Demonstration of a Direct Interaction between Residue 22 in the Carboxy-terminal Half of Secretin and the Amino-terminal Tail of the Secretin Receptor Using Photoaffinity Labeling*

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An understanding of the molecular basis of hormonal activation of receptors provides important insights for drug design. Toward this end, intrinsic photoaffinity labeling is a powerful tool to directly identify the ligand-binding domain. We have developed a new radioiodinatable agonist ligand of the secretin receptor that incorporates a photolabile p-benzoyl-L-phenylalanine (Bpa) into the position of Leu22 and have utilized this to identify the adjacent receptor domain. The rat [Tyr10, Bpa22]secretin-27 probe was a fully efficacious agonist, with a potency to stimulate cAMP accumulation by Chinese hamster ovary SecR cells similar to that of natural secretin (EC50 = 68 ± 22 pm analogue and 95 ± 25 pm secretin). It bound specifically and with high affinity (Kd = 5.0 ± 1.1 nM) and covalently labeled the Mr = 57,000–62,000 secretin receptor. Cyanogen bromide cleavage of the receptor yielded a major labeled fragment of apparent Mr = 19,000 that shifted to Mr = 9,000 after deglycosylation. This was most consistent with either of two glycosylated domains within the amino-terminal tail of the receptor. Immunoprecipitation with antibody directed to epitope tags incorporated into each of the candidate domains established that the fragment at the amino terminus of the receptor was the site of labeling. This was further localized to the amino-terminal 30 residues of the receptor by additional proteolysis of this fragment with endoproteinase Lys-C. This provides the first direct demonstration of a contact between a secretin-like agonist and its receptor and will contribute to the modeling of this interaction.

The secretin receptor is prototypic of a recently recognized family (Class II) of guanine nucleotide-binding protein (G protein)1-receptor (1). Members of this family are believed to have the seven-transmembrane segment topology typical of the superfamily, but they share <12% homology with the extensively studied Class I receptors in the rhodopsin/β-adrenergic receptor family, and they lack the signature sequences of this family (2, 3). Secretin family receptors have long amino-terminal domains incorporating six highly conserved Cys residues, believed to contribute to disulfide bonds that help define the family (3, 4). Indeed, this complex domain has been suggested to play a key role in agonist binding, as suggested by receptor mutagenesis studies (5–9). Other extracellular loop domains have also been implicated in complementary roles for agonist binding and receptor activation (4, 5, 7, 10). Natural ligands for this family of receptors are all peptides longer than 27 residues, with structure-activity series suggesting the presence of diffuse pharmacophoric domains (3). Although this large diffuse pharmacophore nicely complements the multiple domains predicted to be outside the membrane bilayer, there is no working model to predict how the two molecules might interact.

In this work, we attempt to establish an initial constraint that will contribute to the development of a model for the interaction of secretin with its receptor. We do this through photoaffinity labeling. This has the theoretical advantage of directly probing the domain adjacent to the photolabile residue within the probe after it binds to the receptor. Using this approach, we have successfully identified two binding contacts between photolabile analogues of cholecystokinin and its receptor (11, 12).

In this work, we have developed an analogue of secretin that incorporates a site for radioiodination and a photolabile residue intrinsic to the pharmacophore for establishment of a covalent bond to a domain of the receptor adjacent to it as it resides in its binding site. We have characterized this as a fully efficacious and potent agonist, likely to reside in the natural secretin-binding site within the secretin receptor. We have characterized its high affinity, saturable, and specific binding to the receptor, and we have demonstrated that it efficiently covalently labels the secretin receptor in a single distinct domain. This domain was identified as the amino-terminal 30 residues of the amino-terminal tail of the secretin receptor. Although it is too early to know how generalizable such a contact may be within the secretin receptor family, this is likely since themes for the impact of mutagenesis for various members of this family have been generally consistent.

EXPERIMENTAL PROCEDURES

Materials—Wheat germ agglutinin-agarose was from EY Laboratories, Inc. (San Mateo, CA). Endoproteinase Lys-C and anti-hemagglutinin (HA) epitope monoclonal antibody were from Boehringer Mannheim. Iodoacetic acid was from Pierce. Endoglycosidase F was prepared in our laboratory, as we have reported (13). Other reagents were analytical grade.

