The amino-terminal domain of class B G protein-coupled receptors contains six conserved cysteine residues involved in structurally- and functionally-critical disulfide bonds. The mapping of these bonds has been unclear, with one pattern based on biochemical and NMR structural characterizations of refolded, non-glycosylated amino-terminal fragments, and another pattern derived from functional characterizations of intact receptors having paired cysteine mutations. In the present study, we determined the disulfide bonding pattern of the prototypic class B secretin receptor by applying the same paired cysteine mutagenesis approach and confirming the predicted bonding pattern with proteolytic cleavage of intact functional receptor. As expected, systematic mutation to serine of the six conserved cysteine residues within this region of the secretin receptor singly and in pairs resulted in loss of function of most constructs. Notable exceptions were single mutations of the fourth and sixth cysteine residues and paired mutations involving the first and third, second and fifth, and fourth and sixth conserved cysteines, with secretin eliciting statistically significant cAMP responses above basal levels of activation for each of these constructs. Immunofluorescence microscopy confirmed similar levels of plasma membrane expression for each of the mutated receptors. Furthermore, cyanogen bromide cleaved a series of wild type and mutant secretin receptors, yielding patterns that agreed with our paired cysteine mutagenesis results. In conclusion, these data suggest the same pattern of disulfide bonding as that predicted previously by NMR, and thus support a consistent pattern of amino-terminal disulfide bonds in class B G protein-coupled receptors.
with the signal recognition particle, to the surface of the endoplasmic reticulum (ER) for cotranslational translocation and membrane insertion (10). Within seconds of the nascent polypeptide entering the ER lumen, folding intermediates establish the secondary and tertiary structures necessary for the formation of preliminary disulfide bonds and the addition of core N-linked oligosaccharides. Combined with the help of chaperones, disulfide and proline isomerases, and other folding catalysts, these early cotranslational modifications widely are thought to transition the emerging polypeptide through a vectorial progression of conformations that functions to improve the overall efficiency of the folding process (11, 12). Efficient folding becomes particularly relevant in the cellular context, wherein the biosynthetic folding processes are linked to the maturation of cell signaling molecules and plasma membrane receptors. For example, detailed studies of acetylcholine, insulin, and epidermal growth factor receptors suggest that most mammalian receptors follow a common mechanism in which the ER-localized processes of folding, disulfide bond formation/rearrangement, and N-linked glycosylation, but not subunit assembly or Golgi-associated posttranslational processing steps, are prerequisite for acquiring ligand-binding function (13).

Grace et al. (14) very recently provided the first high-resolution NMR structure of the amino-terminal domain of a class B GPCR, the corticotropin-releasing factor receptor. In that structure, three disulfide bonds connecting the first and third, second and fifth, and fourth and sixth conserved cysteine residues established a protein interaction fold containing two antiparallel β-sheets. It was concluded that because the key structural residues of the fold were conserved among family members, class B receptors likely possess similarly-structured amino-terminal domains and thus bind and signal through the same or closely-related mechanisms. These very important findings solidified the conclusions of four earlier studies that had deduced the same pattern of disulfide bonds from the sequences and masses of proteolytic fragments derived from corticotropin-releasing factor, glucagon-like peptide 1, and parathyroid hormone receptors (15, 16, 17, 18). Notably, all of these studies, including the NMR study, employed refolded, non-glycosylated amino-terminal fragments that were not associated with either plasma membranes or intact receptor transmembrane domains.

The only study that has proposed a disulfide bonding pattern for a biosynthetically-folded, fully-glycosylated class B GPCR suggested a pattern that disagrees with the consistent pattern deduced from the biochemical and NMR structural studies of refolded receptor fragments described above. In that work, Qi et al. (5) characterized the binding and biological activities of paired cysteine substitution mutants of the intact corticotropin-releasing factor receptor, reasoning that receptor function would better tolerate the removal of certain pairs of cysteines that form disulfide bonds. Aside from providing the advantage of a functional readout of receptor structure, the approach was reasonable considering that the overall conformation of a protein, at least in the case of bovine pancreatic trypsin inhibitor, can tolerate the mutational removal of a disulfide bond (19). Furthermore, the method had been applied successfully to a class A GPCR, the type IA angiotensin II receptor, to determine which of its extracellular domains were connected by disulfide bonds (20). Remarkably, corticotropin-releasing factor receptors having paired substitutions of the first and third conserved cysteines bound ligand saturably, and constructs with similar mutations of the second and sixth or the fourth and fifth cysteines activated cAMP production to levels comparable to or better than wild type receptor. These findings led the authors to conclude that the intact receptor possessed disulfide linkages between the first and third, second and sixth, and fourth and fifth conserved cysteines. This tenable but different disulfide pattern illuminates the important question of whether the extracellular amino-terminal domain of an intact, fully-glycosylated class B receptor possesses the same (or different) disulfide pattern as that determined from the refolded amino-terminal fragments of corticotropin-releasing factor, glucagon-like peptide 1, and parathyroid hormone receptors.

We are interested particularly in the structural and ligand-binding properties of the G protein-coupled secretin receptor (SecR), the prototypic member of class B receptors that functions primarily in the secretion of pancreatic
and biliary bicarbonate (21, 22). Like other family members, SecR is stimulated by a relatively large peptide agonist, secretin, whose binding determinants appear to be distributed across the length of the basic 27-amino acid peptide. A detailed understanding of how the receptor binds secretin recently has gained clinical importance upon the discovery of a misspliced SecR variant in a pancreatic tumor that in cancer cell lines inhibited wild type receptor function (23, 24). Substantial progress has been made in this area, where photoaffinity labeling studies to date have identified eight structural constraints between bound secretin agonist analogues and SecR (25, 26, 27, 28, 29, 30, 31). Nearly all of these ligand-receptor contacts occur at the distal amino terminus of the receptor. The emerging picture is that the high-affinity binding determinants at the carboxyl end of the ligand interact primarily with the distal regions of the receptor extracellular domain, whereas the receptor selectivity determinants at the amino end of the ligand interact with (and perhaps penetrate) the extracellular loops of the receptor. Although the photoaffinity labeling constraints have provided extremely useful information for modeling the SecR amino-terminal domain, these models have been difficult to prepare without knowledge of the overall fold that is established by the disulfide linkages within this region.

