Direct Identification of a Distinct Site of Interaction between the Carboxyl-terminal Residue of Cholecystokinin and the Type A Cholecystokinin Receptor Using Photoaffinity Labeling

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Mechanisms of ligand binding and activation of G protein-coupled receptors are particularly important, due to their ubiquitous expression and potential as drug targets. Molecular interactions between ligands and these receptors are best defined for small molecule ligands that bind within the transmembrane helices. Extracellular domains seem to be more important for peptide ligands, based largely on effects of receptor mutagenesis, where interference with binding or activity can reflect allosteric as well as direct effects. We now take the more direct approach of photoaffinity labeling the active site of the cholecystokinin (CCK) receptor, using a photolabile analogue of CCK having a blocked amino terminus. This probe, 125I-desaminotyrosyl-Gly-Nle38,39-pNO2-Phe39(CCK-26-33), binds specifically, saturably, and with high affinity ($K_i = 3.3 \text{ nm}$) and has full agonist activity. This makes likely its being sited in a natural position within the receptor. As substrate, we used CHO-CCK receptor cells overexpressing functional recombinant rat type A CCK receptor. Covalent labeling of the appropriate $M_r = 85,000–95,000$ plasma membrane glycoprotein with core $M_r = 42,000$ was established by SDS-polyacrylamide gel electrophoresis and autoradiography. A single domain adjacent to transmembrane 1 was labeled, as established by cyanogen bromide cleavage and separation by gel and/or high pressure liquid chromatography. The site of interaction was further defined by additional proteolysis with trypsin, with purification of the labeled fragment, followed by manual Edman degradation and radiochemical sequencing. This demonstrated that Trp39 specifically labeled and likely resides proximate to the carboxyl-terminal $p\text{NO}_2$-$\text{Phe}^{39}$ residue of the probe. A model of this ligand-bound receptor has been constructed and will be used to plan future experiments to refine our understanding of this interaction.

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Guanine nucleotide-binding protein (G protein)$^1$-coupled receptors are the largest group of plasma membrane receptors, representing a superfamily with a remarkable diversity of activating ligands. Our best understanding of the molecular basis for ligand binding to members of this superfamily is the binding of the chromophore to rhodopsin and the binding of biogenic amines to adrenergic receptors. These insights come from complementary studies of receptor mutagenesis, photoaffinity labeling, and reciprocal chemical modification of ligand and receptor (1–6). All available data focus the relevant interactions to sites at the core of the coalescence of transmembrane helices, in the outer third of the bilayer. Even with this extensive information, the constrained nature of the ligands, and the relatively confined space for ligand docking, the debate continues regarding the specific siting of the agonist ligands in some of these receptor systems (7, 8).

Understanding the interactions between peptide ligands and their G protein-coupled receptors represents an even greater challenge. By first principles, these ligands are quite flexible and can achieve many conformations. Whereas some peptides appear to have some preferred conformation in solution (9), there is little information regarding how such structures relate to the receptor-bound states of these ligands. Most of our insights into binding domains for peptide ligands have come from receptor mutagenesis studies, which have focused attention on receptor domains predicted to be outside the membrane (7, 8). Given the extended size of the pharmacophoric domains and the solubilities of the peptide ligands, these regions of interaction seem plausible. We know, however, that receptor mutagenesis can modify receptor function nonspecifically, interfering with biosynthetic processing or trafficking or having an allosteric effect, rather than necessarily directly interfering with a site of ligand-receptor interaction. For a very limited number of peptide receptors in this family, direct sites of contact have been recently described using photoaffinity labeling approaches (10–13).

Cholecystokinin (CCK) is a peptide hormone and neurotransmitter that has a wide spectrum of physiologic actions (14). These relate largely to control of nutrient assimilation, through regulation of gallbladder contraction, pancreatic exocrine secretion, gastric emptying and gut motility, and satiety. The type A CCK receptor is expressed on the gallbladder muscularis smooth muscle cell, the pancreatic acinar cell, smooth muscle and neurons at different positions along the gastrointestinal tract, and on select central nervous system nuclei. There is a large amount of primary structure-activity information defining the pharmacophoric domain of the CCK peptide to its carboxyl-terminal heptapeptide (15). This includes critical

