Refinement of the Structure of the Ligand-occupied Cholecystokinin Receptor Using a Photolabile Amino-terminal Probe*

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Affinity labeling is a powerful tool to establish spatial approximations between photolabile residues within a ligand and its receptor. Here, we have utilized a cholecystokinin (CCK) analogue with a photolabile benzoylphenylalanine (Bpa) sited in position 24, adjacent to the pharmacophoric domain of this hormone (positions 27–33). This probe was a fully efficacious agonist that bound to the CCK receptor saturably and with high affinity (Ki = 8.9 ± 1.1 nM). It covalently labeled the CCK receptor either within the amino terminus (between Asn10 and Lys37) or within the third extracellular loop (Glu345), as demonstrated by proteolytic peptide mapping, deglycosylation, micropurification, and Edman degradation sequencing. Truncation of the receptor to eliminate residues 1–30 had no detrimental effect on CCK binding, stimulated signaling, or affinity labeling through a residue within the pharmacophore (Bpa24) but resulted in elimination of the covalent attachment of the Bpa24 probe to the receptor. Thus, the distal amino terminus of the CCK receptor resides above the docked ligand, compressing the portion of the peptide extending beyond its pharmacophore toward the receptor core. Exposure of wild type and truncated receptor constructs to extracellular trypsin damaged the truncated construct but not the wild type receptor, suggesting that this domain also may play a protective role. Use of these additional insights into molecular approximations provided key constraints for molecular modeling of the peptide-receptor complex, supporting the counterclockwise organization of the transmembrane helical domains.

Guanine nucleotide-binding protein (G protein)1-coupled receptors represent a remarkable group of structurally homologous membrane proteins that can bind and be activated by widely diverse ligands. The molecular details of how ligands as structurally dissimilar as photons, biogenic amines, peptides, and glycoproteins can elicit similar conformational changes in the cytosolic face of their receptors (where G protein-coupling occurs) are far from clear. Our best understanding of this process relates to the smallest ligands that appear to bind within the confluence of helices within the lipid bilayer (1), where we have analogous low resolution crystal structures on which to rely (2, 3). As the ligands get larger and more structurally complex, the binding domains tend to move toward the extracellular face of the membrane, with extracellular loop and amino-terminal tail domains becoming more important (1, 4). These are receptor domains for which we have minimal meaningful structural data.

We have been quite interested in the molecular basis of ligand binding to the type A cholecystokinin (CCK) receptor (5–10). This receptor is a member of the class I family of G protein-coupled receptors, along with rhodopsin and the β-adrenergic receptor (11). CCK occurs as a series of linear peptides, having lengths ranging from 8 to 58 residues (12). These all share their carboxyl-terminal domain, with the carboxyl-terminal heptapeptide-amide representing the minimal region that has full potency and efficacy for stimulating targets of this hormone. Two molecular approximations have been experimentally determined for residues within this region of CCK and residues within the ligand-binding domain of this receptor (8–10). Extension or structural modification of the amino terminus of CCK-8 has been well tolerated, without interfering with receptor binding or activation (13, 14). One provocative report has recently suggested that the naturally occurring amino-terminal extension that is present in CCK-58 can affect the conformation of the biologically active carboxyl-terminal octapeptide and can have a positive effect on the action of this hormone (15).

With these observations in mind, we initiated the current group of studies. We were particularly interested in the structural details of how an amino-terminal extension from the CCK pharmacophore might be positioned relative to the CCK receptor. This work has given us new insights into the structure and function of the amino-terminal tail of this receptor. Unlike affinity labeling through photolabile residues that are positioned within the pharmacophore of CCK that have yielded only focused sites of covalent attachment to the receptor (8, 9), the present work with a photolabile benzophenone residue positioned outside of this domain has resulted in demonstration of the ability to label either of two sites in distinct regions of the CCK receptor. This suggests that this position within the receptor-bound ligand may retain substantial mobility and not be held tightly in a single position relative to the receptor.

One of the sites of covalent labeling was in a position in the amino-terminal tail of the CCK receptor that can be eliminated without having any negative impact on receptor binding or
signaling. The other site of labeling was within the third extracellular loop domain. However, by truncation of the region of the receptor amino terminus in which the first contact was present, this photolabile residue no longer came in contact with or labeled the CCK receptor. These observations suggest that the amino-terminal domain of the receptor might provide a protective cover for the peptide-binding domain within the receptor. Indeed, when comparing the sensitivity of wild type and truncated receptor constructs to extracellular protease treatment, we found the latter to be much more amenable to damage by trypic protease.