Several important pieces of information have set the stage for the elucidation of the disulfide linkages within the SecR amino terminus. Vilardaga et al. (3) concluded that of the 10 extracellular cysteine residues within rat SecR, the six cysteines C24, C44, C53, C67, C85, and C101 were necessary for receptor function. These amino-terminal residues corresponded to those that are highly conserved within the family (21) and that have been shown in other receptors to be involved in disulfide bonds (Table I). Recently, we confirmed these findings by creating a series of receptors having alanine point mutations for each of the conserved cysteines (4). Despite proper sorting to the plasma membrane, all of the cysteine-mutated receptors were unable to bind secretin or elicit a secretin-stimulated cAMP response. Secretin binding also was diminished after treating cells expressing wild type receptors with cell-impermeant reducing reagents. More importantly, this study also demonstrated directly the presence of three disulfide bonds within the amino-terminal domain, none of which linked this domain to the extracellular loops or membrane-bound body of the receptor. Combined, these data emphasize the importance of extracellular disulfide bonds that exist within the highly-folded, structurally-independent amino-terminal ligand binding domain of SecR.

Here we expand upon our initial efforts in a comprehensive search for paired cysteine mutations that may rescue the loss of function observed previously with single alanine substitutions. This approach was chosen for the following reasons: a) provides functional readout of the structure of intact, fully-processed receptors, b) allows for direct comparisons to our previous site-directed mutagenesis results, as well as to those of Qi et al. (5) derived from paired cysteine mutants of corticotropin-releasing factor receptors, and c) offers the potential to resolve conflicting patterns of disulfide bonding among class B GPCRs. Also presented are cyanogen bromide cleavage results that support a disulfide linkage pattern that agrees with the pattern determined from refolded receptor fragments. Cumulatively, our findings provide both the definitive disulfide bonding pattern for the clinically important SecR and a needed link between determinations of patterns of disulfide bonds for class B GPCRs that were folded in the cell or refolded in vitro.

**EXPERIMENTAL PROCEDURES**

**Materials.** Molecular biology reagents were purchased from New England Biolabs (Beverly, MA), Stratagene (La Jolla, CA), Bio-Rad Laboratories (Hercules, CA), and Qiagen (Valencia, CA). Cell culture growth media and antibiotics were obtained from Invitrogen (San Diego, CA) and supplemented (where appropriate) with Fetal Clone II from HyClone Laboratories (Logan, UT). Formaldehyde was supplied by Ted Pella (Redding, CA), and primary and fluorophore-conjugated secondary antibodies were supplied by Roche Diagnostics (Basel, Switzerland) and Molecular Probes (Eugene, OR), respectively. Phenylmethylsulfonyl fluoride and 3-isobutyl-1-methylxanthine were from Sigma-Aldrich (St. Louis, MO). Chemical cleavage reactions utilized cyanogen bromide (CNBr) purchased from Pierce Biotechnology (Rockford,
All peptides and receptor constructs were based on the sequences of natural rat secretin and secretin receptor. Secretin, [Tyr\textsuperscript{10}]secretin, and photolabile [Tyr\textsuperscript{10},Bpa\textsuperscript{26}]secretin (Bpa\textsuperscript{26} probe) were synthesized in our laboratory (32) and have been shown to bind and activate (all three peptides) and covalently label (Bpa\textsuperscript{26} probe only) secretin receptors in intact cells (27). Procedures describing the oxidative radioiodination of the Tyr\textsuperscript{10} residue are published elsewhere (32). All other reagents were of the highest quality appropriate for the given experiment.

Receptor mutagenesis. The disulfide pattern of the rat secretin receptor was examined by systematically mutating to serine each of the six conserved cysteine residues within the extracellular domain. These mutations generated a complete series of SecR mutants having either single or paired C24S, C44S, C53S, C67S, C85S, and/or C101S changes (numbering starts with the first amino acid residue after the signal sequence). Additional SecR mutants were prepared for CNBr cleavage experiments to strategically test the presence of specific disulfide bonds. These included constructs having M73I, as well as this mutation along with both A41M and L99M.

All receptor mutations were integrated into the coding sequence of a hemagglutinin (HA) epitope-tagged version of SecR that was placed downstream of the constitutive cytomegalovirus enhancer/promoter in the eukaryotic expression vector pcDNA3.1 (Invitrogen) (25). Single-codon mutations were introduced into this pcDNA3.1/HA37-SecR template in whole-plasmid PCR reactions using the QuikChange site-directed mutagenesis kit (Stratagene) and appropriate sets of complementary forward and reverse mutagenic primers. Multiple codon exchanges were introduced into HA37-SecR coding sequences possessing an appropriate single mutation either through successive rounds of QuikChange mutagenesis or through excision and subsequent ligation of restriction fragments that utilized a vector-derived KpnI site and/or naturally-occurring BspEI or EcoNI sites within the receptor open reading frame. Mutated coding sequences were confirmed by automated dye-terminator cycle sequencing.

Cell cultures and transfections. Two different cell types obtained from the American Type Culture Collection (Manassas, VA) were employed. African green monkey kidney (COS) cells propagated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% (v/v) Fetal Clone II were maintained in a humidified chamber with 5% (v/v) CO\textsubscript{2} at 37 °C. Syrian baby hamster kidney (BHK) cells were maintained under the same conditions, except that the culture medium consisted of equal parts DMEM and Ham’s F-12 nutrient mixture. Both cultures were passaged twice per week on Corning (Acton, MA) tissue culture flasks.

The various cysteine mutants of SecR were expressed transiently in COS cells using a modified diethylaminoethyl (DEAE)-dextran transfection method described previously (33). Roughly 500,000 cells adhered to a 10-cm Petri dish were treated for one to two hours with a serum-free DMEM mixture containing 0.2 mg/ml DEAE-dextran and 3 µg plasmid DNA (1.5 ml total volume). The cells then were shocked for exactly 2 min in 10% (v/v) dimethylsulfoxide (DMSO) in serum-free culture medium prior to another one to two hour incubation in 0.1 mM chloroquine in serum-free DMEM. Transfected cells were washed and cultured for 24 h in DMEM with serum before lifting with 0.05% (w/v) trypsin and inoculating plates for subsequent analyses.

