THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 25, Issue of June 21, pp. 14944–14949, 1996

Printed in U.S.A.

Multiple Extracellular Loop Domains Contribute Critical Determinants for Agonist Binding and Activation of the Secretin Receptor*

(Received for publication, March 1, 1996, and in revised form, April 8, 1996)

Martin H. Holtmann, Subhas Ganguli, Elizabeth M. Hadac, Vesile Dolu, and Laurence J. Miller‡

From the Center for Basic Research in Digestive Diseases, Mayo Clinic and Foundation, Rochester, Minnesota 55905

Distinct themes exist for ligand-binding domains of G protein-coupled receptors. The secretin receptor is prototypic of a recently described family in this superfamily which binds moderate-sized peptides possessing a diffuse pharmacophore. We recently demonstrated the importance of the N terminus and first loop of this receptor for secretin binding (Holtmann, M. H., Hadac, E. M., and Miller, L. J. (1995) *J. Biol. Chem.* 270:14394–14398). Here, we extend those findings to define another receptor domain important for agonist recognition and to focus on critical determinants within each of these domains. Extending the secretin-vasoactive intestinal polypeptide (VIP) chimeric receptor approach, we confirmed and refined the critical importance of the N terminus and the need to complement this with other domains of the secretin receptor. There was redundancy in the complementary determinants required, with the second extracellular loop able to compensate for the absence of the first loop. The first 10 residues of the N terminus of the secretin receptor were critical. Sequential segmental and site replacements permitted focusing on the His^{189}–Lys^{190} sequence at the C terminus of the first extracellular loop, and on four residues (Phe^{257}, Leu^{258}, Asn^{260}, and Thr^{261}) in the N-terminal half of the second loop as providing critical determinants. All receptor constructs which expressed sensitive cAMP responses to secretin (EC_{50} <5 nM) bound this peptide with high affinity. Of note, one construct dissociated high affinity binding of secretin from its biological responsiveness, providing a clue to the conformational "switch" that activates this receptor.

Elucidation of the molecular determinants for an agonist to bind and activate its receptor is a critical process in the understanding of the regulation of cellular activities by circulating soluble factors. The largest group of membrane receptors now recognized is the superfamily of guanine nucleotide-binding protein (G protein)[1]-coupled receptors (1), which includes diverse families with distinct themes for ligand binding (2). These themes correlate with the size and physicochemical characteristics of the pharmacophoric domains of the natural ligands, as well as with the structural features of the receptors themselves (2). The secretin receptor family was recently recognized as a distinct family within this superfamily, which binds moderate sized peptides having diffuse pharmacophoric domains (3). A key structural feature of this family is a long extracellular N terminus containing six conserved cysteine residues (3).

Indeed, this N-terminal domain has been shown to be important for the binding of ligands to the secretin receptor family, with deletions and critical site mutants losing key binding and activation properties (4–7). Our recent work with chimeric secretin/VIP receptor constructs was consistent with this theme, supporting a critical role for the receptor N terminus in determining its structural specificity (8). The binding and biological activity properties of the wild type VIP receptor could be reproduced by a chimeric receptor with the predicted extracellular N terminus of the VIP receptor attached to the remainder of the secretin receptor. Of particular interest, the converse was not true (8). While the secretin receptor N terminus was also critical, it was not sufficient in contributing the necessary determinants for high affinity secretin binding and activation. In that report, two constructs which provided both the secretin receptor N terminus and an additional domain provided the necessary determinants for these activities. These additional domains represented the first extracellular loop in one construct and the remainder of the secretin receptor exclusive of that loop in the other construct.

In the present study, we have extended our previous work to further identify critical determinants for secretin receptor function. For this we have continued to construct and study secretin/VIP receptor chimeras, with the secretin receptor sequence domains representing progressively smaller segments. This has established that, like the first extracellular loop previously identified, the second extracellular loop provided important determinants to complement the secretin receptor N terminus. Within these two domains, we have been able to further define two residues in the C-terminal half of the first loop (His^{189} and Lys^{190}) and four residues in the N-terminal half of the second loop (Phe^{257}, Leu^{258}, Asn^{260}, and Thr^{261}) which contain critical determinants for secretin binding and activation. We have also gained additional insights into specific regions of the N terminus of the secretin receptor which are important. Critical determinants are present in the first 10 N-terminal residues and within the half of this domain adjacent to the plasmalemma.

In this series of chimeric receptors, every construct that exhibited cAMP responsiveness to nanomolar concentrations of secretin, also bound secretin with high affinity. Of particular interest, there was one of this series of constructs which bound secretin with high affinity, while not eliciting a cAMP response. This important dissociation of binding and activation might provide insight into induction of a conformational switch that activates this receptor.