The insights coming from the molecular approximations with a photolabile residue sited in position 24 of a CCK-like ligand, when combined with our previous ligand binding and cross-linking data (8–10), also provided us with the opportunity to propose a distinct topological model for CCK binding to its receptor, having a counterclockwise helix bundle topology. This refined molecular model of the ligand-receptor complex is quite distinct from models recently proposed in the literature based on less direct receptor mutagenesis studies (16–18). The current model is fully consistent with all existing experimental data and will continue to spawn experimentally testable predictions as it is further refined.

**EXPERIMENTAL PROCEDURES**

**Materials—**Synthetic CCK-8 was purchased from Peninsula Laboratories (Belmont, CA). Fura-2AM was from Molecular Probes (Eugene, OR); wheat germ agglutinin-agarose was from EY Laboratories (San Mateo, CA); cytochrome b (CNBr) and phenylisothiocyanate were from Pierce; endoproteinase Lys-C and staphylococcal V8 protease (SAP) were from Roche Molecular Biochemicals; t-1-tosylamido-2-phe

**Preparation of CCK Receptor Probes—**t-Tyr-Gly-[Nle28,31]CCK-(26–33), previously characterized to represent a CCK-like agonist ligand, was prepared as described (20). Des-amino-t-Tyr-Gly-Bpa-Gly-[Nle28,31]CCK-(26–33) (Bpaα analogue) and t-Tyr-Gly-[Nle28,31]Bpaα [CCK-(26–33)] (Bpaβ analogue) are photolabile and radiodinated CCK receptor probes that have been previously described (21). The Bpaα analogue probe has not previously been fully characterized. It was synthesized by solid-phase techniques and purified to homogeneity by reversed-phase HPLC (Fig. 1), as we have previously reported for other CCK analogues (8). The identity of the product was established by mass spectrometry. Each of the ligands was radiodinated using the solid-phase oxidant, IODO-BEADS (Pierce), with Na19F and was purified by thin-layer chromatography and subsequent SDS-polyacrylamide gel electrophoresis using the conditions described by Laemmli (27). After resolution on SDS-polyacrylamide gel electrophoresis, the affinity labeled receptor was visualized by autoradiography, eluted, and lyophilized.

**Identification of the Domains of CCK Receptor Labeling—**Affinity labeled, lectin-purified receptor and relevant receptor fragments were desalted and radioactivity of 3000 Ci/mmol using endoglycosidase F conditions we described previously (19).

The initial level of identification of the domains of labeling was performed using CNBr cleavage. This highly efficient cleavage method theoretically results in 17 receptor fragments having a broad range of expected masses (see Fig. 4). As we described previously (8), this was applied to the affinity labeled, gel-purified native and deglycosylated CCK receptor in 70% formic acid. The resultant fragments were then resolved on 10% NuPAGE gels (Novex, San Diego, CA) with MES running buffer and were visualized by autoradiography.

Each of the gel-purified, labeled CNBr-cleaved fragments was further digested with endoproteinase Lys-C and SAP. Lys-C (200 μg/ml) digestion was performed at 37 °C for 24 h in 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 0.1% SDS. SAP (500 μg/ml) digestion was performed in 0.1 M phosphate buffer, pH 7.8, containing 0.1% SDS at 25 °C for 4 h. The products of digestion were also resolved on 10% NuPAGE gels.

**Identification of the Sites of CCK Receptor Labeling—**The gel-purified receptor fragments were desalted using a Dispo-Biodialyzer (The Nest Group Inc., Southborough, MA) at 25 °C for 48 h prior to HPLC purification. For this, the samples were injected onto a PerkinElmer Lichrosorb RP-18, 5 μm C18 column with a flow rate of 5 μl/min. The system was run isocratically at 50% solution B for 50 min, followed by a linear gradient up to 90% solution B over 320 min (solution A, 0.1% trifluoroacetic acid, and solution B, 0.085% trifluoroacetic acid in acetonitrile). A λ295 nm was monitored with UV absorbance detector. Eluate from the microbore column was collected directly onto a polyvinylidene difluoride membrane, and autoradiography was
used to detect the position of the radioactive product. This position on
the filter was excised and subjected to Edman degradation sequencing
using an Applied Biosystems automated instrument.

