Differential Docking of High-Affinity Peptide Ligands to Type A and B Cholecystokinin Receptors Demonstrated by Photoaffinity Labeling†

Maoqing Dong, Guangming Liu, Delia I. Pinon, and Laurence J. Miller*

Mayo Clinic Scottsdale, Cancer Center and Department of Molecular Pharmacology and Experimental Therapeutics, Scottsdale, Arizona 85259

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ABSTRACT: Type A and B cholecystokinin (CCK) receptors are highly homologous members of the class-I family of G protein-coupled receptors that bind CCK with high affinity. However, they have divergent structural specificities, with the type A receptor requiring seven carboxyl-terminal residues including a sulfated tyrosine and the type B receptor requiring only the carboxyl-terminal tetrapeptide. The aim of this work was to utilize affinity labeling to determine spatial approximations with photolabile *p*-benzoyl-L-phenylalanine (Bpa) residues sited at each end of CCK as docked at the type B CCK receptor, contrasting this with analogous work using similar probes docked at the type A receptor. Both probes were fully efficacious, potent agonists that stimulated intracellular calcium in receptor-bearing CHO-CCKBR cells (EC_{50} values: Bpa^{24} probe, 41 ± 9 pM; Bpa^{33} probe, 15 ± 3.3 pM). They bound specifically, with high affinity (K_i values: Bpa^{24} probe, 0.60 ± 0.17 nM; Bpa^{33} probe, 0.58 ± 0.11 nM). Cyanogen bromide cleavage of the covalently labeled receptor suggested the first extracellular loop as the region of labeling by each probe, distinct from the type A CCK receptor regions labeled using the same probes (third loop and amino-terminal tail, respectively). This was confirmed by subsequent enzymatic and chemical cleavage of labeled wild-type and mutant receptors. Sequential cycles of Edman degradation of labeled receptor fragments identified the specific residues within loop one labeled by each probe (Bpa^{24} probe labeled Phe^{122}; Bpa^{33} probe labeled Thr^{119}). This provides a direct demonstration of distinct modes of docking the same high-affinity ligand to highly homologous receptors.

Hormones and receptors typically interact in a high-affinity, specific manner. Evolution has utilized a limited number of general structural themes for receptor molecules to provide the lattices necessary to bind to the vast structural diversity in hormones, neurotransmitters, and other signaling molecules to provide a fine degree of regulation and integration of physiologic functions. The guanine nucleotide-binding protein (G protein)[1]-coupled receptor superfamily represents the largest group of receptors that also has the broadest variation in structures of their natural ligands. These receptors are characterized structurally by having seven hydrophobic transmembrane segments and characterized functionally by having agonist occupation result in stable association of heterotrimeric G protein with the cytosolic surface of the receptor.

The mechanism of binding is best understood for receptors in this superfamily that are activated by small ligands, such as those binding biogenic amines and rhodopsin (*1*−*3*). Much less is currently understood about the molecular mechanisms for those receptors binding larger and less conformationally constrained ligands, such as peptides.

Within this family, there are some very interesting opportunities to gain insight into the molecular basis for the structural specificity of peptide hormone−receptor interaction, by studying closely related receptors for a single hormone or for closely related hormones. Type A and B cholecystokinin (CCK) receptors represent an example of the latter. Previous analysis of the cDNAs for these receptors and for their natural hormonal ligands have provided strong evidence that these pairs of molecules have evolved from common precursors (*4*, *5*). This suggests that the structural specificity of ligand−receptor interaction at these receptors and therefore the molecular basis of their binding also evolved at the same time.

Further, extensive primary structure−activity analysis of the hormonal ligands has demonstrated that the critical sites of both hormones for binding to their receptors reside within a focused region in the carboxyl terminus of the peptide hormones (*6*, *7*). Of note, both CCK and gastrin, the natural agonist ligands of these receptors, share their carboxyl-terminal pentapeptide-amide sequence. This represents the entire pharmacophoric domain for the type B CCK receptor interaction, and indeed, both gastrin and CCK bind to this

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* To whom correspondence should be addressed: Cancer Center, Mayo Clinic Scottsdale, 13400 East Shea Blvd., Scottsdale, AZ 85259. Telephone: (480) 301-6650. Fax: (480) 301-4596. E-mail: miller@mayo.edu.

[1] Abbreviations: Bpa, *p*-benzoyl-L-phenylalanine; Bpa^{24} probe, des-amino-Tyr-Gly-Bpa-Gly-[($Nle^{28,31}$)CCK-26−33]; Bpa^{33} probe, des-amino-Tyr-Gly-[($Nle^{28,31}$,Bpa^{33})CCK-26−33]; CCK, cholecystokinin; CCK-8, CCK-26−33; CHO, Chinese hamster ovary; CHO-CCKBR, Chinese hamster ovary cell-line stably expressing the hemagglutinin-tagged human type B CCK receptor; CNBr, cyanogen bromide; EF, endoglycosidase F; G protein, guanine nucleotide-binding protein; Iodobead, *N*-chlorobenzenesulfonamide; KRH, Krebs-Ringer/HEPES; Fura-2 AM, Fura-2 acetoxymethyl ester.

