The FEBS Journal

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STRUCTURAL SNAPSHOT

# New views into class B GPCRs from the crystal structure of PTH1R

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**Keywords**
crystal structure; G protein-coupled receptor; osteoporosis; parathyroid hormone 1 receptor; protein engineering

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(Received 9 July 2019, revised 28 September 2019, accepted 29 October 2019)

doi:10.1111/febs.15115

The parathyroid hormone 1 receptor (PTH1R) is a major regulator of mineral ion homeostasis and bone metabolism and is thus considered an attractive drug target for the treatment of disorders in calcium metabolism and bone-related diseases such as osteoporosis. PTH1R is a member of the class B of GPCRs, which all share a dynamic multidomain binding mechanism to the peptide hormone. For a long time, these complexes have been recalcitrant to structural studies despite their great therapeutic relevance. Through extensive engineering of both the receptor and the peptide agonist ligand, we were able to determine the first high-resolution structure of a PTH1R–agonist complex. Comparisons of the PTH1R crystal structure with subsequently reported cryo-electron microscopy structures of the same receptor in complex with a G protein, as well as with other class B GPCR structures bound to antagonists, reveal new insights into the two-step activation mechanism of class B GPCRs and extend our understanding of the precise molecular rearrangements during receptor activation.

## Introduction

G protein-coupled receptors (GPCRs) constitute the largest family of human transmembrane proteins and respond to a diverse range of extracellular stimuli, mediated by small molecules, ions, lipids, peptides, or entire proteins. Since they control a wide variety of physiological processes, these receptors are major drug targets. The relatively small subfamily of secretin-like (class B) GPCRs comprises 15 receptors that regulate organism homeostasis, metabolism, and nervous system functions through interaction with specific peptide hormones. As such, these receptors are of great clinical importance for the treatment of a diverse range of chronic diseases including obesity, diabetes, osteoporosis, migraine, and depression.

The parathyroid hormone 1 receptor (PTH1R) is a key regulator in mineral ion homeostasis, skeletal development, and bone metabolism and can be activated by two endogenous peptide ligands, parathyroid hormone (PTH) and PTH-related protein (PTHrP), which have distinct biological functions (reviewed in ref. [1]). PTH is secreted from the parathyroid glands and controls mineral ion homeostasis by triggering calcium reabsorption and phosphate secretion in the kidney as well as calcium release from the bone. PTHrP is secreted from a diverse range of developing tissues, where it modulates cell proliferation and differentiation. Moreover, PTHrP secreted from tumors promotes metastasis and disease progression and is the most common cause for cancer-associated hypercalcemia [2].

Although endogenous PTH peptides have an inherent catabolic effect on bone, intermittently administered PTH was already found in the early 1930s to

**Abbreviations**
ECD, extracellular domain; ECL, extracellular loop; GCGR, glucagon receptor; GLP1R, glucagon-like peptide 1 receptor; GPCR, G protein-coupled receptor; ICL, intracellular loop; PTH, parathyroid hormone; PTH1R, parathyroid hormone 1 receptor; PTHrP, parathyroid hormone-related protein; TMD, transmembrane domain.

increase bone formation and mineralization [3]. The precise mechanism of the opposing effect of continuous and intermittent PTH action is still not fully understood. Yet, this principle has been successfully applied for the treatment of osteoporosis, a common disease in elderly people, which is defined by a continuous loss in bone mass and density, ultimately leading to spontaneous fractures. Currently, more than 200 million people worldwide are affected—a number which is predicted to expand drastically with the increased life expectancy of the population, especially also in developing countries. The most effective treatments of osteoporosis are based on daily injections of peptide analogues of PTH (teriparatide) or PTHrP (abaloparatide). Although highly potent, these therapies are both costly and inconvenient in application due to the requirement of a complex drug formulation and application by injection. Thus, a detailed understanding of the molecular determinants of PTH1R–hormone interaction could certainly lay the foundation for the development of improved, orally available therapeutics for the treatment of osteoporosis and other dysregulations of mineral ion homeostasis, similar to the successful development of oral semaglutide, a glucagon-like peptide 1 receptor (GLP1R) agonist, which recently completed phase III clinical trials for the treatment of type 2 diabetes and obesity [4].

