Secretin Occupies a Single Protomer of the Homodimeric Secretin Receptor Complex

INSIGHTS FROM PHOTOAFFINITY LABELING STUDIES USING DUAL SITES OF COVALENT ATTACHMENT

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The secretin receptor, a prototypic family B G protein-coupled receptor, forms a constitutive homodimeric complex that is stable even in the presence of hormone. Recently, a model of this agonist-bound receptor was built based on high resolution structures reported for amino-terminal domains of other family members. Although this model provided the best solution for all extant data, including 10 photoaffinity labeling constraints, a new such constraint now obtained with a position 16 photolabile probe was inconsistent with this model. As the secretin receptor forms constitutive homodimers, we explored whether secretin might dock across both protomers of the complex, an observation that could also contribute to the negative cooperativity observed. To directly explore this, we prepared six secretin analogue probes that simultaneously incorporated two photolabile benzoylphenylalanines as sites of covalent attachment, in positions known to label distinct receptor subdomains. Each bifunctional probe was a full agonist that labeled the receptor specifically and saturably, with electrophoretic migration consistent with labeling a single protomer of the homodimeric secretin receptor. No band representing radiolabeled receptor was observed with any bifunctional probe. The labeled monomeric receptor bands were cleaved with cyanogen bromide to demonstrate that both of the photolabile benzoylphenylalanines within a single probe had established covalent adducts with a single receptor in the complex. These data are consistent with a model of secretin occupying a single secretin receptor protomer within the homodimeric receptor complex. A new molecular model accommodating all constraints is now proposed.

The secretin receptor is prototypic of family B G protein-coupled receptors (GPCRs), a group containing several potentially important drug targets. Like many members of this superfamily, the secretin receptor has been shown to form oligomers (1). These form constitutively and remain intact after secretin binding (1). Of note, these complexes are structurally quite specific, forming homodimers along the lipid-exposed face of transmembrane segment 4 (2, 3), and not forming higher order oligomers (4). Furthermore, this dimeric complex has been shown to be important to achieve the high affinity state of secretin binding and to be responsible for the negative cooperativity of hormone binding that has been observed (2). In some receptors, such as the insulin receptor, ligand interaction with both protomers of a homodimeric structure has been shown to contribute to this type of binding behavior (5, 6).

Until the present, all natural peptide ligands of family B GPCRs have been proposed as occupying a single protomer, despite numerous reports of oligomerization of these receptors (1–4, 7, 8). Indeed, to date, 10 spatial approximation constraints have been reported for photolabile analogues of secretin (9), and all of these have been successfully accommodated in a molecular model in which secretin occupied a single receptor protomer (9). Although a number of single ligand-receptor protomer models were capable of accommodating the data, this model provided the best solution for all extant constraints, including cross-linking data, FRET distances, mutagenesis, and ligand structure-activity series (9–11).

In the current report, we have extended and tested the previously proposed molecular model of secretin-occupied secretin receptor (9) using another high affinity, biologically active analogue with a photolabile p-benzoyl-l-phenylalanine (Bpa) moiety in position 16, in the midregion of the ligand. This new spatial approximation constraint was not compatible with the previous best fit model. We therefore elected to explore the possibility that these sites of covalent attachment might come from both protomers of the secretin receptor homodimer, with the hormone docked across both protomers, or that alternate
Molecular Basis of Secretin Binding

Secretin (natural)

\[
\begin{array}{cccccccccc}
 & 1 & 5 & 10 & 15 & 20 & 25 \\
Bpa^{16} \text{ probe} & H-S-D-G-T-F-T-S-E-L-S-R-L-Q-D-S-A-R-L-Q-R-L-Q-G-L-V-NH_{2} \\
 & 125^{I} & & & & & & & & \\
Bpa^{2,26} \text{ probe} & H-S-D-G-T-F-T-S-E-Y-S-R-L-Q-D-#-A-R-L-Q-R-L-Q-G-L-V-NH_{2} \\
 & 125^{I} & & & & & & & & \\
Bpa^{16,21} \text{ probe} & H-S-D-G-T-F-T-S-E-Y-S-R-L-Q-D-#-A-R-L-Q-#-L-L-Q-G-L-V-NH_{2} \\
 & 125^{I} & & & & & & & & \\
Bpa^{16,22} \text{ probe} & H-S-D-G-T-F-T-S-E-Y-S-R-L-Q-D-#-A-R-L-Q-R-#-L-L-Q-G-L-V-NH_{2} \\
 & 125^{I} & & & & & & & & \\
Bpa^{16,26} \text{ probe} & H-S-D-G-T-F-T-S-E-Y-S-R-L-Q-D-#-A-R-L-Q-R-L-Q-G-#-V-NH_{2} \\
 & 125^{I} & & & & & & & & \\
Bpa^{21,26} \text{ probe} & H-S-D-G-T-F-T-S-E-Y-S-R-L-Q-D-#-A-R-L-Q-R-L-Q-G-#-V-NH_{2} \\
 & 125^{I} & & & & & & & & \\
Bpa^{22,26} \text{ probe} & H-S-D-G-T-F-T-S-E-Y-S-R-L-Q-D-#-A-R-L-Q-R-L-Q-G-#-V-NH_{2} \\
 & 125^{I} & & & & & & & & \\
\end{array}
\]

FIGURE 1. Primary structures of photolabile secretin analogues used in this study. Shown are the amino acid sequences of natural secretin and its photolabile analogues. Natural residues are illustrated in gray, whereas modified residues are shown in black. #, positions of incorporation of the photolabile Bpa moiety.

single ligand-protomer models could better accommodate the new data.

