

**Themed Section: Secretin Family (Class B) G Protein-Coupled Receptors –
from Molecular to Clinical Perspectives****REVIEW**

Ligand binding and activation of the secretin receptor, a prototypic family B G protein-coupled receptor

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The secretin receptor is a prototypic member of family B G protein-coupled receptors that binds and responds to a linear 27-residue peptide natural ligand. The carboxyl-terminal region of this peptide assumes a helical conformation that occupies the peptide-binding cleft within the structurally complex disulphide-bonded amino-terminal domain of this receptor. The amino terminus of secretin is directed toward the core helical bundle domain of this receptor that seems to be structurally distinct from the analogous region of family A G protein-coupled receptors. This amino-terminal region of secretin is critical for its biological activity, to stimulate Gs coupling and the agonist-induced cAMP response. While the natural peptide ligand is known to span the two key receptor domains, with multiple residue-residue approximation constraints well established, the orientation of the receptor amino terminus relative to the receptor core helical bundle domain is still unclear. Fluorescence studies have established that the mid-region and carboxyl-terminal end of secretin are protected by the receptor peptide-binding cleft and the amino terminus of secretin is most exposed to the aqueous milieu as it is directed toward the receptor core, with the mid-region of the peptide becoming more exposed upon receptor activation. Like other family B peptide hormone receptors, the secretin receptor is constitutively present in a structurally specific homo-dimeric complex built around the lipid-exposed face of transmembrane segment four. This complex is important for facilitating G protein association and achieving the high affinity state of this receptor.

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Abbreviations

BRET, bioluminescence resonance energy transfer; CRF, corticotrophin-releasing factor; GHRH, growth hormone releasing hormone; GIP, glucose-dependent insulinotropic polypeptide; GLP, glucagon-like peptide; GPCR, G protein-coupled receptor; GppNHp, guanosine-5'-(β -imino)triphosphate; KI, potassium iodide; PACAP, pituitary adenylate cyclase-activating peptide; PTH, parathyroid hormone; RAMP, receptor activity-modifying protein; TM, transmembrane segment; VIP, vasoactive intestinal polypeptide

Secretin and its receptor hold special places in history, with secretin representing the first hormone and giving rise to the field of endocrinology (Bayliss and Starling, 1902), and with the secretin receptor representing the first member of the B family of guanine nucleotide-binding protein (G protein)-coupled receptors (GPCRs) to be cloned (Ishihara *et al.*, 1991). This family includes receptors for vasoactive intestinal polypeptide (VIP), pituitary adenylate cyclase-activating peptide (PACAP), glucagon, glucagon-like peptide (GLP),

glucose-dependent insulinotropic polypeptide (GIP), calcitonin, calcitonin gene-related peptide, parathyroid hormone (PTH), corticotrophin-releasing factor (CRF) and growth hormone releasing hormone (GHRH) (Ulrich *et al.*, 1998; Mayo *et al.*, 2003), many of which represent potentially very important drug targets. Potential applications span a broad spectrum that includes therapeutics for diabetes mellitus, bone disease, pain, inflammation, migraine, anxiety, depression, short bowel syndrome and even neoplastic disease.

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All of the natural agonist ligands for receptors in this family are moderate length peptide hormones that have a diffuse pharmacophoric domain, with critical determinants spread throughout the length of the peptide (Ulrich *et al.*, 1998). While family B GPCRs share the predicted structural motif including transmembrane heptahelical bundle conformation and G protein coupling as the proximal effector event for all other GPCRs, they are structurally distinct, not sharing any of the signature sequences typical of family A GPCRs and being predicted to have a structurally distinct helical bundle conformation (Frimurer and Bywater, 1999; Fredriksson *et al.*, 2003; Foord *et al.*, 2005). The themes that have been established for secretin and its receptor have been consistent for other natural ligands and peptide hormone receptors in this family (Gardella *et al.*, 1994; Holtmann *et al.*, 1995; 1996; Di Paolo *et al.*, 1998; 1999; Solano *et al.*, 2001; Al-Sabah and Donnelly, 2003; Mann *et al.*, 2007). These will be developed in this review.

