Identification of an Interaction between Residue 6 of the Natural Peptide Ligand and a Distinct Residue within the Amino-terminal Tail of the Secretin Receptor*

Maoqing Dong, Yan Wang, Elizabeth M. Hadac, Delia I. Pinon, Eileen Holicky, and Laurence J. Miller‡

From the Center for Basic Research in Digestive Diseases, Departments of Internal Medicine and Biochemistry/Molecular Biology, Mayo Clinic and Foundation, Rochester, Minnesota 55905

Photoaffinity labeling is a powerful tool for the characterization of the molecular basis of ligand binding. We recently used this technique to demonstrate the proximity between a residue within the carboxyl-terminal half of a secretin-like ligand and the amino-terminal domain of the secretin receptor (Dong, M., Wang, Y., Pinon, D. I., Hadac, E. M., and Miller, L. J. (1999) J. Biol. Chem. 274, 903–909). In this work, we have developed another novel radioiodinatable secretin analogue ([Bpa6,Tyr10]rat1 secretin-27) that incorporates a photolabile p-benzoyl-L-phenylalanine (Bpa) residue into position 6 of the amino-terminal half of the ligand and used this to identify a specific receptor residue proximate to it. This probe specifically bound to the secretin receptor with high affinity (IC50 = 13.2 ± 2.5 nM) and was a potent stimulant of cAMP accumulation in secretin receptor-bearing Chinese hamster ovary-SecR cells (EC50 = 720 ± 230 pm). It covalently labeled the secretin receptor in a saturable and specific manner. Cyanogen bromide cleavage of this molecule yielded a single labeled fragment that migrated on an SDS-polyacrylamide gel at Mr = 19,000 that shifted to 10 after deglycosylation, most consistent with either of two glycosylated fragments within the amino-terminal tail. By immunoprecipitation with antibody directed to epitope tags incorporated into each of the two candidate fragments, the most distal fragment at the amino terminus was identified as the domain of labeling. The labeled domain was further refined to the first 16 residues by endoproteinase Lys-C cleavage and by cyanogen bromide cleavage of another receptor construct in which Val19 was mutated to Met. Radiochemical sequencing of photoaffinity-labeled secretin receptor fragments established that Val19 was the specific site of covalent attachment. This provides the first residue-residue contact between a secretin ligand and its receptor and will contribute substantially to the molecular understanding of this interaction.

The secretin receptor is a prototypic member of the Class II family within the superfamily of guanine nucleotide-binding protein (G protein)-coupled receptors (2). These are structurally distinct from the Class I receptors in the rhodopsin/β-adrenergic receptor family, sharing their heptahelical topology but having distinct signature sequences and a unique extended amino-terminal motif with six conserved Cys residues (3, 4). The Class II receptors all bind moderately large peptides that have diffuse pharmacophoric domains (3, 4). The molecular basis of ligand binding to these receptors is not well understood.

At the present time, broad receptor domains of importance in ligand binding have been identified. This is based predominantly on mutagenesis studies, including analysis of chimeric receptors, deletion constructs, and site mutants (5–13). These studies have postulated a critical role for the amino-terminal domain of the Class II receptors in binding their natural peptide ligands. Application of these approaches to the secretin receptor have been consistent with observations with other family members (8–10). The indirect insights provided by the mutagenesis studies have been supported by the more direct analysis of interacting domains by affinity labeling (1, 14, 15). Included among these studies is the first demonstration of the affinity labeling of a segment of the amino terminus of the secretin receptor that represents the first 30 residues using a probe with its photolabile site of attachment in position 22 in the carboxyl-terminal half of the ligand (1).

However, the specific molecular basis of binding of these ligands to their receptors remains unclear. In the present work, we have attempted to define a second domain of interaction between a distinct region of secretin and its receptor and to refine the molecular details of this interaction to the level of a single residue. For this, we have developed another novel photolabile radiodinated agonist probe. This probe, [Bpa6,Tyr10]rat1 secretin-27, bound to the secretin receptor specifically and with high affinity and efficiently covalently labeled a single domain within that receptor. The ligand binding domain was localized by a series of targeted enzymatic and chemical fragmentation reactions, and the labeled residue was identified as Val19 using radiochemical Edman degradation sequencing.