To examine these possibilities, we developed a series of six secretin analogue probes that simultaneously incorporated two photolabile Bpa moieties as sites of covalent attachment in positions that were known to label distinct receptor subdomains. Each of these bifunctional probes was a full agonist that bound to the secretin receptor specifically and saturably. Each was able to efficiently covalently label the secretin receptor. Of note, despite evidence that both photolabile moieties within each of the probes established covalent adducts with the receptor after photolysis, all labeled bands corresponded to the labeling at a receptor protomer within the receptor dimer. This made the postulated ligand docking across the receptor homodimer highly unlikely. We therefore refined the molecular model to establish ligand-receptor interactions that were compatible with all experimental constraints, illustrating the docking of secretin within a single receptor protomer.

EXPERIMENTAL PROCEDURES

Materials—Cyanogen bromide (CNBr), N-chlorobenzenesulfonamide (IDO-BOADs), m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester, disuccinimidyldi carbonate, and 2-(2-nitrophenylsulfenyl)-3-methyl-3-bromoisindoline (skatole) were purchased from Pierce. Phenylmethylsulfonyl fluoride, 3-isobutyl-1-methylxanthine, and N-(2-aminoethyl-1)-3-aminopropyl glass beads were from Sigma. 10% BisTris premixed standards were from Cambrex (Rockland, ME). Endoproteinase Lys-C (Lys-C) and the anti-hemagglutinin (HA) epitope monoclonal antibody were from Roche Applied Science. Soybean trypsin inhibitor, Fetal Clone-2, and tissue culture medium were from Invitrogen. Endoglycosidase F was prepared in our laboratory, as described previously (12). All other reagents were of analytical grade.

Synthetic Peptides—A series of monofunctional secretin probes incorporating a single photolabile Bpa residue that had previously been prepared were used for the current study. These included [Bpa^{2,Gly-1,10,Tyr^{10}}]-rat secretin-27 (Bpa^{2-Gly} probe) (13), [Tyr^{10,Bpa^{21}}]-rat secretin-27 (Bpa^{21} probe) (10), [Tyr^{10,Bpa^{21}}]-rat secretin-27 (Bpa^{21} probe) (14, 15), and [Tyr^{10,Bpa^{26}}]-rat secretin-27 (Bpa^{26} probe) (14). A new monofunctional photolabile secretin probe, [Tyr^{10,Bpa^{26}}]-rat secretin-27 (Bpa^{26} probe), was also synthesized for the current work (Fig. 1). This probe was designed to incorporate a photolabile Bpa in position 16 to replace a serine located within the midregion of the ligand and a tyrosine in position 10 to replace a leucine as a site of radioiodination that has been well tolerated in previous analogous probes (16, 17).

Additionally, six bifunctional photolabile secretin probes were synthesized for this work (Fig. 1). These included [Bpa^{2,Gly-1,10,Tyr^{10,Bpa^{26}}}}]-rat secretin-27 (Bpa^{2,Gly} probe), [Tyr^{10,Bpa^{16,21}}]-rat secretin-27 (Bpa^{16,21} probe), [Tyr^{10,Bpa^{16,22}}]-rat secretin-27 (Bpa^{16,22} probe), [Tyr^{10,Bpa^{16,26}}]-rat secretin-27 (Bpa^{16,26} probe), [Tyr^{10,Bpa^{21,26}}]-rat secretin-27 (Bpa^{21,26} probe), and [Tyr^{10,Bpa^{22,26}}]-rat secretin-27 (Bpa^{22,26} probe). They were designed to simultaneously incorporate two Bpa residues into positions of secretin known to label distinct receptor structural domains. Each of these probes also incorporated a tyrosine in position 10 for radioiodination.

All of the above peptides and another secretin analogue to be used as a radioligand, [Tyr^{10}}]-rat secretin-27, were synthesized using the procedures described previously (18). They were radioiodinated oxidatively using 1 mCi of Na^{125I} and exposure to the IODO-BEAD solid phase oxidant for 15 s and were purified using reversed-phase HPLC to yield specific radioactivities of ~2,000 Ci/mmol (18).

Receptor Sources—Chinese hamster ovary cell lines stably expressing the wild-type rat secretin receptor (CHO-SecR) (19) and HA-tagged rat secretin receptor constructs (CHO-SecR-HA37 and CHO-SecR-HA79) (15) were utilized as sources of receptors. They were cultured in Ham’s F-12 medium supplemented with 5% Fetal Clone-2 on Falcon tissue culture plasticware in a 5% CO_{2} environment at 37 °C. Cells were passaged approximately twice a week and lifted mechanically before use. A particulate fraction enriched in plasma membranes was prepared using the procedures described previously (18). They were used as sources of receptors. The membranes were suspended in Krebs-Ringer/HEPES (KRH) medium (25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 2 mM CaCl_{2}, 1 mM KH_{2}PO_{4}, 1.2 mM MgSO_{4}) containing 0.01% soybean trypsin inhibitor and 1 mM...
phenylmethylsulfonyl fluoride and were stored at −80 °C until they were to be used in ligand binding and photoaffinity labeling studies.

In addition, it was necessary to generate two new secretin receptor mutants that introduced sites for Lys-C and CNBr cleavage in the amino terminus of the secretin receptor. These receptor constructs contained changes of Arg16 to lysine (R96K) and Asn106 to methionine (N106M). These were prepared using an oligonucleotide-directed approach with the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA) with sequences verified by direct DNA sequencing. Both receptor mutants were expressed transiently in COS-1 cells (American Type Culture Collection, Manassas, VA) after transfection using a modification of the DEAE-dextran method (20). Cells were maintained under the same conditions as the CHO cells described above, except the culture medium was Dulbecco’s modified Eagle’s medium supplemented with 5% Fetal Clone-2. Forty-eight hours after transfection, cells were used either directly in binding and biological activity studies or for membrane preparation for photoaffinity labeling studies.

