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

INTRINSIC PHOTOAFFINITY 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 those receptors, current knowledge of the molecular basis of calcitonin binding to its receptor is based only 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 Phe^{137} and Thr^{30}, 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).

The calcitonin peptide consists of 32 amino acids, with a disulfide bond between residues 1 and 7 which is conserved among all species and that is believed to be critical for its agonist activity. The amino acid sequence and biological potency of calcitonin vary considerably from species to species, but the integrity of the disulfide bond and the carboxyl-terminal proline-amide are necessary for full biological activity (1–3). The disulfide bond can be replaced by other covalent bonds that lead to improved biological stability while retaining full potency (1). The sequence within the amino-terminal loop region is highly conserved in a variety of species but demonstrates divergence in the rest of the sequence (4). Like secretin and peptides for other members of class B G protein-coupled receptor family, the amino-terminal region of calcitonin contains key determinants for receptor agonist selectivity, whereas the carboxyl-terminal region contains determinants for high affinity binding. Progressive truncation of residues in the disulfide bond-looped domain leads first to partial and then to antagonist peptides (1, 2, 5, 6). Residues 8–22 tend to form an amphiphilic α-helical structure that is important for high affinity binding (1, 2, 7).

The calcitonin receptor is closely related to the secretin and parathyroid hormone (PTH)[1] 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.

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

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[1] The abbreviations used are: PTH, parathyroid hormone; PTHrP, parathyroid hormone-related peptide; Bpa, *p*-benzoyl-L-phenylalanine; Bpa^{16} analogue or probe, (Ile^{8},Bpa^{16},Arg^{18})calcitonin; Bpa^{26} analogue or probe, (Ile^{8},Arg^{18},Bpa^{26})calcitonin; CTR, calcitonin receptor; Endo F, endoglycosidase F; HEK, human embryonic kidney; MES, 4-morpholineethanesulfonic acid; HPLC, high performance liquid chromatography; VIP, vasoactive intestinal peptide.

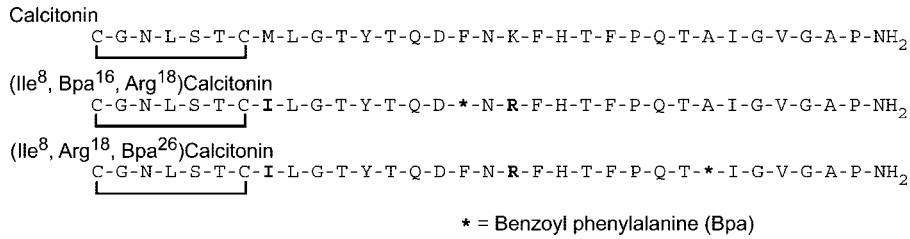

FIG. 1. **Synthetic peptides.** Shown are the sequences of natural human calcitonin as well as the synthetic analogues of this peptide, (Ile^8,Bpa^{16},Arg^{18})calcitonin and (Ile^8,Arg^{18},Bpa^{26})calcitonin, used for photoaffinity labeling studies. Modifications of residues found in the natural peptide are *highlighted* in ***bold***. Peptides were radioiodinated oxidatively on the Tyr residue in position 12.

(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 mid-region 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 Phe^{137} and Thr^{30}, 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 (Ile^8,Bpa^{16},Arg^{18})calcitonin (Bpa^{16} analogue or probe) and (Ile^8,Arg^{18},Bpa^{26})calcitonin (Bpa^{26} 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 as the site for radioiodination and an Ile residue in the position of Met^8 to eliminate a site for potential oxidative damage during radiolabeling. Additionally, Lys^{18} was replaced with an Arg to facilitate the specific digestion of the labeled receptor without the cleavage of the probes themselves (Fig. 1). These changes were demonstrated to be well tolerated without resulting in substantial loss of biological activity of the ligand (19). Both probes were synthesized by manual solid-phase techniques and purified to homogeneity by reversed-phase HPLC using techniques that were previously described (20). The expected molecular masses of the probes were verified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry.

Radioiodination—Fifteen μg of the synthetic probes ((Ile^8,Bpa^{16},Arg^{18})calcitonin or (Ile^8,Arg^{18},Bpa^{26})calcitonin) were radioiodinated oxidatively with Na^{125}I upon exposure to an IODO-BEAD for 15 s and purified by reversed-phase HPLC to yield specific radioactivities of 2000 Ci/mmol (20). In the same way radioiodination of the natural human calcitonin was performed to produce a radioligand for calcitonin receptor binding.

