

Molecular Approximations between Residues 21 and 23 of Secretin and Its Receptor: Development of a Model for Peptide Docking with the Amino Terminus of the Secretin Receptor

Maoqing Dong, Polo C.-H. Lam, Fan Gao, Keiko Hosohata, Delia I. Pinon, Patrick M. Sexton, Ruben Abagyan, and Laurence J. Miller

Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic Scottsdale, Scottsdale, Arizona (M.D., F.G., K.H., D.I.P., L.J.M.); Department of Molecular Biology, Scripps Research Institute and Molsoft LLC, La Jolla, California (P.C.-H.L., R.A.); and Department of Pharmacology, Monash University, Clayton, Victoria, Australia (P.M.S.)

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ABSTRACT

The structurally unique amino-terminal domain of class II G protein-coupled receptors is critically important for ligand binding and receptor activation. Understanding the precise role it plays requires detailed insights into the molecular basis of its ligand interactions and the conformation of the ligand-receptor complex. In this work, we used two high-affinity, full-agonist, secretin-like photolabile probes having sites for covalent attachment in positions 21 and 23 and used sequential proteolysis and sequencing of the labeled region of the receptor to identify two new spatial approximation constraints. The position 21 probe labeled receptor residue Arg¹⁵, whereas the position 23 probe labeled receptor residue Arg²¹. A homology model of the amino-terminal domain of the secretin receptor

was developed using the NMR structure of the analogous domain of the corticotropin-releasing factor receptor. This was attached to a homology model of the secretin receptor transmembrane bundle, with the two domains oriented relative to each other based on continuity of the peptide backbone and by imposing a distance restraint recently identified between the amino-terminal WDN sequence and the region of the helical bundle above transmembrane segment six. Secretin was docked to this model using seven sets of spatial approximation constraints identified in previous photoaffinity labeling studies. This model was found to fully accommodate all existing constraints, as well as the two new approximations identified in this work.

The secretin receptor is a prototypic member of the class II family of G protein-coupled receptors (GPCRs) that contains several potentially important drug targets, such as receptors for calcitonin, vasoactive intestinal polypeptide, glucagon, glucagon-like peptide, corticotropin-releasing factor (CRF), and parathyroid hormone (Ulrich et al., 1998). Despite recent advances in the study of this receptor family, our understanding of receptor structure-function remains limited. Refinement of our understanding of the molecular basis of agonist ligand binding and activation of the secretin receptor

should contribute insights relevant to the entire family and facilitate the design and refinement of potential receptor-active drugs.

Insights into mechanisms of agonist ligand binding and activation of the secretin receptor have come from natural ligand structure-activity relationships and from receptor mutagenesis and photoaffinity labeling studies (Gourlet et al., 1996; Dong et al., 2003). These have identified the critical importance of the long and structurally complex amino terminus of this receptor for agonist ligand binding. This theme has been consistent for multiple other class II GPCR family members (Juppner et al., 1994; Cao et al., 1995; Gourlet et al., 1996). Alignment of the receptor amino terminal sequences reveals conserved features, which include six cysteine residues with three intradomain disulfide bonds (Grauschopf et al., 2000; Lisenbee et al., 2005). Insight into the structure of this region was substantially advanced with

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ABBREVIATIONS: GPCR, G protein-coupled receptor; CRF, corticotropin-releasing factor; CHO, Chinese hamster ovary; CRF2 β , corticotropin-releasing factor receptor; HA, hemagglutinin; Bpa, *p*-benzoyl-L-phenylalanine; KRH, Krebs-Ringers/HEPES; MES, 2-(*N*-morpholino)ethanesulfonic acid; PDB, Protein Data Bank; ICM, internal coordinate mechanics; rmsd, root-mean-square deviation; SecR, secretin receptor; VPAC1, vasoactive intestinal polypeptide receptor.

the solution of an NMR structure of the isolated amino terminus of the corticotropin-releasing factor (CRF2 β) receptor (Grace et al., 2004). However, it remains unclear whether the amino terminus of the intact CRF receptor will have the same or related conformation, how similar the amino terminal region of other receptors in this family will be to that structure, or how ligands dock to these receptors.

Natural ligands for class II GPCRs are moderately long peptides (greater than 25 residues) having diffuse pharmacophoric domains (Ulrich et al., 1998). Like natural ligands for other members of this family, the amino-terminal region of secretin contains key determinants for receptor selectivity and activation, whereas its carboxyl-terminal region contains determinants for high-affinity binding. Considerable experimental evidence indicates that the carboxyl-terminal regions of ligands for class II receptors interact with the amino-terminal regions of their receptors (Juppner et al., 1994; Gourlet et al., 1996; Pham et al., 2004; Tan et al., 2006), whereas the amino terminus of the peptides is critical for agonist activity and interacts with parts of the receptor core and loop regions (Bisello et al., 1998; Dong et al., 2004). Critical to understanding receptor activation is knowledge of how these ligands dock to their receptors. The most useful insights have come from intrinsic photoaffinity labeling studies that provide physical constraints on the relative position of peptide and receptor amino acids. The class II GPCR that has been most extensively studied this way is the secretin receptor, where spatial approximations have been established between residues scattered throughout the pharmacophore of secretin, in positions 6, 12, 13, 14, 18, 22, and 26, and residues within the receptor amino terminus (Dong et al., 1999a,b, 2000, 2002, 2003; Zang et al., 2003).

To date, the only photoaffinity labeling probes shown to covalently interact with receptor residues in regions other than the amino terminus are those having photolabile residues substituted into the amino-terminal end of the peptide ligand (Dong et al., 2004). This type of observation, also reported for the parathyroid hormone receptor, has resulted in the hypothesis that natural peptide ligands of class II GPCRs can act as tethers interacting with both amino terminus and body of their receptors, exerting tension to induce the conformational change associated with activation and G protein association (Bisello et al., 1998). We have proposed an alternative hypothesis (Dong et al., 2006) that describes a ligand-induced conformational change in the receptor amino terminus that exposes a previously "constrained" epitope (WDN in the secretin receptor) that can act as an endogenous agonist that interacts with and activates the body of the receptor.

Evaluation of these two apparently divergent activation mechanisms requires a more detailed understanding of the mechanism of binding and of the conformation of the complex between secretin and the amino terminus of the secretin receptor. We have therefore performed additional photoaffinity labeling experiments with new probes that incorporate photolabile residues into the important carboxyl-terminal region, in positions 21 or 23 of secretin. Both probes bound specifically and saturably to the secretin receptor and were full agonists. They were also able to covalently label single and distinct residues within the amino terminus of the receptor, at residues Arg¹⁵ (for position 21 probe) and Arg²¹ (for position 23 probe). We also have now used the NMR data for

the amino-terminal domain of the structurally related CRF2 β receptor (Grace et al., 2004) to refine that structure and to build a homology model of the analogous domain of the secretin receptor. We have oriented this relative to a homology model of the bovine rhodopsin helical bundle (Palczewski et al., 2000) to yield a preliminary full secretin receptor model. The previously identified spatial approximation constraints from photoaffinity labeling studies were used to provide initial ligand docking. This model was found to also nicely accommodate the two new spatial approximation constraints identified in the current work, with all of the currently available constraints contributing toward the most meaningful model of ligand docking yet proposed.

Materials and Methods

Materials. *N*-chlorobenzenesulfonamide (Iodobeads; a solid-phase oxidant), cyanogen bromide (CNBr), and *m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester were purchased from Pierce Chemical Co. (Rockford, IL). Phenylmethylsulfonyl fluoride, 3-isobutyl-1-methylxanthine, and *N*-(2-aminoethyl)-1-3-aminopropyl glass beads were from Sigma (St. Louis, MO). Endoproteinase Lys-C and the 12CA5 monoclonal antibody that recognizes the hemagglutinin (HA) epitope were from Roche Applied Science (Indianapolis, IN). Secretin and endoglycosidase F were prepared in our laboratory, as described previously (Pearson et al., 1987). All other reagents were of analytical grade.

Synthetic Peptides. Photolabile secretin probes used in this study were [Tyr¹⁰,Bpa²¹]rat secretin (Bpa²¹ probe) and [Tyr¹⁰,Bpa²³]rat secretin (Bpa²³ probe). They incorporated a photolabile *p*-benzoyl-L-phenylalanine (Bpa) to replace Arg²¹ and Leu²³ located at the carboxyl-terminal half of the ligand, respectively. Like the well characterized radioligand [Tyr¹⁰]rat secretin, they incorporated a Tyr to replace Leu¹⁰, which has been shown to be well tolerated. Peptides were synthesized as described previously (Powers et al., 1988), radioiodinated oxidatively using the solid-phase oxidant Iodobeads, and purified to homogeneity using reversed-phase high-performance liquid chromatography to yield specific radioactivities of 2000 Ci/mmol.