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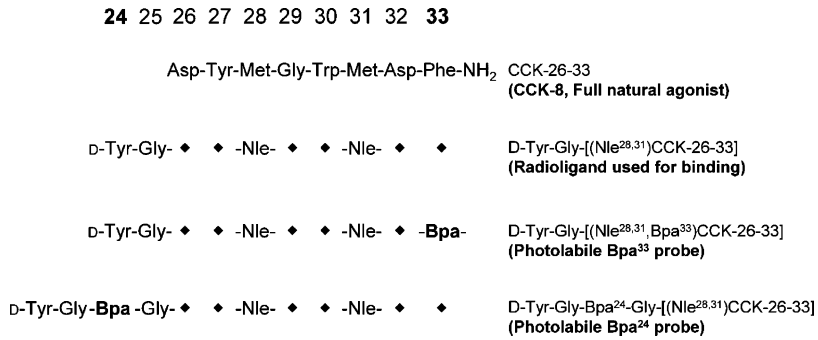

FIGURE 1: Primary structures of CCK analogues used in the present study.

receptor with similar affinities and possess similar high potencies for activation. While this domain will also bind and activate the type A CCK receptor, a sulfated tyrosine residue, seven residues from the carboxyl terminus of CCK, provides substantially improved binding affinity and biological activity at the type A CCK receptor.

To date, cDNAs encoding type B CCK receptors from seven species and type A CCK receptors from four species, as well as a type X CCK receptor, felt to represent a precursor for these receptors, have been cloned and reported (*6*−*10*). Sequence analysis demonstrates that there is 94% identity among the type B CCK receptors and 95% identity among the type A CCK receptors, with the two subtypes having 65% similarity and 49% identity. The sites of sequence identity are particularly concentrated within the regions predicted to represent the transmembrane helices.

The molecular basis of docking CCK to the type A CCK receptor has been extensively studied, including the direct application of a series of photolabile probes to affinity-label specific spatially approximated residues within the active site of the type A CCK receptor (*11*−*14*). These include probes with sites of covalent attachment at the amino terminus, carboxyl terminus, and central to the predicted pharmacophoric domain of the natural CCK ligand. In contrast, much less is currently understood about the docking of high-affinity peptide ligands (CCK or gastrin) to the type B CCK receptor. Only a single report has been published for the application of photoaffinity labeling to establish spatial approximation with a region of the type B CCK receptor (*15*), and that was only extended to the level of a 10-residue fragment of the amino terminus of the receptor, rather than to the identification of a specific residue−residue approximation that could be used as an experimentally derived constraint for molecular modeling.

In the current work, we have utilized probes with photolabile *p*-benzoyl-L-phenylalanine (Bpa) at the amino-terminal and carboxyl-terminal ends of CCK (in positions 24 and 33, respectively) to explore specific molecular approximations with distinct residues within the type B CCK receptor. Both were fully efficacious agonists that bound to this receptor saturably and specifically and that efficiently covalently labeled it in a single distinct domain. Using chemical and enzymatic cleavage and sequential cycles of Edman degradation of labeled wild-type and mutant receptors, the sites of labeling by each probe were identified within the first extracellular loop of the type B CCK receptor. These two pairs of residue−residue approximations should contribute substantially to our understanding of the molecular basis of natural ligand binding to the type A and B CCK receptors.

MATERIALS AND METHODS

Materials. Synthetic CCK-26−33 (CCK-8) was purchased from Peninsula Laboratories (Belmont, CA). Cyanogen bromide (CNBr), solid-phase oxidant, *N*-chlorobenzenesulfonamide (Iodo-beads), and *m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester were from Pierce Chemical Company. Endoproteinase Lys-C was from Roche Molecular Biochemicals, and wheat germ agglutinin-agarose was from EY Laboratories (San Mateo, CA). Fura-2 acetoxymethyl ester (Fura-2AM) was from Molecular Probes (Eugene, OR). Endoglycosidase F (EF) was prepared in our laboratory, as previously described (*16*). Other reagents were analytical-grade.

Peptides. The photoaffinity-labeling probes used in the current study on the type B CCK receptor were des-amino-Tyr-Gly-Bpa-Gly-[(Nle^{28,31})CCK-26−33] (Bpa^{24} probe) and des-amino-Tyr-Gly-[(Nle^{28,31},Bpa^{33})CCK-26−33] (Bpa^{33} probe) (Figure 1). The Bpa^{24} probe contained its photolabile site of covalent attachment at the amino-terminal extension of the CCK pharmacophore, whereas the Bpa^{33} probe incorporated this residue at the carboxyl-terminal end of the CCK ligand. They each contained a Tyr residue for radioiodination within amino-terminal extensions of the ligand. Each probe had its amino terminus blocked, permitting the use of sequential cycles of amino-terminal Edman degradation of the labeled receptor fragments, without concomitant probe cleavage. The Bpa^{24} probe has previously been fully characterized and used in affinity-labeling the type A CCK receptor (*14*). A probe with a photolabilie residue in position 33, des-amino-Tyr-Gly-[(Nle^{28,31},pNO_2-Phe^{33})CCK-26−33], was previously used in analogous studies for the type A CCK receptor (*11*). Both the Bpa^{24} and Bpa^{33} probes and the CCK-like agonist ligand used for binding, D-Tyr-Gly-[(Nle^{28,31})CCK-26−33], were synthesized by solid-phase techniques and purified to homogeneity by reverse-phase HPLC, as we have previously described (*17*, *18*). The identities of the products were established by mass spectrometry. Each of these peptides was radioiodinated using the solid-phase oxidant, Iodo-beads (Pierce), with $Na^{125}I$. These were purified to yield specific radioactivities of 2000 Ci/mmol after purification by reversed-phase HPLC, in a manner analogous to that described previously (*17*).

