Review

Photoaffinity Scanning in the Mapping of the Peptide Receptor Interface of Class II G Protein – Coupled Receptors

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Abstract: The family of G protein-coupled receptors constitutes about 50% of the therapeutic drug targets used in clinical medicine today, although the mechanisms of ligand binding, activation and signal transduction for G protein-coupled receptors are not yet well defined. This review discusses ongoing research using the photoaffinity scanning method to map the bimolecular interface between class II G protein-coupled receptors and their ligands. Furthermore the available computer model of class II peptide ligand docking into the receptor, based on the positional constraints imposed by the photoaffinity scanning analyses, will be discussed briefly. The ultimate goal of these efforts is to understand the molecular basis of receptor binding and therefore to generate a template for rational drug design. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: G protein-coupled receptor; class II G protein-coupled receptor; photoaffinity scanning; peptide ligand binding

INTRODUCTION

G protein-coupled receptors (GPCRs) with seven transmembrane helices (TMHs) represent the largest superfamily of cell surface proteins involved in signal transduction. These receptors bind ligands ranging from ions through biogenic amines, amino acids, peptides, lipids, nucleosides, glycoproteins, light and odorants to selectively transform an extracellular biological signal into a cascade of intracellular responses [1–3]. Therefore GPCRs are involved in a very wide range of physiological systems, including cardiovascular, endocrine, nervous and immune systems and other processes such as drug addiction, mood control and memory. GPCR dysregulation has been identified in a growing number of human diseases, for example; cardiac dysfunction, hypertension, depression, obesity, certain types of cancer, pain, schizophrenia and viral infection [4]. Thus, while GPCRs are only 2%–3% of the human genome, they constitute about 50% of the therapeutic drug targets used in clinical medicine today and consequently are of great interest to the biotechnology and pharmaceutical industry. Understanding how GPCRs function at the molecular level is an important goal of biological research that may be useful in the development of drugs acting at the

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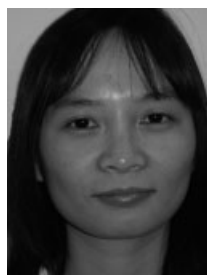
receptor of interest. The elucidation of the details of the bimolecular interaction between ligand and its receptor is an essential step for understanding the basis of molecular recognition and the mechanism of signal transduction by the receptor. Such structural insights may aid in the rational design of analogues/drugs with increased potency and improved selectivity.

Although much progress has been made in the development of molecular models of GPCRs, especially for class I receptors [5], the molecular mechanism of receptor action remains unclear. Although the three-dimensional (3D) structure of rhodopsin, a class I GPCR, has been resolved recently [6, 7], and may serve as a guide for experimental and computational studies in identifying the binding pocket in the receptor, the main problem for most GPCRs remains a lack of high resolution structural

information. Another problem is the collection of the conformational states or structures that ligand and receptor may adopt, and our capacity to identify which are relevant for ligand binding and receptor activation. In the absence of details of the tertiary structure of GPCRs or the bound conformation of their ligands, the only unambiguous practical method for mapping peptide ligand–receptor interactions is a direct one, based on a photoaffinity scanning (PAS) approach. This novel method offers the potential to obtain information about the hormone–receptor interface by identifying the direct points of proximity between the ligand and its receptor.

The goal of the present review is specifically to report the ongoing efforts to map the bimolecular interface between class II GPCRs and their ligands. The ultimate goal of these efforts is to generate an experimentally based model of the ligand–receptor complex that will provide insight into the molecular basis of the recognition and activation processes and generate a template for rational drug design.

CLASSIFICATION OF G PROTEIN-COUPLED RECEPTORS

GPCRs are plasma membrane proteins characterized by a N-terminal extracellular domain (ECD), three hydrophilic extracellular loops (ECLs), seven hydrophobic transmembrane-spanning helices (TMHs), three hydrophilic intracellular loops (ICLs) and an intracellular carboxyl terminus [8] (Figure 1). They are named for their functional interaction with the intracellular heterotrimeric guanine nucleotide-binding regulatory proteins (G-proteins). Agonist occupation by GPCRs allows G proteins to bind to the cytoplasmic surface of the receptor and to become activated [9]. The activated receptor induces a conformational change in the associated G protein α-subunit of the heterotrimer leading to release of GDP followed by binding of GTP. Subsequently, the GTP-bound form of the α-subunit is believed to dissociate from the receptor as well as from the stable $\beta\gamma$-dimer. Both the GTP-bound α-subunit (α-GTP complex) and the released $\beta\gamma$-dimer can interact with target proteins and modulate multiple intracellular signalling pathways [10].

Based on nucleotide and amino acid sequence similarity, the superfamily of mammalian GPCRs may be subdivided into three major families of receptors that share little sequence homology but appear to share the same overall topology
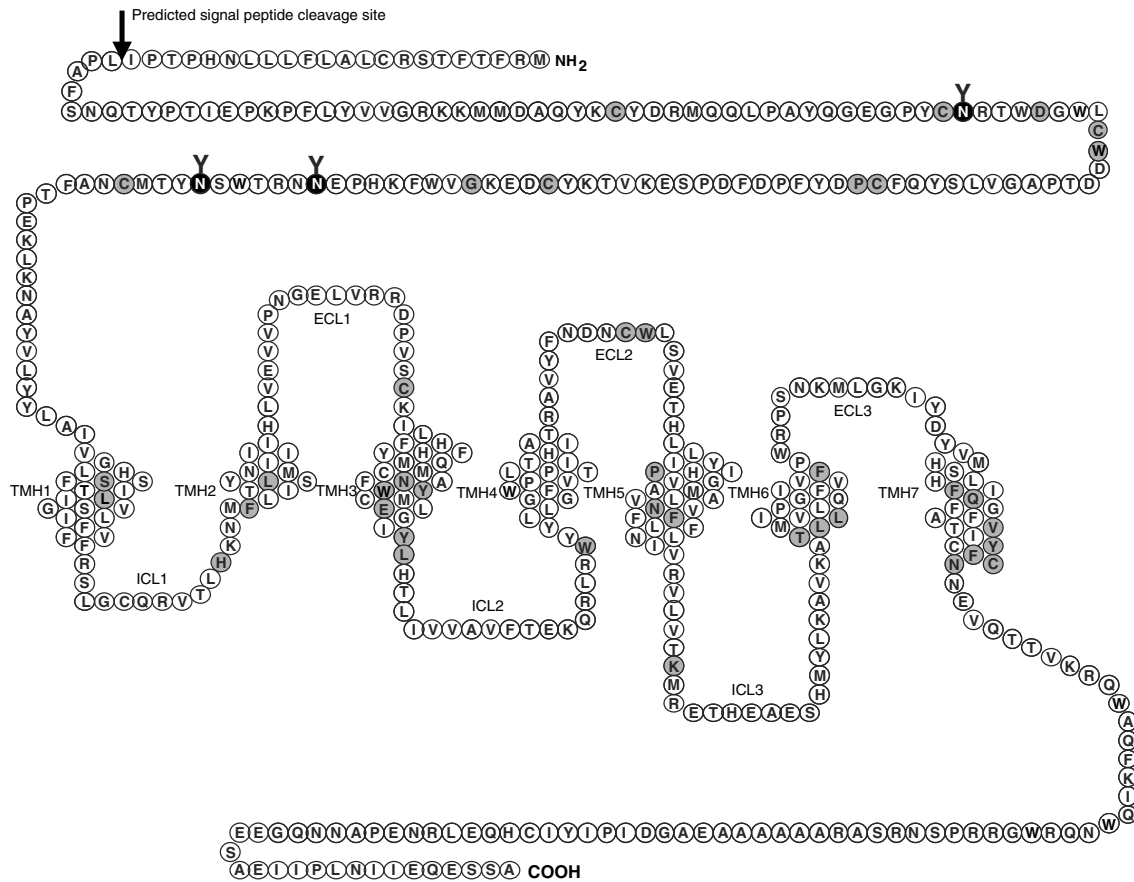
Figure 1 A two-dimensional schematic model of the calcitonin receptor (hCTRa isoform), a class II GPCR. Glycosylated asparagines (N^{73}, N^{124} and N^{130}) within the *N*-terminal ECD of calcitonin receptor are shown. Amino acid residues highlighted in circles are highly conserved in all of the class II GPCRs. Abbreviations: ECD, extracellular domain; TMH, transmembrane helix; ICL, intracellular loop; ECL, extracellular loop.