Secretin family peptides

The natural ligands for the peptide hormone-binding group of family B GPCRs are peptides with 25 or more residues that seem to have substantial tendency to form helical conformations, particularly at their carboxyl-terminal regions (Dong and Miller, 2002). Structure-activity series including alanine-replacement strategies have supported the importance of residues spread throughout the length of these peptides, with many residues at the amino terminus exhibiting structural specificity for high affinity binding and biological activity and with residues spaced every three or four positions in the mid-region and carboxyl terminus exhibiting importance for binding affinity (Figure 1) (Adelhorst *et al.*, 1994; Nicole *et al.*, 2000; Igarashi *et al.*, 2002a,b). This has contributed to the impression that the carboxyl-terminal region of these peptides contributes mainly to binding, with one face of a helical conformation being most important, while the amino-terminal region contributes to both binding and biological activity (Dong *et al.*, 2011). Of note, helix N-capping motifs have been identified in many of these peptides (represented by residues six, seven and ten of many of the peptides, with phenylalanine, threonine and tyrosine in those positions in

many of the natural hormonal ligands), presumably stabilizing the extended helical conformations in the mid-region and carboxyl-terminal region of these ligands (Neumann *et al.*, 2008). In calcitonin, a disulphide bond between cysteine residues in positions one and seven seems to play a similar role to the helix N-capping motif (Neumann *et al.*, 2008). Other interesting experimental approaches have stabilized the helical region using constraints such as lactams, effectively improving ligand binding affinities (Ahn *et al.*, 2001a; Taylor *et al.*, 2002; Willick *et al.*, 2004; Murage *et al.*, 2008).

Much less is understood about the conformation of the amino-terminal regions of these peptide ligands than about their helical carboxyl-terminal regions. The amino-terminal regions of these ligands have not been solved in the co-crystallization studies with receptor amino-terminal domains (Parthier *et al.*, 2007; Pioszak *et al.*, 2008; Pioszak and Xu, 2008; Runge *et al.*, 2008; Underwood *et al.*, 2010). They are clearly not constrained or stabilized in those structures. There is one important report in which the transfer Nuclear Overhauser effect nuclear magnetic resonance approach was successfully utilized to determine the receptor-bound conformation of PACAP as bound to its receptor (Inooka *et al.*, 2001). While that approach did not provide insights into the conformation of that receptor or even what portion of that receptor might contribute to peptide ligand docking, it does provide our only clear insight into the conformation of the amino terminus of the receptor-bound ligand. Of interest, this seems to assume a conformation fully consistent with the disulphide-constrained calcitonin and with the presence of a helix N-capping motif.

Consistent with this general theme for the functional importance of different regions of the natural ligands for many members of this family, truncation of the amino terminus of the peptides results in reduced or absent biological activity and can yield receptor antagonists (Robberecht *et al.*, 1976; Turner *et al.*, 1986; Pozvek *et al.*, 1997; Ahn *et al.*, 2001b). Series of studies with modification of peptide and receptor structures, including powerful chimeric constructs, have predicted that the carboxyl-terminal regions of the peptide ligands interact with the receptor amino-terminal domain, while the amino-terminal region of the peptides interact with the receptor core helical bundle domain (Al-Sabah and Donnelly, 2003; Gardella *et al.*, 1994; Holtmann

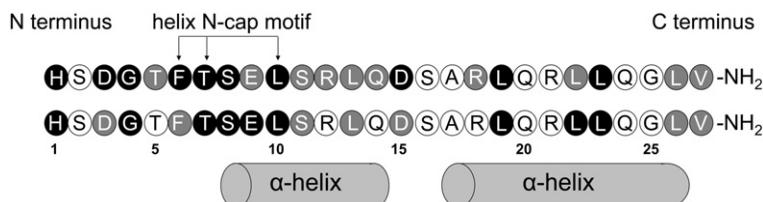


Figure 1

Secretin. Shown is the amino acid sequence of rat secretin from the amino terminus on the left to the carboxyl-terminal amide on the right. The position of the helix N-capping motif and the helical components (in solution) are also labelled. Each of the residues in secretin was replaced with an alanine residue, with the resulting peptide studied for its binding affinity and ability to stimulate a cAMP response from secretin receptor-bearing cells (Dong *et al.*, 2011). Residues in which alanine replacement had major effects (>100-fold reduction in binding affinity (top sequence) and/or biological activity (bottom sequence)) are shown as black-filled circles; those with intermediate effects (>10-fold and <100-fold) are shown as gray-filled circles; those with minimal or no effects (<10-fold) are shown as open circles. This provides a graphic display of the distribution of functionally important residues.

et al., 1996; Di Paolo et al., 1998; Runge et al., 2003; Mann et al., 2007). Further, many other types of studies, including photoaffinity labelling (Bisello et al., 1998; Dong et al., 2004a; Dong et al., 2004b) and co-crystallization of peptide ligands with the soluble receptor amino-terminal domain, support this prediction (Parthier et al., 2007; Pioszak et al., 2008; Pioszak and Xu, 2008; Runge et al., 2008; Underwood et al., 2010).