Receptor Preparations. The receptor-bearing Chinese hamster ovary (CHO) cell line that was previously prepared to express the hemagglutinin (HA)-tagged human type B CCK receptor (CHO-CCKBR) (*19*) was used as a source of wild-type receptor for this work. Cells were grown on tissue-culture plasticware in Ham's F-12 medium supplemented

with 5% fetal clone 2 (Hyclone Laboratories, Logan, UT) in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were passaged approximately twice a week and lifted mechanically before use.

In this work, it was necessary to develop a type B CCK receptor mutant construct in which the Met residue in position 134 within the predicted third transmembrane segment of the receptor was replaced by a Leu (M134L) to eliminate a site for CNBr cleavage. This receptor mutant was prepared using an oligonucleotide-directed approach with the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA), with the products verified by direct DNA sequencing (*20*). It was expressed transiently on COS cells (American Type Cell Collection, Manassas, VA) after transfection using a modification of the DEAE-dextran method (*21*). These cells were harvested mechanically 72 h after transfection.

Plasma membranes were prepared from receptor-expressing cells using methods reported previously (*22*). Membranes were suspended in Krebs-Ringers-HEPES (KRH) medium (25 mM HEPES at pH 7.4, 104 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 2 mM $CaCl_2$, and 1.2 mM $MgSO_4$) containing 1 mM phenylmethylsulfonyl fluoride and 0.01% soybean trypsin inhibitor. Aliquots of membranes were stored at −80 °C until ready for use in receptor-binding and photoaffinity-labeling experiments.

Receptor-Binding Assay. The Bpa^{24} and Bpa^{33} probes were characterized to determine their ability to bind to the type B CCK receptor. This was done in a standard competition-binding assay with CHO-CCKBR cell membranes using conditions that have been previously established for the type A CCK receptor (*14*, *22*). For this, approximately 5−10 μg of enriched plasma membranes was incubated in KRH medium containing 1 mM phenylmethylsulfonyl fluoride, 0.01% soybean trypsin inhibitor, and 0.2% bovine serum albumin, with a constant amount of radioligand, ^{125}I-D-Tyr-Gly-[($Nle^{28,31}$)CCK-26−33] (5 pM, about 20 000 cpm), in the presence of increasing concentrations (0−1 μM) of either the Bpa^{24} or Bpa^{33} probe or CCK, for 1 h at room temperature. Bound and free radioligand were separated using a Skatron cell harvester (Molecular Devices, Sunnyvale, CA) with receptor-binding filtermats, and bound radioactivity was quantified with a γ spectrometer. The same assay was also used to characterize the binding activity of the new receptor mutant transiently expressed in COS cells. Nonspecific binding was determined in the presence of 1 μM CCK and represented less than 6% of the total binding (with a B_0 value of approximately 3400 cpm in a typical assay). Data were graphed using Prism software (GraphPad software, San Diego, CA) and were analyzed using the Ligand program of Munson and Rodbard (*23*).

Intracellular Calcium Stimulation Assay. The agonist activities of the Bpa^{24} and Bpa^{33} probes were studied using a well-characterized assay for stimulation of intracellular calcium release in receptor-bearing CHO-CCKBR cells, as has been described for the type A CCK receptor (*24*). In brief, 2×10^6 CHO-CCKBR cells were loaded with 5 μM Fura-2AM in Ham's F-12 medium for 30 min at 37 °C. They were then washed with KRH medium and stimulated with increasing concentrations of the Bpa^{24} or Bpa^{33} probe at 37 °C. Fluorescence was quantified in a Perkin−Elmer Life Sciences LS50B luminescence spectrometer. Excitation was performed at 340 and 380 nm, and emission was determined at 520 nm, with the calcium concentration calculated from the ratios, as described by Grynkiewicz et al. (*25*). The peak intracellular calcium transient was utilized to determine the agonist concentration dependence of this biological response. Basal levels of calcium were measured in the absence of agonist stimulation, whereas maximum levels were determined as the peak intracellular calcium concentrations measured in the presence of a 1 μM CCK. Stimulated values were calculated as percentages of the range of calcium concentrations between basal and maximum levels. All assays were performed in duplicate and repeated at least 3 times in independent experiments. This assay was also used to characterize the biological activity of the new receptor mutant transiently expressed in COS cells.

Photoaffinity Labeling of the Type B CCK Receptor. This was performed by incubation of 0.1 nM radioiodinated Bpa^{24} or Bpa^{33} probe with 50 μg of CHO-CCKBR plasma membranes in the dark at 25 °C for 60 min in KRH medium in the absence and presence of increasing concentrations of competing CCK. Photolysis was performed in a Rayonet Photochemical Reactor (Southern New England Ultraviolet, Hamden, CT) equipped with 3500 Å lamps at 4 °C for 30 min. Membranes were then washed and solubilized with 1% Nonidet P-40 in KRH medium, prior to wheat germ agglutinin-agarose affinity chromatography and subsequent SDS−polyacrylamide gel electrophoresis (*26*). The affinity-labeled receptor was visualized by autoradiography.

