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Understanding the common themes and diverse roles of the second extracellular loop (ECL2) of the GPCR super-family

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ABSTRACT

The extracellular loops (ECLs) of G protein-coupled receptors (GPCRs) can bind directly to docked orthosteric or allosteric ligands, they can contain transient contact points for ligand entry into the transmembrane (TM) bundle and they can regulate the activation of the receptor signalling pathways. Of the three ECLs, ECL2 is the largest and most structurally diverse reflecting its functional importance. This has been shown through biochemical techniques and has been supported by the many subsequent crystal structures of GPCRs bound to both agonists and antagonists. ECL2 shares common structural features between (and sometimes across) receptor sub-families and can facilitate ligand entry to the TM core or act directly as a surface of the ligand-binding pocket. Structural similarities seem to underpin common binding mechanisms; however, where these exist, variations in primary sequence ensure ligand-binding specificity. This review will compare current understanding of the structural themes and main functional roles of ECL2 in ligand binding, activation and regulation of the major families of GPCRs.
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1. Introduction

G protein-coupled receptors (GPCRs) are a super-family of receptors. There are over 800 different GPCRs in humans and they are involved in almost every physiological process (Fredriksson et al., 2003; Bockaert and Pin, 1999; Rosenbaum et al., 2009). This makes them ideal targets for drugs and thus between 25 and 50% of therapeutics interact with these receptors (Salon et al., 2011). GPCRs undergo conformational changes that result in active and inactive conformations (Cherezov et al., 2007; Rasmussen et al., 2011b). Both drugs and natural agonists function by stabilising a particular conformation (Jazayeri et al., 2015). For most ligands to bind to a specific receptor propagating the signalling response, the extracellular regions of the receptor (specifically the extracellular domain (ECD) and the extracellular loops (ECLs)) are particularly important. It is known that the ECLs of GPCRs can permit or facilitate ligand-entry into the TM bundle, hold ligands within the TM domain, direct ligands towards their binding site, change the shape of the binding pocket or bind directly to orthosteric or allosteric ligands (Wheatley et al., 2012).

GPCR crystal structures and functional studies have shown that ECL2 is particularly important. ECL1 and ECL3 are often short loops that run parallel to the membrane connecting TM 2/3 and TM 6/7 respectively, however ECL2 is longer and shows much greater variation with its structural features (Venkatakrishnan et al., 2013). As well as this structural diversity, ECL2 is vital for ligand binding and the subsequent activation of the receptor (Wheatley et al., 2012).

The importance of ECL2 (connecting TM4 and TM5) was first identified through chimeric receptor experiments and mutagenesis studies of this domain (Olah et al., 1994; Walker et al., 1994; Fitzpatrick and Vandlen, 1994). Even in receptors with known TM ligand-binding pockets, the ECLs were implicated in early stage ligand-binding and presentation to the TM binding site (Colson et al., 1998; Perlman et al., 1997). In many cases ECL2 residues have been implicated in direct or indirect ligand-binding due to the location, flexibility and sequence variability of the loop (Kim et al., 1996; Wheatley et al., 2012).

Given the large recent increase in structural information, highlighting the importance of ECL2 in the extracellular region, this review will specifically focus on ECL2. The crystal structures, together with a large amount of biochemical data, have implicated ECL2 in many key aspects of receptor function (e.g. ligand binding and activation) and this loop often has a role in the current "hot topics" of receptor function (e.g. biased signalling, allosteric

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Abbreviations

5-HT1B	Serotonin 5-HT1B receptor
5-HT2B	Serotonin 5-HT2B receptor
A2AR	Adenosine A2A receptor
AT1R	Angiotensin II type 1 receptor
AT2R	Angiotensin II type 2 receptor
β1AR	β1 adrenergic receptor
β2AR	β2 adrenergic receptor
CCR5	C–C chemokine receptor type 5
CB1	Cannabinoid receptor 1
CLR	Calcitonin receptor-like receptor
CRF	Corticotropin-releasing factor
CRFR1	Corticotropin-releasing factor receptor 1
CXCR	C-X-C chemokine receptor
D3R	Dopamine 3 receptor
ECD	Extracellular domain
ECL	Extracellular loop
ET-1	Endothelin-1
FFAR1	Free fatty acid receptor 1
GCGR	Glucagon receptor
GIP	Gastric inhibitory polypeptide
GIPR	Gastric inhibitory polypeptide receptor
GLP1	Glucagon-like peptide-1
GLP1R	Glucagon-like peptide-1 receptor
GPCR	G protein-coupled receptor
H1R	Histamine H1 receptor
ICL	Intracellular loop
LB	Lobe
mGLUR	metabotropic glutamate receptor
M2R	M2 muscarinic acetylcholine receptor
M3R	M3 muscarinic acetylcholine receptor
NMR	Nuclear magnetic resonance
N/OFQ	Nociception/orphanin FQ peptide
OR	Opioid receptor
PAR1	Proteinase-activated receptor 1
RAMP1 (R1)	Receptor activity modifying protein
S1P1R	Sphingosine-1-phosphate 1 receptor
SCAM	Scanning cysteine accessibility method
TM	Transmembrane
VFT	Venus fly trap

