

REVIEW

Structural and functional insights into the juxtamembranous amino-terminal tail and extracellular loop regions of class B GPCRs

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Class B guanine nucleotide-binding protein GPCRs share heptahelical topology and signalling via coupling with heterotrimeric G proteins typical of the entire superfamily of GPCRs. However, they also exhibit substantial structural differences from the more extensively studied class A GPCRs. Even their helical bundle region, most conserved across the superfamily, is predicted to differ from that of class A GPCRs. Much is now known about the conserved structure of the amino-terminal domain of class B GPCRs, coming from isolated NMR and crystal structures, but the orientation of that domain relative to the helical bundle is unknown, and even less is understood about the conformations of the juxtamembranous amino-terminal tail or of the extracellular loops linking the transmembrane segments. We now review what is known about the structure and function of these regions of class B GPCRs. This comes from indirect analysis of structure–function relationships elucidated by mutagenesis and/or ligand modification and from the more direct analysis of spatial approximation coming from photoaffinity labelling and cysteine trapping studies. Also reviewed are the limited studies of structure of some of these regions. No dominant theme was recognized for the structures or functional roles of distinct regions of these juxtamembranous portions of the class B GPCRs. Therefore, it is likely that a variety of molecular strategies can be engaged for docking of agonist ligands and for initiation of conformational changes in these receptors that would be expected to converge to a common molecular mechanism for activation of intracellular signalling cascades.

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Abbreviations

Bpa, benzoyl-phenylalanine; CALCRL, calcitonin receptor-like receptor; CGRP, calcitonin gene-related peptide; CRF, corticotropin-releasing factor; CT, calcitonin; ECL, extracellular loop; GHRH, growth hormone-releasing hormone; GIP, glucose-dependent insulintropic peptide; GLP-1, glucagon-like peptide-1; NTD, amino-terminal domain; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related protein; RAMP, receptor activity-modifying protein; TM, transmembrane segment; VIP, vasoactive intestinal polypeptide; VPAC₁ or VPAC₂, type 1 or 2 receptor for VIP

Guanine nucleotide-binding protein GPCRs are the largest group of cell membrane receptors in the genome, all sharing heptahelical transmembrane topology with extracellular amino-terminal tail, intracellular carboxyl-terminal tail and loops linking the transmembrane segments (TM), as well as

propensity to couple at their cytosolic face with heterotrimeric G proteins as a prominent proximal effector (Ji *et al.*, 1998). This superfamily has been divided into families based largely on patterns of sequence homology, most evident in their TMs (Ji *et al.*, 1998). To date, crystal structures have been solved

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only for intact receptors in the class A rhodopsin/ β -adrenergic GPCR family (Granier and Kobilka, 2012). These have demonstrated a high degree of similarity for their helical bundles, with typical conformational changes observed between inactive and active structures (Rasmussen *et al.*, 2011). The extracellular tail and loop (ECL) regions in these structures have been notably varied, sometimes closing over an intrahelical ligand and having diverse conformations that have been poorly predicted prior to the crystallization (Stevens *et al.*, 2013). The second extracellular loop (ECL2) region of the class A GPCRs that have been crystallized to date provides a striking example of this variation. This loop in rhodopsin includes two β -sheets forming a β -hairpin that dips into the transmembrane bundle to act as a lid above the chromophore (Palczewski *et al.*, 2000), while this loop in the catecholamine receptors (β_1 -adrenergic, β_2 -adrenergic and dopamine D_3 receptors) has an α -helix that moves it away from the ligand-binding pocket (Wheatley *et al.*, 2012). Of further interest, the peptide receptors cloned to date (CXCR4, μ -opioid, κ -opioid, δ -opioid and neurotensin receptors) also have β -hairpin structures, but in different orientation from rhodopsin, which may help to guide the natural ligands into their normal pocket (Wu *et al.*, 2010; Granier *et al.*, 2012; White *et al.*, 2012).

The class B GPCRs are a relatively small group of receptors for secretin, vasoactive intestinal polypeptide (VIP), glucagon, glucagon-like peptides (GLP), glucose-dependent insulinotropic peptide (GIP), growth hormone-releasing hormone (GHRH), calcitonin (CT), calcitonin gene-related peptide (CGRP), corticotropin-releasing factor (CRF), parathyroid hormone (PTH) and parathyroid hormone-related peptides (PTHrP; Mayo *et al.*, 2003). Members of this group have been proposed as targets for many clinically important disorders, including the management of diabetes, obesity, bone disease, headache, pain and stress (Mayo *et al.*, 2003). The natural ligands for these receptors are all moderate length peptides ranging from 27 to 44 residues, with structure-activity studies demonstrating diffuse pharmacophoric domains (Dong and Miller, 2002). Structural studies demonstrate the propensity for these peptides to form α -helical conformations in membrane-mimicking solvents (Dong and Miller, 2002). A major advance in our understanding of the structure of the class B receptors has come from the ability to determine NMR and crystal structures for their extracellular amino-terminal domains (NTDs; Parthier *et al.*, 2009). These exhibit a highly conserved structural motif including two sets of antiparallel β -sheets with connecting loop regions, three conserved intra-domain disulfide bonds, and a variable amino-terminal α -helical segment. This domain provides a cleft with a hydrophobic base to accommodate the carboxyl-terminal portion of the peptide ligands in their helical conformation (Parthier *et al.*, 2009).

Structure-activity studies of the natural peptide ligands of this family demonstrate that their amino terminus is critical for biological activity, with amino-terminal truncation producing antagonists for most class B GPCRs (Pozvek *et al.*, 1997). A two-domain model has evolved for peptide interaction with these receptors, with the peptide carboxyl terminus interacting with the receptor amino terminus and the peptide amino terminus interacting with what has been called the 'junctional region' of the receptors; this includes the top of the helical bundle and loop domains (Hoare, 2005). However,

there is only limited structural information available to define these interactions, and building molecular models from solved crystal structures of class A receptors is problematic. The prediction has been made, based on primary sequence analysis, that the helical bundle of the class B GPCRs differs substantially from that of the class A GPCRs (Donnelly, 1997; Frimurer and Bywater, 1999; Fredriksson *et al.*, 2003; Foord *et al.*, 2005). Furthermore, there are no clear data that define the orientation of the receptor NTD relative to the helical bundle of these receptors. Multiple diverse orientations have been proposed (Miller and Dong, 2013), but the only real constraint comes from the contiguity of the receptor's peptide backbone linking the carboxyl-terminal end of the receptor amino terminus and the top of its first TM (TM1). It is noteworthy that a conserved hydrophilic network of polar interactions has been proposed to be present within the transmembrane helical bundle of the class B GPCRs (Wootten *et al.*, 2013) that may be important for transition from inactive to active state, as well as for ligand-biased and pathway-biased signalling responses. A full understanding of the intact receptor complex should provide key insights into the molecular mechanisms for regulating this network, such as might be modulated by direct interaction with portions of the ligand, as well as portions of the base of the receptor NTD. The conformations of both of these are likely affected by the docking of the ligand within the peptide-binding cleft within the receptor amino terminus.

In this review, we have collated the published data on the structure and functional significance of the extracellular juxtamembranous regions of the class B GPCRs, more specifically, the three ECLs and the amino-terminal extension of TM1 that connects this with the portion of the receptor amino terminus having known conserved structure. This includes structure-activity data from studies involving receptor mutagenesis, chimeric constructs, photoaffinity labelling, cysteine trapping and structural determinations of isolated components. We have attempted to identify conserved themes that might provide useful insights for the development of small-molecule agonists targeting this group of receptors.

Sequences of natural peptide ligands and juxtamembranous regions of class B receptors

Figures 1 and 2 show the alignments of the sequences of the human representatives of each of the natural peptide ligands (Figure 1) and their receptors (Figure 2) in the class B family of GPCRs, with these ordered as recently proposed by Watkins *et al.* (2012). This groups them into the secretin, CGRP, corticoliberin and PTH subgroups that reflect structural similarities between their natural ligands, positions of structural motifs, such as helix N-capping motifs within the ligands (Neumann *et al.*, 2008) and similarities in associated proteins, such as receptor activity-modifying proteins (RAMPs; Watkins *et al.*, 2012). The GPCR nomenclature used in this review conforms to that preferred by this journal (Alexander *et al.*, 2013).

