

# Structural Insights into the Amino-Terminus of the Secretin Receptor: I. Status of Cysteine and Cystine Residues

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## ABSTRACT

The secretin receptor is prototypic of the class II family of G protein-coupled receptors, with a long extracellular amino-terminal domain containing six highly conserved Cys residues and one Cys residue (Cys<sup>11</sup>) that is present only in the most closely related family members. This domain is critical for function, with some component Cys residues believed to be involved in key disulfide bonds, although these have never been directly demonstrated. Here, we examine the functional importance of each of these residues and determine their involvement in disulfide bonds. Secretin binding was markedly diminished after treating cells with cell-impermeant reducing reagents, supporting the presence of important extracellular disulfide bonds. To determine whether the amino-terminal domain was covalently attached to the receptor body by disulfide linkage, a strategy was implemented that involved introduction of an acid-labile Asp-Pro sequence to enable specific cleavage at the boundary of

these domains. Under nonreducing conditions, the amino terminus was released from the receptor body, supporting the absence of covalent association between these domains. Quantitative [<sup>14</sup>C]iodoacetamide incorporation into the isolated amino-terminal domain of the receptor in the absence and presence of chemical reduction established the ratio of free to total Cys residues as 1:7, consistent with three disulfide bonds. Mutagenesis of each of the amino-terminal Cys residues to Ala was tolerated only for Cys<sup>11</sup>, suggesting that these bonds linked the conserved Cys residues. This was further supported by treatment of intact cells expressing wild-type or C11A mutant secretin receptor with a cell-impermeant sulfhydryl-reactive reagent. Thus, the functionally important amino terminus of the secretin receptor represents a structurally independent, highly folded, and disulfide-bonded domain, with a pattern that is likely critical and conserved throughout this receptor family.

The secretin receptor is a prototypic member of a distinct family (class II) of G protein-coupled receptors that share little amino acid sequence homology (less than 12%) with the larger rhodopsin- $\beta$  adrenergic receptor family (class I) (Segre and Goldring, 1993; Ulrich et al., 1998). A key characteristic of the class II family is that all component receptors have a long (116–147 amino acids) amino-terminal tail that contains six highly conserved Cys residues. This domain of the secretin receptor has been shown to play a critically important role in ligand binding and receptor activation, based on truncation, site-directed mutagenesis, and chimeric receptor studies (Holtmann et al., 1995, 1996; Vilardaga et al., 1995; Gourlet et al., 1996a,b). This theme has been consistent in similar types of studies for multiple members of this family of G protein-coupled receptors (Cao et al., 1995; Stroop et al., 1996; Mannstadt et al., 1998). Although functional disruption in these molecular biological approaches can reflect either a direct interaction between this domain and the ligand

or an allosteric effect, photoaffinity labeling through residues intrinsic to the secretin pharmacophore have also clearly revealed direct spatial approximation with this receptor domain (Dong et al., 1999a,b). This, too, has been consistent with other receptor family members (Cao et al., 1995).

Of particular interest is that receptors in the class II family have been shown to be particularly sensitive to treatment with chemical reductants and sulfhydryl-reactive reagents (Robberecht et al., 1984; Huang and Rorstad, 1989; Vilardaga et al., 1997). This is also true of the secretin receptor (Robberecht et al., 1984; Vilardaga et al., 1997). Unfortunately, these reagents have been cell permeant and could have their deleterious effects explained by reaction with intramembranous or cytosolic Cys residues within the receptor itself or even within its G protein or other components of its signaling cascade. There has not yet been any direct demonstration of disulfide bonds or the delineation of number or pattern of such bonds for receptors in this family. Given the conserved nature of a series of Cys residues within a domain of the receptor that is not only functionally important but also demonstrated to be directly involved in ligand

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**ABBREVIATIONS:** IAA, iodoacetamide; MESNA, 2-mercaptoethanesulfonic acid; MTSET, [2-(trimethylammonium)ethyl] methanethiosulfonate; HA, hemagglutinin; CHO, Chinese hamster ovary; DTT, dithiothreitol.

binding, it is very attractive to postulate the presence of key disulfide bonds in this region that could establish a conserved, highly folded conformation that provides a platform for binding. It is also noteworthy that all the natural ligands for receptors in this family are moderately large peptides, with structural similarities among themselves as well (Ulrich et al., 1998). This adds to the probability of a conserved theme for ligand binding to class II G protein-coupled receptors.

The secretin receptor contains ten extracellular Cys residues. These include seven Cys residues within the amino-terminal tail (11, 24, 44, 53, 67, 85, and 101), two within the first extracellular loop (186 and 193), and one within the second loop domain (263). Cys<sup>193</sup> and Cys<sup>263</sup> are conserved throughout the G protein-coupled receptor superfamily, where they are believed to form an architecturally important disulfide bond connecting the first and second extracellular loop domains (Dohlman et al., 1990). This probably plays a role in the maintenance of the helical bundle. Six of the seven Cys residues within the amino terminus of the secretin receptor are highly conserved throughout the class II family; Cys<sup>11</sup> is only shared with the most closely related vasoactive intestinal polypeptide and growth hormone releasing factor receptors (Segre and Goldring, 1993; Ulrich et al., 1998).

Our ultimate goal is to determine the conformation of the amino terminus of the secretin receptor, and to elucidate the molecular basis of ligand binding and activation of this receptor. In the present work, we used cell impermeant reducing reagents to document the importance of extracellular disulfide bonds within this receptor for its function. We had hoped to also define each of the disulfide bonds that are present and to use these as specific structural constraints to aid in molecular modeling of this receptor. This proved to be extremely difficult because of problems with receptor purification and the nonquantitative cleavage and/or release of cleaved fragments from the native fully intact receptor. However, we achieved much toward this end by demonstrating that the amino-terminal domain was not disulfide-bonded to the body of the receptor, that it contained a finite number of intradomain disulfide bonds, and by experimentally defining the Cys residues involved in those bonds. Key for this accomplishment was the development and validation of a method to cleave and release the intact, folded, and disulfide-bonded amino-terminal domain of this receptor. We inserted an acid-labile Asp-Pro sequence just above the first transmembrane segment and established conditions for its specific cleavage. The released amino terminus is also more amenable to cleavage and release of noncovalently linked fragments than the intact receptor and should therefore be an ideal substrate for the completion of the specific mapping of the disulfide bonds. It also should have obvious usefulness for direct structural analysis.

