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Activation of G protein-coupled receptors : the role of extracellular loops in adenosine receptors

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CHAPTER 2

IMPORTANCE OF THE EXTRACELLULAR LOOPS IN G PROTEIN-COUPLED RECEPTORS FOR LIGAND RECOGNITION AND RECEPTOR ACTIVATION

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ABSTRACT

G protein-coupled receptors (GPCRs) are the major drug target of today's medicines. Therefore, much research is and has been devoted to the elucidation of the function and three-dimensional structure of this large family of membrane proteins, which includes multiple conserved transmembrane domains connected by intra- and extracellular loops. In the last few years the less conserved extracellular loops are becoming of increasing interest, particularly after the publication of several GPCR crystal structures that clearly show the extracellular loops are involved in ligand binding. This review will summarize the recent progress made in the clarification of the ligand binding and activation mechanism of class A GPCRs and the role of the extracellular loops in this process.

INTRODUCTION

G protein-coupled receptors (GPCRs) form a large family of transmembrane proteins that convey an extracellular signal as exerted by a hormone or neurotransmitter to an intracellular response through G proteins. They all have a similar structure, with an extracellular N-terminus, 7 transmembrane helices connected by three extracellular (EL1-3) and three intracellular loops (IL1-3), and an intracellular C-terminus. The GPCR super-family consists of five main classes, of which the class A (or rhodopsin-like) GPCRs form by far the largest subfamily [1]. Next to the N- and C-terminus, the extracellular loops of GPCRs are the most variable structural elements of the receptor, differing greatly in both length and sequence. Even within subfamilies, the extracellular loops often show low sequence homology, if any at all. Also, the early data on receptor architecture stemming from bacterio rhodopsin and bovine rhodopsin provided limited and incomplete information regarding these more flexible GPCR domains. This data paucity and ambiguity meant that structural studies of receptor function and activation (through e.g. mutagenesis) focused on the more conserved and better characterized regions of the receptor such as the transmembrane domains [2]. As a consequence, the average ‘textbook model’ states that mainly two domains are determinants for receptor activation, i.e. the region where the ligand binds and the domain that interacts with the G protein. In this view, the extracellular loops are mainly regarded as peptide linkers to hold the functionally important transmembrane helices together and keep these stably positioned in the cell membrane. However, over the last decade, it has become clear that the extracellular loops fulfill important functional roles in receptor activation and in ligand binding. For example, quite a number of somatic mutations in the loops have been linked to disease [3,4,5]. Therefore, the purpose of this review is to provide evidence that these neglected receptor domains are vital for proper receptor recognition and function.

EXTRACELLULAR LOOPS AS SEEN IN THE CRYSTAL STRUCTURES

GPCR crystallization is extremely challenging. There are at least two reasons for that: GPCRs are unstable outside the cell membrane and they are known to adopt many conformational states. The relatively unstructured loops add to the conformational diversity. This combination of fragility and flexibility is a major hurdle in obtaining good-quality crystals. Nevertheless, we now have access to a handful of GPCR structures [6]. From these structures it is apparent that the extracellular regions can indeed adopt very different structural forms (**Figures 1 and 2**); in particular the unique topologies of the second extracellular loop (EL2) are striking. In rhodopsin, EL2 dives deep into the ligand binding cavity, completely shielding the binding site from solvent access [7]. Within the loop itself, a β-hairpin is present, which together with the N-terminus, forms a four-stranded β-sheet with additional interactions between EL3 and EL1 [8]. In both the β_1- and the β_2-adrenergic receptors, EL2 shows a more open conformation, contains a small α-helix and is given extra rigidity by an intra-loop disulfide bridge [9,10]. The adenosine A_{2A} receptor shows a third structural feature, where EL2 forms an anti-parallel β-sheet with the first extracellular loop and is mainly constrained by the formation of three disulfide bridges with EL1. This anti-parallel β-sheet also causes EL1 to bend more towards EL2 than is seen in the other structures (**Figures 1 and 2**). EL2 has not been completely resolved in the adenosine A_{2A} receptor structure, emphasizing the great flexibility EL2 has, despite the restricting structural features. In addition, a non-conserved disulfide bridge is found within EL3 [11]. Very recently the crystal structure of another GPCR, the chemokine receptor CXCR4, was elucidated by Stevens and coworkers [12]. The authors managed to obtain two separate structures of this chemokine receptor subtype; one with a peptide antagonist bound, the other with a small molecule antagonist. The extracellular region of these structures adapts another, more open, conformation (**Figure 2**). EL2 contains an anti-parallel β-sheet within the loop that can be extended with another strand in the peptide when it is bound to the receptor. The conserved disulfide bridge connects the anti-parallel β-sheet in EL2 with the top of TM3. An additional disulfide bridge can be found between EL3 and the N-terminus. It is here, between the N-terminus and EL2, that the peptide ligand is bound. The small molecule ligand occupies the same binding pocket as the peptide ligand, filling only the deeper part of the binding site. The

presence of the additional disulfide bridge and the extended helix of TM6 make EL3 to be differently positioned compared to the other three structures (**Figure 1**).