Receptor Preparations—Chinese hamster ovary (CHO) cell lines were used as source of receptors for this study. The CHO-secR cell line stably expressing the wild-type rat secretin receptor has been previously established and characterized (14). Two new cell lines were established for this report. These express secretin receptor mutants in which the HA epitope (with sequence YPYDVPDYA) was inserted after residue 36 (secR-HA37) and after residue 78 (secR-HA79) of the wild-type rat

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The abbreviations used are: G protein, guanine nucleotide-binding protein; HA, hemagglutinin; CHO, Chinese hamster ovary; Bpa, p-benzoyl-L-phenylalanine; HPLC, high pressure liquid chromatography; MES, 4-morpholineethanesulfonic acid.
secretin receptor. Constructs were prepared by polymerase chain reaction mutagenesis (15) of the wild-type rat secretin receptor cDNA in the pcDNA3 vector (Invitrogen, Carlsbad, CA) and had their identities checked by directideoxynucleotide chain termination DNA sequencing (16). These cell lines were established in a similar manner to the CHO-SecR cell line (14), transfecting non-receptor-bearing CHO-K1 cells (American Type Culture Collection, Rockville, MD), enriching the population of cells by fluorescence-activated cell sorting, and selecting clonal populations of cells by a series of limiting dilutions. The CHO-SecR-HA37 and CHO-SecR-HA79 cell lines were selected based on their expression of a high density of the relevant secretin receptor constructs and having clear cAMP responses to secretin stimulation. These cell lines had their binding and signaling characteristics fully characterized.

Cell lines were cultured at 37 °C in an environment containing 5% CO2 on Falcon tissue culture plastic ware in Ham’s F-12 medium supplemented with 5% Fetal Clone-2 (HyClone Laboratories, Logan, UT). Cells were passaged twice a week and were lifted mechanically prior to membrane preparation. Enriched plasma membranes were prepared from these cell lines, as we previously reported (17).

**Peptides**—Rat secretin-27, rat [Tyr10]secretin-27, and rat [Tyr10,Bpa22]secretin-27 were synthesized using solid-phase manual techniques, as we previously reported (12, 14). The t-butoxycarbonyl-benzoylphenylalanine residue was synthesized as per Kauer et al. (18) and was incorporated as an intact blocked residue into the appropriate position during synthesis. Design of the rat [Tyr10,Bpa22]secretin-27 probe provided a site for oxidative radioiodination that is known to be accommodated without interfering with secretin activity (14) and a site for cross-linking to a photolabile Bpa incorporated into position 22, where there is a Phe residue in chicken secretin and where a para-nitrophenylalanine has been previously successfully incorporated (14).

Peptides were purified to homogeneity by reversed-phase HPLC. The identities of the peptides were assured by amino acid analysis and Edman degradation sequencing. The Tyr10-containing peptides were radioiodinated oxidatively with Na125I, exposing it to the solid-phase oxidant N-chlorobenzenesulfonylimide (JODO-BEAD, Pierce) for 15 s and purifying the product by reversed-phase HPLC to yield specific radioactivities of 2,000 Ci/mmol. The peptide corresponding to the hemaggutinin epitope (YPDVDPYDA) that was incorporated into the epitope-tagged secretin receptor constructs was also synthesized using solid-phase techniques and purified by reversed-phase HPLC (19).

**Biological Activity Assay**—The agonist activity of rat [Tyr10, Bpa22]secretin-27 was studied using an assay for cAMP in lysates from CHO-SecR cells stimulated with secretin and secretin analogues that was performed with reagents provided by Diagnostic Products Corp. (Los Angeles, CA). The assay was performed as we have previously described (20). The same assay was utilized to determine the signaling characteristics of the two cell lines established to express HA epitope-tagged secretin receptors. In brief, cells were stimulated at 37 °C for 30 min, and the reaction was stopped by adding ice-cold perchloric acid. After adjusting the pH to 6 with KHCO3, cell lysates were cleared by centrifugation at 3,000 rpm for 10 min, and the supernatants were used in the assay. Radioactivity was quantified by scintillation counting in a Beckman LS6000. All assays were performed in duplicate and repeated in at least three independent experiments.

**Receptor Binding Studies**—Binding of rat [Tyr10,Bpa22]secretin-27 to secretin receptors was characterized in a standard assay using cells or membranes from the CHO-SecR cell line as the source of receptor (7, 14). Membranes (1–10 μg) or 106 cells were incubated with a constant amount of rat [125I-Tyr10]secretin-27 (3–5 pm) and increasing concentrations of nonradioactive secretin analogue (0–1 μM) for 1 h at room temperature in Krebs-Ringer-HEPES (KRH) medium (25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 1 mM KH2PO4, 1.2 mM MgCl2, 2 mM CaCl2, 1 mM phenylmethylsulfonyl fluoride, and 0.01% soybean trypsin inhibitor containing 0.2% bovine serum albumin). Bound and free radioligands were separated using a Skatron cell harvester with glass fiber filter mats that had been soaked in 0.3% Polybrene, and bound radioactivity was quantified in a γ-spectrometer. Nonspecific binding was determined in the presence of 1 μM secretin and represented <20% of total binding. The same assay was also utilized to characterize the binding activity of the two cell lines established to express HA epitope-tagged secretin receptors.