## Materials and Methods

**Reagents.** Iodoacetamide (IAA), glutathione, and 2-mercaptoethanesulfonic acid (MESNA) were from Sigma. [<sup>14</sup>C]IAA (55 mCi/mmol) was from American Radiolabeled Chemicals, Inc. (St. Louis, MO). The methanethiosulfonate derivative [2-(trimethylammonium)-ethyl] methanethiosulfonate (MTSET), a positively charged sulfhydryl-reactive reagent, was from Toronto Research Chemicals, Inc. (North York, Ontario, Canada). Cyanogen bromide was from Pierce Chemical Company. 12CA5 monoclonal antibody directed to the

hemagglutinin (HA) epitope tag was from Roche Molecular Biochemicals (Indianapolis, IN). All other reagents were analytical grade.

**Peptides.** Rat secretin-27, (Tyr<sup>10</sup>)rat secretin-27, and (Bpa<sup>6</sup>,Tyr<sup>10</sup>)rat secretin-27 were synthesized in our laboratory. All of these have been shown to be fully biologically active and to bind to the secretin receptor with high affinity (Ulrich et al., 1993; Dong et al., 1999a). (Tyr<sup>10</sup>)rat secretin-27 was designed to provide a site for oxidative radioiodination at position 10, and (Bpa<sup>6</sup>,Tyr<sup>10</sup>)rat secretin-27 was designed to provide both sites for radioiodination and cross-linking with the addition of a photolabile benzoyl-L-phenylalanine incorporated into position 6. These peptides were radioiodinated using IODO-BEADS (Pierce Chemical, Indianapolis, IN) and Na<sup>125</sup>I and were purified by reversed-phase HPLC to specific radioactivities of 2000 Ci/mmol, as we have reported (Ulrich et al., 1993; Dong et al., 1999a).

**Cell Lines.** The wild-type rat secretin receptor-bearing Chinese hamster ovary cell line (CHO-SecR) and HA epitope-tagged secretin receptor-bearing CHO cell line (CHO-SecR-HA37) have been previously established and characterized (Ulrich et al., 1993; Dong et al., 1999b). One new cell line was established for this study. It expresses an HA epitope-tagged secretin receptor mutant in which Glu<sup>11</sup> was changed to (Asp-Pro)<sup>11</sup> (CHO-SecR-HA37-DP111). The mutant constructs were prepared by polymerase chain reaction mutagenesis (Ho et al., 1989) of the SecR-HA37 cDNA in the pcDNA3 vector (Invitrogen, Carlsbad, CA). The presence of the correct mutation in the construct was verified by direct DNA sequencing (Sanger et al., 1977). The purified plasmid construct, pcDNA3/SecR-HA37-DP111, was transfected into CHO cells using lipofectin (Life Technologies, Rockville, MD). Control cells were established by transfecting CHO-K1 cells with the parent pcDNA3 plasmid and selecting for G418 resistance. The population of the SecR-HA37-DP111-bearing cells was enriched after G418 selection and by fluorescence-activated cell sorting using the fluorescent secretin analog that we described previously (Ulrich et al., 1993). This was followed by clonal selection using the limiting dilution method. This cell line was functionally characterized using ligand binding and cAMP response assays.

All of the CHO cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> in Ham's F-12 medium supplemented with 5% (v/v) Fetal Clone 2 (Hyclone Laboratories, Logan, UT). Enriched plasma membranes from these cell lines were prepared as we described previously (Hadac et al., 1996).

**Transiently Expressed Receptor Site Mutants.** We prepared eight different site mutants of the secretin receptor, each representing the replacement of a single Cys residue in the receptor amino terminus or first extracellular loop with an Ala. These included C11A, C24A, C44A, C53A, C67A, C85A, C101A, and C186A. Each of these constructs was also produced incorporating an HA epitope tag in position 37 within the amino-terminal tail, previously shown to not interfere with secretin binding or stimulated biological activity (Dong et al., 1999b). The mutant constructs were prepared and verified as described above. COS-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% (v/v) Fetal Clone 2 at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Transfections were performed on 20 to 25% confluent monolayers in 100-mm dishes using the DEAE-dextran method (Lopata et al., 1984). The transfected cells were lifted with trypsin-EDTA the following day and were plated at a density of 20,000 cells/well into 24-well tissue culture dishes. Radioligand binding and cAMP assays were carried out in these dishes 3 days after transfection.

In other experiments, transfected cells were replated to grow on coverslips for morphological assessment of receptor expression on the cell surface. For this, intact cells were incubated at 4°C with 12CA5 antibody at a dilution of 1:500 in Dulbecco's modified Eagle's medium with 5% Fetal Clone 2 at pH 7.0, and washed three times in phosphate-buffered saline. Cells were then fixed for 30 min at room temperature with 4% paraformaldehyde, washed three more times with PBS, and incubated for 1 h at room temperature with rhodam-

ine-conjugated goat anti-mouse IgG diluted 1:200 in 5% glycerin, 5% fetal bovine serum in PBS. Cells were again washed three times with PBS, mounted, and examined with a Zeiss 510 confocal microscope.

**Secretin-Stimulated cAMP Activity Assay.** The biological activity of secretin receptor constructs was studied using a competition-binding assay for cAMP activity (Diagnostic Products Corporation, Los Angeles, CA), as described previously (Ganguli et al., 1998). For this, intact receptor-bearing cells were stimulated with secretin at 37°C for 30 min. The reaction was stopped by ice-cold 6% perchloric acid. After adjusting the pH to 5.5 to 6.0 with 30% KHCO<sub>3</sub>, cell lysates were cleared by centrifugation at 3000 rpm for 15 min, and the supernatants were used in the cAMP assay. Radioactivity was quantified by scintillation counting in a Beckman LS6000 (Beckman Instruments, Columbia, MD). All assays were performed in duplicate in at least three independent experiments. Concentration-response curves for stimulation of cAMP were plotted using nonlinear regression routines in the Prism software package (GraphPad, San Diego, CA). The cAMP responses of Cys-to-Ala receptor mutants were measured in similar assays, except that the assays were performed on transiently transfected COS cells.