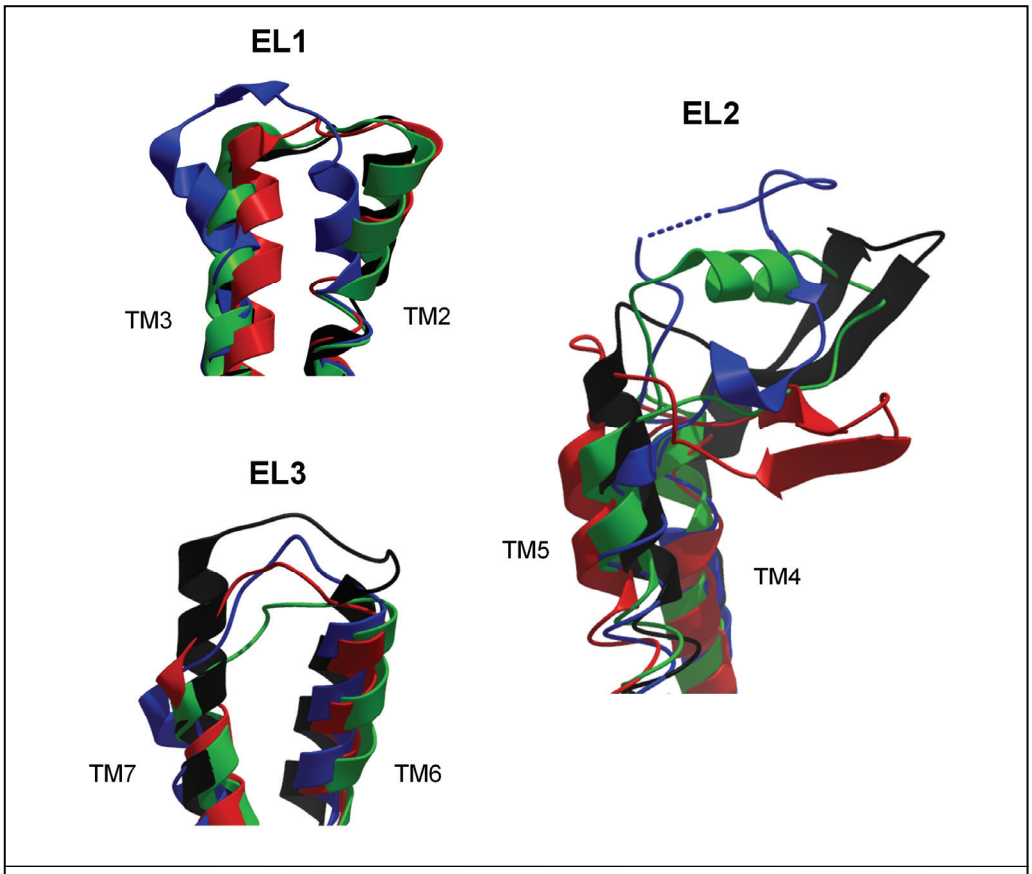

Figure 1. Overlay of extracellular loops of the crystal structures of the β2-adrenergic receptor (green) (PDB: 2RH1), the adenosine A_{2A} receptor (blue) (PDB: 3EML),CXCR4 (black) (PDB: 3ODU), and bovine rhodopsin (red) (PDB: 1U19). The transmembrane helices were superimposed by the backbone of the upper 17 amino acids in each helix while leaving the extracellular loops free during superposition. The superposition was created with Molsoft ICM version 3.6-1 h. The resulting figure shows the different orientation and structure of the loops between the four receptors.

Most GPCRs are expected to have disulfide bridges at their extracellular surfaces, possibly rigidifying the extracellular domains and providing structure to the receptor. It is interesting to note that in the A_{2A} receptor structure all available cysteines in the three extracellular loops are indeed involved in bridge formation. The most conserved disulfide bridge, between the third transmembrane domain and the second extracellular loop, is present in almost all class A GPCRs [7]. The formation of extra disulfide bridges may be an important general mechanism for regulating the activity of GPCRs [13]. Mutagenesis studies of extracellular cysteines have shown that these non-conserved residues are not always essential for receptor structure or binding; however, they might be important in other aspects, e.g. the kinetics of ligand binding

[13,14]. Worth and coworkers have recently listed all structural features present in the crystal structures in the transmembrane domains and the loops [15].