Natural peptide ligands for the secretin subgroup have a highly conserved sequence at their amino terminus followed

Peptide subgroups

Secretin	1	5	10	15	20	25			
Secretin	HSDG	FTSEL	RLREGAR	LQR	LLQGLV	-----			
VIP	HSDA	VTDNY	RLRKQMA	VKKY	ILNS	ILN	-----		
GLP-1	HAEGET	FTSDVS	SYLEGQA	AKEFI	AWLV	KGR	-----		
Glucagon	HSQGT	FTSDYS	SKYLD	SRRAQ	DFVQW	LMNT	-----		
GIP	YAEGT	FISDYS	IAMD	KIHQ	QDFV	NWLLAQKGGK	NDWKHNITQ--		
GHRH	YADA	IF	TNSYK	KVLG	QQLS	ARKLLQD	IMSRQQGESNQERGARARL		
			N-cap						
CGRP	1	5	10	15	20	25	30		
CT	-CGNLS	TC	MLGTYT	QDFN	KFHTFP	QTAIV	GVGAP----		
CGRP	AC-DT	A	TCV	THRL	AGLLS	RSGGV	VKNNFVPTNVGSKAF		
			N-cap						
Corticotiberin	1	5	10	15	20	25	30	35	40
CRF	SEEPPIS	LDL	TFHLL	REV	LEM	ARAE	QLAQQA	HSNRKL	MEII
			N-cap						
PTH	1	5	10	15	20	25	30	35	
PTH	SV	SE	IQLMHN	LK	GH	LN	S		
PTHrP	AV	SE	HQLL	HD	KG	KS	IQDL	RRRFFL	HHLIAEIH

Figure 1

Sequence alignments of natural ligands of human class B GPCRs. These ligands are organized into four subgroups (Watkins *et al.*, 2012). Conserved residues in the subgroups are shown in blue and homologous ones are shown in orange. The N-capping motifs typical of each of the first three subgroups are shown in red boxes and the cysteine residues forming a disulfide bond in the CGRP subgroup are linked via a green line.

by a helix N-capping motif. The peptides in the CGRP subgroup include a disulfide-bonded amino terminus and a distinct N-capping motif, with all of these receptors in this subgroup known to associate with RAMPs in manner affecting their function. The peptides in the corticotiberin group still have a recognizable N-capping motif, but the amino terminus of these peptides is quite different from that of the other ligands. The natural peptide ligands for the PTH subgroup are quite different, typically longer and having no sequence homology with the natural ligands of the other subgroups.

The length of the juxtamembranous amino-terminal region joining the top of TM1 and the most distal, conserved intradomain, disulfide-bonded cysteine within the receptor amino terminus ranges from 15 to 21 residues, with the shortest length in the CGRP subgroup of receptors and the longest length in the secretin subgroup of receptors. This shorter length and sequence homology in the subgroup in which RAMP association is critical may suggest that this region contributes to the functional response of the interaction with this group of receptor-associated proteins. Of the three ECL domains, the longest loop is ECL1, ECL2 is intermediate in length and ECL3 is the shortest loop. The length of ECL1 ranges from 18 to 43 residues, with this loop by far the longest in the PTH subgroup, with the other subgroups being relatively similar in length and sequence homology. Of note, an alternative spliceform (C1b) of the rat CT receptor has been described in which an additional 37 amino acid residues were inserted into ECL1, resulting in altered ligand recognition, with reduced affinity for porcine CT and negli-

gible affinity for human CT, while retaining normal binding of salmon CT (Sexton *et al.*, 1993). All of these receptors retain a cysteine residue at the carboxyl-terminal end, just above the top of TM3, which is involved in a disulfide bond that is present in most receptors in the GPCR superfamily. The receptors most related to glucose homeostasis, receptors for GLP-1, glucagon and GIP, have slightly longer ECL1 loops than the other non-PTH receptors. The length of ECL2 is 17 or 18 residues in all members, with highly conserved motifs, shared by all of the subgroups. This includes the position in the centre of this loop of the cysteine residue that is disulfide bonded to the cysteine residue above TM3. ECL3 ranges from 7 to 12 residues, without clear sequence homology. The short length of this loop limits the distance between TM6 and TM7, keeping them spatially close to each other. ECL2 has the most highly conserved sequence of the loops and, like the role it appears to play in the peptide receptors in the class A GPCR family (Wheatley *et al.*, 2012), it may assume a conserved conformation that is key to interact with and/or guide the natural peptide ligands towards their site of action.

Experimental approaches to evaluate structure and function of extracellular juxtamembranous regions of class B GPCRs

It is important to understand the types of insights possible and the limitations of the various experimental approaches that have been undertaken to understand the structure and function of the extracellular juxtamembranous regions of class B GPCRs that have been published to date. Data in the literature for the juxtamembranous amino-terminal region and each of the three ECL regions are shown in Tables 1–4.

Receptor mutagenesis and chimeric construct studies

Site-directed mutagenesis, in which natural amino acid residues are changed to other residues having distinct characteristics, has been performed on juxtamembranous domains in many members of this receptor family. Most often, natural residues have been changed to alanine residues, but there are also examples of scanning mutagenesis with cysteine residues and examples of purposeful changes of charge or character of specific residues. Most of the changes in amino acid sequence have resulted in loss of function, making it difficult to interpret whether the impact of the mutation was direct or indirect via effect on protein folding, even when the modified construct was shown to traffic normally to the cell surface. For some of these studies, complementary changes were also prepared for the ligands; however, these paired changes rarely achieved normal binding and/or signalling, but rather exhibited shifts in the values of these parameters in ranges of affinity and/or potency that were too low to be confident of relevance to normal structures. Some chimeric receptor constructs, in which the analogous region of one receptor was used to replace that region in a related receptor, resulted in gain-of-function, thereby providing a positive observation that supports the interpretation of functional significance of that sequence.

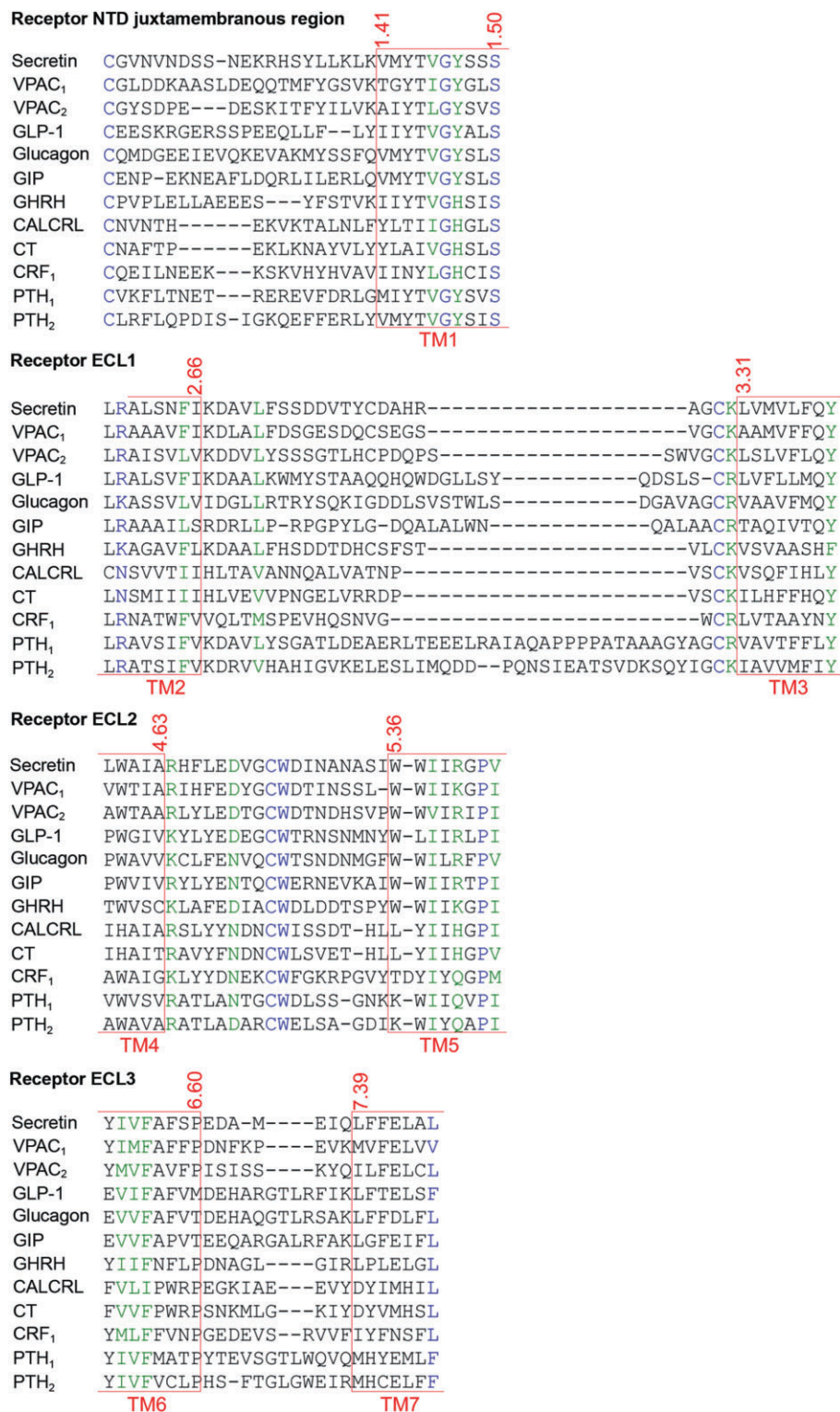


Figure 2

Sequence alignment of the amino-terminal juxtamembranous region and the three ECLs of the human class B GPCR receptors. Conserved residues are shown in blue and homologous ones are shown in green. The proposed boundaries between each region and their adjacent TMs are noted by red lines. The numbering of TM residues in this family analogous to the class A GPCR numbering of Ballesteros reflects that recently reported by Wootten *et al.* (2013).