Receptor-Expressing Cell Lines. Several cell lines that had been established and well characterized previously were used as sources of receptor for this study. These included Chinese hamster ovary (CHO) cell lines stably expressing the wild-type secretin receptor (SecR) (Ulrich et al., 1993), the HA-tagged wild-type secretin receptor (SecR-HA37) (Dong et al., 1999b), and HA-tagged mutant secretin receptors (SecR-V16M-HA37 and SecR-V13M-HA37) (Dong et al., 2000). Numbering of the residues in the secretin receptor reflects the cleavage of the 22-residue signal peptide sequence (Dong et al., 1999a). These cell lines were cultured at 37°C in a 5% CO₂ environment on Falcon tissue culture plasticware in Ham's F-12 medium supplemented with 5% Fetal Clone-2 (HyClone Laboratories, Logan, UT). Cells were passaged twice a week and lifted mechanically before use. Plasma membranes were prepared using discontinuous sucrose gradient centrifugation (Hadac et al., 1996). Membranes were then resuspended in Krebs-Ringers/HEPES (KRH) buffer containing 25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM KH₂PO₄, 1.2 mM MgSO₄, 0.01% soybean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride for storage at -80°C until they were to be used.

Binding Studies. Binding activities of the Bpa²¹ and Bpa²³ probes were performed with enriched plasma membranes from the CHO-SecR cells in standard competition-binding assays, using conditions previously established (Hadac et al., 1996). Membranes (containing ~10 μ g of protein) were incubated with a constant amount of radioligand [¹²⁵I-Tyr¹⁰]rat secretin (5–10 pM) and increasing concentrations of nonradioactive ligand (0–1 μ M Bpa²¹ or Bpa²³ probe)

in KRH buffer containing 0.2% bovine serum albumin for 1 h at room temperature (final volume, 500 μ l). Separation of bound from free radioligand was performed with a Skatron cell harvester (Molecular Devices, Sunnyvale, CA), using receptor-binding filter mats that had been presoaked in 0.3% Polybrene. Bound radioactivity was quantified using a gamma-spectrometer. Nonspecific binding was determined in the presence of 1 μ M unlabeled secretin and represented less than 15% of total binding. Data were graphed using Prism software (GraphPad Software, San Diego, CA) and were analyzed using the nonlinear least-squares curve-fitting routine in LIGAND (Munson and Rodbard, 1980).

Biological Activity Assay. Biological activities of the Bpa²¹ and Bpa²³ probes were studied using assays for stimulation of intracellular cAMP in the receptor-bearing CHO-SecR cell line, as described previously (Ganguli et al., 1998). CHO-SecR cells were incubated with increasing concentrations of the probe (0–1 μ M) for 30 min at 37°C in KRH buffer containing 0.2% bovine serum albumin, 0.01% soybean trypsin inhibitor, 0.1% bacitracin, and 1 mM 3-isobutyl-1-methylxanthine. Reactions were stopped by adding 6% (w/v) perchloric acid, and the pH was adjusted to 6.0 with 30% KHCO₃. The cAMP concentrations in cell lysates were measured using a competition-binding assay (Diagnostic Products Corporation, Los Angeles, CA) (Ganguli et al., 1998). Radioactivity was quantified by scintillation counting in a Beckman LS6000 (Beckman Coulter, Fullerton, CA).

Photoaffinity Labeling. The detailed procedure has been described previously (Dong et al., 1999b). Plasma membranes (~50 μ g) were incubated with the ¹²⁵I-labeled Bpa²¹ or Bpa²³ probes (~0.1 nM) in KRH buffer for 1 h in the dark at room temperature in the presence of increasing concentrations of unlabeled secretin. The reaction was 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. Membranes were then washed with KRH buffer, solubilized in SDS sample buffer, and separated by gel electrophoresis on 10% SDS-polyacrylamide gels (Laemmli, 1970). The apparent molecular masses of radiolabeled receptors, visualized by autoradiography, were determined by interpolation on a plot of the mobility of ProSieve protein standards (Lonza Rockland, Inc., Rockland, ME) versus the log values of their apparent masses.

Peptide Mapping. Photoaffinity-labeled secretin receptor was prepared in larger scale using ~200- μ g aliquots of plasma membranes and 0.5 nM ¹²⁵I-labeled Bpa²¹ or Bpa²³ probes. Labeled bands were cut from gels, eluted, lyophilized, and precipitated with ethanol before subsequent cleavage. Purified labeled receptor was deglycosylated with endoglycosidase F and digested with CNBr and endoproteinase Lys-C under conditions described previously (Dong et al., 1999b). Cleavage products were separated on 10% NuPAGE gels (Invitrogen, Carlsbad, CA) using 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 (Invitrogen) versus the log values of their apparent masses.

The identity of the affinity-labeled secretin receptor fragment resulting from CNBr cleavage was further established by immunoprecipitation with the 12CA5 anti-HA monoclonal antibody using the affinity-labeled HA-tagged receptor construct (SecR-HA37), previously shown to bind and signal identically to wild-type receptor (Dong et al., 1999b). The immunoprecipitated material was eluted into sample buffer and resolved by NuPAGE gel electrophoresis.

Radiochemical Edman degradation sequencing (Dong et al., 1999b) was used to determine the specific sites of attachment after achieving definitive identification of the receptor fragments labeled with each probe. This involved the cross-linking of the receptor fragment of interest through cysteine residues to *N*-(2-aminoethyl-1)-3-aminopropyl glass beads. We used V13M-HA37 and V16M-HA37 mutant receptor constructs expressed in CHO cell lines for the Bpa²¹ and Bpa²³ probes, respectively. Labeled receptor was purified and cleaved by CNBr and the resultant fragments were gel-purified

to radioactive homogeneity before being covalently coupled through cysteine residues to maleimidobenzoyl succinimide-activated *N*-(2-aminoethyl-1)-3-aminopropyl glass beads. After this, repetitive cycles of manual Edman degradation were performed with quantitation of radioactivity released in each cycle. This procedure was repeated three times in independent experiments.

Molecular Modeling. All molecular modeling activities were conducted using a stochastic global energy optimization procedure implemented in Internal Coordinate Mechanics (ICM) (Abagyan et al., 1994). This procedure consisted of the following iterative steps: 1) random conformational change of a dihedral angle according to the biased-probability Monte Carlo method (Abagyan and Totrov, 1994); 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 (Metropolis et al., 1953). This procedure can generate and search through diverse sets of conformations by actively sampling a selected set of dihedral angles. All calculations were carried out on computers equipped with 3.4-GHz Intel XEON-EMT processors.

Refinement of the NMR Models of the Mouse CRF2 β Receptor Amino-Terminal Domain. The 20 NMR models of the mouse CRF2 β receptor amino-terminal domain and the associated NMR distance restraints that were deposited into Protein Data Bank (PDB code 1U34) were obtained. The highly flexible regions at both ends of this domain were removed, retaining only residues 37 to 122. The models were then completed by attaching missing hydrogens, assigning ECEPP/3 atom types and charges (Nemethy et al., 1992), followed by regularization to impose ideal covalent geometries on all bond angles and bond lengths. Eight hundred and three NMR restraints were converted to ICM-readable format and were imposed on the models using a quadratic restraint energy with a strength of 10 kcal/(mol Å²) (Bordner and Abagyan, 2006). Each of the 20 models was then subjected to local minimization, followed by 10 independent cycles of Monte Carlo sampling of both the backbone and side-chain dihedral angles at 600 K in ICM, each simulation lasting for more than 144 h (Abagyan and Totrov, 1999). The lowest energy conformation for each of the 20 models was retained.