* This work was supported in part by National Institutes of Health Grant DK46577 (to L. J. M.), the Fiterman Foundation (to L. J. M.), and Studienstiftung des Deutschen Volkes (to M. H. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence and reprint requests should be addressed: Center for Basic Research in Digestive Diseases, Guggenheim 17, Mayo Clinic, Rochester, MN 55905. Tel.: 507-284-0680; Fax: 507-284-0762.

[1] The abbreviations used are: G protein, guanine nucleotide-binding protein; VIP, vasoactive intestinal polypeptide; PCR, polymerase chain reaction; KRH, Krebs-Ringer-HEPES.

MATERIALS AND METHODS

The rat secretin receptor cDNA was previously cloned and characterized (9, 10), and the rat VIP receptor cDNA was kindly provided by Professor Nagata (Osaka Bioscience Institute, Osaka, Japan) (11). Rat secretin-27, the secretin analogue, [Tyr^{10},pNO_2-Phe^{22}]secretin-27, and VIP were synthesized in our laboratory, using techniques we have previously described (10). Enzymes for mutagenesis were purchased from Boehringer Mannheim, except for *Bsr*GI and *Spe*I, which were from New England Biolabs. All other reagents were analytical grade.

Receptor Constructs—The receptor mutants studied in this work represented chimeric constructs of wild type rat secretin and VIP receptors (see Figs. 1–4). Chimeras were designed to replace portions of one wild type receptor cDNA with the corresponding portions of the other receptor. Mutagenesis strategies included utilization of ligations at naturally occurring restriction sites, polymerase chain reaction (PCR) overlap extensions (12), and applications of the method of Kunkel (13).

Receptor constructs were built in the eukaryotic expression vector, pBK-CMV (Stratagene, La Jolla, CA). The wild type secretin receptor cDNA was inserted into the *Bam*HI and *Hin*dIII sites, and the wild type VIP receptor cDNA was inserted into the *Hin*dIII site. The nomenclature for the chimeras utilizes e_1, e_2, e_3, and e_4 as the predicted N terminus and first, second, and third extracellular loop domains, with amino acid residues identified by number, according to the numbering schemes of the wild type receptors (9, 11). Numbers not specifically attributed to the VIP receptor (*i.e.* following a "V") correspond to numbers of secretin receptor (S) residues.

Se_1V was generated by PCR mutagenesis, as described previously (8). V(1–45)S was produced by overlap extension. Initial PCR products represented the 5′-end of the VIP receptor sequence through codon 45, and secretin receptor sequence representing codons 62 through 149. These were purified, used in a round of PCR overlap extension, and cut with *Bam*HI and *Bsr*GI. The resultant fragment was then ligated into the wild type secretin receptor cDNA construct which was similarly prepared.

S(1–10)V(7–45)S was also prepared by overlap extension, this time using the V(1–45)S construct and wild type secretin receptor construct as templates. The overlap provided replacement of VIP receptor codons 1 through 7 with the corresponding secretin receptor codons 1 through 10.

S(1–10)V was generated using the Kunkel (13) method. An 87-oligomer was designed to replace the VIP receptor codons 1–7 with the corresponding secretin receptor codons 1–10. Simultaneous mutagenesis with a second oligo introduced a silent *Bsr*GI site at position 116 of the VIP receptor, to allow for subsequent mutagenesis by restriction digestion.

S(1–10)V(7–117)S was produced by replacing the *Bsr*GI-*Hin*dIII fragment of the S(1–10)V construct with the corresponding fragment of the wild type secretin receptor construct.

Se_1e_2(179–185)V; Se_1e_2(184–190)V, Se_1e_2(189, 190)V, Se_1e_2(190)V, Se_1e_3V, Se_1e_4V, Se_1e_3(257,258,260,261)V, and Se_1e_3(266–268)V were generated by the method of Kunkel (13), using appropriate mutagenesis oligos. The single-stranded template for this was produced from the Se_1V construct, representing secretin receptor codons 1 through 123 and VIP receptor codons 116 through 429.

All PCR reactions were performed in a thermocycler with *Taq* DNA polymerase running 35 cycles: 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 3 min. PCR and restriction digestion products were separated on 1% agarose gels and purified on Qiaex resin. Transformation of receptor constructs was performed in XLI-Blue MRF′ cells. Proper mutagenesis was confirmed by direct DNA sequencing, using the method of Sanger *et al.* (14).

Receptor Expression—COS-7 cells (American Type Culture Collection) served as the expression system for the receptor constructs. Cells were maintained in culture in Dulbecco's modified Eagle's medium with 5% Fetal Clone 2 (HyClone Laboratories, Logan, UT), and transfected with 2–4 μg of DNA. For this, a protocol based on the DEAE-dextran method was used including dimethyl sulfoxide shock and treatment with 0.1 mM chloroquine diphosphate (8, 15). Cells were harvested mechanically 72 h after transfection for subsequent studies.