MATERIALS AND METHODS

Reagents—Cyanogen bromide (CNBr), phenylisothiocyanate, and the solid phase oxidant, N-chlorobenzenesulfonamide (Iodo-beads) were from Pierce. Soybean trypsin inhibitor was from Worthington. Phenylmethylsulfonyl fluoride and N-2-aminoethyleth-1-3-aminopropyl glass beads were from Sigma. Concanavalin A-agarose was from EY Laboratories, Inc. (San Mateo, CA). Endoproteinase Lys-C and the 12CA5 monoclonal antibody directed to the hemagglutinin epitope (HA) were

‡ To whom correspondence should be addressed: Center for Basic Research in Digestive Diseases, Guggenheim 17, Mayo Clinic, Rochester, MN 55905. Tel.: 507-284-0680; Fax: 507-284-0762; E-mail: miller@mayo.edu.

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1 The abbreviations used are: Bpa, p-benzoyl-L-phenylalanine; CNBr, cyanogen bromide; HA, hemagglutinin; CHO, Chinese hamster ovary; endo F, endoglycosidase F; SecR, secretin receptor; HPLC, high performance liquid chromatography; MES, 4-morpholineethanesulfonic acid.
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from Roche Molecular Biochemicals. Endo F was prepared in our laboratory, as we have reported (16). Other reagents were analytical grade.

**Receptor Preparations**—Chinese hamster ovary (CHO) cell lines stably expressing the rat wild-type secretin receptor (CHO-SecR) and hemagglutinin epitope-tagged secretin receptor constructs SecR-HA37 and SecR-HA79, which have been previously established and characterized (1, 17), were used as sources of receptors for the current study. An additional cell line was established for this report that expresses a hemagglutinin-tagged secretin receptor construct in which Val16 was mutated to Met (SecR-V16M-HA37) to introduce an additional site for CNBr cleavage. The construct was prepared by site-directed mutagenesis, as previously reported (1). This cell line was established in a similar manner to the above cell lines and had its binding and ability to transmit fully characterized. Cells were passaged twice a week and were lifted mechanically before membrane preparation. Enriched plasma membranes from these cell lines were prepared as we previously described (18).

**Synthesis of Peptides**—Rat secretin-27, [Tyr-10]rat secretin-27, [Bpa-6,Tyr-10]rat secretin-27, and HA epitope (YPYDVPDYA) peptides were synthesized by manual solid-phase techniques and purified to homogeneity by reversed-phase HPLC (19). Identities of the peptides were determined by quantitative amino acid analysis and mass spectrometry.

**Radioiodination of Probe**—[Tyr-10]rat secretin-27 and [Bpa-6,Tyr-10]rat secretin-27 were radioiodinated oxidatively with Na125I, upon exposure to the solid phase oxidant, N-chlorobenzenesulfonamide (Iodo-beads, Pierce) for 15 s, and product was purified by reversed-phase HPLC to yield specific radioactivities of 2000 Ci/mmol (19).

**Biological Activity Determination**—The agonist activity of [Bpa-6,Tyr-10]rat secretin-27 was studied using a competition binding assay for stimulation of cAMP activity in the CHO-SecR cells (Diagnostic Products Corporation, Los Angeles, CA). The assay was performed as we have previously described (20). For this, intact cells were stimulated with hormone at 37 °C for 30 min. The reaction was stopped by adding ice-cold perchloric acid. After adjusting the pH to 6 with 30% 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 at least three times in independent experiments.