PTH1R comprises a relatively large N-terminal extracellular domain (ECD), a hallmark of all class B GPCRs, in addition to the canonical hepta-helical bundle (helices I–VII) of the transmembrane domain (TMD), which when activated couples to intracellular effector proteins. In all class B GPCRs, the ECD is critically involved in peptide hormone binding as evidenced by earlier crystal and nuclear magnetic resonance structures of isolated ECD–ligand complexes (reviewed in ref. [5]). However, in the absence of the TMD, these structures could not provide insights into the precise determinants for hormone interactions within the orthosteric pocket of the TMD, which is required for receptor activation.

Despite their enormous potential to treat metabolic diseases, there has been a considerable lag in obtaining structures of class B GPCRs, predominantly owing to the limited expression levels and the inherent instability of these receptors. Initially, structural insights into class B GPCRs have become available through crystal structures of the isolated TMDs in complex with small-molecule antagonists. Compared to class A GPCRs (the largest and best-characterized class of GPCRs), these early structures revealed an extracellularly more open, V-shaped architecture of the TMD, and they allowed to identify both orthosteric and allosteric small-molecule drug binding sites (reviewed in ref. [6]). Subsequent crystal structures containing both the TMD and ECD in complex with small-molecule ligands provided a first glimpse into the two-domain architecture of these receptors. However, since the receptors were not bound to ligands that engaged both TMD and ECD, these structures could not explain the contribution of the ECD to full-length ligand binding [7,8]. This important information was made available only recently through the determination of structures of PTH1R and several other class B GPCRs in complex with bipartite peptide ligands.

## Engineering of a PTH1R: agonist complex for crystal structure determination

Biophysical and structural studies of PTH1R as well as of many other GPCRs have been severely hampered by the poor yield and quality of protein purified from recombinant expression systems. However, for crystallization-based structural biology projects, milligram quantities of pure, homogenous protein are indispensable. To overcome these limitations, we employed a directed evolution-based approach for membrane proteins, which had been developed in our laboratory, encompassing a combination of microbial cell surface display using randomized DNA libraries coding for a specific GPCR and subsequent selection of improved receptor variants by flow cytometry [9]. To select highly expressing receptors, fluorescent ligands are used which give a direct measure of functional receptor expression on the cell surface (as correct protein folding is required for ligand binding). This approach not only can boost receptor expression by several orders of magnitude in different heterologous expression systems, but many of the evolved receptor variants also exhibit higher thermostability [10,11].

While this method had been successfully applied to solve several structures of rhodopsin-like (class A) GPCRs [12,13], for PTH1R several additional steps had to be taken. Since the isolated N-terminal ECD of PTH1R itself is highly stable [14], we set out to selectively improve the TMD of PTH1R. This required a ligand that only binds to the orthosteric binding pocket within the TMD. As native PTH peptides require the ECD for binding and no potent small-molecule ligands for PTH1R are available, we employed an agonist consisting of the engineered N-terminal portion of the native PTH peptide, which we had labeled with a fluorescent dye. Despite high affinity and specificity of this ligand, several attempts to evolve PTH1R in an *E. coli* display system failed, most likely due to the high toxicity of expressed receptor for the bacterial host.

To overcome this limitation, we performed directed evolution of this membrane protein in yeast (*Saccharomyces cerevisiae*-based receptor evolution, SaBRE), which combines the advantages of the secretory eukaryotic quality control machinery with the capacity to display DNA libraries of great diversity [15]. By this strategy, we were ultimately able to evolve the PTH1R-TMD toward higher functional expression levels. Screening of the enriched selection pool of PTH1R-TMD yielded a variant (PTH1R-SaBRE) with significantly enhanced levels of functional receptor in yeast, insect cells, and mammalian cells combined with improved thermostability (unpublished data). With PTH1R-SaBRE, for the first time, purification of the isolated TMD of PTH1R from insect cell membranes in quantities and quality adequate for crystallization trials became possible. To ultimately solve the structure of full-length PTH1R, we further engineered the ECD by removing a large unstructured loop region of 44 amino acids, which connects α1 with β1 within the ECD, to avoid interference of this probably highly flexible part with crystallization. Importantly, this region of the ECD had not been resolved in a previous crystal structure of the isolated ECD [14], and it was furthermore shown to be dispensable for ligand binding and receptor function [16,17].