modulation). This review will start by summarising the structural information known for this loop. This will be followed by a comparison of the functional characteristics of ECL2 in the super-family. Finally, we will discuss whether this loop acts as a molecular "gatekeeper" controlling the course of activation of the receptor.

2. Structure of ECL2

A number of GPCR crystal structures have now been published. These have provided important information with respect to both structure and contact sites with the bound ligand. In this section these structures are illustrated with ECL2 specifically highlighted. Key structural information has also been summarised. The crystal structures often include additional, useful 'non-structural' information (e.g. contact sites with a bound ligand), which will be discussed later in the review. For clarity the receptors have been grouped according to phylogenetic similarity or in some cases a shared structural or functional property.

2.1. An ECL2 β-hairpin structure forms an extracellular cap over the TM bundle in receptors with hydrophobic or covalently attached ligands

The unique structural properties of ECL2 were first highlighted in the crystal structure of bovine rhodopsin (see Fig. 1 and Table 1), which found ECL2 to be an antiparallel β-sheet in a hairpin formation (β-hairpin) extending deep into the centre of the TM bundle (Palczewski et al., 2000). ECL2 makes extensive contacts between both the retinal ligand and the extracellular regions. It also associates with the extracellular N-terminus and ECLs to form an extracellular cap over the TM bundle, closing off the ligand-binding pocket to the extracellular side (the "closed" ECL2 position).

This closed extracellular cap involving ECL2 is a feature that is also present in the sphingosine-1-phosphate 1 (S1P1) receptor (see Fig. 1 and Table 1) and the GPR40 (free fatty acid receptor 1, FFAR1). In both these structures, ECL2 contains a β-sheet structure. In the S1P1R the N-terminal ECD folds over and packs tightly with ECL1 and ECL2 forming a cap over the receptor. This creates the top surface to a ligand-binding pocket (Hanson et al., 2012). In GPR40 the β-sheet structure is preceded by an auxiliary loop (Srivastava et al., 2014). The GPR40 ECL2 has low flexibility and functions as a cap over the binding site, similar to that observed with the S1P1R.

2.2. The ECL2 β-hairpin structure is "open" in the family A GPCR peptide, opioid and chemokine receptor sub-types

The ECL2 β-hairpin structure is common in many GPCRs and is a feature shared across receptor sub-families. However, compared to its "closed" position in the extracellular capped receptors (with hydrophobic or covalently attached ligands) the loop is open for receptors with soluble ligands. "Open" means that the loop "allows" or mediates extracellular access to the TM bundle. These include the peptide-binding receptors as well as the opioid and chemokine receptors (both in the same sub-family (γ) of the family A receptors; see Fig. 1 and Table 1) (Wu et al., 2010, 2012; Park et al., 2012; Wu et al., 2012; Granier et al., 2012; Tan et al., 2013; Manglik et al., 2012). The nociceptin/orphanin FQ (N/OFQ) peptide receptor shares high sequence homology with the opioid GPCRs but has distinct pharmacology. ECL2 has the same β-sheet hairpin structure as the opioid and chemokine receptors (Thompson et al., 2012). The peptide binding endothelin ET_B receptor also shares this β-hairpin structure (Shihoya et al., 2016).

The β-hairpin structure is also present in the protease activated receptor 1 (PAR1). This is part of the δ-subfamily of the family A GPCRs, which include the glycoprotein receptors, the purinergic receptors and the olfactory receptors (Zhang et al., 2012). However PAR1 has been included in this section due to its activation through a (tethered) peptide ligand and a similar ECL2 structure.

2.3. There are a variety of ECL2 structures within the aminergic receptors

The aminergic receptors of the histamine H1 receptor (H1R), dopamine D3 receptor (D3R), muscarinic (M1, M2, M3, M4) receptors, serotonin receptors, β1AR and β2AR all have an open ECL2 conformation with this loop forming the top of a ligand-binding pocket (see Fig. 2 and Table 1). There are some structural differences between the receptors in this group. β1AR and β2AR have an

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