**Secretin Receptor Binding Studies.** Receptor-bearing CHO-SecR-HA37-DP111 cells or transfected COS cells were plated in 24-well dishes 2 days before the binding assay. Cells were incubated with a constant amount of radioligand, [<sup>125</sup>I-(Tyr<sup>10</sup>)rat secretin-27 (3–5 pM), and increasing concentrations of unlabeled secretin (0–1 μM) for 1 h at room temperature in KRH medium (25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 2 mM CaCl<sub>2</sub>, 1 mM phenylmethylsulfonyl fluoride, 0.01% soybean trypsin inhibitor) containing 0.2% BSA. These conditions were adequate to achieve steady-state binding. After washing the cells twice with ice-cold KRH medium, cells were lysed with 0.5 ml of 0.5 M NaOH and the radioactivity in the lysate was determined using an ICN Series 100 gamma counter. Nonspecific binding was determined in the presence of 1 μM unlabeled secretin and represented less than 20% of total binding. Binding data were plotted using the nonlinear

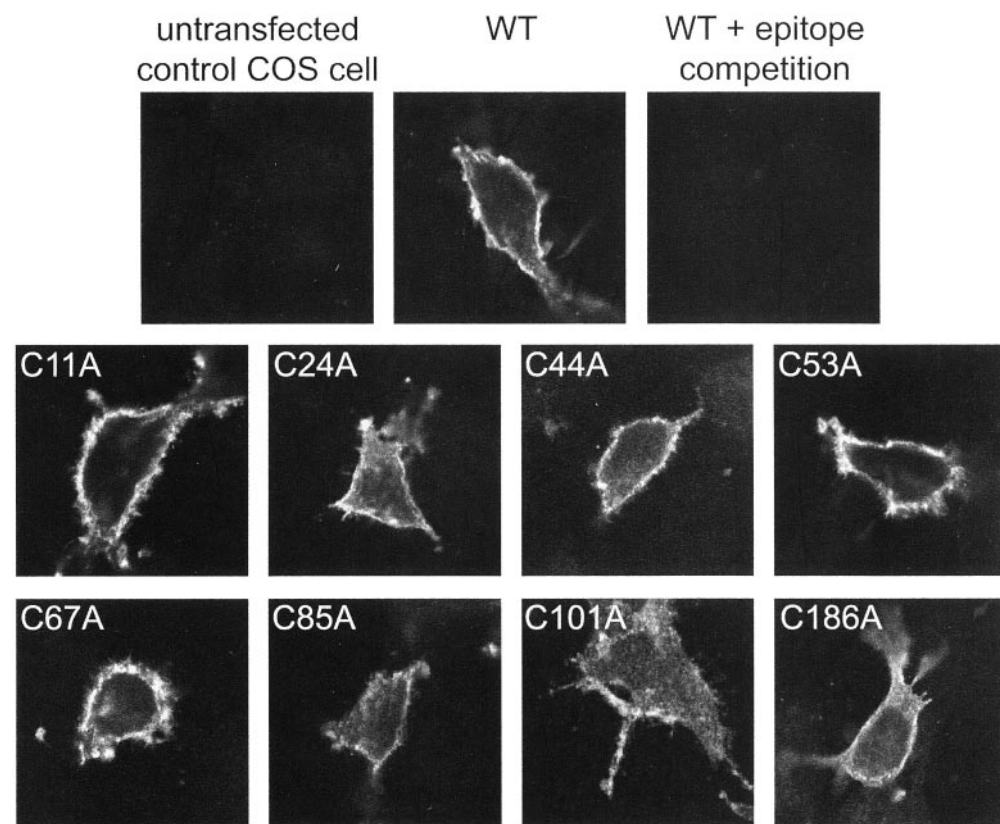
regression routines in the Prism software package (GraphPad, San Diego, CA) and were analyzed using the LIGAND program of Munson and Rodbard (Munson and Rodbard, 1980).

In a series of studies, the binding activities of CHO-SecR cells were characterized after treatment with MTSET or glutathione/MESNA. This used a similar assay, except that the intact cells were preincubated with 5 mM MTSET or 10 mM glutathione/MESNA for 20 min at room temperature and washed before performing radioligand binding.

**Photoaffinity Labeling of the Secretin Receptor.** Photoaffinity labeling of secretin receptor-bearing membranes was performed as we have described previously, with [<sup>125</sup>I-(Bpa<sup>6</sup>,Tyr<sup>10</sup>)rat secretin-27 as probe (Dong et al., 1999a). After affinity labeling, membrane proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels using the method of Laemmli (1970). For selected experiments, the affinity-labeled receptor or relevant fragments were deglycosylated with endoglycosidase F, as we previously reported (Dong et al., 1999b).

**Chemical and Enzymatic Cleavage of the Secretin Receptor.** Affinity-labeled and gel-purified SecR-HA37-DP111 or SecR-HA37 were digested with 8 M acetic acid (in 0.1% SDS, 10 mM Tris-HCl, pH 8.0, 2 mM EDTA) in a water bath that was slowly heated from room temperature to 100°C over 1 h. The products of cleavage were separated on a 10% NuPAGE gel (Novex, San Diego, CA) using 4-morpholineethanesulfonic acid running buffer, and the labeled products were visualized by autoradiography. Digestions of the gel-purified receptor or its relevant fragments with cyanogen bromide or endoproteinase Lys-C were performed as described previously (Hadac et al., 1998; Dong et al., 1999a,b). The apparent molecular masses of the radiolabeled receptor and its fragments were determined by interpolation on a plot of the mobility of prestained Multimark protein standards (Novex) versus the log values of their apparent masses.

**[<sup>14</sup>C]IAA Alkylation of the Secretin Receptor Amino Terminus.** [<sup>14</sup>C]IAA alkylation was carried out as described previously



**Fig. 1.** Immunohistochemical evidence for cell surface expression of secretin receptor constructs. Shown are representative immunofluorescent images of cells incubated with anti-HA epitope antibody, with reactivity demonstrated using rhodamine goat anti-mouse IgG. Shown are untransfected COS cells and COS cells transfected with wild-type (WT) secretin receptor (and stained in the presence or absence of competing HA peptide) or transfected with secretin receptor mutants.