Receptor Preparations—The receptor-bearing human embryonic kidney 293 (HEK293) cell line stably expressing the human calcitonin isoform II receptor (HEK293-CTR) was provided by GlaxoSmithKline and was used as the source of receptors for the current study. Cells were cultured at 37 °C in a 5% CO_2 environment on Falcon tissue culture plasticware in Dulbecco's modified Eagle's medium supplemented with 5% fetal clone-2 (Hyclone Laboratories, Logan, UT). Cells were passaged twice a week and lifted mechanically before use.

Development of new calcitonin receptor mutants was necessary for the current study. One of these incorporated an additional site for CNBr cleavage in a key position for localization of the site of labeling for the Bpa^{26} probe. This represented mutation of Ser^{27} of the calcitonin receptor to Met (S27M). A triple-mutant calcitonin receptor construct was developed to introduce a new CNBr cleavage site while simultaneously eliminating a pair of naturally occurring Met residues, representing Ser^{27} to Met, Met^{48} to Ile, and Met^{49} to Ile (S27M/M48I/M49I) receptor mutant. This was used for radiochemical sequencing to identify the site of labeling with the Bpa^{26} probe. In addition, two more calcitonin receptor constructs were generated that included Phe^{137} to Ala (F137A) and Thr^{30} to Ala (T30A), each representing mutation of the site of labeling by the Bpa^{16} and Bpa^{26} probes, respectively. All above constructs were prepared using an oligonucleotide-directed approach with the QuikChange™ site-directed mutagenesis kit from Stratagene (La Jolla, CA). They were subcloned into the eukaryotic expression vector, pcDNA3 (Invitrogen), and their sequences were verified by direct DNA sequencing (21). All mutant calcitonin receptor constructs were expressed transiently in COS cells (American Type Cell Collection, Manassas, VA) after transfection using a modification of the DEAE-dextran method (22). These cells were harvested mechanically 72 h after transfection. Receptor-enriched plasma membranes were prepared from the above stable HEK293 cell line and transiently transfected COS cells using methods that we previously reported (23).

Ligand Binding—Receptor binding of calcitonin, the Bpa^{16} analogue, and the Bpa^{26} 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, ^{125}I-calcitonin (3–5 pM), in the presence of increasing concentrations of non-radiolabeled calcitonin or the Bpa^{16} analogue or the Bpa^{26} 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 KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 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 filtermats 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 Bpa^{16} and Bpa^{26} analogues were studied for stimulation of cAMP in the HEK293-CTR cells using a competition binding assay (Diagnostic Products Corp., Los Angeles, CA). Cells were stimulated with increasing concentrations of calcitonin or the Bpa^{16} or Bpa^{26} 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 $KHCO_3$, cell lysates were cleared by centrifugation at 3000 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 nM ^{125}I-(Ile^8,Bpa^{16},Arg^{18})calcitonin or ^{125}I-(Ile^8,Arg^{18},Bpa^{26})calcitonin in the presence of increasing concentrations of calcitonin (0–1 μM) for 1 h at room temperature before photolysis for 30 min at 4 °C in a Rayonet photochemical reactor (Southern New England Ultraviolet, Hamden, CT) equipped with 3500-Å lamps. To scale up receptor purification, a larger amount of membranes (~150–200 μg) was incubated with each radiolabeled probe (~0.5 nM) in the absence of competing calcitonin. After photolysis, 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

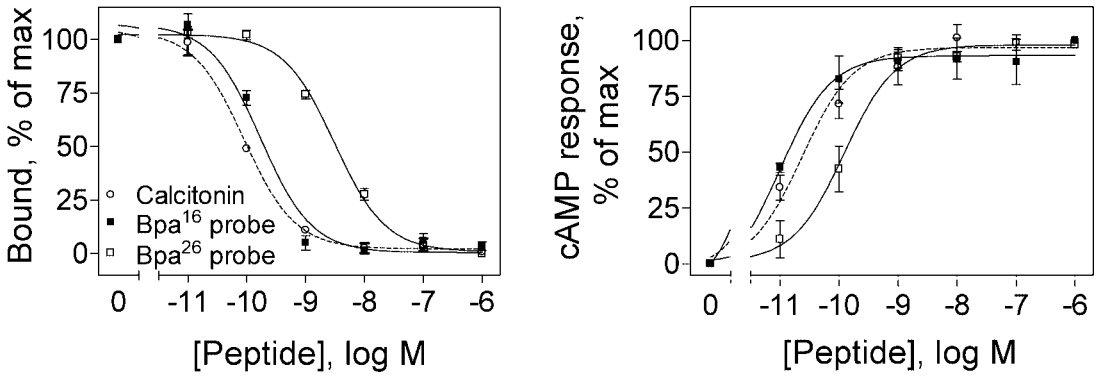

FIG. 2. **Characterization of the Bpa[16] and Bpa[26] probes.** The *left panel* demonstrates the abilities of increasing concentrations of calcitonin or the Bpa[16] and Bpa[26] probes to compete for binding of the radioligand ^{125}I-calcitonin to HEK293-CTR membranes. Values represent saturable binding as percentages of maximal binding observed in the absence of competitor. Data are expressed as the means ± S.E. of duplicate data from a minimum of three independent experiments. The *right panel* shows concentration-dependent intracellular cAMP responses to these peptides in the HEK293-CTR cells. Values are expressed as the means ± S.E. of at least three independent experiments, with data normalized relative to the maximal response to natural calcitonin.