Biological Activity Studies—The biological activity of our receptor constructs was assessed by their ability to elicit intracellular cAMP generation in response to agonist stimulation. Transfected COS cells were harvested mechanically, washed in phosphate-buffered saline, and resuspended in Krebs-Ringer-HEPES (KRH) medium containing 25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 1 mM KH_2PO_4, 0.2% bovine serum albumin, and 0.01% soybean trypsin inhibitor, which was supplemented with 1 mM 3-isobutyl-1-methylxanthine. Cells were stimulated with peptide for 10 min at 37 °C, and lysed by vortexing after the addition of ice-cold 6% perchloric acid. The pH was adjusted to 6 with $KHCO_3$, and lysates were cleared by centrifugation at 2000 rpm for 10 min. Supernatants were used for determination of cAMP levels with a [^{3}H]cAMP kit from Diagnostic Products Corp. (Los Angeles, CA), following the manufacturer's instructions. Assays were performed in duplicate and repeated in at least three independent experiments. Radioactivity was quantified in a Beckman LS6000 scintillation counter.

Ligand Binding Studies—For binding studies, receptor-bearing COS cell membranes were prepared 72 h after transfection, as described previously (10). Radioligands were prepared by oxidative radioiodination of the secretin analogue, [Tyr^{10},pNO_2-Phe^{22}]secretin-27, and VIP, with purification by reversed-phase high peformance liquid chromatography to specific radioactivities of 2000 Ci/mmol, as we described elsewhere (10). Incubations included 1–10 μg of relevant receptor-bearing membranes with a constant amount of radiolabeled ligand (3–5 pM), and increasing concentrations of analogous unlabeled ligand for 1 h at room temperature in KRH medium. Free radioligand was then removed by rapid filtration and washing using a Skatron cell harvester with glass fiber filter mats that had been presoaked in 0.3% polybrene. Nonspecific binding was determined in the presence of excess unlabeled analogous peptide (1 μM secretin and 0.1 μM VIP).

Statistical Analysis—All observations were repeated at least three times in independent experiments and are expressed as means ± S.E. For analysis of binding data, the Prism software package (GraphPad Software, San Diego, CA) was applied using the nonlinear regression analysis routine for radioligand binding. Differences were determined by using the Mann-Whitney test for unpaired values, with $p < 0.05$ considered to be significant.

RESULTS

This work explores the secretin receptor determinants that are critical for ligand binding and the resultant stimulation of signaling cascades. This represents a direct extension of our previous work (8) which also utilized secretin/VIP receptor chimeras, but in which we exchanged much larger domains. Here, we have focused on subdomains within the extracellular N terminus and within each of the extracellular loop regions. Biological activity data are illustrated in Figs. 1–4, and are summarized along with binding data in Table I. Constructs were expressed at similar levels, based on radioligand binding analysis (17 ± 7 pmol/mg of protein) and the quantitative increases in cAMP stimulated by agonists.

The wild type secretin receptor responded to stimulation by increasing intracellular cAMP, with EC_{50} values of 1.6 ± 0.4 nM secretin and 218 ± 109 nM VIP. This represents a selectivity of 2 orders of magnitude for this receptor's native ligand. The chimeric receptor that incorporated the extracellular N terminus of the secretin receptor with the remainder corresponding to the VIP receptor (Se_1V) lost its selectivity for secretin. It was less responsive to secretin than the wild type receptor (EC_{50} = 33 ± 3 nM; $p = 0.01$), while maintaining its responsiveness to VIP (EC_{50} = 45 ± 9 nM; $p = 0.1$). These results provided the basis for identifying additional determinants which were necessary to complement the extracellular N terminus of the secretin receptor in ligand recognition (Fig. 1).

Critical Determinants in the First Extracellular Loop Region of the Secretin Receptor—Replacement of the N-terminal half of the first loop region of Se_1V with the corresponding sequence of the secretin receptor (Se_1e_2(179–185)V) did not improve secretin responsiveness (EC_{50} = 25 ± 8 nM; $p = 0.4$ relative to the Se_1V construct) (Fig. 2). Of note, despite low responsiveness to secretin, this construct bound secretin with the same high affinity (K_i = 1.3 ± 0.1 nM) as was observed for the wild type secretin receptor (K_i = 2.2 ± 0.7 nM; $p = 0.4$). High affinity binding to this receptor construct was thus dissociated from biological responsiveness.

