

Possible Endogenous Agonist Mechanism for the Activation of Secretin Family G Protein-Coupled Receptors

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ABSTRACT

The class B family of G protein-coupled receptors contains several potentially important drug targets, yet our understanding of the molecular basis of ligand binding and receptor activation remains incomplete. Although a key role is recognized for the cysteine-rich, disulfide-bonded amino-terminal domain of these receptors, detailed insights into ligand docking and resultant conformational changes are not clear. We postulate that binding natural ligands to this domain results in a conformational change that exposes an endogenous ligand which interacts with the body of the receptor to activate it. In this work, we examined whether a synthetic peptide corresponding to a candidate region between the first and third conserved cysteines could act as an agonist. Indeed, this peptide was a weakly potent but fully efficacious agonist, stimulating a concentration-dependent cAMP response in secretin receptor-

bearing cells. This effect was maintained as the peptide length was reduced from 30 to 5, and ultimately, three residues focused on the conserved residue Asp⁴⁹. The agonist potency was enhanced by cyclization through a diaminopropionic acid linker and by amino-terminal fatty acid acylation. Both ends of the cyclic peptide were shown to interact with the top of transmembrane segment 6 of the receptor, using probes with a photolabile benzoyl-phenylalanine on each end. Analogous observations were also made for two other members of this family, the vasoactive intestinal polypeptide type 1 and calcitonin receptors. These data may provide a unique molecular mechanism and novel leads for the development of small-molecule agonists acting at potential drug targets within this physiologically important receptor family.

G protein-coupled receptors represent the largest group of receptor molecules, with natural ligands ranging from small photons, odorants, and biogenic amines to larger peptides and glycoproteins, and even very large viral particles (Ji et al., 1998). Agonist ligands bind to receptor domains ranging from the intramembranous confluence of helices to the surface loops and amino-terminal domains that vary substantially among distinct groups of receptors within this superfamily (Schwartz and Rosenkilde, 1996). In general, as ligand size increases, the site of its binding moves from the intramembranous domain to the surface and to external domains of its receptor, yet all agonists elicit a conformational change that is reflected in G protein association with the cytosolic face of the receptor. Best understood are the molecular mechanisms for binding the smallest ligands, such as those which occur at rhodopsin (Palczewski et al., 2000).

The secretin receptor is prototypic of the class B family of G protein-coupled receptors that includes numerous potentially important drug targets, such as the receptors for parathyroid hormone, calcitonin, glucagon, and vasoactive intestinal polypeptide (VIP) (Ulrich et al., 1998). The natural ligands for these receptors are all moderately large peptides having diffuse pharmacophoric domains. Consistent with the general rule described above, receptor mutagenesis and direct photoaffinity-labeling studies have identified the receptor amino terminus as the domain that is key for binding these ligands (Holtmann et al., 1995; Dong et al., 1999a,b, 2000).

Within this large domain of the secretin receptor are six highly conserved cysteine residues and three functionally critical, conserved disulfide bonds (Asmann et al., 2000; Lisenbee et al., 2005). Recent NMR analysis of the amino-terminal domain of another family member, the corticotropin-releasing factor (CRF) receptor, has demonstrated the key roles played by these bonds and two antiparallel β -sheet regions to establish a stable base for peptide ligand binding

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ABBREVIATIONS: VIP, vasoactive intestinal polypeptide; BHK-VPAC1, rat vasoactive intestinal polypeptide type 1 receptor-bearing baby hamster kidney cell line; Bpa, *p*-benzoyl-L-phenylalanine; CHO-SecR, rat secretin receptor-bearing Chinese hamster ovary cell line; CNBr, cyanogen bromide; CRF, corticotrophin-releasing factor; HEK293-CTR, human calcitonin receptor-bearing human embryonic kidney 293 cell line; HA, hemagglutinin.

(Grace et al., 2004). The carboxyl region of natural peptide ligands for class B receptors is believed to dock within a groove in this portion of the receptor (Juppner et al., 1994; Gourlet et al., 1996; Grace et al., 2004). For secretin, this mode of docking has been directly demonstrated using intrinsic photoaffinity labeling. These studies have established spatial approximation between residues scattered throughout the secretin pharmacophore, in positions 6, 12, 13, 14, 18, 22, and 26, and residues within the receptor amino terminus (Dong et al., 1999a,b, 2000, 2002, 2003; Zang et al., 2003). It is interesting that, to date, only the affinity-labeling probes with photolabile site of covalent attachment at their amino-terminal end have covalently labeled residues within the secretin receptor body, predicted to be located at the top of or above transmembrane segment 6 (Dong et al., 2004a).

Indeed, ligand binding to span these two receptor domains, amino terminus and body, has been postulated as a mechanism for activation of class B G protein-coupled receptors (Bisello et al., 1998). Parathyroid hormone and calcitonin have also been shown to dock in approximation with these two domains (Bisello et al., 1998; Dong et al., 2004b,c; Pham et al., 2004). Tethering could exert tension to change the conformation of the receptor body that would be consistent with what might be necessary to expose the cytosolic region of the receptor that interacts with its G protein. Recent data from our laboratory in which we modified the charge of the amino terminus of secretin, redirecting its site of affinity labeling back to the receptor amino terminus without affecting its ability to initiate signaling, suggest that ligand tethering of these two domains may not be necessary for biological activity of secretin (Dong et al., 2005).