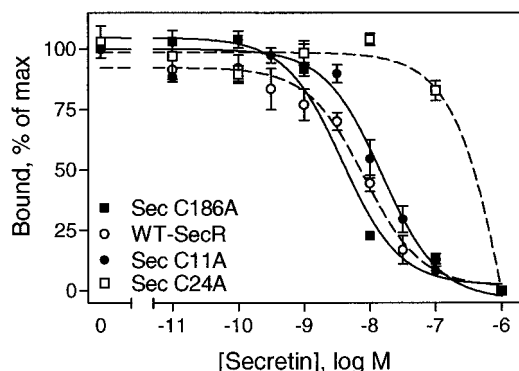
(Dohlman et al., 1990). In this study, affinity-labeled, gel-purified samples representing the amino terminus of the secretin receptor (20  $\mu$ l) were diluted 5-fold with denaturation buffer (7 M guanidine hydrochloride, 0.5 M Tris-HCl, pH 8.5, and 2 mM EDTA), in the absence or presence of 5 mM dithiothreitol (DTT). Samples were incubated for 20 min at 60°C, then cooled to 23°C. Twenty microliters of [ $^{14}$ C]IAA (50  $\mu$ Ci/ml, 55 mCi/mmol, in ethanol) was then added to the solution. After 1 h at 23°C, an equal volume of 200 mM nonradioactive IAA in the absence or presence of 125 mM DTT was added. The reaction was incubated for an additional hour at 23°C and it was stopped by precipitation of the receptor fragments in 85% iced ethanol. Pellets were then resuspended and solubilized in immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM  $\text{MgCl}_2$ , 1 mM EDTA, 0.5% Nonidet P40, 0.2% BSA, and 0.1% SDS) and tagged receptor fragments were purified by immunoprecipitation with anti-HA 12CA5 antibody as reported previously (Dong et al., 1999b). The immunoprecipitated samples were solubilized in SDS sample buffer, resolved by electrophoresis on 10% NuPAGE gels, and visualized by autoradiography, as described above.

**Data Analysis.** All experiments were repeated at least three independent times, with results expressed as means  $\pm$  S.E.M. Significant differences were determined by the Tukey-Kramer test of differences, with  $P < .05$  considered to be statistically significant.

## Results

**Site-Directed Mutagenesis of the Extracellular Cys Residues in the Secretin Receptor.** To explore the importance of the extracellular Cys residues of the secretin receptor, we changed each of the seven Cys residues (11, 24, 44, 53, 67, 85, and 101) within the amino terminus and Cys<sup>186</sup> in the first extracellular loop to alanines. The mutated receptor constructs were transfected into COS cells, where immunohistochemistry was used to examine cell surface expression (Fig. 1). Appropriately negative controls included untransfected COS cells and immunolabeling of receptor-bearing cells in the presence of excess competing HA peptide, and each receptor construct was shown to be expressed on the cell surface in similar density.

Each of these receptor constructs was characterized for secretin binding and secretin-stimulated cAMP responses. We demonstrated that only the Cys residues that are not conserved within the secretin receptor family could be changed without profound negative impact on receptor func-

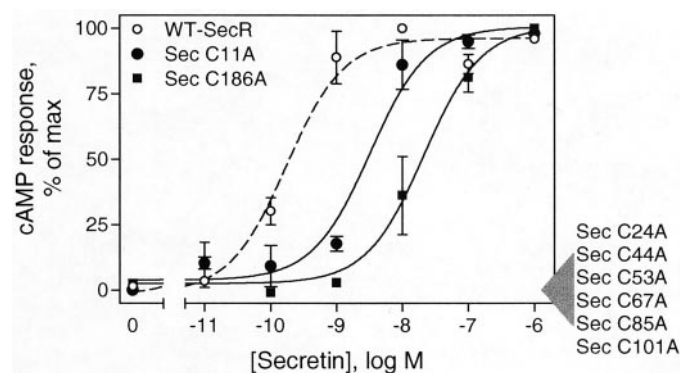


**Fig. 2.** Competition binding to secretin receptor constructs. Shown are curves representing the ability of secretin to compete for secretin radioligand binding to COS cells expressing Cys-to-Ala receptor mutants and wild-type secretin receptor. Values represent means  $\pm$  S.E.M. of assays performed in duplicate at least three independent times. The Cys-to-Ala receptor mutations in positions 44, 53, 67, 85, and 101 demonstrated no consistent saturable binding of radioligand using competing secretin concentrations up to 1  $\mu$ M.

tion. These included C11A and C186A (Figs. 2 and 3). These receptor constructs maintained high affinity binding (Fig. 2) ( $K_i$  values, nanomolar secretin: wild-type,  $16 \pm 1$ ; C11A,  $10 \pm 3$ ; C186A,  $3 \pm 1$ ). Of the other constructs, only C24A demonstrated any significant saturable binding of radioligand using concentrations of secretin up to 1  $\mu$ M ( $\text{IC}_{50} = 0.7 \mu\text{M}$  secretin).

Biological activity data was parallel with the binding (Fig. 3). The  $\text{EC}_{50}$  values (nM secretin) for these constructs to stimulate cAMP include the following: wild-type,  $0.2 \pm 0.1$ ; C11A,  $6 \pm 2$ ; C186A,  $20 \pm 6$ . None of the other constructs had any recognizable biological activity in response to secretin concentrations as high as 1  $\mu$ M. Similar effects have been reported for the mutation of these same Cys residues within the rat secretin receptor to Ser residues (Villardaga et al., 1997) and for analogous Cys residues within the human vasoactive intestinal peptide (VIP) receptor to Gly residues (Gaudin et al., 1995). The results led us to propose that all of the six conserved Cys residues within the amino-terminal domain of the secretin receptor were involved in critical disulfide bonds, whereas the two nonconserved Cys residues, Cys<sup>11</sup> and Cys<sup>186</sup>, were either free or involved in noncritical disulfide bond(s).

**Existence of Disulfide Bonds in the Secretin Receptor.** We have shown above, using site-directed mutagenesis, that each of the conserved Cys residues was critical for receptor function, suggesting that these Cys residues might be involved in disulfide bonds. The existence of disulfide bonds within the receptor was confirmed by the difference in electrophoretic migration between the affinity-labeled native and reduced secretin receptor (Fig. 4). We were able to make this observation because of the extended geometry of the molecule after reduction of its disulfide bonds, interfering with its migration. Further evidence for disulfide bonds came from the difference in the electrophoretic migration of the affinity labeled fragment of this receptor generated by Lys-C cleavage when separated under reducing and nonreducing conditions. The (Bpa<sup>6</sup>,Tyr<sup>10</sup>)rat secretin-27 probe is known to affinity label Val<sup>4</sup> in the amino terminus of the secretin receptor (Dong et al., 1999a). Because this protease cleaves at the carboxyl-terminal side of Lys residues, its action should yield a band of approximate 6000 Da, matching the sum of the masses of the first Lys-C fragment (residues one through 30) (3245 Da) and the covalently attached probe. Although



**Fig. 3.** Impact on biological activity of secretin receptor Cys site mutants. Shown are curves representing the secretin-stimulated cAMP responses of COS cells expressing eight Cys-to-Ala receptor mutants and wild-type receptor. Values represent means  $\pm$  S.E.M. of assays performed in duplicate at least three independent times.

this was apparent after reduction, it was not released in the absence of DTT, demonstrating that this fragment of the receptor was bound through disulfide bond to the receptor body (Fig. 4).

