Review

Passing the baton in class B GPCRs: peptide hormone activation via helix induction?

Christoph Parthier[1], Steffen Reedtz-Runge[2], Rainer Rudolph[1,3,4] and Milton T. Stubbs[1,3,4]

[1] Institut für Biochemie und Biotechnologie, Martin-Luther-Universität Halle-Wittenberg, Kurt-Mothes-Straße 3, D-06120 Halle (Saale), Germany
[2] Department of Structure and Biophysical Chemistry, Novo Nordisk, 2760 Måløv, Denmark
[3] Mitteldeutsches Zentrum für Struktur und Dynamik der Proteine, Martin-Luther-Universität Halle-Wittenberg, D-06120 Halle (Saale), Germany
[4] ZIK HALOmem, Martin-Luther-Universität Halle-Wittenberg, D-06120 Halle (Saale), Germany

G-protein-coupled receptors (GPCRs) represent the largest constellation of validated drug targets. Crystal structures of class A GPCRs have facilitated major advances in understanding the principles underlying GPCR activation. By contrast, relatively little is known about class B GPCRs, a family of receptors for a variety of therapeutically relevant peptide hormones. Encouraging progress has recently been made through the structural elucidation of several extracellular hormone-binding domains of class B GPCRs in complex with their natural ligands or synthetic analogues. The structures reveal similar modes of ligand binding, with concomitant α-helical structuring of the ligand. The latter suggests an attractive mechanical model for class B GPCR activation.

Class B GPCRs: a family of peptide hormone receptors

G-protein-coupled receptors (GPCRs) constitute a large family of transmembrane receptors that mediate transduction of an enormous variety of extracellular stimuli across cell membranes. Stimuli range from light, ions, nucleotides, organic volatiles, neurotransmitters and hormones through to peptides and proteins. Phylogenetically, GPCRs can be divided into at least five receptor classes (or families), of which class A represents the largest group (~700 members, also termed the rhodopsin family). Exhibiting low apparent sequence homology to the rhodopsin family, class B receptors (with 15 members, also termed class 2 or the secretin receptor family) share the same general architecture: seven membrane-spanning α-helices interconnected by intracellular and extracellular loops with a C-terminal intracellular domain that interacts with the G protein. Class B receptors are distinguished by the presence of a large N-terminal extracellular domain (ECD; ~100 to 160 residues) that has an important role in ligand binding [1,2].

The natural ligands of class B GPCRs are endogenous peptide hormones, including glucagon, the incretins glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), parathyroid hormone (PTH), corticotropin-releasing factor (CRF), growth-hormone releasing factor (GRF), pituitary adenylate cyclase activating polypeptide (PACAP), vasoactive intestinal peptide (VIP), secretin and calcitonin. These hormones along with their analogues have attracted considerable pharmaceutical interest for their potential in the treatment of human pathologies including diabetes (glucagon, GLP-1 and GIP) [3,4], osteoporosis (PTH and calcitonin) [5], neurodegeneration (PACAP) [6], inflammation (VIP) [7], dwarfism (GRF) [8], in addition to chronic stress, anxiety and depression (CRF) [9,10]. A substantial obstacle to the therapeutic administration of agonistic peptide hormones is their rapid deactivation by endogenous proteases, resulting in a very short half-life of the bioactive hormones *in vivo* [11,12]. A detailed understanding of ligand recognition and receptor activation would facilitate the design of stable peptide or non-peptide ligand analogues.

Structural studies on class A GPCRs have contributed greatly to a molecular understanding of this important class of signal transducers. The ground-breaking structure of bovine rhodopsin [13] has been augmented in the past year by those of the ligand-bound avian and human β_1- and β_2-adrenoceptors [14–17] and the human A_{2A} adenosine receptor [18]. These structures were solved in the presence of inverse agonists or antagonists and, therefore, represent various inactive states of the receptor. Most recently, the crystal structure of ligand-free rhodopsin, termed opsin, with a bound fragment of the α-subunit of a heterotrimeric G protein was elucidated [19], revealing for the first time the structure of a GPCR representing the active, G-protein-bound conformation. Thus, the stage has been set for a near atomic representation of the events involved in GPCR signal transduction across the transmembrane domain.

Although no experimentally determined full-length class B receptor structure has been achieved to date, the structure elucidation of individual class B GPCR ECDs represents a considerable step towards a molecular understanding of their action. Six representative ECD structures of the secretin family of GPCRs have been determined by X-ray crystallography or nuclear magnetic resonance (NMR) spectroscopy in complex with bound

Corresponding authors: Parthier, C. (parthier@biochemtech.uni-halle.de); Stubbs, M.T. (stubbs@biochemtech.uni-halle.de).

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ligands: the murine type-2β CRF receptor (CRFR2β) [20], a subtype of human PACAP receptor ($PAC1R_s$) [21], human GIP receptor (GIPR) [22], human GLP-1 receptor (GLP-1R) [23], human PTH receptor (PTH1R) [24] and the human type-1 CRF receptor (CRFR1) [25]. The structures provide a common picture for the interaction of class B GPCRs with their cognate peptide hormones and first indications on the origins of ligand selectivity. Surprisingly, the latter seems to be linked to a ligand folding step, with implications for regulation of GPCR activation.