In the current report, we therefore explored an alternative mechanism that has not been proposed previously for this receptor family. In this, the ligand docking to the receptor amino terminus results in a change in conformation of that domain, exposing a previously hidden epitope that can act as

an endogenous agonist ligand. Indeed, our working molecular model of the secretin receptor demonstrated a candidate region between receptor residues 24 and 53. Here, we describe the discovery of a synthetic tripeptide within this region that can function as a full endogenous agonist by binding to the top of transmembrane segment 6 of the secretin receptor. The possibly more general theme of endogenous agonist activity residing within this region of the amino terminus of class B G protein-coupled receptors was also observed with two other members of this family, the calcitonin and VPAC1 receptors.

Materials and Methods

Reagents. Rat VIP and human calcitonin were purchased from Bachem (Torrance, CA). Rat secretin and endoglycosidase F were prepared in our laboratory as described previously (Pearson et al., 1987; Ulrich et al., 1993). Amino acids for peptide synthesis were purchased from Advanced ChemTech (Louisville, KY). Cyanogen bromide (CNBr) and the solid-phase oxidant *N*-chlorobenzenesulfonamide (ODO-Beads) were from Pierce Chemical (Rockford, IL). The mouse 12CA5 monoclonal antibody against the hemagglutinin (HA) epitope was from Roche Applied Science (Indianapolis, IN). All other reagents were of analytical grade.

Peptides. Figure 1 describes the peptides used in this study. They were designed to represent the sequences of varied lengths of the regions of interest within the secretin, VPAC1, and calcitonin receptors. This list includes both linear and cyclic peptides, with the latter prepared by cross-linking the side chains of diaminopropionic acid and aspartic acid moieties. Synthetic strategies involved routine manual peptide synthesis on solid phase using a Pal resin (Advanced ChemTech) and Fmoc-protected amino acids (Powers et al., 1988). For the cyclic peptides, special side-chain protection was applied, using *N*- α -Fmoc-*N*- β -4-methyltrityldiaminopropionic acid and *N*- α -Fmoc-L-aspartic acid- β -2 phenylisopropyl ester. For these peptides, at the stage of being fully protected and still attached to the resin, the methyltrityl and 2-phenylisopropyl groups were selectively removed using 1.8% trifluoroacetic acid in CH₂Cl₂, as described by Li

| SecR peptides | Sequences |
|---|--|
| SecR(24-53), linear | CLQQLSKEKKGALGPETASG [*] SEGLWDMSC |
| LWDNM, linear and cyclic pentapeptide | X ⁻ LWDNM ^b |
| WDN, linear and cyclic tripeptide, also with cyclic tripeptide myristoylated, and with Tyr-Bpa on N-terminus or Bpa-Tyr on C-terminus | X ⁻ WDN ^b |
| DWN, cyclic scrambled tripeptide 1 | X ⁻ DWN ^b |
| NWD, cyclic scrambled tripeptide 2 | X ⁻ NWD ^b |
| WD, linear dipeptide | X ⁻ WD ^b |
| DW, linear scrambled dipeptide | X ⁻ DW ^b |
| VPAC1 peptide | |
| VPAC1(51-71), linear | X ⁻ LEEAQLENETTGS [*] SKMWDNL ^D |
| CTR peptide | |
| CTR(73-80), linear | X ⁻ NRTWDGWL ^D |

* Internal Cys was changed to Ser. X, diaminopropionic acid

Fig. 1. Sequences of peptides used in cAMP assays. Shown are the sequences of the endogenous peptides corresponding to regions of the amino terminus of class B G protein-coupled receptors. Each of the listed peptides, except for SecR(24-53), was synthesized with a diaminopropionic acid moiety at the amino terminus of the listed sequence and an aspartic acid residue at the carboxyl terminus of the listed sequence, with cyclic peptides linking these two residues through their side chains. The myristoylated, Tyr-Bpa, and Bpa-Tyr WDN peptides had these moieties attached outside of the cyclized diaminopropionic acid-WDN-aspartic acid core.

and Elbert (2002). For closing of the loop, benzotriazole-1-yloxy-tris (dimethyl amino)phosphonium hexafluorophosphate was used as a coupling reagent with 1-hydroxybenzotriazole and *N,N*¹-diisopropylethylamine, reacting this for 2 h and checking with ninhydrin to be certain that the reaction was complete. The resin was then washed, and the amino-terminal Fmoc protection was removed using 20% piperidine in dimethylformamide. For synthesis of the myristoylated cyclic peptide, while still on the resin with the amino terminus free, myristic acid was added along with *O*-benzotriazole-*N,N,N*¹-tetramethyl uranium hexafluorophosphate (as coupling agent), 1-hydroxybenzotriazole, and *N,N*¹-diisopropylethylamine, and this was allowed to react overnight. The peptides were removed from the resin using the standard trifluoroacetic acid mixture containing 6.25% (w/v) phenol, 2% (v/v) triisopropylsilane, 4% (v/v) thioanisole, 4% (v/v) distilled water, and 83% (v/v) trifluoroacetic acid. All peptides were purified to homogeneity by reverse-phase high-performance liquid chromatography using techniques that have been described previously (Powers et al., 1988). The expected molecular masses of the peptides were verified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry.