[10] (for review see reference [5]): (1) class I (or family A) receptors, including rhodopsin and adrenergic receptors, comprise more than 90% of the GPCRs identified to date; (2) class II (or family B) includes receptors for peptide hormones such as secretin, calcitonin (CT), glucagon, corticotrophin-releasing factor (CRF) and parathyroid hormone (PTH); (3) class III (or family C) is the smallest receptor subfamily and comprises receptors for glutamate, the major excitatory neurotransmitter in the central nervous system, as well as $GABA_B$ receptors and the Ca^{2+} sensory receptor.

GENERAL STRUCTURE OF CLASS II G PROTEIN-COUPLED RECEPTORS

Class II receptors can be subclassified into three groups on the basis of sequence; those with GPCR proteolytic sites (GPS), those with cysteine-rich domains (CRD) such as smoothened and frizzled and the rest. The GPS subgroup is the most numerous and least well studied, deriving its nomenclature from an unusual mode of processing at the *N*-termini, which are cleaved during transit through the endoplasmic reticulum at a defined GPS and rejoined by non-covalent linkage. The remaining receptors, defined by the absence of CRD or GPS domains are the best characterized and comprise receptors for many peptide hormones (Table 1) [11]. It is this latter group of receptors and their ligands that form the focus of this review.

The two-dimensional topology of a typical member of this subfamily, a receptor for CT, is illustrated in Figure 1. These class II GPCRs share 30%–50% amino acid identity but have less than 12% sequence homology with members of other GPCR families. The class II peptide hormone receptors are characterized

Table 1 Class II Subgroup of Mammalian GPCRs

Receptor	Species from which the receptors have been cloned	Peptide ligands for class II receptors	Truncated peptide antagonists	References
Parathyroid hormone type 1 receptor (PTH1R)	Human, rat, mouse, pig and opossum	PTH(1–34) PTHrP(1–36)	PTH(7–34) PTHrP(7–34) PTH(3–34)	[99, 105, 106]
Parathyroid hormone type 2 receptor (PTH2R)	Human and rat	Tuberoinfundibular peptide TIP(1–39)	TIP(7–39)	[46, 47, 98]
Secretin receptor (SecR)	Human, rat and rabbit	Sec(1–27)	NA	[12, 107]
Calcitonin receptor (CTR)	Human, rat, mouse, rabbit, pig and guinea-pig	CT(1–32)	CT(8–32)	[41, 93, 108]
Calcitonin receptor-like receptor (CLR)	Human, rat, mouse, pig and cow	Amylin(1–37) Calcitonin gene-related peptide CGRP(1–37) Adrenomedullin AM(1–52)	CGRP(8–37) AM(22–52)	[50, 109, 110]
Vasoactive intestinal peptide receptor (VPAC1R and VPAC2R) and pituitary adenylate cyclase activating peptide type 1 receptor (PAC1R)	Human, rat, mouse and pig	VIP(1–28) PACAP(1–27) PACAP(1–38)	PG 97–269 [VIP(3–7)/GHRH(8–27)] VIP(10–28)	[12, 48, 49, 104, 111]
Glucagon receptor (GR)	Human, rat and mouse	Glucagon(1–29)	NA	[112, 113]
Glucagon-like peptide 1 receptor (GLP1R)	Human, rat and mouse	GLP1(7–37) GLP1(7–36) Exendin-4	Extendin(9–39)	[44, 45, 112, 113]
Glucagon-like peptide 2 receptor (GLP2R)	Human and rat	GLP2(1–33)	GLP2(3–33)	[43, 112, 113]
Gastric inhibitory peptide or glucose-dependent insulinotropic peptide receptor (GIPR)	Human and rat	GIP(1–42)	GIP(6–30) GIP(7–30) GIP(10–30)	[42, 112, 113]
Growth hormone releasing hormone receptor (GHRHR)	Human, rat, mouse, pig, bovine and ovine	GHRH(1–29)	NA	[74, 113]
Corticotropin-releasing factor receptor (CRFR1, CRFR2 and CRFR3)	Human, rat, mouse and sheep	CRF(1–41); CRFR-like peptides: Urocortin, Sauvagine, and Urotensin I; CRFR2-selective peptides stresscopin (SCP) and SCP-related peptide	NA	[114, 115]

by (1) a large *N*-terminal ECD (150–180 amino acids) that includes six conserved cysteine residues; (2) the *N*-terminal ECD also contains several potential *N*-linked glycosylation sites that play a role in cell surface receptor expression and high affinity binding [12–14]; (3) a conserved cysteine residue in each of the first two ECLs that likely contribute to a disulfide bridge, and may be important for stabilizing the receptor in the correct conformation for ligand binding and activation [15]; (4) the absence of the amino acid sequence DRY (or ERW) in the *N*-terminal part of the second ICL (highly conserved in class I GPCRs) [5]; (5) a distinctive distribution of transmembrane proline residues and the presence of a unique set of conserved transmembrane residues [16].

The mechanism of hormone binding, activation and signal transduction for class II GPCRs is poorly understood. Structure–function studies of these receptors using chimeric receptors and site-directed mutagenesis point to a prominent role of the receptor's *N*-terminal ECD for peptide binding [17, 18] and ligand discrimination [19] and indeed moderate to high affinity binding of some peptides to isolated, purified ECD can be demonstrated for a number of receptors [20–24]. However, it is clear that other receptor domains are required for receptor activation and most high affinity agonist binding.

MOLECULAR CHARACTERIZATION OF COMMON HORMONE-BINDING DOMAINS OF CLASS II GPCRs

In general, evidence from mutational and photoaffinity labelling studies have shown that most class II peptide ligands bind to their cognate receptors by primarily interacting with the large *N*-terminal ECD [19, 25–34]. However, the amino terminus is not sufficient for binding of these ligands and additional interactions are found in the ECLs [19, 26, 30, 32, 35–37] and most likely within the TMHs [38, 39]. Thus, receptor binding and activation by these peptides appears to involve a large pharmacophore, which, in the absence of structural distance constraints is difficult to model. Nonetheless, the data are suggestive of significant similarities in the mode of ligand–receptor interaction for each of the peptide hormone receptors. In support of a conserved mode of receptor–ligand interaction are studies in which the *N*-terminal domain is exchanged between the PTH1 and CT receptors, whose ligands share essentially no amino acid identity. In this case the

loss of functional activity of native PTH and CT peptides observed for the receptor chimeras can be partly recovered with reciprocal peptide chimeras. Thus the peptide with *N*-terminal residues of CT and mid-*C*-terminal residues of PTH was the most potent activator of the receptor with the PTH1 receptor *N*-terminus and CT receptor core. The *N*-terminal PTH/*C*-terminal CT peptide was the most potent on the reverse receptor chimera [40]. Accordingly, the receptor domains involved in contact with *N*- and *C*-regions of the peptide appear to be well conserved for these receptors. Summaries of the available mutational and receptor chimera studies are presented in Tables 2 and 3.

Studies on Ligand Structure

The characterized ligands of class II receptors are typically peptides ranging in length from 27 to 41 amino acids (Figure 2). Many of the peptides including glucagon, glucagons-like peptide 1 (GLP1), secretin, vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating peptide (PACAP) share significant amino acid homology, particularly within the amino terminus, however, others such as CT, PTH and CRF share relatively little identity with the other peptides. Nonetheless, the peptides are able to adopt similar secondary structure, including one or two regions of amphipathic α-helical secondary structure, and this is likely to be important in the mode of ligand binding. Also constant among the peptides is the location of the principle activation domain within the *N*-terminus of the peptide, with truncation of this domain often leading to high affinity antagonist peptides (Table 1) [41–50].