**Functional Importance of Secretin Receptor Disulfide Bonds.** To investigate the importance of the extracellular disulfide bonds, experiments were designed using the cell-impermeant reducing reagents glutathione and MESNA. The results showed that preincubation of the wild-type receptor-bearing CHO-SecR cells with 10 mM glutathione or MESNA markedly decreased secretin-binding activity, with dramatic reduction in radioactivity bound and shifts to the right in the peptide competition curves (Fig. 5). If these data are interpreted using LIGAND and maintaining conservation of the receptor molecules on the cell surface, they are consistent with a reduction in receptor affinity from a  $K_i$  value of  $16 \pm 0.6$  nM (control) to  $125 \pm 18$  nM for glutathione ( $P < .01$ ) and  $137 \pm 2$  nM for MESNA ( $P < .001$ ).

**Possibilities of Intra- versus Interdomain Disulfide Bonds.** Key to determining whether the extracellular disulfide bonds spanned major receptor domains was the ability to specifically cleave the amino-terminal domain from the receptor body. Our strategy for this involved the construction of a receptor mutant that did not interfere with function but permitted the efficient and selective cleavage near the intersection between these two domains. We introduced an acid-labile Asp-Pro (DP) sequence by insertional mutagenesis into three different positions, at 108, 111, and 116, based on alignments between related receptors and on the character of amino acid residues normally present at a given position. The insertion of DP in position 111 was best tolerated for both ligand binding and biological activity, both seeming similar to that of wild-type receptor (Fig. 6).

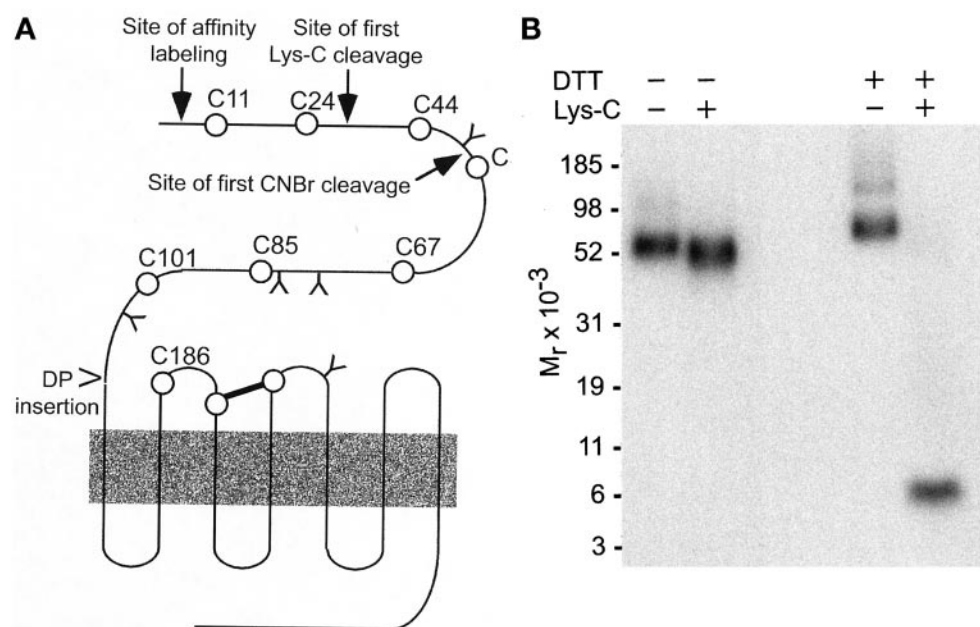
Acetic acid treatment was used to selectively cleave this receptor construct at the position of the DP111 sequence. This was validated, with the cleavage occurring under non-reducing conditions, but with the samples then reduced with DTT and run on gels under reducing conditions. After acetic acid treatment, a band migrating at 40- to 45-kDa was re-

leased from the intact SecR-HA37-DP111 receptor, but not from the non-DP-containing SecR-HA37 construct (Fig. 7). Of particular interest was that this labeled band was also released and demonstrated when run on a nonreducing gel (Fig. 7), supporting the absence of disulfide bonds linking this region with the body of the secretin receptor.

To demonstrate that the acid-released band represented the amino terminus of the receptor, as expected from the location of the acid-labile DP sequence, we deglycosylated the released 40- to 45-kDa band with endoglycosidase F and performed proteolytic cleavage of it. The deglycosylation of the released band resulted in a shift of approximately 18 kDa, corresponding to the sum of the masses of the protein core of the amino-terminal tail and the affinity-labeling probe (Fig. 8). Lys-C cleavage of the 40- to 45-kDa band shifted migration of the labeled fragment to approximately 6,000 Da, identical with that of the Lys-C fragment of the intact receptor (Fig. 8).

**Cysteine/Cystine Ratio within the Receptor Amino-Terminal Domain.** Quantitative analysis of free and total Cys residues within the secretin receptor amino-terminal domain was performed using the irreversible radioactive sulfhydryl-reactive alkylating reagent [ $^{14}$ C]IAA. Reactions were performed as described under *Materials and Methods*, using samples of gel-purified receptor amino terminus in the absence or presence of chemical reduction. This showed that the stoichiometry of the free versus total Cys residues within this domain of the receptor was 1:7 (Fig. 9). This result supported the presence of one of seven Cys residues being free, with the others involved in three intradomain disulfide bonds.