Ligand Binding—The newly synthesized monofunctional photolabile Bpa16 probe was characterized to test its ability to bind to the secretin receptor. This was performed with receptor-bearing CHO-SecR cells using conditions that have been reported previously (12). In brief, increasing concentrations of the nonradiolabeled Bpa16 ligand probe or control secretin ranging from 0 to 1 µM were incubated with ~10 µg of membranes prepared from CHO-SecR cells in the presence of a constant amount of the radioligand, 125I-[Tyr10]rat secretin-27 (5–10 pm, ∼20,000 cpm) in 500 µl of KRH medium containing 0.01% soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, and 0.2% bovine serum albumin for 1 h at room temperature. After incubation, the membrane-bound radioligand was separated from free radioligand with a Skatron cell harvester (Molecular Devices, Sunnyvale, CA), using receptor-binding filter mats that had been presoaked in 0.3% hexadimethrine bromide (Polybrene). Bound radioactivity was quantified using a γ-counter. Nonspecific binding was determined in the presence of 1 µM unlabeled secretin and represented less than 15% of total binding. This assay was also used for characterization of the two new secretin receptor mutants expressed in COS-1 cells in a similar manner.

Biological Activity—The monofunctional Bpa16 and the six bifunctional photolabile secretin probes described above were characterized to test their abilities to stimulate biological responses in CHO-SecR cells by quantification of intracellular cAMP accumulation. For this, 80,000 cells were plated in each well of 96-well plates and cultured for 48 h. Cells were washed twice with phosphate-buffered saline and stimulated for 30 min at 37 °C with increasing concentrations of each of the secretin analogues to be characterized or control secretin (0–1 µM) in 50 µl of KRH medium containing 0.1% soybean trypsin inhibitor, 0.2% bovine serum albumin, 0.1% bacitracin, and 1 mM 3-isobutyl-1-methyloxanthine. The reaction was terminated by removing the peptide solution and adding 6% (w/v) ice-cold perchloric acid. After vigorous agitation for 15 min, the cell lysates were adjusted to pH 6.0 with 30% KHCO3 before being introduced directly into a time-resolved FRET immunoassay for cAMP quantification in a 384-well plate using a LANCE™ cAMP kit from PerkinElmer Life Sciences. The assay was performed as per the manufacturer’s instructions and repeated in at least three independent experiments. This assay was also used for characterization of the two new secretin receptor mutants expressed in COS-1 cells.

Photoaffinity Labeling—Photoaffinity labeling of the secretin receptor by the monofunctional Bpa16 and the six bifunctional photolabile secretin probes was performed as described previously (15). In brief, ∼50 µg of enriched CHO-SecR plasma membranes were incubated with ∼0.1 nm 125I-labeled Bpa16 probe or one of the bifunctional photolabile secretin probes in 500 µl of KRH medium containing 0.01% soybean trypsin inhibitor and 1 mM phenylmethylsulfonyl fluoride in the presence of increasing amounts of competing secretin (0–1 µM). Incubations were performed in the dark for 1 h at room temperature, and the reactions were then exposed to photolysis for 30 min at 4 °C using a Rayonet photochemical reactor (Southern New England Ultraviolet Co., Bradford, CT) equipped with 3500-Å lamps. After being washed twice with ice-cold KRH medium, membranes were solubilized in SDS sample buffer before being applied to 10% SDS-polyacrylamide gels. Gels were stained with Coomassie Blue R-250 and bands were visualized by autoradiography. The apparent molecular masses of the radioactive bands were determined by interpolation on a plot of the mobility of the ProSieve protein standards versus the log values of their apparent masses. The Kᵢ values were calculated from concentration-dependent curves. The position on the gels of secretin receptor homodimer was definitively established using Western blotting of an HA epitope-tagged secretin receptor construct ([1247C]SecR-HA37) that was cross-linked with cuprous phenanthroline, as we described previously (2).

Peptide Mapping—For localization of receptor regions and specific sites of covalent labeling, larger aliquots of membranes (∼200 µg) and ∼0.5 nm 125I-labeled probes were used. After gel electrophoresis, labeled bands prepared on this large scale were excised, eluted, lyophilized, and ethanol-precipitated before being used for deglycosylation and peptide mapping by chemical and enzymatic cleavages. Deglycosylation of the labeled secretin receptor was performed with endoglycosidase F, using procedures that we described previously (12). CNBr and Lys-C were used to cleave the labeled secretin receptor, following procedures described previously (15). Immunoprecipitation of digested HA-tagged secretin receptors labeled with the Bpa16 probe was performed using anti-HA monoclonal antibody to determine the identities of some of the labeled fragments (15). Products of cleavage were separated on 10% BisTris NuPAGE gel using MES running buffer, and labeled bands were detected by autoradiography. The apparent molecular weights of the radiolabeled receptor fragments were determined by interpolation on a plot of the mobility of Multimark multicolored or SeeBlue Plus-2 prestained standards versus the log values of their apparent masses.

