A Peptide Agonist Acts by Occupation of a Monomeric G Protein-Coupled Receptor: Dual Sites of Covalent Attachment to Domains Near TM1 and TM7 of the Same Molecule Make Biologically Significant Domain-Swapped Dimerization Unlikely

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Membrane receptor dimerization is a well-established event for initiation of signaling at growth factor receptors and has been postulated to exist for G protein-coupled receptors, based on correction of nonfunctional truncated, mutant, or chimeric constructs by coexpression of appropriate normal complementary receptor domains. In this work, we have directly explored the molecular composition of the minimal functional unit of an agonist ligand and the wild-type G protein-coupled cholecystokinin (CCK) receptor, using photoaffinity labeling with a CCK analogue probe incorporating dual photolabile benzoylphenylalanine (Bpa) residues as sites of covalent attachment. This probe, ^{125}I-D-Tyr-Gly-[(Nle^{28,31}, Bpa^{29,33})CCK-26-33], was shown to represent a full agonist and to specifically label the CCK receptor. Like probes incorporating individual photolabile residues in these positions,[1,2] the two Bpa residues in the dual photoprobe covalently labeled receptor domains in the amino-terminal tail outside TM1 and in the third extracellular loop outside TM7. Absence of demonstrable receptor dimerization after the establishment of dual sites of covalent attachment supports the presence of these two domains within a single receptor molecule. Demonstration of the covalent adduct of a single probe molecule with the two cyanogen bromide fragments of the CCK receptor representing the expected domains further supports this interpretation. Thus, while domain-swapped dimerization of G protein-coupled receptors may be possible as a mechanism of rescue for nonfunctional molecules, it is not necessary for ligand binding and initiation of signaling at a wild-type receptor in this superfamily. The functional unit for CCK action is normally a ligand−receptor monomer.

Introduction

Membrane receptor dimerization is best established and understood for the single transmembrane growth factor and cytokine receptors, where agonist ligand binding may induce receptor dimerization, autophosphorylation, and initiation of signaling.[3,4] A crystal structure has even been solved demonstrating the occupation of such a dimeric receptor complex by a ligand.[5] Application of the concept of receptor dimerization to guanine nucleotide-binding protein (G protein)-coupled receptors has also recently attracted much interest[6−12] but is much less well-understood.

G protein-coupled receptor dimerization (or oligomerization) could exist at two distinct levels: one level involves the swapping of domains between two distinct receptor molecules to contribute to the binding domain of a single ligand molecule, and the second involves the clustering or association of 1:1 stoichiometric molecular complexes of ligand-occupied receptors. In this work, we have focused on exploring the former possibility, which has been supported by a variety of experimental approaches and has been applicable to several G protein-coupled receptors.[7,9−12] These approaches include the coexpression of complementary structurally and functionally deficient receptors to yield a functional unit. This has particular interest and significance to the molecular modeling of the ligand-binding domain, since such domain swapping might expose different surfaces of involved helices to a ligand.

In the current work, we have utilized photoaffinity labeling with a unique receptor ligand incorporating dual photolabile residues that directly probe binding determinants on both the carboxyl-terminal and amino-terminal halves of the G protein-coupled cholecystokinin (CCK) receptor. Using this ligand that has full agonist activity, we have demonstrated that both labeled domains normally reside within a single receptor molecule. This will be helpful in providing key constraints useful for the modeling of the ligand-binding domain of this receptor.

While domain-swapped dimerization has been demonstrated in artificially engineered in vitro cell systems for truncated and mutant forms of receptors in this superfamily,[6,7,9] the current work suggests that this may represent only a potential mechanism for "rescue" when the transmembrane segments of a single molecule that are tethered by loop domains do not normally associate with each other within the plasmalemma. On this basis, we suggest that the normal physiological structural unit