Table 1

Functionally important residues in the juxtamembranous region of the NTD of class B GPCRs

Proposed functional interaction				
Receptor	Peptide	Receptor regions or residues	Methods	References
Secretin	[Bpa ¹³]sec	Val ¹⁰⁶	Photoaffinity labelling	Zang <i>et al.</i> , 2003
	[Bpa ¹⁶]sec	Leu ⁹⁹	Photoaffinity labelling	Dong <i>et al.</i> , 2010
	[Bpa ²⁰]sec	Pro ⁹⁷	Photoaffinity labelling	Dong <i>et al.</i> , 2011
	Asp ³ of sec	Tyr ¹²⁸	Mutagenesis	Di Paolo <i>et al.</i> , 1999a
VPAC ₁	His ¹ of VIP	Lys ¹⁴³ , Thr ¹⁴⁴ , Thr ¹⁴⁷	Molecular modelling, mutagenesis	Ceraudo <i>et al.</i> , 2012
	[Bpa ⁻¹]VIP	Gln ¹³⁵	Photoaffinity labelling	Ceraudo <i>et al.</i> , 2012
	[Bpa ⁶], [Bpa ²²], [Bpa ²⁴] and [Bpa ²⁸]VIP	Residues between 107 and 135	Photoaffinity labelling, mutagenesis	Tan <i>et al.</i> , 2003; 2004; 2006; Ceraudo <i>et al.</i> , 2008; 2012
	[[Bpa ⁶]PG97-269	Residues between 67 and 108	Photoaffinity labelling, mutagenesis	Ceraudo <i>et al.</i> , 2012
	[Bpa ²⁴]PG97-269	Residues between 121 and 133	Photoaffinity labelling, mutagenesis	Ceraudo <i>et al.</i> , 2012
GLP-1	[Bpa ¹²]GLP1(7–36)	Tyr ¹⁴⁵	Photoaffinity labelling	Chen <i>et al.</i> , 2010
	[Bpa ¹⁶]GLP1(7–36)	Leu ¹⁴¹	Photoaffinity labelling	Miller <i>et al.</i> , 2011
	[Bpa ²⁴]GLP1(7–36)	Glu ¹³³	Photoaffinity labelling	Chen <i>et al.</i> , 2009
	[Bpa ³⁵]GLP1(7–36)	Glu ¹²⁵	Photoaffinity labelling	Chen <i>et al.</i> , 2009
GIP	GIP	TM1	GIP-GLP-1 receptor chimeras	Gelling <i>et al.</i> , 1997
GHRH	Position 12 of GHRH	NTD above TM1 (1–132)	Photoaffinity labelling	Gaylinn, 2002
CT	[Bpa ¹⁶]hCT	Phe ¹³⁷	Photoaffinity labelling	Dong <i>et al.</i> , 2004b
	[Bpa ¹⁹]sCT(1–32); [Bpa ¹⁹]sCT(8–32)	Cys ¹³⁴ -Lys ¹⁴¹	Photoaffinity labelling, mutagenesis	Pham <i>et al.</i> , 2004; 2005
	Glaxo compounds 2d, 2e, 2f and 2 g	Tyr ¹⁵⁰ -Ile ¹⁵³	CT-Secretin receptor chimeras, truncation, deletion and mutagenesis	Dong <i>et al.</i> , 2009
PTH ₁	PTH residue (3–14)	Phe ¹⁸⁴ , Arg ¹⁸⁶ , Leu ¹⁸⁷ , Ile ¹⁹⁰	Mutagenesis	Carter <i>et al.</i> , 1999
	[(BzBz)Lys ¹³]PTH	Arg ¹⁸⁶	Photoaffinity labelling and mutagenesis	Zhou <i>et al.</i> , 1997; Adams <i>et al.</i> , 1998
	Arg ²⁵ , Lys ²⁶ , Lys ²⁷ , Asp ³⁰ and His ³² of PTH	Glu ¹⁷⁷ , Arg ¹⁷⁹ , Arg ¹⁸¹ , Glu ¹⁸² , Asp ¹⁸⁵ , Arg ¹⁸⁶	NMR	Pellegrini <i>et al.</i> , 1998
	[Bpa ¹¹ , ¹⁵ , ¹⁸ or ²¹]PTH	Residues 165–298	Photoaffinity labelling	Wittelsberger <i>et al.</i> , 2006

As shown in Table 1, mutagenesis studies have suggested that the amino-terminal tail region above TM1 contributes to the function of the secretin receptor (Tyr¹²⁸; Di Paolo *et al.*, 1999a), the CT receptor (Tyr¹⁵⁰-Ile¹⁵³; Dong *et al.*, 2009) and the PTH receptor (Phe¹⁸⁴, Arg¹⁸⁶, Leu¹⁸⁷ and Ile¹⁹⁰; Carter *et al.*, 1999). More specific support for the importance of this region comes from receptor-specific gain-of-function that was observed in chimeric GIP-GLP-1 receptor constructs (Gelling *et al.*, 1997). In this work, providing the first 132 residues of the GIP receptor to the chimeric construct yielded high-affinity GIP binding; however, this hormone did not elicit any significant cAMP response. In contrast, extending the GIP receptor component of the chimeric construct by another 19 amino acids, to include three additional residues in the juxtamembranous NTD as well as 16 residues in TM1, yielded 38% of the cAMP response of wild-type receptor-

bearing cells with an EC₅₀ value approximately 30-fold that of the wild-type receptor. This may reflect an effect of either the juxtamembranous region or the top of TM1.

As shown in Table 2, the first ECL region (ECL1) appears prominently in mutagenesis and chimeric receptor studies. As is typical of all groups of GPCRs, including most class A GPCRs, there is a predicted disulfide bond linking a cysteine residue at the carboxyl-terminal end of this loop, near the junction with TM3, with a cysteine residue in ECL2. Disruption of this bond by mutagenesis of either cysteine has been reported to result in loss-of-function of GPCRs (Qi *et al.*, 1997; Vilardaga *et al.*, 1997; Mann *et al.*, 2010). The residues adjacent to the cysteine in ECL1 and at both ends of this loop also appear to be important for receptor function, with mutagenesis of these residues resulting in loss of function. However, mutagenesis has not provided any other consistent