It is noteworthy that the photolabile *p*-benzoyl-L-phenylalanine (Bpa)-containing peptides were designed to be used in photoaffinity-labeling studies. These probes incorporated a Bpa at either the amino-terminal or carboxyl-terminal end of the cyclic WDN tripeptide. A tyrosine was incorporated at the noted position at the end of these peptides for oxidative radioiodination. These probes were radioiodinated and purified to radiochemical purity by reverse-phase high performance liquid chromatography to yield specific radioactivities of approximately 2000 Ci/mmol as described previously (Powers et al., 1988).

Receptor Preparations. Receptor-bearing Chinese hamster ovary and human embryonic kidney 293 cell lines stably expressing the rat secretin receptor (CHO-SecR) and human calcitonin isoform II receptor (HEK293-CTR), respectively, were used as the source of receptors for the current study. The former has been established previously and characterized in our laboratory (Ulrich et al., 1993), and the latter was provided by GlaxoSmithKline (Uxbridge, Middlesex, UK). Cells were cultured at 37°C in a 5% CO₂ environment on Falcon tissue culture plastic ware in Ham's F-12 or Dulbecco's modified Eagle's medium supplemented with 5% fetal clone-2 (Hyclone Laboratories, Logan, UT) and were passaged twice a week. Plasma membranes from CHO-SecR cells were prepared using methods described previously (Hadac et al., 1996).

An additional receptor-bearing baby hamster kidney cell line stably expressing the rat VPAC1 receptor (BHK-VPAC1) was established using a calcium phosphate-mediated transfection protocol, as described previously (Lisenbee et al., 2005). Cells were maintained under the same conditions as described above, except that the culture medium consisted of equal parts Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture (Invitrogen, San Diego, CA).

In addition, two secretin receptor mutants were generated that included Asp⁴⁹ to a glycine residue (D49G) or Trp⁴⁸ to a leucine residue (W48L), each representing the mutation of residues within the conserved WDN sequence of the receptor. Both were prepared using an oligonucleotide-directed approach with the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA) with their sequences verified by direct DNA sequencing. Both mutants together with the I334M secretin receptor mutant prepared and characterized previously (Dong et al., 2004a) were expressed transiently in COS-1 cells (American Type Culture Collection, Manassas, VA) after transfection using a modification of the DEAE-dextran method (Holtmann et al., 1996). Cells were maintained under the same conditions as the HEK293 cells described above.

Biological Activity Assay. The agonist activity was studied by measuring the intracellular cAMP accumulation of CHO-SecR, BHK-VPAC1, and HEK293-CTR cells stimulated by each tested peptide using a competition-binding assay (Diagnostic Products Corporation, Los Angeles, CA). In the legend for each relevant figure, the

absolute measured cAMP concentrations in both basal and maximally stimulated states in that series of experiments are reported. Concentration-response curves were also analyzed to determine EC₅₀ values. For these assays, adherent cells that were 75% confluent were incubated with increasing concentrations of each tested peptide for 30 min at 37°C in Krebs-Ringer-HEPES buffer (25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 1 mM KH₂PO₄, 1.2 mM MgSO₄, and 2 mM CaCl₂) containing 0.2% bovine serum albumin, 0.01% soybean trypsin inhibitor, 0.1% bacitracin, and 1 mM 3-isobutyl-1-methylxanthine, and the reaction was stopped by adding ice-cold perchloric acid. After adjusting the pH to 6 with KHCO₃, cell lysates were cleared by centrifugation at 3000 rpm for 10 min, and the supernatants were used in assays, as described previously (Ganguli et al., 1998). Radioactivity was quantified by scintillation counting in a Beckman LS6000. Each assay was performed in duplicate in at least three independent experiments.

Photoaffinity-Labeling Studies. Photoaffinity labeling was conducted with CHO-SecR cell membranes (100 μg) and the radioiodinated photolabile [¹²⁵I-Tyr]Bpa-WDN or WDN-Bpa[¹²⁵I-Tyr] (100 pM) in the absence and presence of competing WDN concentrations up to 1 μM using procedures described previously (Dong et al., 1999a). Labeled membranes were then washed, solubilized using 1% Nonidet P-40, and component glycoproteins were adsorbed to concanavalin A-agarose (EY Laboratories, Inc., San Mateo, CA). These beads were then loaded onto SDS-polyacrylamide gels in which the glycoproteins were eluted by SDS-sample buffer and separated by electrophoresis. Labeled products were visualized by exposure to X-ray film at -80°C.

Preparation of labeled receptor in large scale was necessary for mapping the labeled receptor domain. For this, 200 μg of receptor-bearing membranes and 1 nM [¹²⁵I-Tyr]Bpa-WDN or WDN-Bpa[¹²⁵I-Tyr] was incubated in the absence of competing WDN before photolysis. Labeled receptor bands were excised, eluted, lyophilized, and precipitated with ethanol before being used for chemical and enzymatic cleavage experiments. Immunoprecipitation of the HA-tagged secretin receptor with anti-HA monoclonal antibody in the presence of excess competing HA peptide was performed to further identify the labeled product. For selected experiments, the labeled receptor was deglycosylated with endoglycosidase F. CNBr was used to cleave the labeled wild-type or the I334M mutant secretin receptor to further localize the region of labeling. The cleavage products were resolved on 10% NuPAGE gels using 2-(*N*-morpholino)ethanesulfonic acid running buffer (Invitrogen). All of the experimental procedures have been well-described previously (Dong et al., 1999a).