exposure to x-ray film with intensifying screens at −80 °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 Bpa[16] or from cleavage of the S27M/M48I/M49I mutant receptor labeled with the Bpa[26] probe was coupled to *N*-(2-aminoethyl-1)-3-aminopropyl 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 γ-spectrometer.

Statistical Analysis—All observations were repeated at least three times in independent experiments and are expressed as the means ± 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 Bpa[16] and Bpa[26] 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 Bpa[16] probe bound to its receptor with similar affinity to that of natural calcitonin (calcitonin, K_i = 83 ± 6 pM; Bpa[16] probe, K_i = 168 ± 18 pM), whereas the Bpa[26] probe had affinity more than an order of magnitude lower (K_i = 3.1 ± 0.3 nM). They both represented full agonists, simulating cAMP accumulation in HEK293-CTR cells in a concentration-dependent manner, with the Bpa[16] probe having higher potency than the Bpa[26] probe (calcitonin, EC_{50} = 30 ± 7 pM; Bpa[16] probe, EC_{50} = 28 ± 6 pM; Bpa[26] probe, EC_{50} = 97 ± 14 pM; Fig. 2).

Photoaffinity Labeling of the Calcitonin Receptor—Both the Bpa[16] and Bpa[26] 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 saturably. 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 (Bpa[16] probe, IC_{50} = 81 ± 9 nM; Bpa[26] probe, IC_{50} = 5.0 ± 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 Bpa[16] 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 radioiodinated Bpa[16] 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 (Cys[134]-Met[187], molecular mass = 6138 Da). As also shown in Fig. 4, CNBr cleavage of the calcitonin receptor labeled with the Bpa[26] 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 radioiodinated Bpa[26] 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 Bpa[16] probe. As shown in Fig. 5, endoproteinase Lys-C cleavage of the intact calcitonin receptor labeled with the Bpa[16] 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 His[121]-Lys[141]. Taken together with the above CNBr data, the labeling domain for the Bpa[16] probe was now narrowed to the segment Cys[134]-Lys[141]. 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 Cys[134]-Lys[141]).

Endoproteinase Lys-C was also used sequentially with CNBr to refine the labeled receptor domain for the Bpa[26] probe. As also shown in Fig. 5 (*bottom right panel*), endoproteinase Lys-C

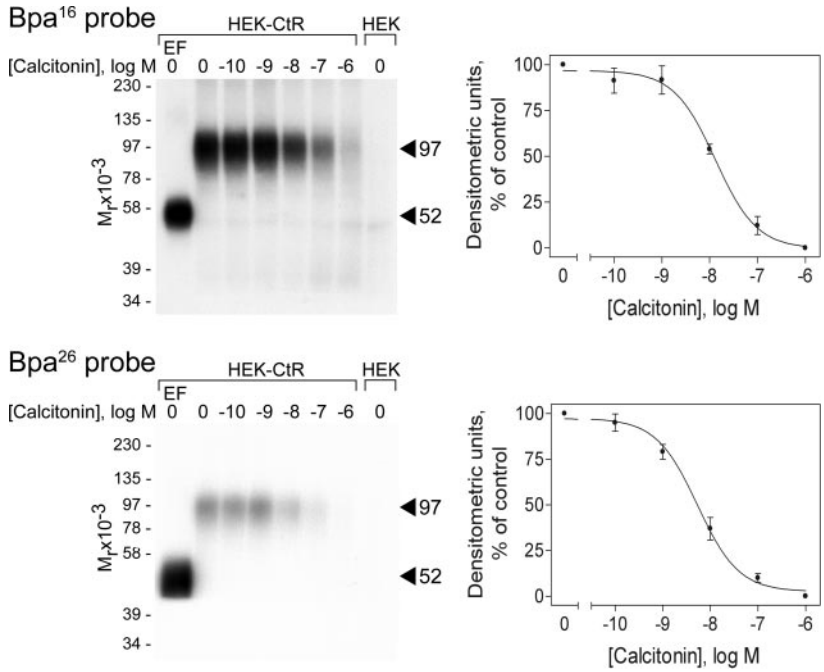

FIG. 3. **Photoaffinity labeling of the calcitonin receptor.** Shown are typical autoradiographs of 10% SDS-polyacrylamide electrophoresis gels used to separate the products of affinity labeling of HEK293-CTR cell membranes by the Bpa[16] (*top panel*) and Bpa[26] (*bottom panel*) probe in the presence of increasing concentrations of calcitonin. Shown in the *right panels* are the densitometric analyses of four similar independent experiments by each probe (means ± S.E.). The receptor labeled with each probe migrated at approximate M_r = 97,000 and shifted to approximate M_r = 52,000 after deglycosylation with endoglycosidase (*EF*). No bands were detected in affinity-labeled non-calcitonin receptor-bearing HEK293 cell membranes.