Although the thermostability of PTH1R-SaBRE was significantly increased in comparison with the wild-type PTH1R, numerous crystallization attempts with various fusion proteins tested at multiple insertion positions failed. This suggested that PTH1R-SaBRE required additional stabilization to support crystal formation in the lipidic cubic phase. Based on this assumption and previous similar experiences with the neurokinin 1 receptor, we designed and screened single point mutations throughout the predicted α-helices of the TMD in an attempt to identify positions that increase TMD thermostability. Together, these efforts yielded the thermostabilized PTH1R-TMD, which, together with an optimally inserted *Pyrococcus abysii* glycogen synthase (PGS) domain replacing the flexible intracellular loop 3 (ICL3) of the receptor, displayed an additional ~8 °C increase in thermostability when compared to PTH1R-SaBRE.

Yet, crystallization trials of the so-engineered PTH1R in complex with several peptide ligands, including the therapeutic peptide agonists teriparatide and abaloparatide, remained unsuccessful. We thus aimed to maximally constrain the conformational heterogeneity of the PTH1R:ligand complex. The first 14 amino acids of the peptide ligand are located in the TMD, but, when used as a 14-mer, show a decrease of several orders of magnitude in binding affinity and signaling potency, compared to the full-length peptide. With the aim to minimize the PTH pharmacophore, a modified 14-mer peptide, which incorporates non-natural amino acids, has been discovered through extensive structure–activity relationship studies [18], and it largely restores binding and signaling to that of wild-type ligand levels. To generate a bipartite ligand connecting both TMD and ECD of PTH1R, we then joined this engineered PTH N terminus with the C-terminal residues 15–34 of PTH and included two additional side-chain substitutions as well as a C-terminal amide, based on earlier reports of enhanced agonist affinity and potency [14,19,20]. The resulting 34-amino acid agonist, termed ePTH, covers the full sequence of PTH involved in PTH1R binding. Indeed, it helped to increase the thermostability by a further 7 °C when compared to receptor complexes with teriparatide (equivalent to the N-terminal 34 amino acids of endogenous PTH) or abaloparatide.

With these combined engineering efforts (Fig. 1), the purified PTH1R:ePTH complex finally yielded initial crystallization hits in lipidic cubic phase that could subsequently be optimized to diffract to a resolution of 2.5 Å, thereby enabling us to determine the first atomic resolution structure of PTH1R [21].

## The two-step activation mechanism of class B GPCRs

Upon activation, class B GPCRs undergo large conformational rearrangements involving both the ECD and the TMD of the receptor. Generally, the series of events can be broadly divided into two steps: (i) rapid, high-affinity trapping of the ligand C-terminal region by the receptor ECD, followed by (ii) interaction of the ligand N terminus with the TMD, leading to receptor activation. For PTH1R, the serial involvement of the ECD and TMD in ligand binding and receptor activation, as well as the kinetics of these events (time constants of 140 ms and 1 s for step (i) and step (ii), respectively), has been determined by Förster resonance energy transfer (FRET) studies in living cells [17]. The first step is governed by hydrophobic interactions of the ECD's structurally conserved contact surface with the ligand C terminus, adopting an amphipathic helix which binds with its hydrophobic surface portion to the ECD, while its hydrophilic surface remains solvent-exposed (Fig. 2A). The second step requires a defined orientation of ECD and TMD to each other, thereby allowing the hormone N terminus to insert and bind into the orthosteric pocket, which is centrally located in the hepta-helical bundle (Fig. 2A). Simultaneously, the ligand N terminus