THE FIRST EXTRACELLULAR LOOP PROVIDES STRUCTURE TO THE EXTRACELLULAR COMPLEX

The first extracellular loop of class A GPCRs is usually very small, consisting of only a few amino acids. As in all three loops, its amino acid sequence is highly variable among family members. However, the length of the first extracellular loop is highly conserved (**Figure 3**), with over 70% of class A GPCRs having 52 amino acids separating the two most conserved residues in helices 2 and 3 (2.50 and 3.50) according to Ballesteros and Weinstein notation [16]. Similar analyses on other GPCR families showed that the EL1 of class C GPCRs is also highly conserved in length, with only one amino acid difference between the longest and smallest loop. Interestingly, among class B GPCRs, the first extracellular loop is highly divergent. It is even more variable in length than the second extracellular loop, which is the most divergent loop in class A GPCRs.

Even though EL1 is quite small and not directly involved in the binding of small ligands, several reports mention that the loop influences the shape of the binding pocket [17,18,19,20,21]. Together with other extracellular regions, the loop can provide rigidity and structure essential in receptor activation. Härterich et al. described an aromatic π-stacking region in the neurotensin 1 receptor that provides rigidity to the loop, keeping TM2 and TM3 together. Disturbance of the π-π interactions between aromatic residues within EL1 interfered with receptor activation and strongly reduced ligand binding [18]. A recent mutagenesis study of the neuropeptide S receptor showed that polar residues within the loop seem especially to influence receptor conformation [17]. This had been observed previously in the CCR2 receptor, where an asparagine and a glutamic acid residue were found to be essential for high affinity binding [22]. Charged residues in the loop were suggested to be important in vasopressin receptor activation; in particular, the positive charge of an arginine residue appeared required for stabilizing the active conformation of the receptor [23]. Very recently, two residues in EL1 of the M_4 muscarinic acetylcholine receptor, an isoleucine and a lysine, were identified as important for the signaling efficacy of the allosteric agonist LY2033298. This indicates that EL1 might also

influence signaling originating from a site distinct from the orthosteric binding site [24].

In contrast to most class A GPCRs, the binding site of receptors that bind large protein ligands is most likely found predominantly at the extracellular surface of the receptor. The large N-terminal part of glycoprotein hormone receptors may form the main contact for binding the hormones, as suggested from the crystal structure of the N-terminal domain of the follicle stimulating hormone receptor (FSHR) with its ligand [25]. In another study, the authors concluded that the extracellular loops in the thyroid stimulating hormone receptor (TSHR) work closely together in activation, as shown in the combined action of constitutively active mutants (CAMs) in these regions of the receptor. The strongest influence on the combined signaling activity was provided by EL1 [26]. The first extracellular loop might also contact (peptide) ligands directly. An indication for this was recently shown by a photo-affinity labeling study of the angiotensin II type 1 receptor (AT_1R). For this research, the authors labeled each consecutive residue of the endogenous ligand AngII with a photo-reactive label that can form a covalent complex with the site of interaction at the receptor. They found that the N-terminal part of AngII simultaneously interacts with several regions of the AT_1R, including the N-terminal domain and EL1 [21].

WXFG and DXXCR motifs in EL1

Two conserved structural motifs, WXFG and DXXCR, were identified within the first extracellular loop. The WXFG motif was first described by Klco et al. [19] and is present in 80% of class A GPCRs, including rhodopsin and the β-adrenergic receptors. Interestingly, the adenosine A_{2A} receptor is one of the few receptors that does not contain the WXFG motif. Several studies have shown that mutations in the WXFG motif disrupt receptor activation but not ligand binding [17,19,23]. Klco et al. [19] propose that the WXFG motif may be important in translating the ligand-binding signal directly to movements within the TM bundle. In addition, the motif might play a role in regulating the percentage of receptors that are in the high affinity ligand binding state. In the angiotensin II receptor, the WXFG motif has been postulated to form a type II β-turn that is involved in angiotensin II binding [27]. The second motif, DXXCR, is located in the carboxyl-terminal part of EL1 at the interface with TM3 and is highly conserved among peptidergic GPCRs. This region is probably engaged in

the formation of the classical disulfide bond with EL2 and has been shown to be involved in signal transduction and receptor activation in the V_{1A} vasopressin receptor [23,28]. In marked contrast, GPCRs that bind aminergic ligands, favour a negative charge (D or E) in the last position of the motif [23].