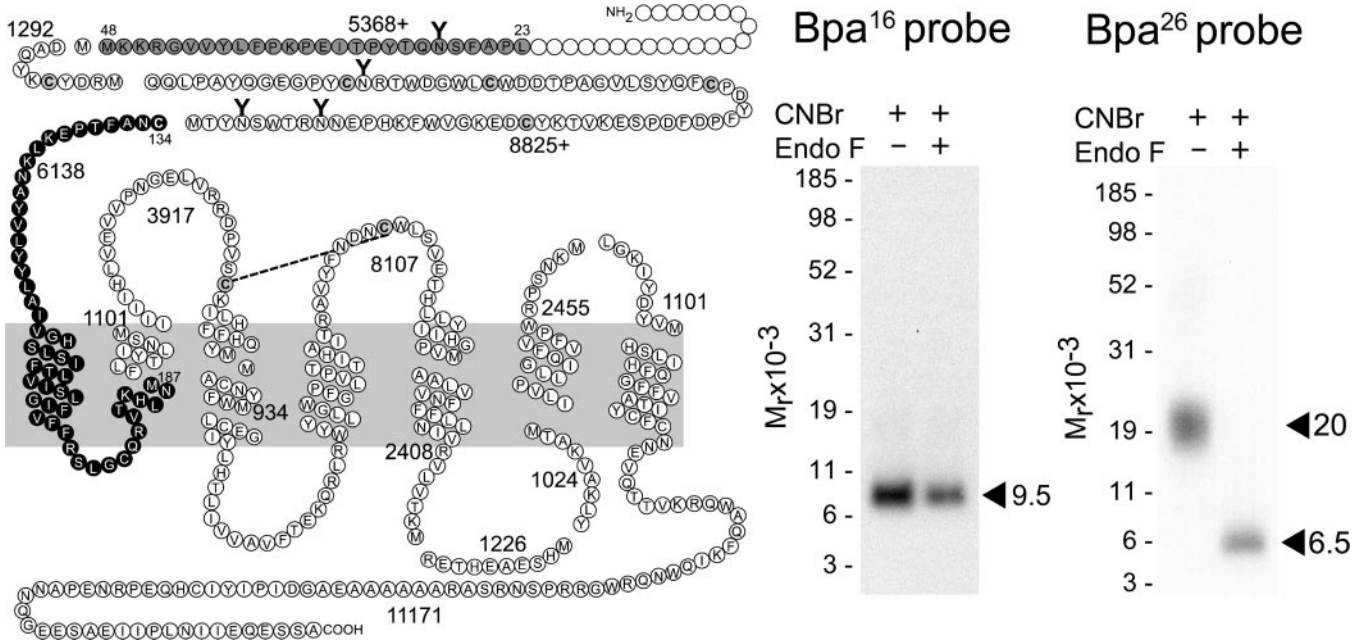

FIG. 4. **CNBr cleavage of the affinity-labeled calcitonin receptor.** Theoretically, CNBr cleavage of the calcitonin receptor would result in 16 fragments ranging in molecular mass from 0.1 to 11 kDa, two of which contain potential sites of glycosylation (*left panel*). Shown in the *middle* and *right panels* are representative autoradiographs of 10% NuPAGE gels used to separate the products of CNBr cleavage of the calcitonin receptor labeled with the Bpa[16] (*middle panel*) and Bpa[26] (*right panel*) probes. CNBr cleavage patterns were different for both probes. Cleavage of the calcitonin receptor labeled with the Bpa[16] probe yielded a band migrating at approximate M_r = 9,500 that was not affected by treatment with Endo F. Taking into account the molecular mass of the probe (3657 Da), the best candidate to represent the receptor domain of labeling for the Bpa[16] probe is the fragment Cys[134]-Met[187], as highlighted on the diagram (*black circles*). CNBr cleavage of the calcitonin receptor labeled with the Bpa[26] probe yielded a band migrating at approximate M_r = 20,000 that shifted to approximate M_r = 6,500 after deglycosylation by Endo F. Taking consideration of the molecular mass of the probe (3732 Da) and clear evidence of glycosylation, the fragment Leu[23]-Met[48] (*highlighted* in *gray circles*) at the distal amino terminus of the calcitonin receptor was the domain of labeling for the Bpa[26] probe.