Three BHK cell lines stably expressing HA37-SecR, HA37-M73I-SecR, or HA37-A41M-M73I-L99M-SecR were created for CNBr cleavage experiments. Briefly, ~500,000 cells adhered to a 10-cm Petri dish were washed with phosphate-buffered saline (PBS) and then incubated for 15 min in a buffered CaCl\textsubscript{2} (125 mM) solution (25 mM HEPES, pH 7.1, 0.75 mM Na\textsubscript{2}HPO\textsubscript{4}, 70 mM NaCl) containing 20 µg plasmid DNA (1 ml total volume). The cells then were cultured for 24 h in serum-containing DMEM/F-12 medium before lifting with trypsin and transferring to flasks for selection. Transfected, receptor-bearing cells selected as G418-resistant colonies were enriched through a single round of clonal selection by limiting dilution. The resulting clonal cultures were screened for secretin binding, and those clones exhibiting attributes most like wild type receptors were characterized fully for secretin ligand binding and receptor activation.

Immunofluorescence microscopy. Transiently-transfected COS cells seeded on 25mm round coverslips in 6-well plates were prepared for indirect immunofluorescence
microscopy ∼72 h post-transfection, according to established procedures (34). Specifically, cells were fixed in fresh 2% (w/v) formaldehyde in PBS for 15 min immediately after removal from the growth incubator and without washing. After two 10-min washes in PBS, non-specific binding sites were blocked during two additional 10-min washes in PBS containing 1% (v/v) normal goat serum. Cell surface antigens were immunolabeled without permeabilization by inverting coverslips onto drops of primary (mouse anti-HA epitope, clone 12CA5, 1:500) and secondary (Alexa 488-conjugated goat anti-mouse, 1:500) antibodies diluted in PBS with normal goat serum for one to two hours in a humidified chamber. The cells were washed with three 10-min exchanges of PBS with normal goat serum and four 10-min exchanges of PBS between antibody incubations and after application of secondary antibodies, respectively, before mounting on microscope slides in 40% (v/v) glycerol. All steps were performed at room temperature. Labeled cells were observed and photographed with a Zeiss (Thornwood, NY) LSM 510 confocal microscope. Micrographs were adjusted for contrast and assembled into figures using Adobe Photoshop (Mountain View, CA).

Membrane preparations. Plasma membranes from BHK cell cultures were prepared for receptor binding analyses and photoaffinity labeling experiments according to well-described methods used routinely for receptor-bearing Chinese hamster ovary (CHO) cell cultures (35). The procedure involved ultrasonic disruption of cells followed by isolation of plasma membrane vesicles within sucrose flotation gradients. Membranes retrieved from the gradients were washed and pelleted prior to resuspension with a Dounce homogenizer for storage at -80 °C.

Receptor binding assays. Each of the secretin receptor cysteine mutants was tested for its ability to bind a secretin agonist radioligand in intact cell binding assays. In these experiments, COS cells distributed among 24-well plates were incubated 72 h post-transfection with increasing concentrations (from 0 to 1 µM) of secretin and a constant amount of 125I-[Tyr10]secretin diluted in a Krebs-Ringers-HEPES (KRH) solution (25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 1 mM KH2PO4, 1.2 mM MgSO4, 2 mM CaCl2) containing 0.2% (w/v) bovine serum albumin (BSA), 0.01% (w/v) soybean trypsin inhibitor and 1 mM phenylmethylsulfonyl fluoride. Following a 1-h incubation at room temperature, cells were washed with KRH, lysed with 0.5 M NaOH, and then transferred to a γ-spectrometer for quantification of bound radioactivity. Non-specific binding at saturating levels of unlabeled secretin (1 µM) typically represented less than 30% of the maximum radioligand bound.

The binding activities of the M73I and A41M-M73I-L99M SecR mutants were determined in similar binding assays with isolated BHK cell membranes. Each assay received ∼5 µg of receptor-bearing membranes. Membranes incubated with labeled and unlabeled secretin as described above were collected by pelleting, washed extensively, and then surveyed for bound radioactivity.

Receptor activity (cAMP) assays. Each of the SecR mutants employed in this study was characterized for its ability to elicit production of cyclic AMP (cAMP) second messenger upon stimulation with secretin (36). Stimulation of PBS-washed COS cells in 24-well plates was performed 72 h post-transfection with either increasing concentrations (from 0 to 1 µM) or a single dose (0.1 µM) of secretin diluted in KRH containing 0.2% (w/v) BSA, 0.01% (w/v) soybean trypsin inhibitor, 0.1% (w/v) bacitracin, and 1 mM 3-isobutyl-1-methylxanthine (from a freshly-prepared 100 mM stock solution in DMSO). Cells were incubated with peptide for 30 min at 37 °C and then lysed in ice-cold 6% (w/w) perchloric acid for 15 min with vigorous shaking. The resulting lysates were neutralized to pH 6.0 with 30% (w/v) KHCO3 and then assayed for the presence of cAMP with a competition-binding assay purchased from Diagnostic Products Corp. (Los Angeles, CA) according to the manufacturer’s instructions. Bound 3H-cAMP competitor was quantified with a Beckman (Fullerton, CA) LS 6000SC liquid scintillation counter.

Photoaffinity labeling and chemical cleavage. Coupled with the strategic placement of methionine residues in secretin receptor mutants, predicted disulfide linkages were confirmed or refuted through CNBr cleavage of radiolabeled receptors in the presence or absence of reducing agent. Secretin receptors were radiolabeled
utilizing an established photochemical cross-linking procedure, as previously described (30). Briefly, receptor-bearing BHK cell plasma membranes (~200 µg) were incubated with \(^{125}\text{I-}\left[\text{Tyr}^{10},\text{Bpa}^{26}\right]\text{secretin} (~0.5 \text{ nM}) in KRH containing 0.01% (w/v) soybean trypsin inhibitor and 1 mM phenylmethylsulfonyl fluoride in the dark for 1 h at room temperature. The photolabile \(\text{Bpa}^{26}\) probe was chosen because of its established site of covalent attachment to receptor residue L36, located between the first two conserved cysteine residues (27). The reactions were transferred to siliconized glass tubes and then photolyzed for 30 min at 4 °C in a pre-chilled Rayonet photochemical reactor (Southern New England Ultraviolet, Hamden, CT) fitted with 3500-Å lamps. Membranes were washed with KRH, solubilized for at least 30 min at room temperature in SDS sample buffer with or without dithiothreitol, and then subjected to SDS-PAGE separation in 10% polyacrylamide gels for subsequent detection of labeled proteins by autoradiography. Specific binding to secretin receptors was confirmed in separate reactions in the presence of 1 µM unlabeled secretin.