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§ The abbreviations used are: G protein, guanine nucleotide-binding protein; CCK, cholecystokinin; CCKR, cholecystokinin receptor; CNBr, cyanogen bromide; PITC, phenylisothiocyanate; CHO, Chinese hamster ovary; MES, 4-morpholineethanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; endo F, endo-$\beta$-N-acetylglucosaminidase F; HPLC, high pressure liquid chromatography; Nle, norleucine.
Identification of the Cholecystokinin Receptor Active Site

contributions by an unusual tyrosine sulfate and the carboxyl-terminal phenylalanine-amide, and important contributions by almost every residue within this domain. The structurally related type B CCK (gastrin) receptor has less extensive structural requirements for its ligands, with the carboxyl-terminal tetrapeptide shared by CCK and gastrin adequate for high affinity binding and full agonist activity at that receptor (16). Gastrin is a very weak agonist at the type A CCK receptor, where it has a binding affinity greater than 3 orders of magnitude lower than CCK (14).

Our current insights into the binding domain of the type A CCK receptor have come exclusively from mutagenesis of that receptor (17–19). While a large series of photolabile probes for this receptor have been developed and applied to identify the receptor molecule (20–24), they have not yet been applied to define distinct sites of interaction up to the present time. Like the peptide receptors described above (7, 8), predicted extracellular loop regions of the CCK receptor seem to be important. In this report, we have utilized photoaffinity labeling with a CCK analogue probe that incorporates a photolabile residue intrinsic to its pharmacophoric domain to identify a distinct site of interaction with the receptor. This was achieved by the application of chemical and enzymatic cleavage of the affinity labeled receptor, with the subsequent manual Edman degradation and radiochemical sequencing of the labeled fragment. Of particular interest, a domain within this receptor, which was previously shown to be important by receptor truncation and mutagenesis studies, does contain the residue we now identify as interacting with the carboxyl terminus of CCK. However, the previous work suggested that this was a site of interaction with the amino-terminal domain of CCK, based on the indirect correlation of effects on different CCK analogues (19). The model previously proposed placed the carboxyl terminus of CCK deep within the bilayer, among the transmembrane helices (19), something not possible with the current finding. We now have one clear site of contact between distinct residues of agonist ligand and receptor. We present a new model that incorporates this new contact data and is consistent with a large body of agonist structure-activity relationship data for CCK A receptors.

EXPERIMENTAL PROCEDURES

Materials—Synthetic CCK-8 was purchased from Peninsula Laboratories (Belmont, CA). Previously characterized CCK analogues, 9-Tyr-Gly-[Nle28,31]CCK-(26–33) and 9-Tyr-Gly-[Nle28,31,pNO2-Phe33]CCK-(26–33), were synthesized in our laboratory as we previously described (22). Desaminotyrosyl-Gly-[Nle28,31,pNO2-Phe33]CCK-(26–33) (CCK analogue).

This is quite analogous to the 9-Tyr-Gly-[Nle28,31,pNO2-Phe33]CCK-(26–33) probe previously reported (22), and characterized to represent a high affinity, full agonist at the CCK receptor, except for having its amino terminus blocked. This modification was critical to permit the radiochemical sequencing of the labeled receptor fragment, without the cleavage of the probe itself. The probe was synthesized and purified in our laboratory by solid and solution phase techniques, as we have previously reported (22, 26). The chemical identity of this product was confirmed by amino acid analysis and mass spectrometry. The CCK analogue was radioiodinated using the solid phase oxidant, Nchlorobenzensulfonamide (IODO-BEADS, Pierce), and purification of the probe by reverse-phase high performance liquid chromatography to 2000 Ci/mmol, as we have described (26).