Based on the recent ECD complex structures, we outline common and divergent principles of ligand binding to class B GPCRs. The ECDs share the same 'secretin family recognition fold', with all peptidic ligands binding in a predominantly α-helical manner. The peptide orientation implies that the ligand N terminus is exposed to the transmembrane domain of the receptor. Upon consideration of this and other available structural and biochemical data, we postulate that α-helix formation takes place upon binding to the ECD. Coupling of ligand structuring to conformational changes in the transmembrane domain would enable transmission of the signal to the cytoplasmic signalling cascade.

The class B GPCR ligands: peptide hormones with α-helical propensities

The first X-ray crystal structure determination of glucagon in 1975 revealed a helical conformation for the hormone [26]. Later, NMR structural analyses, however, indicated that glucagon was disordered in solution [27]. This behaviour was also observed for PTH, which in solution exhibits limited secondary structure [28,29] but is helical in protein crystals [30]. It is now established that most class B ligands, including GLP-1 and its lizard homologue exendin-4 [31,32], GIP [33], PACAP [21], CRF [34] and the related urocortins, in addition to the derivative synthetic ligands stressin and astressin [35], show little, if any, ordered structure in aqueous solutions but can be induced to form α-helices under mild ambient conditions such as in the presence of organic solvents (e.g. trifluorethanol) or lipids or upon crystallization. A notable exception is provided by calcitonin, in which a largely α-helical conformation seems to be constitutive owing to stabilization by an intramolecular disulphide bridge that fixes the α-helix to the N terminus of the isolated hormone [36,37]. This propensity to form helices reveals itself for individual hormones in the form of a continuous α-helix, a kinked α-helix or an extension of a helical 'nucleus', although the helices rarely span the entire peptide ligand.

The sequence requirements conferring bioactivity to the hormone ligands have been investigated extensively for several class B GPCRs. N-terminal truncations generate competitive antagonists, marking these residues as essential for receptor activation, as shown for CRF [38], exendin-4 [39], GIP [40,41] and PTH [42,43]. By contrast, C-terminally truncated ligands remain active, although they exhibit a significantly decreased affinity for the receptor [40,44–47].

Hormone recognition by class B GPCRs is believed to follow a 'two domain model' of binding, in which the C-terminal portion of the ligand is captured by the receptor ECD and the N-terminal portion of the ligand is delivered to the membrane-bound domains of the receptor, where it interacts with extracellular loops and the transmembrane α-helices [48]. The role of the class B GPCR ECDs in binding their cognate ligands was first established based on studies of chimeric receptor constructs and corresponding ligands, including PTH1R and calcitonin receptor [49], GIPR and GLP-1R [50], GLP-1R and glucagon receptor [51,52] and several other class B GPCR members [53–55].