The purified products of CNBr digestion of the CCK receptor that
were labeled with the Bpa analogue were also manually sequenced
using Edman degradation chemistry, quantifying radioactivity released
in each cycle. For this, purified fragments were coupled to N-(2-amino-
ethyl)-3-aminopropyl glass beads through the sulphydryl side chain
of a Cys residue. This was accomplished by derivatizing the amino
groups on the beads with m-maleimidobenzoyl-N-hydroxysuccinimide
ester at pH 7.0, quenching remaining amino reactivity with Tris, and
then adding a purified, labeled receptor fragment. Cycles of Edman
degradation were repeated manually in a manner that has been de-
scribed previously in detail (8).

Characterization of Amino-terminally Truncated CCK Receptor Con-
struct—Existing literature (28) has suggested that truncation of the
first 37 residues of the amino-terminal tail of the CCK receptor does not
affect CCK binding or biological action at the CCK receptor. We per-
formed binding and biological activity studies with the cell line express-
ing our truncated receptor construct (eliminating residues 1–30), using
the methodology described above. This construct was also used as a
target of affinity labeling using the Bpa and Bpa probes described
above.

Protease Resistance of CCK Receptor Constructs—Truncated and wild
type CCK receptor constructs were studied to determine their abilities to resist attack by proteases from outside the cell. Trypsin was
used since there are seven tryptic cleavage sites within this receptor
that are theoretically exposed to the extracellular milieu. Intact recep-
tor-bearing cells were lifted from their culture dishes using protease-
free cell dissociation medium, washed in KRH medium, and suspended
in this medium in the presence or absence of 0.05% trypsin. This was
allowed to react for 20 min at 37 °C. At that time, the cells were washed
with KRH medium and standard CCK radioligand-binding assays were
performed (as described above).

Molecular Modeling—Three-dimensional models were generated for
the type A CCK receptor using the low resolution rhodopsin structure
(3) as a template. Both clockwise and counterclockwise seven-helix
bundles were generated, and extracellular and cytosolic loops were
added as described previously (29). The ligand, N-Tyr-Gly-
[Nle28,31]Bpa[29]CCK(26–33), was docked manually in the receptor
models, using available photoaffinity labeling and other ligand binding
data as constraints to facilitate exact placement and orientation. The
solution phase conformation reported for CCK(26–33) (30) was ini-
tially adopted for the ligand, although this conformation was permitted
to relax in subsequent energy minimization calculations. All manual
d-three-dimensional model building was performed with the interactive
molecular graphics program, PSSHOW (31). Docked ligand-receptor
complexes were refined with limited energy minimization and low tem-
perature molecular dynamics calculations using the AMBER 5.0 suite
of programs (32).

RESULTS

Characterization of the Amino-terminal CCK Receptor
Probe—The synthetic Bpa analogue of CCK was purified to
homogeneity (Fig. 1) and was analyzed by mass spectrometry
to ensure its identity. This was oxidatively radiiodinated and purified by reversed-phase HPLC to yield a specific radioactivity
of 2000 Ci/mmol. The probe bound to the CCK receptor—purified by reversed-phase HPLC to yield a specific radioactiv-
ty of 2000 Ci/mmol. The probe bound to the CCK receptor
and labeling was absent in nonreceptor bearing membranes from CHO-CCKR cells saturably, specifi-
cally in a concentration-dependent manner by competition with unla-
abeled CCK, and labeling was absent in nonreceptor bearing
CHO cell membranes. As previously reported for the CCK
receptor, deglycosylation with endoglycosidase F shifted the
migration of the labeled band to \( M_r = 42,000 \), the position of migration of the receptor core protein. Fig. 3 also shows the
densitometric quantitation of the receptor labeling in the pres-
ence of increasing concentrations of CCK, supporting the ex-
pected saturability and high affinity interaction between li-
gand and receptor.

Identification of the Domains of Receptor Labeling—CNBr
cleavage of the affinity labeled CCK receptor was used as a first
indication of the receptor domains being covalently labeled by
the Bpa analogue of CCK. Fig. 4 shows a typical autoradiogram
of a NuPAGE gel used to separate the products of CNBr
cleavage of the affinity labeled native and deglycosylated CCK
receptor. Two distinct mixtures of molecular weight markers
were used, due to substantial differences in the migration of the
lower mass markers (migration positions noted). This cleavage of the intact receptor reproducibly generated labeled
fragments migrating in the range of \( M_r = 4,000–7,000 \) (the
smaller fragment) and \( M_r = 25,000 \) (the larger fragment). In
addition, as seen in this figure, larger labeled bands represent-
ing products of partial digestion were also sometimes observed.
The smaller fragment was always predominant, representing
75 ± 2% of the labeling.