METHODS FOR DEFINITION OF LIGAND–RECEPTOR BIMOLECULAR INTERACTIONS

Conventional Methods to Investigate Ligand–Receptor Interface

Three primary mechanisms are used to investigate the nature of ligand–receptor interaction: (1) alteration to ligand structure; (2) alteration to receptor structure (e.g. by mutation or formation of receptor chimeras or receptor truncation); and (3) physiochemical investigation of points of contact between the ligand and receptor. While all three approaches provide potentially important information for understanding of how ligands interface with

Table 2 Molecular Mechanisms of Ligand–receptor Interaction from Site-directed Mutagenesis Studies

Class II GPCR ligand	Construct	Candidate interaction sites/domains	References
Parathyroid hormone (PTH)	PTH(1–34) substitution	L^{24}, L^{28} and V^{31} in the amphipathic α-helix in the *C*-terminal region of PTH(1–34) contribute important receptor-binding interaction	[116]
	Mutation of $S^{370}A$, $V^{371}I$, and $L^{427}T$ of rPTH1R or mutation of $A^{364}S$, $I^{365}V$, and $T^{421}L$ of oPTH1R	R^2 of hPTH likely interacts with receptor residues rV^{371} and rL^{427} near the EC ends of TMH5 and TMH6 of rPTH1R, respectively to alter the ligand binding and with receptor residue rS^{370} in the TMH5 to alter the signalling specificity	[87]
	Deletion mutants of PTH1R	Residues near the receptor's *N*-terminus (residues 31–47) and within ECL3 (residues 431–440) are necessary for proper ligand–receptor interaction	[75]
	$C^{48}S$, $C^{109}S$, $C^{117}S$, $C^{131}S$, $C^{148}S$, $C^{170}S$, $C^{281}S$ and $C^{351}S$	The conserved Cys residues in the *N*-terminal ECD, and to a lesser degree in the first two ECLs are important for intracellular processing and/or cell surface expression	[75]
	Mutation of W^{437} and Q^{440}	W^{437} and Q^{440} in the 3rd ECL of rPTH1R (together with the membrane-spanning helices) form a part of the ligand-binding pocket that recognizes residues S^1 and V^2 of the ligand	[84]
	Mutation of $R^{233}H$ and $Q^{451}K$ in the TMH2 and TMH7 of the rPTH1R respectively	R^{233} and Q^{451} are important in receptor function by contributing to the interaction with residues 1 and 2 in PTH(1–34). Residues in TMH2 and TMH7 (R^{233}-Q^{451}, intramolecular interaction) are functionally linked and this interaction can be critical in transmitting the hormone's signal across the cell membrane	[38]
	Mutation of H^{223} in the 2nd TMH and T^{410} in the 6th TMH (Jansen's metaphyseal chondrodysplasia)	A positive charge at residue 233 is necessary for efficient receptor expression, and required for agonist-independent receptor activation. Residue T^{410} appears to constrain the receptor in an inactive conformation which can be relieved by either agonist binding or mutation to any other amino acid	[117]
	Mutation of I^{244}, Y^{318} in PTH2R to L^{289} and I^{363} in the hPTH1R (a PTH2R-to-PTH1R mutation)	I^{244} and Y^{318} at the EC ends of TMH3 and TMH5 respectively functionally interact with residue 5 of the ligand	[36, 118]
	Single mutation in the juxtamembrane base of the amino-terminal ECD (residues 182–190)	F^{184}, R^{186}, L^{187} and I^{190} are important determinants of maximum binding of bPTH(1–34) and bPTH(3–34); and are determinants of responsiveness to the *N*-terminal analogue PTH(1–14) in cAMP stimulation assay. Side chain hydrophobicity at F^{184} and L^{187} functionally interacts with the *N*-terminal region (3–14) of PTH	[64]
Secretin (Sec)	Amino acid mutation in TMH2	D^{174}, K^{173} and R^{166} in the 2nd TMH interact with D^3 in the peptide *N*-terminus	[119]

Table 2   (*Continued*)

Class II GPCR ligand	Construct	Candidate interaction sites/domains	References
	Mutations of aromatic residues in TMH1	Y^{128} in the 1st TMH interacts with D^3 of secretin	[120]
	Mutations of conserved charged amino acids	Residues D^{49}, R^{83}, K^{194} in *N*-terminus and R^{255} in the 2nd ECL are important for the secretin receptor structure or function	[121]
	Mutations of extracellular Cys residues	C^{24}, C^{44}, C^{53}, C^{67}, C^{85}, C^{101} are necessary for receptor function. Three suggested disulfide bridges are C^{11}-C^{186}, C^{24}-C^{101} and C^{193}-C^{263}	[13]
	Mutations of Cys residues	C^{24}, C^{44}, C^{53}, C^{67}, C^{85}, C^{101} may form disulfide bonds that are important for receptor function. No disulfide bonds between the *N*-terminus and the ECL/TMH	[122]
	Deletions or mutations of highly conserved basic amino acids in ICL3	K^{301} is important for G protein coupling	[123]
Calcitonin (CT)	Mutations of three potential *N*-linked glycosylation sites in the *N*-terminal ECD	Glycosylation in hCTR$_a$ plays an important role in high-affinity binding and potency of CT	[14]
Corticotropin-releasing factor (CRF)	Mutations of Cys residues to Ser or Ala	Disulfide bridges formed by C^{44}, C^{68}, C^{87}, C^{102} in the ECD, C^{188} (in the 1st ECL) and C^{258} (in the 2nd ECL) are important for ligand–receptor interaction, but C^{30} and C^{54} mutations do not affect ligand binding and signalling	[124]
	Mutations of residues in hCRFR1 with amino acids from the corresponding position in the *N*-terminal region of hVPAC2R	*N*-terminal ECD (43–50) and (76–84) of hCRFR1 are crucial for the binding of CRF agonists and antagonists	[125]
	Mutations of hCRF$_{2A}$R and xCRF$_2$R to their corresponding receptors	An amino acid triplet in the 2nd ECL ($D^{262}L^{263}V^{264}$ in hCRF$_{2A}$R or $K^{264}Y^{265}I^{266}$ in xCRF$_2$R) and a two-amino acid motif in the *N*-terminal ECD ($E^{66}Y^{67}$ in hCRF$_{2A}$R or $D^{69}S^{70}$ in xCRF$_2$R) mediate ligand selectivity differences	[126]
Glucagon (G) and glucagon-like peptide (GLP)	Glucagon mutation	S^8 and D^{15} are important determinants of receptor binding. H^1, D^9 and S^{16} constitute a putative triad responsible for activation of the receptor	[127–129]
	Mutations of D^{64}	D^{64} in the ECD may play a key role in glucagon binding	[130]
	Deletion mutant and truncation mutant of glucagon receptor	All seven TMHs are important for the proper folding, processing and cell surface expression of GR. The *N*-terminal ECD is required for ligand binding. Glycosylation is not important for the receptor to reach the cell surface and therefore may not be involved in ligand binding. The *C*-terminal tail is unnecessary for adenylate cyclase coupling	[131]
	Truncated/mutated GLP1R	A single amino acid block (K^{334}-L^{335}-K^{336}) in the ICL3 of GLP1R is required for efficient coupling of rGLP1 receptor to adenylate cyclase	[132]

Table 2 (*Continued*)

Class II GPCR ligand	Construct	Candidate interaction sites/domains	References
	Receptor mutation	V^{327}, I^{328} and V^{331} located at the junction of the 5th TMH and the 3rd ICL form part of a hydrophobic face that directly interacts with G protein and activates adenylate cyclase	[133]
Vasoactive intestinal peptide (VIP)	Mutation of all Cys in VPAC1R to Gly	C^{50}, C^{63}, C^{72}, C^{86}, C^{105} and C^{122} in the *N*-terminus are functionally crucial by forming intramolecular disulfide bonds which may help to maintain the topology for ligand binding in hVPAC1R. Disulfide bonds between C^{208}/C^{215} in the 2nd ECL and C^{285} in the 3rd ECL are not formed	[134]
	Mutation of C^{215} and C^{285} to Ala or Ser	A disulfide bond between C^{215} and C^{285} is required for securing ECL2 in the correct conformation for ligand binding and activation	[15]
	Mutation in *N*-terminal ECD	Highly conserved D^{68}, W^{73} and G^{109} in the *N*-terminus of hVPAC1R are important for its intrinsic binding activity to VIP	[25]
	Mutagenesis of *N*-glycosylation sites (Asn to Thr)	N^{58} and N^{69} in the *N*-terminal ECD of hVPAC1R are essential for correct delivery of the receptor to plasma membrane	[135]