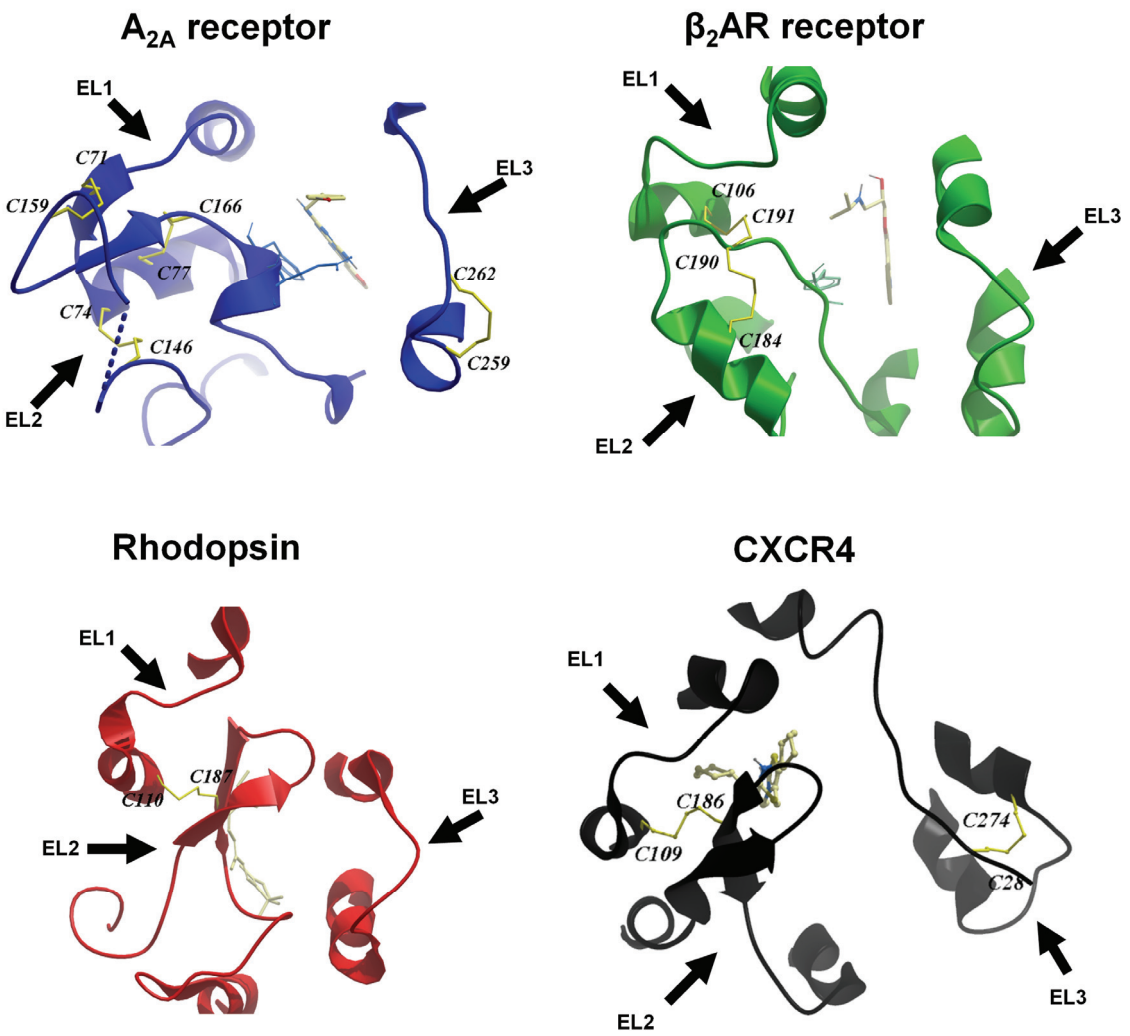

Figure 2. Top view of the crystal structures of the adenosine A_{2A} receptor (blue) (PDB: 3EML), the β2-adrenergic receptor (green) (PDB: 2RH1), CXCR4 (black) (PDB:3ODU), and bovine rhodopsin (red) (PDB: 1U19). The crystal structures are positioned so that all three extracellular loops are in view with their structural features (ribbons), the disulfide bridges (yellow), and the ligand bound in the ligand binding pocket. The conserved cysteine bridge between EL2 and TM3 is seen in all four structures: in the A_{2A} receptor betwee Cys77 and Cys166; in the β2-adrenergic receptor between Cys106 and Cys191; in CXCR4 between Cys 109 and Cys186; and in rhodopsin between Cys110 and Cys187. The figure was created with Molsoft ICM version 3.6-1 h.