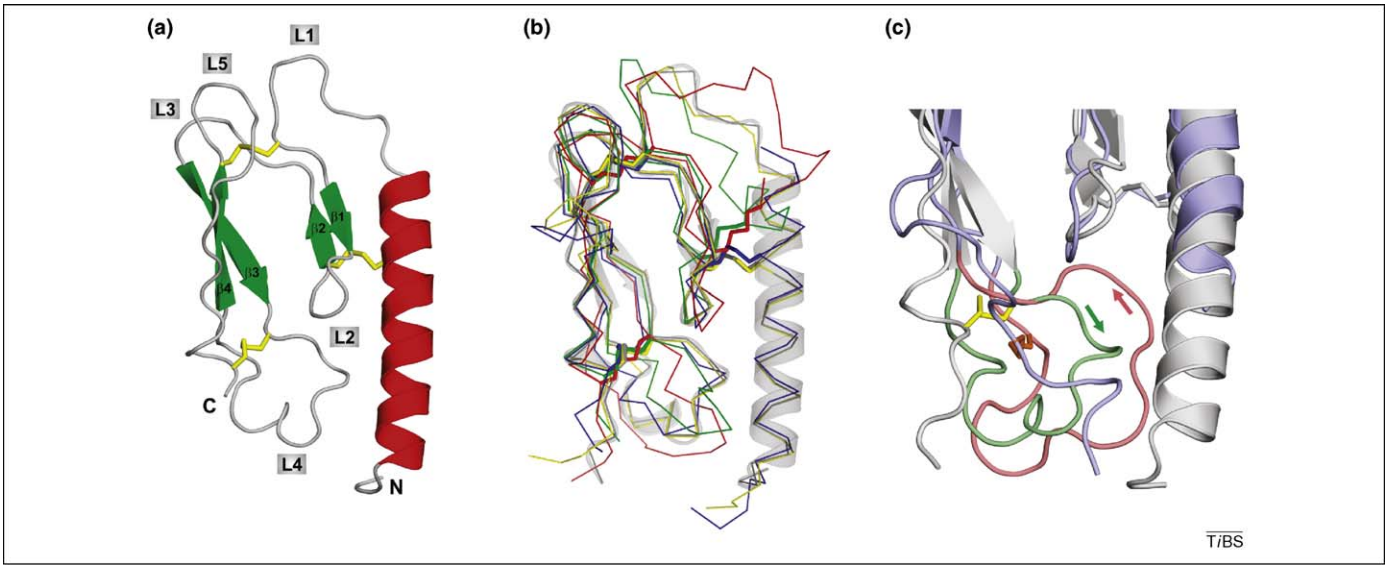

Figure 1. Structures of class B GPCR ECDs. Each ECD structure solved to date exhibits the 'secretin family recognition fold'. **(a)** Common structural elements of class B GPCR ECDs as observed in the GIPR-ECD [22]: an N-terminal α-helix (red), two β-sheets composed of strands β1 to β4 (green), with loop regions L1 to L5 (grey). The domains are stabilized by three conserved disulphide bridges (yellow sticks). **(b)** Superposition of the polypeptide backbones of CRFR2β-ECD [20] (red; PDB code: 2JND), CRFR1-ECD [25] (green; PDB code: 3EHU), GIPR-ECD [22] (grey, in cartoon representation; PDB code: 2QKH), GLP-1R-ECD [23] (yellow; PDB code: 3C5T) and PTH1R-ECD [24] (blue, PDB code: 3C4 M). Disulphide bonds are shown as sticks in the respective colour. **(c)** The aberrant topology of loop 4 in $PAC1R_s$-ECD [21] (pink and lilac; PDB code: 2JOD) superimposed on the GIPR-ECD (light green and grey). The direction of the main chain of loop 4 (indicated by arrows) in $PAC1R_s$-ECD is opposite to that in GIPR-ECD and the other ECDs. Note the position of the disulphide bridge, which in $PAC1R_s$-ECD (orange) lies 'below' the loop, whereas in GIPR-ECD (yellow) it is 'above' the loop. Structural alignment carried out using the program STAMP (STructural Alignment of Multiple Proteins) [70]. All figures prepared using PyMOL (http://www.pymol.org).

Detailed characterization of the isolated ECDs of GLP-1R-ECD [56–58], PTH1R-ECD [59], CRFR1-ECD [60] and CRFR2β-ECD [46] have confirmed that the ECDs contribute substantially to the affinity of the full-length receptor towards their cognate ligands.

The extracellular ligand-binding domains: one fold serves all?

Elucidation of the NMR structure of CRFR2β-ECD [61] revealed a core domain structure consisting of two central antiparallel β-sheets stabilized by three intramolecular disulphide bridges – a topology that resembles the short consensus repeat fold commonly found in proteins of the complement system [62]. With the structure determination of two further ECDs, PAC1R_s-ECD (using NMR) [21] and GIPR-ECD [22], the first crystal structure within this class, it became apparent that the ECD fold includes an additional N-terminal α-helix linked to the β-sheet core by one of the three disulphide bonds; we termed this the 'glucagon hormone family recognition fold' (Figure 1a). Three further crystal structures, GLP-1R-ECD [23], PTH1R-ECD [24] and CRFR1-ECD [25], confirmed that the secretin receptor family hormone-binding domains adopt a distinct fold; therefore, a more valid terminology would be 'secretin family recognition fold'. The apparent structural similarity of the different class B ECDs is particularly striking considering that sequence conservation is limited to the six cysteine residues and five additional residues that are crucial for domain stability [63] (Figure 1b). The strongly conserved fold observed in the ECD structures suggests that common mechanisms underlie ligand recognition.

Although the compact core is conserved between the ECDs, individual loops (in particular loops 1 and 4) deviate significantly, a finding that is apparent from both the NMR structure ensembles and the high B-factors in the crystal structures. The exceptionally long loop 1 of PTH1R-ECD is disordered in the crystals [24], whereas the conformation of loop 4 of CRFR1-ECD and CRFR2β-ECD seems to adapt to ligand binding [20,25]. Intriguingly, the solution structure of PAC1Rs-ECD in complex with $PACAP_{6–38}$ [21] exhibits a conspicuous difference in loop 4 topology (Figure 1c). Although the overall structure of PAC1R_s-ECD is similar to the other ECDs, loop 4 proceeds to β-strand 4 'above' the terminal disulphide bond; in all other ECD structures, this loop proceeds 'below' this disulphide, resulting in an inverse direction of loop 4 compared with the other ECDs. The same aberrant topology was also present in the initial CRFR2β-ECD NMR structure ensemble [61] but, later, was revised in a refined structure of the domain [20].

Ligand binding by the ECDs: gripping the baton

Six of the recently solved class B ECD structures were elucidated in complex with a ligand: the solution structures of murine CRFR2β-ECD bound to the synthetic antagonist astressin [20]; PAC1R_s in complex with the antagonist $PACAP_{6–38}$ [21]; the crystal structures of GIPR-ECD bound to its natural peptide hormone $GIP_{1–42}$ [22]; GLP-1R-ECD in complex with the antagonist exendin-$4_{9–39}$ [23]; PTH1R-ECD bound to the truncated ligand $PTH_{15–34}$ [24]; and two structures of CRFR1 in complex with the truncated ligands $CRF_{22–41}$ and $CRF_{27–41}$ [25]. With the exception of PAC1Rs-ECD, each ECD interacts with its cognate ligand in an equivalent orientation, binding C-terminal residues of the ligand, whereas N-terminal residues, if present, do not interact with the ECD. In each case, the bound ligand adopts an α-helical conformation, sandwiched between the two β-sheets of the ECD, with the ligand C terminus 'buffer stopped' by intermolecular hydrogen bonds to ECD side chains (Figures 2a and 2b).

