Crystal structures of endothelin ET_B receptors revealed the activation mechanism by endothelin-1

Endothelin-1 (ET-1) is the most potent and long-lasting vasoconstrictor ever discovered, which is a 21-amino-acid-long peptide hormone isolated from the supernatant of porcine endothelial cells [1]. ET-1 and its related isopeptides ET-2 and ET-3 participate in various physiological processes, such as neural development, cell proliferation, sodium homeostasis and vascular regulation, by activating cytoplasmic G-protein signals through two subtype receptors, ET_A and ET_B, both of which belong to the class A G-protein-coupled receptors (GPCRs) [2].

ET_A and ET_B share 60% sequence similarity and both receptors bind to ET-1 in a quasi-irreversible manner with sub-nanomolar affinities and extremely low dissociation rates (on the order of days). However, the physiological roles of the two receptors are quite distinct. For example, in vasoregulation, ET_A induces primary vasoconstriction, whereas ET_B mainly induces vasorelaxation via NO-mediated signaling (Fig. 1). The endothelin system, including the three isopeptides and the opposing and balanced actions of the two receptors, is related to the progression of various diseases such as arterial hypertension, atherosclerosis, heart failure and cancer [3]. Therefore, non-selective and subtype-selective antagonists for the endothelin receptors (bosentan, macitentan and ambrisentan) have been developed and indeed clinically used for the treatment for pulmonary arterial hypertension (PAH). Despite such importance, little has been understood about how these receptors perceive endothelin peptides and activate cytoplasmic G-protein signals.
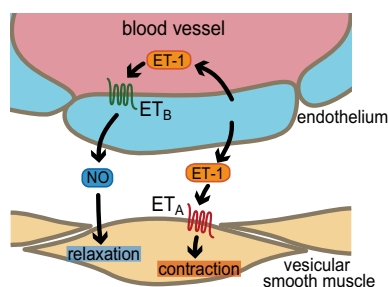

Fig. 1. Schematic diagram of the vascular tone regulation by endothelin. ET-1 is produced in the endothelial cells by multistep processing, and the mature peptides are secreted into extracellular spaces. While the secreted ET-1 can act on distant organs through the blood flow, most of the secreted ET-1 act in autocrine and paracrine manners. The ET_A receptor is predominantly expressed in the vesicular smooth muscle cells and mediates vasoconstriction, whereas the ET_B receptor in the endothelium cells mediates the NO release and thus induces vasodilation.

Recent advances in crystallographic techniques, such as lipidic cubic phase (LCP) crystallization and the use of the fusion-protein strategy to promote crystallization, have led to an increasing number of high-resolution GPCR structures [4]. Together with protein dynamics studies, these structures have allowed better understanding of the molecular mechanism of the GPCRs, such as agonist or antagonist binding, agonist-induced conformational changes and consequent G-protein activation. However, only a limited number of peptide-activated GPCR structures have been reported so far. Therefore, how peptide molecules, especially those with high molecular weights, bind and activate their receptors still remains poorly understood.

The intrinsic flexible nature of GPCRs generally hampers their crystallization. To overcome this problem, a thermostabilized ET_B receptor was established by mutagenesis screening [5]. Five point mutations were introduced to increase the stability of the receptor in the ligand-unbound state as compared with the wild-type receptor. This construct still retained the ability of agonist binding and G-protein activation and thus appeared suitable for visualizing multiple conformations of the receptor. T4 lysozyme (T4L) or modified T4 lysozyme (mT4L) was fused into the third intracellular loop (ICL3) to promote crystallization. The LCP crystals of these constructs were obtained in the absence or presence of the endogenous agonist ET-1, and the diffraction datasets were collected at the micro-focused beamline **BL32XU**, SPring-8. Finally, the structures of the ET_B receptor in the complex with ET-1 (Fig. 2(a)) and in the ligand-free form were determined at 2.5 Å and 2.8 Å resolutions, respectively [6] (Fig. 2(b)).

