Crystal structure of an antagonist-bound ghrelin receptor

Ghrelin, a peptide hormone consisting of 28 amino acids, was originally discovered in the stomach as an endogenous ligand for the growth hormone secretagogue receptor (GHSR, now called the ghrelin receptor), which belong to class A G protein-coupled receptors (GPCRs) [1]. The name "ghrelin" comes from the root word "ghre" in Proto-Indo-European languages meaning "grow", since ghrelin exhibits a potent growth hormone releasing activity. Moreover, ghrelin has a wide range of physiological functions that play roles in appetite stimulation, adiposity, energy homeostasis, memory formation, and hippocampal neurogenesis (Fig. 1) [2]. A salient feature of ghrelin is the *O*-acyl modification at Ser3, which is essential for its activity; des-acyl ghrelin (i.e., ghrelin lacking the acyl modification) is inactive. No other peptide hormone is known to require such an acyl modification for its activity. Despite such an interesting feature, because of the lack of structural information about the ghrelin receptor, it is unclear how the ghrelin receptor recognizes the acyl modification of ghrelin.

To facilitate crystallization, 28 and 20 residues were removed from the N- and C-termini of the ghrelin receptor, respectively, and the thermostabilized apocytochrome b_{562}RIL (bRIL) protein from *Escherichia coli* was fused to the deleted N-terminus. In addition, two mutations have been introduced: Thr130$^{3.39}$Lys (superscripts are the standard residue numbers for GPCRs in accordance with the Ballesteros-Weinstein nomenclature) to improve thermostability and Asn188Gln in the second extracellular loop (ECL2) to avoid glycosylation. Moreover, a Fab antibody fragment specific for the ghrelin receptor (Fab7881) was generated to increase thermostability and to promote the crystallization of the bRIL-conjugated truncated ghrelin receptor. We obtained microcrystals of the ghrelin receptor in a complex with Fab7881 and the antagonist Compound21 [3]. The structure of this complex was determined at 3.3 Å resolution using SPring-8 **BL32XU**.

Like other class A GPCRs, the ghrelin receptor has a canonical seven-transmembrane helical architecture and an intracellular amphipathic helix 8 (Fig. 2(a)). Similarly to other peptide hormone receptors, ECL2 forms antiparallel β-strands with a short hairpin and is stabilized by a highly conserved disulfide bond between Cys116$^{3.28}$ and Cys198^{ECL2}. Fab7881 binds to the third intracellular loop (ICL3) and seems to stabilize ICL3 and improve the thermostability of the ghrelin receptor, allowing its crystal formation.

An antagonist-bound ghrelin receptor has two main characteristics (Fig. 2(b)). There are a bifurcated ligand-binding pocket and the hydrophobic wide gap of transmembrane helices (TM) 6 and 7 (Fig. 2(b)). The binding pocket is separated into two cavities by a salt bridge between Glu124$^{3.33}$ and Arg283$^{6.55}$. Mutations of residues in the salt bridge, Glu124$^{3.33}$Ala and Arg283$^{6.55}$Ala, completely abolished the ghrelin-induced receptor function, whereas after replacement with the cognate amino acid mutants Glu124$^{3.33}$Asp and Arg283$^{6.55}$Lys, the receptor remains functional, although its activity is significantly reduced. Recent NMR and modeling studies suggest that the N-terminus of ghrelin extends down into the bottom of the ligand-binding pocket of the receptor, where it interacts with Glu124$^{3.33}$. Furthermore, the alanine mutation of two other polar amino acids in the ligand-binding pocket, Asp99$^{2.60}$ and Arg102$^{2.63}$, abolishes the receptor activity. Polar amino acids in the ligand-binding pocket are also important in other peptide hormone GPCRs, such as NTSR1 (PDB code; 4grv), ET_B (PDB code; 5glh), and AT2 (PDB code; 5xjm). These polar amino acids likely interact with the peptide main-chain and side-chain atoms in the binding pockets. Four polar amino acids in the ligand-binding pocket of the ghrelin receptor likely play a similar function.

Another characteristic feature of the ghrelin receptor is the gap between TM6 and TM7. We call this gap "the crevasse". Such a wide gap is often found in lipid GPCRs, such as EP4 (PDB code; 5yhl), GPR40 (PDB code; 4phu), and $S1P_1$ (PDB code; 3v2y). On the other hand, no peptide GPCRs with a gap structure have yet been reported. The crevasse of the ghrelin receptor

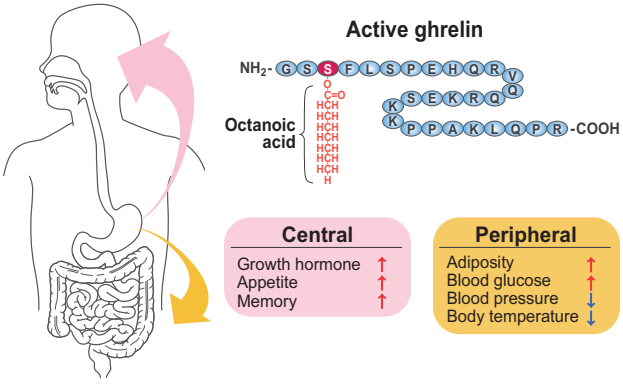

Fig. 1. Structure of human ghrelin and its physiological functions. Ghrelin secreted from the stomach is modified by octanoic acid to the active form, which exhibits various central and peripheral effects.