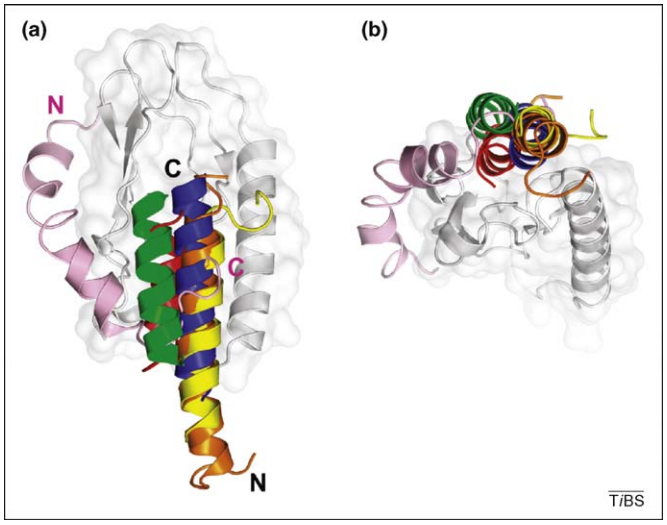

Figure 2. Structures of class B GPCR ligands bound to the ECDs. Each ligand binds in an α-helical conformation. Only the C-terminal residues of the ligand interact with the ECD, leaving the N-terminal sections free for interactions with other parts of the receptor. **(a)** Superposition of the ECD-ligand complexes after structural alignment of the ECDs (GIPR-ECD shown as grey cartoon with surface representation). The bound ligands are coloured as follows: astressin [20] (red; PDB code: 2JND), $PACAP_{6–38}$ [21] (pink; PDB code: 2JOD), $GIP_{1–42}$ [22] (orange; PDB code: 2QKH), exendin-$4_{9–39}$ [23] (yellow; PDB code: 3C5T), $PTH_{15–34}$ [24] (blue; PDB code: 3C4 M), $CRF_{22–41}$ [25] (green; PDB code: 3EHU). Note the binding mode of ECD-bound $PACAP_{6–38}$, which is substantially different to those of the other ligands (N and C termini are labelled). **(b)** View rotated about a horizontal axis by 90°. All peptides exhibit an α-helical conformation; note that N-terminal residues of GIP, exendin and CRF do not contact the ECD.

In each structure, residues of the ligand in contact with the ECD form an amphipathic α-helix, with three or more hydrophobic residues occupying a complementary ligand-binding groove on the surface of the ECD (Figures 3a and 4a). Alanine-scanning experiments have established the important contribution of hydrophobic interactions to ligand binding [20–22,24,25]. The ligand-binding groove is lined by hydrophobic residues from loops 2 and 4 and from the ECD C terminus (Figures 3a and 3b). Interestingly, the conformations of loops 2, 4 and part of loop 5 of CRFR1-ECD and CRFR2β-ECD are different in the ligand-free and ligand-bound states [20,25] (Figure 3c). Although the magnitude of the conformational shift in CRFR1-ECD loop 4 might be influenced by crystal packing effects, the NMR data indicate that ligand binding induces changes in loops 2 and 4. If these loops were to juxtapose the membrane and/or transmembrane helix domain, the ligand-induced conformational changes in the ECD could be transmitted to the membrane core of the full-length GPCR.

The only exception to this otherwise common binding mode is provided by the PAC1Rs-ECD in complex with the truncated hormone $PACAP_{6–38}$ [21]. Whereas the PACAP C terminus interacts with ECD residues of the ligand-binding groove, the peptide wraps around the ECD: N-