Structure of family B GPCRs

Primary sequence analysis has clearly identified a series of seven segments of 18 or more relatively hydrophobic residues, thought to represent transmembrane helices contributing to an intramembranous helical bundle. Of note, the signature sequences found in the large and well-characterized family A GPCRs, are completely absent in the family B GPCRs. Theoretical analysis of these segments, noting specifically conserved residues and positions of charged residues, has resulted in predictions of a helical bundle quite distinct from that of the family A GPCRs (Fredriksson et al., 2003; Foord et al., 2005; Frimurer and Bywater, 1999). While we now have several examples of crystal structures of family A GPCRs (Cherezov et al., 2007; Jaakola et al., 2008; Scheerer et al., 2008; Warne et al., 2008; Chien et al., 2010; Wu et al., 2010; Rosenbaum et al., 2011), and we have a relatively clear understanding of that structure, no such information yet exists for family B GPCRs. Using computational predictive techniques, such as 'cold spot' analysis, transmembrane helical bundle structures for family B GPCRs have been proposed, recognizing that these represent general structures to be tested.

A major recent advance in our insights into structure of family B GPCRs has come from the ability to directly solve structures of soluble amino-terminal domains of several members of this family that bind peptide hormones (Grace et al., 2004; 2007; Parthier et al., 2007; Pioszak et al., 2008; Koth et al., 2010; Pioszak and Xu, 2008; Sun et al., 2007; Runge et al., 2008; ter Haar et al., 2010; Underwood et al., 2010). This is an extremely important domain of these receptors that contributes critical determinants of ligand binding (Miller et al., 2007; Parthier et al., 2009). It also includes many of the signature sequences for family B GPCRs, including the six conserved cysteine residues predicted to contribute to conserved functionally important intradomain disulphide bonds (Miller et al., 2007; Parthier et al., 2009). This new insight has been accomplished using nuclear magnetic resonance for the CRF2 β receptor, the PACAP receptor and the calcitonin receptor-like receptor (CLR)-receptor activity-modifying protein-1 (RAMP-1) complex, as well as more recent crystal structures for the GIP, GLP-1, PTH and CRF1, and the CLR-RAMP1 complex (Grace et al., 2004; 2007; Parthier et al., 2007; Pioszak et al., 2008; Koth et al., 2010; Pioszak and Xu, 2008; Sun et al., 2007; Runge et al., 2008; ter Haar et al., 2010; Underwood et al., 2010). This has resulted in confirmation of the disulphide bond pattern that had been predicted (Lisenbee et al., 2005). This structure includes a highly conserved core that includes two anti-parallel β -sheets, three disulphide bonds, and variable amino-terminal α -helix and multiple loop regions (Parthier et al., 2009). The structure also includes a conserved hydrophobic

peptide-binding cleft above the stable core and between the helix and loop regions (Parthier et al., 2009).

With several of these structures including associated peptide ligands, the prediction of a peptide-binding cleft has also been realized (Grace et al., 2004; 2007; Parthier et al., 2007; Pioszak et al., 2008; Koth et al., 2010; Pioszak and Xu, 2008; Sun et al., 2007; Runge et al., 2008; ter Haar et al., 2010; Underwood et al., 2010). However, while these structures have been highly consistent, there has been less consistency in the details of the docking of the peptide ligands within these structures. This raises concern whether the hydrophobic nature of the binding cleft has resulted in non-physiological association with the hydrophobic face of the helical peptides in these complexes. Also, none of these structures have included any direct experimental insights into the orientation of the receptor amino terminus relative to the receptor core helical bundle domain. The orientation of these domains has been predicted in several of these structures; however, they have been markedly divergent and inconsistent. At this point, this relative orientation of domains must be considered as currently unknown.

The other aspect of receptor structure that must also be considered unknown is the conformation of loop regions, including both extracellular and intracellular loops. Some structure has been predicted based on nuclear magnetic resonance analysis of lipid-linked loop sequences (Pellegrini et al., 1998; Piserchio et al., 2000), but no confirmation of such structures is yet available. In fact, the ends of the intramembranous helical segments are not definitively mapped either.