Complementation of the secretin receptor N terminus with the C-terminal half of the first loop region in the Se_1e_2(184–

TABLE I
Receptor activity and binding

Receptor construct	Intracellular cAMP response, EC_{50}			Secretin binding, K_i
	Secretin	VIP	Relative increase over basal (×)	
	nM			
WTSecR	1.6 ± 0.4	218 ± 109	2.2 ± 0.2	2.2 ± 0.7
Se_1V	33 ± 3	45 ± 9	2.7 ± 0.3	ND[a]
Se_1e_2(179–185)V	25 ± 8	45 ± 23	3.1 ± 0.4	1.3 ± 0.1
Se_1e_2(184–190)V	3.3 ± 0.4	157 ± 72	2.3 ± 0.5	3.1 ± 0.3
Se_1e_2(189,190)V	4.5 ± 0.6	65 ± 11	2.2 ± 0.4	1.6 ± 0.3
Se_1e_2(190)V	24 ± 3	29 ± 11	2.1 ± 0.2	ND
Se_1e_3V	3.5 ± 0.6	80 ± 15	3.0 ± 0.5	1.4 ± 0.6
Se_1e_3(257,258,260,261)V	4.9 ± 0.1	93 ± 44	2.3 ± 0.3	1.6 ± 0.2
Se_1e_3(266–268)V	95 ± 48	230 ± 115	2.7 ± 1.4	ND
Se_1e_4V	147 ± 50	>1000	2.4 ± 0.2	ND
V(1–45)S	74 ± 21	148 ± 48	2.8 ± 0.3	ND
S(1–10)V(7–45)S	3.2 ± 0.5	303 ± 203	2.3 ± 0.4	15.3 ± 3.8
S(1–10)V(7–117)S	81 ± 11	15 ± 6	2.3 ± 0.2	ND
S(1–10)V	675 ± 48	2.2 ± 0.5	2.6 ± 0.2	ND

[a] ND, not detected.

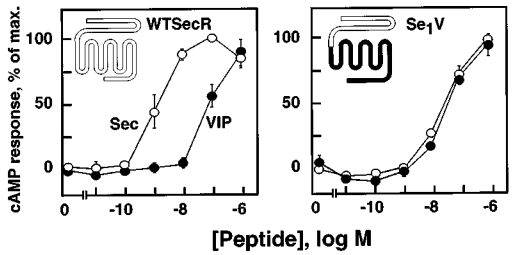

FIG. 1. **Biological responses of the wild type secretin receptor and the chimeric Se_1V construct expressed in COS cells.** Shown are cAMP responses to secretin (○) and VIP (●). Values are expressed as the means ± S.E. of four independent experiments, with data normalized relative to the maximal responses to agonist stimulation.

190)V construct substantially improved responsiveness to secretin (EC_{50} = 3.3 ± 0.4 nM; p = 0.03 relative to the Se_1 construct), such that it was not statistically different from the wild type secretin receptor (p = 0.1) (Fig. 2). VIP stimulated cAMP responses in this construct with an EC_{50} of 157 ± 72 nM. This construct also displayed high affinity binding of secretin, not different from that for the wild type secretin receptor (K_i = 3.1 ± 0.3 nM; p = 0.1).

The critical contribution of the C-terminal half of the first loop region to secretin recognition appears to be dependent on two basic residues, His^{189} and Lys^{190}. Insertion of these two residues at codon position 183 of the VIP receptor portion of Se_1V, resulting in the Se_1e_2(189, 190)V construct, was also able to improve responsiveness to secretin (EC_{50} = 4.5 ± 0.6 nM; p = 0.01 relative to the Se_1V construct) (Fig. 2). Responsiveness to VIP was unaltered (EC_{50} = 65 ± 11 nM). Secretin bound to this construct with high affinity (K_i = 1.6 ± 0.3 nM). Interestingly, insertion of only one basic residue (Lys^{190}) at this same position in the Se_1e_2(190)V construct had no effect on secretin responsiveness (EC_{50} = 24 ± 3 nM, p = 0.1 relative to the Se_1V construct) (Fig. 2).

Critical Determinants in the Extracellular Second and Third Loop Regions—Successful complementation of the extracellular N terminus of the secretin receptor could also be achieved by replacement of determinants in the second extracellular loop region of the secretin receptor. Replacement of the entire second loop region in addition to the extracellular N terminus in the Se_1e_3V construct resulted in an EC_{50} of 3.5 ± 0.6 nM secretin (Fig. 3). This represented a 10-fold improvement compared to the Se_1V construct (p = 0.01), and secretin responsiveness was not different from that of the wild type receptor (p = 0.1). VIP stimulated cAMP responses with an EC_{50} of 80 ± 15 nM. Consistent with its biological activity, Se_1e_3V bound secretin with high affinity (K_i = 1.4 ± 0.6 nM).

Sequential bisection of this domain revealed that the critical determinants for recognition of secretin were located in the N-terminal half of the second loop. The effect seen by replacement of the entire loop region could be mimicked by incorporation of four secretin receptor-specific residues (Phe, Leu, Asn, Thr) in the Se_1e_3(257,258,260,261)V construct. This construct responded to secretin with an EC_{50} of 4.9 ± 0.1 nM (p = 0.1 compared to Se_1e_3V) (Fig. 3). Cyclic AMP responses to VIP were unaltered (EC_{50} = 93 ± 44 nM). Binding of secretin occurred with a K_i of 1.6 ± 0.2 nM. Conversely, the C-terminal half of the second loop was not capable of complementing the extracellular N terminus. Replacement of the relevant three residues in that domain in the Se_1e_3(266–268)V construct did not improve responsiveness to secretin (EC_{50} = 95 ± 48 nM; p = 0.2 relative to the Se_1V construct) (Fig. 3). When the third loop region of the Se_1V was changed to secretin receptor-specific sequence in the Se_1e_4V construct, no effect was seen in secretin responsiveness which remained low (EC_{50} = 147 ± 50 nM) (Fig. 3).