Table 2

Functionally important residues in ECL1 of class B GPCRs

Proposed functional interaction				
Receptor	Peptide	Receptor regions or residues	Methods	References
Secretin	Asp ³ of sec	Lys ¹⁷³	Mutagenesis	Vilardaga <i>et al.</i> , 1996
	Asp ³ of sec	Arg ¹⁶⁶ (TM2); Lys ¹⁷³ , Asp ¹⁷⁴	Mutagenesis	Di Paolo <i>et al.</i> , 1998
		Asp ¹⁷⁴ , Lys ¹⁹⁴	Mutagenesis	Di Paolo <i>et al.</i> , 1999b
		Cys ¹⁸⁶ , Cys ¹⁹³	Mutagenesis	Vilardaga <i>et al.</i> , 1997
	Residues 1–10 of sec	His ¹⁸⁹ -Lys ¹⁹⁰	Secretin-VPAC ₁ receptor chimeras	Holtmann <i>et al.</i> , 1996
VPAC ₁	[N ₃ -Phe ²]sec	Phe ¹⁹⁹	Photoaffinity labelling	Dong <i>et al.</i> , 2011
	Asp ³	Arg ¹⁸⁸ , Lys ¹⁹⁵ (TM2)	Mutagenesis	Solano <i>et al.</i> , 2001
	VIP	Asp ¹⁹⁶	Mutagenesis	Du <i>et al.</i> , 1997
	Peptide histidine isoleucine	Gln ²⁰⁷ , Gly ²¹¹ , Met ²¹⁹ (ECL1/TM3)	Mutagenesis	Couvineau <i>et al.</i> , 1996
	Asp ³	Arg ¹⁷² (TM2)	Mutagenesis	Vertongen <i>et al.</i> , 2001
VPAC ₂	Asp ³	Arg ¹⁷² (TM2)	Mutagenesis	Vertongen <i>et al.</i> , 2001
GLP-1	N-terminal region of GLP1	Asp ¹⁹⁸ , Met ²⁰⁴ , Tyr ²⁰⁵	Mutagenesis	Lopez de Maturana and Donnelly, 2002; Lopez de Maturana <i>et al.</i> , 2004
		Cys ²²⁶	Alanine scanning	Mann <i>et al.</i> , 2010
	Thr ¹⁴ of GLP1	Ile ¹⁹⁶ (TM2); Leu ²³² , Met ²³³ (ECL1)	GLP-1-GIP receptor chimeras, molecular modelling	Moon <i>et al.</i> , 2012
	[Bpa ⁶]GLP1(7–36)	Tyr ²⁰⁵	Photoaffinity labelling	Chen <i>et al.</i> , 2010
	Gln ³	Ile ¹⁹⁴ (TM2)	Glucagon-GLP-1, receptor chimeras, mutagenesis	Perret <i>et al.</i> , 2002; Runge <i>et al.</i> , 2003a,b
Glucagon	Glucagon	ECL1, TM3	Glucagon-GLP-1 receptor chimeras	Buggy <i>et al.</i> , 1995
	C-terminal 17 residues	Arg ²⁰² , residues 206–219,	GLP-1-secretin receptor chimeras, mutagenesis	Unson <i>et al.</i> , 2002
	Glucagon	Residues (197–223)	Cysteine scanning	Roberts <i>et al.</i> , 2011
	GHRH	All ECLs and TMs	GHRH/secretin and GHRH/VPAC ₁ receptor chimeras	DeAlmeida and Mayo, 1998
CALCRL	CGRP	Leu ¹⁹⁵ , Val ¹⁹⁸ , Ala ¹⁹⁹ (top of TM2); Ala ²⁰³ , Ala ²⁰⁶ (ECL1); His ²¹⁹ , Leu ²²⁰ , Leu ²²² (TM3)	Mutagenesis	Barwell <i>et al.</i> , 2011
CRF ₁	CRF, urocortin, sauvagine	Residues 175–178, His ¹⁸⁹	CRF ₁ -CRF ₂ receptor chimeras with point mutations	Liaw <i>et al.</i> , 1997a,b
	Small-molecule antagonist NBI 27914	His ¹⁹⁹ (TM3)	CRF ₁ -CRF ₂ receptor chimeras with point mutations	Liaw <i>et al.</i> , 1997a
	CRF	Cys ¹⁸⁸	Mutagenesis	Qi <i>et al.</i> , 1997
PTH ₁	[Bpa ¹⁹]PTH	Lys ²⁴⁰ (TM2-ECL1)	Photoaffinity labelling, mutagenesis	Gensure <i>et al.</i> , 2003
	[(BzBz)Lys ²⁷]PTH	Leu ²⁶¹	Photoaffinity labelling, mutagenesis	Greenberg <i>et al.</i> , 2000
	Lys ²⁷	Leu ²⁶¹	NMR	Piserchio <i>et al.</i> , 2000
		Asn ¹⁹² (TM2)	PTH ₁ -secretin receptor chimeras, mutagenesis	Turner <i>et al.</i> , 1996
	Arg ¹ and Ser ² of PTH	Arg ²³³ (TM2)	Mutagenesis	Gardella <i>et al.</i> , 1996
PTH ₂	Ile ⁵ of PTH	Ile ²⁴⁴ (TM3)	PTH ₁ -PTH ₂ receptor chimeras, mutagenesis	Turner <i>et al.</i> , 1998

Table 3

Functionally important residues in ECL2 of class B GPCRs

Proposed functional interaction				
Receptor	Peptide	Receptor regions or residues	Methods	References
Secretin	Residues 1–10 of sec	Phe ²⁵⁷ -Leu ²⁵⁸ , Asn ²⁶⁰ -Thr ²⁶¹ Arg ²⁵⁵ Cys ²⁶³	Secretin-VAPC ₁ receptor chimeras Mutagenesis Mutagenesis	Holtmann <i>et al.</i> , 1996 Di Paolo <i>et al.</i> , 1999b Villardaga <i>et al.</i> , 1997
	[Cys ²]sec	Phe ²⁵⁸ , Trp ²⁷⁴	Cysteine trapping	Dong <i>et al.</i> , 2012b
	His ¹	Trp ²⁷⁴	Complementary mutagenesis and molecular modelling	Dong <i>et al.</i> , 2012b
	Asp ³	Asn ²⁶⁸	Complementary mutagenesis and molecular modelling	Dong <i>et al.</i> , 2012b
	Gly ⁴	Phe ²⁵⁸	Complementary mutagenesis and molecular modelling	Dong <i>et al.</i> , 2012b
VPAC ₁		Cys ²⁸⁸	Mutagenesis	Gaudin <i>et al.</i> , 1995; Knudsen <i>et al.</i> , 1997
GLP-1	N-terminal region of GLP1	Lys ²⁸⁸ (TM4)	Mutagenesis	Al-Sabah and Donnelly, 2003
	GLP1, exendin-4, oxyntomodulin	Lys ²⁸⁸ , Glu ²⁹² , Asp ²⁹³ , Cys ²⁹⁶ , Trp ²⁹⁷ , Arg ²⁹⁹ , Asn ³⁰⁰ , Asn ³⁰² , Met ³⁰³ , Asn ³⁰⁴ , Tyr ³⁰⁵	Alanine scanning	Mann <i>et al.</i> , 2010; Koole <i>et al.</i> , 2012a,b
	His ⁷ of GLP1	Asn ³⁰²	GLP-1-GIP receptor chimeras, molecular modelling	Moon <i>et al.</i> , 2012
	[Bpa ²⁰]GLP1(7–36)	Trp ²⁹⁷	Photoaffinity labelling	Miller <i>et al.</i> , 2011
Glucagon	Lys ¹² of glucagon	ECL2	Glucagon-GLP-1 receptor chimeras, mutagenesis	Runge <i>et al.</i> , 2003a,b
GHRH	Glucagon	TM4	Glucagon-GLP-1 chimeras	Buggy <i>et al.</i> , 1995
	GHRH	All ECLs and TMs	GHRH/Secretin and GHRH/VPAC ₁ receptor chimeras	DeAlmeida and Mayo, 1998
CALCRL	CGRP	Arg ²⁷⁴ , Tyr ²⁷⁷ , Tyr ²⁷⁸ , Asp ²⁸⁰ , Cys ²⁸² , Trp ²⁸³ , Ser ²⁸⁵ , Thr ²⁸⁸	Alanine scanning	Conner <i>et al.</i> , 2007; Barwell <i>et al.</i> , 2012
	CGRP	Gly ²⁵⁹ , Trp ²⁶⁰ (TM4); Asn ³⁰⁵ (TM5)	Molecular modelling, mutagenesis	Vohra <i>et al.</i> , 2013
CRF ₁	Bpa ¹ [urocortin(ucn)]	Phe ²⁶⁰ -Met ²⁷⁶	Photoaffinity labelling	Kraetke <i>et al.</i> , 2005
	Bpa ¹² [ucn]			
	Lys ¹⁶ of sauvagine	Lys ²⁵⁷	Photoaffinity labelling	Assil-Kishawi and Abou-Samra, 2002
	Amino-terminal residues 8–10 of sauvagine and corresponding region of CRF	Trp ²⁵⁹ , Phe ²⁶⁰	Mutagenesis	Gkountelias <i>et al.</i> , 2009
	CRF, urocortin, sauvagine	Asp ²⁵⁴ , Val ²⁶⁶ , Tyr ²⁶⁷ , Thr ²⁶⁸	CRF ₁ /CRF ₂ receptor chimeras with point mutations	Liaw <i>et al.</i> , 1997a,b
	Small-molecule antagonist NBI 27914	Met ²⁷⁶ (TM5)	CRF ₁ /CRF ₂ receptor chimeras with point mutations	Liaw <i>et al.</i> , 1997a
	CRF	Cys ²⁵⁸	Mutagenesis	Qi <i>et al.</i> , 1997
	CRF	Asp ²⁶² -Leu ²⁶³ -Val ²⁶⁴ (ECL2) in hCRF2A	Mutagenesis	Dautzenberg <i>et al.</i> , 2002
PTH ₁	Arg ² of PTH	Ser ³⁷⁰ , Val ³⁷¹ (TM5)	rPTH ₁ -opPTH ₁ receptor chimeras, mutagenesis	Gardella <i>et al.</i> , 1994
PTH ₂	[Cys ¹]PTH	Leu ³⁶⁸ (TM5)	Cysteine trapping	Monaghan <i>et al.</i> , 2008
	Ile ⁵ of PTH	Tyr ³¹⁸	PTH ₁ -PTH ₂ chimeras, mutagenesis	Bergwitz <i>et al.</i> , 1997