Probe Binding and Biological Activity—The ability of the CCK analogue probe to bind to the type A CCK receptor was established in a standard binding assay (25). This utilized a CCK-like radioligand (125I-9-Tyr-Gly-[Nle28,31]CCK-(26–33)) which has previously been extensively validated (27), and rat type A CCK receptor-bearing membranes from the CHO-CCKR cell line (25). Assay tubes included approximately 10 pM radioligand, approximately 2–5 μg of membrane protein, and 0.5 ml of Krebs-Ringer/Hepes medium containing 25 mM Hepes, pH 7.4, 1 mM KH2PO4, 104 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1.2 mM MgSO4, 0.2% bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride, and 0.01% soybean trypsin inhibitor. This was carried out in the absence or presence of variable concentrations of desaminotyrosyl-Gly-[Nle28,31,33-pNO2-Phe33]CCK-(26–33). Incubations were performed for 60 min at 25 °C, conditions adequate to achieve steady-state binding. Rapid separation of bound from free radioligand was accomplished with a Skatron cell harvester (Sterling, VA) using receptor-binding filter mats. Bound radioactivity was quantitated with a gamma spectrometer, and data were analyzed using the competition-binding program in the Prism software suite (GraphPad Software, San Diego, CA).

The agonist activity of the CCK analogue probe was studied using an assay for stimulation of intracellular calcium activity in the CHO-CCKR cell line (25). In this assay, approximately 2 million cells were loaded with 5 μM Fura-2AM (Molecular Probes, Eugene, OR) in Krebs-Ringer/Hepes medium for 30 min at 37 °C. They were then washed and stimulated with variable concentrations of desaminotyrosyl-Gly-[Nle28,31,33-pNO2-Phe33]CCK-(26–33) at 37 °C, with fluorescence quantified in a Perkin-Elmer LS50B luminescence spectrometer (Norwalk, CT). Excitation was performed at 340 and 380 nm, and emissions were determined at 520 nm, with calcium concentration calculated from the ratios, as described by Grynkiewicz et al. (28). The peak intracellular calcium transient was utilized to determine the concentration dependence of the biological response.

Photoaffinity Labeling of the CCK Receptor—For the covalent labeling of the binding domain of the CCK receptor, receptor-bearing membranes from the CHO-CCKR cells were incubated with the 125I-desaminotyrosyl-Gly-[Nle28,31,33-pNO2-Phe33]CCK-(26–33) under the same conditions described above for the binding assay, except using larger amounts of membrane (50–100 μg) and radioligand (0.1–1 mM). Affinity labeled, lectin-purified CCK receptors were photolabeled using a photochemical reactor (Southern New England Ultravoilet Co., Hamden, CT) equipped with 3000-Å lamps for 30 min at 4 °C. After photolysis, membranes were washed and solubilized using 1% digitonin at 4 °C overnight.

The radiolabeled and solubilized CCK receptors were enriched by adsorption to wheat germ agglutinin-agarose beads for 4 h at 4 °C, followed by washing and electrophoretic elution in 2% sodium dodecyl sulfate (SDS) sample buffer and electrophoresis through a 10% SDS-polyacrylamide gel according to the conditions described by Laemmli (29). Aliquots of affinity-adsorbed receptor were deglycosylated by treatment for 2 h at 37 °C with 2 units of endo-β-N-acetylgalcosaminidase F (endo F) in buffer containing 0.1 mM sodium phosphate, pH 6.1, 50 mM EDTA, 1% Nonidet P-40, 0.1% SDS, and 1% N-mercaptoethanol. This was also then separated on a 10% SDS-polyacrylamide gel. After identification of the radiolaabeled CCK receptor band by autoradiography, it was excised and eluted by suspension in water and Dounce homogenization. This material was then lyophilized, and the radiola- beled receptor was precipitated in 85% ethanol at −20 °C overnight.

Active Site Identification—Affinity labeled, lectin-purified CCK receptor was loaded into native or CNBr-digested state, in Ham's F-12 medium supplemented with 5% Fetal Clone-2 (HyClone Laboratories, Logan, UT) and was passed twice per week. For study, cells were lifted mechanically and were triturated and washed. Enriched plasma membranes from these cells were prepared as described (25).

Affinity Labeling Probe—The major new probe used in this work was desaminotyrosyl-Gly-[Nle28,31,pNO2-Phe33]CCK-(26–33) (CCK analogue).
Affinity labeled CCK receptor or its purified CNBr fragments were also cleaved with trypsin. For this, up to 8 mg/ml trypsin was utilized in 100 mM Tris buffer, pH 8.8, treating for 24 h at 25 °C. Products of digestion were then separated on a Tris-Tricine gel (30) or on a 10% NuPAGE gel, as described above. Products were again visualized by autoradiography.