Results and Discussion

We explored whether a synthetic peptide corresponding to the region between the first and third conserved cysteines (residues 24–53) of the secretin receptor amino terminus that has no structural homology with any part of the natural agonist peptide (Fig. 1) could act as an endogenous agonist ligand. Indeed, data in Fig. 2a demonstrate that this peptide was a weakly potent agonist, stimulating a concentration-dependent cAMP response in secretin receptor-bearing CHO-SecR cells but having no effect on the nonreceptor-bearing parental CHO cell line in concentrations as high as 100 μM. Likewise, there was no cAMP response to this peptide in CHO cell lines engineered to express high concentrations of a structurally distinct G protein-coupled receptor, the class A cholecystokinin receptor (data not shown).

It is particularly important to note that, despite its very low potency, the efficacy of this endogenous amino-terminal segment peptide was almost that of natural secretin. Because the volume of distribution of the endogenous peptide would be limited by its linkage to another region of the same mol-

ecule that it is postulated to act upon, its effective concentration at its site of action is quite high in that location. Therefore, the observed low potency may be consistent with the postulated molecular mechanism.

A more detailed definition of the receptor sequence relevant for this effect is quite important. There are clues from recent structural studies (Grace et al., 2004) and from the receptor mutagenesis literature (Couvineau et al., 1995) to focus on a subdomain within this receptor segment. NMR analysis of the amino terminus of the CRF receptor in the absence and presence of equimolar concentrations of a high-affinity peptide antagonist, astressin, showed that a portion of this region of interest that resides between the second and third conserved cysteine residues was one of the segments that underwent the largest chemical shift perturbations (Grace et al., 2004). It was assumed from these data that it represented a site of direct interaction with the ligand, but it could also represent a domain that has its conformation changed in a consistent and systematic manner in response to ligand binding. Because these studies were performed with the isolated amino-terminal tail region of the receptor rather than with the intact receptor, the orientation of this relative to the receptor body is not clear. This type of change in conformation could also be consistent with what might be necessary to mediate the mechanism we are postulating to exist. It is also interesting that this study was done with an antagonist rather than with an agonist. Binding of an agonist could lead to a conformational change that is distinct or more marked from that observed in this NMR study.

In an attempt to focus further on the shortest sequence responsible for this effect, we used a series of synthetic peptides (Fig. 1) in analogous activity assays (Fig. 2b and Table 1). The endogenous agonist effect was maintained as the peptide was reduced in length from 30 residues to 5, and ultimately, three residues in the region focused on an aspartic acid residue (corresponding to position 49 in the secretin receptor and position 65 of the CRF receptor) that is highly conserved among class B G protein-coupled receptors (Fig. 2b). However, as the peptide was further shortened to a dipeptide, no agonist activity was observed. Because the region of interest corresponds to the region of the CRF receptor that assumes a relatively tight loop structure bounded by one side of a disulfide bond and by an antiparallel β -sheet structure (Grace et al., 2004), we also examined whether a constrained loop would enhance potency (Fig. 2c and Table 1). Indeed, the potency of the endogenous agonist was enhanced by presenting this three-residue sequence as part of a constrained loop using a diaminopropionic acid linker across the ends. In addition, this cyclic peptide maintained full efficacy. It is noteworthy that scrambling the sequence of these three residues eliminated all agonist activity at the secretin receptor (Fig. 2c).

This highly conserved aspartic acid residue in the center of this segment has been demonstrated to be critically important for biological function of some class B receptors. This represents Asp⁶⁰ in the mouse growth hormone-releasing factor receptor, in which a mutation in this residue leads to a dwarf phenotype known as the *little mouse* (Godfrey et al.,