ECD, extracellular domain; ICL, intracellular loop; ECL, extracellular loop; TMH, transmembrane helix; r, rat; b, bovine; x, xenopus; h, human; o, opossum; s, salmon.

their receptors, the first two approaches have limitations in the interpretation of data because they can provide only indirect and inferential information about the nature of the bimolecular interaction and cannot precisely define the complementary domains in the apposing partner involved in the interaction. This is particularly true for ligands with diverse pharmacophores such as those for class II GPCRs, where significant potential exists for conformational changes affecting to affect sites distal to the point of modification. The third approach provides a mechanism for directly defining the ligand–receptor interface by using photoactive amino acid derivatives spaced along the peptide to define proximity of particular amino acids in the receptor and ligand. Subsequent identification of the specific amino acid-to-amino acid contact points between hormone and receptor may facilitate the rational design of novel analogues and generate insights into the mechanism of ligand–receptor interaction.

Overview of Benzophenone (BP) Photochemistry in Photoaffinity Scanning (PAS)

A number of groups have used a photoaffinity scanning (PAS) approach that utilizes the unique photochemical properties of the benzophenone (BP) moiety as a photophore spaced along peptides to probe the bimolecular interface between the ligand and its macromolecular partner [51, 52]. BP substituents in the ligand (t-tuboxycarbonyl-benzoylphenylalanine, Bpa and ε-p-benzoylbenzoyl, pBz$_2$) have the ability to form stable intermolecular covalent bonds in the presence of UV irradiation when in close proximity (within 3.1 Å) to an interacting receptor [52]. This approach allows the purification of a covalent ligand–receptor complex, which can be subsequently subjected to exhaustive, specific, chemical and enzymatic cleavage to generate a cross-linked ligand–receptor domain. The basic principle on which PAS operates is that the photophore-modified ligand binds to the target receptor in a similar fashion to the

Table 3   Molecular Mechanisms of Ligand–receptor Interaction from Chimeric Ligand and Chimeric Receptor Studies

Class II GPCR ligand	Constructs	Candidate interaction site	References
Parathyroid hormone (PTH)	Rat/opossum PTHR chimeras	Three residues (V^{371}, L^{427} and S^{370}) in the TMH5 and 6 of the rPTHR contribute to the different binding and signalling responses to $[R^2]$PTH(1–34)	[87]
	Chimeric rat/human/opossum PTH1R chimeras	The *N*-terminal ECD of the receptor determines the binding affinity of *C*-terminal fragments of PTH(1–34)	[136]
	PTH/PTHrP hybrid ligand	Residue 5, His in PTHrP and Ile in PTH, is the major selectivity switch between the two PTHR subtypes and modulate ligand-induced receptor activation because the presence of H^5 in PTHrP blocked cAMP signalling.	[137]
	PTHR/SecR chimeras with point mutation	Mutation of SecR N^{192} in TMH2 to the corresponding residue in the PTHR ($N^{192}I$) responds to PTH, suggesting a critical role of residues in the 2nd TMH in maintaining ligand selectivity	[138]
	PTH2R/PTH1R	Residues, I^{244} near the end of TMH3 and Y^{318} in ECL2, determine H^5/I^5 signalling selectivity	[36]
	PTHR/SecR	*N*-terminal domains of PTHR (1–62 and 105–186) are important for binding PTH	[16]
Secretin (Sec)	SecR/VPAC1R	*N*-terminus (1–123) and ECL1 (175–190) are critical for ligand binding. More specifically, H^{189}, K^{190}, F^{257}, L^{258}, N^{260} and T^{261} are important determinants for Sec binding and activation	[18, 27, 139]
	SecR/VPAC1R	*N*-terminus (1–121) plays a key role in ligand binding	[140]
	SecR/VPAC1R chimeras with point mutation of D^{98}	D^{98} seems to interact with Sec residue K^{15}	[27]
	SecR/VPAC1R	*N*-terminus (1–10), ECL1 (H^{189}-K^{190}), and ECL2 (L^{258}, N^{260}-T^{261}) are critical for ligand binding	[27]
	SecR/VPAC1R	*N*-terminus (1–121) interacts with secretin *C*-terminus	[141]
	SecR/VPAC1R	*N*-terminus (1–121) interacts with residues 8–15 of Sec	[142]
	SecR/VPAC1R	*N*-terminus (1–121) interacts with residue 16 in Sec, VIP and PACAP	[143]
	SecR/VPAC1R	*N*-terminus (1–143) and ECL1 (174–199) are crucial for ligand binding	[81]
	SecR/VPAC1R	*N*-terminus (124–144) is critical for ligand recognition	[144]
	SecR/VPAC1R chimeras with point mutations	*N*-terminus (103–110) and (116–120) are implicated in secretin and VIP recognition. VIP residues 8 and 10 are positioned in the vicinity of the receptor domain 116–120	[145]
	SecR/GlucagonR	Residue D^3 of Sec interacts with K^{173} in ECL1	[146]
Calcitonin (CT)	Reciprocal hybrid ligands CT/PTH and CTR/PTH1R chimeras	The *C*-terminus of PTH or CT binds to *N*-terminal ECD of their receptor, while the *N*-terminal domain of the ligand interacts with trunk of the receptor and its associated loops	[40]
	hCTR/hGR	Two ligand binding sites on the CTR that can be physically dissociated: The high-affinity binding of sCT at site-one in the receptor *N*-terminus and activation of adenylate cyclase at site-two in the receptor trunk. The helical portion (8–22) of sCT is important for high-affinity binding to the receptor *N*-terminus	[17, 19]

(*continued overleaf*)

Table 3 (*Continued*)

Class II GPCR ligand	Constructs	Candidate interaction site	References
Growth hormone-releasing hormone (GHRH)	Truncated GHRHR; GHRHR/SecR or GHRHR/VPAC1R	*N*-terminal ECD, TMHs and associated ECLs of GHRH receptor form the principal determinants for specific interaction with GHRH. Chimeric GHRHR *N*-terminus-SecR or VPAC1R *C*-terminus neither bound GHRH nor signalled, whereas chimeric SecR or VPAC1R *N*-terminus-GHRHR *C*-terminus did. Neither constructs signalled in response to secretin or VIP	[147]
Corticotropin-releasing factor (CRF)	CRFR1/CRFR2 chimeras with point mutations	ECL2 (175–178), H^{189} at the junction of ECL1 and TMH3, and V^{266}, Y^{267} and T^{268} at the junction of ECL2 and TMH5 are important for optimal binding and receptor activation of CRF, urocortin and sauvagine	[148, 149]
	CRFR1/CRFR2 chimeras with point mutations	H^{199} in the 3rd TMH and M^{276} in the TMH5 are crucial for binding the non-peptide high-affinity CRFR1 antagonist NBI27914, suggesting TMHs play an important role in forming the binding pocket for the non-peptide antagonist	[148]
	xCRFR1/hCRFR1 chimeras with point mutations	*N*-terminal portion (70–89) of CRFR1 is important for the ligand binding and ligand selectivity	[31]
	CRFR/GHRHR/activin IIB receptor	The ECD of CRFR1 is involved in high affinity binding to CRF agonists and antagonists	[150]
	hCRFR1/hCRFR2α or xCRFR2 chimeras	Q^{76}, G^{81}, V^{83}, H^{88} and L^{89} in ECL1 of xCRFR1 are important for ligand selectivity, whereas ECLs other than ECL1 of hCRFR2α or xCRFR2 also contain determinants for ligand selectivity	[151]
	rCRFR1/rGR/hPAC1R	The ECL3 of rCRFR1 plays a major role in CRF high affinity binding, in which the polar residues T^{346}, F^{347} and N^{348} may interact directly with the polar *N*-terminus of CRF. Intramolecular ionic interactions of receptors participate in ligand binding	[152]
	mCRFR1/rPTH1R chimeras	C^{68}-E^{109} portion which is proximal to TMH1 of CRFR1 is essential for high-affinity binding and for recognition of CRF and sauvagine. Residues flanked by C^{87}-C^{102} are critical for CRFR1 activation	[153]
Glucagon (G) and glucagon-like peptide (GLP)	hGR/hGLP1R chimeras	The membrane-proximal half of the *N*-terminal extension, the 1st ECL, and the 3rd, 4th, and 6th TMDs are important for high affinity glucagon binding	[26]
	GLP1R/GIPR	*N*-terminus of GIP is required for ligand selectivity and the 1st TMH is crucial for receptor activation	[154]
	GR/GLP1R or secretin receptor chimeras with point mutation	*N*-terminus and ECL1 of GR is required for high-affinity binding to glucagon, with ligand specificity residing predominantly in the ionic binding pocket (206–219) of ECL1. Particularly, R^{202} in ECL1 may be involved in specific ligand binding	[155]
	GR/GLP1R chimeras with GR mutation and glucagon substitution	S^2 of glucagon is proximal to the EC end of TMH7 of GR (D^{385}) Q^3 of glucagon is close to the EC end of TMH2 of GR (I^{194}) K^{12} is proximal to ECL2	[156, 157]