The cleavage of the deglycosylated CCK receptor always
produced only two labeled bands, migrating at \( M_r = 4,000–7,000 \)
and \( M_r = 8,500 \) (Fig. 4). The former was shown to come from
the band migrating in the same position from the digestion
of the native receptor, thus representing a nonglycosylated
fragment. The latter was shown to come from deglycosylation
of the glycosylated larger products of CNBr digestion of the
intact receptor.

The specific covalent labeling of multiple bands was different
from our previous experience with affinity labeling this recepto-
rt through photolabile residues sited within the pharmacophoric
domain of the ligand in which only a single site of labeling was
observed (8, 9). Fig. 4 also shows the structure of the rat
CCK receptor with theoretical sites of cleavage by CNBr and
the characteristics of the expected fragments. The fragment
extending from residue 10 to 72 best matches the \( M_r = 25,000 \)
fragment, as supported by its shift in migration after deglyco-
sylation and the sum of the masses of that fragment and the
covalently attached probe (1,506 Da). Fragments 7, 9, 11, and
14 were all potential candidates for representing the smaller
labeled fragment, based on topology, apparent size, and ab-
sence of glycosylation.

The identity of the \( M_r = 25,000 \) fragment was further de-
fined by enzymatic cleavage of the purified fragment. The receptor fragment extending from residues 10 to 72 contains sites of N-linked glycosylation and two lysine residues that can be cleaved by endoproteinase Lys-C (Fig. 5). One Lys is very close to the carboxyl terminus of this fragment, and the other is positioned effectively to separate a nonglycosylated fragment.

**FIG. 2.** Binding and biological activity of the photoreactive Bpa analog of CCK. The probe, desamino-Tyr-Gly-Bpa-Gly-[Nle<sup>28,31</sup>]-CCK-(26–33), displaced CCK radioligand binding to CCK receptor-bearing membranes from CHO-CCKR cells (left) and stimulated an increase in intracellular calcium in Fura2AM-loaded CHO-CCKR cells (right) in a concentration-dependent manner. Data are expressed as means ± S.E. of values from three independent experiments. Inset is a typical intracellular calcium transient response to 100 nM peptide over time in these cells.

**FIG. 3.** Photoaffinity labeling of the CCK receptor with desamino-Tyr-Gly-Bpa-Gly-[Nle<sup>28,31</sup>]-CCK-(26–33). Shown on the left is a typical autoradiograph of an SDS-polyacrylamide gel used to separate products of the labeling of receptor-bearing membranes in the absence and presence of increasing amounts of competing unlabeled CCK. The labeled band migrated at the expected position of Mr = 85,000–95,000 that shifted to Mr = 42,000 after deglycosylation with endoglycosidase F. Absence of significant labeling of nonreceptor-bearing CHO cell membranes is also shown. Shown on the right is the densitometric analysis of the receptor labeling in the presence of increasing concentrations of competing CCK in four independent experiments (means ± S.E.).