**Receptor Binding Studies**—The ability of [Bpa-6,Tyr-10]rat secretin-27 to bind to the secretin receptor was explored in a standard radioligand competition binding assay using the secretin receptor-bearing membranes from the CHO-SecR cells (17). Membranes (1–10 μg of protein) were incubated with a constant amount of radioligand [Tyr-10]rat secretin-27 (3–5 nM) and increasing concentrations of nonradiolabeled secretin analogue (0–1 μM) for 1 h at room temperature in KRH medium (25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 1 mM KH2PO4, 1.2 mM MgSO4, 2 mM CaCl2, 1 mM phenylmethylsulfonyl fluoride, 0.01% soybean trypsin inhibitor) containing 0.2% bovine serum albumin. Bound and free radioligand were separated using a Skatron cell harvester with glass fiber filtermats that had been soaked in 0.3% polybrene, with bound radioactivity quantified in a gamma counter. Nonspecific binding was determined in the presence of 1 μM secretin and represented less than 20% of total binding. A similar assay was used to characterize the binding activity of the cell line expressing the SecR-V16M-HA37 secretin receptor mutant.

**Photoaffinity Labeling of the Secretin Receptor**—For covalent labeling, receptor-bearing membranes from the CHO-SecR-HA37 cells containing approximately 100 μg of protein were incubated with 100 pm [Bpa-6,125I-Tyr-10]rat secretin-27 in KRH medium in the absence or presence of various concentrations of unlabeled secretin (from 0–1 μM). After incubation for 1 h at room temperature in the dark, the incubation...
mixture was photolyzed for 30 min at 4 °C in a Rayonet photochemical reactor (Southern New England Ultraviolet, Hamden, CT) equipped with 3500-Å lamps. Membranes were pelleted, washed, and exposed to reduction and alkylation as we previously described (1). Membrane proteins were either separated directly by electrophoresis on a 10% SDS-polyacrylamide gel (21) or solubilized with 1% Nonidet P-40 and bound to concanavalin A-agarose before SDS-polyacrylamide gel electrophoresis. For selected experiments, the affinity-labeled secretin receptor and its relevant fragments were deglycosylated with endo F under the conditions we previously reported (22).

**Chemical and Enzymatic Cleavage of the Secretin Receptor**—Gel-purified, affinity-labeled native and deglycosylated secretin receptor were digested with CNBr in 70% formic acid according to the procedure previously described (22). Endoproteinase Lys-C digestion was performed as we recently reported (1). The products of cleavage were separated on 10% NuPAGE gels (Novex, San Diego, CA) using MES buffers.

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**Fig. 3.** CNBr cleavage of photoaffinity labeled SecR-HA37. Shown is a typical autoradiograph of a 10% NuPAGE gel used to separate the products of CNBr digestion of the native and deglycosylated SecR-HA37 secretin receptor that had been labeled with [Bpa<sup>6</sup>,<sup>125</sup>I-Tyr<sup>10</sup>]rat secretin-27 and with [Bpa<sup>6</sup>,<sup>125</sup>I-Tyr<sup>22</sup>]rat secretin-27. The CNBr cleavage patterns were identical for both probes, with labeled native fragments migrating at approximate $M_r = 19,000$ and deglycosylated fragments shifting to approximate $M_r = 10,000$. This is representative of six experiments. Also shown are theoretical sites of CNBr cleavage and masses of resulting fragments, independent of carbohydrate (sites of potential glycosylation marked). Fragments 1 and 3 are possible matches for the apparent migration of the labeled fragments observed on the gel, given the masses of these fragments, the mass of the covalently attached probe (3,307 Da), and the presence of glycosylation.

**Fig. 4.** Immunoprecipitation of epitope-tagged secretin receptor constructs, SecR-HA37 and SecR-HA79. Shown in the upper panel is the graphic representation of the sites of CNBr cleavage of the amino terminus of the epitope-tagged secretin receptor constructs. Shown in the lower left panel is an autoradiograph of a typical 10% SDS-polyacrylamide electrophoresis gel used to separate the products of immunoprecipitation of intact SecR-HA37 and SecR-HA79 secretin receptor constructs labeled with [Bpa<sup>6</sup>,<sup>125</sup>I-Tyr<sup>22</sup>]rat secretin-27 in the absence and presence of competing HA. Shown in the lower right panel is an autoradiograph of a typical 10% NuPAGE gel used for separation of the immunoprecipitated products of CNBr cleavage of the same labeled receptor constructs.
running buffer, with labeled products visualized by autoradiography. 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.