**Photoaffinity Labeling of the Secretin Receptor**—For covalent labeling, receptor-bearing membranes from the secretin receptor-bearing cells containing ~100 μg of protein were incubated with rat [125I-Tyr10,Bpa22]secretin-27 (0.1–1 nm) in KRH medium in the absence or presence of concentrations of nonradioabeled secretin ranging up to 1 μM. Incubations were performed for 1 h at room temperature. The incubation mixture was frozen and exposed to photolysis in a Rayonet photochemical reactor (Southern New England Ultraviolet, Hamden, CT) equipped with 3500-Å lamps for 30 min at 4 °C. Membranes were then pelleted, washed, and exposed to reduction and alkylation. Reduction was accomplished by suspension of the membranes in KRH medium containing 5 mM dithiothreitol in a nitrogen environment at room temperature for 1 h. This was followed by alkylation with 2 mM iodoacetic acid under the same conditions for another 1 h. Membrane proteins were then either directly applied to a 10% SDS-polyacrylamide gel for electrophoresis (21) or solubilized with 1% Nonidet P-40 in KRH

![Fig. 1. Chemical characterization of synthetic rat [Tyr10, Bpa22]secretin-27.](image1)

![Fig. 2. Binding and biological activity characteristics of rat [Tyr10, Bpa22]secretin-27.](image2)
medium, prior to wheat germ agglutinin-agarose affinity chromatography and subsequent SDS-polyacrylamide gel electrophoresis.

**Deglycosylation**—The affinity-labeled secretin receptor and relevant receptor fragments were deglycosylated with endoglycosidase F using techniques previously described (17).

**Chemical and Enzymatic Cleavage of the Secretin Receptor**—Gel-purified, affinity-labeled native and deglycosylated secretin receptors were digested with cyanogen bromide in 70% formic acid according to the procedure previously described (12). Endoproteinase Lys-C digestion (20 μg/ml) was performed at 37 °C for 24 h in 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 0.1% SDS. Receptor fragments were resolved on 10% NuPAGE gels (Novex, San Diego, CA) with MES running buffer. The apparent molecular masses of radiolabeled receptor fragments were determined by interpolation on a plot of the mobility of Multimark™ protein standards (Novex) versus the log values of their apparent masses.

**Segment Identification**—The radiolabeled fragments of the affinity-labeled, epitope-tagged secretin receptors resulting from cyanogen bromide cleavage were resolved on 10% NuPAGE gels (Novex) with MES running buffer. The apparent molecular masses of radiolabeled receptor fragments were determined by interpolation on a plot of the mobility of Multimark™ protein standards (Novex) versus the log values of their apparent masses.

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**Fig. 3.** Photoaffinity labeling of the secretin receptor on CHO-SecR cells with rat [125I-Tyr10,Bpa22]secretin-27. 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 unlabeled secretin (left panel). The saturably labeled band migrated at Mr 57,000–62,000, as previously observed for the secretin receptor (14). Shown also is the densitometric quantitation of receptor labeling in the presence of increasing concentrations of secretin (middle panel). Results reflect the means ± S.E. of data from three similar competition labeling experiments. After deglycosylation with endoglycosidase F (Endo F), the labeled band migrated in the expected position of Mr 42,000 (right panel).

**Fig. 4.** Cyanogen bromide cleavage of the photoaffinity-labeled secretin receptor. Shown on the left is a diagram of the predicted sites of cyanogen bromide cleavage of the rat secretin receptor, along with the masses of the protein cores of the fragments and the consensus sites for possible glycosylation of the receptor. Shown on the right is a typical autoradiograph of a 10% NuPAGE gel used to separate the products of cyanogen bromide cleavage of the native and deglycosylated secretin receptor that had been labeled with rat [125I-Tyr10,Bpa22]secretin-27. Cleavage of the native receptor yielded a labeled fragment migrating at apparent Mr 19,000. This shifted to Mr 9,000 after deglycosylation. These results are representative of five similar experiments. The first and third fragments best match the apparent migration of the labeled fragments observed, given the mass of the covalently attached probe (3,341 Da) and the presence of glycosylation.
mide digestion were unambiguously identified by immunoprecipitation. For this, fragments were solubilized for 15 min at room temperature in 200 μl of 0.5% Nonidet P-40 in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 0.1% SDS, 0.2% bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride, and 0.01% soybean trypsin inhibitor. Samples were divided in half, with one tube containing competing HA peptide (25 μM) and all tubes having anti-HA monoclonal antibody (2 μg). After 2 h of incubation at room temperature with gentle shaking, heat-inactivated, fixed Staphylococcus aureus (10 μl) cells were added to bind antigen-antibody complexes. After an additional hour, the adsorbent was pelleted by centrifugation at 6,000 rpm for 2 min and washed with 1 ml of solubilization buffer without bovine serum albumin. Proteins bound to the washed pellet were eluted in SDS-containing sample buffer and separated by NuPAGE gel electrophoresis.