Radiochemical Sequencing—This procedure was used to determine the specific receptor residue covalently labeled by the Bpa16 probe. The labeled Pro177–Lys119 fragment from Lys-C cleavage of the wild type secretin receptor was gel-puri-
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ried to radioactive homogeneity before being covalently coupled through Cys\textsuperscript{101} to maleimidobenzoyl succinimide-activated N-(2-aminoethyl-1)-3-aminopropyl glass beads. This was followed by repetitive cycles of manual Edman degradation with quantitation of radioactivity released in each cycle, using the method reported previously (21).

Molecular Modeling—All molecular modeling was conducted using a stochastic global energy optimization procedure in Internal Coordinate Mechanics (ICM) using the ICM-Pro package version 3.6 (MolSoft LLC, San Diego, CA) (22). This procedure consisted of three iterative steps: 1) random conformational change of a dihedral angle according to the biased probability Monte Carlo method (23); 2) local minimization of all free dihedral angles; and 3) acceptance or rejection of the new conformation based on the Metropolis criterion at the simulation temperature, usually at 600 K (24).

The initial homology model of the rat secretin receptor amino-terminal domain was generated using the crystal structure of the amino terminus of the gastric inhibitory polypeptide receptor (25). A pentasaccharide, Man\textsubscript{3}GlcNAc\textsubscript{2}, was attached to secretin receptor residues Asn\textsubscript{50}, Asn\textsubscript{51}, Asn\textsubscript{141}, and Asn\textsubscript{150} to mimic their glycosylated state. The initial conformation of the secretin peptide was generated using the NMR structure of receptor-bound PACAP\textsubscript{(1–21)}NH\textsubscript{2} as template and aligning this with gastric inhibitory polypeptide in the gastric inhibitory polypeptide-gastric inhibitory polypeptide receptor complex to determine its initial docking pose. It is noted that this initial pose did not satisfy all experimentally determined photoaffinity labeling constraints. The whole complex was therefore globally optimized in the presence of photoaffinity labeling constraints between the peptide and the amino-terminal domain of the receptor (respectively) (Arg\textsubscript{12} to Val\textsubscript{6}, Leu\textsubscript{13} to Val\textsubscript{103}, Ser\textsubscript{16} to Leu\textsubscript{26} to Leu\textsubscript{36}) and four FRET distance constraints (11). The best fits occurred when the backbone dihedral angles surrounding the first and third disulfide bonds of the amino-terminal domain were relaxed to allow for some rotational freedom. Twenty of the lowest energy complexes were retained.

The peptide-amino-terminal receptor complexes were then docked onto 60 diverse receptor core domain models, arising from 10 distinct helical bundles, with each completed with 10 different loop conformations. This domain docking took advantage of two additional photoaffinity labeling constraints involving the receptor core (peptide to receptor residues, respectively), His\textsuperscript{1} to Phe\textsuperscript{338} and Thr\textsuperscript{2} to Phe\textsuperscript{349}, and 12 FRET distance constraints between the peptide and the transmembrane domain, as previously described (9). The previously used soft restraint between the WDN sequence within the amino-terminal receptor domain and the top of the helical bundle domain was not utilized in this modeling effort. All of the resultant models were clustered and ranked by their ICM energetics and their health as established by PROCHECK and WHAT_CHECK evaluations (26, 27). The best models were selected.

Statistical Analysis—All of the above 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 (28) and were plotted using the nonlinear regression analysis routine for radioligand binding in the Prism program version 3.02 package (GraphPad Software, San Diego, CA).

RESULTS

Characterization of the Bpa\textsuperscript{16} Probe—The Bpa\textsuperscript{16} probe was synthesized by manual solid phase techniques and purified by reversed-phase high pressure liquid chromatography, and its identity was verified by mass spectrometry. It bound to the secretin receptor specifically and saturably (secretin, $K_i =$ 4.3 ± 0.9 nM; Bpa\textsuperscript{16} probe, $K_i =$ 10.2 ± 1.2 nM). It was a full agonist (secretin, $EC_{50} =$ 36 ± 8 pm; Bpa\textsuperscript{16} probe, $EC_{50} =$ 53 ± 10 pm), stimulating cAMP accumulation in CHO-SecR cells in a concentration-dependent manner. The binding affinity and biological activity of this probe were similar to that of natural secretin (Fig. 2).

Photoaffinity Labeling of the Secretin Receptor by the Bpa\textsuperscript{16} Probe—The Bpa\textsuperscript{16} probe was further examined for its ability to covalently label the secretin receptor. Fig. 3 shows that it
labeled the secretin receptor specifically and saturably, with the labeling being inhibited by natural secretin in a concentration-dependent manner (IC$_{50}$ = 52 ± 11 nM). The labeled receptor band migrated at approximate $M_r = 70,000$ and shifted to approximate $M_r = 42,000$ after deglycosylation. No radioactive band was observed in the affinity-labeled non-receptor-bearing CHO cell membranes.

**Identification of the Site of Labeling by the Bpa$^{16}$ Probe**—CNBr was used to provide an initial indication of the region of labeling by the Bpa$^{16}$ probe based on its ability to quantitatively cleave a protein at the carboxyl-terminal side of methionine residues. The secretin receptor contains nine methionine residues, and CNBr cleavage would theoretically result in 10 receptor fragments ranging in molecular mass from 1 to 11 kDa, with three of these containing sites of glycosylation (Fig. 4). As shown in Fig. 4, CNBr cleavage of the labeled secretin receptor resulted in a band that migrated at approximate $M_r = 31,000$ and shifted to approximate $M_r = 9,000$ after deglycosylation. Taking into account the molecular mass of the attached Bpa$^{16}$ probe (3,242 Da) and the glycosylated nature of the labeled band, the receptor fragment labeled could be limited to two candidates. Both are within the amino terminus of the receptor, with one representing the most
distal fragment at the amino terminus (fragment one) and the other representing the segment adjacent to the first transmembrane segment (fragment three) (Fig. 4). Previous experience with the electrophoretic migration of each of these CNBr fragments makes the third fragment most likely (15, 29).