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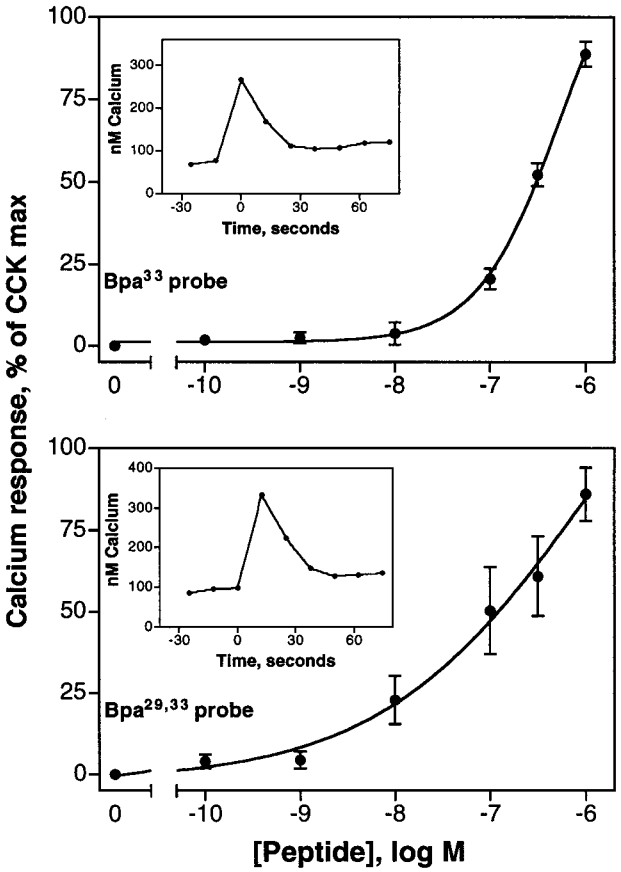

Figure 1. Biological activity of photolabile CCK receptor probes. Shown are graphs of peak intracellular calcium responses of CHO-CCKR cells stimulated by various concentrations of D-Tyr-Gly-[(Nle^{28,31},Bpa^{33})CCK-26-33] and D-Tyr-Gly-[(Nle^{28,31},Bpa^{29,33})CCK-26-33], with the results expressed relative to responses in the same cells to a maximal concentration of CCK (0.1 μM). Values represent means ± SEM of data from three independent experiments performed in duplicate.

for G protein-coupled receptors likely represents a single ligand occupying a single receptor molecule.

Methods

Peptides. Photolabile cholecystokinin (CCK) analogues were synthesized by solid-phase manual techniques, as we previously described.[13,14] D-Tyr-Gly-[(Nle^{28,31},Bpa^{29})CCK-26-33] was previously reported.[15] For the current project, we also synthesized d-Tyr-Gly-[(Nle^{28,31},Bpa^{33})CCK-26-33] and D-Tyr-Gly-[(Nle^{28,31},Bpa^{29,33})CCK-26-33] using similar strategies and methods. All synthetic peptides were purified to homogeneity by reversed-phase HPLC and were characterized by amino acid analysis and mass spectrometry.

Each of these peptides was radioiodinated oxidatively, using the solid-phase oxidant *N*-chlorobenzenesulfonamide (Iodobeads; Pierce Chemical Co., Rockford, IL).[15] Products were purified to a specific radioactivity of 2000 Ci/mmol by reversed-phase HPLC.

Receptor Preparations. A CCK receptor-bearing Chinese hamster ovary cell line (CHO-CCKR) was utilized for this work.[16] These cells express 125 000 receptors per cell and bind CCK and signal in a manner similar to rat pancreatic acinar cells. Cells were cultured at 37 °C in a 5% CO_2 environment on Falcon tissue culture plasticware, in Ham's F-12 medium supplemented with 5% fetal clone-2 (Hyclone Laboratories, Logan, UT). Cells were typically passaged two times per week and were lifted mechanically in preparation for study.

Biological activity was assessed using an intracellular calcium assay. Signaling was studied in intact CHO-CCKR cells that had been loaded with FURA-2AM.[17] Intracellular calcium responses to CCK and its analogues were determined as previously described.[1] All incubations were performed in Krebs-Ringers-HEPES (KRH) medium, containing 25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 1 mM KH_2PO_4, 0.2% (w/v) bovine serum albumin, and 0.01% soybean trypsin inhibitor. All assays included both full concentration−response curves for an analogue and a maximally stimulatory concentration of natural CCK, to provide a control maximal response for normalization.

Table 1. Biological Activity of CCK Analogue Probes

probes	EC_{50} values, nM
D-Tyr-Gly-[(Nle^{28,31},Bpa^{29})CCK-26-33]	28 ± 6
D-Tyr-Gly-[(Nle^{28,31},Bpa^{33})CCK-26-33]	280 ± 23
D-Tyr-Gly-[(Nle^{28,31},Bpa^{29,33})CCK-26-33]	150 ± 66
CCK-8	0.009 ± 0.001

Receptor binding and photoaffinity labeling studies were performed with enriched plasma membranes prepared from the CHO-CCKR cells.[1] Binding studies were performed with 10 pmol/L radioligand and 10 μg of membranes in KRH medium, incubated at 25 °C for 60 min in the absence or presence of increasing concentrations of competing unlabeled CCK. Bound and free radioligand were separated using a cell harvester apparatus (Skatron Instruments, Inc., Sterling, VA) with receptor-binding filtermats. The photolabile analogues were quite sticky, having high nonsaturable binding in this type of traditional competition binding assay. However, the saturability, specificity, and high-affinity nature of the binding of the radioligands to the CCK receptor were clearly demonstrated in affinity labeling studies.