Much of the current understanding of the structure of family B GPCRs has come from computational studies and the application of techniques like photoaffinity labelling that has defined spatial approximations between distinct residues within the docked ligand and residues within the receptor (Dong et al., 2004b; 2010; Chen et al., 2010). For this technique to be most meaningful, the ligand probe that includes a photolabile site of covalent attachment and a radiolabel to track through the subsequent purification must be fully biologically active. This ensures its positioning similar to the natural ligand on which it is based. The best such probes are also high affinity ligands and are efficient in establishing a covalent bond with a single distinct receptor residue. We have successfully developed and applied nine such probes that have incorporated a photolabile moiety into positions between six and 26 (Dong et al., 1999a,b; 2000; 2002; 2003; 2007; Zang et al., 2003). We have also developed amino-terminal probes with photolabile moieties in positions five, one, minus one and minus two (Dong et al., 2004a; 2008). The latter probes were developed because it was difficult to maintain high affinity binding and agonist activity with incorporation of a photolabile benzoyl-phenylalanine residue at the critical amino terminus. Of note, all of the mid-region and carboxyl-terminal probes labelled only the receptor amino terminus (Dong et al., 1999b; 2000; 2002; 2007; Zang et al., 2003). The sites of covalent attachment are described in Table 1. Only the amino-terminal probes labelled residues within the receptor core helical bundle domain (Dong et al., 2004a; 2008). This provided the first clue that the natural peptide ligand binding site was largely accommodated within the receptor amino terminus, with only the ligand amino terminus extending toward the receptor core (Figure 2).

Table 1

Photoaffinity labelling constraints determined for the rat secretin receptor

Secretin: site of photolabile residue	Secretin receptor residue labelled	Labelled receptor domain
His ¹	Phe ³³⁸	EL3
Thr ⁵	Phe ³⁴⁹	EL3
Phe ⁶	Val ⁴	NT
Arg ¹²	Val ⁶	NT
Leu ¹³	Val ¹⁰³	NT
Ser ¹⁶	Leu ⁹⁹	NT
Arg ¹⁸	Arg ¹⁴	NT
Arg ²¹	Arg ¹⁵	NT
Leu ²²	Leu ¹⁷	NT
Leu ²³	Arg ²¹	NT
Leu ²⁶	Leu ³⁶	NT

EL3, extracellular loop 3; NT, amino-terminal tail.

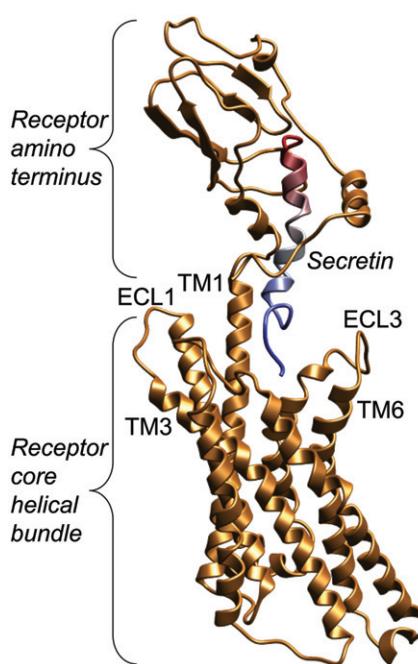
This pattern of photoaffinity labelling is shared by several members of family B GPCRs (Bisello *et al.*, 1998; Dong *et al.*, 2004a,b; Chen *et al.*, 2010). This continues to support a common theme for docking natural peptide ligands for these receptors. However, despite the extensive residue-residue constraint data and the high resolution structures of the receptor amino terminus that include several of these peptides, this still does not provide adequate constraint to resolve the orientation of these major receptor structural domains. This likely reflects the high degree of flexibility of these long linear peptides and the flexibility of the receptor loop domains, with the receptor amino terminus linked to the receptor core only through the peptide backbone, with the carboxyl terminus of the receptor amino-terminal domain in contiguity with the top of transmembrane segment one.