Statistical Analysis—All observations were repeated at least three times in independent experiments and are expressed as the means ± S.E. Binding curves were analyzed using the LIGAND program of Munson and Rodbard (22) and were plotted using the nonlinear regression analysis routine for radioligand binding in the Prism software package (GraphPAD Software for Science, San Diego, CA).

RESULTS

Probe Characterization—The rat [Tyr¹⁰,Bpa²²]secretin-27 analogue of secretin was synthesized, purified to homogeneity, and characterized by amino acid analysis and Edman degradation sequencing (Fig. 1). It was a fully efficacious and potent agonist at the secretin receptor, as demonstrated by its ability to stimulate cAMP accumulation in receptor-bearing CHO-SecR cells in a concentration-dependent manner (Fig. 2). The analogue stimulated cAMP with an EC₅₀ of 68 ± 22 pm, not statistically different from natural secretin (95 ± 25 pm). Consistent with this, the analogue bound to the secretin receptor saturably, specifically, and with high affinity (Fig. 2). Analysis of the binding data with this analogue best fit a single site with a Kᵣ of 5.0 ± 1.1 nM. This also was not different from natural secretin.

Photoaffinity Labeling of the Secretin Receptor—The photoaffinity analogue covalently labeled a plasma membrane protein from the CHO-SecR cells that migrated on an SDS-polyacrylamide gel at apparent Mᵣ 55,000–62,000 (Fig. 3). This labeling was saturable and competed in a concentration-dependent manner with unlabeled secretin. Densitometric analysis of three similar experiments is also shown in Fig. 3. The IC₅₀ for this competition was 6.5 ± 2.4 nM, in the range expected from the binding affinity given the large amount of radioligand used in these experiments. As previously reported for the secretin receptor (3), deglycosylation with endoglycosidase F shifted the migration of the labeled band to Mᵣ = 42,000 (Fig. 3).

Active Site Identification—Cyanogen bromide cleavage of the affinity-labeled secretin receptor was used as a first indication of the receptor domain being covalently labeled. Shown in Fig. 4 is a graphical representation of the sites of cyanogen bromide cleavage of this receptor and the characteristics of the expected fragments. Protein cores of these fragments range in mass from 1 to 11 kDa, with three of the fragments also containing potential sites of N-linked glycosylation. Also shown in Fig. 4 is a

![Fig. 5. Characterization of the cell lines expressing epitope-tagged secretin receptors.](image-url)
typical autoradiograph of a 10% NuPAGE gel used to separate the products of cyanogen bromide cleavage of the labeled native and deglycosylated receptor. This consistently demonstrated a specifically labeled band migrating at $M_r \sim 19,000$ that shifted to $M_r \sim 9,000$ after deglycosylation. Given that the ligand probe has a mass of 3,341 Da, the core protein of the labeled fragment should be in the 5,700-Da range. With this and the requisite need for glycosylation, the only candidates represent the first and third fragments, both within the amino-terminal tail of the secretin receptor. One candidate fragment is at the end of this domain, and the other is adjacent to the first transmembrane domain. These have characteristics that are too similar for them to be distinguished by this simple manipulation.

To determine which of these domains included the site of covalent attachment, two secretin receptor constructs were prepared that incorporated HA epitope tags within each of these cyanogen bromide fragments. Each of these constructs was expressed stably in a CHO cell line. These were characterized to demonstrate normal secretin binding and signaling characteristics (Fig. 5). Each of these epitope-tagged receptor-bearing cell lines was effectively affinity-labeled with the rat $[^{125}\text{I}-\text{Tyr}^{10},\text{Bpa}^{22}]$secretin-27 probe (Fig. 6), with the labeled epitope-tagged secretin receptor migrating at a slightly higher apparent $M_r$ than the wild-type receptor (14). Both intact epitope-tagged receptor constructs were well recognized by the anti-HA monoclonal antibody, as demonstrated by their saturable immunoprecipitation in the absence and presence of competing HA peptide. However, after cyanogen bromide cleavage, only the epitope-tagged first fragment was radioactive, reflecting the presence of covalently attached rat $[^{125}\text{I}-\text{Tyr}^{10},\text{Bpa}^{22}]$secretin-27.