Homology Modeling of the Amino-Terminal Domain of the Rat Secretin Receptor. The multiple sequence alignment of class II GPCRs from the Swissprot database was used as a guide to align the sequence of the rat secretin receptor to the mouse CRF2 β receptor amino-terminal domain (Boeckmann et al., 2003). The 20 refined models of this domain of the CRF2 β receptor were then used to generate 20 homology models of the amino terminus of the secretin receptor (Cardozo et al., 1995). A subset of 115 NMR restraints of the CRF2 β receptor amino terminus was imposed on the residues that are conserved between the secretin receptor and the CRF2 β receptor. Each of the 20 models was then subjected to local minimization, followed by 10 independent cycles of Monte Carlo sampling of backbone and side-chain conformations at 600 K, each simulation lasting for more than 216 h. The lowest energy conformation for each of the 20 models was retained and further refined by Monte Carlo simulation at 100 K for 36 h.

Homology Modeling of the Transmembrane Domain of the Rat Secretin Receptor. The sequence of the transmembrane helices of the rat secretin receptor were aligned to those of bovine rhodopsin (PDB code 1U18) using the "cold spot" method (Frimurer and Bywater, 1999). A homology model of the transmembrane helical bundle of the rat secretin receptor was then built in ICM. The packing of the model was further optimized by side-chain sampling and backbone minimization.

Alignment of the Amino-Terminal Domain of the Secretin Receptor with Its Transmembrane Helical Bundle. A distance restraint was imposed between the WDN epitope and the top of the sixth transmembrane segment. A second distance restraint was imposed between the carboxyl end of the amino-terminal domain and the amino-terminal end of helix 1. The amino-terminal domain was then docked onto the helical bundle by Monte Carlo sampling of the

six positional variables of the amino-terminal domain. The contact between the two domains was further optimized by side chain sampling to generate a preliminary model of the secretin receptor incorporating both the amino terminus and receptor core (Fernández-Recio et al., 2003).

Docking of Secretin to the Full Model of the Secretin Receptor. The initial conformation of secretin to be used in docking studies was taken from a previous solution-phase NMR determination of the porcine secretin structure (differs from the rat sequence only with a glutamine in position 14 in place of an arginine) (Clore et al., 1988). A maximum distance of 3.5 Å was imposed between residues Phe⁶, Arg¹², Leu¹³, Gln¹⁴, Arg¹⁸, Leu²², and Leu²⁶ of secretin and residues Val⁴, Val⁶, Val¹⁰³, Pro³⁸, Arg¹⁴, Leu¹⁷, and Leu³⁶ of the secretin receptor, respectively. Secretin was first docked to the grid potentials derived from the model of the intact secretin receptor in 10 independent runs. During the grid docking, the six positional variables of secretin were actively sampled, with rigid backbone and flexible side chain during local minimization cycles. All 10 independent runs converged to a single solution after 8 h of docking. Subsequently, the secretin/secretin receptor complex was refined. During refinement, the side chains of both secretin and the secretin receptor were actively sampled, while the backbone of secretin and residues 1 to 43 and 102 to 110 of the secretin receptor were allowed to be more flexible during cycles of local minimization. The refinement process typically lasted for 70 h, with the lowest energy conformation among the 10 independent runs retained. Finally, the two new residue-residue spatial approximation constraints determined by photoaffinity labeling in the current work were assessed in the model. The health of the models was established by PROCHECK and WHAT_CHECK evaluations (Laskowski et al., 1993; Hooft et al., 1996).

Results

Characterization of Photolabile Probes. Both the Bpa²¹ and Bpa²³ probes were synthesized by manual solid phase techniques and purified by reversed-phase HPLC, and their identities were verified by mass spectrometry. They were functionally characterized in a competition radioligand-binding assay. As shown in Fig. 1, both probes bound to the secretin receptor specifically and saturably, although with

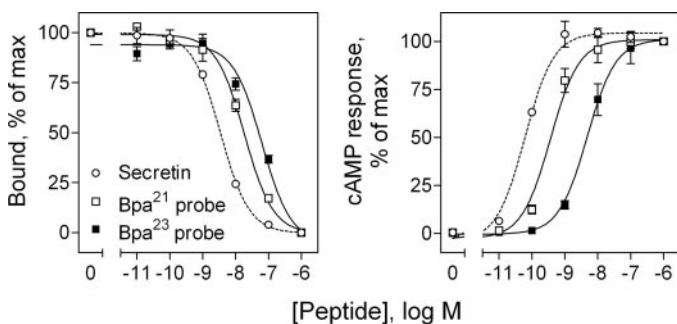


Fig. 1. Functional characterization of the Bpa²¹ and Bpa²³ probes. Left, the competition-binding curves of increasing concentrations of unlabeled Bpa²¹ and Bpa²³ probes to displace the binding of radioligand, [¹²⁵I]-[Tyr¹⁰]rat secretin, to membranes from CHO-SecR cells. Values represent percentages of maximal saturable binding that were observed in the absence of competitor. They are expressed as means \pm S.E.M. of duplicate data from three independent experiments. The absolute values for maximal binding in these experiments were 3282 ± 45 cpm. Right, intracellular cAMP responses to increasing concentrations of the Bpa²¹ and Bpa²³ probes in CHO-SecR cells. There were no significant differences in basal and maximal intracellular cAMP levels in CHO-SecR by both probes and secretin. The basal levels of intracellular cAMP were 1.3 ± 0.4 pmol/million cells, and the maximal levels reached 198 ± 31 pmol/million cells. Values are expressed as means \pm S.E.M. of data from three independent experiments, with data normalized relative to the maximal response to secretin.

affinities lower than that of natural secretin (K_i values: secretin, 2.7 ± 0.1 nM; Bpa²¹ probe, 17.9 ± 1.2 nM; Bpa²³ probe, 47.1 ± 14.2 nM).

The probes were then tested for their ability to stimulate intracellular cAMP accumulation in CHO-SecR cells. As shown in Fig. 1, both probes were fully efficacious agonists, stimulating full intracellular cAMP responses that were not different from those achieved in response to natural secretin. They were, however, less potent than natural secretin (EC_{50} values: secretin, 0.06 ± 0.01 nM; Bpa²¹ probe, 0.37 ± 0.02 nM; Bpa²³ probe, 5.22 ± 0.97 nM).

Photoaffinity Labeling of the Secretin Receptor. We explored the ability of the Bpa²¹ and Bpa²³ probes to covalently label the secretin receptor. As shown in Fig. 2, both probes specifically and saturably labeled the secretin receptor, with the labeled protein bands migrating at approximate $M_r = 70,000$ and shifting to approximate $M_r = 42,000$ after deglycosylation with endoglycosidase F. This labeling was competed by secretin in a concentration-dependent manner (Bpa²¹ probe, $IC_{50} = 18.1 \pm 2.8$ nM; Bpa²³ probe, $IC_{50} = 3.8 \pm 0.5$ nM). No radioactive band was observed in the affinity-labeled nonreceptor-bearing CHO cell membranes.

Identification of Sites of Labeling by the Bpa²¹ and Bpa²³ Probes. To gain insights into the domains of labeling by each of the probes, CNBr cleavage was first used. The secretin receptor contains nine Met residues and, in theory, CNBr cleavage would yield 10 fragments ranging in molecular mass from 1 to 11 kDa, three of which contain sites of glycosylation (Fig. 3). As shown in Fig. 3, CNBr cleavage of the secretin receptor labeled by each probe resulted in a fragment migrating at approximate $M_r = 19,000$ and shifting to $M_r = 10,000$ after deglycosylation. This pattern was the same for both probes, indicating that they might label the same region of the receptor. Based on the molecular masses of the attached probes (Bpa²¹ probe, 3173 Da; Bpa²³ probe, 3216 Da) and on the glycosylated nature of the labeled bands, two fragments were felt to be candidates (segments including amino acid residues 1–51 and 74–123). Both of these fragments are within the amino-terminal tail of the secretin receptor, with one at the amino-terminal end of the receptor (fragment 1) and the other (fragment 3) adjacent to the first transmembrane segment (Fig. 3). Previous experience with the electrophoretic migration of each of these CNBr fragments makes the first fragment most likely (Dong et al., 1999b; Zang et al., 2003).

To definitively establish receptor residues 1 to 51 as the region of labeling, a well characterized receptor mutant that incorporates an HA epitope within this fragment (SecR-HA37) (Dong et al., 1999b) was used in immunoprecipitation experiments. Figure 4 shows that the $M_r = 19,000$ CNBr fragment radiolabeled by Bpa²¹ and Bpa²³ probes was immunoprecipitated by the anti-HA monoclonal antibody and precipitation was prevented in the presence of excess HA peptide.