AN ALL-ENCOMPASSING ROLE FOR THE SECOND EXTRACELLULAR LOOP (EL2)

The second extracellular loop in class A GPCRs is the largest and most divergent of the three. Both in length and in sequence, it can differ greatly even within subfamilies (**Figure 3**). In class B and C GPCRs, this difference in length is much less pronounced. These families are much smaller than class A GPCRs, especially family C only consists of 23 human receptors. The receptors in class B and C GPCRs all have large N-termini that mainly determine ligand selectivity between receptors. In class A GPCRs, EL2 might be associated with ligand selectivity, which would explain their large variety. Contrary to the concept that 'more conserved means more important', the second extracellular loop has been reported to be essential in normal receptor behaviour. EL2 is often the site for glycosylation; over 32% of class A GPCRs possess at least one consensus N-glycosylation site in the second extracellular loop [29]. Glycosylated or not, EL2 is thought to play a role in receptor structure, signaling, and ligand recognition, as well as ligand binding, both orthosteric and allosteric. Constraining the loop seems to be essential for receptor activation among all class A GPCRs, because disturbance of the conserved disulfide bridge between EL2 and TM3 largely diminishes receptor function [30].

EL2 as a "gatekeeper"

Despite the presence of a restricting disulfide bridge, the second extracellular loop needs a certain amount of conformational flexibility for efficient receptor activation [31,32,33]. In the M_2 muscarinic acetylcholine receptor, constraining EL2 loop flexibility by introducing extra disulfide bridges between the loop and TM7 had a profound inhibitory effect on intracellular signaling [31]. Forcing the EL2 of the receptor into a "locked" state impeded the binding of orthosteric ligands and, interestingly, had a great influence on the binding kinetics in both reducing association and dissociation rates [31,34]. The loop might adopt different conformations during the activation mechanism, starting with an open conformation to enable the entry of the ligand. After the ligand has moved into the binding site within the transmembrane helices, EL2 closes over the ligand and is further stabilized by engaging in additional interactions [31,35]. The stability and orientation of the EL2 lid is dependent on the extreme ends of the loop, though glycosylation sites within

the loop may also determine its positioning [29]. The proposed change from an open to a closed conformation was recently corroborated by cysteine scanning studies on the angiotensin II type I receptor. Cysteines introduced in two segments of EL2 were accessible by the cysteine-reactive biotin probe from the extracellular environment in the empty receptor, indicating the open conformation. These segments, positioned on either side of the conserved cysteine, were inaccessible when an agonist or antagonist was bound to the receptor. The residues that were inaccessible in the open conformation might regulate low basal activity of the ligand-free receptor [34]. Sum et al. suggested the presence of ionic locks at the extracellular surface, similar to the one seen at the cytoplasmic region of the crystal structure of rhodopsin [7,36]. These locks between EL2 and TM5 and between EL2 and TM7, would keep the receptor in its basal inactive state and cause constitutive activity when disturbed [36]. Klco et al. performed saturation mutagenesis of the complement factor 5a receptor (C5aR) that revealed many constitutively active mutant receptors. These results led to the conclusion that EL2 is a negative regulator of the receptor that keeps the receptor in a silent state prior to agonist-induced activation [33,37]. Also, alanine-scanning mutagenesis of the M_1 muscarinic acetylcholine receptor revealed that the access of ligands to the binding site was increased by mutation of EL2 residues [38]. Contrary to these findings, several other EL2 mutagenesis studies did not yield constitutively active mutants. However, also in these studies, specific residues that were shown to be important in stabilizing the active conformation of the receptor were identified [32,39].

The recent advances in nuclear magnetic resonance (NMR) technology enable us to look more closely at protein dynamics and structure and will help us greatly in unraveling conformational changes during receptor activation. So far, no whole GPCR structure has been resolved by NMR. However, parts of the receptor, including the extracellular regions, have been elucidated which provided similar results to what is seen in the crystal structures [40]. In crystallography the receptor structures are highly constrained due to crystal packing. This is not so much the case in NMR spectroscopy, rendering it possible to gain insight in receptor dynamics and activation. Solid state NMR studies revealed that EL2 of rhodopsin might form a reversible gate that opens during the activation process. Upon activation, a coupling between movements of EL2 and TM5 has been observed, as well as a rearrangement in the hydrogen-bonding networks connecting EL2 with the

extracellular ends of TM4, TM5, and TM6 [41]. Even more recently, Bokoch et al. used NMR spectroscopy to investigate ligand-specific conformational changes around a salt bridge in the β_2-adrenergic receptor that links EL2 with EL3. They were able to detect a relative motion between EL3-TM7 and EL2 upon ligand binding and to distinguish three different conformations of the extracellular surface: a ligand-free/antagonist, an inverse agonist, and an agonist conformation [42].