Critical Determinants in the Extracellular N Terminus of the Secretin Receptor—We previously showed the critical role of this domain for ligand specificity (8) and now wanted to better localize critical portions within this domain. Replacement of half of the N terminus of the secretin receptor with the analogous 45 residues of the VIP receptor in the V(1–45)S construct resulted in a marked reduction of secretin responsiveness (EC_{50} = 74 ± 21 nM) compared to the wild type secretin receptor (p = 0.01) (Fig. 4), and resulted in loss of saturable secretin binding. The portion of the VIP receptor incorporated in this construct alone was also unable to provide high responsiveness to VIP (EC_{50} = 148 ± 48 nM).

Of note, reincorporation of the N-terminal 10 secretin receptor specific residues in the S(1–10)V(7–45)S construct resulted in almost full responsiveness to secretin (EC_{50} = 3.2 ± 0.5 nM secretin) (Fig. 4), while responsiveness to VIP remained low (EC_{50} 303 ± 203 nM). While important, this small domain of the secretin receptor was not sufficient by itself to recognize secretin. The S(1–10)V construct displayed low responsiveness to secretin (EC_{50} = 675 ± 48 nM) (Fig. 4), with no saturable binding of secretin. This construct, however, responded well to VIP stimulation (EC_{50} = 2.2 ± 0.5 nM).

It was also not possible to recover secretin responsiveness through complementation of the N-terminal 10 residues, incorporated in the S(1–10)V construct, with the secretin receptor trunk that comprised all loop regions of this receptor proven to contribute to ligand recognition. The resulting construct, S(1–10)V(7–117)S, retained its low responsiveness to secretin (EC_{50}

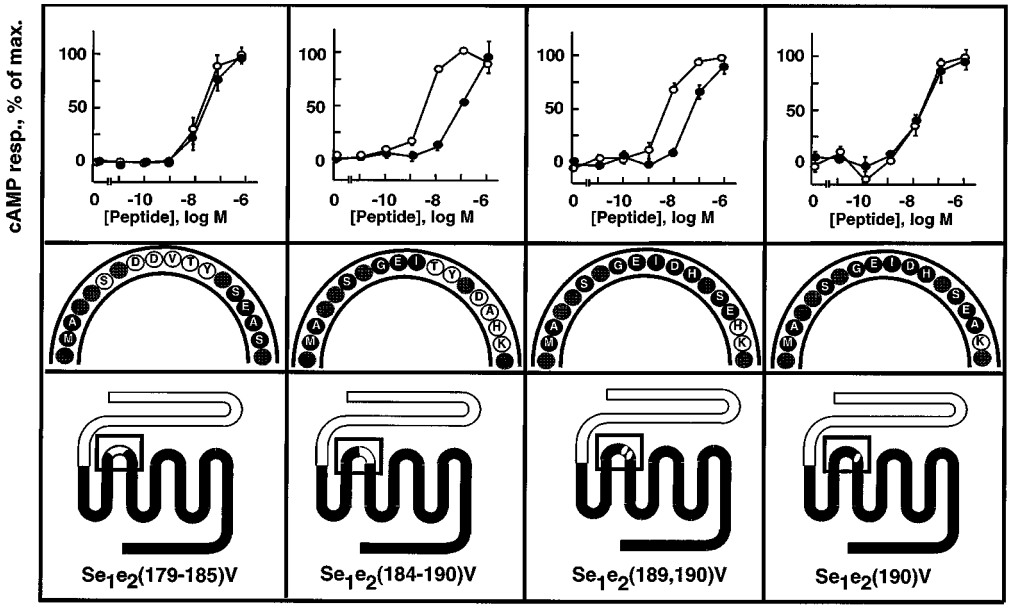

FIG. 2. **Structure and biological responses of chimeric receptor constructs exploring critical determinants in the first extracellular loop region of the receptor.** Displayed are the structures of the receptor constructs showing secretin receptor portions in *white* and VIP receptor portions in *black* (*bottom*). The structure of the first loop region of these constructs is magnified, with gray representing residues conserved between secretin and VIP receptors (*center*). Also shown are cAMP responses to secretin (○) and VIP (●) (*top*). Values are expressed as the means ± S.E. of at least three independent experiments, with data normalized relative to the maximal responses to agonist stimulation.