**Immunoprecipitation**—Definitive identification of the affinity-labeled secretin receptor fragments was achieved by immunoprecipitation of affinity-labeled intact and digested epitope-tagged constructs. Immunoprecipitations were performed using the procedure that we previously reported (1), adsorbing the antigen-antibody complex onto protein G-agarose (Calbiochem) before application to NuPAGE gel electrophoresis.

**Peptide Sequencing**—Radiochemical sequencing was performed using Edman degradation chemistry and quantifying radioactivity released in each cycle. For this, radiolabeled CNBr and endoproteinase Lys-C fragments of the receptor that had been eluted from NuPAGE gels were precipitated with 90% acetone for at least 4 h at −20 °C. Samples were pelleted by centrifugation at 15,800 × g for 30 min at 4 °C and washed with acetone. Purified fragments were coupled to N-(2-aminoethyl-1)-3-aminopropyl glass beads through the sulfhydryl group of a Cys residue. This was accomplished by derivatizing the amino groups on the beads with N-(maleimidobenzoyl-N-hydroxysuccinimide ester at pH 7.0 and quenching remaining amino reactivity with Tris before adding the labeled receptor fragment. Cycles of Edman degradation were repeated manually in a manner that has been previously described in detail (22).

**Statistical Analysis**—All observations were repeated at least three times in independent experiments and are expressed as means ± S.E. Binding curves were analyzed using the LIGAND program of Munson and Rodbard (23) and were plotted using the nonlinear regression analysis routine for radioligand binding in the Prism software package (GraphPad Software, San Diego, CA).

**RESULTS**

**Probe Characterization**—[Bpa⁶,Tyr¹⁰]Rat secretin-27 contains a Tyr residue in position 10 for radioiodination and a photolabile Bpa in the position of Phe⁶ for covalent attachment. This secretin analogue was synthesized by manual solid-phase techniques, purified by reversed-phase HPLC, and characterized chemically by amino acid analysis and mass spectrometry. As expected, it bound to the secretin receptor saturably, specifically, and with high affinity with a $K_i$ of 26.3 ± 3.7 nM (IC⁵₀ = 13.2 ± 2.5 nM; Fig. 1). This analogue was a full agonist, stimulating intracellular cAMP accumulation in the secretin receptor-bearing CHO (CHO-SecR) cells in a concentration-dependent manner with an EC⁵₀ of 720 ± 230 pM (Fig. 1).

**Photoaffinity Labeling of the Secretin Receptor**—We have previously characterized the epitope-tagged secretin receptor mutant, SecR-HA37, and showed that it behaved similarly to the wild-type receptor, with the advantage of having the epitope tag for rapid purification and domain identification (1). This was used as source of receptor for covalent attachment. This secretin analogue was synthesized by manual solid-phase techniques, purified by reversed-phase HPLC, and characterized chemically by amino acid analysis and mass spectrometry. As expected, it bound to the secretin receptor saturably, specifically, and with high affinity with a $K_i$ of 26.3 ± 3.7 nM (IC⁵₀ = 13.2 ± 2.5 nM; Fig. 1). This analogue was a full agonist, stimulating intracellular cAMP accumulation in the secretin receptor-bearing CHO (CHO-SecR) cells in a concentration-dependent manner with an EC⁵₀ of 720 ± 230 pM (Fig. 1).

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labeled receptor resulted in a shift of migration to approximate 10,000. As expected, deglycosylation with endo F of the labeled receptor resulted in a shift of migration to approximate 42,000. Bands of this size were absent in affinity-labeled nonsecretin receptor-bearing CHO cell membranes.