The ET_B receptor adopts the canonical GPCR fold with seven transmembrane (TM) helices and a short amphipathic helix 8 that runs parallel to the membrane. The ET-1 peptide penetrates into the receptor core, with its flexible C-terminal tail inserted in the depths of the binding pocket and specifically recognized by the receptor. This manner of binding is consistent with the essential role of the C-terminal tail of ET-1 for receptor binding and activation. The interacting surface area between ET-1 and the ET_B receptor is the largest ever reported for the GPCR structures. In addition, ECL2 and the N-terminal tail of the receptor together form a lidlike architecture that covers the orthosteric ligand binding pocket, thereby preventing ET-1 dissociation. These structural features well explain the irreversible binding of ET-1. The amino acid residues involved in the receptor interaction are highly conserved in the
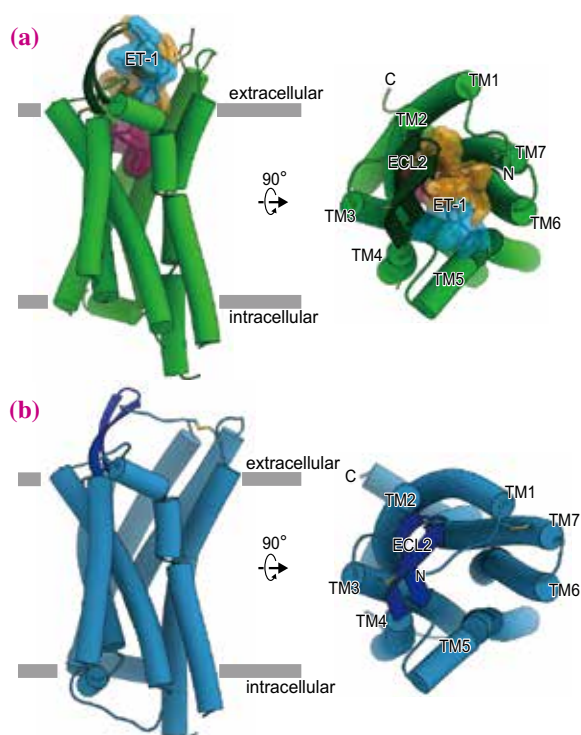
Fig. 2. Overall structures of the ET$_B$ receptor in different conformations. The crystal structures of the ET-1-bound **(a)** and ligand-unbound **(b)** receptors are shown as cylinder models, viewed from within the membrane plane (left panels) and from the extracellular side (right panels). The bound ET-1 peptide is shown as a surface representation with the same colors as those in the original paper; cyan: N-terminal region, yellow: α-helical region, red: C-terminal region. Transmembrane helices, ECL2 and the N- and C-termini are indicated by the labels.

mechanism proposed in this study is similar to those of other GPCRs, including the M2 acetylcholine receptor, the μ-opioid receptor and the β-2 adrenergic receptor, therefore suggesting a conserved mechanism for the G-protein activation in class A GPCRs.

In summary, the current study reported the first crystal structures of the ET$_B$ receptor in the ligand-unbound inactive and ET-1-bound partially active conformations, which revealed the molecular basis for the irreversible binding of ET-1 and the ET-1-dependent activation process of the receptor. These mechanisms are likely conserved in the ET$_A$ receptor. These notions provided a critical view for understanding the mechanism of the endothelin receptors and other peptide GPCRs. The current structures of the ET$_B$ receptor also provided important information for the development of drugs targeting endothelin receptors, thus paving the way for the more effective treatment of PAH and other diseases associated with the endothelin system in the future.
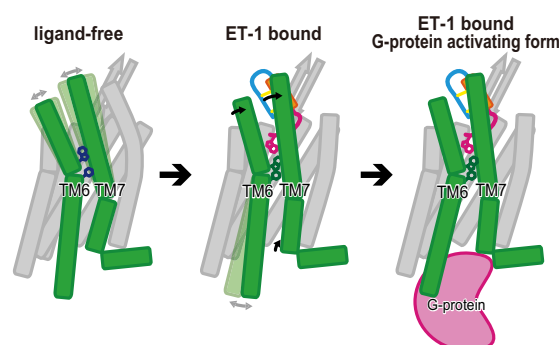

Fig. 3. Schematic diagrams of the receptor activation by ET-1. The extracellular portions are flexible when the receptor is in the apo state (left). ET-1 binding induces a major rearrangement in the orthosteric pocket, which involves the inward shift of the extracellular portions of TM6 and TM7 (middle). These conformational changes propagate to the receptor core and cytoplasmic side, which induces the cytoplasmic segment of the receptor to adopt a flexible conformation in the ET-1 bound state and thus promotes G-protein binding (right).

three isopeptides (ET-1 to ET-3). However, mutation analysis suggested that these three isopeptides have slightly different binding properties, probably caused by the indirect effects of the amino acid residues of their N-terminal regions that are not involved in the receptor binding. These results provided insights into the different selectivity between the two receptor subtypes.

The structural comparison of the ET-1-bound and ligand-free structures of the ET$_B$ receptor revealed agonist-induced dynamic conformational changes in the extracellular orthosteric pocket. These structures, together with the previous biochemical studies, suggested that the inward movement of the extracellular portions of TM6 and TM7 is the critical event for receptor activation. The ET-1-induced conformational changes propagate to the receptor core and also to the cytoplasmic side of the receptor, which involves the collapse of the allosteric Na$^+$ binding site that is conserved among class A GPCRs. These structural features suggested that ET-1 binding induces the flexibility in the cytoplasmic side of the receptor, especially in the cytoplasmic segment of TM6, thus promoting subsequent G-protein binding at the cytoplasmic interface (Fig. 3). The activation

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