To further localize the site of covalent labeling of the receptor with this probe, this cyanogen bromide fragment was further cleaved with endoproteinase Lys-C. As shown in Fig. 7, this proteolytic enzyme cleaves at the carboxyl-terminal side of Lys residues, dividing the first cyanogen bromide fragment of the receptor into a non-glycosylated fragment with a mass of 3,425 Da, a glycosylated fragment with core protein of 1,808 Da, and two tiny fragments. A typical autoradiograph of a 10% NuPAGE gel used to separate various fragments of the affinity-labeled secretin receptor is shown in Fig. 7. As expected, the labeled cyanogen bromide fragment of this receptor migrated at $M_r \sim 19,000$ and shifted to $M_r \sim 9,000$ after deglycosylation with endoglycosidase F. Endoproteinase Lys-C digestion of each of these fragments shifted the labeled fragment to migrate at $M_r \sim 6,000$, with similar migration of both the native and deglycosylated fragments. Given the mass of the attached probe and
the absence of effect of deglycosylation on electrophoretic migration of the labeled products of endoproteinase Lys-C digestion, the data support the covalent attachment of this probe to the secretin receptor through the amino-terminal portion of the first fragment. Further evidence for the identity of the site of labeling as the amino-terminal domain of the first fragment came from the endoproteinase Lys-C digestion of the intact receptor, again yielding a labeled band of this same size (Fig. 7).

### DISCUSSION

The superfamily of G protein-coupled receptors is extensive and diverse, binding and being activated by ligands as different as photons, odorants, biogenic amines, small peptides, and large glycoproteins. Our understanding of the molecular basis of ligand binding is most advanced for small molecules, such as the biogenic amines, where the binding domain is intramembranous (23, 24). Binding of peptide ligands appear to be more complex, with determinants closer to the external face of the bilayer and within external loop domains. The examples of true rational design of drugs acting at peptide hormone receptors are limited, and almost all of these are antagonists. Successful mimicking of natural peptide agonists with small non-peptidyl compounds is only beginning to be successful (25) and only by screening large libraries of compounds. The development of such drugs using structure-based rational design will be markedly advanced by a better understanding of the molecular basis of ligand binding and receptor activation.

In this work, we focus on the molecular basis of agonist binding for a recently recognized group of G protein-coupled receptors, the secretin receptor family (Class II). Members of this family were first identified in 1991 (26–28) and were noted to be structurally quite distinct from the extensively studied rhodopsin/β-adrenergic receptor family (Class I). The secretin family receptors have \(<12\%\) sequence homology to the rhodopsin family and are missing all the signature sequences of that family. Although the predicted seven-transmembrane segment topology was felt to be intact in the secretin receptor family, substantial differences are even predicted to be present in the helical packing motifs (29). A prominent feature of this family is a moderately long and complex amino-terminal tail region, incorporating a series of conserved Cys residues that are predicted to contribute to critical disulfide bonds (3, 4, 30–33).

Because of the indirect nature of most mutagenesis studies, in which a function is lost or impeded, and the paucity of examples of successful unambiguous gain-of-function mutagenesis experiments, we have used the complementary approach of direct photoaffinity labeling of the ligand-binding domain of the receptor. We hoped that this would provide an unambiguous constraint to use in the initial modeling of the secretin-binding domain. This approach is dependent on the spatial proximity between a photolabile group incorporated into a l-
rational structure-based design of drugs acting at this import-
for these receptors (44–46), molecular modeling will be of great
into the conformation of peptides representing natural ligands
In the presence of such constraints and the extensive insights
from photoaffinity labeling studies will be much more mean-
Class II G protein-coupled receptors that have been gleaned
affinity labeling experiments.
 tagenesis (7), although it has not yet been directly identified in
(41). The domain adjacent to the first transmembrane domain
hormone receptor interacting with a photolabile residue in
the very recent localization of the region of the parathyroid
labeling approaches to map the binding domains of the para-
characteristics and substantial experience with this residue
otherwise well tolerated in the same position in another secretin
and for the Phe residue present in position 22 in chicken secretin
and as it resides in its binding site and a portion of the
It is noteworthy that the intrinsic photoaffinity labeling ap-
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