**FIG. 4.** CNBr cleavage of the photoaffinity labeled CCK receptor. The left panel represents a diagram of the rat CCK receptor structure that includes theoretical sites of cleavage by CNBr, with the masses noted for the generated fragments. The first 15 residues of the amino terminus of the rat receptor are shown in parentheses, since this region is not present in the type A CCK receptor cloned from human or other species. The numbering scheme used, therefore, reflects the more highly conserved domains. A typical autoradiograph of a NuPAGE gel used to separate the products of CNBr cleavage of affinity labeled native and deglycosylated CCK receptor is shown on the right. CNBr digestion of affinity labeled CCK receptor molecules generated two distinct fragments, migrating at apparent positions of Mr = 4,000–7,000 and Mr = 25,000, the former being predominant. Fragment 4 best matches the Mr = 25,000 fragment, as evidenced by its migration at apparent Mr = 8,500 after deglycosylation. Fragments 7, 9, 11, and 14 are potential candidates for the Mr = 4,000–7,000 fragment, based on apparent size and absence of glycosylation. Two mixtures of molecular weight markers, Multimark<sup>®</sup> standards (Invitrogen, Carlsbad, CA) and Kaleidoscope<sup>®</sup> polypeptide standards (Bio-Rad), were used to indicate better the apparent mass of the smaller fragment.
with a mass of 3,771 Da from a glycosylated fragment with core protein of 3,045 Da. The gel-purified $M_r = 25,000$ product of CNBr cleavage was further digested with endoprotease Lys-C and deglycosylated with endoglycosidase F. Fig. 5 shows a typical autoradiograph of a NuPAGE gel used to separate the products of these treatments. Lys-C cleavage of this fragment yielded a band migrating at approximate $M_r = 21,500$ that shifted to approximate $M_r = 5,000$ after deglycosylation. Considering the mass of the covalently attached probe (1,506 Da), the amino-terminal part of the amino-terminal receptor fragment, extending from residues 10 to 37, best fit the data. This region of the CCK receptor is known not to be critical for CCK binding, based on the ability to truncate the first 37 residues of the amino-terminal tail of the receptor, while maintaining normal function (28). For this reason, no further specific localization of this site of labeling was pursued.

Identification of the smaller labeled fragment ($M_r = 4,000–7,000$) was more of a challenge, since migration on a gel may not precisely reflect mass (particularly for small peptides, as demonstrated by the differential migration of the molecular weight standards in the two mixtures). Focus on the absolute mass of a single standard marker in a previous report (21) resulted in consideration of too few candidate fragments. Of the potential candidates for the current labeling (fragments 7, 9, 11, and 14), fragments 7, 9, and 14 contain Lys, Asp, or Glu residues that can be cleaved by the specific proteases, Lys-C or SAP. Therefore, these enzymatic reactions were first utilized to gain preliminary insight into the identity of this fragment. Fig. 6 shows a diagram of these CNBr fragments, with theoretical cleavage sites by these proteases. As hoped, both Lys-C and SAP treatment resulted in shifts in the migration of the labeled band on a gel (Fig. 6). Only fragments 7 and 14 contain residues that can be cleaved by both of these enzymes, suggesting that they were the most suitable candidates to represent this smaller labeled fragment. This fragment was definitively identified as CNBr fragment 14 by direct Edman degradation sequencing, in which the first 11 cycles matched the expected sequence (Pro-Ile-Phe-Ser-Ala-Asn-Ala-Trp-Arg-Ala-Tyr).

The site of covalent attachment of this probe to this fragment was further characterized by radiochemical sequencing of the labeled products of CNBr cleavage of wild type and V342M receptor constructs. Although no radioactivity above background was observed through 15 cycles for the labeled CNBr fragment of the wild type receptor, a peak was consistently observed in cycle 3 for this fragment of the V342M construct in four independent experiments (Fig. 7). This corresponds with covalent labeling of Glu$^{345}$ in the third extracellular loop of the receptor.

Characterization and Affinity Labeling of the Amino-terminally Truncated CCK Receptor Construct—Fig. 8 shows that the amino-terminally truncated CCK receptor construct recognizes and responds to CCK in a similar manner to the wild type receptor. This confirms the high affinity binding of CCK previously reported for another truncated CCK receptor construct in which residues 1–37 were eliminated (28). It extends the previous observations by adding insight into normal signaling by such a construct.

Fig. 9 shows the attempt to affinity label the truncated CCK receptor construct with the Bpa$_{24}$ analogue of CCK. Of note, despite high affinity binding, this probe did not covalently label this receptor construct. Even prolonged exposure of the gel for autoradiography failed to demonstrate a labeled receptor band. This means that both sites of covalent attachment were impacted by the absence of the receptor amino-terminal domain. As a control, another analogue of CCK that incorporated the
same photolabile residue within the pharmacophoric domain was also used in an analogous series of affinity labeling experiments. This probe, the Bpa29 analogue of CCK (9), efficiently labeled this receptor construct.

Protease Resistance of CCK Receptor Constructs—Exposure to trypsin of cells expressing similar densities of wild type and truncated CCK receptor constructs that had similar CCK-binding capacities resulted in substantial differences. Despite having multiple sites in the extracellular domain for digestion with this protease, only the truncated construct was negatively affected by this treatment, with its binding markedly inhibited (Fig. 10). The glycosylated amino terminus was able to provide a protective cover for the peptide-binding domain of the fully intact wild type CCK receptor.