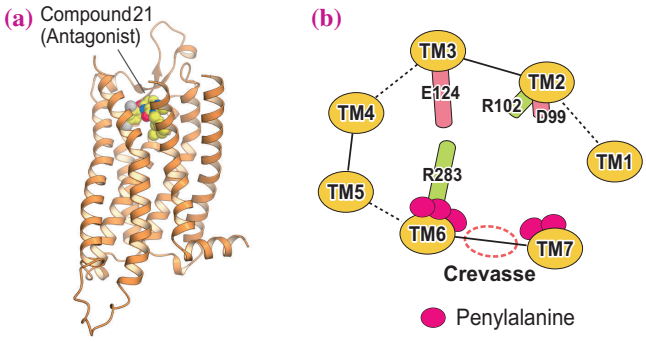

Fig. 2. Structure of the antagonist-bound ghrelin receptor. **(a)** Overall structure of the ghrelin receptor and **(b)** schematic model of the ligand-binding pocket of ghrelin receptor. The ghrelin receptor is shown in cartoon representation and colored in orange. Compound21 (antagonist) is shown as spheres and sticks with carbon atoms in yellow, oxygen in red, and nitrogen in blue. The crevasse is shown as a red dashed line. The five phenylalanine residues are shown as red circles.

contains five phenylalanine residues (Phe279$^{6.51}$, Phe286$^{6.58}$, Phe290$^{6.62}$, Phe309$^{7.39}$, and Phe312$^{7.42}$), indicating that the hydrophobic environment of the crevasse is suitable for receiving the acyl-modified moiety of ghrelin. Results of mutagenesis analyses of the phenylalanine cluster in the crevasse suggest that the cluster is important to the receptor activity. When phenylalanine residues, which are located at the bottom of the crevasse (Phe279$^{6.51}$, Phe309$^{7.39}$, and Phe312$^{7.42}$), are individually mutated to alanine, ghrelin-induced receptor activities are significantly reduced. By contrast, receptor activities of alanine mutants of Phe286$^{6.58}$ or Phe290$^{6.62}$, located near the extracellular surface of the receptor, were only slightly lower than that of the wild-type ghrelin receptor. These results may suggest that the role of phenylalanine residues at the upper part of the crevasse is different from that of the lower part. Some lipid GPCRs accommodate the hydrophobic moieties of their lipid ligands at positions corresponding to the bottom of the crevasse of the ghrelin receptor. For example, the acyl tail of ML056, an antagonist of the $S1P_1$ receptor, and both the tricyclic tetrahydrocannabinol ring and alkyl chain of AM11542, an antagonist of the CB_1 receptor, are placed in this position (Figs. 3(a,b)). These facts suggest that the acyl moiety of ghrelin could be located at the bottom of the ligand-binding pocket where it interacts with phenylalanine residues essential for receptor activation.

This study provides insights into the interactions between ghrelin and its receptor, and our findings may explain why the acyl modification of the ghrelin peptide is necessary for ghrelin receptor activation. Several ghrelin mimetics are under development for the treatment of cancer cachexia or metabolism-linked disorders, and our results may promote the design of more potent and effective ghrelin mimetics.

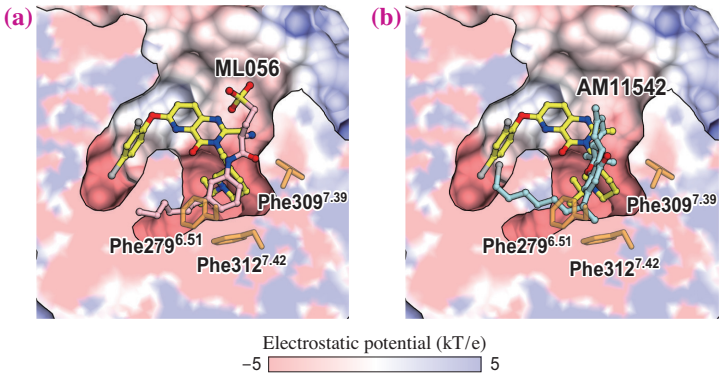

Fig. 3. Ligand binding modes of the ghrelin receptor, $S1P_1$ receptor, and CB_1 receptor. The ghrelin receptor surface and its cross section were colored with respect to electrostatic potential from red (negative) to blue (positive) using APBS tools. **(a)** $S1P_1$ receptor (PDB code; 3v2y) and **(b)** the CB_1 receptor (PDB cord; 5xra) are superposed onto the ghrelin receptor. Phe279$^{6.51}$, Phe309$^{7.39}$, and Phe312$^{7.42}$ of the ghrelin receptor and Compound21 are depicted as orange and yellow sticks, respectively. The sphingolipid mimic $S1P_1$ antagonist (ML056) and the CB_1 antagonist (AM11542) are depicted as pink and cyan sticks, respectively.

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