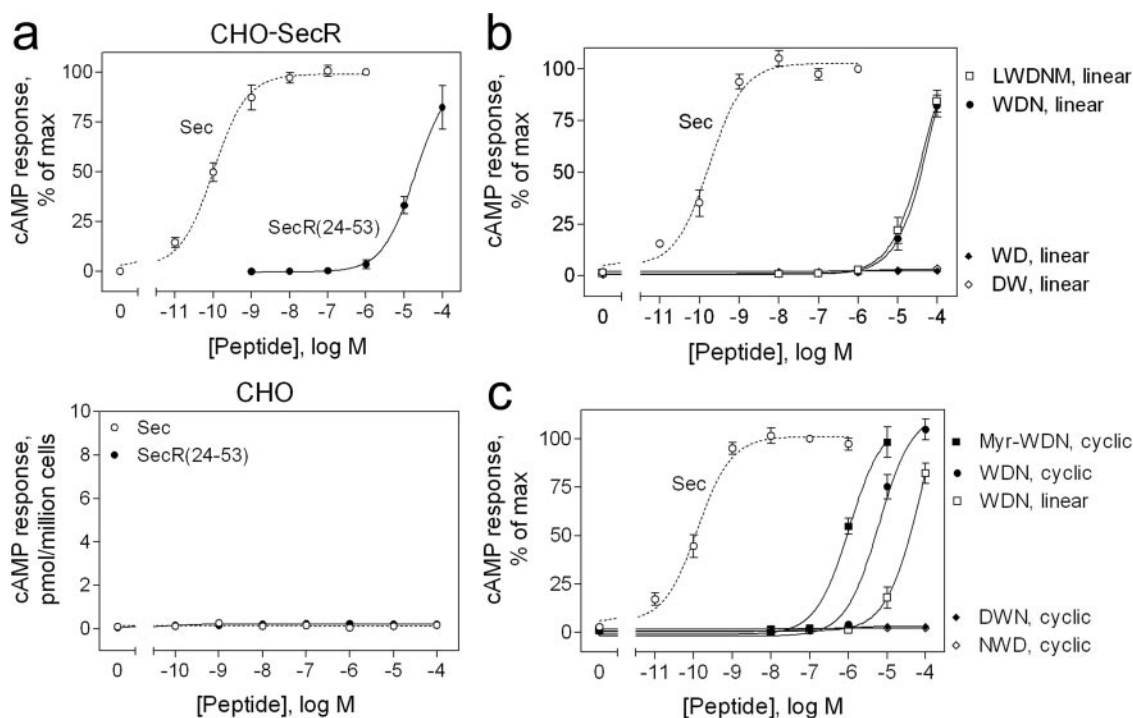


Fig. 2. Biological activities of endogenous secretin receptor peptides. **a**, curves reflecting the abilities of natural secretin and the synthetic peptide representing the region of the secretin receptor amino terminus extending between the first and third conserved cysteine residues, Cys²⁴ and Cys⁵³ [SecR(24–53)], to stimulate cAMP accumulation in secretin receptor-bearing CHO-SecR cells and in the nonreceptor-bearing parental CHO cells. Basal levels of cAMP were 2.6 ± 0.2 pmol/million cells, and maximal levels in response to secretin reached 198 ± 26 pmol/million cells. EC₅₀ values are summarized in Table 1. **b**, the abilities of shorter component sequences of this region of the secretin receptor amino terminus to stimulate cAMP responses in the CHO-SecR cells. Basal levels of cAMP were 3.2 ± 0.4 pmol/million cells, and maximal levels in response to secretin reached 188 ± 30 pmol/million cells. EC₅₀ values are summarized in Table 1. **c**, the effect of cyclization of the critical tripeptide on its agonist activity and the effect of fatty acid acylation on this activity. Basal levels of cAMP were 3.0 ± 0.3 pmol/million cells, and maximal levels reached 202 ± 35 pmol/million cells. EC₅₀ values are summarized in Table 1. The two scrambled sequences had no detectable agonist activity. Values represent means \pm S.E.M. of data from a minimum of three independent assays performed in duplicate. Myr, myristoylated cyclic tripeptide.

1993; Lin et al., 1993). A similar disruptive effect on function has been demonstrated for the VPAC1 receptor in an analogous mutagenesis experiment (Couvineau et al., 1995). However, mutation of the analogous Asp⁴⁹ of the secretin receptor to a glycine residue had little negative effect on the receptor biological activity (data not shown). Therefore, we focused on the highly conserved residue Trp⁴⁸ adjacent to this aspartic acid residue. Indeed, mutation of Trp⁴⁸ to a leucine residue resulted in a secretin receptor having a markedly impaired response to its natural agonist ligand (Fig. 3). However, this mutant receptor responded with full efficacy to stimulation with the endogenous secretin receptor peptide (Fig. 3).

It is noteworthy that the analogous aspartic acid residue (Asp⁶⁵) within the CRF receptor, another receptor in this family, is reported to be involved in an intradomain salt bridge with basic residue Arg¹⁰¹ based on the NMR structure determined for its isolated amino terminus (residues 39–133) (Grace et al., 2004). Although it may be unlikely that such a bond would be broken by a conformational change induced by ligand binding, it is possible that this bond is not present in the natural conformation of the fully intact receptor that includes both amino-terminal and helical bundle domains.

The potency of the cyclic peptide was further enhanced by fatty acid acylation (myristoylation) to make it more hydrophobic and to direct it to the membrane, the presumed site of action (Fig. 2c and Table 1). To better establish the mechanism of action of this peptide, we used photoaffinity labeling, modifying the cyclic peptide with a photolabile benzoyl-phenylalanine and a radioiodinatable tyrosine residue on each end. Both photoprobes maintained the biological activity of the cyclic peptide (Fig. 4a and Table 1). Both were able to covalently label the HA-tagged secretin receptor in a saturable and specific manner, as demonstrated in Fig. 4b, left, in which competing unlabeled WDN peptide eliminated the covalent labeling of the 70-kDa receptor band. This figure also provides a good approximation of the binding affinity of the labeled cyclic peptide based on the concentration-dependent

competitive inhibition of the covalent labeling of the receptor by the WDN peptide (IC_{50} of $0.9 \pm 0.4 \mu\text{M}$). Figure 4b, right, illustrates that this same autoradiographic band was present after immunoprecipitation of the solubilized labeled membrane proteins using anti-HA antibody, with this similarly eliminated by competition with HA peptide. Figure 4b, right, also shows that deglycosylation of this labeled band using endoglycosidase F yielded the expected mass of approximately 42 kDa of the core receptor protein, further confirming the identity of the photoaffinity-labeled product (Fig. 4b).