Table 4

Functionally important residues in ECL3 of class B GPCRs

Proposed functional interaction				
Receptor	Peptide	Receptor residues	Methods	References
Secretin	[Bpa ⁻²]sec	Phe ³³⁶ (ECL3/TM6)	Photoaffinity labelling	Dong <i>et al.</i> , 1999a
	[Bpa ⁻¹]sec	Tyr ³³³ (ECL3/TM6)	Photoaffinity labelling	Dong <i>et al.</i> , 1999a
	[Bpa ¹]sec	Phe ³³⁸ (ECL3/TM6)	Photoaffinity labelling	Dong <i>et al.</i> , 1999a
	[Bpa ⁵]sec	Phe ³⁴⁹	Photoaffinity labelling	Dong <i>et al.</i> , 2008b
	[Cys ²]sec	Phe ³³⁹ , Ser ³⁴⁰ , Pro ³⁴¹	Cysteine trapping	Dong <i>et al.</i> , 2012b
	[Cys ⁵]sec	Ala ³³⁸ , Phe ³³⁹ , Gln ³⁴² , Ile ³⁴⁷ , Gln ³⁴⁸ , Phe ³⁵¹ , Glu ³⁵²	Cysteine trapping	Dong <i>et al.</i> , 2012b
GLP-1	WDN	Val ³³⁵ -Met ³⁴⁴ (ECL3/TM6)	Photoaffinity labelling	Dong <i>et al.</i> , 2006
	NRTFD	Asp ³⁷² -Lys ³⁸³ (ECL3/TM6)	Photoaffinity labelling	Dong <i>et al.</i> , 2012a
	Ser ²	Asp ³⁸⁵ (beginning of TM7)	Glucagon-GLP-1 receptor chimeras, mutagenesis	Runge <i>et al.</i> , 2003a,b
Glucagon	Glucagon	ECL3; TM6	Glucagon-GLP-1 receptor chimeras	Buggy <i>et al.</i> , 1995
	NTD of the receptor (note: not related to natural ligand)	Gln ³⁷⁴ , Ser ³⁷⁹ , Ala ³⁸⁰	Crystal structure, Ab mapping	Koth <i>et al.</i> , 2012
CT	Bpa ⁸ of hCT	Leu ³⁶⁸	Photoaffinity labelling	Dong <i>et al.</i> , 2004c
CALCRL	CGRP	Pro ³⁵³ (TM6)	Molecular modelling, mutagenesis	Vohra <i>et al.</i> , 2013
	CGRP	Leu ³⁵¹ , Glu ³⁵⁷ , Ile ³⁶⁰	Mutagenesis	Barwell <i>et al.</i> , 2011
	Adrenomedullin	ECL3	CALCRL-VPAC ₂ receptor chimeras	Kuwasako <i>et al.</i> , 2012
	CGRP, adrenomedullin	Pro ³²¹ , Pro ³³¹ (TM6)	Mutagenesis	Conner <i>et al.</i> , 2005
GHRH	GHRH	All ECLs and TMs	GHRH/secretin and GHRH/VPAC ₁ receptor chimeras	DeAlmeida and Mayo, 1998
CRF ₁	CRF	Thr ³⁴⁶ , Phe ³⁴⁷ , Asn ³⁴⁸	rCRF-rGlucagon and CRF-hPAC ₁ receptor chimeras, mutagenesis	Sydow <i>et al.</i> , 1999
PTH ₁	Arg ² of PTH	Leu ⁴²⁷ (TM6)	rPTH ₁ -opPTH ₁ receptor chimeras, mutagenesis	Gardella <i>et al.</i> , 1994
	Position 1 and 2 of PTH	Trp ⁴³⁷ , Gln ⁴⁴⁰	Mutagenesis	Lee <i>et al.</i> , 1994; 1995
	Position 1 and 2 of PTH	Gln ⁴⁵¹ (TM7)	Mutagenesis	Gardella <i>et al.</i> , 1996
	[Bpa ¹]PTH	Met ⁴²⁵ (TM6)	Photoaffinity labelling	Bisello <i>et al.</i> , 1998; Behar <i>et al.</i> , 2000
	[Bpa ²]PTH	Met ⁴²⁵ (TM6)	Photoaffinity labelling	Behar <i>et al.</i> , 2000
	[Bpa ¹]PTHrP	Met ⁴²⁵ (TM6)	Photoaffinity labelling	Behar <i>et al.</i> , 2000
	[Bpa ²]PTHrP	Met ⁴²⁵ (TM6)	Photoaffinity labelling	Behar <i>et al.</i> , 2000
	[Cys ¹]PTH	Tyr ⁴²¹ , Phe ⁴²⁴ , Met ⁴²⁵ (TM6)	Cysteine trapping	Monaghan <i>et al.</i> , 2008
	[Bpa ¹]PTH	Met ³⁸⁰ (TM6)	Photoaffinity labelling, mutagenesis	Behar <i>et al.</i> , 1999
PTH ₂	[Bpa ¹]PTH	Met ³⁸⁰ (TM6)	Photoaffinity labelling, mutagenesis	Behar <i>et al.</i> , 1999

themes across the class B receptor family for amino acids within this loop. Of note, the effect of mutagenesis of loop residues may also vary for different peptide ligands binding to the same receptor (Couvineau *et al.*, 1996). When the role of amino acids within this loop of the glucagon receptor was examined systematically by cysteine-scanning mutagenesis, 15 of 27 mutants exhibited loss of function, with the most prominent losses at both ends of this loop (Roberts *et al.*, 2011). In evaluating the accessibility of these cysteines to large hydrophilic cysteine-reactive reagents, there were three periodic patches of residues capable of being derivatized, including positions of Leu¹⁹⁸, Arg²⁰¹, Tyr²⁰², Asp²⁰⁸, Ser²¹³, Ser²¹⁷

and Asp²¹⁸, perhaps reflecting the importance of one face of a helical segment. It was suggested that these residues would also be available for binding to the natural peptide ligand, although that was not directly demonstrated. Of interest, agonist binding was shown to change the accessibility of a cysteine inserted at the amino-terminal end of this loop, in the position of Leu¹⁹⁸.

As shown in Table 3, mutation of ECL2 reveals consistent evidence for the functional importance of this domain across the family, although again, the specific role of individual amino acids often varies from receptor to receptor and for different peptides acting at the same receptor. As noted

above, the importance of the cysteine predicted to be involved in the conserved disulfide bond with the cysteine above TM3 is supported by mutagenesis (Gaudin *et al.*, 1995; Knudsen *et al.*, 1997; Qi *et al.*, 1997; Vilardaga *et al.*, 1997; Conner *et al.*, 2007; Barwell *et al.*, 2012). Many other regions of this loop are also highlighted as important for peptide binding and subsequent downstream signalling in studies of various members of this family (Koole *et al.*, 2012a,b). There have been systematic analyses of each of the residues along this loop, providing insights into regions in which modification of a series of successive residues affects function. This could indicate lack of helical conformation for those regions or could be compatible with ligand-induced changes in conformation and accessibility, such as was described above. Again, the details of importance of specific residues or positions in the loop have not been consistent across the class B GPCRs, including lack of specific themes even in the component subgroups of receptors in this family.

As shown in Table 4, mutagenesis of several members of this family, including glucagon, calcitonin receptor-like receptor (CALCRL), CRF and PTH receptors, supports the functional importance of residues along ECL3, however, the positions of functionally important residues vary from one receptor to another. Residues Trp⁴³⁷ and Gln⁴⁴⁰ in the middle and carboxyl-terminal end of ECL3 of the PTH receptor were of particular interest, with mutations resulting in 9- to 16-fold reductions in PTH(1–34) binding affinity with parallel reductions in biological potency and efficacy, while PTH(3–34) binding was unaffected by these mutations (Lee *et al.*, 1995). This was interpreted to suggest possible sites of interaction with the biologically active amino terminus of this hormone. In contrast, residue Pro³³¹ at the top of TM6 at the amino-terminal end of ECL3, was shown to be important for CGRP binding and activation of CALCRL (Conner *et al.*, 2005). Experience with systematic scanning mutagenesis of all residues within this loop, such as characterizing alanine replacements, has not yet been reported.