Peptide Mapping. This required a larger scale preparation of affinity-labeled receptor. For this, a larger amount of receptor-bearing membranes (200 g) and radioiodinated Bpa^{24} or Bpa^{33} probe (0.5 nM) was incubated in the absence of competing CCK prior to photolysis and electrophoresis.

Radiolabeled receptor bands were excised from polyacrylamide gels, eluted, lyophilized, and precipitated using ethanol. The resultant radiochemically pure receptor was cleaved with 2.5 mg of CNBr in 70% formic acid, as previously described (*22*). The products of cleavage were washed in water and dried in a vacuum centrifuge prior to being resolved on 10% NuPAGE gels with MES running buffer (Invitrogen, Carlsbad, CA), with labeled fragments visualized by autoradiography. Subsequent endoproteinase Lys-C cleavage of the gel-purified, labeled CNBr fragment was performed as we previously reported (*27*). The products of digestion were also resolved on 10% NuPAGE gels. For selected experiments, gel-purified labeled receptor or its relevant fragment was deglycosylated with endoglycosidase F using conditions as we have described (*22*).

Determination of the Residue Labeled. The receptor residue labeled by each photolabile probe was determined after definitive identification of the labeled fragment by using amino-terminal Edman degradation. In brief, radiochemically pure labeled fragments from CNBr cleavage of the type B CCK receptor were exposed to sequential cycles of Edman degradation chemistry, quantifying radioactivity released in each cycle. For this, purified fragments were immobilized through the thiol group of Cys^{127} using the bifunctional cross-linker, *m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester, and *N*-(2-aminoethyl-1)-3-aminopropyl glass beads (Sigma). Modification of the amino groups on the beads with the bifunctional cross-linker was followed by quenching all amino-reactivity, leaving only sulfhydryl reactivity to ensure

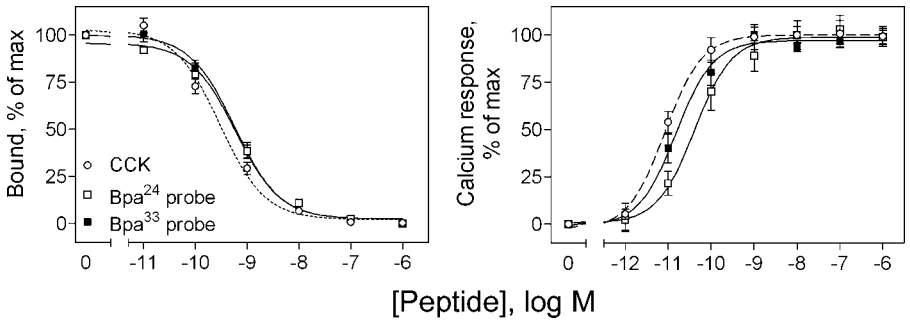

FIGURE 2: Characterization of the Bpa[24] and Bpa[33] probes. (Left) Abilities of increasing concentrations of the Bpa[24] and Bpa[33] probes to compete for binding of the radioligand, [125]I-D-Tyr-Gly-[(Nle[28,31])CCK-26−33], to plasma membranes from receptor-bearing CHO-CCKBR cells. Values are calculated as percentages of maximal saturable binding observed in the absence of the competitor. They are expressed as means ± SEM of duplicate data from three independent experiments. (Right) Both the Bpa[24] and Bpa[33] probes stimulated intracellular calcium responses in CHO-CCKBR cells in a concentration-dependent manner. The basal level of intracellular calcium concentration was 119 ± 12 nM, with the stimulated levels reaching a maximum of 419 ± 22 nM for CCK, 427 ± 46 nM for the Bpa[24] probe, and 414 ± 39 nM for the Bpa[33] probe. Values are expressed as the means ± SEM of at least three independent experiments, with data normalized relative to maximal responses.

derivatization of the only cysteine residue within the purified fragment. Cycles of Edman degradation were repeated up to 8 times, in a manner previously reported (*11*), and the radioactivity released in each cycle was quantified in a γ spectrometer.

RESULTS

Probe Characterization. The synthetic Bpa[24] probe was developed to incorporate a photolabile Bpa at the amino-terminal extension of the CCK pharmacophore, and it was previously characterized to bind to the type A CCK receptor with high affinity and to stimulate intracellular calcium release in type A CCK receptor-bearing CHO-CCKR cells with similar potency to natural CCK (*14*). The Bpa[33] probe was developed to replace pNO_2-Phe with Bpa at the carboxyl terminus, in position 33, of the analogous photolabile CCK agonist analogue, des-amino-Tyr-Gly-[(Nle[28,31],pNO_2-Phe[33])-CCK-26−33] that we used previously for the type A CCK receptor (*11*). This new probe was synthesized by manual solid-phase techniques and purified by reverse-phase HPLC. It was characterized to demonstrate the expected molecular mass by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

In this study, both the Bpa[24] and Bpa[33] probes were functionally characterized using type B CCK receptor-bearing CHO-CCKBR cells. As shown in Figure 2, both probes bound to plasma membranes from these cells saturably, specifically, and with affinity not different from that of natural CCK (K_i values: Bpa[24] probe, 0.60 ± 0.17 nM; Bpa[33] probe, 0.58 ± 0.11 nM; CCK, 0.30 ± 0.05 nM). They both were full agonists, stimulating increases in intracellular calcium concentrations in Fura-2AM-loaded CHO-CCKBR cells in a concentration-dependent manner (Figure 2), with efficacies similar to that of natural CCK (EC_{50} values: Bpa[24] probe, 41 ± 9 pM; Bpa[33] probe, 15 ± 3.3 pM; CCK, 8.4 ± 2.1 pM). The binding affinity and agonist activity of both probes at the type B-CCK receptor were not significantly different from those at the type A CCK receptor (*11*, *14*).