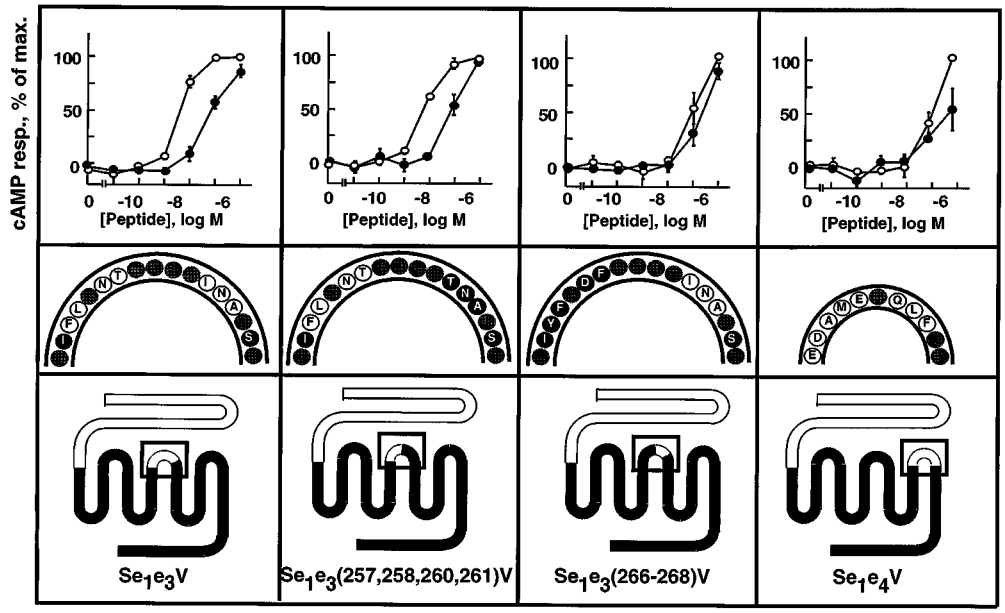

FIG. 3. **Structure and biological responses of chimeric receptor constructs exploring critical determinants in the second and third extracellular loop regions of the receptor.** Displayed are the structures of the receptor constructs showing secretin receptor portions in *white* and VIP receptor portions in *black* (*bottom*). The structure of the second and third loop regions of these constructs are magnified with gray representing residues conserved between secretin and VIP receptors (*center*). Also shown are cAMP responses to secretin (○) and VIP (●) (*top*). Values are expressed as the means ± S.E. of at least three independent experiments, with data normalized relative to the maximal responses to agonist stimulation.

= 81 ± 11 nM). When the inability of this construct to recognize secretin is contrasted with the S(1–10)V(7–45)S construct, it is clear that the portion of the secretin receptor in the N terminus adjacent to the plasma membrane also contains determinants critical for secretin recognition.

Fig. 5 shows direct competition-binding data for key chimeric receptor constructs. These represent those constructs which resulted in sensitive responsiveness to secretin by providing both the N terminus and critical loop domains of the secretin receptor. Also shown are data for the construct which bound secretin with high affinity, but did not have a sensitive biological response to this hormone (Se_1e_2(179–185)V).

DISCUSSION

Increasing evidence suggests that the sites which best discriminate ligand-receptor interactions in the recently discovered secretin receptor family are located in the extracellular portions of these receptors, reflecting the characteristic structural features of these receptors and the distinct properties of their peptide ligands (3). In our recent study of chimeric secretin/VIP receptors, we demonstrated the pivotal role of the extracellular N terminus of these receptors for ligand specificity (8). In the secretin receptor, however, this domain was not sufficient by itself to provide full specificity and sensitivity to