Photoaffinity labelling studies

Photoaffinity labelling is a powerful technique in which a photolabile moiety is introduced into a ligand and, upon photolysis, can form a covalent bond with any adjacent residue in the receptor. Using a series of manipulations, such as proteolysis and sequencing, it is possible to define the receptor residue that is covalently labelled, establishing spatial approximation between the two residues. When the photolabile probe used in such studies binds with its receptor with reasonable affinity and efficacy (if studying agonists), the docking of the modified peptide is highly likely to be reflective of the interactions that occur between the receptor and natural peptide, allowing the determined spatial approximations of ligand and peptide residues to guide understanding of ligand–receptor complexes. These data are also included in Tables 1–4.

Two of the extracellular juxtamembranous regions have been labelled with photolabile probes intrinsic to the pharmacophore of natural ligands for several of the class B GPCRs. These are regions at the top or above TM1 and TM6. This direct evidence for spatial proximity of these regions with the ligands for multiple class B receptors is clearly important. It is key that both receptor regions have been labelled using pho-

tolabile probes with sites of attachment at their amino terminus (Bisello *et al.*, 1998; Dong *et al.*, 2004a; Ceraudo *et al.*, 2012), but it is also noteworthy that the same regions have been labelled with probes having sites of attachment in other regions of the peptides, such as their mid-regions (Zang *et al.*, 2003; Dong *et al.*, 2004b; Wittelsberger *et al.*, 2006). Other extracellular juxtamembranous regions, including both ECL1 and ECL2, have also been covalently labelled through selected photolabile ligands for a few receptors, but since this technique has not been applied systematically with ligands for all the receptors or with a broad sampling of positions within many of the ligands, it is less clear how broadly applicable these approximations might be.

The juxtamembranous region around the top of TM1 has been labelled in studies with secretin (Zang *et al.*, 2003; Dong *et al.*, 2010; 2011), VIP (Tan *et al.*, 2003; 2004; 2006; Ceraudo *et al.*, 2008; 2012), GLP-1 (Chen *et al.*, 2009; 2010; Miller *et al.*, 2011), GHRH (Gaylinn, 2002), CT (Dong *et al.*, 2004b,c; Pham *et al.*, 2004; 2005) and PTH (Adams *et al.*, 1998; Wittelsberger *et al.*, 2006). Included in this list are examples in which a focused region of a single receptor was labelled with a series of photolabile analogues of its natural ligand. The region between Asp¹⁰⁷ and Gln¹³⁵ of the type 1 receptor for VIP (VPAC₁) was identified as the site of cross-linking by probes with the photolabile group substituted into positions -1, 6, 22, 24 and 28 (Tan *et al.*, 2003; 2004; 2006; Ceraudo *et al.*, 2008; 2012). The region of the GLP-1 receptor between Glu¹²⁵ and Tyr¹⁴⁵ was also covalently labelled in photoaffinity studies, this time using probes with a photolabile group substituted into positions 12, 16, 24 and 35 (Chen *et al.*, 2009; 2010; Miller *et al.*, 2011). Although it appears that different regions of these two peptide ligands are spatially approximated with analogous regions of their receptors, it is more likely that all of these studies support the importance of this receptor region and that the details of covalent labelling will ultimately require an understanding of the secondary structures of the ligands as docked.

The second site of labelling to appear in multiple studies is ECL3, with the region near the top of TM6 clearly representing a hot spot. These studies include the covalent labelling of this region of class B receptors with photolabile analogues of secretin (Dong *et al.*, 2004a; 2006; 2008b), PTH (Bisello *et al.*, 1998; Behar *et al.*, 1999; 2000) and CT (Dong *et al.*, 2004c). For the secretin receptor, probes with site of covalent attachment in positions -2, -1, 1 and 5, within the biologically active region of this peptide, all labelled this region. Similarly, a series of probes with sites of covalent attachment in positions 1 and 2 within the amino terminus of PTH and PTHrP all labelled this region of the types one and two PTH receptors. Additionally, a CT probe with photolabile residue in position 8 also labelled this region.

The other loop regions were also labelled in selected studies of members of this family. ECL1 of the secretin receptor was covalently labelled using the aryl-azide, azidophenylalanine, in position 2 of secretin, a more reactive chemistry than was applied to the other amino-terminal positions [the benzophenone, benzoyl-phenylalanine (Bpa)] in secretin that labelled the area at the top of TM6 (Dong *et al.*, 2011). This loop was also labelled in the GLP-1 receptor through the amino terminus of GLP-1(7–36), with a Bpa in position 6 (Chen *et al.*, 2010). ECL1 was labelled with probes

of the PTH receptor that had a site of covalent attachment in positions 19 and 27 (Greenberg *et al.*, 2000; Gensure *et al.*, 2003). ECL2 was covalently labelled in the GLP-1 receptor through position 20 of GLP-1(7–36) (Miller *et al.*, 2011). This loop was also labelled in the CRF receptor using position 1 and 12 urocortin probes and position 16 sauvagine probe, again emphasizing the differences observed for the docking and spatial approximations with different peptide ligands (Assil-Kishawi and Abou-Samra, 2002; Kraetke *et al.*, 2005).

Cysteine trapping studies

In cysteine trapping studies, cysteine residues substituted into the ligand and select regions of the receptor form covalent disulfide bonds if they are within approximately 2 Å of each other (S^{γ} – S^{γ} distance 2.04 ± 0.07 Å) and if the side chains are oriented towards each other and able to achieve the correct geometry (C^{β} – S^{γ} – S^{γ} – C^{β} dihedral angle $90 \pm 12^{\circ}$; Dickson and Finlayson, 2009). Since the geometry governing disulfide bond formation is more constraining than those from photoaffinity cross-linking groups, this approach can provide more refined information to guide modelling of receptor–ligand complexes. This approach can be applied extensively to examine the dynamic proximity of receptor and ligand during ligand binding. Before interpretation of data, both the cysteine-incorporated probe and the cysteine-incorporated receptor construct need to be characterized to be certain of biosynthesis, trafficking and proper folding to achieve meaningful data that can be used to refine models of ligand–receptor interaction. Additionally, in these studies, it is critical to include controls to be certain that only the cysteine of interest in a particular study is forming the disulfide bond. To date, this approach has been applied to two members of this family, the PTH receptor (Monaghan *et al.*, 2008) and the secretin receptor (Dong *et al.*, 2012b). For the PTH receptor, cysteines incorporated into the positions of 11 residues at the top of TM5 and 13 residues at the top of TM6 were examined (Monaghan *et al.*, 2008), while it was applied to cysteines incorporated into 61 positions, including residues at the tops of all of the TMs and all residues within all three ECLs of the secretin receptor (Dong *et al.*, 2012b).

Incorporation of a cysteine into position 1 at the amino terminus of PTH resulted in strong disulfide bond formation with cysteine mutants substituted for Tyr⁴²¹ or Phe⁴²⁴ at the top of TM6 of the PTH receptor. For secretin, incorporation of cysteine in positions 2 and 5 resulted in labelling of multiple residues in ECL2 and ECL3. Peptides substituted at position 2 labelled residues in the amino-terminal region of the loops with greater efficiency, while position 5-substituted peptides tended to label carboxyl-terminal regions of the loops with higher efficiency. The labelling of successive residues in distinct regions suggests that these receptor segments do not form helical secondary structure. These spatial constraints have been used to refine evolving molecular models of secretin docking with its receptor (Dong *et al.*, 2012b). Furthermore, testing of the refined model using receptor mutagenesis and complementary modifications within the ligand provide support for receptor–ligand interactions proposed by this model (Dong *et al.*, 2012b).

Structural studies

While there are no existing high-resolution structural studies of an intact class B GPCR, there are structures for fragments of receptors in this family that have been proposed to provide clues to the conformation of these regions in the intact receptor. High-resolution NMR structures have been determined for isolated soluble regions of the PTH receptor (juxtamembranous amino-terminal tail, ECL1 and ECL3; Pellegrini *et al.*, 1998; Piserchio *et al.*, 2000; Mierke *et al.*, 2007) and ECL3 of the glucagon receptor (Koth *et al.*, 2012) with associated 'linkers', representing portions of adjacent TM segments, in the membrane mimetic environment of zwitterionic micelles of dodecylphosphocholine. As an example, a fragment of the PTH receptor including ECL1 (residue 241 through 285) exhibited three α -helices, with the first and third helices corresponding to the tops of TM2 and TM3, and the middle helix corresponding to residues 256 to 264. This was predicted to associate with the membrane. How this might relate to the structure of this region in the intact receptor is not yet established. Unfortunately, this approach has not been amenable to determining structures of natural peptide ligands associated with these receptor fragments.