Photoaffinity Labeling of the Type B CCK Receptor. Both the Bpa[24] and Bpa[33] probes were also used to explore their ability to covalently label the type B CCK receptor. As shown in Figure 3, they both covalently labeled the receptor saturably and specifically, and the covalent labeling was inhibited in a concentration-dependent manner by CCK (IC_{50} values: Bpa[24] probe, 8.3 ± 2.0 nM; Bpa[33] probe, 1.7 ± 0.5 nM). Consistent with our previous labeling of the type A CCK receptor, the receptor migrated at approximately M_r = 85 000−95 000 and shifted to M_r = 42 000 after deglycosylation. No significant labeling was observed in samples prepared from nonreceptor-bearing parental CHO cell membranes. The high efficiency for covalent labeling of the receptor allowed us to proceed with further mapping of the regions of labeling and identification of the specific residues labeled by each probe.

Peptide Mapping of the Labeled Domains. We chose CNBr cleavage of the labeled receptor as the first indication of the regions of labeling by the Bpa[24] and Bpa[33] probes, because of its ability to cleave quantitatively. The type B CCK receptor contains 10 Met residues, and theoretically, CNBr cleavage of this receptor would yield 11 fragments, ranging in molecular masses from 0.1 to 11 kDa, with 1 fragment containing potential sites of N-linked glycosylation (Figure 4). As shown in Figure 4, CNBr cleavage of the type B CCK receptor labeled with each probe resulted in nonglycosylated fragments migrating consistently at approximately M_r = 4000. Given the molecular masses of the attached probes (Bpa[24] probe, 1744 Da; Bpa[33] probe, 1541 Da) and the nonglycosylated nature, the fragment Gly[118]−Met[134] within the first extracellular loop was the candidate that best matched these data. This was supported by subsequent endoproteinase Lys-C cleavage. As shown in Figure 5, the M_r = 4000 CNBr fragment labeled by each probe shifted to approximately M_r = 3500 after endoproteinase Lys-C cleavage. This further confirmed that the Gly[118]−Met[134] fragment represented the region of labeling by each probe because this was the only CNBr fragment within the extracellular loop domains that contains a Lys residue for endoproteinase Lys-C cleavage.

To further confirm this identification, a type B CCK receptor mutant construct was prepared in which Met[134] was changed to Leu (M134L) to eliminate a site of CNBr cleavage and it was transiently expressed in COS cells. This receptor had similar affinity to bind CCK (K_i = 0.5 ± 0.1 nM), and it responded to CCK to elicit intracellular calcium