Photoaffinity labeling was performed using 75−100 pmol/L radioligand and 50−100 μg of membranes incubated at 25 °C for 60 min in KRH medium, in the absence or presence of competing unlabeled CCK. Bound and free radioligand were separated by centrifugation and washing. Nonspecific binding was determined in the presence of 1 μM CCK. Photolysis was performed in a Rayonet model RP-100 apparatus (Southern New England Ultraviolet, Hamden, CT) equipped with 350-nm lamps, with the sample in siliconized borosilicate glass tubes 5.7 cm from the light source, at 4 °C for 30 min. Membranes were collected by centrifugation and analyzed initially by autoradiography after electrophoresis on either 10% or 4−15% gradient sodium dodecyl sulfate (SDS)−polyacrylamide slab gels. Some receptor preparations were partially enriched by adsorption to wheat germ agglutinin-agarose prior to electrophoresis. For this, membranes were solubilized with 1% Nonidet P-40 and diluted to yield 0.1% detergent prior to adsorption to the lectin, and the lectin-bound glycoproteins were washed with 0.5 M NaCl and water. The apparent molecular weights of affinity labeled proteins were determined by interpolation of migration relative to that of standard proteins, using a plot of log of molecular weights versus migration. Concentrations of CCK that inhibit 50% of affinity labeling (IC_{50}) were determined by densitometric scanning of autoradiographs.

Determination of Receptor Domains Labeled. Cyanogen bromide cleavage of the affinity-labeled CCK receptor preparations was performed as previously described.[1] Radiochemically purified CCK receptor was eluted from the SDS−polyacrylamide gel and reduced and alkylated by treatment with 5 mM dithiothreitol and 20 mM iodoacetic acid prior to cleavage. Cleavage was performed for 16 h at 22 °C in the dark, using 2.5 mg of cyanogen bromide in 70% formic acid. The sample was brought to dryness under vacuum in a Speed-Vac concentrator (Savant Instruments, Holbrook, NY). Products of digestion were separated on a 10% NuPAGE gel (Novex, San Diego, CA) with MES running buffer and detected by autoradiography. The apparent molecular masses of radiolabeled receptor fragments were determined by interpolation on a plot of the log of the molecular weights of MultiMark (Novex) or Kaleidoscope (BioRad) standards versus their migration.

Molecular Modeling of Ligand−Receptor Complex. The previously published model of the CCK receptor occupied