Consolidated view of structure and function of extracellular juxtamembranous regions of class B GPCRs

While there are data to support the functional importance of each of the extracellular juxtamembranous regions of the class B GPCRs that are the focus of this review, our insights into the structures of these regions continue to be limited. Although we now recognize a highly conserved structural motif for the amino-terminal tail of these receptors (Parthier *et al.*, 2009) and there are structures proposed for the helical bundle region of these receptors (Wootten *et al.*, 2013), the structures of the portions between these domains and even how the domains are oriented relative to each other remain poorly understood. These regions are prime candidates to contribute to the junctional complex, referred to as the site of action of the biologically active amino terminus of the natural agonist ligands of these receptors. There are compelling data to support each of the extracellular juxtamembranous regions as being spatially adjacent, under certain conditions, with the amino terminus of a natural ligand in this family. It is possible that each subfamily or even each receptor in the class B GPCR group will follow a theme unique to that receptor or receptor group, and possibly even unique to a given ligand acting at that receptor. It is conceivable that a relatively open extracellular end of the helical bundle could accommodate a wide variety of molecular structures that could induce the conformational changes associated with receptor activation. This may help to explain the challenges that have been experienced by pharmaceutical companies in developing small-molecule agonists for receptors in this family that possess high affinity and potency. The diversity of structures of small-molecule agonist ligands that have been successfully developed for one of these receptors,

the GLP-1 receptor (Koole *et al.*, 2013), suggest that many solutions to this problem will be possible.

Search for consistent themes in subgroups of class B GPCRs

While no clear themes have yet emerged for the interaction of the class B GPCR ligands with the juxtamembranous regions of their receptors, an attempt was made to consolidate what is known about the functional importance of these regions for each of the class B GPCR subfamilies.

Secretin receptor subgroup

The natural ligands of the secretin receptor subgroup share a high degree of sequence homology in their distal amino terminus, just before a conserved N-capping motif. This homology in the biologically active region of these ligands may support a consistent theme for interaction with the junctional region of their receptors.

For the secretin receptor, mutagenesis and chimeric receptor studies have shown the importance of the juxtamembranous region of the amino terminus and contributions of ECL1 and ECL2 for peptide ligand binding and/or function (Tables 1–3). The direct application of photoaffinity labelling has established spatial approximations between residues in multiple positions along the secretin ligand and residues within its receptor (Figure 3). Secretin probes incorporating a

photolabile residue [benzoylbenzoyl lysine ((BzBz)Lys) or Bpa or N₃-Phe] into the carboxyl-terminal half (positions 18, 20, 21, 22, 23, 25 and 26; Dong *et al.*, 1999b; 2000; 2002; 2007; 2011), mid-region (positions 12, 14 and 15; Dong *et al.*, 2003; 2011) and the amino-terminal half (position 6; Dong *et al.*, 1999a) of the peptide all labelled the first 40 residues of the amino terminus of the receptor (after the predicted signal peptide), supporting the importance of this domain for ligand binding. Of note, two mid-region probes with photolabile residues in position 13 and 16, and a carboxyl-terminal probe with a Bpa in position 24 labelled receptor residues Val¹⁰³, Leu⁹⁹ and Pro⁹⁷ (Zang *et al.*, 2003; Dong *et al.*, 2010; 2011), respectively, representing sites still within the receptor amino terminus, as it approaches the top of TM1 (Table 1). Importantly, secretin probes incorporating a photolabile residue in position 1, 2 and 5 labelled extracellular juxtamembranous regions of the receptor, with position 1 and 5 probes labelling residues Phe³³⁸ and Phe³⁴⁹ at the top of TM6 and within ECL3, respectively (Table 4; Dong *et al.*, 2004a; 2008b), and the position 2 probe labelling residue Phe¹⁹⁹ at the carboxyl-terminal end of ECL1 (Table 2; Dong *et al.*, 2011). The top of TM6 is also the target of a short Trp-Asp-Asn (WDN) peptide agonist (Table 4; Dong *et al.*, 2006), further suggesting the possibility of drug interactions with this region of the receptor to activate it. Acetylation of the amino-terminal photolabile probes to neutralize the charge of the primary amino group in each probe resulted in sites of labelling shifting to the distal amino terminus from the top of TM6 (Dong *et al.*, 2005), suggesting proximity of these two

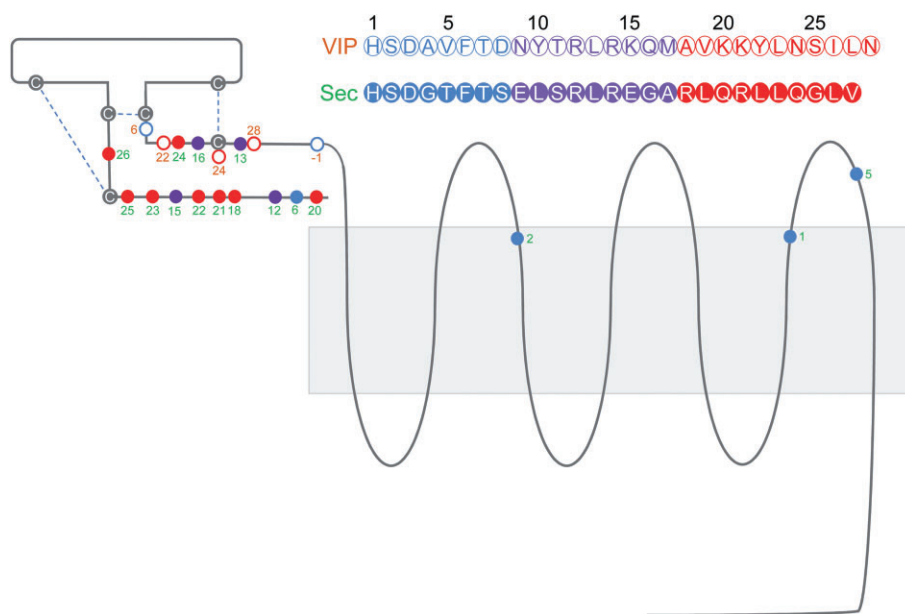


Figure 3

Comparison of patterns of receptor photoaffinity labelling class B GPCRs using probes based on natural ligands, VIP (VPAC₁ receptor) and secretin (secretin receptor). These two structurally related probes, which also can bind to both structurally related receptors, have been shown to covalently label their respective receptors with characteristic patterns. Shown are amino acid sequences of the natural peptides and a schematic diagram of a prototypic class B GPCR, with its disulfide-bonded amino terminus off to the side, and the heptahelical core of the receptor shown with typical topology. Peptides are coloured blue-to-red, from amino terminus to carboxyl terminus, with the VIP residues and their sites of labelling the VPAC₁ receptor shown in open circles, and secretin residues and their sites of labelling the secretin receptor shown in solid circles. Each site of labelling is identified with the position of the photolabile group within the peptide (VIP in orange and secretin in green).

regions. The cysteine trapping efforts described above have also demonstrated spatial approximation between positions 2 and 5 within the biologically active end of secretin and multiple residues within ECL2 and ECL3 of the receptor (Tables 3 and 4; Dong *et al.*, 2012b). Combining with molecular modelling and complementary mutagenesis, this approach has also identified molecular approximations between positions 1, 3 and 4 of secretin and Trp²⁷⁴, Asn²⁶⁸, Phe²⁵⁸ within ECL2 of the receptor respectively (Table 1; Dong *et al.*, 2012b).

Mutagenesis studies of the VPAC₁ receptor have demonstrated an important role of ECL1 for the binding and activity of peptide agonists, VIP and peptide histidine isoleucine (Table 1). In contrast to the mutagenesis data for this receptor, photoaffinity labelling using VIP probes to more directly determine interactions between ligand and receptor, using VIP analogues with photolabile Bpa in positions -1, 6, 22, 24 and 28, all labelled receptor residues in the segment between Asp¹⁰⁷ and Gln¹³⁵, within the juxtamembranous amino-terminal region (Tan *et al.*, 2003; 2004; 2006; Ceraudo *et al.*, 2008; 2012). This pattern of covalent labelling again emphasizes the importance of the receptor amino terminus in peptide docking, while suggesting possible differences in the approximations with the peptide amino terminus (Figure 3). It is interesting that these ligands and receptors are so closely related to each other that both receptors are able to bind both ligands (albeit with different affinities) and each ligand is able to fully activate both receptors (Dickson and Finlayson, 2009). This may provide an example of distinct agonists of a single receptor utilizing differential molecular strategies to form different interactions with the receptor that ultimately yield similar biological responses. Additionally, structurally related agonist and antagonist peptide probes either dock differently or induce different conformations of the receptor, as illustrated by data for photoaffinity labelling through positions 6 and 24 of the PG97-269 VPAC₁ antagonist that establish covalent bonds with the same juxtamembranous amino-terminal region of the receptor as the analogous agonist probes, while the position -1 antagonist probe labelled receptor residue Gly⁶² within the amino terminus, distant in primary structure from the juxtamembranous region labelled by the analogous agonist probe (Ceraudo *et al.*, 2012).