Identification of the site of covalent attachment of the probes using cyanogen bromide cleavage of the labeled native and deglycosylated receptor bands yielded a mass of approximately 7 kDa (Fig. 4c). Based on the nonglycosylated nature and apparent mass of the cyanogen bromide fragments of this receptor covalently bound to this ligand (1154 Da), the most likely candidate represented the fragment extending from the third intracellular loop, through transmembrane segment 6, to the third extracellular loop. Using a receptor mutant constructed previously in which the isoleucine residue in position 334 within this cyanogen bromide fragment was changed to a methionine to produce an additional site of cyanogen bromide cleavage (Dong et al., 2004a), a clear shift in the electrophoretic migration of the labeled fragment was observed (Fig. 4d). This provides definitive evidence for the region of covalent labeling with both probes to be the decapeptide at the top of transmembrane segment 6 and the loop, extending between residues 335 and 344.

These data provide a mechanism for the endogenous ligand within the receptor amino terminus to exert leverage or tension on the region of the receptor that needs to undergo a conformational change to lead to coupling with a G protein. This theme has similarities with that of the class A G protein-coupled protease-activated receptors in which a proteolytic cleavage by the ligand exposes an endogenous tethered ligand (Nystedt et al., 1994; Lerner et al., 1996). The secretin receptor theme seems to achieve the same result via conformational change without an irreversible modification such as proteolysis. This has important implications for receptor resensitization and regulation. There are also similarities between the molecular mechanism being proposed here and the mechanism proposed for the glycoprotein hormone receptors, in which hormone recognition and activation are accomplished by separate domains of the receptor. Hormone binding to the receptor amino terminus has been postulated to switch that domain from a tethered inverse agonist to a full agonist of the helical bundle domain of the receptor (Vassart et al., 2004).

To examine how broadly applicable the proposed molecular mechanism might be for the activation of other members of

TABLE 1
Stimulation of cAMP in CHO-SecR cells

| Peptides | cAMP Stimulation EC_{50} |
|-----------------|-----------------------------|
| Secretin | $139 \pm 20 \text{ pM}$ |
| SecR (24–53) | $24 \pm 5 \mu\text{M}$ |
| LWDNM, linear | $32 \pm 3.8 \mu\text{M}$ |
| WDN, linear | $55 \pm 16 \mu\text{M}$ |
| WDN, cyclic | $5.9 \pm 1.2 \mu\text{M}$ |
| Myr-WDN, cyclic | $1.1 \pm 0.3 \mu\text{M}$ |
| WDN-Bpa | $7.9 \pm 2.0 \mu\text{M}^a$ |
| Bpa-WDN | $8.8 \pm 2.1 \mu\text{M}^a$ |
| VPAC1(51–71) | $4.1 \pm 1.6 \mu\text{M}$ |

^a EC_{40} values were used for these compounds because limited solubilities precluded the curves reaching 50% of maximal levels of cAMP with achievable concentrations.

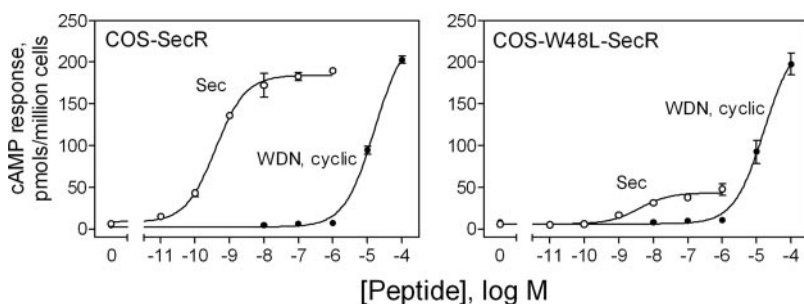


Fig. 3. Biological activity of the endogenous secretin receptor peptide at functionally impaired mutant secretin receptor. Shown are biological activity curves for secretin and the endogenous secretin receptor peptide acting at wild-type (left) and W48L (right) secretin receptor transiently expressed in COS cells. EC_{50} values: COS-SecR, Sec, $395 \pm 48 \text{ pM}$; WDN, cyclic, $15.8 \pm 2.0 \mu\text{M}$; COS-W48L-SecR, WDN, cyclic, $16.4 \pm 3.1 \mu\text{M}$. Values represent means \pm S.E.M. of data from a minimum of three independent assays performed in duplicate.

this physiologically and pharmacologically important class B family of G protein-coupled receptors, we tested the shortest endogenous secretin peptide possessing agonist activity (the WDN peptide) at two other family members, the VPAC1 and calcitonin receptors. Because of the low-potency agonist re-

sponses, it was critical to work with cell lines expressing high concentrations of receptor to be able to demonstrate agonist activity in these assays. Data in Fig. 5 show that the WDN peptide was also active at the VPAC1 receptor in which this sequence is conserved within its amino terminus, whereas it