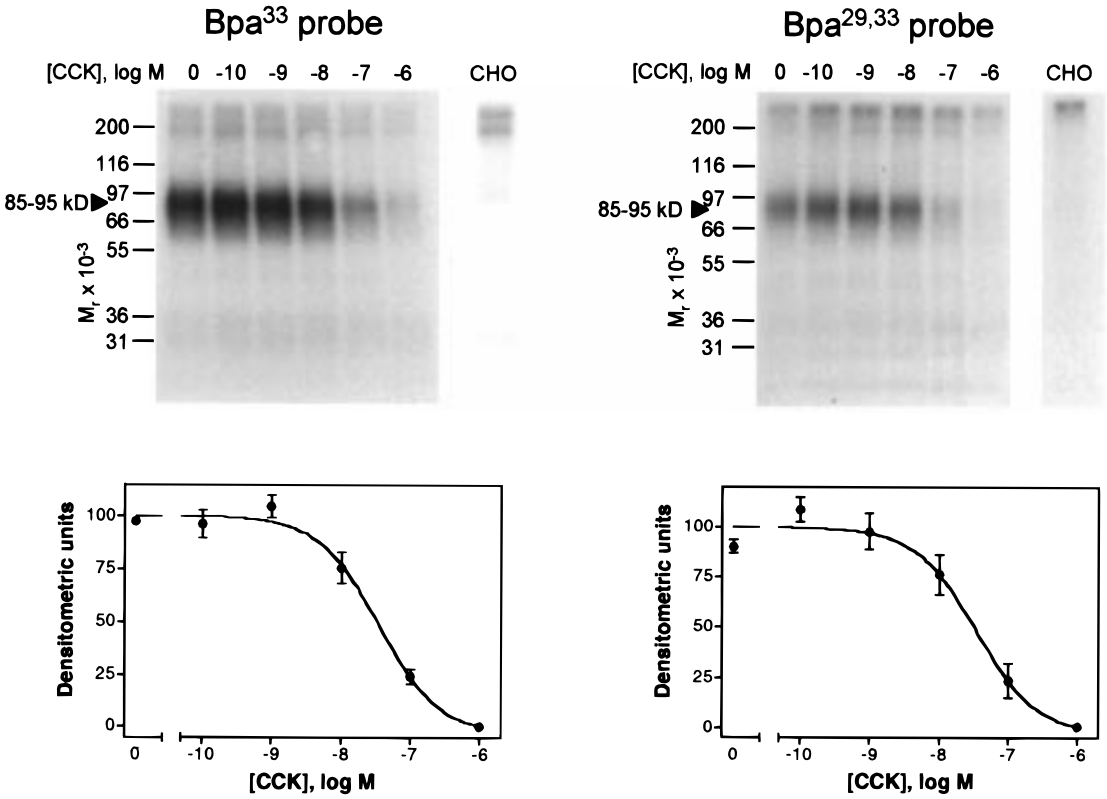

Figure 2. Affinity labeling of the CCK receptor with the photolabile CCK analogues incorporating Bpa residues in position 33 and in both positions 29 and 33. Shown are typical autoradiographs of SDS−polyacrylamide gels used to separate the products of photoaffinity labeling the CCK receptor on CHO-CCKR cells with ^{125}I-D-Tyr-Gly-[(Nle^{28,31},Bpa^{33})CCK-26-33] and ^{125}I-D-Tyr-Gly-[(Nle^{28,31},Bpa^{29,33})CCK-26-33], in the absence or presence of increasing concentrations of CCK. Control lanes are also included in which non-receptor-bearing CHO cell membranes were affinity labeled in the absence of competitor, demonstrating only two high-molecular-weight bands. These do not represent receptor and were also seen as nonsaturably labeled bands in the same position on the gel in labeling the receptor-bearing membranes. Shown also are graphs of the densitometric quantitation of receptor labeling performed in three similar experiments. Values represent means ± SEM.

by a CCK analogue[1] was modified sequentially by the following: (i) rebuilding the ligand to accurately reflect the probe incorporating dual photolabile Bpa residues used in the current work; (ii) manually docking the peptide, establishing appropriate proximity between the photolabile residues and the covalent sites of attachment, using interactive molecular graphics methods; and (iii) relaxing the docked complex using limited energy minimization and low-temperature molecular dynamics calculations, as reported previously.[1]

Results

Each of the synthetic, photolabile CCK analogues was purified to homogeneity and was chemically characterized to ensure its identity.

The three photolabile CCK analogues (D-Tyr-Gly-[(Nle^{28,31},Bpa^{29})CCK-26-33], D-Tyr-Gly-[(Nle^{28,31},Bpa^{33})-CCK-26-33], and D-Tyr-Gly-[(Nle^{28,31},Bpa^{29,33})CCK-26-33]) were biologically active agonists. The Bpa^{29} analogue was previously reported to represent a full agonist, stimulating rat pancreatic acinar cells to secrete amylase in a concentration-dependent manner and with maximal secretion identical to that stimulated by CCK.[2] The two new analogues (Bpa^{33} and Bpa^{29,33}) stimulated intracellular calcium responses in CHO-CCKR cells in a concentration-dependent manner (Figure 1). They were also full agonists, as demonstrated by their abilities to stimulate intracellular calcium responses not statistically different from the maximal responses stimulated by maximal concentrations of natural CCK in the same assays. All three analogues were, however, substantially less potent than CCK (concentrations that stimulated half-maximal responses (EC_{50}) are shown in Table 1).

These photolabile CCK analogues also bound to and affinity labeled the CCK receptor saturably, specifically, and with high affinity. Figure 2 illustrates the data for the Bpa^{33} and the dual Bpa^{29,33} analogues. The Bpa^{29} analogue has previously been demonstrated to share these same properties.[2] Like the probes individually incorporating a Bpa residue into positions 29 or 33, the dual probe covalently labeled the CCK receptor, migrating at M_r = 85 000−95 000 on an SDS−polyacrylamide gel. Labeling of the CCK receptor was saturable and specific and was inhibited in a concentration-dependent manner with CCK. There was nonspecific covalent attachment to two bands that migrated above M_r = 200 000, but these were clearly not receptor, based on their lack of saturability and on their presence in the lane representing the affinity labeling of non-receptor-bearing CHO cell membranes.