ECD, extracellular domain; ICL, intracellular loop; ECL, extracellular loop; TMH, transmembrane helix; r, rat; b, bovine; x, xenopus; h, human; o, opossum; s, salmon.

(A)

Peptide	Amino acid sequence	Conditions for NMR analysis of structure
hSecretin	HSDGTFTSELSRLREGARLQRLLQGLV	40% TFE [77,78]
hVIP	HSDAVFTDNYTRLRKQMAVKKYLNSILN	25% methanol [159] 50% methanol [159] OSIRIS software[160]
hPACAP27	HSDGIFTDSYSRYRKQMAVKKYLAAVL	25% methanol [161]
hPACAP38	HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNK	50% TFE [162]
hGlucagon	HSQGTFTSDYSKYLDSRRAQDFVQWLMNT	Dodecylphosphocholine micelle [163]
hGLP1	HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR	Dodecylphosphocholine micelle [164]
hGHRH	YADAIFTNSYRKVLGQLSARKLLQDIMSRQQGESNQERGARARL	Water, pH 3 [165] 75% ethanol [165] 30% TFE / SDS [166]
hPTH	SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNF	Near-physiological solution [167] Crystal structure [90] Water / 40% TFE [168] 40% TFE [169]
hPTHrP	AVSEHQLLHDKGKSIQDLRRRFFLHHLIAEIHTA-EI	Near-physiological solution [170] Crystal structure [89]
hCRF	SEEPPISLDLTFHLLREVLEMARAEQLAQQAHSNRKLMEII	66% TFE [171] 50% TFE [172]

(B)

Peptide	Amino acid sequence	Conditions for NMR analysis of structure
hCGRP1	ACDTATCVTHRLAGLLSRSGGVVKNNFVPTNVGSKAF	50% TFE [173]
hAmylin	KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY	
hAdrenomedullin	YRQSMNNFQGLRSFGCRFGTCTVQKLAHQIYQFTDKDKDNVAPRSKISPQGY	
hCT	CGNLSTCMLGTYTQDFNKFHTFPQTAI-----GVGAP	SDS [174] 60% water / 40% TFE [175]
sCT	CSNLSTCVLGKLSQELHKLQTYPRTNT-----GSGTP	90% methanol [176] and TFE / water [177] SDS [178]

Figure 2 Amino acid alignments of class II receptor ligands. The location of α-helical structure from NMR studies is indicated by (——). (A) Shaded box shows identical amino acids to human secretin. The helical conformation of secretin, VIP, glucagon, PACAP and GHRH is remarkably similar. (B) Calcitonin and related peptides have little sequence homology to the other class II receptor ligands. These are aligned separately. Identical amino acids are highlighted.

unmodified ligand, namely, with high affinity, and induces the same profile of bioactivity. The cross-linking site of the BP photophore may represent either an actual contact point between ligand and receptor or a site in the immediate spatial proximity of such a contact point.

In the absence of solution or solid-state structures of ligand–GPCR complexes, PAS is a direct approach to study ligand–receptor bimolecular interactions, and complements the indirect assays of interaction based on altering ligand or receptor. This PAS approach has been used successfully in the determination of peptide ligand–receptor interaction sites for other receptor systems, for example the receptors for substance-P, vasopressin, cholecystokinin, luteinizing hormone and natriuretic peptide [53–61].

PHOTOAFFINITY SCANNING IN MAPPING OF THE INTERFACE OF CLASS II PEPTIDE HORMONE RECEPTORS

To date, site specific interaction data from photoaffinity labelling are limited to relatively few class II GPCRs, with detailed information restricted to the PTH and secretin receptors. Currently, the PTH1 receptor is the most extensively characterized and serves as an important model system for this receptor family. Proximity sites for ligand–receptor interaction have been identified for amino acids 1, 13 and 27 in PTH and amino acids 1, 2, 23, 27, 28 and 33 in PTH-related protein (PTHrP) with the PTH1 receptor and also amino acids 1 and 13 in PTH with the PTH2 receptor (Table 4) [33, 34, 37, 62–68]. Using the same methodology, proximity sites for amino acids 6, 13, 18, 22 and 26 of secretin and its receptor have been established (Table 4) [69–71].

The use of photoactive PTH/PTHrP and secretin analogues as chemical cross-linking probes is providing a complementary approach to the mapping of ligand receptor interactions by mutational methods. As mentioned above, conventional studies on mutated, chimeric or truncated receptors have suggested a basic template for the ligand–receptor interaction in which the mid- and carboxyl-terminal portions of the peptide ligand interact with the amino-terminal ECD of the receptor and the amino-terminal region of the ligand interacts with the receptor region comprising the membrane-spanning helices and ECLs (Tables 2 and 3). As reported below, the photoaffinity labelling studies have broadly supported this theme.

Contact Sites Within the *N*-terminal ECD of the Receptor

The first application of photoactive peptides to identify proximal sites within the receptor came from the laboratory of Chorev and colleagues who revealed that a PTH(1–34) analogue having the pBz_2 functional group attached to the ε-amino group of Lys^{13} (in the peptide mid-region; $[K^{13}pBz_2]PTH$) is in contact with the 17-amino-acid hPTH1 receptor fragment Phe^{173}-Met^{189}, adjacent to the *N*-terminal ECD/TMH 1 junction [63]. This cross-linked domain was resolved to the 8 amino acid fragment Glu^{182}-Met^{189} and site-directed mutagenesis implicated Arg^{186} as the site of cross-linking between PTH amino acid 13 and the receptor [34]. A virtually equivalent 10 amino acid fragment, delimited by residues Gln^{138}-Met^{147} was identified

as the site of cross-linking of the same analogue to the PTH2 receptor [72], indicating a high degree of conservation in the binding of this segment of PTH to both receptors. However, although alignment of the sequences of the PTH1 and PTH2 receptors revealed a homologous Arg [Arg^{143}] to Arg^{186} in the PTH1 receptor, mutation of this site (or the nearby $V^{144}A$ and $L^{146}A$) did not eliminate cross-linking to the PTH2 receptor [72]. The authors suggest that there is a minor structural feature within the $[K^{13}pBz_2]PTH$ binding domain of the PTH2 receptor, which is distinct from the homologous domain in PTH1 receptor. Recently Zhang and colleagues synthesized an analogue of secretin substituted at position 13 and identified its site of cross-linking to the secretin receptor. Similar to binding of PTH to the PTH1 and PTH2 receptors, $[Bpa^{13}]$secretin interacted with the receptor *N*-terminal ECD in a region proximal to TMH1; cross-linking to Val^{103} located ~22 amino acids from the boundary with TMH1 [73]. Consistent with the photoaffinity cross-linking of position 13 in PTH and secretin, preliminary studies of growth hormone-releasing hormone (GHRH) which has a photoactive group at amino acid 12 of the peptide, indicates that it is in close proximity to a 4 kDa *N*-terminal ECD of GHRH receptor, near the TMH1 [74].