cleavage of the labeled M_r = 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 M_r = 19,000 (Fig. 5, *bottom right panel*, *fourth lane*). Moreover, endoproteinase Lys-C cleavage of the deglycosylated M_r = 6,500 CNBr fragment (Fig. 5, *bottom right panel*, *second lane*) from the intact calcitonin receptor labeled with the Bpa[26] probe yielded a radioactive band shifting to approximate M_r = 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 Leu[23]-Lys[37] at the distal amino terminus of the calcitonin receptor represented the domain of labeling for the Bpa[26] probe.

To further refine the region of labeling for the Bpa[26] probe, a receptor mutant was developed to introduce an additional site for CNBr cleavage, representing the Ser[27] to Met (S27M) calcitonin receptor mutant, and was transiently expressed in COS

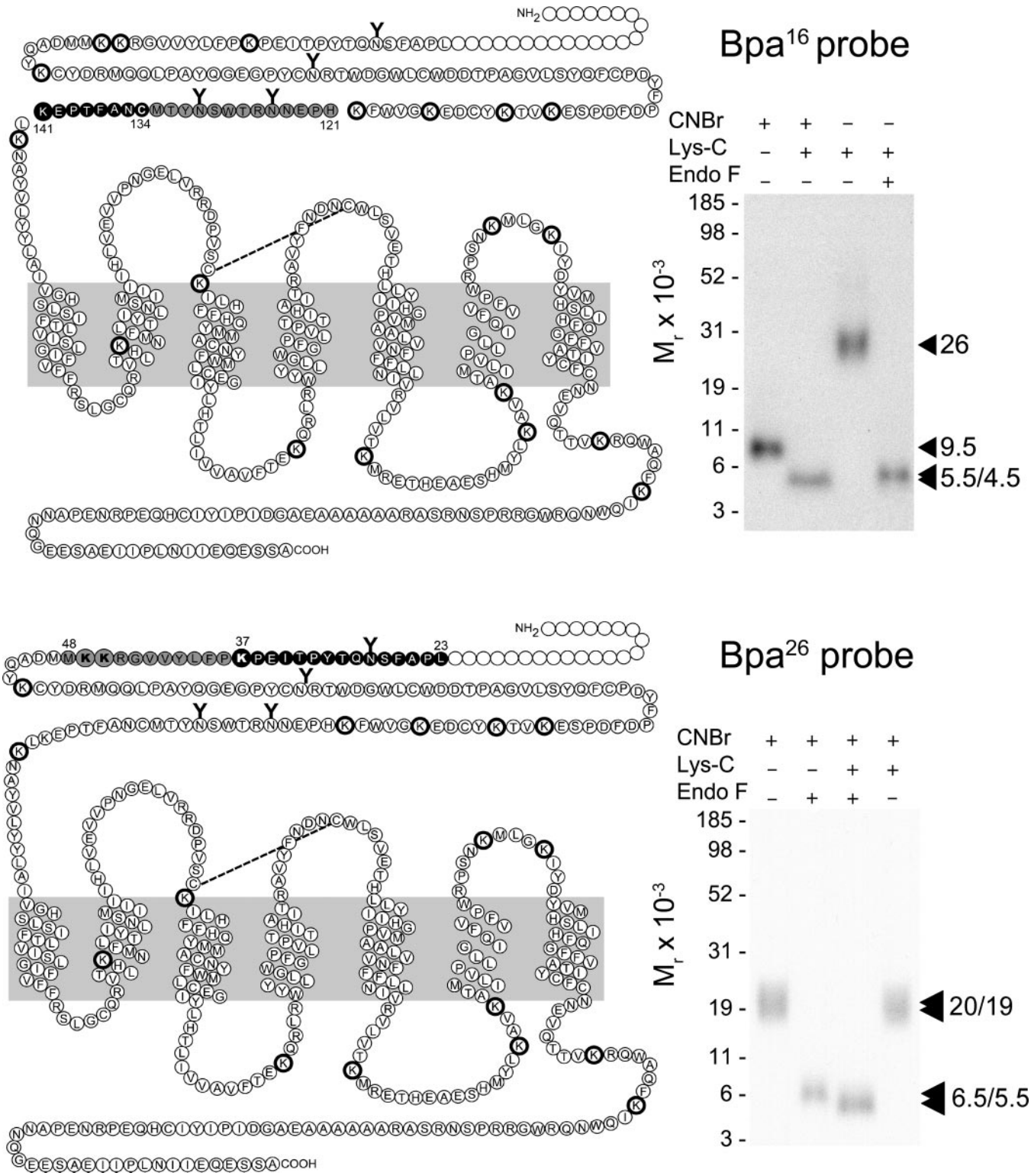

FIG. 5. **Endoproteinase Lys-C cleavage of the affinity-labeled calcitonin receptor and its CNBr fragment.** Shown are typical autoradiographs of 10% NuPAGE gels used to separate the products of endoproteinase Lys-C digestion of both intact calcitonin receptor and its CNBr fragments labeled with the Bpa^{16} (*top panel*) and Bpa^{26} (*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 Bpa^{16} 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 His^{121}-Lys^{141} (*gray* and *black circles* in the *top left diagram*). Sequential Lys-C cleavage of the M_r = 9,500 CNBr fragment labeled with the Bpa^{16} probe (*first lane*) resulted in a shift of this fragment to approximate M_r = 4,500 (*second lane*, the labeled segment Cys^{134}-Lys^{141}). Taken together, these data identified the 8-residue segment Cys^{134}-Lys^{141} as the domain of labeling for the Bpa^{16} 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 Bpa^{26} 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 Leu^{23}-Lys^{37} at the distal amino terminus of the calcitonin receptor was the domain of labeling for the Bpa^{26} probe (*black circles* in the *bottom left diagram*).

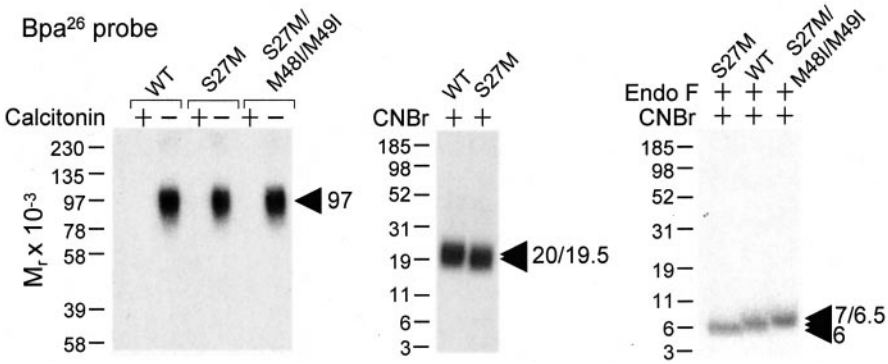