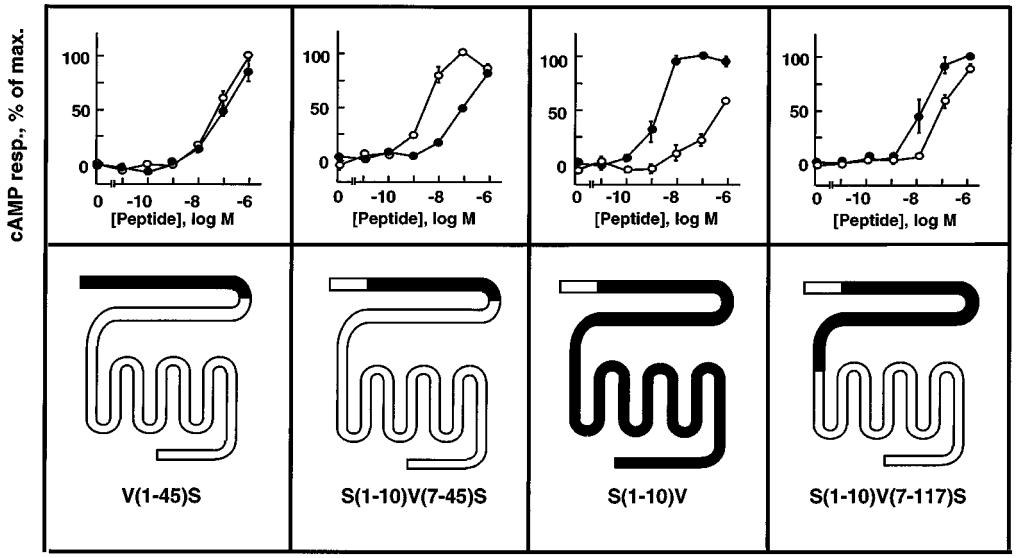

FIG. 4. **Structure and biological responses of chimeric receptor constructs exploring critical determinants in the predicted extracellular N terminus of the receptors.** Displayed are the structures of the receptor constructs showing secretin receptor portions in *white* and VIP receptor portions in *black* (*bottom*), and cAMP responses to secretin (○) and VIP (●) (*top*). Values are expressed as the means ± S.E. of at least three independent experiments, with data normalized relative to the maximal responses to agonist stimulation.

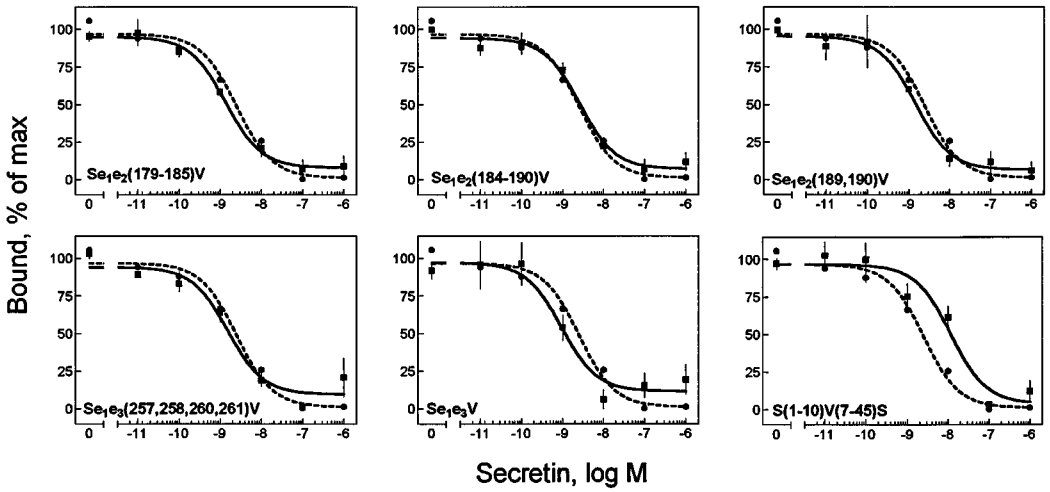

FIG. 5. **Binding data for key chimeric receptor constructs.** Shown are curves representing secretin competition for binding the secretin radioligand to membranes from COS cells transfected with the noted constructs. For reference, the same competition-binding curve for the wild type secretin receptor construct is also shown with a *broken line*. Values represent means ± S.E. of assays performed in duplicate at least three independent times.

secretin. In the chimeric constructs, this domain had to be complemented by other secretin receptor-specific determinants. There was, however, redundancy among the receptor determinants that were able to effectively complement the extracellular N terminus. Identification of this was only possible using chimeric receptor constructs. The high degree of structural homology between the receptors for secretin and VIP, having clearly distinct ligand specificity, provided the basis for this approach. Such chimeric constructs may be more likely to fold properly on the cell surface and to maintain their functional conformations than approaches involving receptor mutations such as deletions or replacements with nonconserved residues. The chimeric approach may also reduce the likelihood of allosteric artifacts interfering with receptor-ligand interaction, and may provide an opportunity to identify receptor determinants directly involved in ligand discrimination.

In the present work we extended our previous studies (8) and directed our attention to the elucidation of those determinants that were able to complement the extracellular N terminus of the secretin receptor for ligand recognition. Only 12 residues in the first loop region were potential candidates for contributing to secretin specificity. Sequential bisections and site mutant construction revealed that a sequence of two basic residues, His^{189} and Lys^{190}, but not one of them alone, in the C-terminal half of the first loop region was capable of complementing the N terminus of the secretin receptor in recognizing the native ligand. Evidence exists that an acidic residue in the center of the secretin peptide (Asp^{15}) is involved in binding of this ligand to the receptor molecule (16). The strongly basic nature of the epitope identified would make a direct interaction of the ligand and the receptor at this site plausible, although an intramolecular ion-pairing function has also been suggested for the secretin-Asp^{15} residue (17).

