular basis of calcitonin binding to its receptor. However, the molecular basis of ligand binding to the calcitonin receptor is largely unknown. Our current understanding of the functional basis of ligand binding to the calcitonin receptor is limited and has been hindered because of the inability to employ high-resolution methods. 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Among these studies many used aryl azide-containing moieties such as N-(β-aminomethyl)-4-azido-2-nitroaniline (30, 31, 35) and N-hydroxysuccinimide-4-azidobenzoate (36). However, the labeling efficiency through these photoreactive cross-linkers was poor, likely due to the fact that this group of photolabile residues generates highly reactive electrophilic species, leading to low yield photo-insertion reactions (37). Benzophenones have been the preferred chemical moiety for higher yield photolabeling (37). Suva et al. (19) have successfully incorporated an (p-benzyoxbenzoyl)lysine into a series of salmon calcitonin analogues and demonstrated high efficiency labeling of the calcitonin receptor expressed endogenously in cultured cell lines and transiently in transfected COS cells. However, whether these benzophenone-containing calcitonin analogues could be useful for exploring the ligand binding domains of the calcitonin receptor is not clear. In this work we not only developed two high efficiency photolabile human calcitonin probes incorporating a Bpa but also were able to use them for further mapping the domains of labeling within their receptor. The Bpa16 and Bpa26 probes used in this study incorporated the photolabile residue Bpa in the mid-region and carboxyl-terminal half in positions 16 and 26 of the ligand, respectively, both within regions that are important for high affinity binding (2, 7).

It is noteworthy that the intrinsic photoaffinity labeling approach identified two residues within the amino-terminal domain of the calcitonin receptor as the sites of covalent attachment to these probes. In fact this domain has been shown to be critical for ligand binding by analysis of calcitonin-glucagon (9, 10) and calcitonin–PTH (8) receptor chimeras. The importance of the amino-terminal domain in ligand binding has been consistent for other members in the class B G protein-coupled receptor family, including receptors for secretin (22, 38–41), vasoactive intestinal polypeptide (VIP) (38, 40), PTH (42), and pituitary adenylate cyclase-activating polypeptide (43, 44). This domain is also the domain labeled in analogous photoaffinity labeling studies for mapping of the binding domains of the secretin receptor (11, 12, 14–17) (for review, see Ref. 13), the PTH1 receptor (45–47) (for review, see Ref. 48), and the VIP receptor (49).

Of particular interest, this work demonstrated spatial approximation between the mid-region of calcitonin (position 16) and receptor residue Phe137, a position within the extracellular amino terminus adjacent to the first transmembrane domain of the calcitonin receptor. Sexton and co-workers recently showed that this region was the domain of labeling for a salmon calcitonin probe incorporating a Bpa at position 19 of the peptide.2 As summarized in Table I, this identification is quite similar to the localization of the analogous region of the secretin receptor interacting with a photolabile residue in the mid-region, in position 13 of secretin (14), and that of the VIP receptor interacting with a carboxyl-terminal residue of the VIP ligand (49). It is also the domain of the PTH 1 receptor interacting with a photolabile residue in the mid-region, in position 13 of PTH (45, 50), and in the carboxyl-terminal region, in position 33 of PTH-related peptide (PTHrP) (46) (Table I). In this work we also demonstrated the proximity between the carboxyl-terminal half (position 26) and receptor Thr30, a residue within the distal amino-terminal tail of the calcitonin receptor. The identification of this domain is similar to covalent labeling of the distal amino-terminal tail of the secretin receptor by secretin probes incorporating photolabile residues in positions 6, 12, 14, 18, 22, and 26 (11, 12, 14–17) (Table I). It is also similar to covalent labeling of the distal amino-terminal tail of the PTH 1 receptor by PTH/PTHrP probes, incorporating a photolabile residue in positions 23 (47) and 28 (46) (Table I). The identification of the amino-terminal domain of the calcitonin receptor as the labeling domain for both Bpa16 and Bpa26 probes is distinct from photoaffinity labeling of the first extracellular loop domain of the PTH 1 receptor by a position 27 probe (51) and from that of the top of the sixth transmembrane domains of the PTH 1 receptor by PTH/PTHrP probes incorporating a photolabile residue at their amino termini (52, 53) (Table I).