Radiolabeled receptor bands excised from polyacrylamide gels were prepared for CNBr cleavage by gel elution, lyophilization, and ethanol/acetone precipitation. Approximately 1000 cpm of labeled, purified receptors were digested under nitrogen with 25 mg/ml CNBr in 70% (v/v) formic acid containing 5 mM dithiothreitol (reduced samples only) in the dark for 2 to 3 days at room temperature with constant agitation. Cleavage products were separated in 10% NuPAGE gels under appropriate reducing or non-reducing conditions using an MES buffer system (Invitrogen). Labeled fragments were detected by autoradiography and sized by interpolation on a graph of the migration of Multimark protein standards (Invitrogen).

**Statistical analyses.** Binding and biological activity curves generated in nonlinear regression analyses performed with Prism (GraphPad Software, San Diego, CA) were evaluated relative to wild type receptor. Binding kinetics were calculated with the LIGAND program (37) and in all cases data are reported as the means ± S.E.M. of duplicate assays from at least three independent experiments. For the biological activity data presented in Fig. 2, absolute responses recorded in the absence or presence of 100 nM secretin were examined carefully with one-way analysis of variance calculations (assuming Gaussian distributions) performed with GraphPad's InStat software package. Statistically significant responses (p < 0.05) were determined post-test with Bonferroni comparisons of each secretin-stimulated response to the average non-specific response achieved from all receptor constructs (0.71 ± 0.024 pmol cAMP).

**RESULTS**

**Creation of secretin receptors having single or paired cysteine mutations.** Each of the six conserved cysteine residues in the extracellular domain of SecR was replaced with a serine residue either singly or in pairs in order to assess the functional implications of losing the ability to form a disulfide bond at each conserved cysteine position. The full set of single or paired cysteine replacements amounted to a total of 21 different mutant receptors, including six unique single mutant receptors (C24S, C44S, C53S, C67S, C85S, and C101S) plus the 15 different combinations of paired mutations that were possible among these six positions. Serine substitutions were employed instead of alanine substitutions because of the better structural match between the alcohol and sulfhydryl side chains of serine and cysteine, respectively, although one paired mutation (C24A-C44S) employed an alanine substitution at position C24 as a matter of convenience (see below).

COS cells expressing transiently each of the SecR cysteine mutants were fixed and then labeled with anti-HA primary and fluorophore-conjugated secondary antibodies to assess the sorting and insertion of each receptor variant to/into the plasma membrane. Fig. 1 shows representative confocal fluorescence micrographs of non-permeabilized COS cells expressing several examples of mutant receptors possessing single or paired cysteine substitutions. Similar to HA37-SecR (wild type), each of the mutant receptors shown was localized to the plasma membrane and inserted such that the amino-terminal HA epitope tag was oriented toward the cell exterior. No fluorescence was observed in negative control cells that had been transfected
with an empty expression vector (Fig. 1). Analagous results were obtained for each of the mutant receptors not pictured in Fig. 1, indicating that levels of cell surface expression were comparable among all of the mutants (data not shown). These findings demonstrated that at least some portion of the newly-synthesized, cysteine-mutated receptors met the minimal structural requirements necessary cell surface expression.

**Biological activity assays of secretin receptor cysteine mutants.** Basal and maximal secretin-stimulated cAMP responses were measured as an initial screen for single and/or paired cysteine mutants that could rescue the signaling properties of wild type SecR. As shown in Fig. 2, wild type HA37-SecR exhibited typical accumulations of cAMP within COS cells, where stimulation with 100 nM secretin increased intracellular cAMP from 1 ± 0.2 pmol (basal) to 55 ± 4 pmol (maximal) (absolute responses per 25,000 cells). Nearly all mutant receptors also elicited some detectable level of cAMP above basal levels when treated similarly, although most of these responses were less than five times those of the corresponding signals recorded from unstimulated cells (Fig. 2, shaded area). Interestingly, two single and three paired cysteine mutants, namely C67S, C101S, C24S-C53S, C44S-C85S, and C67S-C101S, stimulated statistically significant (p < 0.05) levels of cAMP compared to an average basal response of 0.7 ± 0.02 pmol (Fig. 2, asterisks). Of these, the C67S-C101S mutant was capable of the highest levels of stimulation (16 ± 4 pmol) with responses that approached one-third of stimulated levels achieved in wild type receptor-bearing cells. These observations were consistent across several repetitions, indicating that secretin-stimulated receptor activation can tolerate only a very few, specific alterations in the disulfide bond structure of the amino-terminal domain.

Insight into the relative importance of the conserved cysteine residues can be derived from the observed signaling properties of the mutant receptors. For example, four of the six single mutations (C24S, C44S, C53S, and C85S) abolished secretin-stimulated cAMP accumulation in COS cells expressing these receptors (Fig. 2). Most combinations of paired mutations among these residues also impaired signaling activity. However, the paired mutations C24S-C53S and C44S-C85S rescued a significant portion of wild type signal compared to the average basal response, achieving cAMP accumulations of 5 ± 0.7 pmol (p < 0.001) and 4 ± 0.8 pmol (p < 0.01), respectively. That these cysteine mutations were tolerated preferentially as pairs but not singly suggests the individual cysteines of each pair participate in a common structural element that relates to the signaling mechanism of the secretin receptor.

Single mutations in either C67 or C101 were tolerated much better than the other single mutations. These receptors were capable of intracellular cAMP accumulations that were similar to those recorded for the C24S-C53S and C44S-C85S paired mutants: C67S, 4 ± 0.5 pmol (p < 0.05); C101S, 4 ± 0.7 pmol (p < 0.05). Paired replacement of C67 and C101 led to the highest signal recorded from any of the SecR cysteine mutants, reaching 16 ± 4 pmol (p < 0.001). This signal consistently was three to five times larger than those achieved from the C24S-C53S and C44S-C85S paired mutants, suggesting that C67 and C101 form a unique pair among the various combinations. All other paired mutations that included a C67S or C101S substitution abolished signaling to within five times the basal response, lending support to the notion that C67 and C101 participate in amino-terminal structural elements that do not involve C24, C44, C53, and C85. In summary, these results led us to hypothesize that disulfide bonds connect C24 and C53, C44 and C85, and C67 and C101, and that C67 and C101 participate in the bond that is the least critical to the mechanism of receptor activation.