When it was clear that the expected product was present and in good yield, it was then purified by reversed phase-high performance liquid chromatography. This was performed on a Beckman system equipped with a Vydac (214TP5415) analytical C-4 column, utilizing a 1 ml/min flow rate and a gradient of increasing acetonitrile with a background of 0.1% trifluoroacetic acid. The gradient consisted of the following: 1% solution B for 5 min, advancing from 1 to 25% solution B over 5 min, advancing from 25 to 60% solution B over 25 min, advancing from 60 to 99% solution B over 5 min, and retaining the 99% solution B flow for another 5 min. A<sub>254 nm</sub> was monitored on line and 1-ml fractions had radioactivity quantified with a gamma spectrometer. Select peak fractions were further characterized by separation on an appropriate gel by electrophoresis, with visualization by autoradiography.

Purified fragments were sequenced using manual Edman degradation chemistry. For this, the purified fragment was dissolved in 100 mM MES, pH 5.0, containing 10 mg/ml 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and 20% acetonitrile. Coupling to 20 mg of N-(2-aminoethyl)-1-3-amino propyl glass beads was allowed to proceed for 2 h at 25 °C. The beads were then washed sequentially with the following: 200 µl of methanol, 200 µl of methanol:water (1:1, v:v) two times, 200 µl of methanol:water (1:1, v:v) two times, and 200 µl of methanol two times. This was followed by treatment with 50 µl of trifluoroacetic acid followed by 200-µl methanol washes, repeating this three times. The solvents were then eliminated under vacuum, and the beads were manually cycled through steps of alkalization, covalent modification with PITC, washing, cleavage with trifluoroacetic acid, and elution. The cycles included the following: (i) beads were suspended in 50 µl of triethylamine and brought to dryness under vacuum, repeating three times; (ii) 60 µl of triethylamine:methanol:PTIC (1:7:1, v:v) were added and incubated for 5 min at 50 °C; (iii) three sequential 400 µl of ethyl acetate washes were performed; (iv) beads were brought to dryness under vacuum in a Speed Vac centrifuge, and 50 µl of trifluoroacetic acid were added and incubated for 5 min at 25 °C; and (v) elution was performed with three successive washes using 200 µl of methanol. Radioactivity in each pooled eluate was quantified by gamma spectrometer. A cycle (number 0) was performed excluding the PITC step to ensure elution of all non-covalently bound radiolabeled fragments, before initiating cycle 1.

Molecular Modeling—Three-dimensional models for rat CCK-A receptor were constructed using methods described previously for adrenergic receptors (31, 32). Briefly, seven-helix bundles were constructed, using our previous models for α-1 and β-adrenergic receptors as templates. Both clockwise and counterclockwise helical bundle clusters were built. Extracellular and cytosolic loop segments were then attached in an extended conformation to a target helix in the bundle, and weak harmonic constraints were applied to ligate the free end of each loop segment with the second target helix during a short, low temperature molecular dynamics simulation. The peptide was docked manually into the extracellular loop region of the receptor models using interactive molecular graphics methods. The peptide was initially docked in a standard β-hairpin conformation in the receptor models. After manual docking, limited energy minimization and low temperature molecular dynamics calculations were used to relieve any residual bad steric contacts and to optimize the peptide-receptor interactions.

All energy minimization and molecular dynamics calculations were performed using AMBER 4.1 (33), with a united atom potential function (34). Interactive graphics model building was performed using the FSSHOW package (35).

RESULTS

Probe Characterization—Desaminotyrosyl-Gly-[Nle<sup>28,31</sup>, pNO<sub>2</sub>-Phe<sup>33</sup>]-CCK(26–33) was synthesized and purified and corresponded to the expected structure as demonstrated by amino acid analysis and mass spectrometry. Also, as expected, it bound to the type A CCK receptor saturably and with high affinity (K<sub>i</sub> = 3.3 ± 0.3 nM) (Fig. 1). Nonspecific binding, determined in the presence of 1 µM CCK, represented less than 15% total binding.

This analogue of CCK was a full agonist, stimulating an increase in the intracellular calcium concentration in a concentration-dependent manner (Fig. 2). This response was not different from that stimulated by natural CCK in the same cells (25).