Endoproteinase Lys-C that cleaves at the carboxyl-terminal side of lysine residues was next used to further localize the region of covalent labeling by each probe. Figure 5 shows that endoproteinase Lys-C cleavage of labeled CNBr fragment 1 yielded a labeled fragment migrating at approximate $M_r = 6000$ that did not shift further after deglycosylation. This identified the nonglycosylated 30-residue fragment at

the distal amino terminus of the secretin receptor as the domain of labeling by each of the probes.

To further localize the region of labeling by each of the probes, two previously established secretin receptor mutant constructs, V13M-HA37 and V16M-HA37 (Dong et al., 2000), were used. Both receptor mutants were specifically and saturably labeled with each probe (Fig. 6). CNBr cleavage of the V13M-HA37 mutant labeled with the Bpa²¹ probe yielded a fragment migrating on a 10% NuPAGE gel at approximate $M_r = 19,000$ that shifted to $M_r = 9000$ after deglycosylation, representing the fragment between Arg¹⁴ and Met⁵¹. CNBr cleavage of the V16M-HA37 mutant labeled with the Bpa²¹ probe yielded a nonglycosylated fragment migrating at approximate $M_r = 5000$, representing the fragment between Ala¹ and Met¹⁶ (Fig. 6). Taken together, these data indicate that the site of labeling with the Bpa²¹ probe was within the small segment between Arg¹⁴ and Val¹⁶.

CNBr cleavage of the V13M-HA37 and the V16M-HA37 receptor mutants labeled with the Bpa²³ probe yielded frag-

ments migrating similarly on a 10% NuPAGE gel at approximate $M_r = 19,000$ that shifted to $M_r = 9000$ after deglycosylation, representing the fragment between Arg¹⁴ and Met⁵¹. Taking into account the endoprotease Lys-C cleavage data above, the site of labeling with the Bpa²³ probe was within the region between Leu¹⁷ and Lys³⁰.

Radiochemical Edman degradation sequencing of the purified labeled fragments resulting from the CNBr cleavage of the labeled V13M-HA37 (for the Bpa²¹ probe) and V16M-HA37 (for the Bpa²³ probe) receptor mutants was performed. Shown in Fig. 7 are the profiles of eluted radioactivity in which a peak was found in cycle 2 for the Bpa²¹ probe and in cycle 5 for the Bpa²³ probe. This identified Arg¹⁵ and Arg²¹ of the secretin receptor as the sites of labeling with the Bpa²¹ probe and for the Bpa²³ probe, respectively.

Refinement of the NMR Models of the Mouse CRF2 β Receptor Amino Terminus. PROCHECK and WHAT-CHECK were used to compare the quality of the ensemble of 20 models of receptor residues 37 to 122 before and after

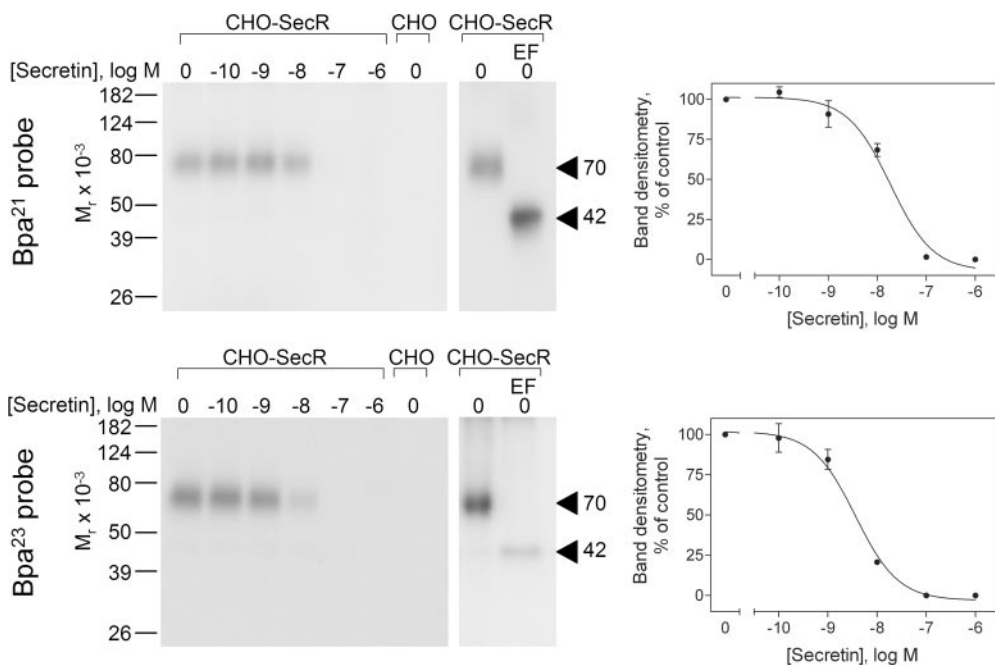


Fig. 2. Photoaffinity labeling of the secretin receptor. Shown are representative 10% SDS-PAGE gels used to separate the products of photoaffinity labeling of CHO-SecR membranes by the Bpa²¹ (top) and Bpa²³ (bottom) probes in the absence and presence of increasing concentrations of competing unlabeled secretin. The labeled secretin receptor with each probe migrated similarly at approximate $M_r = 70,000$ and shifted to approximate $M_r = 42,000$ after deglycosylation by endoglycosidase F (EF). Shown also are the densitometric analyses of three similar independent experiments by each probe, with data points expressed as means \pm S.E.M. No bands were detected in affinity-labeled nonreceptor-bearing CHO cell membranes.

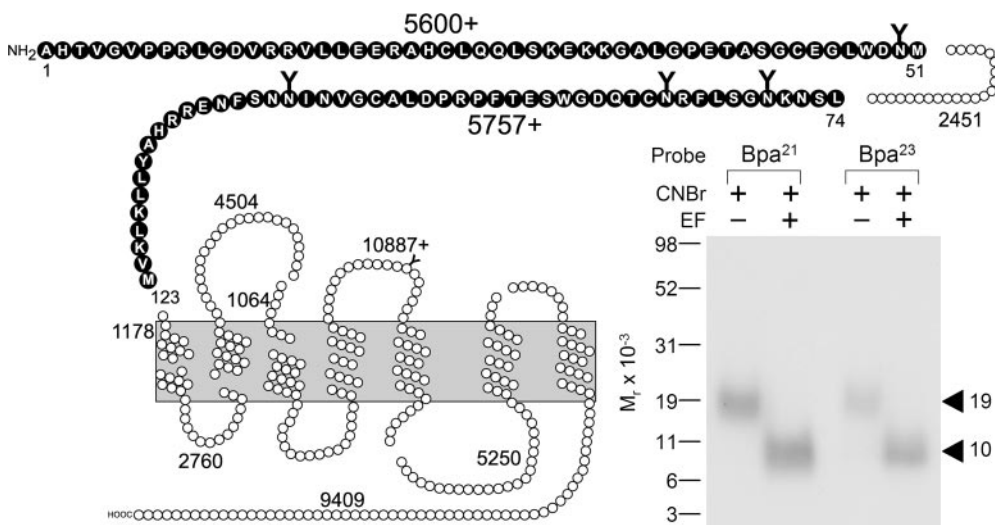


Fig. 3. CNBr cleavage of the labeled secretin receptor. Left, shows a diagram of the predicted sites of CNBr cleavage of the rat secretin receptor, along with the masses of protein cores of these fragments. Residues are labeled sequentially from 1, after cleavage of the 22-residue signal peptide (Dong et al., 1999a). Right, shows a representative autoradiograph of a 10% NuPAGE gel used to separate the products of CNBr cleavage of the secretin receptor labeled with the Bpa²¹ and Bpa²³ probes. Cleavage of the native receptor labeled with each probe resulted in a band migrating at approximate $M_r = 19,000$ that shifted to $M_r = 10,000$ after deglycosylation with endoglycosidase F (EF). The best candidates representing this are the first fragment at the amino terminus of the receptor and the third fragment (bold circles).

Monte Carlo simulation (Table 1). The results from PROCHECK show that after simulation, 25% more residues occupied the most favored regions, whereas the percentages of residues in the generously allowed and disallowed regions dropped from 11.6 and 3.3% to 1.9 and 1.0%, respectively. Likewise, results from WHAT_CHECK indicated significant improvement in quality of second generation packing, Ramachandran plot appearance, χ^1/χ^2 rotamer normality, and backbone conformation after simulation.