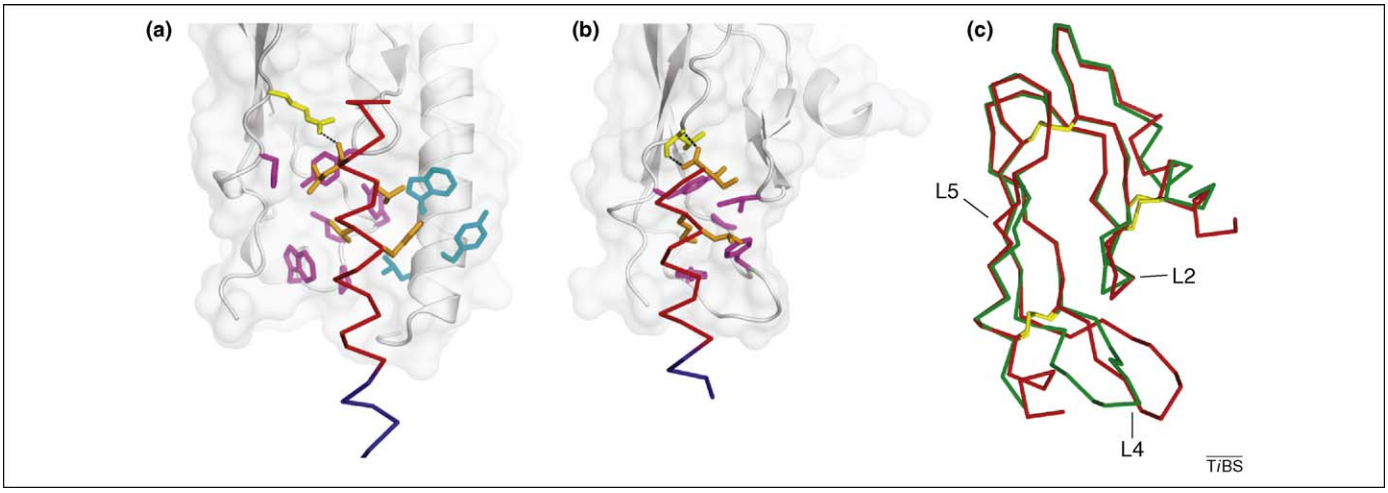

Figure 3. Binding of class B GPCR ligands to the ECDs. Two distinct binding modes are observed, each employing a similar cluster of hydrophobic interactions between the ligand and ECD loops but differing in the contribution from the ECD N-terminal α-helix. **(a)** The binding mode of GIP (shown as C_α trace, red and blue) to GIPR-ECD [22] (grey; PDB code: 2QKH). Hydrophobic residues in the ECD core from loop 2, loop 4 and the C terminus (magenta) and from the N-terminal helix (cyan) interact with corresponding residues (orange sticks) from the C-terminal region of the ligand (C_α trace, red). The C-terminal end of the ligand is stabilized by an intermolecular hydrogen bond (black dots) between the ligand backbone and a polar ECD residue in loop 5 (yellow sticks). N-terminal residues of the ligand not in contact with the ECD are depicted as a blue C_α trace. **(b)** Ligand binding by the CRF receptor ECDs: $CRF_{22–41}$ (shown as C_α trace, red and blue) in complex with CRFR1-ECD [25] (grey, same colour coding of residues; PDB code: 3EHU). Hydrophobic interactions between the ECD and the ligand are similar, but they lack contributions from the vestigial N-terminal α-helix of the ECD. The CRF C-terminal amide group is involved in two intermolecular hydrogen bonds to the ECD. **(c)** The CRFR1-ECD undergoes a conformational change upon ligand binding [25]. Backbone superposition of ligand-free (red; PDB code: 3EHS) and ligand-bound (green; PDB code: 3EHU) CRFR1-ECD (for simplicity, the ligand, $CRF_{22–41}$, is not shown). Disulphide bonds are shown as yellow sticks. Structural differences are observed in loop 2 (L2), loop 4 (L4) and at the end of loop 5 (L5).

terminal residues contact the 'outer' face of β-strands β3 and β4, followed by a pronounced kink in the center of the ligand towards the ligand-binding groove. This divides $PACAP_{6–38}$ into two approximately α-helical segments, with strikingly different positions of the N-terminal and central portions of the ligand (Figure 2a and 2b). Although this fundamentally different binding mode of $PACAP_{6–38}$ to $PAC1R_s$-ECD has fascinating implications for alternative recognition of peptide agonists and antagonists, the aforementioned discrepancy in the $PAC1R_s$-ECD loop 4 topology raises questions regarding the validity of this structure.

Despite the overall similarity in ECD ligand binding, noteworthy differences can be seen between the binding modes of GIP, exendin-4 and PTH compared with CRF and astressin. Superposition of the ECDs reveals that the positions of the bound $CRF_{22–41}$ and astressin α-helices deviate significantly from those of GIP, exendin-4 and PTH (5–7 Å; Figure 2). Displacements of the two CRF receptor ligands seem to originate from the much shorter N-terminal ECD helices of CRFR1-ECD and CRFR2β-ECD; in GIPR-ECD, GLP-1R-ECD and PTH1R-ECD, the extensive N-terminal helices contribute several hydrophobic con-