For the GLP-1 receptor, the juxtamembranous amino-terminal region and the first and second ECL regions have all been identified as being important for ligand binding and receptor activation (Tables 1–3). Photoaffinity labelling with GLP-1 probes incorporating a Bpa in the peptide mid-region (position 12 and 16; Chen *et al.*, 2010; Miller *et al.*, 2011) and its carboxyl-terminal half (position 24 and 35; Chen *et al.*, 2009) all labelled the juxtamembranous amino-terminal region of the receptor (Table 1), while probes with sites of cross-linking at the peptide amino terminus (position 6) labelled a residue in ECL1 (Table 2; Chen *et al.*, 2010) and another mid-region (position 20) probe labelled residues in ECL2 (Table 3; Miller *et al.*, 2011). Systematic alanine scanning of ECL2 has demonstrated a critical role for this region in receptor activation by peptide agonists, and in determining signalling pathway-specific effects of these agonists (Table 3; Koole *et al.*, 2012a,b). Interestingly, the actions of a small-molecule agonist of this receptor were not affected by the ECL2 mutations (Koole *et al.*, 2012b), suggesting a dis-

tinct mechanism of action for allosteric agonist interaction and receptor activation. In contrast, the pentapeptide agonist, Asn-Arg-Thr-Phe-Asp (NRTFD) (Dong *et al.*, 2008a; 2012a), labelled ECL3, but it is not clear how the site of action of that molecule might relate to other small-molecule agonists of the GLP-1 receptor (Willard and Sloop, 2012).

For the other receptors in this subgroup, the data are less extensive, but generally consistent with the observations described above. A recent study has suggested that the surface of the glucagon receptor amino terminus that faces the helical bundle may interact with ECL3 of that receptor (Koth *et al.*, 2012), adding another level of complexity to the analysis of these studies. In that work, a monoclonal antibody that is known to act via binding the amino terminus and that displays inverse agonist action, had its inverse agonist activity, but not its binding affinity, disrupted by mutation of residues within ECL3. It is not yet established whether such an interaction exists for other members of this subfamily or even the class B GPCR family more generally.

CGRP receptor subgroup

The natural ligands for the receptors in the CGRP subgroup also share sequence homology and an N-capping motif, both of which are distinct from those in the secretin subgroup (Figure 1). Another characteristic feature of the CGRP receptor subgroup is their functionally important association with RAMPs, often defining the receptor-binding phenotype (McLatchie *et al.*, 1998). The RAMPs are a family of three single transmembrane proteins that can associate with and regulate the trafficking of some class B GPCRs to the cell surface where they can change the ligand binding and signalling properties. While a number of class B GPCRs have been shown to be capable of binding RAMPs, the functional impact of such associations is most profound in the members of the CGRP subfamily. RAMPs are required for CALCRL to traffic to the cell surface, where the phenotype is dependent on the specific RAMP protein associated. RAMP1/CALCRL is the CGRP receptor, while RAMP2/CALCRL and RAMP3/CALCRL exhibit distinct adrenomedullin receptor characteristics. RAMP association with the CT receptor yields distinct amylin receptor characteristics as well. Co-crystal structures of the NTD of CALCRL and its associated RAMP have clearly demonstrated a drug-binding pocket nestled between these structures that utilizes the juxtamembranous amino-terminal region above TM1 (ter Haar *et al.*, 2010; Koth *et al.*, 2010).

For the CT receptor, photoaffinity labelling has identified the juxtamembranous amino-terminal region and ECL3 as proximate to the bound ligand, while mutagenesis has also highlighted the functional importance of the amino-terminal region of this receptor (Table 1). Photoaffinity labelling with mid-region CT position 16 and 19 probes has demonstrated labelling of receptor residues within the juxtamembranous amino-terminal region between Cys¹³⁴ and Lys¹⁴¹ (Table 1; Dong *et al.*, 2004b; Pham *et al.*, 2004), while labelling with a position 26 probe identified Thr³⁰ near the amino terminal end of the receptor (Dong *et al.*, 2004b). Similar to the labelling of the secretin receptor, the amino-terminal position 8 CT probe labelled receptor residue Leu³⁶⁸ within ECL3 (Table 4; Dong *et al.*, 2004c). Of note, the amino-terminally truncated position 19 CT antagonist probe labelled the same region (Cys¹³⁴-Lys¹⁴¹) as did the analogous full length agonist

probe (Pham *et al.*, 2005), whereas the truncated position 8 antagonist probe labelled Met⁴⁹ near the amino terminal end of the CT receptor (Pham *et al.*, 2005). The differences in sites of labelling observed between the two position 8 probes can be explained by a relatively small change in peptide orientation, assuming these regions are close to each other in space. Interestingly, the juxtamembranous amino-terminal region (the segment extending from Tyr¹⁵⁰ to Ile¹⁵³) has also been proposed to contribute to a small-molecule agonist binding pocket for the CT receptor (Dong *et al.*, 2009). Site-directed mutagenesis and chimeric receptor studies of the CALCR1 identified all three loop regions as functionally important for peptide interaction and receptor activation (Kraetke *et al.*, 2005; Conner *et al.*, 2007; Barwell *et al.*, 2011; 2012).

Corticoliberin receptor subgroup

The peptide ligands for the corticoliberin subgroup are different from the previous two groups and may exhibit distinct themes for interaction with the junctional region and for stimulating biological activity. Like other subgroups of class B receptors, mutagenesis and chimeric receptor studies have identified areas of importance in all three ECLs for this subgroup. Extensive mutagenesis of the CRF₁ receptor has identified residues within all three ECLs that are important for binding peptide ligands, CRF, urocortin and sauvagine, as well as the small-molecule antagonist, NBI 27914 (Tables 2–4). Limited photoaffinity labelling studies have demonstrated spatial approximation with ECL2. Photoaffinity labelling studies using probes incorporated into position -1 and 12 of urocortin (Kraetke *et al.*, 2005) and position 16 of sauvagine (Assil-Kishawi and Abou-Samra, 2002) each labelled amino acids in the region between Lys²⁵⁷ and Met²⁷⁶ within ECL2 of this receptor (Table 3).

PTH receptor subgroup

The natural ligands for the receptors in the PTH subgroup are quite distinct from all of those for the other subgroups, being longer and having no sequence homology, while the receptors in this subgroup exhibit all of the signature sequences typical of the other class B GPCRs. For the PTH receptor subgroup, mutagenesis and chimeric receptor studies identified regions within the juxtamembranous amino terminus as the major determinant for natural ligand binding and action, with all three loops also making contributions. Photoaffinity labelling using photolabile PTH probes with Bpa in the carboxyl-terminal half (positions 22, 27, 28 and 33; Gensure *et al.*, 2001) labelled residues within the extracellular NTD, whereas probes with Bpa in the mid-region (positions 11, 13, 15, 18 and 21; Adams *et al.*, 1998; Wittelsberger *et al.*, 2006) labelled the amino-terminal juxtamembranous region of its receptor. PTH probes with Bpa in amino-terminal positions 1 and 2 labelled a single residue Met⁴²⁵ located within the top of TM6 (Bisello *et al.*, 1998; Behar *et al.*, 2000). Cysteine trapping analysis confirmed spatial approximations between ligand position 1 and the residues surrounding Met⁴²⁵, as well as spatial approximation with Leu³⁶⁸ at the top of TM5 (Monaghan *et al.*, 2008). These data generally support the proposed two domain mechanism for binding and activation of the PTH receptor, although they also emphasize the functional contributions of multiple regions within the junctional

region. It is also of interest that focused cysteine trapping studies demonstrated changes in the labelled residues only after agonist occupation, and not after antagonist occupation, consistent with conformational changes associated with receptor activation.

Summary and conclusions

The juxtamembranous amino-terminal tail region and all three ECLs of class B GPCRs contribute to their function. These regions likely make important contributions to the structurally poorly defined junctional region that has been considered to be the target of the biologically active amino terminus of natural ligands for the class B GPCRs in the two domain hypothesis. There seems to be a diversity of molecular strategies to interact with different regions of this part of the receptors, with no simple and consistent theme for receptor activation yet emerging, true even when considering subgroups of these receptors. There is the suggestion that different peptide agonists of a single receptor can use differential molecular strategies to activate that receptor and stimulate the same biological response, with each exhibiting distinct spatial approximations with the various juxtamembranous regions and often having their function differentially affected by various receptor mutants. With the diversity in molecular mechanisms possible for the activation of these receptors by peptide ligands, it is not surprising that structurally distinct small-molecule agonists for a single receptor are also capable of activation of these receptors using distinct molecular strategies.

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Conflict of interest

None.

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