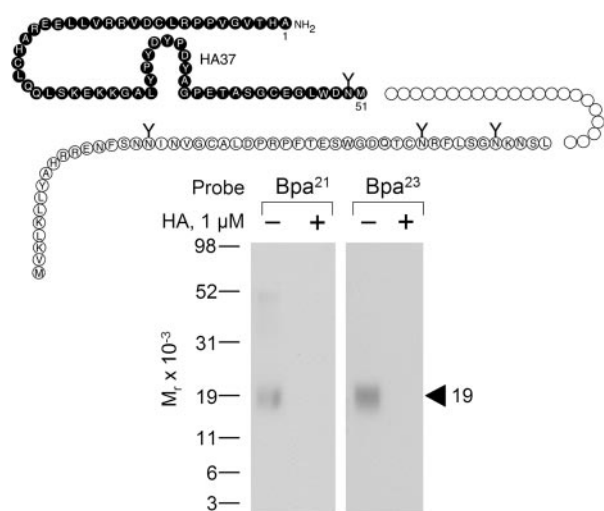


Fig. 4. Immunoprecipitation of the CNBr fragments of the labeled secretin receptor. Top, a diagram illustrating the theoretical sites of CNBr cleavage of the amino terminus of the HA-tagged secretin receptor construct (SecR-HA37). Bottom, representative autoradiographs of 10% Nu-PAGE gels used for separating products of immunoprecipitation with monoclonal anti-HA antibody of CNBr fragments from cleavage of HA37-tagged secretin receptor labeled with each of the probes in the presence and absence of competing HA peptide. This provides the definitive identification of the fragment at the most distal end of the amino terminus as the affinity-labeled receptor domain for both the Bpa²¹ and Bpa²³ probes.

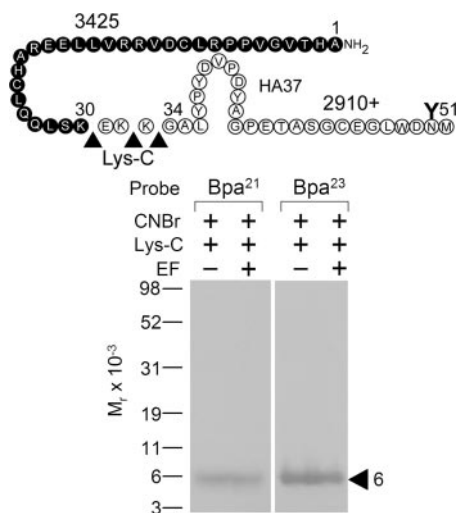


Fig. 5. Endoproteinase Lys-C cleavage of the labeled secretin receptor. Top, a diagram illustrating the theoretical sites of endoproteinase Lys-C cleavage and masses of expected fragments from sequential endoproteinase Lys-C cleavage of the CNBr fragment 1 (Ala¹-Met⁵¹) of the secretin receptor. Bottom, that endoproteinase Lys-C cleavage of CNBr fragment 1 from both native and deglycosylated secretin receptor labeled with the Bpa²¹ or Bpa²³ probe yielded nonglycosylated fragments migrating similarly at approximate $M_r = 6000$. This is most consistent with labeling of the amino-terminal nonglycosylated fragment with each of the probes, representing the region of the receptor between residue Ala¹ and Lys³⁰.

Nederveen et al. (2005) published the RECOORD database, in which more than 500 NMR models from the Protein Data Bank were recalculated. The largest improvement in the model quality was achieved by restrained molecular dynamics in a hydrated environment. Comparing their study with ours shows that refinement in ICM improved the model quality similar to that of Nederveen's study in all categories in PROCHECK and WHAT_CHECK, bringing the overall quality of the amino terminus of the CRF2 β receptor to that of an average NMR model. Note that although the Z-score of the backbone conformation after refinement was still -8.4 , it represented an improvement over the Z-score of the original models of -11.4 . Consistent with this, Nederveen's refinement also did not improve the backbone conformations significantly.

To see how the refinement affected the overall structure of the models, we calculated the pairwise C_α root-mean-square deviation (rmsd) by superimposing the C_α of the stable central core residues 59 to 64, 70 to 81, and 100 to 113. For each of the 20 models, we superimposed the model before and after refinement; the resultant C_α rmsd for the central core and the remaining disordered region were 1.22 ± 0.16 Å and 7.8 ± 1.6 Å, respectively. These values reflected the degree of movement in the central core and the disordered region during refinement. As a reference, the pairwise C_α rmsd for the central core and the disordered region within the ensemble of the original 20 NMR models were 0.84 ± 0.17 Å and 7.7 ± 1.9 Å, respectively, which indicate the intrinsic flexibility/uncertainty in the two regions in the original NMR models. These results demonstrate that our simulation enables large movement in the disordered region while limiting the movement of the stable central core within experimental uncertainty, as shown in Fig. 8.

Homology Modeling of the Amino Terminus of the Secretin Receptor. The aligned amino terminal regions of secretin and CRF2 β receptors share 19% sequence identity and 36% sequence homology with the three disulfide bonds that are conserved throughout this family (Grace et al., 2004; Lisenbee et al., 2005). An initial homology model of the amino terminus of the secretin receptor was produced using ICM, and was refined based on a subset of the original NMR distance restraints of the CRF2 β receptor amino terminus that included 115 distance restraints between residues that are conserved among secretin and CRF2 β receptors. The backbone and side-chain dihedral angles of the secretin receptor models were then sampled by biased-probability Monte Carlo method in the presence of these distance restraints. This refinement was necessary because of the low sequence identity and the highly undefined nature of the amino-terminal domain outside of its central core, as shown in the CRF2 β receptor in Fig. 8.

The ensemble of 20 rat secretin receptor amino terminus models after homology modeling and refinement is shown in Fig. 9. We calculated the pairwise C_α rmsd within the ensemble by superimposing the C_α of the stable central core residues 43 to 48, 53 to 64, and 82 to 93. The C_α rmsd for the central core and the remaining disordered region were 2.11 ± 0.60 Å and 11.3 ± 2.0 Å, respectively. A comparison of these numbers with those of the original NMR models of the amino terminus of the CRF2 β receptor (0.84 ± 0.17 and 7.7 ± 1.9 Å) shows a wider spread of the supposed stable central core,

probably reflecting the smaller number of distance restraints used in the modeling of this region of the secretin receptor.

Only seven of the 20 models were found to contain an intact salt bridge between Asp⁴⁹ and Arg⁸³, corresponding to the salt bridge between Asp⁶⁵ and Arg¹⁰¹ proposed to be present in the amino terminus of the mouse CRF2 β receptor. We inspected the distance restraints deposited in Protein Data Bank and found that there was no direct distance restraint connecting Asp⁶⁵ and Arg¹⁰¹ in the original NMR models, even though this salt bridge was reported as a prominent feature of those conformations.

Molecular Model of the Intact Secretin Receptor. To study how secretin interacts with its receptor, we first constructed an initial model of the receptor including the receptor amino terminus and the seven-transmembrane segment

bundle. A preliminary model of the transmembrane helical bundle domain was generated using a previously reported "cold-spot" method (Frimurer and Bywater, 1999). We attached the seven secretin receptor amino-terminal domain models having an intact Asp⁴⁹ to Arg⁸³ salt bridge (that were most similar to the reported conformation of the CRF2 β receptor) to this receptor transmembrane domain. The relative orientation of the two domains was restrained by the connection of the two domains at the amino-terminal end of helix 1 and by the proposed interaction between the WDN sequence and the transmembrane domain (Dong et al., 2006). Seven models of the receptor that were consistent with these restraints were generated.