An additional cross-linking site has been identified by Gardella, Juppner and colleagues using an analogue of PTHrP(1–36) containing Bpa at position 23, in place of the native Phe. They demonstrated proximity of this site to an 18 amino acid segment delimited by residues Y^{23}-M^{40} and T^{33}-M^{63} of the extreme amino-terminal rat and human PTH1 receptors, respectively [33, 68] (where Tyr^{23} is the presumed *N*-terminus of the receptor following cleavage of the signal peptide). These results indicated that residue 23 of PTHrP(1–36) likely contacts the receptor between residues 33–40. Subsequent alanine-scanning mutagenesis of this receptor region supported this conclusion revealing two amino acid residues, Thr^{33} and Gln^{37}, as possible functional contact sites for binding, at least for PTHrP where both agonist PTHrP(1–36) and antagonist PTHrP(7–34) binding was markedly attenuated [33]; binding of PTH(1–34) was only weakly affected. Support for functionally important contacts between ligand and receptor in the extreme *N*-terminus arises from earlier deletion studies where removal of residues 26–60 or 31–47 in the PTH1 receptor was detrimental to effective binding of PTH(1–34) [75].

Table 4   Molecular Mechanisms of Ligand-receptor Interaction by using Photoaffinity Cross-linking Studies

Class II GPCR ligand	Photoactive analogues[a]	Contact points on ligand	Contact sites/domains on receptor	References
Parathyroid hormone (PTH)	[Bpa¹]bPTH or [Bpa¹]bPTHrP or [Bpa²]bPTH (potent agonist)	1 and 2	M^{425} at the ectopic portion of TMH6 of hPTH1R	[37, 65]
	[Bpa²]PTHrP is a potent antagonist for hPTH1R, but is an agonist for PTH2R	2	M^{425} and P^{415}-M^{425} at the ectopic portion of TMH6 of hPTH1R	[65]
	[K^{13} pBz_2]bPTH and its antagonist	13	R^{186} of hPTH1R (located at the juxtamembrane end of N-ECD) and Q^{138}-M^{147} of hPTH1R	[34, 63, 86]
	[Bpa²³]PTHrP	23	33-40 of r/hPTHrP (located at the very N-terminal end of PTH1R)	[33, 66, 67]
	[K^{27} pBz_2]PTH	27	L^{261} of hPTH1R in the first ECL	[66]
	[Bpa²⁷]PTH or [Bpa²⁷]PTHrP	27	96-102 of hPTH1R located at the N-terminal ECD	[67]
	[Bpa²⁸]PTHrP	28	64-95 of hPTH1R located at the N-terminal ECD	[67]
	[Bpa³³]PTHrP	33	151-172 of hPTH1R located at the N-terminal ECD	[67]
Secretin (Sec)	[Bpa⁶]rSec	6	V^4 located at the N-terminal ECD	[70]
	[Bpa¹³]rSec	13	V^{103} located at the N-terminal ECD	[73]
	[Bpa¹⁸]rSec	18	R^{14} located at the N-terminal ECD	[76]
	[Bpa²²]rSec	22	L^{17} located at the N-terminal ECD	[69]
	[Bpa²⁶]rSec	26	L^{36} located at the N-terminal ECD	[71]
Calcitonin (CT)	[Bpa¹⁹]sCT	19	C^{134}-K^{141} in the N-terminal ECD, close to the 1st TMH	(Pham et al., 2003) submitted
	[Bpa¹⁶]hCT	16	F^{137} in the N-terminal ECD	(Dong et al., 2003) submitted
Growth hormone-releasing hormone (GHRH)	—	12	Near the TMH1 (in close proximity to a 4 kDa N-terminal ECD)	[74]
	—	1 and 21	Close to different points in the C-terminal half of the receptor sequence	[74]
Corticotropin-releasing factor (CRF)	Using chemical cross-linker disuccinimidyl suberate (DSS)	K^{16}	K^{257} of CRFR1 in the 2nd ECL	[158]
Vasoactive intestinal peptide (VIP)	[Bpa²²]VIP	22	G^{109}-I^{120} in the N-terminal ectodomain of the hVPAC1R	[79]

Bpa, benzoylphenylalanine; pBz_2, ε-benzoylbenzoyl.
[a] Many analogues contain additional amino acid substitutions (see original references for details). For simplicity of reading, only the position of the photoactive moiety is listed.

Again, a similar pattern of interaction was seen for secretin (1–27) and its receptor, where an analogue containing Bpa at position 22 in the carboxyl-terminal half of the peptide also cross-linked to the far N-terminus of its cognate receptor; within 30 amino acids of the predicted start of the receptor (assuming cleavage of the signal peptide) [69]. It was later confirmed that residue 22 of secretin interacts with Leu[17] (again assuming cleavage of the signal peptide) of the secretin receptor [71]. Secretin amino acid 18 cross-linked to Arg[14] of the receptor, close to the cross-linking site of amino acid 22 [76], consistent with NMR structural models of secretin where these amino acids are ∼1 helical turn apart [77,78]. Additional work by the same group, illustrated that position 26 of secretin located close to the carboxyl-terminus of the ligand interacts with residue Leu[36] of the secretin receptor [71].

Progressive substitution of amino acids 27, 28 and 33 of PTHrP(1–36) indicated that each of these amino acids resides in close proximity to parts of the amino-terminal ECD of the PTH1 receptor [67]. Residue 27 of the ligand cross-linked within amino acids 96–102 of the receptor, residue 28 cross-linked within amino acids 64–95 and residue 33 cross-linked within amino acids 151–172. Thus, for PTH/PTHrP and secretin, residues in the C-terminal half of the peptide that are required for high affinity binding of the peptides interact predominantly with the N-terminal domain and is consistent with the wide body of data supporting the N-terminus as the principal binding domain.

Recent data from a number of other receptors are also supportive of this general arrangement. [Bpa[22]]VIP cross-links to a short receptor fragment delimited by G[109] and I[120] (∼25 amino acids from the TMH1 boundary) [79]. This contact domain is consistent with a preliminary homology model developed for the VIP N-terminal domain, using a yeast lipase as the template [80]. VIP has significant homology with secretin (particularly in the N-terminal 15 amino acids), and it is therefore intriguing that this contact point is quite distinct from the proximity site for secretin 22 described above, where the interaction was mapped to 'L[17]' in the far N-terminus of the receptor. However, as only one proximity site for VIP is currently available it is difficult to speculate on the extent to which positioning of the two peptides within the N-terminus differs. For the CT receptor, two very recent studies have examined the site of cross-linking of [Bpa[16]]hCT (Dong et al., 2003, submitted) and [Bpa[19]]sCT (Pham et al., 2003, submitted).

Both these are proximal to a short receptor fragment (C[134]-K[141]) close to the TMH1 border. For [Bpa[16]]hCT, the cross-linking site was refined to F[137] (Dong et al., 2003, submitted). Amino acids 16 and 19 of calcitonin peptides are one turn apart within the predicted α-helix of the peptides (see Figure 2) and the cross-linking data are therefore consistent with the occurrence of the predicted helix during high affinity binding to the hCTR.

Interestingly, secretin amino acid 6, which resides within the 'activation' domain of the peptide also cross-linked to the ECD of the receptor, mapping to Val[4] at the extreme N-terminus of the receptor [70]. As other residues (more N-terminal) in the peptide activation domain are thought to interact principally with the trunk of the receptor (see below); this may provide an important constraint on the positioning of the receptor ECD with respect to the body of the receptor.

Interactions with Extracellular Loop Regions and the Receptor Core

ECL1. A role for ECL1 in agonist binding and selectivity within the type II subfamily of GPCRs has been demonstrated for VIP [18, 81], secretin [27] and PACAP receptors [30] in studies utilizing chimeric receptors. Similarly, altering the length of this loop can change affinity [75,82] and/or specificity of ligand binding [82] of CT and PTH1 receptors.