FIG. 6. **CNBr cleavage of the calcitonin receptor mutants labeled with the Bpa[26] probe.** The *left panel* shows a representative autoradiograph of a 10% SDS-polyacrylamide electrophoresis gel used to separate the products of affinity labeling of plasma membranes from COS cells transiently expressing the wild type (*WT*), S27M, and S27M/M48I/M49I calcitonin receptor constructs labeled by the Bpa[26] in the presence and absence of 1 μM calcitonin. The labeled wild type and mutant receptors also migrated at approximate M_r = 97,000, as did that from HEK293-CTR cell membranes. The *middle panel* shows that the labeled glycosylated M_r = 20,000 CNBr fragment from the wild type calcitonin receptor (*first lane*) shifted to approximate M_r = 19,500 in the S27M receptor mutant (*second lane*), whereas the *right panel* shows that the labeled deglycosylated M_r = 6,500 fragment from the wild type calcitonin receptor (*second lane*) shifted to M_r = 6,000 in the S27M mutant receptor (*first lane*). These data further narrowed the domain of labeling for the Bpa[26] probe to the 10-residue segment Asn[28]-Lys[37] within the distal amino-terminal tail of the calcitonin receptor (see the *diagram* in Fig. 4). The *right panel* also shows the CNBr fragment from cleavage of the labeled S27M/M48I/M49I receptor mutant migrated at M_r = 7,000, running slightly slower than that from the labeled wild type receptor and the S27M receptor construct.

cells. The S27M construct bound calcitonin with high affinity (K_i = 4.0 ± 1.1 nM) and had a normal cAMP response to calcitonin (EC_{50} = 96 ± 15 pM). It was also labeled by the Bpa[26] probe saturably and specifically (Fig. 6, *left panel*). CNBr cleavage of the labeled S27M receptor construct resulted in a band migrating at approximate M_r = 19,500, migrating slightly faster than the labeled glycosylated M_r = 20,000 band from CNBr cleavage of the labeled wild type receptor (Fig. 6, *middle panel*). After deglycosylation, the M_r = 6,500 CNBr fragment from the labeled wild type receptor (Fig. 6, *right panel*, *second lane*) shifted to approximate M_r = 6,000 in the labeled S27M receptor construct (Fig. 6, *right panel*, *first lane*). Together with the above data from the endoproteinase Lys-C cleavage, these data clearly indicated that segment Asn[28]-Lys[37] within the distal amino terminus of the calcitonin receptor contained the site of labeling for the Bpa[26] probe.