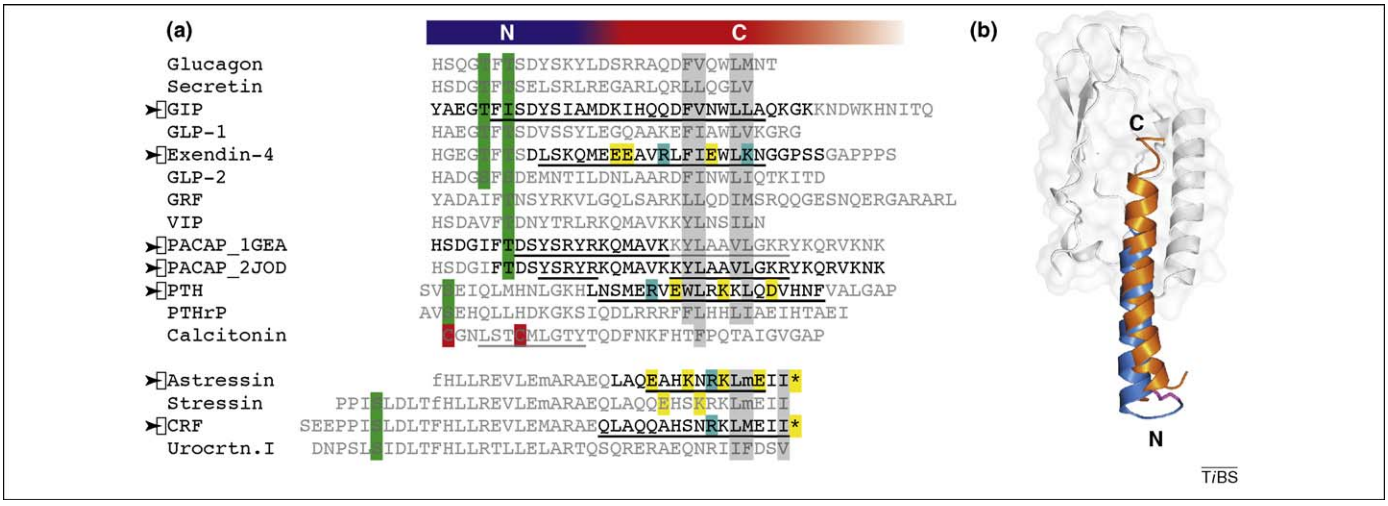

Figure 4. Class B GPCR ligands: destined for α-helix formation. The ECD complex structures enable structure-based alignment of known class B receptor peptidic ligands. Each sequence can be divided into an N- and a C-terminal part, the latter being responsible for interactions with the respective ECD. **(a)** Structure-based sequence alignment of class B GPCR ligands, indicating hydrophobic residues involved in ECD-binding (shaded in grey). The colour bar above the sequences illustrates division into N- (blue) and C-terminal (red) regions of the ligands. Ligands with known structure in ECD- or receptor-bound conformation are marked with an arrowhead; black letters represent structurally resolved residues, grey letters represent those not visible in the complex structures. Underlined residues are found in an α-helical conformation in the complex. Charged residues involved in ECD binding are shaded in cyan, putative helix-capping residues are shaded in green, residues involved in α-helix stabilization are shaded in yellow, cysteines involved in a disulphide-bridge (calcitonin) are shaded in red. Peptide modifications within the CRF group of ligands: asterisk, C-terminal amidation; f, D-phenylalanine, m, norleucine. Two different complex structures exist for PACAP: $PACAP_{1–21}$ bound to full-length receptor [69] (PDB code: 1GEA) and $PACAP_{6–38}$ in complex with $PAC1R_s$-ECD [21] (PDB code: 2JOD). **(b)** Structural superposition of free calcitonin [37] (blue, with the intramolecular disulphide bond in pink; PDB code: 2GLH) to $GIP_{1–42}$ (orange) in complex with GIPR-ECD [22] (grey; PDB code: 2QKH). Note that the conjectured ECD-binding C-terminal residues of calcitonin are unstructured in the free ligand.

tacts to the ligands (Figure 3). In addition, loop 2 of the CRF receptors contains an additional glycine residue, leading to restrictions in the ligand-binding groove. Thus, the structures indicate a further subclassification of class B GPCR ECDs into glucagon-like and CRF-like, which could be related to the much longer N-terminal sections within the CRF ligand group (Figure 4).

In addition to hydrophobic burial, a variety of polar and ionic interactions are observed that presumably fine-tune affinity and selectivity. Complementary structural and chemical properties of the ECDs and their cognate ligands are superimposed upon a common ligand scaffold of an amphipathic α-helix, with major contributions from the burial of hydrophobic residues. Nevertheless, the similarity in binding modes and determinants begs the question: what is it that governs ligand selectivity in the class B GPCRs?

Presenting the baton: evidence for α-helix formation during receptor binding

To recapitulate, the secretin family peptide hormones tend to be disordered in aqueous solution, but they are prone to adopt α-helical structures depending upon the ambient conditions or molecular environment. The ECD complex structures clearly show that this helical propensity of the isolated ligands translates into well-defined α-helical structures upon binding to the receptor ECD (Figure 2a). For most of the complexes, truncated peptides were used for structure determination (e.g. $PTH_{15–34}$, astressin and $CRF_{22–41}$; Figure 4a); in each case, peptide residues in contact with the ECD are completely helical from their (truncated) N termini (Figure 4). Several class B GPCRs display a correlation between ligand α-helicity with affinity and bioactivity. Intrinsic stabilization of α-helical conformations through intramolecular salt bridges has been observed in exendin-4 [23], PTH [24] and astressin [20]. The C-terminal amidation of CRF and its analogues (a prerequisite for bioactivity [64]) serves the same purpose, enabling an intramolecular hydrogen bond to form between the terminal amide and a preceding carbonyl group within the helix [20,25]. The design of synthetic ligands in which intramolecular lactam-bridges restrain the α-helical ligand conformation or α-helical linker segments has facilitated the generation of several potent agonists and antagonists with increased affinity and efficacy [35,37,65–67].

Most noticeably, the α-helix in $GIP_{1–42}$ extends towards the N terminus (residues $GIP_{6–14}$), although only the C-terminal residues $GIP_{15–32}$ are in contact with the GIPR-ECD; similarly, residues exendin-$4_{9–14}$ project away from the GLP-1R-ECD, with residues exendin-$4_{9–38}$ forming a continuous α-helix (Figure 2a). Despite the fact that the N-terminal residues do not contact the ECDs, progressive N-terminal truncation of the ligands has an adverse effect on the affinity to the ECDs, as observed in binding