**Functional characterizations of secretin receptor cysteine mutants.** Table II compares the ligand binding and biological activity properties of each of the SecR cysteine mutants created for the present study. Replacement of any of the conserved cysteine residues singly or in pairs resulted in the complete loss of saturable secretin binding (Ki value: HA37-SecR, 43 ± 5 nM). Binding kinetics for these SecR cysteine mutants are, therefore, not relevant and could not be calculated. The more sensitive cAMP assay did demonstrate secretin-stimulated cAMP accumulations that were significantly greater than
basal responses for two single and three paired cysteine mutants (Fig. 2, Table II).

Binding and activity curves for the paired cysteine mutants C24S-C53S, C44S-C85S, and C67S-C101S are shown in Fig. 3. As was summarized in Table II, these three paired mutations abolished binding of radiolabeled secretin such that non-specific radioactivity accounted for most of the signal that was detected at all concentrations of competing ligand. In contrast, the same mutations allowed for saturable or near saturable accumulations of cAMP within COS cells that approached 50% of wild type responses at the highest secretin concentration tested (1 µM) (Fig. 3). The EC₅₀ values for these responses were as follows: HA37-SecR (wild type), 15 ± 2 pM secretin; C24S-C53S, 180 ± 68 nM; C44S-C85S, 440 ± 330 nM; C67S-C101S, 13 ± 8 nM. Dose-response curves were not generated for the other cysteine mutants because none of them (except for single mutants C67S and C101S) displayed significant biological activity in response to stimulation with 100 nM secretin (Fig. 2). Cumulatively, these quantitative data demonstrate that secretin binding is highly dependent upon the disulfide structure of the amino-terminal domain. Moreover, secretin-stimulated biological activity can be rescued by paired mutations of conserved cysteines C44 and C85, C24 and C53, and C67 and C101 (in order of increasing effectiveness), thus supporting our hypotheses that these pairs form disulfide bonds and that the bonds between C44-C85 and C67-C101 are more and less important, respectively, to the overall structure of the receptor.

**Chemical cleavage of photoaffinity-labeled secretin receptors.** To provide further evidence supporting or refuting the disulfide bonding pattern predicted from the functional analyses of the SecR cysteine mutants, a separate biochemical approach was employed. This approach utilized the ability to detect differences in the electrophoretic migrations of affinity-labeled fragments generated by CNBr cleavage of intact native or reduced receptors. Fig. 4A compares to wild type HA37-SecR the amino-terminal domains of two new SecR mutants, HA37-M73I-SecR and HA37-A41M-M73I-L99M-SecR, that were created for these experiments in order to change strategically the sites for CNBr cleavage. Both of these SecR mutants bound secretin with high affinity ($K_i$ values: M73I, 6 ± 1 nM; A41M-M73I-L99M, 82 ± 3 nM) and stimulated cAMP production with responses comparable to wild type (EC₅₀ values: M73I, 40 ± 8 pM secretin; A41M-M73I-L99M, 190 ± 6 pM).

Previous work has demonstrated that the Bpa²⁶ probe, a radiiodinated photolabile secretin agonist analogue, labels receptor residue L36 immediately to the amino-terminal side of the HA37 epitope tag (27). Fig. 4B shows this site of labeling (asterisks) in schematic representations of the peptide fragments predicted from CNBr digestions of affinity-labeled, non-reduced HA37-A41M-M73I-L99M-SecR. The fragments are connected in the two drawings according to the two patterns of disulfide bonding determined from studies of the intact corticotropin-releasing factor receptor (Pattern I) and studies of refolded amino-terminal domains of several class B receptors (Pattern II) (references listed in Table I). Shown in bold are the fragments that would remain covalently attached to the affinity-labeled fragment under non-reducing conditions and thus contribute to its electrophoretic mobility during polyacrylamide gel separations. According to Pattern I, only the fragment spanning S52 and M99 would remain bonded to the labeled fragment at the distal amino terminus, whereas according to Pattern II, all CNBr fragments would remain attached and thus migrate as an intact amino-terminal domain. It should be noted that none of the CNBr fragments would remain bonded to the labeled fragment after cleavage and separation under reducing conditions.

Fig. 4C shows that in a 10% NuPAGE gel, Bpa²⁶ probe-labeled wild type and M73I SecR migrated similarly as single bands at approximately $M_r = 70,000$ under reducing and non-reducing conditions (lanes 1, 3, 5, 7). CNBr cleavage of these receptors under reducing conditions created two bands that migrated to approximately $M_r = 40,000$ and 18,000 (lanes 2, 6). The former likely represents incomplete digestion of the amino-terminal fragment released at M123 and is consistent with the migration of a similar acid-released fragment of SecR (4). Likewise, the band at $M_r = 18,000$ is consistent with the demonstrated migration of the first 51
amino acid residues, including the HA37 epitope tag and the covalently-attached radiolabeled probe (27). In similar CNBr digestions of A41M-M73I-L99M SecR, the band at \( M_r = 18,000 \) instead migrated to \( M_r = 7,500 \) (lane 9), again consistent with the CNBr digestion of an A41M SecR mutant that yielded a single band at \( M_r = 7,500 \) corresponding to the amino-terminal 41 residues of the receptor (27). However, CNBr cleavage of all three receptors under non-reducing conditions produced a single labeled fragment that migrated at approximately \( M_r = 40,000 \) (lanes 4, 8, 10), similar to the reduced fragment thought to represent the entire amino-terminal domain. The migration of the non-reduced fragment from A41M-M73I-L99M receptors (lane 10) could be achieved only if all of the CNBr fragments were connected by disulfide bonds, and thus more closely matches that predicted by Pattern II in Fig. 4B. Although the migrations of the labeled CNBr fragments do not reveal a specific pattern of disulfide bonding, these results provide convincing support for a pattern that is consistent with our functional characterizations of SecR cysteine mutants.