Active Site Identification—CNBr has long been used for quantitative cleavage of membrane proteins (1, 22, 24). Theoretically, CNBr cleavage of the secretin receptor would result in 11 fragments ranging in molecular weight from 1 to 11 thousand (1) (Fig. 3). The spectrum of molecular masses and the presence of glycosylation at distinct sites may provide evidence for the domain being labeled using this single manipulation. Therefore, CNBr was used as a first indication of the domain being covalently labeled by \([\text{Bpa}_6,^{125}\text{I-Tyr}_{10}]\text{rat secretin-27}\). As a control, the CNBr cleavage of the same receptor that was affinity-labeled with \([^{125}\text{I-Tyr}_{10},\text{Bpa}_{22}]\text{rat secretin-27}\) was shown in Fig. 3 as a typical autoradiograph of a 10% NuPAGE gel used to separate the products of CNBr digestion of the affinity-labeled SecR-HA37 secretin receptor. As a control, the CNBr cleavage of the same receptor that was affinity-labeled with \([^{125}\text{I-Tyr}_{10},\text{Bpa}_{22}]\text{rat secretin-27}\) was shown in Fig. 3 in parallel with that of the intact SecR-V16M-HA37 receptor affinity-labeled with \([\text{Bpa}_6,^{125}\text{I-Tyr}_{10}]\text{rat secretin-27}\). Shown in Fig. 3 is a typical autoradiograph of a 10% NuPAGE gel used to separate the products of CNBr digestion of the affinity-labeled SecR-HA37 secretin receptor. As a control, the CNBr cleavage of the same receptor that was affinity-labeled with \([^{125}\text{I-Tyr}_{10},\text{Bpa}_{22}]\text{rat secretin-27}\) was shown in parallel with that of the intact SecR-V16M-HA37 receptor affinity-labeled with \([\text{Bpa}_6,^{125}\text{I-Tyr}_{10}]\text{rat secretin-27}\). Shown in Fig. 3 is a typical autoradiograph of a 10% NuPAGE gel used to separate the products of CNBr digestion of the affinity-labeled SecR-HA37 secretin receptor. As a control, the CNBr cleavage of the same receptor that was affinity-labeled with \([^{125}\text{I-Tyr}_{10},\text{Bpa}_{22}]\text{rat secretin-27}\) was shown in parallel with that of the intact SecR-V16M-HA37 receptor affinity-labeled with \([\text{Bpa}_6,^{125}\text{I-Tyr}_{10}]\text{rat secretin-27}\). The spectrum of molecular masses and the presence of glycosylation at distinct sites may provide evidence for the domain being labeled using this single manipulation. Therefore, CNBr was used as a first indication of the domain being covalently labeled by \([\text{Bpa}_6,^{125}\text{I-Tyr}_{10}]\text{rat secretin-27}\).

To identify which of these domains contained the site of covalent attachment, two well characterized secretin receptor mutants, SecR-HA37 and SecR-HA79 (1), were again used in immunoprecipitation experiments to definitively identify the domain being labeled. As shown in Fig. 4, both intact secretin receptor mutants affinity labeled with \([\text{Bpa}_6,^{125}\text{I-Tyr}_{10}]\text{rat secretin-27}\) were recognized by anti-HA monoclonal antibody as demonstrated by their saturable immunoprecipitation in the absence and presence of excess competing HA peptide. However, immunoprecipitation of CNBr fragments of both affinity-labeled receptor mutants revealed that only the fragment from the HA37-tagged construct was radioactive (Fig. 4). This provides definitive identification of fragment one at the amino-terminal tail as the affinity-labeled receptor domain.