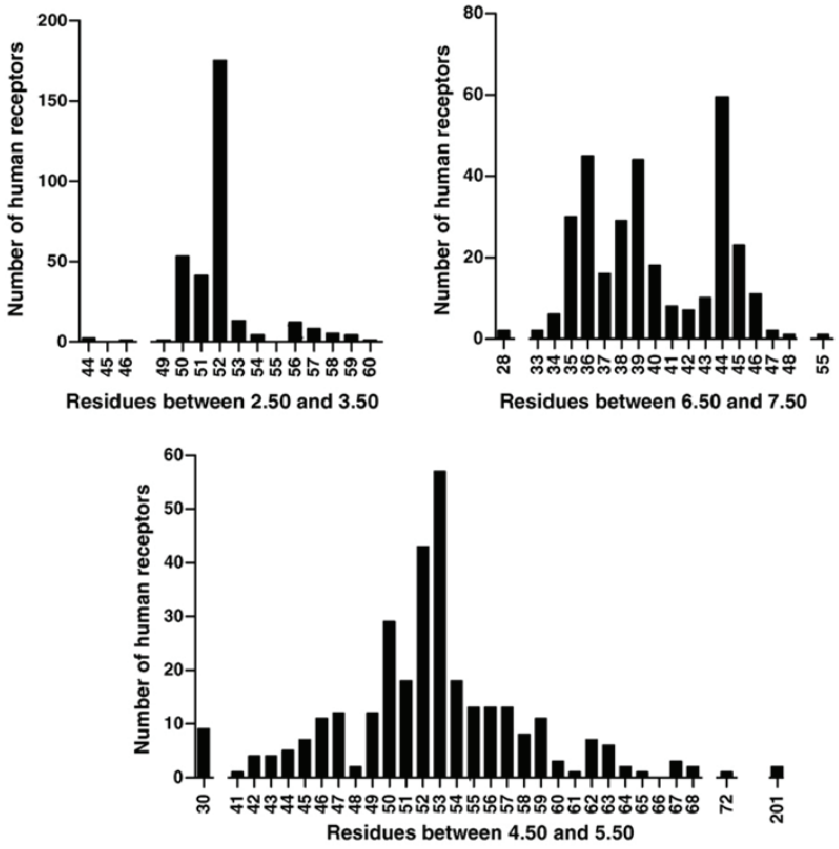

Figure 3. Distribution of the length of the extracellular loops among Class A GPCRs. The distance between the conserved Ballesteros and Weinstein marks in the transmembrane domains that are connected by the extracellular loops were taken as a measure for the distribution of the length of the loops. The distance between positions 2.50 and 3.50 was calculated for EL1, between 4.50 and 5.50 for EL2, and between 6.50 and 7.50 for EL3. The graph represents all human Class A GPCRs present in the GPCRDB (http://www.gpcr.org/7tm/), with on the x-axes the distance between the conserved marks. The average number of amino acids between the marks is 52 ± 2 (EL1), 53 ± 13 (EL2), and 40 ± 4 (EL3).

EL2 in ligand recognition and binding

The second extracellular loop can contribute to the specificity of ligand binding by directly forming part of the ligand binding cavity. This had been shown in studies on ligand selectivity in aminergic and other small molecule binding GPCRs [30,31,32,37,43]. Direct interactions of EL2 with the bound ligand are observed in the crystal structures, in which the EL2s interact directly with the ligand by a phenylalanine in the centre of the loop [7,9,10,11]. In the structure of the adenosine A_{2A} receptor, a glutamic acid residue in EL2 also contributes to the ligand binding pocket (**Figure 2**) [11]. The newest crystal structure of CXCR4 is an exception. The ELs shape the binding pocket with electrostatic and hydrophobic interactions, but no direct contact is made with the small ligand. The peptide ligand does form hydrogen bond connections with the EL2 backbone residues Asp187 and Tyr190. Also, the peptide forms a β-strand that extends the β-sheet present in EL2 [12]. The agonist binding pocket is most likely different from the antagonist binding domain, but considerable overlap should exist between the different cavities with EL2 as a participant. Consistent with this suggestion, mutagenesis studies of the prostacyclin receptor showed distinct but also overlapping residues in EL2 that are important for agonist and antagonist recognition [44]. EL2 was identified in the gonadotropin releasing hormone (GnRH) receptor, the cannabinoid 1 receptor and the adenosine receptors as a determinant in recognizing a ligand as an agonist, antagonist, or inverse agonist. In particular, the C-terminal part of the loop appears to influence the signaling ability of a ligand [20,45,46,47].