**Nonconserved Cys<sup>11</sup> Is Free.** These results and the results of our Cys mutagenesis experiments led us to postulate that the free Cys represented the only nonconserved Cys residue (Cys<sup>11</sup>) in the amino-terminal domain, whereas each of the other six conserved residues was involved in a disulfide bond. A series of experiments was designed to further confirm this. In this, we treated both wild-type secretin receptor-bearing CHO-SecR cells and C11A mutant receptor-bearing



**Fig. 4.** Existence of disulfide bonds in the secretin receptor. Shown is a cartoon reflecting the positions of key residues within the secretin receptor that are relevant to the interpretation of labeling and cleavage data. Shown also is a representative autoradiograph of a NuPAGE gel used to resolve, in the presence or absence of DTT, the intact and Lys-C digested wild-type secretin receptor affinity labeled with the Bpa<sup>6</sup> analog of secretin (Dong et al., 1999b). The intact affinity-labeled secretin receptor had reduced mobility after reduction (approximately 60 kDa) relative to its nonreduced state (approximately 52 kDa). After reduction with DTT, Lys-C digestion released a labeled fragment migrating at approximately 6000 Da that represented the region of the receptor between residue Ala<sup>1</sup> and Lys<sup>30</sup> (Dong et al., 1999b). In the absence of reduction, this fragment remained disulfide-bonded to the body of the receptor, with the mobility affected slightly by cleavage at the carboxyl terminus of the labeled receptor.

cells with the positively charged, cell-impermeant sulfhydryl-reactive reagent MTSET. This resulted in markedly reduced secretin binding for the wild-type receptor but minimal impairment of the mutant receptor (Fig. 10). These data were consistent with a reduction in receptor affinity from a  $K_i$  value of  $7 \pm 1.4$  nM (wild-type control) to  $24 \pm 6$  nM after treatment with MTSET ( $P < .05$ ) and from a  $K_i$  value of  $10 \pm 3$  nM (C11A control) to  $13 \pm 3$  nM after treatment with MTSET ( $P > .05$ ). The relatively small impairment of the C11A mutant was probably caused by the alkylation of Cys<sup>186</sup> within the first extracellular loop, because the level of impact was similar to that seen with the mutation of that residue to an Ala (Fig. 2). The inability to alkylate Cys<sup>11</sup> in the mutant receptor (because of its absence) clearly protected this construct from the marked negative effect observed with the wild-type receptor.

## Discussion

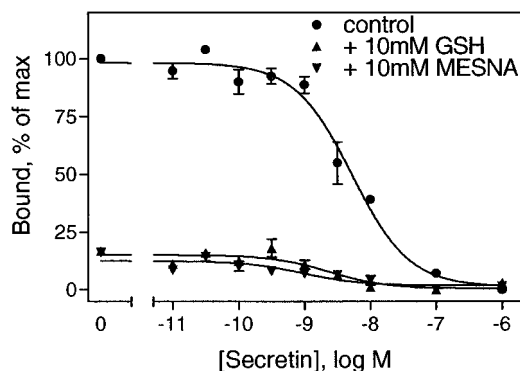
The G protein-coupled receptor superfamily is remarkable for its diversity of natural ligands, ranging from extremely small photons, odorants, and biogenic amines to peptides and even large glycoproteins and viral particles (Kolakowski, 1994). The general themes for the binding and activation by these molecules are similarly varied (Schwartz, 1994; Schwartz and Rosenkilde, 1996). The binding of the smallest ligands are best understood and occur within the confluence of helices in the membrane. As ligands increase in size, their sites of binding move toward the extracellular surface of the lipid bilayer and even to loop and tail domains outside the

bilayer. As this occurs, our understanding of the molecular basis of propagating the conformational change that is necessary to facilitate G protein coupling at the cytosolic face of the receptor becomes less clear.

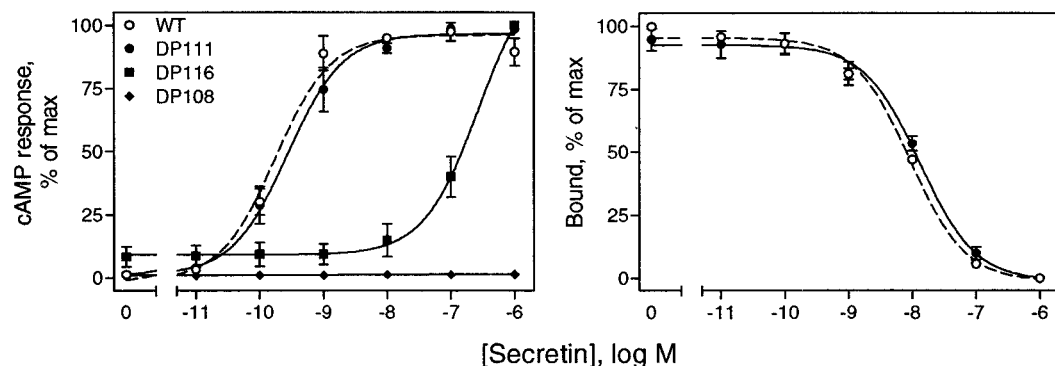
Natural ligands for the class II G protein-coupled receptors are moderately large peptides having amino- and carboxyl-terminal helical domains (Ulrich et al., 1998). There is substantial conformational homology among many of these ligands (Bodanszky and Bodanszky, 1986). Also remarkable is the diffuse nature of the pharmacophoric domains of these ligands (Bodanszky and Bodanszky, 1986; Ulrich et al., 1998). Although the selectivity for their binding and activation resides predominantly within the amino-terminal regions of these hormones, the carboxyl-terminal regions are also important to provide the information necessary for high affinity binding (Juppner et al., 1994). Therefore, it is unsurprising that receptor mutagenesis efforts have pointed toward the importance of the amino-terminal tail and extracellular loop domains for ligand binding and initiation of signaling (Holtmann et al., 1996). Photoaffinity labeling has also demonstrated the direct involvement of the amino-terminal domain of the receptor in ligand binding (Dong et al., 1999a,b). These themes have been quite consistent for several receptors in the class II family.

Class II G protein-coupled receptors share the heptahelical transmembrane topology and signaling via heterotrimeric G proteins with essentially all members of the G protein-coupled receptor superfamily. However, they have their own unique features (Ishihara et al., 1991; Juppner et al., 1991; Lin et al., 1991; Segre and Goldring, 1993; Ulrich et al., 1998). One of the most distinct features of the class II family is the moderately long amino-terminal domain that includes six conserved Cys residues and putative key disulfide bonds. Disulfide bonds can play a major role in the structure and stability of proteins. Such bonds within these receptors, however, have never been directly demonstrated, quantified, or mapped.