Site Identification by Radiochemical Sequencing—Manual Edman degradation sequencing of the purified labeled fragment (Cys[134]-Met[187], see Fig. 4) resulting from CNBr cleavage of the calcitonin receptor was performed to identify the specific residue labeled by the Bpa[16] probe. A radioactive peak eluted consistently in cycle 4 as shown in Fig. 7, *left panel*. This result indicates that the site of labeling for the Bpa[16] probe was residue Phe[137], which is located in the extracellular amino terminus of the calcitonin receptor adjacent to the first transmembrane domain.

To identify the specific residue labeled by the Bpa[26] probe by radiochemical sequencing, an additional Met calcitonin receptor mutant was generated that represented the S27M/M48I/M49I receptor construct. It was designed to couple the CNBr fragment Asn[28]-Met[59] through Cys[55] to *N*-(2-aminoethyl-1)-3-aminopropyl glass beads (26). This receptor mutant bound calcitonin with high affinity (K_i = 3.4 ± 1.4 nM) and had similar cAMP responses to calcitonin stimulation as the wild type receptor (EC_{50} = 109 ± 29 pM). The S27M/M48I/M49I calcitonin receptor mutant was saturably and specifically labeled by the Bpa[26] probe (Fig. 6, *left panel*). CNBr cleavage of the deglycosylated S27M/M48I/M49I calcitonin receptor mutant labeled with the Bpa[26] probe yielded a labeled fragment migrating at approximate M_r = 7,000 (Fig. 6, *right panel*), representing the fragment Asn[28]-Met[59], distinct in migration from the M_r = 6,000 and M_r = 6,500 CNBr fragments resulting from cleavage of the labeled S27M mutant and wild type receptor, respectively (Fig. 6, *right panel*). Radiochemical sequencing of the labeled fragment Asn[28]-Met[59] from the S27M/M48I/M49I calcitonin receptor mutant identified Thr[30] as the site of labeling by the Bpa[26] probe (Fig. 7, *right panel*).

Characterization of Calcitonin Receptor Site Mutants—The F137A and T30A calcitonin receptor mutants were expressed transiently in COS cells and studied in that cell system to explore the potential impact on the binding and biological activity of calcitonin. Mutation of the residues that had been covalently labeled in the photoaffinity labeling studies were found to not interfere with the normal binding of calcitonin (F137A, K_i = 344 ± 28 pM; T30A, K_i = 263 ± 70 pM) or with its ability to elicit a full biological response with normal potency (F137A, EC_{50} = 117 ± 29 pM; T30A, EC_{50} = 157 ± 20 pM). These observations confirm that there is adequate space between the relevant residues in calcitonin and its receptor when normally docked to accommodate the photoprobes. Because the benzoylphenylalanine residues in the probes are larger than the natural calcitonin residues in those locations, such space is important so as not to interfere with normal binding and activation. These observations are fully consistent with the photoaffinity labeling data described above.

DISCUSSION

G protein-coupled receptors represent the largest group of drug targets in the body. Efforts in identification of the structural basis of ligand binding of receptors have long been a focus for developing receptor-active drugs. However, because of the sparsity and physicochemical nature of these molecules, such efforts have been hindered because of the inability to employ high resolution methods. Our current understanding of the molecular basis of calcitonin binding to its receptor is largely limited to analysis of receptor chimeras (8–10). Photoaffinity labeling is a more direct approach to identify interactions between a ligand and its receptor. In this work we have successfully developed two agonist probes with photolabile residues in the mid-region and carboxyl-terminal half of calcitonin analogues and identified specific residues within the amino-terminal domain of the calcitonin receptor that are in approximation to these residues when the agonist probes were docked.

Photoaffinity labeling has been used for labeling calcitonin receptors from cultured cell lines (19, 28, 30, 31, 35), kidney plasma membranes (36), and transfected cell lines (19, 29).