Secretin was first docked to the grid representation of the seven full models to maximize sampling efficiency. Because

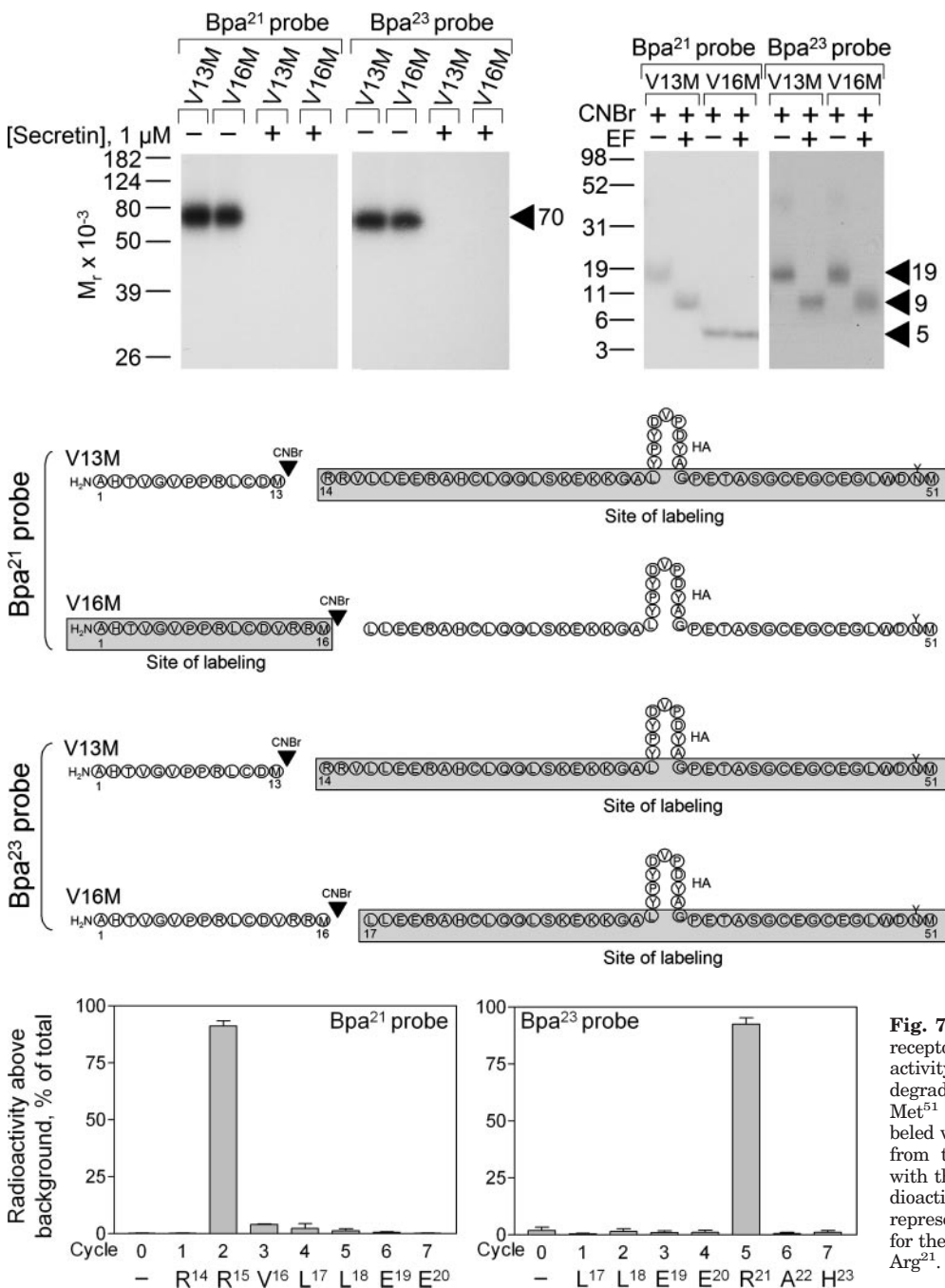


Fig. 6. Photoaffinity labeling and CNBr cleavage of the mutant secretin receptors. Top left, both the V13M-HA37 and V16M-HA37 mutant secretin receptors were able to be affinity-labeled saturably and specifically by the Bpa²¹ and Bpa²³ probes. Top right, CNBr cleavage of the V13M-HA37 and V16M-HA37 mutants labeled with the Bpa²¹ probe yielded a glycosylated fragment with the core migrating at approximate $M_r = 9000$ (Arg¹⁴-Met⁵¹) and a nonglycosylated fragment migrating at approximate $M_r = 5000$ (Ala¹-Met¹⁶), respectively. These data indicate that the site of labeling with the Bpa²¹ probe was between Arg¹⁴ and Val¹⁶. However, CNBr cleavage of these two mutant receptors labeled with the Bpa²³ probe both yielded fragments migrating at approximate $M_r = 19,000$ and shifting to approximate $M_r = 9000$, indicating the site of labeling being within the fragment Leu¹⁷ to Met⁵¹. Bottom, theoretical sites of CNBr cleavage of the Ala¹ to Met⁵¹ fragment of the V13M-HA37 and V16M-HA37 receptor mutants labeled with each of the noted probes.

Fig. 7. Identification of the photoaffinity-labeled receptor residues. Shown are representative radioactivity elution profiles from radiochemical Edman degradation sequencing of the fragments Arg¹⁴ to Met⁵¹ from the V13M-HA37 mutant receptor labeled with the Bpa²¹ probe (left) and Leu¹⁷-Met⁵¹ from the V16M-HA37 mutant receptor labeled with the Bpa²³ probe (right). A peak eluted in radioactivity appeared in cycle 2 for the Bpa²¹ probe, representing receptor residue Arg¹⁵, and in cycle 5 for the Bpa²³ probe, representing receptor residue Arg²¹.

of the rigid nature of the receptor in grid docking, we subsequently carried out flexible backbone refinement. The C_{α} distances between cross-linked residues in secretin and the secretin receptor from photoaffinity labeling studies are shown for three of the best models in Table 2. Note that the two photoaffinity labeling groups, *p*-benzoyl-L-phenylalanine and *p*-benzoylbenzoyl-L-lysine, can span distances of 10 and 15 Å, respectively. Therefore, the seven distance restraints between secretin and the amino-terminal domain of the secretin receptor coming from previous photoaffinity labeling studies were all satisfied.

Most importantly, the last two entries of Table 2 show that

the two pairs of cross-linked residues being demonstrated for the first time in the current study (Arg²¹ of secretin to receptor residue Arg¹⁵ and Leu²³ of secretin to receptor residue Arg²¹) were found to be within cross-linking distances in our models, even though these two sets of distance restraints had not been used during the peptide docking and refinement. Figure 10 shows the best working model for secretin docking to the receptor amino terminus and highlights the potential accessibility of the WDN sequence within the amino terminus of the receptor previously postulated as playing an endogenous agonist role by interacting with the body of the secretin receptor (Dong et al., 2006).

TABLE 1

Improvement in structural quality of the mouse CRF2 β receptor amino-terminal domain after Monte Carlo simulation; comparison with RECOORD

	Original Ensemble of 20 NMR Modes, Residues 37–122	Ensemble of 20 Models after Monte Carlo Simulation	545 Original NMR Models in RECOORD	545 NMR Models after Water Refinement in RECOORD
WHAT_CHECK Z-scores				
Second-generation packing quality	-4.3 ± 0.3	-2.2 ± 0.4	-3.5 ± 1.9	-2.5 ± 2.0
Ramachandran plot appearance	-7.1 ± 0.6	-3.6 ± 0.7	-4.6 ± 1.6	-3.4 ± 1.4
c1/c2 rotamer normality	-6.0 ± 0.3	0.9 ± 0.9	-3.7 ± 1.5	
Backbone conformation	-11.4 ± 2.1	-8.4 ± 1.1	-4.0 ± 3.0	-3.8 ± 2.7
PROCHECK results				
Most favored regions	$43.3 \pm 5.7\%$	$68.4 \pm 5.6\%$	$69.3 \pm 14.2\%$	$76.1 \pm 11.3\%$
Allowed regions	$41.7 \pm 5.4\%$	$28.7 \pm 5.4\%$	$25.4 \pm 10.6\%$	$19.6 \pm 8.5\%$
Generously allowed regions	$11.6 \pm 3.8\%$	$1.9 \pm 2.1\%$	$3.9 \pm 3.8\%$	$2.5 \pm 2.1\%$
Disallowed regions	$3.3 \pm 2.2\%$	$1.1 \pm 1.0\%$	$1.3 \pm 1.7\%$	$1.8 \pm 1.8\%$

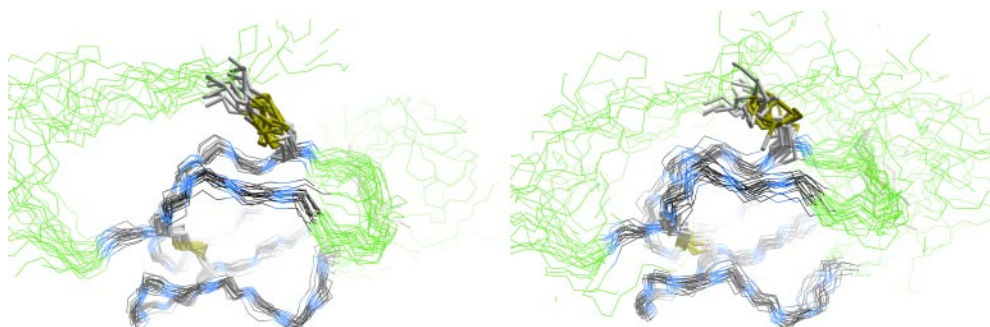


Fig. 8. Ensemble of 20 models of the amino terminus of the CRF2 β receptor. The structurally stable central core of the CRF2 β receptor amino terminus consisting of residues 59 to 64, 70 to 81, and 100 to 113 is colored in black for backbone carbon and blue for backbone nitrogen. The flanking flexible regions are colored green. The disulfide bonds are shown in yellow. *Left*, the original NMR models. The C_{α} rmsd of the central core and the flexible regions among the 20 models were 0.84 ± 0.17 and 7.7 ± 1.9 Å, respectively. *Right*, models after refinement in ICM. During refinement, the C_{α} rmsd change for the central core and the flexible regions were 1.22 ± 0.16 and 7.8 ± 1.6 Å, respectively.