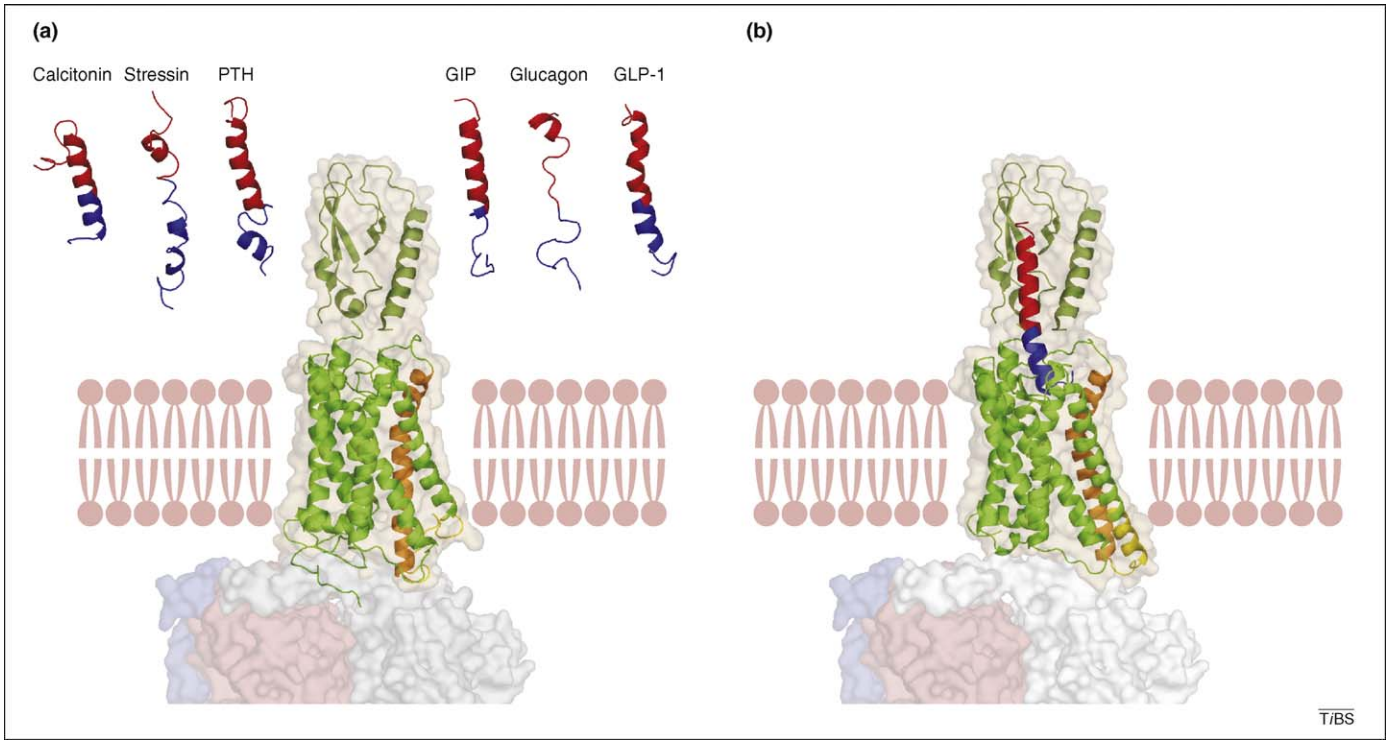

Figure 5. A model for class B GPCR activation. Structural model of a full-length class B GPCR, comprising an ECD (GIPR-ECD [22]) and the transmembrane domain (bovine rhodopsin [71] and opsin [19]) interacting with a heterotrimeric G protein [72]; for clarity, the intracellular domain of the GPCR is not shown. In addition to the peptide linkage between the ECD C terminus and the transmembrane domain N terminus, the orientation of the ECD was adjusted manually according to distance constraints between the three N-terminal residues of GIP and residues of the transmembrane helices 2, 6 and 7 of GIPR, as experimentally derived for corresponding residues in other class B ligands and their cognate receptors (glucagon and glucagon receptor [73], secretin and secretin receptor [74], GLP-1 and GLP-1R [75], and PTH and PTH1R [76]). Nevertheless, other orientations of the ECD to the transmembrane domain are conceivable (Box 1). Owing to a lack of experimental constraints, we have neglected potential dimerization of the receptor [77]. **(a)** The free ligands are only partially structured in solution (N-terminal region coloured blue, C-terminal region coloured red). The inactive GPCR (green; transmembrane helix 6 in orange) is depicted as a preformed complex with the intracellular G protein (subunits G_α, G_β and G_γ coloured white, red and blue, respectively). **(b)** Binding of the C-terminal region of the ligand (red) to the ECD induces α-helix formation or extension to the N-terminal ligand region (blue). The rigidified N terminus of the ligand can insert into the transmembrane domain of the GPCR, inducing extension of transmembrane helix 5 (yellow) and an outward shift of transmembrane helix 6 (orange) as observed for the activated opsin. This mechanism would trigger receptor activation, initiating subsequent steps in signal transduction. PDB codes: GIPR-ECD, 2QKH; rhodopsin, 1U19; opsin, 3DQB; G-protein, 1GOT; calcitonin, 2GLH; stressin, 2RME; PTH, 1BWX; GIP, 2B4N; glucagon, 1KX6; GLP-1, 1D0R.

studies with GIPR-ECD and CRFR1-ECD [22,25]. Correlating with the reduced affinity is a marked decrease in helical propensity of the remaining peptide ligands.

Passing the baton: a model for class B GPCR activation

In combination with the current data on class A GPCRs, the helix formation observed upon ECD binding enables the formulation of an attractive mechanical model for class B GPCR activation (Figure 5). We suggest that binding of a peptide hormone to its cognate ECD, driven largely by the burial of hydrophobic residues within the ligand-binding groove, would result in α-helix formation in the peptide. Indeed, it has recently been suggested that the presence of helix-capping residues in the N-terminal region of numerous class B GPCR hormone ligands [68] (Figure 4a) might be crucial to receptor activation. The structure of $PACAP_{1–21}$ in the PAC1R-bound conformation [69], which shows an α-helix spanning residues $PACAP_{8–21}$, provides strong support for such a scenario. Affinity of the hormone for the receptor would be achieved through a combination of folding cooperativity, hydrophobic burial and disposition to helix formation; as each of these contributions will vary between different peptide ligand sequences, this provides a complex (and therefore highly specific) degree of selectivity.