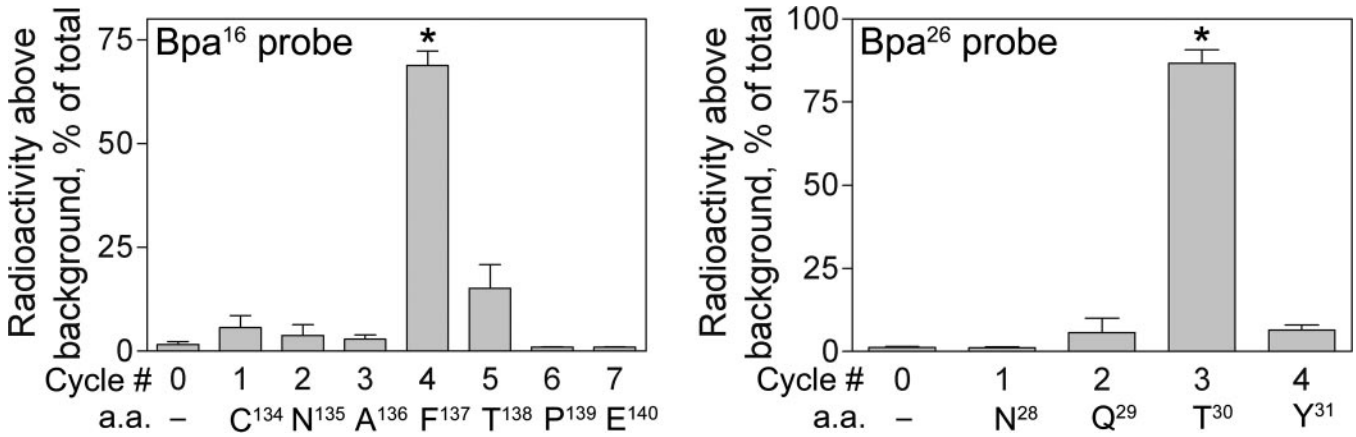

FIG. 7. **Identification of the affinity-labeled receptor residues by Edman degradation sequencing.** Shown in the *left panel* is the profile of eluted radioactivity from sequencing of the purified CNBr fragment (Cys^{134}-Met^{187}) of the wild type calcitonin receptor labeled with the Bpa^{16} probe. A radioactive peak consistently eluted in cycle 4 in 3 independent experiments. This corresponds to the covalent attachment of the Bpa^{16} probe to Phe^{137} of the calcitonin receptor. Shown in the *right panel* is the radioactive elution profile of sequencing of the purified CNBr fragment (Asn^{28}-Met^{59}) from the S27M/M48I/M49I calcitonin receptor construct labeled with the Bpa^{26} probe. A radioactive peak consistently eluted in cycle 3 that represents covalent labeling of residue Thr^{30} of the calcitonin receptor with this probe. *a.a.*, amino acid.

Among these studies many used aryl azide-containing moieties such as *N*-(β-aminoethyl)-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 photo-insertion (37). Suva *et al.* (19) have successfully incorporated an (ε-*p*-benzoylbenzoyl)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 Bpa^{16} and Bpa^{26} 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 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 Phe^{137}, 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 Thr^{30}, 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 Bpa^{16} and Bpa^{26} 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 Phe^{137} 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

[2] V. Pham, J. D. Wade, B. W. Purdue, and P. M. Sexton, in press.

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.

Receptors	Position of photolabile residue in ligand	Labeled receptor regions	Labeled receptor residues	References
Calcitonin	16	*N*-ECD adjacent to TM1	Phe^{137}	This work
	19	*N*-ECD adjacent to TM1	Cys^{134}-Lys^{141}	Pham[2]
	26	Distal *N*-ECD	Thr^{30}	This work
Secretin	13	*N*-ECD adjacent to TM1	Val^{103}	14
	6	Distal *N*-ECD	Val^{4}	15
	12	Distal *N*-ECD	Val^{6}	17
	14	Distal *N*-ECD	Pro^{38}	17
	18	Distal *N*-ECD	Arg^{14}	12
	22	Distal *N*-ECD	Leu^{17}	11, 16
	26	Distal *N*-ECD	Leu^{36}	11
VIP	22	*N*-ECD adjacent to TM1	Gly^{109}-Lys^{120}	49
PTH	13	*N*-ECD adjacent to TM1	Arg^{186}	45, 50
	33	*N*-ECD adjacent to TM1	Asn^{151}-Lys^{172}	46
	28	Distal *N*-ECD	Glu^{64}-Lys^{95}	46
	23	Distal *N*-ECD	Thr^{33}, Gln^{37}	47
	27	ECL1	Lcu^{261}	51
	1,2	Top of TM6	Mct^{425}	52, 53

transmembrane domains of the receptor, and (iii) the carboxyl-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^{30} 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.[3] 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^{137} and Thr^{30}) 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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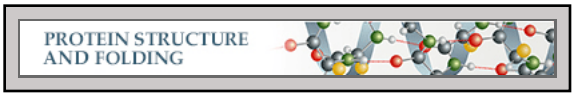
Protein Structure and Folding:



Importance of the Amino Terminus in Secretin Family G Protein-coupled Receptors: INTRINSIC PHOTOAFFINITY LABELING ESTABLISHES INITIAL DOCKING CONSTRAINTS FOR THE CALCITONIN RECEPTOR

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