Alternatively, a microdomain of 4 residues in the N-terminal half (Phe^{257}, Leu^{258}, Asn^{260}, and Thr^{261}) of the second extracellular loop in the presence of the extracellular N terminus of the secretin receptor was also able to yield high affinity binding of secretin, and to increase receptor responsiveness to secretin stimulation by one order of magnitude. The third extracellular loop, on the other hand, did not seem to be involved in ligand

discrimination.

With this series of constructs we can clearly demonstrate that the determinants that provide secretin receptor specificity are located in extracellular portions of the receptor molecule. However, it would be too simplistic to conclude that ligand interaction with the receptor is confined to these domains. Residues which are conserved between the secretin and VIP receptors, will by definition not contribute to ligand specificity, however, they are likely to contribute key structural features to the receptor conformation, and some could also be involved in ligand binding and receptor activation.

We also focussed on the extracellular N terminus of the secretin receptor, which was previously demonstrated to be important (8), trying to more specifically identify critical regions within this domain. A large region in the half of the N terminus most distal from the plasmalemma could be replaced without effect, but the presence of the first 10 residues at the distal end of this region was necessary to recognize secretin. Critical determinants also seem to be present within the other half of the secretin receptor N terminus, because the first 10 residues alone were not able to provide full secretin responsiveness, even when complemented with the secretin receptor-specific loop regions. Interestingly, deletional mutagenesis of the corresponding portions in the parathyroid hormone receptor showed critical effects on cell surface expression of that receptor (6).

One of the chimeric constructs in which the extracellular N terminus of the secretin receptor was complemented with the N-terminal half of the secretin receptor first extracellular loop, while the rest of the construct represented VIP receptor sequence (Se_1e_2(179–185)V), did not show high responsiveness to secretin. However, this construct bound secretin with the same high affinity as the wild type secretin receptor. The dissociation of receptor activation from high affinity binding is a characteristic feature of antagonists and has supported the concept that ligand binding and receptor activation are separate and distinct events. Activation likely requires a highly specific conformational change in the receptor. The finding in this chimeric construct is particularly interesting, since it demonstrates the dissociation of these two events for a naturally occurring agonist. This observation might provide a valuable clue as to how high affinity binding of agonist ligand and the subsequent induction of the conformational switch that activates the receptor, could be structurally related in the secretin receptor.

Acknowledgments—We acknowledge the excellent technical assistance of E. Holicky and D. Pinon, and the excellent secretarial assistance of S. Erickson.

REFERENCES

1. Kolakowski, L. F. (1994) *Recept. Channels* **2,** 1–7
2. Coughlin, S. R. (1994) *Curr. Opin. Cell Biol.* **6,** 191–197
3. Segre, G. V., and Goldring, S. R. (1993) *Trends Endocrinol. Metab.* **4,** 309–314
4. Jüppner, H., Schipani, E., Bringhurst, F. R., McClure, I., Keutmann, H. T., Potts, J. T., Jr., Kronenberg, H. M., Abou-Samra, A. B., Segre, G. V., and Gardella, T. J. (1994) *Endocrinology* **134,** 879–884
5. Houssami, S., Findlay, D. M., Brady, C. L., Myers, D. E., Martin, T. J., and Sexton, P. M. (1994) *Endocrinology* **135,** 183–190
6. Lee, C., Gardella, T. J., Abou-Samra, A.-B., Nussbaum, S. R., Segre, G. V., Potts, J. T., Jr., Kronenberg, H. M., and Jüppner, H. (1994) *Endocrinology* **135,** 1488–1495
7. Couvineau, A., Gaudin, P., Maoret, J.-J., Rouyer-Fessard, C., Nicole, P., and Laburthe, M. (1995) *Biochem. Biophys. Res. Commun.* **206,** 246–252
8. Holtmann, M. H., Hadac, E. M., and Miller, L. J. (1995) *J. Biol. Chem.* **270,** 14394–14398
9. Ishihara, T., Nakamura, S., Kaziro, Y., Takahashi, T., Takahashi, K., and Nagata, S. (1991) *EMBO J.* **10,** 1635–1641
10. Ulrich, C. D., II, Pinon, D. I., Hadac, E. M., Holicky, E. L., Chang-Miller, A., Gates, L. K., and Miller, L. J. (1993) *Gastroenterology* **105,** 1534–1543
11. Ishihara, T., Shigemoto, R., Mori, K., Takahashi, K., and Nagata, S. (1992) *Neuron* **8,** 811–819
12. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) *Gene* (*Amst.*) **77,** 51–59
13. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 488–492
14. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5463–5467
15. Lopata, M. A., Cleveland, D. W., and Sollner-Webb, B. (1984) *Nucleic Acids Res.* **12,** 5707–5717
16. Bodanszky, M., Fink, M. L., and Boden, G. (1977) *Gastroenterology* **72,** 801–802
17. Bodanszky, M., Natarajan, S., Gardner, J. D., Makhlouf, G. M., and Said, S. I. (1978) *J. Med. Chem.* **21,** 1171–1173