Using an analogue of PTH modified with a pBz$_2$ group on Lys[27], Greenberg and colleagues demonstrated an interaction with amino acid 261 in the first ECL of the PTH1 receptor [66]. This contrasts with the work of Gensure and colleagues described above where Bpa[27] substituted analogues of PTH and PTHrP cross-linked to the ECD of the receptor. The divergence of these results most likely reflects the different spatial properties of the photoreactive benzoyl group in the ligands used by the two groups. In the Lys[27]-pBz$_2$ analogue, the functional group is attached distally to the lysine side chain, whereas it is incorporated into the benzoylphenylalanine amino acid. These data, therefore, are indicative of a close proximity between residues 96–102 of the receptor ECD and amino acid 261 in ECL1 of the PTH1 receptor, which are close to amino acid 27 of the bound peptide ligand. However, the relevance of this finding to other class II receptors is less clear. Amino acids 96–102 of the ECD are encoded by Exon 2 of the PTH1 receptor gene, and are not conserved across the receptor family. Furthermore, deletion of Exon 2 (residues

61–105 in the *N*-terminal ECD) has no effect on either ligand binding or activity [75,83] indicating that this region of the receptor does not contribute functionally to ligand binding. Similarly, ECL1 of the PTH1 receptor is longer than the equivalent domain of most other class II receptors and deletion of parts of this domain in the PTH1 receptor (including amino acid 261) has little impact on ligand binding (yielding only a small increase in affinity) [84].

ECL2. In an alternative strategy to benzophenone substituted ligands, cross-linking of the CRF receptor and the 41 amino acid peptide sauvagine using the bifunctional cross-linking agent disuccinimidyl suberate (DSS), identified a proximity contact site between Lys^{16} of the peptide and Lys^{257} in ECL2 of the receptor. Unlike the benzophenone substituted analogues, DSS requires free amino groups and therefore cross-links only at the epsilon amino group of available lysine residues (within ~11.4 Å).

ECL3. Specific sites of interaction between the *N*-terminal regions of PTH(1–34) or PTHrP(1–36) and the PTH1 or PTH2 receptors have been determined from site-directed mutagenesis and photoaffinity cross-linking studies. For example, receptor deletion mutants indicate that residues in the third ECL and the adjacent sixth TMH are critical for hormone binding and signal transduction [75, 85]. Consistent with these mutational data, Chorev and colleagues have shown that Bpa introduced at amino acid 1 of the PTH1 receptor agonists PTH(1–34) and PTHrP(1–36) and Bpa introduced at amino acid 2 of PTH(1–34) cross-link to Met^{425} in the extracellular end of TMH6 of the PTH1 receptor [37,65]. Met^{425} is a non-functional contact as mutation of this residue to Ala abolishes cross-linking but does not affect ligand binding. These findings support the prevailing notion that PTH and PTHrP interact with the hPTH1 receptor in an almost identical manner [65]. Furthermore, analysis of the photoaffinity cross-linking of ^{125}I-[Bpa^1]PTH to the PTH2 receptor identified the equivalent amino acid (Val^{380}), to Met^{425} as a contact point [72]. Thus, as seen with peptide residue 13, the PTH1 and PTH2 receptor subtypes use analogous sites for interaction with position 1 in PTH.

Although not well refined, preliminary analysis indicates that amino acid 1 of GHRH cross-links within the *C*-terminal half of the receptor (encompassed by TMH4 to TMH7) [74], and this is broadly in-line with the findings from the PTH receptors.

Unlike, other amino acids from the mid-*C*-terminus of PTH, preliminary analysis of the PTH1 receptor site proximal to amino acid 19 of PTH indicated that [Bpa^{19}]PTH cross-linked to a region containing ECL3 and TMH6 [72]. Although, yet to be confirmed, this would potentially be consistent with findings from the GHRH receptor, where amino acid 21 interacted within the region delimited by TMH4 and TMH7.

Comparison of the Interaction of Agonist and Antagonist Analogues with Receptors

One of the major questions in GPCR biology relates to how receptors are activated by ligands and the different conformational states that govern active and inactive receptors. In an attempt to probe these differences, investigators have started to compare the interaction of photoactive substituted agonist and antagonist peptides. For class II receptors, the ability to do this is enhanced by maintenance of high affinity binding for peptides that are *N*-terminally truncated to form antagonists. Thus, side by side comparison between full-length and truncated analogues with equivalent photoactive substitution is possible, although only limited studies have been done to date.

In addition to the photoactive agonist, [Bpa^{13}] PTH(1–34), Chorev and coworkers also developed a photoactive antagonist, PTH(7–34), substituted at position 13 to directly study the nature of the bimolecular interface interaction of PTH antagonist with its receptor [86]. In this case, equivalent sites of cross-linking occurred for both peptides (R^{186} in the ECD), indicating that orientation of this segment of the receptor does not alter dramatically during agonist activation of the receptor.

Position 2 of PTH is known to be important for binding to active state receptor and receptor activation [87]. Bpa^2 substituted PTH(1–34) retained high affinity agonist binding and activation of the PTH1 receptor and cross-linked to Met^{425} of the receptor, similar to Bpa^1 substituted PTH and PTHrP analogues (Table 4). In contrast, Bpa^2 substituted PTHrP was a potent PTH1 receptor antagonist. Unlike [Bpa^2]PTH, [Bpa^2]PTHrP cross-linked to both Met^{425} and a second, more proximal region (within Pro^{415} and Met^{425}), suggesting differences in the conformation of the receptor that interacts with the antagonist peptide. Although Met^{425} is passive in the interaction and agonist activity of PTH, [Bpa^2]PTH and Bpa^1 substituted analogues of PTH and PTHrP, the data suggest that this residue

plays a significant role in the antagonist activity of [Bpa^2]PTHrP. Mutation of M^{425} to Leu recovers agonist activity of [Bpa^2]PTHrP at the PTH1 receptor. Similarly, in the PTH2 receptor where the analogue retains agonist activity, the corresponding amino acid is a valine (V^{386}). This work is also consistent with earlier work with non-human species of the PTH1 receptor, where the Arg^2 analogue of PTH ranged from partial agonist at the rat receptor and full agonist at the opossum receptor. In this case mutation of L^{427} to Thr, together with V^{371} to Ile led to specific increases in binding of [R^2]PTH to the rat receptor [87]. In the case of [Bpa^2]PTHrP there is likely a degree of steric hindrance imparted by Met^{425} that prevents key interactions required for agonist activity, although direct interaction with this residue to maintain inactive state receptor is also possible.

Additional insight into the process of PTH1 receptor activation arises from the work of Gensure and colleagues (2001) [68], who looked at cross-linking of [Bpa^2]PTHrP to a constitutively active receptor mutant that contains the substitution H^{223}R. At this receptor, denoted PTH1R$_{CAM-HR}$, [Bpa^2]PTHrP is an inverse agonist, in contrast to native PTHrP, which is an agonist. [Bpa^2]PTHrP cross-linked to the PTH1R$_{CAM-HR}$ at two sites; one within the TMH6/ECL boundary (P^{415}-M^{441}; possibly at M^{425}), the second site is within the region delineated by A^{313} and M^{414} [68]. It is likely that this latter site is at the extracellular border of TMH5 as earlier mutational studies implicated S^{370} and I^{371} as being functionally important interaction points for residue 2 in PTHrP [87]. Mutation of M^{425}L in the PTH1R$_{CAM-HR}$ mutant converted [Bpa^2]PTHrP from an inverse agonist into a weak partial agonist and led to loss of cross-linking in the P^{415}-M^{441} fragment with a corresponding increase in binding to the more proximal receptor fragment [68]. Close proximity to M^{425} is therefore required for the inverse agonist activity of the [Bpa^2]PTHrP analogue and the bulky Met may provide steric hindrance against functional interaction with residues within TMH5 that are required for agonist activity. However, as speculated above, it is also possible that direct interaction of Bpa^2 and M^{425} is necessary for this analogue to constrain the receptor in an inactive state, thus giving rise to the inverse agonist activity of the analogue. The difference in cross-linking pattern between the PTH1R$_{CAM-HR}$ and wild-type receptor is also indicative of differences in the orientation of the peptide ligand and receptor helices in active and inactive conformations. However, caution is needed in interpreting these data as models of ligand-induced receptor activation as the property of the [Bpa^2]PTHrP analogue differs for distinct CAM mutants of the PTH1 receptor; it is an inverse agonist at the H^{223}R PTH1 receptor, but not at the T^{410}P PTH1 receptor [88].