**DISCUSSION**

Considering the relatively limited amount of structural information for class B GPCRs folded during normal biosynthesis in the cell, our priority was to determine if an intact, fully-processed SecR assumes a disulfide bond structure that is the same as or different from that determined from refolded receptor fragments (14, 15, 16, 17, 18). To this end, the paired cysteine mutagenesis and chemical cleavage approaches employed in the current study establish the disulfide bonding pattern of the functional intact SecR, and in doing so provide interesting avenues for discussion of how these disulfide bonds create and/or stabilize both the overall fold and the ligand-binding contacts of the amino-terminal domain.

Single and paired serine substitutions of all six conserved cysteines abolished saturable secretin binding by receptor-expressing COS cells. This result for the single cysteine mutants is consistent with previous studies of serine (3) and alanine (4) substituted receptors. Some competitive binding by a C24A SecR mutant had been observed, but the \( K_i \) for this signal was more than 100 times greater than for wild type receptor (4). The lack of secretin binding was not due to improper trafficking or membrane insertion of the mutant receptors, because each single or paired mutant was detected on the surface of non-permeabilized cells. Receptors having single alanine substitutions also have been detected on the surface of receptor-expressing cells by similar immunofluorescence methods (4). These results indicate that although the mutant receptors are unable to bind secretin at the concentrations tested, their cysteine mutations do not prevent potentially misfolded receptors from reaching the plasma membrane.

Our binding results for single and paired SecR cysteine mutants agree with those determined from nearly all cysteine-substituted class B receptors, including receptors for vasoactive intestinal peptide (6), parathyroid hormone (7), and corticotropin-releasing factor (5). In all of these cases, cysteine mutations abolished the abilities of these receptors to saturably bind their respective ligands. An exception was mutations of the first and/or third conserved cysteines of corticotropin-releasing factor receptor (5). In this case, mutant receptors carrying serine substitutions for C30, C54, or C30-C54 displayed 35%, 91%, and 90% of the binding of wild type receptors. However, it should be noted that the expression level of the C54 mutant was about twice that of the other two mutants and 50% greater than wild type. On the other hand, single cysteine mutants of parathyroid hormone receptor were very poorly expressed, and it was argued that this fact was the cause of the low binding signals for these mutants (7). Although not quantitative, our immunofluorescence results indicate that this was not the case for the SecR cysteine mutants. Taken together, it is clear that the amino-terminal cysteine residues of class B receptors, with the possible exceptions of the first and third cysteines of corticotropin-releasing factor receptor, are required for high-affinity binding, probably because of their involvement in structurally- and functionally-important disulfide bonds.

Many receptor systems normally are present in excess on the cell surface, with only a very small percentage of receptors needing to be occupied to elicit a maximal signaling response. With this in mind, it is not surprising that second
messenger responses can be more sensitive indicators of an active complex of agonist and receptor than a saturable radioligand binding assay. Despite this opportunity for amplification of response, the vast majority of the single and paired SecR cysteine mutations prepared for this study abolished secretin-stimulated signaling. Likewise, all of the single serine and alanine mutations introduced by Vilardaga et al. (3) and Asmann et al. (4), respectively, created non-functional receptors, although in the former study the mutant receptors could not be detected on the surfaces of the CHO cell lines generated for biological activity assays. Interestingly, we observed significant cAMP accumulations in response to secretin for the two single mutants C67 and C101 and the three paired mutants C24-C53, C44-C85, and C67-C101. With respect to the non-functional SecR mutants created in previous studies (3, 4), the significant biological responses of the C67 and C101 mutants observed in the current study may have been due to better plasma membrane sorting and/or structural preservation of ligand-mediated signaling afforded by isosteric serine substitutions. These two single and the three paired mutants were the only ones that provided a structural basis for statistically significant cAMP stimulations (p < 0.05), with all other single or paired mutants unable to stimulate signals that were statistically different from basal levels. Of note, each of the six conserved cysteine residues within the SecR amino-terminal domain are represented once in the three paired cysteine mutants C24-C53, C44-C85, and C67-C101. Our paired cysteine mutagenesis results suggest that the SecR amino-terminal domain possesses disulfide bonds between the conserved cysteines C24-C53 (first to third), C44-C85 (second to fifth), and C67-C101 (fourth to sixth). Using the same approach, Qi et al. (5) concluded that two disulfide bonds connect C44-C102 (second to sixth) and C68-C87 (fourth to fifth) in the amino-terminal domain of corticotropin-releasing factor receptor. In that study, paired mutations of these residues were the only ones that allowed improved signaling capabilities as compared to their respective single mutant counterparts. These two paired mutants do not correspond to our partially functional C24-C53, C44-C85, and C67-C101 SecR mutants, which also exhibited improved signaling capabilities as compared to their respective single mutants. Despite this difference, the paired SecR and corticotropin-releasing factor receptor mutants in question each rescued biological activity to varying degrees, suggesting that the paired mutagenesis approach is a sensitive indicator of how alterations in disulfide bonds affect the structure/function relationships that dictate ligand-mediated signaling. In support of this assertion, functional comparisons of paired cysteine mutants have identified the highly conserved disulfide bond that connects the first and second extracellular loops of most GPCRs, including SecR and parathyroid hormone receptor (3, 7, 20). It is also interesting to note that like Qi et al. (5), we identified two single cysteine mutations that did not fully impair stimulation of cAMP, suggesting that although the residues involved do not coincide between the two receptors studied, class B receptors utilize similar signaling mechanisms. The CNBr cleavage results presented in Fig. 4 supported assignment of disulfide bonds between the first and third, second and fifth, and fourth and sixth cysteines of SecR. Of the 15 disulfide bonding patterns that are possible among the six cysteine residues, only six of the patterns could have produced the observed cleavage products from non-reduced A41M-M73I-L99M receptors covalently labeled with the Bpa26 probe (data not shown). One of these six eligible patterns fit the assignment noted above, but none of the six corresponded to the pattern predicted by Qi et al. (5). Moreover, five of the six patterns included two or more disulfide bonds that were not supported by the functional analyses of paired cysteine mutants. It should be stressed that the conditions of the cleavage experiments were chosen deliberately to provide evidence that would support/refute one of the two disulfide bonding patterns reported in the literature for class B GPCRs. Other conditions were explored in an attempt to apply this method in a way that would reveal individual disulfide bonds. For example, several other SecR methionine mutants were created, but these mutants either were non-functional or were resistant to predictable patterns of CNBr cleavage. Several other photolabile secretin probes also provided interesting alternatives, but their positions of labeling on the receptor did not offer the same discriminatory
capabilities of the Bpa²⁶ probe. Nonetheless, the cleavage results presented here support a disulfide bonding pattern for SecR that is the same as that of class B receptor fragments folded in vitro, but different from that described for corticotropin-releasing factor receptors folded in the cell.