The covalent attachment of calcitonin residue 16 to receptor residue Phe137 within the amino terminus is also consistent with chimeric calcitonin-glucagon receptor studies that suggested the helical portion of the hormone within residues 8–22 of calcitonin as the principal determinant for binding to the receptor amino terminus (10). In that work, it was also demonstrated that residues 2–6 of calcitonin interact with the receptor transmembrane loop region and are critical for activation of adenylate cyclase (10). It was based on this study that Sexton et al. (1) proposed a model for ligand-receptor interaction for the calcitonin receptor that (i) the α-helix of the peptide (residues 8–22) interacts with the amino-terminal extracellular domain of the receptor, (ii) the amino-terminal disulfide bridged loop of the peptide (residues 1–7) interacts with the

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TABLE I  
Summary of domains of photoaffinity labeling of secretin family G protein-coupled receptors

| N-ECD, amino-terminal extracellular domain; ECL, extracellular loop domain, TM, transmembrane domain. | T
|---|---|---|
| Receptors | Position of photolabile residue in ligand | Labeled receptor regions | Labeled receptor residues | References |
|---|---|---|
| Calcitonin | 16 | N-ECD adjacent to TM1 | Phe<sup>137</sup> | This work |
| | 19 | N-ECD adjacent to TM1 | Cys<sup>234</sup>, Lys<sup>141</sup> | Pham<sup>7</sup> |
| | 26 | N-ECD | Thr<sup>100</sup> | This work |
| Secretin | 13 | N-ECD adjacent to TM1 | Val<sup>110</sup> | 14 |
| | 6 | Distal N-ECD | Val<sup>9</sup> | 15 |
| | 12 | Distal N-ECD | Val<sup>9</sup> | 17 |
| | 14 | Distal N-ECD | Pro<sup>118</sup> | 17 |
| | 18 | Distal N-ECD | Arg<sup>14</sup> | 12 |
| | 22 | Distal N-ECD | Leu<sup>17</sup> | 11, 16 |
| | 26 | Distal N-ECD | Leu<sup>17</sup> | 11 |
| | 29 | N-ECD adjacent to TM1 | Gly<sup>109</sup>, Lys<sup>120</sup> | 49 |
| | 33 | N-ECD adjacent to TM1 | Arg<sup>156</sup> | 45, 50 |
| | 28 | Distal N-ECD | Asn<sup>155</sup>, Lys<sup>172</sup> | 46 |
| | 23 | Distal N-ECD | Gly<sup>64</sup>, Lys<sup>95</sup> | 46 |
| | 27 | ECL1 | Thr<sup>23</sup>, Gly<sup>77</sup> | 47 |
| | 2 | Top of TM6 | Leu<sup>261</sup> | 51 |
| | 1, 2 | | Met<sup>425</sup> | 52, 53 |

transmembrane domains of the receptor, and (iii) the carboxy-terminal region of the peptide (residues 22–32) interacts with other extracellular loop domains. The covalent attachment of calcitonin residue 26 to receptor residue Thr<sup>100</sup> within the amino-terminal tail of the calcitonin receptor may suggest some differences from this model. To further test this hypothesis by photoaffinity labeling studies, development of novel probes incorporating photolabile residues at their amino termini is also needed.

Like that of the secretin receptor and all other members of the class B G protein-coupled receptors, the amino-terminal extracellular domain of the calcitonin receptor contains six conserved Cys residues, differing from members of the class A receptors in the rhodopsin/β-adrenergic receptor family. These Cys residues are predicted to form disulfide bonds that are thought to be important for ligand binding. Although there are no direct data to demonstrate their involvement in forming intra-domain disulfide bonds in the calcitonin receptor as in receptors for secretin (54), PTH (55), glucagon-like peptide 1 (56), and corticotropin-releasing factor (57), progressive truncation of this domain resulted in loss of ligand binding and receptor activation. Clearly, these conserved Cys residues that probably all involve forming disulfide bonds within the amino-terminal extracellular domain are important to constrain the conformation of the calcitonin receptor. Such constraints should be complementary to those coming from photoaffinity labeling studies for the elucidation of the molecular basis of ligand binding.

It should be noted that the mutation of the residues that were covalently labeled in the current photoaffinity labeling studies (Phe<sup>137</sup> and Thr<sup>100</sup>) did not interfere with the normal binding and biological activity of calcitonin. This confirms the presence of adequate space in those positions when the natural agonist peptide is normally docked, thus permitting the siting of the photolabile benzoylphenylalanine residue in the photoprobes in the positions of smaller natural residues in calcitonin. The photoaffinity labeling studies provide the constraints of spatial approximation but not necessarily positions of direct residue-residue interactions. In fact, if such interactions were present and critical, the modifications in the photoprobes would likely interfere with their use.

In conclusion, having identified two specific receptor residues that are proximate to two specific residues within a calcitonin agonist ligand, we have provided two valuable constraints for the molecular modeling of the agonist-bound calcitonin receptor. As the number of pairs of approximated ligand-receptor residues grows and as other key constraints such as disulfide bonding patterns become available, a meaningful model can then be proposed. Such a model can provide insights into whether a common ligand binding mechanism exists for all members of the class B G protein-coupled receptors.

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