EL2 might also be an important determinant in subtype selectivity of ligands. The bound antagonist in the crystal structure of the adenosine A_{2A} receptor, ZM241385, is clearly protruding out of the transmembrane regions into the extracellular domain with a long side chain (**Figure 4**). Structure-activity relationship (SAR) studies performed in our laboratory revealed that smaller substituents at this part of the ligand greatly reduced the A_{2A} receptor selectivity [48]. The conformation EL2 can adopt to accommodate these large side chains might be receptor-specific and can be used in the design of subtype-selective ligands.

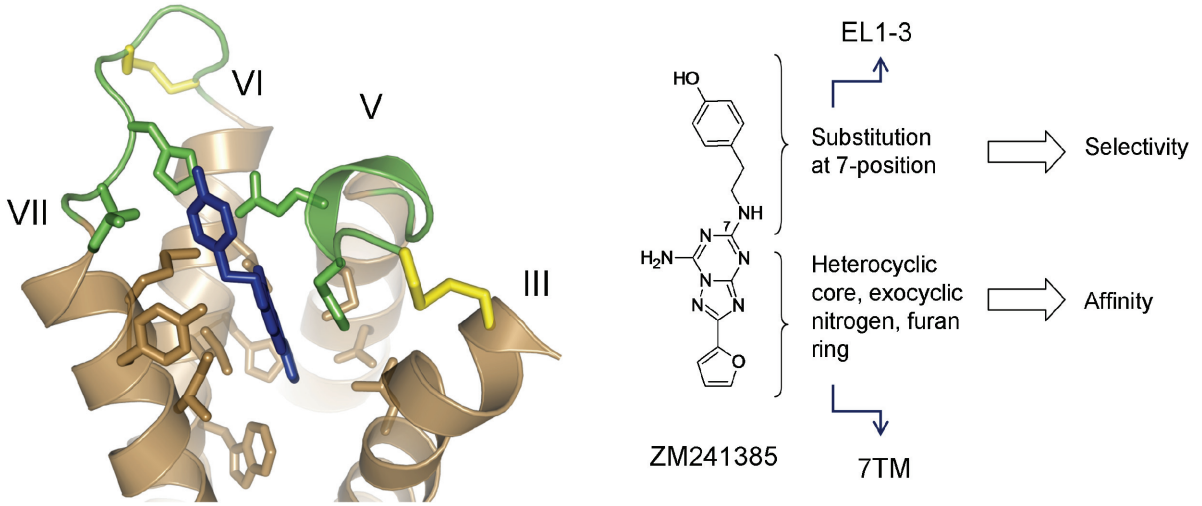

Figure 4. The binding pose of ZM241385 in the adenosine A_{2A} receptor crystal structure. Tan: TM domains, green: EL domains, yellow: disulfide bridges, blue: ZM241385. For clarity only helices III, V, VI and VII are shown, as they make up most of the binding site. The 4-hydroxyphenylethylamine side chain of ZM241385 interacts with the extracellular loops and is largely responsible for receptor subtype selectivity, whereas the rest of the ligand molecule is located within the TM domains of the receptor.

EL2 in allosteric modulation

GPCRs are subject to allosteric modulation [35], suggesting the presence of ligand binding sites other than the orthosteric site. These alternative binding sites are able to bind non-endogenous molecules in a specific manner and can influence the binding and function of an orthosteric ligand (e.g., a neurotransmitter or hormone). Allosteric binding sites may be found in non-conserved receptor regions that originated by chance during evolution. Therefore, it is entirely feasible that such binding pockets may be found at the extracellular surface of the receptor. The muscarinic acetylcholine receptors (mAChR) have been studied most in the search for binding sites of allosteric modulators in GPCRs. Several residues in EL2 have been shown to contribute to the allosteric binding of prototypical mAChR modulators, in particular the EDGE motif centrally located in the loop of the M_2 mAChR [31,49]. Also, a phenylalanine in EL2 of the M_4 mAChR has been shown to interact with the allosteric agonist LY2033298, whereas it did not influence binding of orthosteric agonists [24]. In a recent study on the adenosine A_1 receptor, bivalent ligands were used that connect an orthosteric ligand with an allosteric modulator to probe the location of the allosteric site relative to the orthosteric site. The authors speculated that the allosteric binding site of the adenosine A_1 receptor is located within the

boundaries of the second extracellular loop [50]. The ability of many modulators to slow down orthosteric ligand dissociation has been explained by a “capping” mechanism of the EL2 lid, stabilizing its closure [30,31,51].