DEVELOPMENT OF MODELS OF THE LIGAND-RECEPTOR INTERFACE

There are substantial structural data available for class II peptide ligands (Figure 2), with considerable overlap in the proposed secondary structure for the peptides. Also apparent is the extent and range of secondary structures that may be formed under differing experimental conditions. This is exemplified in the study of Pellegrini *et al.* (1998) [89], who examined the solution-based structure of PTH(1–34) by NMR. This work indicates that PTH exhibits a high degree of conformational flexibility with differing levels of helix and position of flexible domains depending upon solvent conditions. In contrast, x-ray crystallography was indicative of a highly ordered structure that was predominantly α-helix throughout the extent of the peptide [90]. The choice of structure to use in docking studies is therefore problematic and while it is probable that the peptides form 1 to 2 regions of amphipathic α-helix, the extent to which these helices will form and the tertiary structure of peptides bound to active and inactive state receptors is not clear. The positional constraints imposed by the photoaffinity scanning analyses will therefore be important in understanding the mode(s) of receptor binding.

Structural data on class II receptors is sparse and is primarily limited to the solution-based NMR-derived structure of short peptide receptor fragments of the PTH1 receptor [90, 91]; these are discussed below. Nonetheless, homology modelling of the transmembrane helices using the rhodopsin crystal structure as a template is possible and this can be made more relevant to class II receptors through the use of computer modelling of non-canonical elements within the helices [92]. The structure of the ECD and ECLs is more speculative.

As discussed earlier, the ECD of all class II peptide hormone receptors has six highly conserved cysteines. Recently a number of groups have succeeded in purifying and refolding isolated ECDs for the PTH1, CRF1 and CRF2β receptors [20–22]. Biochemical analyses have revealed a common pattern

of disulfide bond formation for each of these receptors; Cys[I]-Cys[III], Cys[II]-Cys[V] and Cys[IV]-Cys[VI] and this is likely to be common for receptors of this class. Thus the ECD is highly constrained. For the CRF2β receptor ECD, high affinity binding of some peptides was maintained, although the relative specificity of peptide interaction was different to the full length receptor [21] indicating a requirement for cooperative binding between receptor microdomains that is more important for some ligands. Interestingly, circular dichroism analysis indicates that the ECD undergoes significant conformational change upon binding of astressin, and these observations may support the proposals for multiple receptor states for binding and activation that have been put forward for the CT receptor [19, 93–95] and the PTH receptors [96–99].

For the PTH1 receptor, a micelle-derived conformation for residues 168–198, containing part of TMH1 and the proximal ECD, has been established by NMR [89]. This work indicates that the membrane proximal ECD comprises predominantly an amphipathic α-helix; G^{188}-E^{180} and N^{176}-E^{169} and that it lies parallel to the hydrophobic membrane. This receptor domain contacts PTH[13] that resides within a helical portion of the peptide and consequently Chorev and colleagues have postulated that the peptide and ECD lie parallel to each other along the membrane, at least during initial ligand binding [99]. Likewise, the ECL1 domain of the PTH1 receptor has been suggested to comprise predominantly α-helical secondary structure, based on NMR analysis of a synthetic peptide of ECL1 and the ectopic parts of TMH2 and 3 [91]. The predicted contact domain of PTH[27] (L^{261}) lies approximately mid-helix. The utility of the ECL1 structure for modelling of other class II receptors is unclear. The ECL1 of the PTH receptors is considerably longer than the equivalent domain of most other class II receptors (≥ 10 a.a.s), and indeed, much of this domain in the PTH1 receptor can be deleted without detriment to ligand binding and efficacy [75]. This loop also has a Cys near the border of TMH3 that is believed to form a conserved disulfide linkage with a Cys in ECL2. How this constraint would impact on tertiary structure is unclear.

For the PTH1 receptor two preliminary computer models of ligand docking have been proposed [90, 91, 100]. Both accommodate mutational and photoactive cross-linking data for the interaction of the peptide *N*-terminus and the mid-molecule contact, but lack resolution or consistency with *C*-terminal contact constraints. Rolz and colleagues

have presented the interactions of the *N*-terminal portion of the ligand with ECL3 and extracellular ends of TMH6 and TMH7, while the *C*-terminal region of the ligand is engaged principally with the *N*-terminus of the receptor [100]. They suggest that the role of agonist binding is to bring TMH6 and TMH7 in juxtaposition to the bundle of the remaining TMHs. Such motions of the TMHs would have a direct effect on the conformation of the third ICL, which has been shown to be coupled with both Gs and Gq [101]. Similar models for receptor activation have been proposed for other GPCRs such as NK1 receptor [102, 103]. The model of Jin *et al.* (2000) is based on docking of the crystallographically derived structure of PTH that is almost entirely α-helical and has only the membrane proximal segment of the ECD modelled. These authors supposed that the *N*-terminal portion of PTH(1–34) binds to a pocket consisting of the extracellular region of TMH3, TMH4 and TMH6 and the second and third ECLs of the receptor [89]. The middle of the ligand is sandwiched between the first ECL and the *N*-terminal extracellular region of the receptor adjacent to TMH1. In this model, the *C*-terminal region of ligand forms extensive interactions with a putative binding domain of the membrane proximal ECD of the receptor. The alignment interface predicted a hydrophobic interaction between residues Trp[23], Leu[24], and Leu[28] of PTH(1–34) and Phe[173], Leu[174] of the PTH1R; a polar interaction between Lys[27] of PTH(1–34) and Glu[169] of the PTH1R as well as between Arg[20] of PTH(1–34) and Glu[180] and Glu[177] of the receptor [90]. Unfortunately, the above cross-linking data of position 23 [33] and 27 of PTH [66] do not provide support for these components of the model. On balance, it seems unlikely that PTH (and indeed other class II receptor peptides) will exhibit the degree of constrained structure suggested in the derived crystal structure of PTH, which was used by Jin to model ligand binding. It has been suggested that the PTH1 receptor may adopt an open structure when in the inactive state, but may undergo transition to a more closed structure upon agonist binding to active state receptor. Significant structural differences between active and inactive state receptor is supported by the cross-linking data with constitutively active PTH1 receptors described above [68] and from the pseudo-irreversible binding of agonists to the PTH receptor [98, 99]. Pseudo-irreversible binding is also seen with helical CT agonists at CTRa receptors [93–95].

For the VPAC1 receptor, Laburthe and colleagues have established a homology model of the ECD,

using a yeast-lipase template, containing an electronegative groove that forms a putative binding pocket for VIP [79, 80, 104]. The initial cross-linking constraint of VIP[22] is internally consistent with this model, which is also reported to accommodate disulfide bridges between the conserved cysteines. The utility of this model for the ECD will be tested and refined as additional photoaffinity scanning contacts are resolved.

CONCLUSION

The highly constrained nature of the *N*-terminal ECD of class II peptide receptors, together with the general similarity in potential peptide secondary structure, implies a conserved mode of ligand receptor interaction that is broadly supported by mutational and photoaffinity scanning analyses. As a consequence it is likely that models of individual ligand–receptor interaction and receptor activation will provide general insights into class II receptor function. Photoaffinity scanning of the peptide receptor interface provides valuable constraints in the development of such models and workers in the field are increasingly turning to this methodology to help understand the diverse pharmacophore of the peptide ligands. Together with incremental advances in our knowledge of receptor structure, such as those derived from solution-based analyses of receptor fragments, and the potential for crystal structures of the complex ECD that comes from successful purification of the isolated ECD, this research promises to provide major insights into class II receptor function over the next 5 years.

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