To definitively establish the third fragment (receptor residues 74–123) as the region of labeling, two well characterized receptor mutants that incorporate an HA epitope within the two candidate fragments (SecR-HA37 and SecR-HA79) (15) were used in immunoprecipitation experiments. Immunoprecipitation of the CNBr fragments from the HA37- and HA79-tagged secretin receptor constructs labeled with the Bpa16 probe revealed that only the fragment from the HA79-tagged receptor was radioactive, and its precipitation was prevented in the presence of excess HA peptide (Fig. 4). This provided definitive evidence that the site of covalent labeling with the Bpa16 probe was within CNBr fragment three. It should be noted that the immunoprecipitated band resulting from CNBr cleavage of the labeled SecR-HA79 receptor ($M_r = 25,000$) migrated differently from that labeled with the wild type secretin receptor ($M_r = 31,000$). This difference in migration probably reflects the introduction of the HA tag after Asn78 in the HA79 construct that eliminated the consensus site for its glycosylation (29).

To further localize the region of the secretin receptor labeled with the Bpa16 probe, the labeled CNBr fragment was further cleaved at tryptophan residues with skatole. Theoretically, this reagent would cleave this fragment of the receptor into two glycosylated fragments having core proteins of 1,940 and 3,834 Da. Fig. 5 shows that skatole cleavage of the labeled wild type secretin receptor ($M_r = 31,000$ shifting to $M_r = 9,000$ after deglycosylation), indicating that the fragment Leu74–Asn106 was the region of labeling (bottom). Considered together, the site of labeling with the Bpa16 probe was within the region between Pro97 and Asn106 of the secretin receptor (heavy circles).

FIGURE 6. Cleavage of the R96K and N106M secretin receptor mutants labeled with the Bpa16 probe. Top, Lys-C cleavage of the labeled wild type secretin receptor (WT) and the R96K mutant resulted in bands migrating at approximate $M_r = 30,000$ and $M_r = 12,500$ that further shifted to approximate $M_r = 8,000$ and $M_r = 6,500$ after deglycosylation with endoglycosidase F (EF), respectively, indicating that the site of labeling was within the Pro97–Met123 fragment. Bottom, CNBr cleavage of the labeled N106M receptor yielded a band migrating at approximate $M_r = 23,000$ and shifting to approximate $M_r = 7,000$, distinct from the pattern of cleavage of the wild type secretin receptor ($M_r = 31,000$ shifting to $M_r = 9,000$ after deglycosylation), indicating that the fragment Leu74–Asn106 was the region of labeling (bottom). Considered together, the site of labeling with the Bpa16 probe was within the region between Pro97 and Asn106 of the secretin receptor (heavy circles).

FIGURE 7. Identification of the receptor residue labeled with the Bpa16 probe. Shown is the radioactive elution profile of Edman degradation sequencing of the purified Lys-C fragment (Pro97–Met123) resulting from cleavage of the R96K secretin receptor mutant labeled with the Bpa16 probe. There was a consistent peak in cycle 3 that corresponds with covalent labeling of residue Leu99 of the secretin receptor.
To further localize the site of labeling by the Bpa16 probe, two new secretin receptor mutants, R96K and N106M, were prepared. Both receptor mutants bound secretin (wild type, \(K_i = 4.1 \pm 0.9 \text{ nM} \); R96K, \(K_i = 6.7 \pm 1.3 \text{ nM} \); N106M, \(K_i = 3.1 \pm 0.8 \text{ nM} \)) and signaled similarly to the wild type receptor (wild type, \(EC_{50} = 87 \pm 17 \text{ pM} \); R96K, \(EC_{50} = 47 \pm 10 \text{ pM} \); N106M, \(EC_{50} = 101 \pm 30 \text{ pM} \)). They were also specifically labeled with the Bpa16 probe (data not shown).

As shown in the upper panel of Fig. 6, Lys-C cleavage of the labeled wild type secretin receptor yielded a band migrating at approximate \(M_r = 30,000 \) and shifting to approximate \(M_r = 8,000 \) after deglycosylation, consistent with the fragment between Asn78 and Lys119. Lys-C cleavage of the labeled R96K receptor resulted in a band migrating at approximate \(M_r = 12,500 \) and shifting to approximate \(M_r = 6,500 \) after deglycosylation, indicating that the fragment between Pro97 and Lys119 was the region of labeling. As shown in the bottom panel of Fig. 6, CNBr cleavage of the labeled N106M receptor mutant yielded a band migrating at approximate \(M_r = 23,000 \) and shifting to approximate \(M_r = 7,000 \), distinct from the migration of the bands resulting from cleavage of the labeled wild type receptor. This localized the region of covalent labeling as the fragment between Leu74 and Asn106. Considering the data from both mutants, the region between Pro97 and Asn106 was identified as containing the site of labeling with the Bpa16 probe.

To identify the specific site of labeling with the Bpa16 probe, the radiochemically pure Lys-C fragment (Pro97–Lys119) from the photoaffinity-labeled secretin receptor R96K mutant was utilized for manual radioactive Edman degradation sequencing. Fig. 7 shows a representative radioactive profile of eluted radioactivity in which a peak eluted in cycle 3. This corresponds to the covalent labeling of receptor residue Leu99, in the carboxyl terminus of the amino-terminal tail of the secretin receptor.