The current data provide strong confirmation that the pattern of disulfide bonds present in the recently reported NMR structure of the corticotropin-releasing factor receptor (14) is correct, and that this pattern is conserved throughout the class B family of GPCRs. More importantly, this new information provides additional insights into the functional roles of these three disulfide bonds and their impact on mechanisms of receptor maturation, binding and signaling. The observed differences in the biological activities of the C24-C53, C44-C85, and C67-C101 mutants establish a clear hierarchy of the functional importance of these bonds, based on the paired cysteine mutagenesis approach. For example, the C24-C53 and C44-C85 paired mutations were less capable of rescuing cAMP production than the C67-C101 paired mutation, although their restorative effect was slightly better than that provided by the C67 and C101 single mutations. Receptors with either of these single mutations were the only single mutants that exhibited cAMP levels significantly above basal. Furthermore, the single mutants C67 and C101 corresponded to the C67-C101 paired mutant that provided the largest biological responses observed among any of the mutants, restoring about one-third of wild type cAMP production. Thus, the disulfide bonds predicted from these functional data may be ranked in descending order of importance as second to fifth, first to third, and fourth to sixth.

These new structure/activity results can be summarized into an evolving mechanism for receptor activation that depends heavily upon the disulfide-bonded conformation of the extracellular domain. In this mechanism, the bonds linking the first to third and second to fifth conserved cysteines are most critical, as can be seen by the roles they play in establishing the stable base for ligand interaction. These two bonds flank the ligand binding pocket in the NMR structure of the corticotropin-releasing factor receptor (14), bringing into apposition two β-sheet structures that include the most highly conserved amino-terminal residues (other than the cysteines) among the class B receptors. As the least critical of the three, the bond linking the fourth to sixth conserved cysteines helps to situate the well-folded ligand-binding platform in correct orientation with the body of the receptor. Although this interaction probably is key for transmitting the conformational change to the region of coupling with the heterotrimeric G protein, this spatial approximation already is established independent of this disulfide bond because the amino terminus of the receptor also is tethered to the receptor body through the peptide chain. This connection through the peptide backbone probably explains why the C67-C101 paired SecR mutant restored the highest levels of cAMP accumulation among all of the cysteine mutants.

In conclusion, the paired mutagenesis approach employed in the current study has advanced our understanding of class B GPCR structure through the functional validation of a disulfide bonding pattern that until now had been demonstrated only with receptor fragments folded in vitro. Combined with supporting evidence from chemical cleavage experiments, these results build the strong argument that all class B receptors possess a similar pattern of disulfide bonds within their extracellular ligand-binding domains. Taken further, the emerging picture is that these functionally-important domains have very similar global structures that are based upon the short consensus repeat motif of the newly-described amino-terminal domain of corticotropin-releasing factor receptor (14). The framework now is in place for directed studies aimed at determining the local structural elements responsible for ligand-receptor specificity, with the eventual goal of mimicking these elements in the form of rationally-designed therapeutics.

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FOOTNOTES

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1The abbreviations used are: Bpa, benzoyl phenylalanine; CNBr, cyanogen bromide; HA, hemagglutinin epitope; GPCR, G protein-coupled receptor; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SecR, rat secretin receptor.

FIGURE LEGENDS

Fig. 1. Localization of secretin receptor cysteine mutants. Each panel shows a representative confocal micrograph (single optical section) of one or more COS cells fixed in formaldehyde and labeled for indirect immunofluorescence localization of HA epitope-tagged cell surface receptors. Similar to the wild type receptor in (A), the single or paired cysteine mutant receptors C85S (C), C101S (D), C67S-C85S (E), C24S-C53S (F), C44S-C85S (G), and C67S-C101S (H) sorted properly to the plasma membrane at levels comparable to wild type. All receptor constructs were expressed from the pcDNA3 eukaryotic expression vector (B) as described in Experimental Procedures. Bar in (H) = 25 µm.

Fig. 2. Biological activities of secretin receptor cysteine mutants. The white bars indicate basal levels and the black bars indicate maximal levels of intracellular cAMP produced upon stimulation with 0 or 100 nM secretin, respectively, of roughly 25,000 COS cells expressing the indicated single or paired cysteine mutants. Values are expressed as the means ± S.E.M. of duplicate data points from three independent experiments. The asterisks denote responses significantly different from an averaged background of all basal responses in the absence of ligand, as determined by one-way analysis of variance and Bonferroni post-test comparisons (* p < 0.05, ** p < 0.01, *** p < 0.001). The shaded area marks an arbitrary threshold of five times basal responses within which were all non-significant responses recorded in the presence of ligand.

Fig. 3. Functional analyses of the secretin receptor cysteine mutants C24S-C53S, C44S-C85S, and C67S-C101S. The top panel demonstrates the abilities of increasing concentrations of natural secretin agonist to compete for the binding of a radioligand, 125I-[Tyr10]secretin, to intact COS cells expressing the paired mutant receptors indicated. The bottom panel illustrates intracellular cAMP accumulated in the same cells stimulated with increasing concentrations of natural secretin agonist. A single data point (triangle) represents the average cAMP accumulations elicited by all other cysteine mutants after stimulation of cells with 100 nM secretin. Data were normalized to wild type responses and are presented as means ± S.E.M. from three experiments performed in duplicate.
Fig. 4. **CNBr cleavage of photoaffinity-labeled secretin receptors.** (A) Schematic diagrams indicating the relative locations of the HA epitope tag and key cysteine and methionine amino acid residues in the amino-terminal domains of three secretin receptor constructs engineered for CNBr cleavage experiments. The predicted CNBr cleavage patterns of wild type HA37-SecR were altered by replacing the endogenous methionine M73 with isoleucine (HA37-M73I-SecR) and then inserting the new methionines at positions A41 and L99 (HA37-A41M-M73I-L99M-SecR). (B) Schematic diagrams showing proteolytic fragments predicted from CNBr cleavage of non-reduced HA37-A41M-M73I-L99M-SecR. Disulfide bonds are drawn between cysteine residues according to the patterns determined for the intact corticotropin-releasing factor receptor (Pattern I) or from refolded amino-terminal domains of this and other class B receptors (Pattern II). **Bold lines** denote the radioactive fragments that would be visualized by autoradiography due to photochemical cross-linking of the $^{125}$I-labeled Bpa$^{26}$ probe (asterisks). (C) Representative autoradiographs of CNBr cleavage products of secretin receptors labeled with the Bpa$^{26}$ probe and separated in 10% NuPAGE gels. The fragments from the labeled reduced HA37-SecR and HA37-M73I-SecR receptors migrated at approximately $M_r = 18,000$ (lanes 2, 6), representing the amino-terminal 51 residues, whereas those of HA37-A41M-M73I-L99M-SecR receptors migrated at $M_r = 7,500$ (lane 9), representing only the amino-terminal 41 residues. The fragments from all three labeled receptors migrated at approximately $M_r = 40,000$ under non-reducing conditions (lanes 4, 8, 10). That the fragment from non-reduced HA37-A41M-M73I-L99M-SecR did not migrate to a smaller $M_r$ supports the pattern of disulfide bonds indicated by Pattern II in (B).