Photoaffinity Labeling of the CCK Receptor—The photolabile CCK analogue covalently labeled the CCK receptor, as demonstrated by autoradiography of SDS-polyacrylamide gels used to separate the products after binding in the presence of increasing amounts of competing unlabeled CCK (Fig. 3). The electro-
phoretic migration of the specifically labeled Mr 85,000–95,000 band was identical to that previously observed with a series of photolabile analogues of this hormone (22, 23). The electrophoretic migration of the deglycosylated band was also as expected from previous studies (36) (Mr = 42,000).

**Active Site Identification**—Cyanogen bromide cleavage of the affinity labeled CCK receptor yielded a single labeled fragment migrating at apparent Mr = 25,000 (Fig. 4A). The same cleavage of the deglycosylated receptor yielded a single band with apparent Mr = 8,500 (Fig. 4A). There is only one possible cyanogen bromide fragment of the CCK receptor that could account for this electrophoretic migration (Fig. 4B). Only the fragment extending from residue 10 to 72 (using a numbering scheme common to the type A CCK receptors cloned from other species, recognizing that this represents a domain between residues 25 and 87 from the predicted start site for the rat type A CCK receptor that was originally reported (37)), which includes the amino-terminal tail adjacent to transmembrane 1 and the first transmembrane segment, has the appropriate mass, as well as predicted sites of N-linked glycosylation. We know from previous work (36) that at least three sites of complex, N-linked glycosylation, and possibly four, are used during the biosynthesis of this receptor. The only other cyanogen bromide fragment that could be glycosylated is the fragment extending from residue 174 to 195, which is much too small to represent the observed affinity labeled fragment. This provides strong evidence that the current CCK analogue probe is covalently attached to the CCK receptor somewhere between residues 10 and 72.

**FIG. 3.** Photoaffinity labeling of the CCK receptor on CHO-CCKR cells using 125I-desaminotyrosyl-Gly-[Nle28,31,pNO2-Phe33]CCK-(26–33). Shown is a typical autoradiograph of an SDS-polyacrylamide gel used to separate products of the labeling of receptor-bearing membranes in the presence of increasing amounts of competing non-radiolabeled CCK. The CCK receptor migrated at Mr = 85,000–95,000, as previously observed. After deglycosylation with endo F, this band migrated in the expected position of Mr = 42,000. Shown also is quantitation of the labeled CCK receptor in three similar experiments (means ± S.E. shown, but errors are within the data points).

**FIG. 4.** Cyanogen bromide cleavage of the photoaffinity labeled CCK receptor. Shown is a typical autoradiograph of a 10% NuPAGE gel used to separate the products of CNBr digestion of the native and deglycosylated CCK receptor that had been labeled with 125I-desaminotyrosyl-Gly-[Nle28,31,pNO2-Phe33]CCK-(26–33), which is representative of three experiments. Also shown are the theoretical sites of cleavage of the rat type A CCK receptor (including all residues originally reported, note that this includes the first 15 residues in parentheses that do not enter the current numbering scheme, since they are not present in the CCK receptor of other species) and the masses of the expected fragments, independent of carbohydrate (+ marks fragments with consensus sequences for N-linked glycosylation). The fragment extending from residues 10 to 72 is an excellent match for the apparent migrations of the native and deglycosylated fragments observed on the gel, given the mass of the peptide component, the presence of sites of glycosylation, and the mass of the covalently bound receptor probe (1481 Da).
FIG. 5. Trypsin cleavage of the photoaffinity labeled CCK receptor. Shown is a typical autoradiograph of a Tris-Tricine gel used to separate the products of trypsin digestion of the native and deglycosylated CCK receptor that had been labeled with $^{125}$I-desaminotyrosyl-Gly-[Nle$_{28,31}$,pNO$_2$-Phe$_{33}$]CCK-(26–33). The different amounts of trypsin used are noted. With amounts of protease as high as 8 mg/ml, the naturally glycosylated receptor was incompletely cleaved. After endo F deglycosylation, the site of labeling was apparent on a fragment migrating at approximate $M_r = 5,000$.