Incompatibility of New Photoaffinity Labeling Constraint with Previous Molecular Models—Shown in Fig. 8 is the previously proposed molecular model of secretin-occupied secretin receptor (9) in which the site of the photolabile moiety (Bpa in position 16 of the probe) and its site of covalent labeling the receptor (receptor residue Leu99) have been highlighted. Note that this new experimentally derived spatial approximation constraint is not compatible with the previously proposed best fit model because of the excessive distance between these residues and because part of the receptor amino terminus obstructs the direct access of these residues to each other. This suggests three possibilities: 1) the assumption that...
the secretin receptor has the same structural folds as other family B GPCR members could be wrong; 2) the photoaffinity labeling spatial approximation constraints could reflect labeling of two receptor protomers rather than one; 3) one or more of the other constraints that were utilized to dock the peptide in its location in the previous model could have skewed the results. The evidence for conservation of structure of the amino terminus of family B GPCRs is compelling, making the first stated possibility highly unlikely. We therefore next moved to critically evaluate the second stated possibility using bifunctional photolabile probes.

**Characterization of the Bifunctional Photolabile Secretin Probes**

The six bifunctional probes were synthesized and purified, and their chemical identity was verified. They were functionally characterized using CHO-SecR cells. As shown in Fig. 9, all probes were fully efficacious agonists, stimulating maximal intracellular cAMP responses that were not different from those achieved in response to natural secretin. They were, however, less potent than natural secretin, except for the Bpa16,26 probe (secretin, EC50 = 25 ± 7 pm; Bpa16,21 probe, EC50 = 94 ± 21 pm; Bpa16,22 probe, EC50 = 169 ± 36 pm; Bpa16,26 probe, EC50 = 15 ± 6 pm; Bpa16,26 probe, EC50 = 306 ± 77 pm; Bpa16,22 probe, EC50 = 523 ± 103 pm; Bpa16,26 probe, EC50 = 541 ± 88 pm). Due to the hydrophobic nature of these dual Bpa peptides, there was very high nonspecific binding in a standard radioligand binding assay, precluding its use for determination of binding affinity. Instead, the affinities of these peptides for this receptor were approximated based on secretin competition for photoaffinity labeling described below.

**Photoaffinity Labeling of the Secretin Receptor by Bifunctional Photolabile Secretin Probes**

These bifunctional probes were next explored for their ability to covalently label the secretin receptor. Fig. 10 shows that each of these probes labeled the
Photoaffinity labeling of the secretin receptor with bifunctional secretin probes. Absence of secretin receptor homodimer after cross-linking with cuprous phenanthroline (CuP) was previously demonstrated to be Phe\(^{316}\) (13). The regions of the secretin receptor that were affinity labeled with each of the bifunctional probes were also identified by CNBr cleavage. As shown in Fig. 12, CNBr cleavage of the secretin receptor labeled by the bifunctional Bpa\(^{16,21}\), Bpa\(^{16,22}\), and Bpa\(^{16,26}\) probes each resulted in a band migrating at approximate \(M_r = 46,500\) and shifting to approximate \(M_r = 15,500\). This pattern is consistent with the first (Ala\(^1\)–Met\(^{344}\)) and third (Leu\(^{74}\)–Met\(^{123}\)) CNBr fragments being covalently linked by the bifunctional Bpa\(^{16,21}\), Bpa\(^{16,22}\), and Bpa\(^{16,26}\) probes. CNBr cleavage of secretin receptor labeled by the bifunctional Bpa\(^{21,26}\) and Bpa\(^{22,26}\) probes each resulted in a labeled band migrating at approximate \(M_r = 19,000\) and shifting to approximate \(M_r = 10,000\). This migration pattern was not different from that of CNBr cleavage of the secretin receptor labeled by the monofunctional Bpa\(^{21}\), Bpa\(^{22}\), and Bpa\(^{26}\) probes. This is not surprising, because each of these monofunctional probes has been shown to label the same receptor fragment, Ala\(^1\)–Met\(^{344}\) (10, 14, 15). CNBr cleavage of the receptor labeled with the bifunctional Bpa\(^{2,26}\) probe yielded a band migrating at approximate \(M_r = 24,500\) and shifting to approximate \(M_r = 15,500\) after deglycosylation. This pattern is consistent with the first CNBr fragment (Ala\(^1\)–Met\(^{344}\)) and the fragment containing the sixth transmembrane segment and the beginning of the third extracellular loop (Arg\(^{300}\)–Met\(^{344}\)) of the secretin receptor being linked by the bifunctional Bpa\(^{2,26}\) probe.

To further define the regions of labeling by the bifunctional Bpa\(^{21,26}\) and Bpa\(^{22,26}\) probes, Lys-C was used to cleave the first CNBr fragment (Ala\(^1\)–Met\(^{344}\)) labeled by each of the probes. As shown in Fig. 13, Lys-C cleavage of this fragment labeled by the monofunctional Bpa\(^{22}\) and Bpa\(^{26}\) probes each yielded a non-glycosylated band migrating at approximate \(M_r = 6,000\), representing the segment Ala\(^1\)–Lys\(^{30}\) at the amino terminus of the first CNBr fragment of the receptor (10, 15). Fig. 13 also shows that the Lys-C cleavage of the Ala\(^1\)–Met\(^{51}\) fragment labeled by the Bpa\(^{26}\) probe resulted in a glycosylated fragment migrating at approximate \(M_r = 15,500\) and shifting to approximate \(M_r = 6,000\), representing segment Gly\(^{34}\)–Met\(^{51}\) at the carboxyl terminus of the first CNBr fragment of the receptor (14). However, Lys-C cleavage of the first CNBr fragment labeled by either the bifunctional Bpa\(^{21,26}\) or the Bpa\(^{22,26}\) probe yielded a band migrating at approximate \(M_r = 19,000\) and shifting to approximate \(M_r = 10,000\). This is most consistent with the establish-
Molecular Basis of Secretin Binding