The CCK receptor band labeled with each of these probes shifted appropriately to an apparent M_r = 42 000 after deglycosylation with endoglycosidase F (Figure 3). Note that for each probe, there was no covalent labeling in the position of a receptor dimer on the gels. This was true even when the autoradiographs were heavily over−exposed. Any dimer that might be present would therefore have to represent far less than 1% of the monomer that was clearly apparent on the autoradiograph.

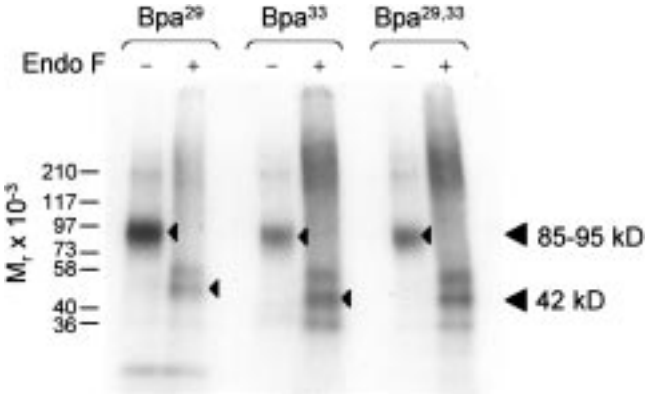

Figure 3. Photoaffinity labeling of the CCK receptor with probes having single and dual photolabile residues. Shown is a typical autoradiograph of a 4−15% gradient SDS−polyacrylamide gel used to separate products of photoaffinity labeling of membranes from CHO-CCKR cells with ^{125}I-D-Tyr-Gly-[(Nle^{28,31},Bpa^{29})CCK-26-33], ^{125}I-D-Tyr-Gly-[(Nle^{28,31},Bpa^{33})-CCK-26-33], and ^{125}I-D-Tyr-Gly-[(Nle^{28,31},Bpa^{29,33})CCK-26-33]. In noted lanes, receptor was deglycosylated by treatment with endoglycosidase F. No evidence of receptor dimerization was observed. Images are typical of at least seven similar experiments.

The domains of the CCK receptor that were affinity labeled with each of the probes were identified by cyanogen bromide cleavage. Figure 4 illustrates the theoretical sites of cyanogen bromide cleavage of the type A rat CCK receptor. As previously reported,[2] the Bpa^{29} analogue covalently labeled a receptor fragment migrating at $M_r = 6\,700$, representing the third extracellular loop linking the sixth and seventh transmembrane segments (Figure 4). The specific site of labeling the receptor through Bpa^{29} was previously demonstrated to be His^{347} and Leu^{348}.[2]

Like our previous work with the pNO$_2$-Phe^{33} analogue of CCK that labeled Trp^{39} of the CCK receptor, just outside of the first transmembrane segment,[1] the Bpa^{33} analogue labeled a cyanogen bromide fragment of the size expected (apparent migration at $M_r = 25\,000$) (Figure 4). This represented a glycopeptide that shifted to approximate $M_r = 8\,500$ after treatment with endoglycosidase F.

The dual Bpa^{29,33} analogue affinity labeled a receptor fragment that migrated at apparent $M_r = 30\,000$, which shifted to apparent $M_r = 15\,000$ (Figure 4). This is most consistent with the establishment of two covalent bonds linking the probe to both of the receptor fragments labeled by each of the individual Bpa analogues.

A model of the CCK receptor occupied by this ligand is illustrated in Figure 5. This shows the simultaneous dual sites of covalent attachment between this ligand and the receptor. Earlier modeling studies[1] suggested that it should be possible to simultaneously form two covalent cross-links between the CCK receptor and a ligand with photolabile residues at positions 29 and 33. The current results support the earlier modeling hypothesis, and also suggest that there are no unusual conformational changes in the receptor induced by the introduction of either separate cross-link. Thus, this dual cross-linking result provides a more stringent spatial and topological constraint than has been available previously for modeling the binding domain and