Fluorescence studies of secretin peptides

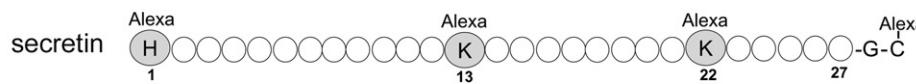
Fluorescence studies can provide unique complementary insights to the types of studies described above. Approaches involving fluorescence have the advantage of providing not only static insights into the microdomain occupied by a fluorophore incorporated into a receptor ligand as it is normally docked at the receptor, but also dynamic insights into the agonist-induced changes in such a micro-environment. Some key insights come from collisional quenching studies using a hydrophilic molecule like potassium iodide (KI) or a hydrophobic molecule like Tempo; this provides information about the accessibility of the fluorophore to the quenching reagent. Steady-state anisotropy provides insights into the freedom of rotational movement of the fluorophore within the probe during fluorescence decay. Time-correlated single photon counting can provide information about the fluorescence lifetime of the fluorophore within the probe.

As in the photoaffinity labelling studies, these studies require a probe that binds and activates the receptor normally in order to provide meaningful data that can be extrapolated to the natural ligand. Indeed, we have developed a series of fluorescent probes that fulfil these criteria. These have involved the positioning of an alexa fluorophore at the amino terminus adjacent to position one, in the mid-region in positions 13 and 22, and at the carboxyl terminus in position 29 (Harikumar *et al.*, 2006a) (Figure 3). All of these probes were full agonists and bound to the secretin receptor saturably and specifically, with reasonably high affinity (Harikumar *et al.*, 2006a). Studies were performed with intact receptor-bearing cells or membranes that had been incubated with the fluorescent ligand probes. Important controls included the concurrent competition with 100-fold molar excess of non-fluorescent secretin and incubations with CHO cells not expressing the secretin receptor. Both of these provided similar low levels of signals that were subtracted from the meaningful signals reported (Harikumar and Miller, 2009).

Fluorescence quenching with hydrophilic KI revealed that the probe with the amino-terminal fluorophore was more accessible than the other three probes. For each of the probes, the fluorophore was quenched more easily when the

**Figure 2**

Three-dimensional molecular model of secretin-occupied secretin receptor. Shown is a working molecular model of the secretin receptor (shown in gold) occupied by natural secretin (coloured blue-to-red from its amino terminus, directed toward the receptor core helical bundle domain, to its carboxyl terminus, occupying the peptide-binding cleft within the receptor amino terminus). Selected transmembrane helices (TM) and extracellular loop regions (ECL) are labelled for orientation.



Fluorescence properties	Receptor conformation	Quenching with KI ($K_q \times 10^9, M^{-1}S^{-1}$)	Lifetime (τ, ns)	Anisotropy	
Quenching with KI ($K_q \times 10^9, M^{-1}S^{-1}$)	Active	++++ (2.5)	++ (1.7)	++ (1.8)	++ (1.3)
	Inactive	+++* (2.1)	+* (0.8)	+* (0.8)	+* (0.9)
	Active	++++ (4.3)	++ (3.9)	++ (3.8)	++++ (4.5)
	Inactive	++++ (4.3)	++++* (4.3)	++++* (4.6)	++++ (4.5)
	Active	++	+++	+++	++
	Inactive	++	++++*	++++*	++

* p<0.05 for inactive state vs active state

Figure 3

Fluorescence analysis using secretin probes bound to the secretin receptor. Shown are the positions of incorporation of fluorescent alexa⁴⁸⁸ into the amino terminus of secretin (adjacent to position 1), the carboxyl terminus of secretin (as a two-residue extension beyond position 27), and in the mid-region of secretin in positions 13 and 22. These probes were utilized in studies of the ability to quench the fluorescence with hydrophilic KI, the fluorescence lifetimes and the fluorescence anisotropy, as indications of the characteristics of the microdomains occupied by each fluorophore in the probes while bound to the secretin receptor (Harikumar *et al.*, 2006a). The G protein-uncoupled low affinity state was achieved by incubation with GppNHp, and significant differences in each of the fluorescence characteristics relative to those when the receptor was in the high affinity state are noted. Fluorescence at the amino terminus of the peptide ligand was more readily quenched than that at any other position. The fluorescence of each of the probes was more readily quenched when the receptor was in its G protein-coupled high affinity state than in its low affinity state. For both lifetimes and anisotropy, only the mid-region probes reflected differences relative to receptor conformation, with the probe fluorescence while the receptor was in its high affinity state exhibiting shorter lifetimes and lower anisotropy than when the receptor was in its low affinity state.