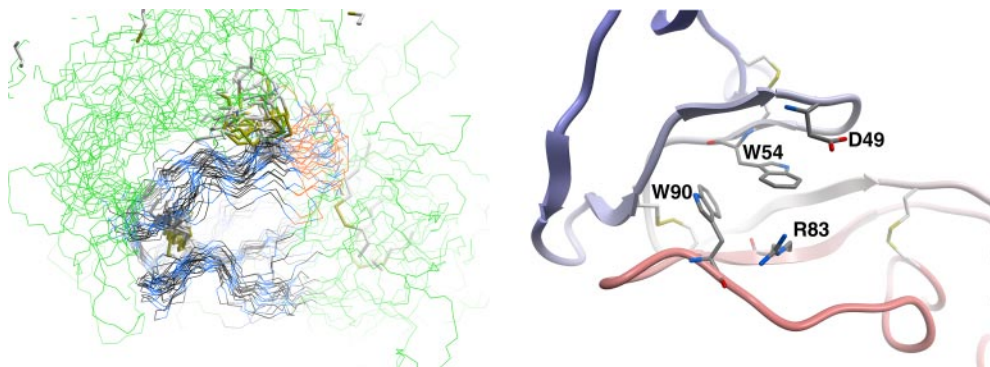


Fig. 9. Homology models of the amino-terminal domain of the rat secretin receptor. *Left*, ensemble of 20 models of the amino terminus of the secretin receptor. The structurally stable central core, consisting of residues 43 to 48, 53 to 64, and 82 to 93 is colored in black for backbone carbon and blue for backbone nitrogen; the tip of the β -hairpin, residues 49 to 52, is colored in orange for backbone carbon and blue for backbone nitrogen, and the flanking flexible regions are colored green. The C_{α} rmsd of the central core and the flexible regions among the 20 models were 2.11 ± 0.60 Å and 11.3 ± 2.0 Å, respectively. *Right*, lack of salt bridge between Asp⁴⁹ and Arg⁸³ in models of the amino terminus of the rat secretin receptor. Of the 20 final models of the amino terminus of the secretin receptor, 13 did not contain a salt bridge between Asp⁴⁹ and Arg⁸³. Shown is one representative model.

Discussion

The molecular basis of natural peptide ligand binding to the amino-terminal domain of class II G protein-coupled receptors and the conformation of this domain are of substantial current interest. This is based on a series of observations showing that this region is key for agonist binding and receptor activation for multiple members of this family (Jupner et al., 1994; Cao et al., 1995; Gourlet et al., 1996). The current work has focused on these aspects of the prototypic secretin receptor. This receptor was the first member of the class II GPCR family to be cloned and displays conservation of important domains in the natural peptide agonist ligand, as well as receptor structural signatures and functional mechanisms examined. This model system was chosen, because extensive experimental data are already available to facilitate the meaningful docking of secretin to this receptor.

Useful structural insights have come from the solution of the NMR structure of the amino-terminal domain of another class II GPCR, the CRF2 β receptor (Grace et al., 2004). In the current work, we have carefully examined those reported

structures and the distance constraints that had contributed to the reported models. In the current report, the CRF2 β receptor model was refined, and the insights coming from it were applied to the analogous region of the structurally related secretin receptor. This probably represents a more meaningful template for a homology model for this region of the secretin receptor than the amino-terminal region of ribonuclease Mc1, which was previously used as template in the absence of the CRF2 β receptor structure (Dong et al., 2003). Indeed, the core regions of the amino-terminal structures of the secretin receptor and of the CRF2 β receptor have now been found to be very similar.

Most of the experimentally defined sites of residue-residue approximation that have been determined for the secretin receptor are within the first 38 residues of the distal amino terminus in poorly constrained regions of this structure. Of particular interest is that the regions of the secretin receptor that are homologous to the relatively stable core of the amino terminus of the CRF2 β receptor have not been covalently labeled by any of positions of photolabile residues spread throughout the pharmacophoric domain of secretin receptor probes that have been used to date. This raises the possibility that this stable core provides a lattice deep in the structure with the portions of the receptor that interact with the natural peptide ligands situated above it.

Although placement of the peptide ligands within the amino-terminal domain is crucial to understanding high-affinity binding for class II receptors, an additional key factor to resolving the mechanism of receptor activation by agonists is the positioning of both the peptide ligand and receptor amino-terminal domain relative to the heptahelical transmembrane core.

There are currently few empirical data to direct the organization of the receptor amino-terminal domain and the transmembrane receptor core, and this has led to disparate models of the relative positioning of these two domains. For

TABLE 2

C α distances between cross-linked residues in secretin and the secretin receptor in three of the best models after grid docking and flexible backbone refinement.

Secretin	Secretin Receptor	Model 1	Model 2	Model 3
			Å	
Phe ⁶	Val ⁴	12.5	9.2	6.9
Arg ¹²	Val ⁶	4.8	6.0	5.6
Leu ¹³	Val ¹⁰³	15.4	18.9	16.0
Gln ¹⁴	Pro ³⁸	12.1	10.3	12.8
Arg ¹⁸	Arg ¹⁴	6.8	6.6	5.5
Leu ²²	Leu ¹⁷	6.1	9.1	5.7
Leu ²⁶	Leu ³⁶	5.2	5.8	6.2
Arg ²¹	Arg ¹⁵	10.4	10.6	10.3
Leu ²³	Arg ²¹	12.6	10.8	11.0

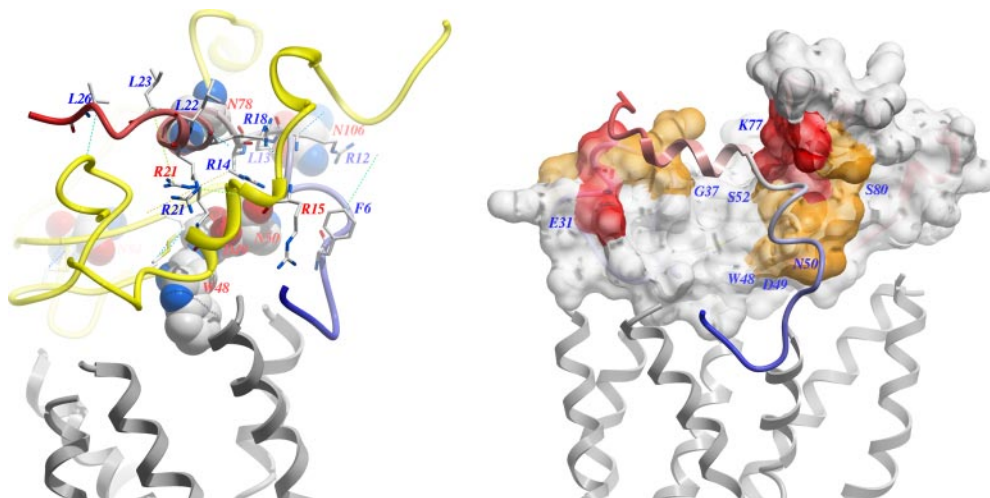


Fig. 10. Refined conformation of the amino terminus of the secretin receptor. This structure accommodates nine photoaffinity labeling constraints and the three disulfide bonds shown to exist in the secretin receptor (model 1 from Table 2). It also demonstrates the potential availability of the WDN sequence (highlighted) as a possible endogenous agonist ligand that could interact with the body of the secretin receptor. This is illustrated two different ways: the left panel illustrates the entire sequence; the right panel removes the distal ends of the receptor amino terminus to highlight the peptide-binding surface. *Left*, the amino-terminal domain is represented in yellow, the transmembrane domain in gray. The secretin peptide is colored blue-red from the amino terminus to the carboxyl terminus. The WDN motif and the three solvent accessible, glycosylated Asn⁷⁸, Asn⁸⁴, and Asn¹⁰⁶ are displayed in CPK representation: blue, nitrogen; red, oxygen; white, carbon. Cross-linking residues on the secretin peptide are displayed in xstick, the distance restraints are displayed as dotted lines. Residues labels are blue on the secretin peptide and red on the receptor. *Right*, the final docking pose of the secretin peptide in our secretin receptor model is displayed. The WDN motif is labeled in blue. Red patch represents residues in direct contact with secretin peptide. Orange patch represents residues within 3 Å of secretin peptide.