In the present work, we provide definitive evidence for the existence of disulfide bonds on the extracellular face of the secretin receptor. These have clear functional importance, based on the severe impact of their disruption by cell impermeant reducing reagents. Although we have not yet achieved the specific mapping of each of these bonds, we have made substantial progress toward that end. We now know that three disulfide bonds exist as intradomain bonds within the receptor amino-terminus and that this domain is not disulfide-bonded to the receptor body. We know that these bonds



**Fig. 5.** Effect of cell-impermeant reducing reagents on wild-type secretin receptor. Shown are the curves representing secretin competition for binding of the secretin radioligand to CHO-SecR cells in the presence or absence of 10 mM MESNA or glutathione (GSH). Data are expressed as the means  $\pm$  S.E.M. of at least three independent experiments.



**Fig. 6.** Characterization of the SecR-HA37-DP108, SecR-HA37-DP111, and SecR-HA37-DP116 constructs. Shown are the abilities for these three receptor constructs to stimulate cAMP accumulation (left) and for the cell line expressing the SecR-HA37-DP111 construct to bind secretin (right). Values represent means  $\pm$  S.E.M. of at least three independent experiments.

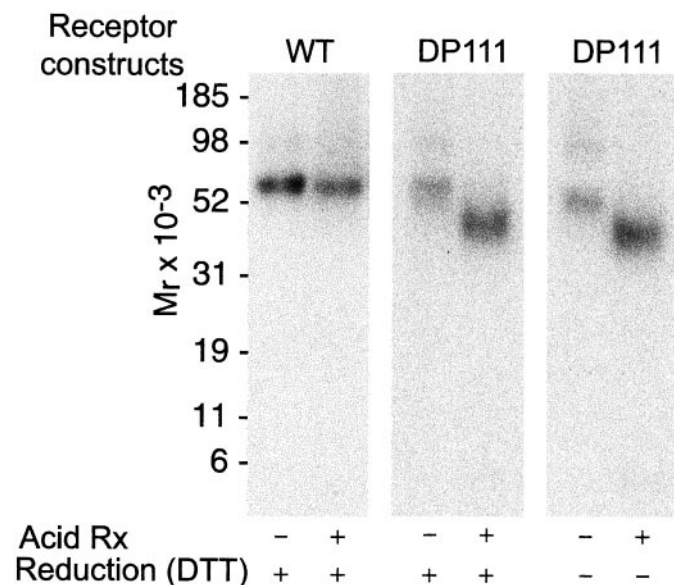


involve the six highly conserved Cys residues in this interesting and important receptor domain.

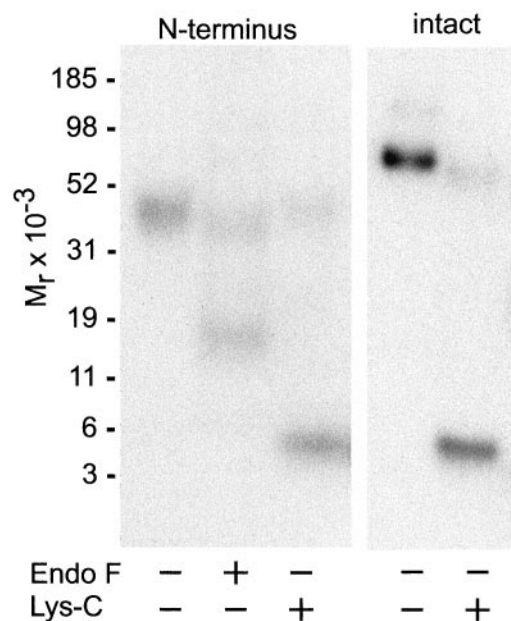
We also now know that the less conserved Cys<sup>11</sup> residue that is only present in closely related class II receptors, is not

normally involved in a disulfide bond. This insight came from the use of a cell-impermeant methanethiosulfonate derivative that has been demonstrated to be both highly reactive and selective for extracellular free sulfhydryl groups (Stauffer and Karlin, 1994). This class of reagents has major theoretical advantages over other cysteine-reactive reagents in regard to its kinetics, the mild nature of its reaction conditions, and its efficiency of derivatization. If an important Cys residue is accessible to this reagent, the extra mass and modification of the charge it provides may alter function. Based on mutagenesis and truncation data, the distal end of the amino terminus of the secretin receptor, where Cys<sup>11</sup> is located, is of critical importance (Holtmann et al., 1996). This is further supported by direct photoaffinity labeling data. This may explain why the modification of this residue with MTSET had such negative impact on receptor function.

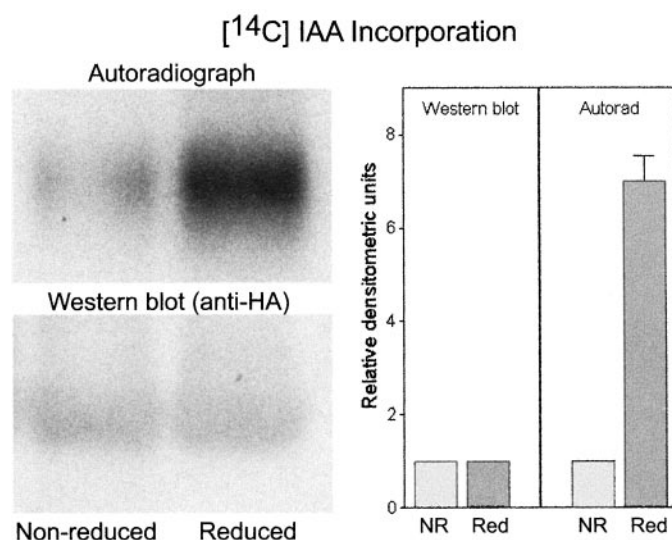
Traditional methods of mapping complex disulfide bonds typically require a large amount of highly purified protein that subsequently undergoes cleavage and analysis by direct amino acid sequencing or mass spectrometry for bond assignment. Even using the richest source of secretin receptors available to us [the recombinant receptor-bearing CHO-SecR cell line (Ulrich et al., 1993)], the level of receptor expression was not high enough for us to achieve an adequate degree of purification and yields to allow these approaches. We therefore genetically engineered a series of sites for the action of specific proteases in various critical locations within the receptor amino terminus, along with epitope tags to provide the opportunity to utilize immunorecognition for the identification of small amounts of less pure material. These sites were well tolerated, having minimal impact on ligand binding and signaling (data not shown). Unfortunately, this approach was also unsuccessful for mapping the disulfide bonds, because of difficulties in achieving quantitative cleavage at these sites. Presumably, the intact receptor is highly folded and compact, interfering with access for proteolytic enzymes to cleave and