### TABLE I

**Evidence for the disulfide bonding pattern of class B G protein-coupled receptors**

| Experimental approach            | Receptor$^a$ | Disulfide pattern | Ref.      |
|----------------------------------|--------------|-------------------|-----------|
| **Intact receptors**             |              |                   |           |
| Functional, paired Cys mutants   | CRFR1        | 1-3, 2-6, 4-5     | 5         |
| Functional, paired Cys mutants   | SecR         | 1-3, 2-5, 4-6     | this study|
| **Refolded receptor fragments**  |              |                   |           |
| Biochemical, E.coli-expressed    | CRFR1        | 1-3, 2-5, 4-6     | 15        |
| amino-terminal domain            |              |                   |           |
| Biochemical, E.coli-expressed    | CRFR2β       | 1-3, 2-5, 4-6     | 16        |
| amino-terminal domain            |              |                   |           |
| NMR structural, E.coli-expressed | CRFR2β       | 1-3, 2-5, 4-6     | 14        |
| amino-terminal domain            |              |                   |           |
| Biochemical, E.coli-expressed    | PTHR1        | 1-3, 2-5, 4-6     | 17        |
| amino-terminal domain            |              |                   |           |
| Biochemical, E.coli-expressed    | GLP-1R       | 1-3, 2-5, 4-6     | 18        |
| amino-terminal domain            |              |                   |           |

$^a$CRFR1/2β, Corticotropin-releasing factor receptors 1 and 2β; SecR, Secretin receptor; PTHR1, Parathyroid hormone receptor 1; GLP-1R, Glucagon-like peptide 1 receptor.
**TABLE II**

*Functional characteristics of secretin receptors with single or paired cysteine mutations*

| Receptor        | Binding | Biological activity |          |
|-----------------|---------|---------------------|----------|
|                 | % WT    | Basal (pmol)        | Max. (pmol) | EC50 (nM) |
| WT              | 100.0 ± 0.00 | 0.96 ± 0.16 | 55.30 ± 4.25 | 0.015 ± 0.002 |
| C24S            | 5.59 ± 1.71 | 0.71 ± 0.10 | 1.74 ± 0.09 | ND*       |
| C44S            | 0.89 ± 0.89 | 0.63 ± 0.10 | 2.24 ± 0.23 | ND        |
| C53S            | 4.48 ± 2.18 | 0.61 ± 0.04 | 1.82 ± 0.05 | ND        |
| C67S            | 3.63 ± 0.68 | 0.53 ± 0.14 | 3.52 ± 0.47 | ND        |
| C85S            | 10.06 ± 1.78 | 0.65 ± 0.06 | 1.55 ± 0.16 | ND        |
| C101S           | 6.79 ± 2.76 | 0.49 ± 0.03 | 3.74 ± 0.69 | ND        |
| C24A-C44S       | 8.83 ± 2.02 | 0.61 ± 0.06 | 1.88 ± 0.16 | ND        |
| C24S-C53S       | 2.92 ± 0.03 | 0.87 ± 0.13 | 4.93 ± 0.72 | 183 ± 68  |
| C24S-C67S       | 7.43 ± 3.34 | 0.71 ± 0.10 | 0.75 ± 0.10 | ND        |
| C24S-C85S       | 3.71 ± 1.83 | 0.68 ± 0.15 | 1.75 ± 0.09 | ND        |
| C24S-C101S      | 5.48 ± 2.46 | 0.63 ± 0.04 | 1.76 ± 0.15 | ND        |
| C44S-C53S       | 3.16 ± 1.55 | 0.81 ± 0.11 | 0.88 ± 0.11 | ND        |
| C44S-C67S       | 6.08 ± 1.60 | 0.75 ± 0.13 | 3.05 ± 0.28 | ND        |
| C44S-C85S       | 6.67 ± 1.68 | 0.78 ± 0.09 | 4.19 ± 0.79 | 444 ± 327 |
| C44S-C101S      | 5.37 ± 1.56 | 0.62 ± 0.14 | 1.88 ± 0.12 | ND        |
| C53S-C67S       | 6.57 ± 0.73 | 0.81 ± 0.17 | 2.81 ± 0.28 | ND        |
| C53S-C85S       | 6.09 ± 1.99 | 0.79 ± 0.08 | 2.75 ± 0.17 | ND        |
| C53S-C101S      | 1.73 ± 0.90 | 0.61 ± 0.08 | 1.85 ± 0.13 | ND        |
| C67S-C85S       | 9.90 ± 1.42 | 0.89 ± 0.18 | 2.28 ± 0.32 | ND        |
| C67S-C101S      | 3.98 ± 0.40 | 0.78 ± 0.10 | 15.64 ± 4.06 | 12.8 ± 8.3 |
| C85S-C101S      | 7.27 ± 3.98 | 0.80 ± 0.04 | 2.71 ± 0.20 | ND        |

*ND, Not determined.*
Fig. 1. Lisenbee et al.
Fig. 2. Lisenbee et al.
Fig. 3. Lisenbee et al.
Paired cysteine mutagenesis to establish the pattern of disulfide bonds in the functional intact secretin receptor
Cayle S. Lisenbee, Maoqing Dong and Laurence J. Miller

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