Molecular Modeling—Based on results from our earlier photoaffinity labeling studies (8–10), we initially positioned the peptide ligand such that a photolabile residue (Bpa) at position 29 would be well situated to form covalent cross-links with receptor residues His347 and Leu348 in the third extracellular loop, whereas a photolabile residue at position 33 would form cross-links with Trp39 in the amino terminus of the receptor. Since structure-activity relationship data for CCK peptides indicate that residues positioned as an extension from the amino terminus of the pharmacophoric domain (residues Tyr24 and Gly25) have no impact on receptor binding (5, 20, 33), we oriented the peptide ligand so that its amino terminus made no specific contacts with the receptor but was instead exposed to solvent. Given that the amino terminus and extracellular loops of the receptor likely possess some conformational flexibility, it is quite plausible that a photoaffinity label substituted in the position of Tyr24 (shown in Fig. 11) would alkylate the amino terminus and possibly the third extracellular loop of the receptor.

The experimental results reported here are completely consistent with our models for the CCK peptide-receptor complex. The specific photoaffinity labeling patterns observed in this study (i.e. labeling of Glu345 in the third extracellular loop plus residue(s) in the amino terminus), together with results from previous ligand binding and photoaffinity labeling studies, allow us for the first time to propose a specific helix bundle topology for the CCK receptor. If we assume that the bound conformation of the CCK peptide ligand is not dramatically different from the solution conformation for CCK-(26–33) (30), then a counterclockwise helix bundle arrangement for the CCK receptor best accommodates the available ligand binding data and the full set of covalent cross-links we observe in our photoaffinity labeling experiments.

DISCUSSION

G protein-coupled receptors can provide pockets for binding small ligands within the confluence of their seven intramembranous helical segments and for binding large glycoprotein ligands in specialized structural domains configured within large amino-terminal domains (1, 4). These receptors can also bind and be activated by peptide ligands, such as CCK. The emerging themes for such binding suggest critical contributions by extracellular loop and amino-terminal tail domains that are near the external face of the membrane, as well as including examples of portions of ligands dipping down into the confluence of helices.

Our current understanding of the molecular basis of CCK binding is based on receptor mutagenesis and affinity labeling studies. Both support the importance for peptide binding of regions just outside of the first transmembrane segment and in the extracellular loop domains (8, 9, 16). There has, however, been substantial controversy related to the details of CCK peptide docking to these domains. Very distinct models of the peptide-occupied receptor have been proposed (8, 16, 17). Nonpeptidyl ligand binding to this receptor seems to occur deeper in the membrane within the confluence of helices (34).
Established structure-activity relationships for CCK have localized the pharmacophoric domain to the carboxyl-terminal heptapeptide-amide (12, 13). Almost every residue within this domain makes an important contribution to binding and activity. In contrast, almost any modification to the amino terminus of CCK-8 that has been attempted has been well tolerated, without modifying receptor binding or signaling. It was this feature that encouraged our positioning of the amino terminus of CCK-8 above the ligand-binding domain of the CCK receptor in our evolving model, such that an extension would not make contact with the regions of this receptor that are known to be important for function (8, 10). It is noteworthy that another molecular model has placed the amino terminus of CCK much closer to the membrane and directed toward the other side of the helical bundle (16, 17). Such a model is inconsistent with the residue-residue approximations that have been directly established by photoaffinity labeling studies (8, 10).

To date, there has been the successful photoaffinity labeling of distinct spatially approximated residues within the CCK receptor through two positions within the pharmacophoric domain of CCK (8–10). This has been accomplished with two different photoactive moieties in position 33 of CCK (8, 10) and with a benzophenone moiety in position 29 of CCK (9). The position 33 photoprobes covalently labeled receptor residue Trp39 just above the first transmembrane segment. The position 29 photoprobe established a covalent bond with receptor residues His347 and Leu348 just above the seventh transmembrane segment. It is noteworthy that both of these represented focused contacts with a single receptor domain, as might be expected from the high affinity tight interaction between native agonist ligand and the ligand-binding domain of the receptor.

In contrast, in the present work, the Bpa residue in position 24 of CCK established covalent bonds to either of two distinct domains of the CCK receptor. This probe was fully characterized as a high affinity ligand that had full efficacy relative to natural CCK. While one of the covalently labeled receptor domains was within a region that is known to be important, the third extracellular loop, it is notable that this contact was lost when the amino terminus of the receptor was shortened by truncation. The second labeled domain was a portion of the amino-terminal tail of the CCK receptor that can be eliminated by truncation, without any detrimental effects on CCK binding or agonist-stimulated signaling. This contact is therefore not critical for ligand affinity or function.