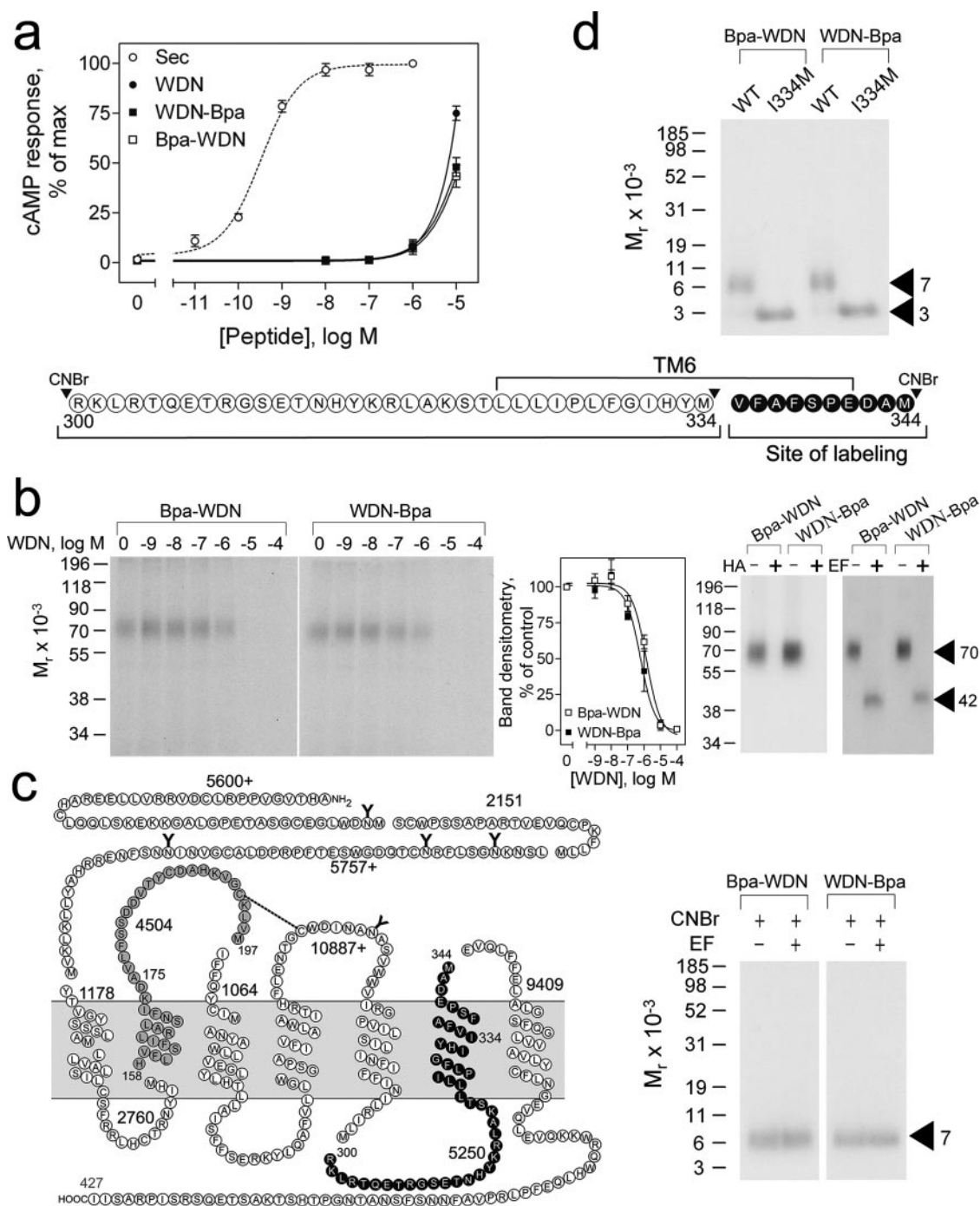


Fig. 4. Localization of the site of action of the endogenous secretin receptor peptide. **a**, the abilities of photolabile analogs of the secretin receptor peptide (WDN-Bpa represents the cyclic WDN peptide with Bpa-Tyr on its carboxyl terminus; Bpa-WDN represents the cyclic WDN peptide with Tyr-Bpa on its amino terminus) to stimulate cAMP activity in CHO-SecR cells. Basal levels of cAMP were 3.3 ± 0.4 pmol/million cells, and maximal levels in response to secretin reached 211 ± 43 pmol/million cells. Values represent means \pm S.E.M. of data from three independent assays performed in duplicate. EC_{50} values are summarized in Table 1. **b**, the ability of these radioiodinated photoprobes to covalently label the HA-tagged secretin receptor. Shown are autoradiographs of photoaffinity labeling of CHO-SecR membranes in the absence or presence of competing concentrations of WDN cyclic peptide (left), densitometric analyses of such receptor competition labeling performed in three similar experiments (middle), proteins immunoprecipitated with anti-HA antibody in the absence or presence of competing HA peptide (right), and the migration of the labeled band after deglycosylation with endoglycosidase F (EF) (right). **c**, the migration of the labeled band after CNBr cleavage. **d**, analogous CNBr reaction performed with the I334M mutant secretin receptor and the sequence of the relevant receptor fragment.