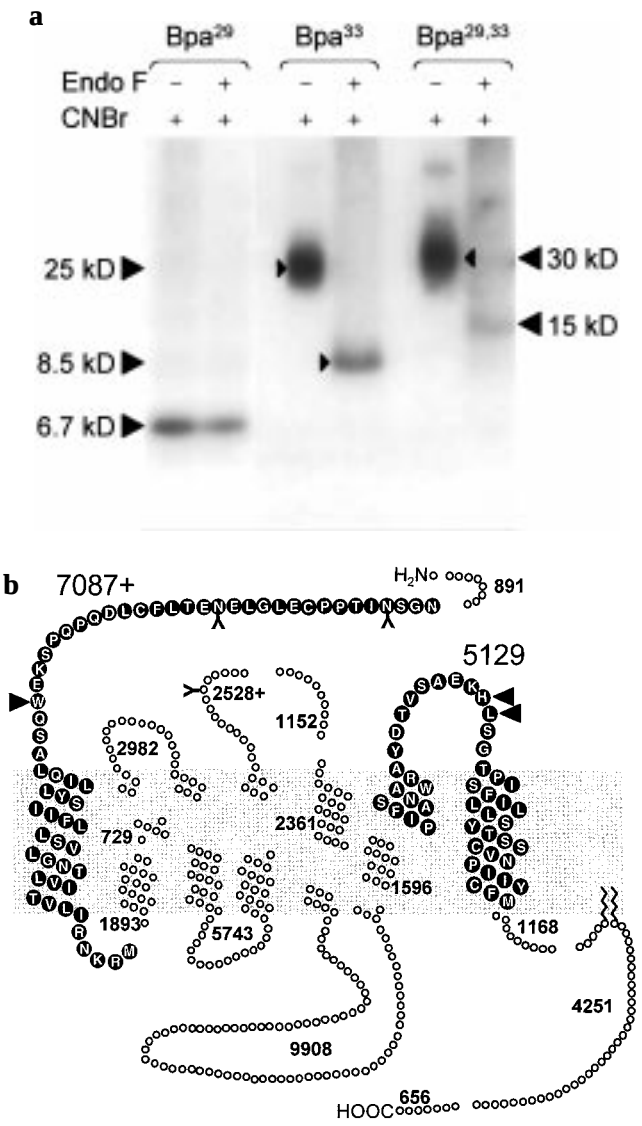

Figure 4. Cyanogen bromide digestion of the CCK receptor. Shown is a diagram of the sites of cyanogen bromide cleavage of the rat type A CCK receptor, along with the masses of the protein cores of the resultant fragments. Consensus sites for potential glycosylation are noted. Also shown is a typical autoradiograph of a NuPAGE gel used to separate the products of cyanogen bromide digestion of radiochemically pure photoaffinity-labeled CCK receptor, before and after deglycosylation. Affinity labeling was performed with each of the three probes: ^{125}I-D-Tyr-Gly-[(Nle^{28,31},Bpa^{29})CCK-26-33], ^{125}I-D-Tyr-Gly-[(Nle^{28,31},Bpa^{33})CCK-26-33], and ^{125}I-D-Tyr-Gly-[(Nle^{28,31},-Bpa^{29,33})CCK-26-33]. The probes with individual photolabile residues in positions 29 and 33 labeled fragments that migrated identically to those we have recently fully characterized, including the identification of the specific residues labeled.[1,2] The dual photolabile probe predominantly labeled a band representing the simultaneous establishment of dual covalent bonds to each of the receptor fragments labeled by each of the single photolabile probes. The migration of the cyanogen bromide digests of native and deglycosylated receptor labeled with the dual probe was higher on the gel than either of the single probes alone and reflected the sum of masses of the probe with each individual receptor fragment. Results are typical of a minimum of 10 similar experiments.

the relative positions of transmembrane helices in the type A CCK receptor.