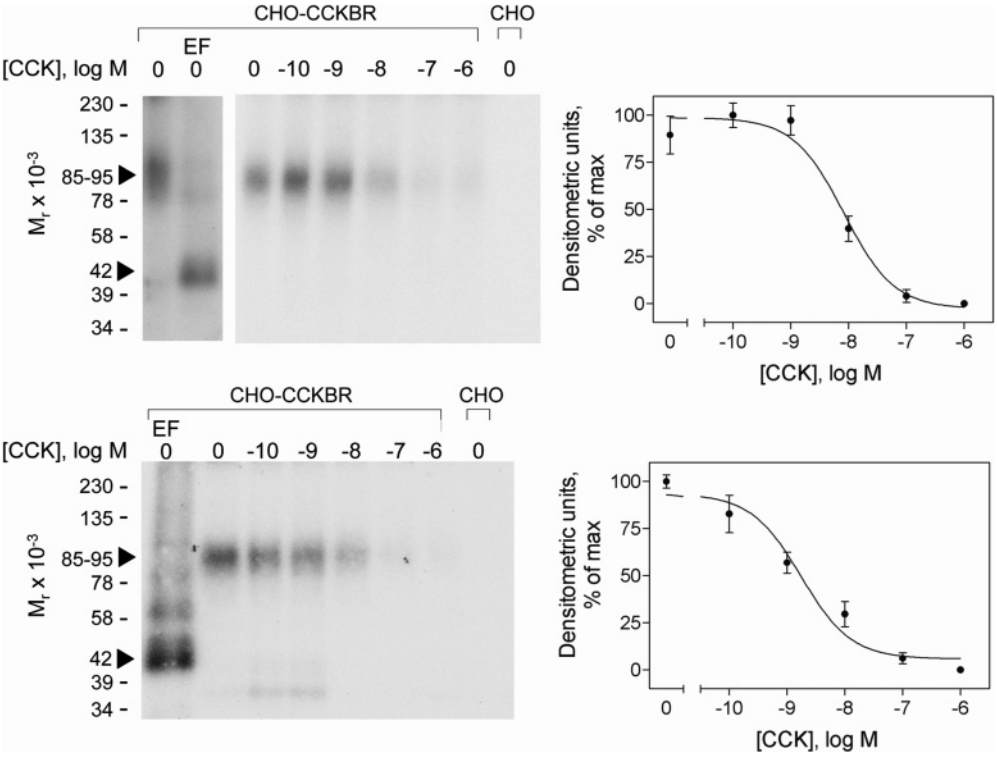

FIGURE 3: Photoaffinity labeling of the type B CCK receptor. Shown are typical autoradiographs of 10% SDS−polyacrylamide electrophoresis gels used to separate products of affinity labeling of CHO-CCKBR cell membranes by the Bpa^{24} (top) or Bpa^{33} (bottom) probes in the absence or presence of increasing concentrations of competing unlabeled CCK. The affinity-labeled type B CCK receptor migrated at approximately M_r = 85 000−95 000 and shifted to M_r = 42 000 after deglycosylation with EF. No radioactive bands were observed in affinity-labeled nonreceptor-bearing CHO cell membranes. Shown also are densitometric analyses of such receptor competition labeling performed in three similar experiments (means ± SEM).

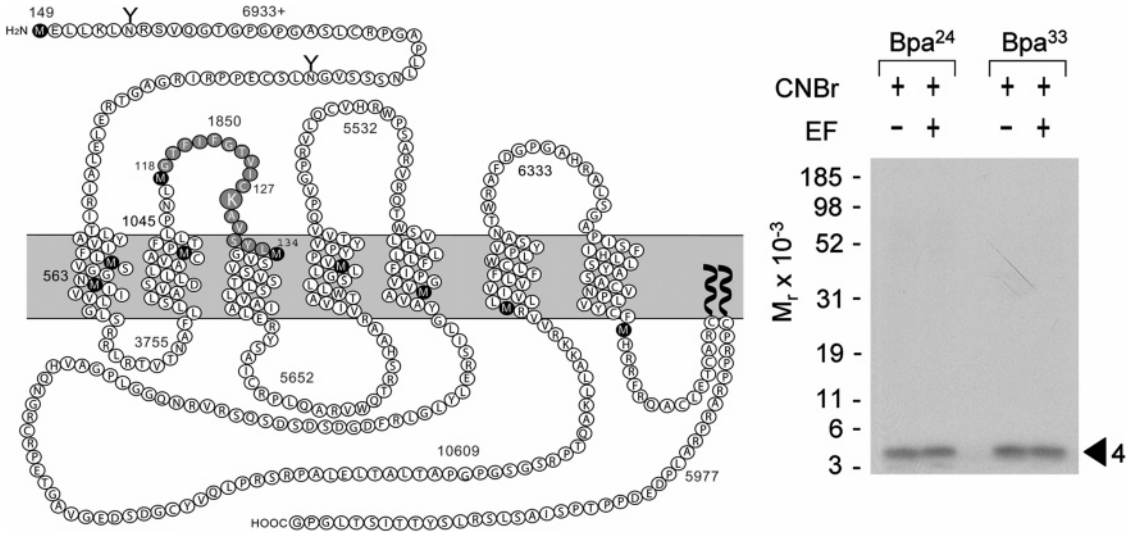

FIGURE 4: CNBr cleavage of the affinity-labeled type B CCK receptor. (Left) Diagram that shows theoretical sites of CNBr cleavage within the type B CCK receptor, with the masses of the predicted fragments. (Right) Representative autoradiograph of a 10% NuPAGE gel used to separate the products of CNBr cleavage of the native and deglycosylated type B CCK receptor that had been labeled with the indicated probe. Such cleavage yielded bands migrating consistently at approximate M_r = 4000 for both probes that were not affected by treatment with EF. The fragment Gly^{118}−Met^{134} within the first extracellular loop of the receptor that was highlighted in gray in the diagram at the left is the candidate fragment that is most consistent with being the receptor region of labeling for both probes.

responses with similar efficacy and potency (EC_{50} = 16 ± 4.6 pM) as the wild-type receptor (Figure 6). Furthermore, this construct was specifically and saturably labeled by the Bpa^{24} and Bpa^{33} probes, with similar efficiencies to the covalent labeling of the wild-type receptor (Figure 6). This construct labeled with each probe was then submitted to CNBr cleavage. As shown in Figure 6, CNBr cleavage of the M134L mutant labeled with each of the probes yielded a much bigger fragment (M_r = 8500) than the fragment resulting from CNBr cleavage of the labeled wild-type receptor (M_r = 4000). This further confirmed that Gly^{118}−Met^{134} contained the sites of labeling for both the Bpa^{24} and Bpa^{33} probes.

Identification of the Specific Receptor Residues Labeled. The sites of labeling of both the Bpa^{24} and Bpa^{33} probes were further identified by exposing the purified labeled CNBr

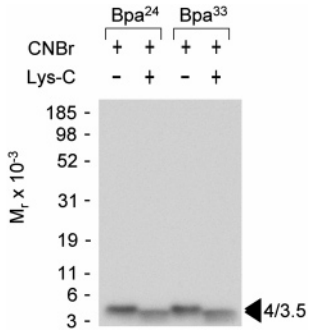

FIGURE 5: Endoproteinase Lys-C cleavage of CNBr fragments from labeled type B CCK receptor. Shown is a typical autoradiograph of a 10% NuPAGE gel used to separate the products of sequential endoproteinase Lys-C cleavage of the CNBr fragment from the type B CCK receptor labeled with the Bpa^{24} and Bpa^{33} probes. The cleavage patterns were identical for both probes, with the resulting fragments labeled with each probe migrating at approximately $M_r = 3500$, significantly distinct from the $M_r = 4000$ CNBr fragment. This supports the identification of the fragment Gly^{118}−Met^{134} within the first extracellular loop of the receptor as the region of labeling for both probes.