These spatial approximations, however, provide the basis to postulate that the docked peptide is tucked under the protective cover of the amino terminus of the CCK receptor. This function was further supported by the demonstration that this domain protected the receptor from extracellular proteolytic attack. Eliminating this region of the receptor by truncation had no detrimental effect on ligand binding affinity or agonist-stimulated signaling, but resulted in a more labile receptor that was much more sensitive to proteolysis. The glycosylation of the amino-terminal domain of the CCK receptor is probably most responsible for this resistance to proteolysis. This is a recognized function of the glycosylation of membrane proteins (35, 36). Another established function for membrane protein glycosylation relates to assisting in solubility and folding during biosynthesis that is also likely relevant to the CCK receptor.

Although position 24 in the CCK ligands is external to the established pharmacophore, cross-linking data generated by

**Fig. 9.** Affinity labeling of the truncated CCK receptor with Bpa$^{24}$ and Bpa$^{29}$ probes. Shown is a typical autoradiograph of an SDS-polyacrylamide gel used to separate products of the labeling of the truncated receptor-bearing membranes using Bpa$^{24}$ and Bpa$^{29}$ probes in the absence and presence of competing unlabeled CCK (1 μM) (representative of three similar experiments). The Bpa$^{24}$ probe failed to label covalently the truncated receptor, whereas the Bpa$^{29}$ probe efficiently labeled this preparation.

**Fig. 10.** Protease resistance of CCK receptor constructs. Shown are CCK competition-binding curves for CHO cell lines expressing wild type (WT) and truncated CCK receptor constructs that had been treated with trypsin. Cells were exposed to trypsin digestion for 20 min at 37 °C, followed by extensive washing and standard CCK radioligand binding assay. The binding capacity of the truncated receptor was significantly negatively impacted by trypsin treatment, whereas the wild type receptor was resistant to this treatment. Values are expressed as the means ± S.E. of data from three independent experiments.
substitution of a photolabile residue at this position provides extremely useful constraint data for three-dimensional model building studies. The results reported here provide clear evidence that our earlier decision to position the amino terminus of the ligand facing away from receptor binding site (8, 10) was appropriate. The photoaffinity labeling data for the ligand amino terminus, combined with ligand binding studies and cross-linking data for positions within the peptide pharmacophore, also enable us for the first time to propose a distinct topological model for the peptide ligand-CCK receptor complex. Based on the full set of photoaffinity labeling and ligand binding data now available, it appears likely that the CCK receptor possesses a counterclockwise helix bundle topology. More definitive biophysical data will be needed to ultimately verify this prediction.

With this refinement in our working model, the tyrosine-sulfate residue in position 27 of CCK that has been shown to be so important in structure-activity studies (37) is now in direct contact with Arg197. These residues, therefore, represent potential partners for charge-charge interaction. Indeed, Arg\textsuperscript{197} is one of only three basic residues in the extracellular domain of the CCK receptor that has been reported to have a marked negative impact on CCK binding when replaced with an Ala residue (38).

Thus, using an analogue of CCK with a photolabile Bpa moiety in position 24, we have learned much about the binding domain for this hormone within its receptor. While the pharmacophoric domain of the peptide is held in constant approximation with the regions of the CCK receptor close to the membrane, the amino-terminal extension from the pharmacophore likely moves further away from the bilayer and is overlaid by a region of the receptor amino terminus that has no direct effect on binding affinity or on biological activity. Instead, this region of the receptor serves a protective function over the more critical domain below. The distinct positions of covalent labeling with this probe also provide additional detail to refine the molecular model of the docked peptide agonist. This evolving model now best supports a counterclockwise helical bundle topology for the receptor and residue approximations that are fully consistent with all existing experimental data.

Addendum—Since submission and review of this manuscript, a preliminary crystal structure for bovine rhodopsin has appeared (Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le, Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Science 289, 739–745) that exhibits a counterclockwise helix bundle topology. Since the CCK receptor is also a member of the rhodopsin \(\beta\)-adrenergic receptor family, it is quite likely that the helix bundle topology will be similar.

![Molecular model of peptide-receptor complex](image-url)
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