was not active at the calcitonin receptor in which a WDG sequence replaces the WDN found in the secretin and VPAC1 receptors (sequences of the amino terminal regions of interest of these receptors shown in Fig. 1). The peptide representing the analogous broad region of the amino terminus of the VPAC1 receptor was also tested for agonist activity at the three receptors in this family (Fig. 5). Despite having unrelated sequences outside of the WDN segment, the VPAC1 peptide had full agonist activity at the secretin receptor and efficacy similar to that of the WDN peptide at the VPAC1 receptor. It is noteworthy that, like the WDN peptide, it had

no agonist activity at the less closely related calcitonin receptor. The only endogenous amino terminal peptide found to have agonist activity at the calcitonin receptor in these assays was its own endogenous peptide (Fig. 5). Taken together, these data support a general mechanism of action focused on the region of the tryptophan-aspartic acid sequence within the amino terminus of these receptors.

These observations provide new and important insights that support a unique molecular mechanism of action for class B G protein-coupled receptors. In addition, and perhaps most importantly, this provides small-molecule leads for the development of agonists acting at receptors in this family, which includes high-priority targets for drug development. These data also provide insights into a relevant site of docking for small-molecule agonists acting at receptors within this family. With these insights and given the current abilities for high-throughput modification of a lead compound and for rational refinement of drug candidates, it is likely that such leads can be used to develop more potent and highly useful drugs.

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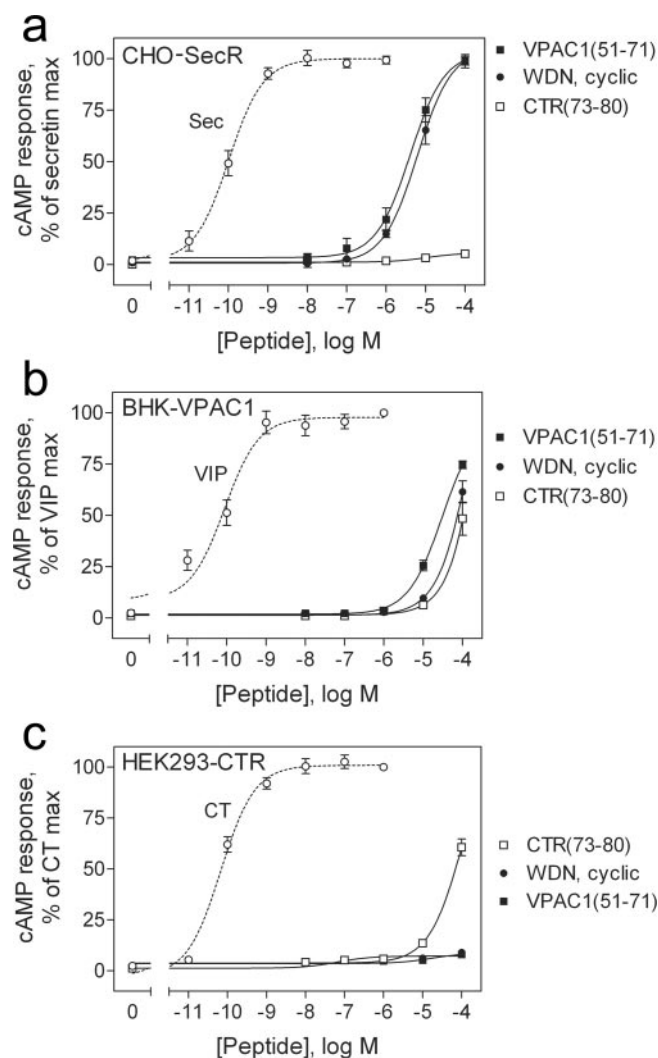


Fig. 5. Generalizability and cross-reactivity of endogenous agonist peptides in other class B G protein-coupled receptors. Shown are biological activity curves for the WDN tripeptide and analogous amino-terminal peptides from the VPAC1 (residue 51–71) and calcitonin (residues 73–80) receptors acting at the secretin (a), VPAC1 (b), and calcitonin (c) receptors. Each of the endogenous secretin receptor and VPAC1 receptor peptides exhibited agonist activity at both receptors (a and b) but had no activity at the calcitonin receptor (c). In addition, the endogenous calcitonin receptor peptide had no agonist activity at the secretin receptor (a) but was active at the VPAC1 receptor (b). Absolute cAMP values (in picomoles per million cells): CHO-SecR (a), basal, 3.5 ± 0.6 , maximal in response to secretin, 217 ± 39 ; BHK-VPAC1 (b), basal, 2.3 ± 1.0 , maximal in response to VIP, 249 ± 55 . HEK293-CTR (c), basal, 25 ± 6 , maximal in response to calcitonin, 237 ± 44 . EC_{50} values: CHO-SecR (a), Sec, 139 ± 20 pM; VPAC1(51–71), 4.1 ± 1.6 μ M; WDN, cyclic, 5.9 ± 1.2 μ M. BHK-VPAC1 (b), VIP, 78 ± 11 pM; VPAC1(51–71), 32 ± 2 μ M; WDN, cyclic, 71 ± 10 μ M; CTR(73–80), 101 ± 12 μ M. HEK293-CTR (c), CT, 71 ± 8 pM; CTR(73–80), 72 ± 9 μ M.

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