the CRF2 β receptor, the orientation used was based on a proposed interaction between a negatively charged surface of the receptor amino-terminal domain and positively charged residues present in the extracellular loops of the receptor core (Grace et al., 2004), whereas the VPAC1 receptor amino-terminal domain was arbitrarily placed above the receptor core in the recently proposed model of the intact VPAC1 receptor (Tan et al., 2006). In both cases, the docking of peptides to their receptors relied significantly on general consideration of the functional roles of the amino- and carboxyl-terminal ends of the peptide ligands across class II receptors that are dependent upon the orientation of the receptor amino-terminal domain and receptor core. These models are further complicated by lack of direct knowledge of regions of the amino terminus that are disordered in the NMR structure that form the basis for receptor amino-terminal domain modeling.

In the current report, we have used two restraints in determining how to best position the amino-terminal domain of the secretin receptor with its helical bundle domain. The first restraint is the integrity of the peptide backbone involved in connecting the two domains, bringing the amino-terminal end of the first transmembrane helix to the carboxyl-terminal end of the receptor amino terminus. This is a common feature of each of the receptor models; however, the innate flexibility of the distal end of the receptor amino-terminal domain allows multiple surfaces to potentially abut the receptor core. The second restraint is specific to the secretin receptor and assumes that the WDN sequence within the amino terminus of the receptor interacts with the helical bundle above transmembrane segment six (Dong et al., 2006). This was based on a series of studies demonstrating the ability of this receptor sequence to act as an endogenous agonist ligand, with its site of interaction demonstrated by covalent labeling of the receptor body using two distinct photoaffinity labeling probes derived from the critical WDN tripeptide (Dong et al., 2006). It is noteworthy that similar activation of VPAC1 and calcitonin receptors by homologous endogenous peptide sequences has also been shown to occur, suggesting that such activation may be a common mechanism within the class II GPCR family (Dong et al., 2006).

Secretin was docked to this preliminary model of the secretin receptor using the seven published photoaffinity labeling spatial approximation constraints. Of particular interest is that, with the peptide docked this way and given the proposed orientation of the two domains of the secretin receptor, the amino terminus of the peptide was found to be adjacent to the helical bundle in the region it has been independently shown to photoaffinity label (Dong et al., 2004). In addition, the two spatial approximation constraints newly identified in the current report were also compatible with this docking and orientation. It is encouraging that these independently derived observations are fully compatible with the working model.

Critical comparison of the currently proposed model of the mode of peptide docking to the amino-terminal domain of the secretin receptor with those of the astressin/CRF-occupied CRF2 β receptor (Grace et al., 2004) and of the vasoactive intestinal polypeptide-occupied VPAC1 receptor (Tan et al., 2006), shows clear differences. As discussed above, the orientations of the amino-terminal domains with the helical bundle domains of these receptors are all different. The se-

cretin receptor orientation was guided by the WDN motif and was, therefore, fully consistent with its interaction with the relevant region of the receptor body. However, in the CRF2 β receptor model, the amino-terminal domain interacts with the transmembrane domain through the "back side" of the model displayed in Figs. 8 and 9. As a consequence, Leu⁶⁴ to Gln⁶⁶ of the CRF2 β receptor, corresponding to the WDN motif, is facing upward and away from the analogous region of that receptor. In the VPAC1 receptor model, the amino-terminal domain makes contact with the transmembrane domain near the end of the second β -sheet containing residues Ile⁶¹ to Cys⁶³ and Pro⁷⁷ to Gly⁷⁹. In this orientation, the region analogous to the WDN motif of the secretin receptor is facing sideward. However, there was no clear rationalization for this orientation with respect to the helical bundle.

Comparison of the peptide-binding regions of these receptors also reflects differences. In the current model of the secretin receptor, the peptide is situated between the distal amino terminus, the region containing the majority of the sites of cross-linking, and part of the stable core. Glu⁹ to Ala¹⁷ of secretin interacts with the tip of the first β -sheet, including residues Asp⁴⁹ to Ser⁵², and the disordered loop comprising residues Lys⁷⁷ to Ser⁸⁰. Gln²⁰ to Val²⁷ of secretin interacts with the disordered loop, including residues Glu³¹ to Gly³⁷ (Fig. 10). In the proposed CRF2 β receptor structure, regions proposed to represent the peptide-binding site based on chemical-shift perturbations upon antagonist (astressin) binding include Ile⁶⁷ to Thr⁶⁹, Gly⁹⁰ to Asn⁹³, Glu¹⁰² to Cys¹⁰³, and Arg¹¹² to Ser¹¹⁶. The peptide-binding region in both the secretin and CRF2 β receptor models includes the tip of the first β -sheet and the disordered loop near the palm of the second β -sheet. In the proposed VPAC1 receptor structure, the peptide-binding interface was inferred from three pairs of cross-linked residues, at positions 6, 22, and 24 of the peptide ligand. Here, this region includes receptor residues Gln⁸⁰ to Leu⁸⁴, Val¹⁰¹ to Cys¹⁰⁵, and Trp¹¹⁰ to Cys¹²². It is noteworthy that this interface would be at the "bottom side" of the model of the secretin receptor displayed in Figs. 8 and 9, opposite the proposed peptide-binding sites in both the currently proposed model of the secretin receptor and that of the CRF2 β receptor. At present, we do not have sufficient empirical data to confirm whether the alternate models proposed for different receptors reflect divergence in mechanism of action or potential problems in generation of working models.

Another structural motif of substantial interest is the proposed intradomain salt bridge between Asp⁶⁵ and Arg¹⁰¹ of the CRF2 β receptor. Of particular interest, the region of the secretin receptor amino terminus that has been shown to have endogenous agonist activity is centered on the residue analogous to CRF2 β receptor residue Asp⁶⁵. If such a bond were present and stable in the secretin receptor (it would involve the secretin receptor residues Asp⁴⁹ and Arg⁸³), it might make the endogenous agonist hypothesis less likely as a mechanism of activation of these receptors. In the secretin receptor amino terminus models that were built based on homology with the CRF2 β receptor structure, the salt bridge between Asp⁴⁹ and Arg⁸³ actually did not exist in the majority of conformations. Whether the absence of this salt bridge in the secretin receptor models is an artifact caused by an inadequate number of distance restraints in modeling or a genuine feature of the secretin receptor amino terminus re-

mains to be explored. It should be noted, however, that there is no explicit salt-bridge present from the CRF2 β NMR distance restraints, whereas mutation of Asp⁴⁹ to glycine has no negative effect on the biological activity of the secretin receptor (Dong et al., 2006). It is also recognized that solvent-accessible salt bridges are easily broken. Such a breakable salt-bridge between Asp⁴⁹ and Arg⁸³ of the secretin receptor is still consistent with the endogenous agonist hypothesis (Dong et al., 2006). However, in the best conformation of the secretin-occupied secretin receptor currently being proposed, the WDN motif is readily accessible to its proposed target of action, even with the salt-bridge incorporated.

It is encouraging that, in the current work, multiple experimentally derived residue-residue distance constraints were easily accommodated within the molecular model of the secretin receptor. With initial peptide docking accomplished based on the previously described seven constraints, it was even more encouraging that the currently derived two additional constraints were also then well accommodated. In the best working model, all existing experimentally derived constraints were fully accommodated, and the WDN motif was available for interaction with the body of the receptor.

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Address correspondence to: Laurence J. Miller, M.D., Mayo Clinic, 13400 East Shea Blvd., Scottsdale, AZ 85259. E-mail: miller@mayo.edu