THE THIRD EXTRACELLULAR LOOP (EL3) INFLUENCES LIGAND BINDING AND ACTIVATION

The third extracellular loop (EL3) is perhaps the least investigated of the three loops. Like EL1, it is small in all class A GPCRs (**Figure 3**). This is also the case in class B and class C GPCRs. Nonetheless, the third extracellular loop has been proposed to be important in GPCR signaling [19,26,52]. Claus et al. identified the presence of a hydrophobic cluster of amino acid residues within EL3 of the thyrotropin receptor that strongly influences signal transduction and G protein activation [53]. This confirmed the earlier described presence of such a hydrophobic cluster in EL3 of the δ-opioid receptor, in which it was suggested to form a hairpin-like structure essential in the early steps of receptor activation [39]. Claus et al. also showed evidence supporting an interaction between EL2 and EL3 in the form of a hydrogen bond that is necessary for proper folding and signaling of the receptor [53]. An interaction between aromatic residues in EL2 and EL3 has recently been proposed to play a key role for allosteric/orthosteric binding and activation cooperativity in the muscarinic M_2 acetylcholine receptor [49,51]. One particular residue in EL3 of the human M_4 mAChR, D432, appears to be involved in the functional cooperativity between the allosteric modulator and the endogenous agonist [24]. EL3 has been shown to form interactions with other extracellular regions as well; in rhodopsin, EL3 is connected to the N-terminal domain through hydrogen bonds with the oligosaccharide chain on N2 [7]. In the angiotensin II receptor, a cysteine bridge seems to link the two domains together. Such interactions must be receptor-specific as the presence of cysteine residues and glycosylation patterns vary considerably [52].

For the neurotensin 1 receptor, the urotensin receptor and the CC chemokine 2 receptor (CCR2), EL3 has been suggested to be part of the ligand binding site that is supposed to form a tunnel between EL3, TM6 and TM7 [18,20,54,55]. The crystal structure of the chemokine receptor CXCR4 indeed shows that EL3, together with the N-terminus, shapes the entrance to the ligand-binding pocket [12]. In the structure of

the adenosine A_{2A} receptor, a histidine in EL3 and a leucine at the interface between EL3 and TM7 are in close proximity to the bound ligand ZM241385 [11].
These results clearly imply an important role of the third extracellular loop in GPCR function. It has yet to be determined whether EL3 individually is essential in ligand binding and receptor activation, or whether it is a vital and integral part of the extracellular domain.

ANTIBODIES AGAINST THE EXTRACELLULAR LOOPS

Many commercially available monoclonal antibodies for GPCRs are raised against epitopes located at the extracellular surface of the receptor, most often EL2. However, these antibodies frequently are receptor conformation-dependent and have met with limited success, for example in immuno-histochemistry [56]. Owing to subtle changes in receptor conformation, segments of the loop become more or less accessible. If the flexible EL2 moves to interact with the ligand, then the necessary epitopes for antibody recognition are potentially shielded [34,56,57]. The changes in receptor conformation may also explain how auto-antibodies against human GPCRs are involved in auto-immune diseases, such as preeclampsia and malignant hypertension [34,56,58]. These endogenous antibodies functionally interfere with the target, showing an agonist-like effect and thus also contribute to changes in receptor conformation. Several reports have also described monoclonal antibodies directed against EL2 of the M_2AChR and the β_2-adrenergic receptor with functional effects, acting as agonists or inverse agonists [59,60,61].

CONCLUSION: A CLOSE COLLABORATION AMONG THE EXTRACELLULAR LOOPS

All three extracellular loops are relevant for the activation mechanism of class A GPCRs. EL1 probably provides structure to the extracellular region of the ligand binding site and enables movement of the transmembrane helices upon ligand binding. EL2 seems to play the most important role in activation, because it is involved in direct ligand binding, ligand recognition and ligand entry. It might also host allosteric binding sites. EL3 seems essential for proper folding and signaling of

the receptor, might be a binding site itself, and may also contribute to allosteric binding sites. However, the strength of the extracellular loops lies in their collaboration. Proper receptor activation appears to be greatly dependent on a complex network of interactions at the extracellular region that recognizes and transmits the initial activation signals. The extracellular region should be looked at as a whole, in which the extracellular loops together provide structure and cooperative signal triggering that is required for full receptor activation [26].

Although all loops play an important role in the activation mechanism of GPCRs, details of their mode of action may differ among the family members. The great divergence of the loops and their flexibility provide each receptor with its own structural interactions and conformations to reach selectivity and specificity in binding their ligands and subsequent signaling.

In conclusion, the complex intra- and intermolecular interactions that develop and wane over time at the extracellular region of class A GPCRs, are vital for receptor activation as well as ligand binding.

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