FIGURE 12. CNBr cleavage of the secretin receptor labeled with the bifunctional secretin probes. Shown is a diagram illustrating sites of CNBr cleavage of the secretin receptor, along with the masses of the protein cores of the resultant fragments (top left) as well as the sites of labeling by each of the monofunctional Bpa\textsuperscript{16}, Bpa\textsuperscript{21}, Bpa\textsuperscript{22}, and Bpa\textsuperscript{26} probes (top right) (10, 13–15). Also shown are typical autoradiographs of 10% NuPAGE gels used to separate the products of CNBr cleavage of the radiochemically pure secretin receptor labeled with each of the monofunctional (bottom left) and bifunctional (bottom right) secretin probes, before and after deglycosylation. The monofunctional probes with Bpa in positions 2, 16, 21, 22, and 26 labeled fragments that migrated identically to those we have fully characterized, including the identification of the specific residues labeled (10, 13–15). Each of the dual photolabile Bpa\textsuperscript{16,21}, Bpa\textsuperscript{16,22}, and Bpa\textsuperscript{2,26} probes labeled bands representing the simultaneous establishment of two bonds, representing the covalent attachment to two fragments of a single receptor protomer. The migration of the CNBr digests of the receptor labeled with the bifunctional Bpa\textsuperscript{16,21}, Bpa\textsuperscript{16,22}, and Bpa\textsuperscript{2,26} probes 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. However, the migration patterns of the CNBr digests of the receptor labeled with the Bpa\textsuperscript{16,21} and Bpa\textsuperscript{2,26} probes were not different from those patterns of the receptor labeled with each of the monofunctional probes because they each labeled the same Ala\textsuperscript{1}–Met\textsuperscript{51} CNBr fragment (see subdomain identification in Fig. 13). The molecular weight standards used in the bottom left panel of this figure and Figs. 3–5 were the discontinued Multimark multicolored standards. The SeeBlue Plus-2 prestained standards were used in the bottom right panel of this figure and Fig. 13.

Molecular Modeling of Secretin Occupation of a Single Secretin Receptor Protomer Accommodating All Extant Constraints—

The third stated possibility to explain the incompatibility of the new photoaffinity labeling spatial approximation constraint with the previously proposed molecular model was the possibility that one of the constraints utilized had been misleading. The softest such constraint had been the imposed spatial approximation between the WDN sequence within the secretin receptor amino terminus and the receptor third extracellular loop, where photolabile analogues of WDN had photoaffinity-labeled the receptor. That constraint was felt to be relatively soft because it is unclear that the demonstrated spatial approximation actually exists in normal physiology. We therefore proceeded with utilization of all other experimentally derived constraints, independent of this one.

Indeed, the peptide could be effectively docked at the opposite side of the receptor amino terminus from what had previously been proposed, while accommodating all of the other experimentally derived constraints. However, the docking of the peptide carboxyl-terminal region within the receptor amino-terminal domain was quite consistent in all high scoring models, there was considerable variability in the orientation of the receptor amino terminus relative to the receptor helical bundle domain in these models. Table 1 shows the distance between cross-linked residues in three of the best representative models, each of which satisfies all experimental data.
Molecular Basis of Secretin Binding

Recent data support the structural and functional importance of homodimeric structures of family B GPCRs (2, 3, 8). The structural details of this type of complex for the secretin receptor have been carefully explored, and symmetry along the interface of the lipid-exposed face of transmembrane segment four has been demonstrated (2, 3). Of note, this homodimeric receptor complex is able to express a higher affinity state of secretin binding and a higher potency of secretin action than a disrupted complex or than a non-dimerizing mutant receptor (2). The homodimeric complex also expresses prominent negative cooperativity of secretin binding (2).

A classical model expressing negative cooperativity is the insulin receptor in which hormone is bound to a symmetrical homodimeric receptor complex (5, 6). The docking of one ligand across both receptor protomers in that system is believed to contribute to its negative cooperativity. Two distinct sites on the receptors contribute to the insulin binding, probably resulting in less optimal orientation of the other pair of sites for binding the second (low affinity) ligand. In the current report, after we identified a new spatial approximation constraint that was found to be incompatible with the previously proposed molecular model of peptide binding to a single secretin receptor molecule, we sought to carefully examine whether binding across both protomers might be occurring at the secretin receptor homodimer as well. In such a model, some constraints might come from spatial approximation with one protomer, whereas others might come from the second protomer. This would not be recognized when only a single photolabile site of covalent attachment was utilized. Instead, it would require the incorpo-