In this context, the disulphide-stabilized calcitonin N-terminal α-helix, which is essential for receptor activation, provides an example of a 'constitutive' α-helical conformation of the ligand [37]. Superposition of calcitonin with the ECD-bound ligands $PTH_{15–34}$ and $GIP_{1–42}$ reveals a strikingly similar conformation and position of the N-terminal regions of calcitonin and $GIP_{1–42}$, respectively (Figure 4b). By contrast, the C-terminal residues of calcitonin destined to bind to the ECD ligand-binding groove are disordered in the free hormone, suggesting that ECD binding would also result in concomitant peptide folding. This last example, together with the clear differences between glucagon-like and CRF-like binding modes (Figure 3), suggests that individual hormone–GPCR pairs will utilize variations on the theme of hydrophobic burial, α-helix formation and ECD binding to achieve selectivity.

After helix formation, the N-terminal residues responsible for receptor activation can be presented to the transmembrane helix domain. A combination of a stiffening of the hormone and a contraction of the distance between the N terminus and the ECD-binding residues, possibly enhanced by ligand-induced conformational changes in ECD loops adjacent to the membrane domain, would enable accurate positioning of the crucial N-terminal residues at the 'activation site' (i.e. the location of retinal in rhodopsin [13] or the ligand-binding sites in the class A receptors [14–17]). This, in turn, would enable structural rearrangements in the transmembrane domain itself similar to those observed for activated opsin [19], leading to crosstalk with the intracellular G-proteins.

Although this scenario satisfies the biophysical constraints discussed in this article, much remains uncertain. Several models have been proposed (Box 1) based on a variety of experimental approaches. Until more structural information of the ligand N-terminal 'activation residues' is available, the detailed nature of the activation process will remain underdetermined. This, of course, would have ramifications for the positioning of the ECD relative to the transmembrane domain, as would ligand-induced conformational changes in the ECD. Finally, a pronouncement on whether the formation of a rigid helical rod is necessary and sufficient for receptor activation must await further experimentation.

Box 1. Alternative models of class B GPCR activation

Several proposals have been made based on peptide ligand structures combined with cross-linking data and analysis of mutant and chimeric receptors. Each model expands upon the 'two domain' hypothesis, with the ligand N terminus buried within the transmembrane domain (involving extensive contacts to extracellular loop 3 of the receptor) and C-terminal residues in contact with the ECD. The most pervasive of these models, each bearing implications for the relative orientation of the ECD to the transmembrane domain, are:

(i) The PTH receptor model [76]. This early model, based on the NMR structure of PTH, which indicated a discontinuous helix bent between residues 9 and 17 [28], was called into question after the crystal structure of $PTH_{1–34}$ [30] revealed a continuous α-helix. The PTH–PTH1R complex structure [24] confirmed the helical nature of residues 15 to 34, so that the 'bent helix' model seems unlikely in light of the present structures.

(ii) The VIP/PACAP receptor model [78]. Mapping of photoaffinity data to a model of the human VIP/PACAP (VPAC1) receptor ECD yielded a binding mode similar to the controversial PACAP–PAC1Rs NMR structure [21] (i.e. with a reverse orientation of the ligand helix). This proposal, which would imply a relative orientation of the ECD to the transmembrane domain diametrically opposed to ours, is at odds with the remaining structural data described in this review.

(iii) The secretin receptor model [79,80]. The secretin-ECD model, combining cross-linking data and molecular modelling, is in broad agreement with those described here. In incorporating the controversial 'endogenous agonist hypothesis', however, docking of the secretin N-terminal residues to the transmembrane domain yields a distinctly different overall structure. As ECD-derived peptides corresponding to loop 2 show full agonist behaviour, albeit with a reduced potency of more than four orders of magnitude compared to *bona fide* ligands, the authors have suggested that peptide agonist binding induces a conformational change in the ECD, thereby exposing a cryptic agonist within the ECD itself which, in turn, interacts with and activates the transmembrane domain. Considering the burial of the 'endogenous agonist' loop 2 region and its presumed role in the structural integrity of the ECDs, such a major conformational rearrangement within the ECD is difficult to reconcile with the present structural data.

Going into the next lap: concluding remarks and future prospects

The recent flurry of class B ECD structures sheds light on their interactions with cognate ligands and provides first insights into the initial steps of class B GPCR activation. The common secretin family recognition fold acts as a capturing module for class B GPCR ligands, facilitating their simultaneous folding into an α-helix. This, in turn, suggests an activation mechanism in which the ECD presents a well-structured α-helical ligand to the receptor transmembrane helix domain, generating signal transmission across the cell membrane.

Clearly, much additional work is required before a thorough understanding of the interactions between class B GPCRs and their cognate hormones and non-peptidic ligands can be reached. Further structural studies on isolated ECDs will help to resolve open questions concern-

ing ligand selectivity, the precise nature of helix formation and the extent of ligand-induced structural changes in the ECDs. A detailed understanding of class B GPCR activation awaits elucidation of full-length receptors: the next leg of the relay.

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