The fragment of the CCK receptor extending from residue 10 to 72 contains four basic residues, as potential sites of tryptic cleavage (Fig. 4B). Three of these sites are very close to the carboxyl terminus of this fragment and, therefore, not very useful. The fourth basic residue is Lys$^{105}$ near the mid-region of this fragment. It is positioned quite effectively to separate the potential sites of glycosylation within the amino-terminal half of this fragment from the last five residues predicted to be proximate to and just outside the bilayer and the transmembrane domain itself. The glycosylation state of the CCK receptor interfered with the ability of the trypsin to quantitatively cleave the intact receptor, but after enzymatic deglycosylation trypsin yielded a single radiolabeled band migrating at apparent $M_r = 5,000$ (Fig. 5). This is consistent with the sum of the expected masses of the region of the receptor between residues 38 and 68 (3530 Da) and the covalently attached probe (1481 Da).

Further evidence for this identification came from the tryptic cleavage of the cyanogen bromide fragments of the affinity labeled native and deglycosylated CCK receptor (Fig. 6). Trypsin was more effective in cleaving this receptor fragment than the intact native receptor. It is noteworthy that the sequential cleavages of the cyanogen bromide fragment of the CCK receptor to homogeneity was achieved after elution of the band on the NuPAGE gel and further purification on high performance liquid chromatography (Fig. 7). This resulted in a single radioactive species with $A_{280}$ below detectability. Rerunning this material on another NuPAGE gel resulted in identical migration ($M_r \approx 5,000$).

The profile from the Edman degradation sequencing of this fragment of the CCK receptor is shown in Fig. 8. This was typical of four independent purifications and sequencing efforts. In each, the radioactive peak corresponded with the second cycle after initiating the PITC modification of the amino-terminal residue. This corresponds with covalent attachment to Trp$^{39}$ of the CCK receptor.

**Molecular Modeling**—The covalent attachment of the peptide carboxyl terminus to Trp$^{39}$ was used as a constraint in the initial manual docking exercises. Previous studies have shown that the peptide amino terminus has no impact or role in receptor binding (21, 38), so initial docking orientations were chosen such that there were no contacts between the amino terminus of the ligand probe and any receptor residues. Previous studies have also shown that Tyr-sulfate$^{27}$ in CCK peptides is important for high affinity binding and activity for the type A CCK receptor (39, 40) but not for the type B CCK receptor (40). Multiple sequence alignments for a series of mammalian type A and B CCK receptors reveal one intriguing difference between these two receptors in the first extracellular loop. The type A CCK receptors all possess an LKD sequence (Leu$^{104}$, Lys$^{105}$, Asp$^{106}$), whereas type B CCK receptors have an MGT sequence at the analogous positions. The flanking regions of this tripeptide segment are highly homologous in both type A and B CCK receptors, and our receptor models suggest that this tripeptide segment is exposed near the top of the interhelical loop. When the peptide ligand is docked in a $\beta$-hairpin conformation, with the carboxyl-terminal Phe$^{33}$ of the ligand near Trp$^{39}$ of the receptor, and the amino terminus of the ligand oriented away from all receptor residues, the Tyr-sulfate$^{27}$ of the ligand is well positioned to form a good interaction with Lys$^{105}$ in the first extracellular loop of the receptor. With these initial docking contacts to help position the peptide in the receptor, the complex was relaxed with limited energy minimization and low temperature molecular dynamics simulation. No explicit constraint functions were used to maintain the initial docking contacts during relaxation. Nonetheless, the pNO$_2$-Phe$^{33}$ at the carboxyl-terminal position of the ligand probe still exhibits good interactions with Trp$^{39}$ of the receptor, including favorable ring stacking contacts. The Tyr-sulfate$^{27}$ in the probe forms a strong charge-reinforced hydrogen bond with receptor Lys$^{105}$ and also forms transient hydrogen bonds with receptor Ser$^{111}$ during dynamics relaxation. The aromatic ring
of Tyr-sulfate \(^{27}\) forms a good stacking interaction with Phe\(^{198}\) in the second extracellular loop of the receptor. The Nle residue at the position of Met\(^{293}\) within CCK-33 forms favorable van der Waals contacts with hydrophobic residues in the second extracellular loop of the receptor, including Leu\(^{200}\) and Pro\(^{201}\). Gly\(^{29}\) in the probe is positioned close to the third extracellular loop of the receptor, near the beginning of transmembrane 7, but does not form specific contacts due to its small size. Trp\(^{39}\) and Nle\(^{31}\) in the probe fit snugly in a hydrophobic pocket formed by residues from the ends of extracellular loops 2 and 3 of the receptor. Trp\(^{39}\) in the probe makes good van der Waals interactions with Leu\(^{200}\) and Ile\(^{202}\). Asp\(^{28}\) and β-carbons from Lys\(^{315}\), and the β-carbon in Ser\(^{308}\). Nle\(^{31}\) in the probe forms good van der Waals interactions with Pro\(^{352}\), Ile\(^{353}\), and Ile\(^{356}\). Asp\(^{38}\) in the probe forms a salt bridge with receptor Lys\(^{315}\) in the relaxed complex. The three amino-terminal residues of the probe (the Tyr and Gly residues attached to the amino terminus of the CCK-26–33 analogue, and Asp\(^{38}\)) are oriented away from the receptor and form no contacts with any receptor residues. The final, minimized complex is shown in Fig. 9.