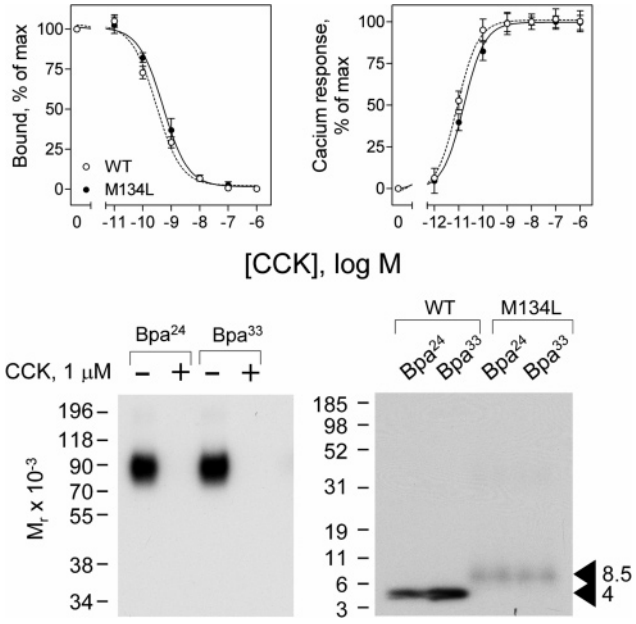

FIGURE 6: Characterization and CNBr cleavage of the M134L type B CCK receptor mutant. (Top) CCK radioligand binding experiments (left) and concentration dependency of CCK-stimulated intracellular calcium response (right) of the M134L construct and the wild-type (WT) receptor expressed in intact COS cells. The basal level of intracellular calcium concentration was 130 ± 24 nM, with the stimulated level reaching a maximum of 430 ± 35 nM. Data represent the means ± SEM of three independent experiments performed in duplicate. (Bottom) Saturable and specific photoaffinity labeling by each indicated probe in the absence and presence of competing CCK (left) and CNBr cleavage of the M134L mutant receptor labeled by each probe (right). The resultant cleavage fragments labeled by each probe migrated consistently at approximate $M_r = 8500$, distinct from the $M_r = 4000$ CNBr fragment resulting from cleavage of the labeled wild-type receptor. This further confirms that the fragment Gly^{118}−Met^{134} within the first extracellular loop was the region of labeling for both probes.

fragment (Gly^{118}−Met^{134}) from the type B CCK receptor labeled with each probe that had been immobilized through the thiol of Cys^{127} to sequential cycles of amino-terminal Edman degradation. Figure 7 shows that the peaks in eluted radioactivity appeared consistently in cycle 5 for the Bpa^{24} probe and in cycle 2 for the Bpa^{33} probe. These correspond with covalent attachment of the Bpa^{24} probe to Phe^{122} and the Bpa^{33} probe to Thr^{119} of the receptor. Both residues are located within the first extracellular loop region of the type B CCK receptor.

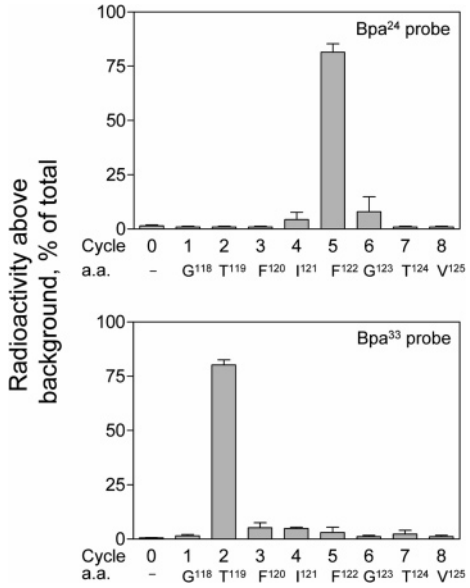

FIGURE 7: Identification of the affinity-labeled receptor residues. Shown are the elution profiles of the radioactivity released (as percentages of the total cpm recovered) in sequential cycles of amino-terminal Edman degradation of the purified CNBr fragment (Gly^{118}−Met^{134}) from cleavage of the type B CCK receptor labeled with each probe. In typical experiments, the total cpm recovered and background cpm were 638 and 44 respectively for the Bpa^{24} probe and 668 and 41 respectively for the Bpa^{33} probe. A radioactive peak consistently eluted in cycles 5 and 2 for the Bpa^{24} (top) and Bpa^{33} (bottom) probes, respectively. Data represent means ± SEM of three independent experiments. This established Phe^{122} and Thr^{119} of the type B CCK receptor as the sites of covalent attachment of the Bpa^{24} and Bpa^{33} probes, respectively.