receptor was in its G protein-coupled high affinity state, than when in its lower affinity state induced by incubation with the non-hydrolysable GTP analogue, guanosine-5'-($\beta\gamma$ -imino)triphosphate (GppNHp). Of particular interest, the micro-environments of the two mid-region probes seemed to be the most sensitive to the conformational changes, reflecting the largest changes in response to GppNHp. The fluorescence lifetimes and anisotropy of the probes varied in concert with each other. The two mid-region probes exhibited significant changes in these parameters in response to GppNHp, with the high affinity state exhibiting shorter lifetimes and lower anisotropy to reflect greater rotational dynamics. It was noteworthy that the two end probes exhibited no change in these parameters in the two states. These data support the model of the carboxyl-terminal end of the natural ligand residing in a protected peptide-binding cleft and the amino-terminal end pointing toward the helical bundle, but in a less protected environment (Harikumar *et al.*, 2006a). No analogous data exist yet for other members of this family.

The major change upon secretin receptor coupling with its G protein and assuming its high affinity state seems to be an opening of the peptide-binding cleft, with indicator residues in that position becoming more exposed to the aqueous milieu. Also, of interest, these data do not support substantial penetration of the amino terminus of the natural ligand into the helical bundle in this state; it may be that a more subtle

change in its approximation with a relevant receptor domain is involved.

Quaternary structure of the secretin receptor

In addition to being associated with their heterotrimeric G proteins, GPCRs can associate with other GPCRs and/or with RAMPs to result in complex quaternary structures. Most of the literature examining GPCR oligomerization has been directed toward family C and family A receptors, with the former having strong evidence for functionally critical dimerization that can even represent covalent complexes. The literature for family A GPCRs has been quite confusing and inconsistent, with examples of constitutive oligomerization as well as agonist-stimulated and agonist-disrupted complexes, and examples of the full spectrum of functional impact from absent to substantial (Milligan *et al.*, 2003). No rules have yet been developed to predict the oligomerization behaviour and functional impact of oligomerization for any receptor in the A family of GPCRs.

In contrast, for family B peptide hormone-binding GPCRs, it appears that dimerization will likely be the rule, with this present constitutively, not affected by agonist binding, and facilitating G protein association and achieving the high affinity state (Gao *et al.*, 2009). The oligomerization

status of the secretin receptor has been studied more extensively than any other member of this family. This has been explored systematically to reveal structurally specific and symmetrical secretin receptor homo-dimers aligning along the lipid-exposed face of the fourth transmembrane segment that are critical for achieving the high affinity state of this receptor. These studies have utilized resonance transfer studies, both in static and saturation modes, fluorescence complementation, and establishment of covalent disulphide-bonded complexes (Harikumar *et al.*, 2006b; 2007; 2008a; Gao *et al.*, 2009; Lisenbee and Miller, 2006).

The simplest demonstration of receptor association was performed using bioluminescence resonance energy transfer (BRET) with receptors tagged at their carboxyl terminus with *Renilla* luciferase or with yellow fluorescent protein (Milligan and White, 2001). Here, the secretin receptor was shown to associate with itself in the membrane, yielding a significant BRET signal above background. This signal was independent of the presence of secretin and it was observed morphologically to start during biosynthesis in the intracellular compartments (Lisenbee and Miller, 2006). Controls established that this occurred at receptor concentrations as low as are observed physiologically and that this BRET signal was saturable (Harikumar *et al.*, 2007). In extending these studies to determine if the BRET signal reflected a dimeric complex or a higher order oligomeric complex, bimolecular complementation with receptors tagged at their carboxyl terminus with the amino-terminal or carboxyl-terminal non-fluorescent halves of yellow fluorescent protein was utilized to yield a strong fluorescent signal. However, this was not able to act as effective donor or acceptor with a third tagged secretin receptor, as would be present in a higher order complex (Harikumar *et al.*, 2008a).

Consistent with the homo-dimeric secretin receptor complex, competition studies were utilized to disrupt the complex and only a single region was able to do this, suggesting the presence of a single interface. These studies showed no effect of truncation of the receptor amino terminus or carboxyl terminus, and no effect of mixing with transmembrane segments except for TM4. Indeed, the relevant face of TM4 was shown to represent the lipid-exposed face in modified peptide competition studies, and this was confirmed with mutants of the intact receptor (Harikumar *et al.*, 2007; Gao *et al.*, 2009; Lisenbee and Miller, 2006).