**DISCUSSION**

Understanding the molecular determinants of agonist binding provides important insights that may be useful in structure-based drug design and in understanding the basic mechanisms of receptor activation. The cholecystokinin (CCK) receptor is a potentially important drug target, having multiple physiologic actions at many target organs. The type A CCK receptor is a G protein-coupled receptor in the rhodopsin-β-adrenergic receptor family, with structural homology to several receptors that bind small peptides. The theme that seems to be emerging for such receptors is the key contribution of receptor residues predicted to reside outside the membrane within the amino-terminal fragment and loop domains. This comes from both receptor mutagenesis studies and from direct photoaffinity labeling of receptor domains and residues. (1–6).

To date, the only insights into the binding determinants of the CCK receptor come from receptor mutagenesis studies (17–19). Although it is possible for such a mutation to interfere directly with a site of contact between ligand and receptor, it is also well recognized that it can interfere with receptor function indirectly via allosteric mechanisms. This makes such observations difficult to interpret on a molecular level. The most definitive mutagenesis studies have involved the reciprocal changes in receptor and ligand that lead to gain of function, rather than loss of a function after a given mutation is constructed. Unfortunately, no such manipulations have been successfully achieved for the CCK receptor. Also, up to the present time there have been no clear and direct insights into the binding domain of this receptor using the site labeling approach. Often the two approaches can be very complementary and quite useful.

An interesting series of studies (19, 41) has focused attention on the importance of a domain predicted to be within the amino-terminal tail adjacent to the first transmembrane domain, which includes residues Trp\(^{39}\) and Gln\(^{40}\). These residues were in a segment of five residues (38–42) which was identified as key, since elimination of the amino-terminal 37 residues had no effect on CCK binding or biological activity, whereas elimination of the amino-terminal 42 residues eliminated demonstrable binding and activity of this natural agonist ligand (41). An analogous domain has been shown to be important for binding of peptide agonists at the angiotensin 1 receptor (42), the endothelin B receptor (43), the neurokinin 1 receptor (44), and the neurtensin receptor (45). The Trp\(^{39}\) and Gln\(^{40}\) residues in the type A CCK receptor were identified as important in a follow-up site-directed mutagenesis study (19).
Although the specific identification of Trp39 as being a key contact point between the carboxyl-terminal residue of CCK and its receptor in our current report is consistent with the previous mutagenesis data, the interpretation of the data must now be quite different. The previous report built a working model of CCK occupation of its receptor with the amino terminus of CCK interacting with this residue and the carboxyl terminus of the peptide in the middle of the transmembrane region of the receptor (19). This is similar to a model previously proposed for the type B CCK receptor (46). Clearly the exclusive covalent attachment of a photolabile residue in the position of the carboxyl-terminal residue of the full agonist ligand for the type A CCK receptor to Trp39 of that receptor makes that siting of the ligand quite unlikely. Instead, we believe that

![Diagram of peptide-type A CCK receptor complex.](image)

**A**, side view of the peptide-receptor complex. Receptor Trp39 is clearly visible at the right, with the peptide carboxyl-terminal pNO₂-Phe33 forming a direct contact. The three amino-terminal residues of the peptide point away from the receptor and make no specific contacts. Peptide Trp30 and Nle31 sit in a hydrophobic pocket formed by receptor residues Leu348, Pro352, Ile353, and Ile356. Peptide Asp32 forms an ionic interaction with receptor Lys115.