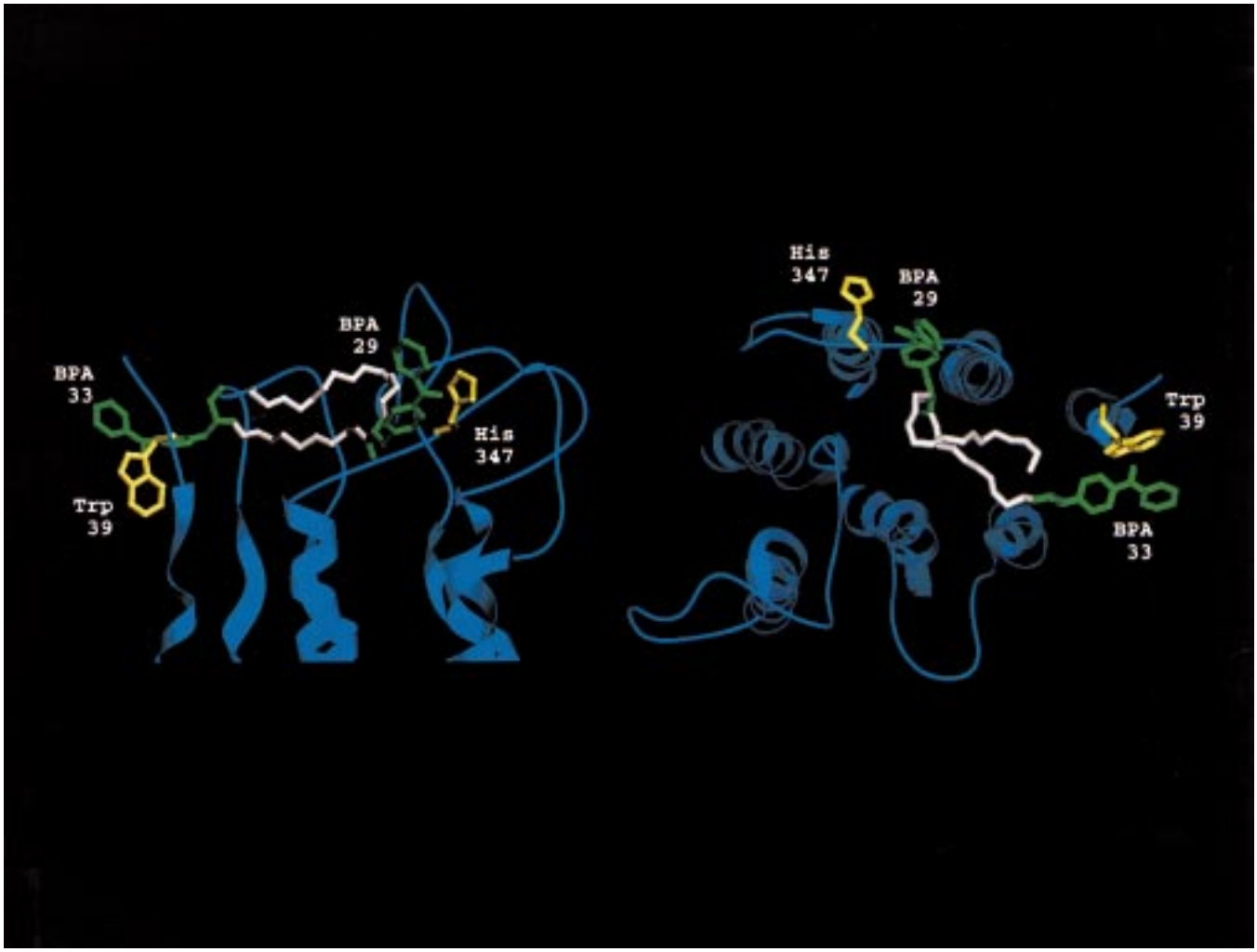

Figure 5. Molecular model of the type A rat CCK receptor occupied by the dual Bpa[29,33] probe. Shown are the backbones of the upper transmembrane and ectodomains of the CCK receptor (light blue) and of the CCK analogue representing a photolabile agonist probe (white). The molecular structures of key residues are expanded, with the photolabile Bpa residues in positions 29 and 33 of the probe shown in green and covalently labeled receptor residues shown in tan. The D-Tyr-Gly-[(Nle[28,31],Bpa[29,33])CCK-26-33] simultaneously established two covalent bonds with the CCK receptor: one through Bpa[29] to the region just above TM7 and one through Bpa[33] to the region just above TM1. This dual cross-linking provides a more stringent spatial and topological constraint than has previously been available for modeling the binding domain and the relative positions of the transmembrane helices in this receptor.

Discussion

It has long been recognized that the heptahelical G protein-coupled receptor molecules may migrate on SDS−polyacrylamide gels as oligomers, as well as monomers. Such observations have come from affinity labeling, immunoblotting, and immunoprecipitation experiments.[18,19] This has traditionally been attributed to the physicochemical nature of these molecules and to their tendency to nonspecifically aggregate, particularly after exposure to elevated temperatures. However, several lines of evidence have demonstrated that receptors in this superfamily are capable of forming functional dimers with swapped domains under certain experimental conditions.[6,7,10,11] These include several strategies to coexpress two mutant receptors, each of which is itself incapable of binding or signaling but when expressed together can achieve functional complementation. This has taken the form of truncated m2- and m3-muscarinic receptors,[7] distinct binding-defective point mutations of the angiotensin receptor,[9] and complementary chimeric α2-adrenergic and m3-muscarinic receptors.[6] These "split receptor" approaches support the concept that there are independent autonomous structural domains within G protein-coupled receptors that may associate upon binding to yield functional receptors.[12,20] The established structural specificity and stability of interaction between two transmembrane helices that normally associate further support this.[21,22]