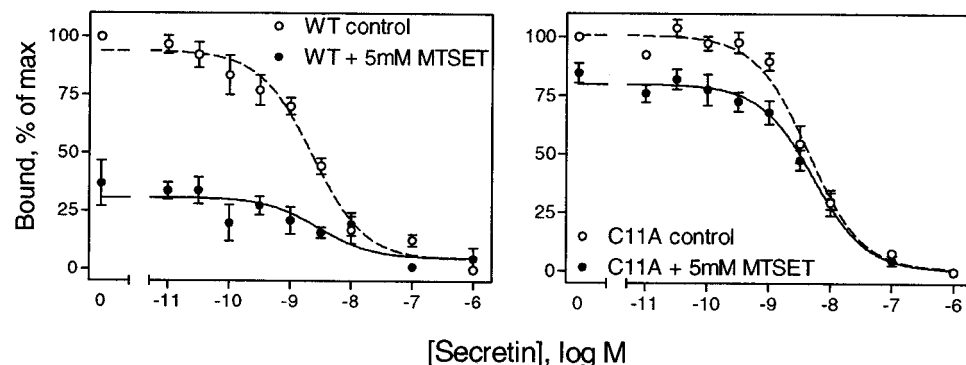
**Fig. 7.** Acid-induced release of a 40 kDa band from the SecR-HA37-DP111 construct. Shown is a representative autoradiograph of a NuPAGE gel used to separate the products of acid cleavage of SecR-HA37-DP111 and SecR-HA37 constructs, when separated in the presence or absence of DTT. A labeled receptor fragment migrating at approximately 40 kDa was released from the DP-containing construct, but not from the same construct that did not include this acid-labile sequence. This fragment was released even in the absence of chemical reduction.



**Fig. 8.** Identification of the acid-released receptor fragment as the amino-terminal domain. Shown is a representative autoradiograph of a reducing NuPAGE gel used to separate the products of deglycosylation and Lys-C digestion of the affinity-labeled acid-released 40-kDa fragment and intact receptor. After deglycosylation, the band shifted to approximately 18 kDa, consistent with the sum of the masses of the expected fragment plus that of the probe. Lys-C digestion resulted in a shift of the labeled bands to 6,000 Da for both the digestion of the released fragment and that of the intact receptor.



**Fig. 9.** Cysteine/cystine ratio within the receptor amino-terminal domain. Shown is a representative western blot and autoradiograph of a gel loaded with equal amounts of secretin receptor amino terminus that had been treated with [<sup>14</sup>C]IAA before and after chemical reduction. Right, quantification of data from five independent experiments (means  $\pm$  S.E.M.), revealing a 1:7 ratio of Cys reactivity in the absence or presence of reduction.



**Fig. 10.** Impact of modification of accessible free Cys residues on receptor binding. Shown are curves representing secretin competition-binding to CHO-SecR (left) and CHO-SecR-C11A (right) cells in the presence or absence of 5 mM MTSET. Values are expressed as the means  $\pm$  S.E.M. of assays performed in duplicate at least four independent times.

even interfering with the release of fragments from the non-reduced molecule.

We had also attempted to express the receptor amino-terminal domain as a truncated, isolated segment in *Escherichia coli*, yeast, and even in CHO cells (data not shown). Although it was possible to produce relatively large amounts of material for analysis this way, we were, unfortunately, unable to achieve and ensure proper folding and disulfide bond formation in such expression systems.

Therefore, we ultimately pursued a new strategy that should be extremely powerful and that should have broad applications to the understanding of the structure and function of extracellular amino-terminal domains of membrane proteins. This involved the novel use of the acid-labile Asp-Pro sequence near the interface between the amino-terminal tail of the secretin receptor and its first transmembrane segment. This provided an opportunity for this domain to be appropriately processed and folded during its biosynthesis, and provided a simple assay to ensure an active conformation before cleaving and releasing it from the body of the receptor. The site of cleavage was easily accessible to hydrogen ions, even though it was less accessible to proteolytic enzymes. The isolated amino-terminal domain was also much more amenable to its own cleavage and release of fragments, ultimately making it a powerful tool for directly mapping the disulfide bonds.

A model has been proposed for the binding of parathyroid hormone to its receptor, a member of the class II family of G protein-coupled receptors (Huang et al., 1996; Turner et al., 1996; Bisello et al., 1998). In this, the carboxyl-terminus of the peptide interacts with the amino-terminal domain of the receptor, whereas the amino-terminus of the peptide dips down into the confluence of helices in the lipid bilayer. This could provide a mechanism for the ligand to exert tension on the body of the receptor that could be transmitted to the cytosolic face of the receptor to facilitate coupling to its G protein. To date, we have identified spatial approximation between residues in both the carboxyl-terminal (residue 22) and amino-terminal (residue 6) halves of secretin and the secretin receptor (Dong et al., 1999a,b). Note that both reagents labeled the amino terminus of the secretin receptor. Once we collect adequate structural constraints to build a molecular model of this receptor or its key amino-terminal domain, these contacts will help to provide initial docking of a secretin agonist ligand into its binding domain. We continue to need additional affinity labeling data to refine such a model. In light of the proposed hypothesis for the molecular basis of parathyroid hormone binding, it may be particularly

interesting to develop a secretin receptor probe with a photolabile residue closer to the amino terminus. It is quite possible that a new hypothesis will have to be proposed to fit these data. It is possible that a highly folded and disulfide-bonded amino-terminal domain will be rigid enough to provide the entire ligand binding surface, with the base of this domain rather than the ligand exerting tension on the receptor body.

In conclusion, the functionally important amino terminus of the secretin receptor represents a structurally independent, highly folded, and disulfide-bonded domain, with a pattern of bonds that is probably critical and conserved throughout this receptor family. The specific nature of these three disulfide bonds should provide useful constraints for the initial molecular modeling of this key receptor domain and will provide a structure that will permit us to begin to dock agonist ligands in a meaningful way.

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