DISCUSSION

Affinity labeling of receptor residues using a photolabile ligand probe provides information regarding the spatial approximation between a specific residue within a ligand as docked to its receptor and a specific region (or residue) of that receptor. Using analogues of natural hormonal ligands that retain full biological activity as probes in such studies, it is possible to gain insights into the molecular mode of hormone binding and receptor activation. In the current work, we have utilized analogues of CCK that incorporate their photolabile sites of covalent attachment at either end of the pharmacophoric domain. Probes with photolabile moieties in these positions have previously been utilized to define the mechanism of binding CCK to the type A CCK receptor (*11*, *13*, *14*). Here, we have utilized CCK probes with sites of covalent attachment in positions 24 and 33 to determine whether the mechanism of binding to the closely related type B CCK receptor is also similar. Of note, in contrast to labeling a residue within the third extracellular loop (Glu^{345}) in the type A CCK receptor (*14*), the amino-terminal position 24 probe labeled a residue within the first extracellular loop of the type B CCK receptor. Additionally, in contrast to labeling a residue within the amino-terminal tail adjacent to the first transmembrane segment (Trp^{39}) of the type A CCK

receptor (*11*), the carboxyl-terminal position 33 probe labeled another residue within the first extracellular loop of the type B CCK receptor. These divergent spatial approximations using probes with equally high affinities for type A and B CCK receptors are indicative of distinct mechanisms of ligand docking to these receptors.

These data are complementary to data that we recently reported in which the environment of a fluorophore at the amino-terminal end of CCK was demonstrated to be different when docked to the type A and B CCK receptors (*28*). At the type B receptor, the amino-terminal end of CCK was more exposed to the extracellular milieu and more easily quenched by hydrophilic reagents than when docked at the type A CCK receptor. Consistent with these observations, the site of covalent labeling of the type B CCK receptor with the amino-terminal Bpa^{24} probe is likely more distant from the membrane than the third loop region labeled within the type A CCK receptor (*14*). Of interest, the position 24 probe with the dominant site of labeling the type A CCK receptor within the third loop was also shown to have a minor site of labeling the receptor amino terminus between Asn^{10} and Lys^{37} that is felt to reside above the peptide ligand-binding cleft (*14*).

The distinct mechanisms of receptor binding at the type A and B CCK receptors are also consistent with the unsuccessful previous attempts to use chimeric receptor constructs with portions of the type A and B CCK receptors to define the regions of these receptors that are responsible for their distinct specificities of binding (*29*, *30*). Such an approach is only useful when the ligand binds to an analogous position in the two receptors, contributing their sequences to the chimeric construct. This does not seem to be true for the subtypes of CCK receptors.

While it is clear that the modes of CCK binding to the type A and B CCK receptors are distinct, there are not yet adequate experimentally derived constraints to effectively model the ligand−receptor complex for the type B CCK receptor. Current insights are considerably better developed for the type A CCK receptor. For the type A receptor, there are not only extensive established structure−activity relationships for peptide ligands but also receptor mutagenesis, site-specific photoaffinity labeling of the receptor, fluorescence resonance energy transfer, and fluorescent probe environment studies (*24*, *31*−*34*). These have all contributed to a working molecular model that accommodates all of these types of data (*32*).

The only previous report identifying a region of photoaffinity labeling of the type B CCK receptor utilized a probe incorporating a *p*-benzoylbenzoyl-Orn(proprionyl) moiety at the amino terminus of CCK-8 (*15*). In that work, the probe (site of covalent attachment approximating position 25 of CCK, extending from the peptide with a flexible spacer) was shown to label the Leu^{52}−Tyr^{61} segment at the junction of the amino terminus and the first transmembrane segment of the human type B CCK receptor. The structural features of the loop and tail regions of G protein-coupled receptors, particularly of the CCK receptor, are not nearly as well-resolved as the structure of the helical bundle region. The combination of inadequate understanding of the structure of the segment labeled in the previous study and the previous inability to refine the site of labeling within that segment make these data difficult to utilize for meaningful model building. Nevertheless, it is quite conceivable that the labeled segment within the amino-terminal tail domain could be spatially adjacent to receptor residue Phe^{122} within the first extracellular loop of this receptor that was covalently labeled by the position 24 probe utilized in the current study. When this specific, experimentally derived residue−residue approximation constraint is provided, this region of the docked ligand−receptor complex can be better refined.

With the addition of the second residue−residue approximation constraint derived from the opposite end of the hormone pharmacophore in this study, the docking of CCK can be clearly established. It is of great interest that the receptor residues that were covalently labeled by the two distinct probes having their sites of attachment at each end of the peptide pharmacophoric domain were linearly so close to each other, at residues Thr^{119} and Phe^{122}. This is most consistent with a turn or loop in the peptide, bringing its two ends toward each other, as it is docked to the type B CCK receptor. This is particularly interesting, because conformationally constrained cyclic peptide analogues of CCK have been previously described that are highly selective for the type B CCK receptor, binding to the type A CCK receptor with low affinity and lacking biological activity there (*35*, *36*). Again, the contrast with the type A CCK receptor is striking, because the photoaffinity labeling of the type A receptor through the analogous positions results in covalent labeling of receptor residues that are far apart in the receptor structure, the third loop, and the amino-terminal tail (*11*, *13*, *14*).

While the current data for the photoaffinity labeling of the type B CCK receptor provide evidence for the peptide-binding domain to include receptor loop and amino-terminal tail regions residing at the extracellular surface of the plasma membrane, the structures of the labeled regions are not yet adequately well-resolved to permit the proposal of a meaningful three-dimensional model for this agonist-bound receptor. Such a model will be proposed with the future addition of complementary experimentally derived constraints.

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