While these studies establish that intermolecular interactions between G protein-coupled receptor molecules can occur, they do not establish that this does occur physiologically in the natural membrane environment and with the typical sparse density of normal receptor molecules. Supporting the potential functional importance of dimerization of these receptors is the observation that the degree of association can be affected by the nature of the ligand, stabilized by agonist and negatively impacted by inverse agonist.[10] These treatments may simply modify the characteristics of the external faces of the heptahelical complex, thereby affecting their tendency to cluster or aggregate, rather than implying true interdigitation of transmembrane

helices. Indeed, early work by Hazum and Keinan[23] demonstrated the ability of a bivalent antibody directed to an epitope tag on an analogue of gonadotropin-releasing hormone to convert an antagonist to an agonist, while the monovalent Fab fragment did not have this effect. Such an observation could be consistent with the antibody-induced association of ligand-occupied G protein-coupled receptors to be involved in their initiation of signaling.

It is clear that we do not yet have a detailed understanding of the conformation of G protein-coupled receptors. While evidence is strong to support the presence of seven transmembrane segments in such molecules as they reside in the plasma membrane, even the orientation and assignment of the individual helices have been debated.[24,25] This has included discussion of sequential counterclockwise and clockwise assignments of the transmembrane segments, as well as nonsequential intra- and intermolecular assignments of these segments. There are arguments for each of these, and it might even be possible for different orientations to exist for different receptors or for the same receptor when occupied by distinct ligands. Within a single receptor molecule the length of each of the loop domains determines the flexibility of assembly in the membrane bilayer. The loop that is most variable and most lengthy is the third intracellular loop that separates the amino-terminal portion of the receptor including transmembrane segments 1−5 and the carboxyl-terminal portion of the receptor including transmembrane segments 6 and 7. Indeed, these two receptor domains have been successfully utilized in "split receptor" studies.[12,20] Recently, it has been shown that the length of the third intracellular loop in receptors can affect its ability to form dimers and to signal.[8]

The most interesting and biologically relevant conformation of a receptor is its active conformation when occupied by a full agonist. We have recently begun to accumulate information about the placement of agonist when bound to the G protein-coupled CCK receptor.[1,2] In these studies, we have incorporated a photolabile residue into different positions within the ligand pharmacophore and defined the receptor residues that are labeled upon photolysis. Siting a photolabile nitrophenylalanyl residue at the carboxyl-terminus of the peptide in position of Phe^{33} established a covalent bond with receptor residue Trp^{39} just above the first transmembrane segment (TM1).[1] Siting a photolabile residue into the midregion of the peptide in position of Gly^{29} established covalent bonds with receptor residues His^{347} and Lys^{348} just above the seventh transmembrane segment (TM7).[2] In this work, we have incorporated two photolabile residues into a single probe molecule, with benzophenone precursor benzoylphenylalanines into both positions 29 and 33 of a CCK-like photoprobe. We have established that this is a full agonist acting at the CCK receptor and that it can form two covalent bonds with each of the two expected regions of a single receptor molecule. It is noteworthy that the model of dual simultaneous cross-links between this ligand and the CCK receptor is nearly unchanged from the original model proposed with a single site of covalent attachment.[1] While achieving dual covalent linkages, there was no evidence of domain-swapped receptor dimers, supporting the clear conclusion that a single peptide agonist ligand can occupy a single monomeric G protein-coupled receptor and can thereby result in functional activity.

It is likely that the "rescue" demonstrated to be feasible in the series of studies with highly expressed truncated, mutant, and chimeric forms of G protein-coupled receptors[6,7,9] represents a remote possibility for normal structure, rather than a common mechanism to achieve functional integrity for these receptors. As we refine our conformational models of these receptors, it will be important to ultimately unambiguously assign helical identities to the seven-helix bundle such that each is contributed by a single receptor molecule. While association between these molecules may indeed occur in vivo or during in vitro preparation of membranes for electrophoresis of component proteins, this would most likely occur through the external faces of associating complete helical bundles. This could contribute to cooperativity, but not to the integrity of the minimal functional unit of agonist ligand and receptor.

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