**B**, top view, with the three peptide amino-terminal residues removed to improve the view of the peptide pharmacophore-receptor interactions. The peptide Tyr-sulfate37 forms an ionic interaction with receptor Lys105 from extracellular loop 1 and a ring stacking interaction with Phe386 in loop 2. These images were made using MOLSCRIPT (50).
the binding data with the series of CCK analogues previously reported (19) was largely overinterpreted. By evaluating relative affinities for binding to the wild type and mutant receptor constructs, the authors failed to focus attention on absolute affinities. The receptor requires the information provided by multiple critical molecular contacts for high affinity binding. Among these is the critical contribution of the carboxyl-terminal phenylalanine-amide. In the JMV-180 analogue and other CCK analogues studied the contribution of that residue that was necessary to achieve the high affinity binding and potency of action was missing, leading to the ligand binding through a smaller number of determinants and possibly in a slightly different site. The fact that mutation of Trp54 did not lead to further deterioration of binding affinity of ligands which lack the phenylalanine-amide in that position cannot prove that this receptor residue does not play a critical role in “normal” full agonist binding. Indeed, its interaction with the photolabile carboxyl-terminal residue of our full agonist probe supports just this interpretation.

The model we have generated in this study differs significantly from some of the earlier models in several ways but is completely consistent with available data for the peptide-CCK A receptor complex. Our present model suggests that the peptide carboxyl-terminal phenylalanine forms specific contacts with Trp54, and previous models suggested this residue was buried deep in the receptor seven-helix bundle. The photoaffinity labeling data reported here is clearly inconsistent with these earlier models. The three amino-terminal residues of the ligand probe (Tyr, Gly, and Asp) form no interactions with the receptor, but our model predicts that the remaining residues form good interactions with the type A CCK receptor and probably contribute to favorable binding. In particular, our model suggests that Tyr-sulfate forms a strong charged-reinforced hydrogen bond with Lys105 in the CCK receptor. This interaction is particularly intriguing and may help explain an interesting difference between the type A and B CCK receptors. This tyrosine sulfate in CCK is important for high affinity binding to type A receptors but not type B receptors. The proposed lysine contact at position 105 in the receptor is a glycine residue in the type B CCK receptor, so our model predicts that Tyr-sulfate should not contribute favorably to peptide binding in type B CCK receptors, consistent with experimental observations.

The model also suggests several other interesting peptide-receptor contacts that can be probed by mutagenesis or photoaffinity labeling experiments. In particular, our model suggests that Asp26 of the peptide forms a salt bridge with Lys8. The model also implies that rather large, hydrophobic residues near the carboxyl terminus of the peptide (Trp30 and Nle31 respectively) may be important for high affinity binding to the type A CCK receptor. Gly29 in the peptide may be an interesting site for modification, as our model indicates that this position will tolerate larger residues that could form favorable interactions with the third extracellular loop. Experimental testing of these hypothesized interactions will yield data that can be used as additional constraints to improve and refine the current model. For example, both clockwise and counterclockwise helical bundle receptor models look equally reasonable, given the limited data available at present for specific peptide-receptor contacts and peptide conformation in the complex. Additional experimental constraints may permit us to clearly identify the correct model topology in the future.

More extensive mutagenesis has been performed and reported for the type B CCK receptor than for the type A CCK receptor (18, 47, 48). These two receptors are quite closely related, with 50% structural identity and 66% similarity. In the type B receptor mutagenesis of all transmembrane residues that are different in the type B and type A CCK receptors only interfered with the binding of non-peptidyl antagonists and had no effect on the binding of peptide agonists (18, 47). This would suggest that the non-peptidyl antagonist has binding determinants that are distinct from those that are critical for peptide ligands. That conclusion has recently been supported for the angiotensin-2 receptor as well (49). There are no data, however, providing insight into whether natural peptide agonists of either receptor have similar or different binding determinants. Presumably both receptors and both ligands have evolved from single evolutionary predecessors. It will be of great interest to finally gain insights into the molecular basis of binding and the relationship of the binding site in two such “neighbors” on the evolutionary tree.
Identification of the Cholecystokinin Receptor Active Site

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