After demonstrating that the lipid-exposed face of TM4 was critical for the secretin receptor dimerization, we were also successful in inducing covalent complexes through siting cysteine residues in particular positions within that interface (Gao *et al.*, 2009). Fourteen successive residues in TM4 were mutated to cysteines, with all except for three helix-facing constructs able to traffick normally to the plasma membrane, where secretin bound and elicited cAMP responses (Gao *et al.*, 2009). Disulphides were then induced by exposure to cuprous phenanthroline. Five constructs with lipid-exposed cysteines in positions 240, 243, 246, 247 and 250 formed covalent dimers, with three of these (243, 247 and 250) stabilized in the high affinity state (Gao *et al.*, 2009). This provided constraints to begin to model the dimeric state of the secretin receptor (Gao *et al.*, 2009).

Of note, every other member of family B peptide hormone-binding GPCRs has been shown to be capable of

forming hetero-dimers with the secretin receptor, while family A GPCRs tested were not (Harikumar *et al.*, 2006b; 2008b). In the few cases that have been studied, the TM4 interface for other members of this receptor family seems to be consistent (Harikumar *et al.*, 2007; 2010).

The dimeric state of the secretin receptor has also been shown to be responsible for the negative cooperativity observed for this receptor (Gao *et al.*, 2009). All indications suggest the structural symmetry of these dimers, yet negative cooperativity suggests an asymmetry with one protomer able to bind secretin with higher affinity than the second protomer. In another important series of studies (Dong *et al.*, 2010), probes having two photolabile sites of covalent attachment were utilized to induce bonds to divergent regions of the secretin receptor to be certain that the bound peptide was not interacting with both receptor protomers (Dong *et al.*, 2010). Indeed, these studies with six such pairs of dual probes confirmed that the peptide was only occupying a single protomer (Dong *et al.*, 2010). The basis for the negative cooperativity is still unknown, but may reflect steric hindrance of the two large extracellular amino-terminal domains or selective coupling to the heterotrimeric G protein. The structural basis for the latter and the stoichiometry of receptor to G protein has not yet been established for this receptor family.

RAMPs are single transmembrane proteins that have been described to interact with several members of family B peptide hormone-binding GPCRs (Hay *et al.*, 2006). There are three RAMPs that share approximately 30% amino acid homology and that selectively interact with receptors. These were first identified when they were found to be responsible for the translocation of the CLR from the biosynthetic intracellular organelles to the plasma membrane, where the complex bound and responded to calcitonin gene-related peptide. This was mediated by RAMP-1 association with this receptor, while its association with RAMP-2 and RAMP-3 resulted in adrenomedullin receptor phenotypes. Similarly, RAMP association with the calcitonin receptor is responsible for its amylin receptor phenotype. Recently, the secretin receptor was shown to be capable of associating with RAMP-3 (Harikumar *et al.*, 2009). In this work, the RAMP-3 construct was translocated to the plasma membrane when co-expressed with the secretin receptor. Competition studies demonstrated that transmembrane segments TM6 and TM7 were capable of disrupting this translocation, suggesting that they contribute to the determinants of this interaction. This is clearly a distinct interface from that involved in establishing receptor homo-dimers. No clear function could be attributed to the secretin receptor-RAMP-3 complex (Harikumar *et al.*, 2009).

Conclusion

The secretin receptor is prototypic of family B peptide hormone-binding GPCRs, following consistent themes of structure and function. It is clear that this family is distinct from the more extensively studied family A receptors, with predicted differences in the conformation of its helical bundle and in the two-step process of natural ligand binding involving the peptide-binding cleft within the disulphide-bonded receptor amino terminus and extension of the peptide toward the core helical bundle domain. Fluorescence

studies suggest that the critical receptor amino-terminal domain undergoes a conformational change upon agonist binding, with this resulting in an opening of the peptide-binding cleft and some movement of the peptide ligand toward its effector domain within the receptor core. The specific structural basis for the activation event and even the relative orientation of the two key receptor domains has yet to be determined. Of note, the secretin receptor is present as a structurally specific homo-dimer in the plasma membrane, where this quaternary complex is critical to facilitate G protein association and the high affinity state of the receptor. This complex is also key for the negative cooperativity observed for this signalling system.

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Conflicts of interest

The authors have no conflicts of interest and no financial links with manufacturers of reagents or ligands relevant to this work.

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