Endoproteinase Lys-C, which specifically cleaves at Lys residues, was used to further refine the labeled receptor domain. As shown in Fig. 5, this protease cleaves CNBr fragment one of the receptor into a nonglycosylated fragment of 3,425 daltons, a glycosylated fragment with core protein of 2,910 daltons, and two tiny fragments. A typical autoradiograph of a NuPAGE gel used to separate the fragments of Lys-C digestion of the CNBr-cleaved affinity-labeled secretin receptor is shown. The fully digested band migrated at approximate \(M_r = 4,500\) and did not shift after treatment with endo F. Given the mass of the attached probe, this was most consistent with the amino-terminal portion of fragment one. To be certain of this, the intact affinity-labeled receptor was also cleaved with Lys-C. If labeling had occurred in the carboxyl-terminal portion, this would have been expected to produce a band of higher molecular mass because of the presence of glycosylation. Instead, this procedure produced a labeled band of the same size as that produced by sequential digestion of CNBr fragment one. This identified the first 30 residues at the extreme amino-terminal tail of the secretin receptor as the domain of attachment of the \([\text{Bpa}_6,^{125}\text{I-Tyr}_{10}]\text{rat secretin-27}\) analogue.

To further refine our understanding of the location of labeling, a stable cell line was established that expressed a secretin receptor mutant in which Val16 was changed to Met to introduce an additional site for CNBr cleavage (SecR-V16M-HA37). This receptor construct was functionally characterized in binding and cAMP assays. Data in Fig. 6 demonstrate specific and high affinity binding as well as stimulation of cAMP accumulation in response to secretin in these cells that is indistinguishable from that in control cells. Plasma membranes were prepared from the CHO-SecR-V16M-HA37 cells and affinity-labeled with \([\text{Bpa}_6,^{125}\text{I-Tyr}_{10}]\text{rat secretin-27}\) under the same conditions used for wild-type secretin and epitope-tagged SecR-HA37 receptors. This construct was also labeled saturably and specifically with \([\text{Bpa}_6,^{125}\text{I-Tyr}_{10}]\text{rat secretin-27}\), with the labeled band migrating on an SDS-polyacrylamide gel at approximate \(M_r = 70,000\) and shifting to \(M_r = 42,000\) after deglycosylation with endo F (Fig. 7). CNBr cleavage of the intact receptor yielded a fragment migrating at approximate \(M_r = 4,500\) that did not shift after further treatment with endo F. Like digestion of the SecR-HA37 receptor, the endoproteinase Lys-C cleavage of the intact SecR-V16M-HA37 mutant receptor yielded a radioactive fragment migrating at approximate \(M_r = 4,500\). Further digestion of this fragment with CNBr resulted in a fragment migrating at \(M_r = 4,500\). These data suggest that the site of labeling resides within the region of residue Ala1 to Met16.

FIG. 7. Cleavage of SecR-V16M-HA37 receptor affinity-labeled with \([\text{Bpa}_6,^{125}\text{I-Tyr}_{10}]\text{rat secretin-27}\). Shown is a typical autoradiograph of a NuPAGE gel used to separate products of labeling of SecR-V16M-HA37 receptor-bearing membranes in the presence and absence of excess competing unlabeled secretin (1 µM). Like the SecR-HA37 secretin receptor, the affinity-labeled secretin receptor migrated at approximate \(M_r = 70,000\). After deglycosylation with endo F, this band migrated at approximate \(M_r = 42,000\). CNBr digestion of both native and deglycosylated receptor mutant yielded fragments migrating similarly at \(M_r = 4,500\). Digestion of the intact affinity-labeled SecR-V16M-HA37 receptor with Lys-C alone yielded a fragment migrating at \(M_r = 6,000\). Further digestion of this fragment with CNBr resulted in a fragment migrating at \(M_r = 4,500\). These data suggest that the site of labeling resides within the region of residue Ala1 to Met16.
The site of covalent labeling of the secretin receptor with \([\text{Bpa}^6,125\text{I}-\text{Tyr}^{10}]\text{rat secretin-27}\) was further localized by radiochemical sequencing using manual Edman degradation. For this, the radiolabeled receptor fragments resulting from CNBr and Lys-C digestions were purified by NuPAGE gel electrophoresis. The profiles from this sequencing are shown in Fig. 8. These were repeated with six independent samples for each fragment, with the radioactive peak eluting consistently in cycle 4 in all experiments. This corresponds with covalent attachment to Val^4 of the secretin receptor. Of note, similar results were observed with radiochemical sequencing of the purified affinity-labeled fragment resulting from endoproteinase Lys-C digestion of a secretin receptor mutant in which we inserted an initiation codon and a Flag epitope sequence (DYKDDDDK) just prior to the predicted start of the mature receptor.