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**TABLE 1**

Interatomic distances between cross-linked residues in the three best models

| Secretin peptide residue | Secretin receptor residue | Secretin receptor atom | Distance in model 1 | Distance in model 2 | Distance in model 3 |
|-------------------------|--------------------------|-----------------------|--------------------|--------------------|--------------------|
| His1                    | Phe338                   | C2                    | 7.3                | 7.5                | 5.9                |
| Thr2                    | Phe339                   | C2                    | 8.2                | 7.6                | 8.9                |
| Phe6                    | Val13                    | C4                    | 8.1                | 7.4                | 8.1                |
| Arg12                   | Val13                   | C4                    | 8.9                | 10.3               | 8.9                |
| Leu13                   | Val1061                  | C4                    | 7.3                | 7.3                | 7.3                |
| Ser16                   | Leu29                    | C3                    | 6.2                | 5.2                | 6.2                |
| Arg18                   | Arg21                   | C2                    | 7.9                | 8.0                | 7.9                |
| Arg18                   | Arg21                   | C2                    | 8.5                | 8.9                | 8.5                |
| Leu22                   | Leu22                   | C2                    | 7.3                | 7.5                | 7.3                |
| Leu23                   | Leu23                   | C2                    | 6.6                | 7.0                | 6.6                |
| Leu24                   | Leu24                   | C2                    | 7.7                | 7.7                | 7.7                |

* Measured from the C4 of the corresponding residue.

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With extensive experimental constraints that have already been generated and applied to the secretin receptor, additional constraints will be necessary to definitively establish the conformation of the secretin-secretin receptor complex.

**DISCUSSION**

Understanding the molecular basis of binding to and activating a receptor by its natural agonist ligand can provide important insights that are useful for the development of receptor-active drugs and for the refinement of drug candidates. To gain a clear picture of these processes, it is useful to integrate data from multiple experimental approaches that are often complementary.

Family B GPCRs contain multiple important potential drug targets for diseases involving bone metabolism, glucose metabolism, pain, and anxiety (30–33). There is clear evidence that the amino terminus of family B GPCRs is important for the binding of natural peptide ligands, with multiple lines of evidence for the carboxyl-terminal region of these peptides to interact with the receptor amino terminus (15, 20, 34–37). This includes NMR and crystallographic evidence for binding of these peptides to the isolated amino-terminal domains of receptors in this family (25, 38–43). Of note, however, are inconsistencies in the mode of binding displayed by the complexes of peptide and isolated amino terminus of these receptors. There are subtle differences in the location of binding and even in the orientation of the docked peptides (25, 38–43). There are also profound differences proposed for the orientation of the amino terminus of the receptors with their helical bundle domains (25, 38–43).

Constraints. Fig. 14 highlights their differences in the juxtaposition of the amino-terminal and transmembrane domains and reflects the remaining uncertainty of the conformations of the extracellular loop regions. This suggests that, even with the
ration of two such sites of covalent labeling into a single probe. We previously utilized this approach with the cholecystokinin receptor to establish that two sites of covalent labeling would be simultaneously established with its receptor (44).

In the current report, we chose sites of incorporation of photolabile residues in positions within secretin that were known to covalently label distinct subdomains within the secretin receptor. We combined all possible combinations of these subdomains to gain global coverage of the possibilities. Each set of experiments was carefully designed and controlled. All probes were able to bind with high affinity and to be full agonists, albeit some exhibited reduced potency. Manipulations were utilized to be certain that both photolabile moieties within a single probe were able to establish covalent bonds with the regions of the receptor adjacent to them after docking. Indeed, with all of this in place, none of the six bifunctional probes was found to label both protomers within the complex. Instead, each probe formed two bonds with a single receptor protomer.

With this strong evidence of the docking of secretin to a single receptor protomer, we repeated the molecular modeling we had done previously, this time adding the additional new, non-conforming experimental restraint. This resulted in a new molecular model in which the site of docking at the amino terminus of the secretin receptor was analogous to that described for the corticotropin-releasing factors 1 and 2, gastric inhibitory polypeptide, glucagon-like peptide 1, and parathyroid hormone receptors (25, 39–42). This model accommodates all 11 photoaffinity labeling constraints, the FRET constraints, and the mutagenesis and structure-activity observations that have previously been made (9). Of note, this model no longer was able to accommodate the direct approximation of the WDN “endogenous agonist” motif with the third extracellular loop that was previously suggested (45). This seems to refute any physiological basis for this region of the receptor to normally interact with the helical bundle region to activate the receptor. However, it clearly does not refute the validity of the earlier observation of cyclic WDN acting as an agonist, and this structure might still represent a lead for the development of a receptor-active agonist drug, although one with low potency and low specificity.

There remains substantial uncertainty regarding how the amino-terminal domain of the secretin receptor or indeed any family B GPCR is oriented relative to the helical bundle of this receptor. Clearly, there must be contiguity of the backbone of the carboxyl-terminal end of the receptor amino terminus with the top of transmembrane segment one. Although this region has recently been shown to have functional importance for the binding and action of a small molecule agonist of the structurally related calcitonin receptor (46), the absence of structural insights limits its usefulness as a constraint. It is somewhat remarkable that of 11 positions of photoaffinity labeling spread throughout the 27-residue secretin peptide, only the amino-terminal end of the natural peptide has been shown to covalently label the helical bundle region of the secretin receptor (13). This, too, provides inadequate constraint for refining the orientation of these two receptor domains. It will be critically important to add more experimental constraints to more meaningfully orient these domains in the future.
We are now confident that secretin docks within a single protomer of the secretin receptor homodimeric complex, and we are confident of the position of the docking of the carboxy-terminal end of this peptide relative to the receptor amino terminus. The orientation of the receptor amino terminus relative to the helical bundle domain and the precise siting of the peptide amino terminus are less clear. Similarly, the molecular basis for the negative cooperativity observed at the secretin receptor homodimer is not yet established. The asymmetry thought to be necessary to elicit the negative cooperativity could come from induced conformational changes or steric effects on the second protomer within the complex or from asymmetry of G protein coupling. This possibility will need to be examined in the future.

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