There is extraordinary structural diversity of ligands that can bind to and activate molecules in the superfamily of G protein-coupled receptors. Of particular interest is the correlation between the structures of groups of ligands and the structural themes expressed within the groups of G protein-coupled receptors that they recognize (25, 26). This organization has been helpful in identifying the ligands for orphan receptor cDNA sequences that have been cloned. It has also been helpful in the identification of general themes for the binding determinants for groups of ligands, such as biogenic amine binding occurring in the intramembranous confluence of helices and glycoprotein hormones binding to large extracellular domains (27). However, we have detailed insights into the molecular basis of ligand binding for only a very small subset of these receptors (28).

All the ligands that bind to and activate the Class II receptor family that have been identified to date have consistent structural features, representing moderately large peptides with diffuse pharmacophoric domains (29). Additionally, these peptides all have prominent areas of a helical content, with the extent of helix dependent on the hydrophobicity of their environment (30–32). The structure of secretin is consistent with these themes (33, 34). Of particular interest is the demonstration that two segments of a helix within secretin are separated by a turn, with this also affected by the environment. It is quite likely that the conformation of these peptides changes as they move from the aqueous milieu of the circulation and bind to their receptor molecules.

We have been particularly interested in the conformation of secretin as it binds to its receptor. To gain insight into this, we are building a series of probes that are structurally similar to secretin while incorporating a photolabile site of covalent attachment in different positions throughout the pharmacophore. These should be useful in a strategy of intrinsic photoaffinity labeling and site determination. The first of these probes had a photolabile Bpa residue in position 22, within the carboxyl-terminal half of the ligand (1). It was demonstrated to covalently label a specific receptor domain consisting of the first 30 residues at the amino terminus (1).

The present work has extended this insight by siting a Bpa in position 6, within the amino-terminal half of the ligand. Importantly, we have been able to substantially extend the level of insight provided by this contact to the level of a single residue of labeling, Val^4, near the amino terminus of the receptor. Such insight will finally provide a solid constraint that can ultimately be built into meaningful conformational models of the ligand-bound receptor.

It is particularly interesting that the domains labeled with the Bpa^22- and Bpa^6-containing secretin analogues were both near the end of the amino terminus of the secretin receptor. This is within a predicted helical domain of the receptor. Helix-helix interaction has been postulated to exist in the recognition of ligands for Class II receptors (30, 31). This is also consistent with the mutagenesis studies, which suggested a critical importance of the amino-terminal tail region of the secretin receptor, although some extracellular loop domains may complement this domain and contribute to secretin binding and activation of its receptor (7–10, 35). This theme has been consistent for other members in Class II receptor family, such as the calcitonin receptor (12), the vasoactive intestinal polypeptide receptor (8, 10), the parathyroid hormone receptor (14, 36), the pituitary adenylyl cyclase-activating peptide receptor (11, 37).

By identifying a specific receptor residue that is proximate to a specific residue within a secretin agonist ligand, we have provided a valuable constraint to contribute to a meaningful molecular model of the agonist-bound receptor. At the present time, however, such a model cannot be proposed because of inadequate insight into the conformation of the important and long amino-terminal domain of the receptor. Delineation of the disulfide bonds that are predicted to exist and involve the highly conserved Cys residues within this domain (38) may provide key constraints to begin the modeling of this region. It will be particularly interesting and important to further localize the receptor residue proximate to the Bpa^22 residue within the probe we used previously (\([\text{I}^{251}\text{I}-\text{Tyr}^{10},\text{Bpa}^{22}]\text{rat secretin-27}\) (1). With such information, two distinct molecular contacts could be introduced. Although there will still be substantial opportunity for flexibility and movement of different...
parts of the ligand relative to the receptor, these insights will generate testable hypotheses to model residue interactions.

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