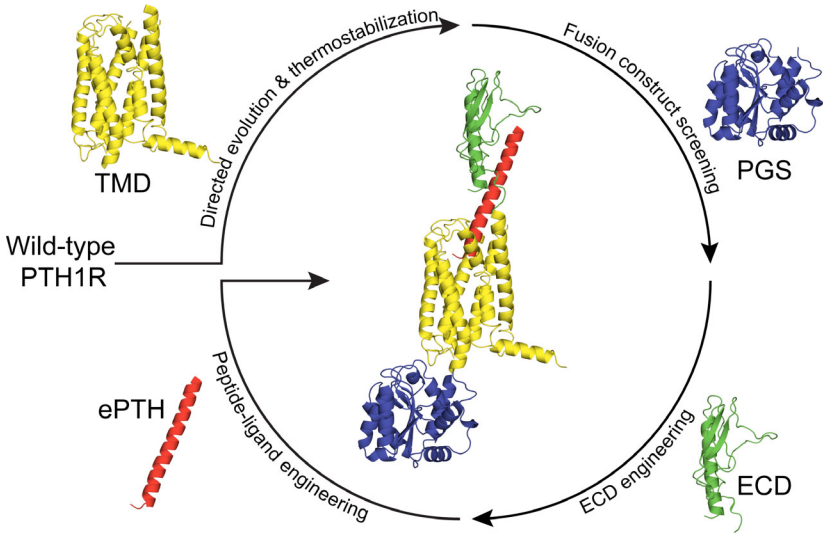


**Fig. 1.** Engineering of a PTH1R: agonist complex for crystal structure determination.

activates the receptor by introducing large conformational changes to the extracellular tips of helices I, VI, and VII (clockwise rotation of helix tips and unwinding of helix VI). These rearrangements culminate in a pronounced outward movement of the intracellular portion of helix VI (a hallmark for all GPCRs), exposing an intracellular binding cavity for the C terminus of Gα, thereby enabling intracellular G protein activation and signal propagation.

A key initial observation during model building and refinement of the PTH1R structure was the absence of electron density for the extracellular loop 1 (ECL1) and high temperature factors in the region that connects the ECD to the extracellular end of helix I of the TMD. In PTH1R, the overall orientation between the ECD and TMD thus appears to be governed solely through the peptide ligand itself, which engages a polar network, cross-linking ligand and receptor at the ECD–TMD junction (Fig. 3B). The identification of this interaction network rationalizes earlier findings that alterations in ligand or receptor side-chain identities in this region of the PTH1R:ePTH complex can drastically affect receptor function [22,23]. In contrast, ECL1 is not required for activation of PTH1R and, similar to the PTH1R crystal structure, a nonresolved ECL1 as well as polar contacts between ligand and ECD has subsequently also been reported in the structure of PTH1R in complex with another engineered agonist (LA-PTH) [16,24].

Prior to the determination of the crystal structure of PTH1R, the orientation between ECD and TMD of class B GPCRs was thought to be determined through interaction of ECL1 and 3 of the TMD with the ECD and a helical extension of helix I (denoted stalk) [25]. This mechanism was formulated based on structures of the glucagon receptor (GCGR), where the ligand was clamped between ECL1 and the stalk, and accordingly, ECL1 was shown to be indispensable for activation of GCGR [8,25,26]. Similarly, an interaction of ECL1 with the ligand and the ECD was reported from the structures of the closely related GLP1R in complex with several peptide agonists [7,27,28]. Therefore, the crystal structure of PTH1R reveals an additional important differentiation in secretin-type GPCRs with respect to the receptor portions involved in receptor activation, thus adding another layer of complexity to the purported two-step activation mechanism of this receptor class.

## Conformational changes during class B GPCR activation

Recent studies of other class B GPCRs had captured either the inactive state (crystal structures of antagonist complexes) or the G protein-bound state (cryo-electron microscopy (cryo-EM) structures of agonist complexes). The crystal structure of the PTH1R:ePTH complex now provides, for the first time, insights into an agonist-engaged, full-length class B receptor in the absence of a G protein. The comparison of the PTH1R:ePTH structure [16] with the subsequently published cryo-EM structures of the $G\alpha_s$-bound PTH1R in complex with LA-PTH [24] and with the crystal structure of the antagonist-bound state of the GCGR bound to the glucagon analogue NNC1702 (the only structure of a class B GPCR bound to a bipartite antagonist) [26] allows us to gain comprehensive insights into the detailed conformational rearrangements happening during receptor activation.

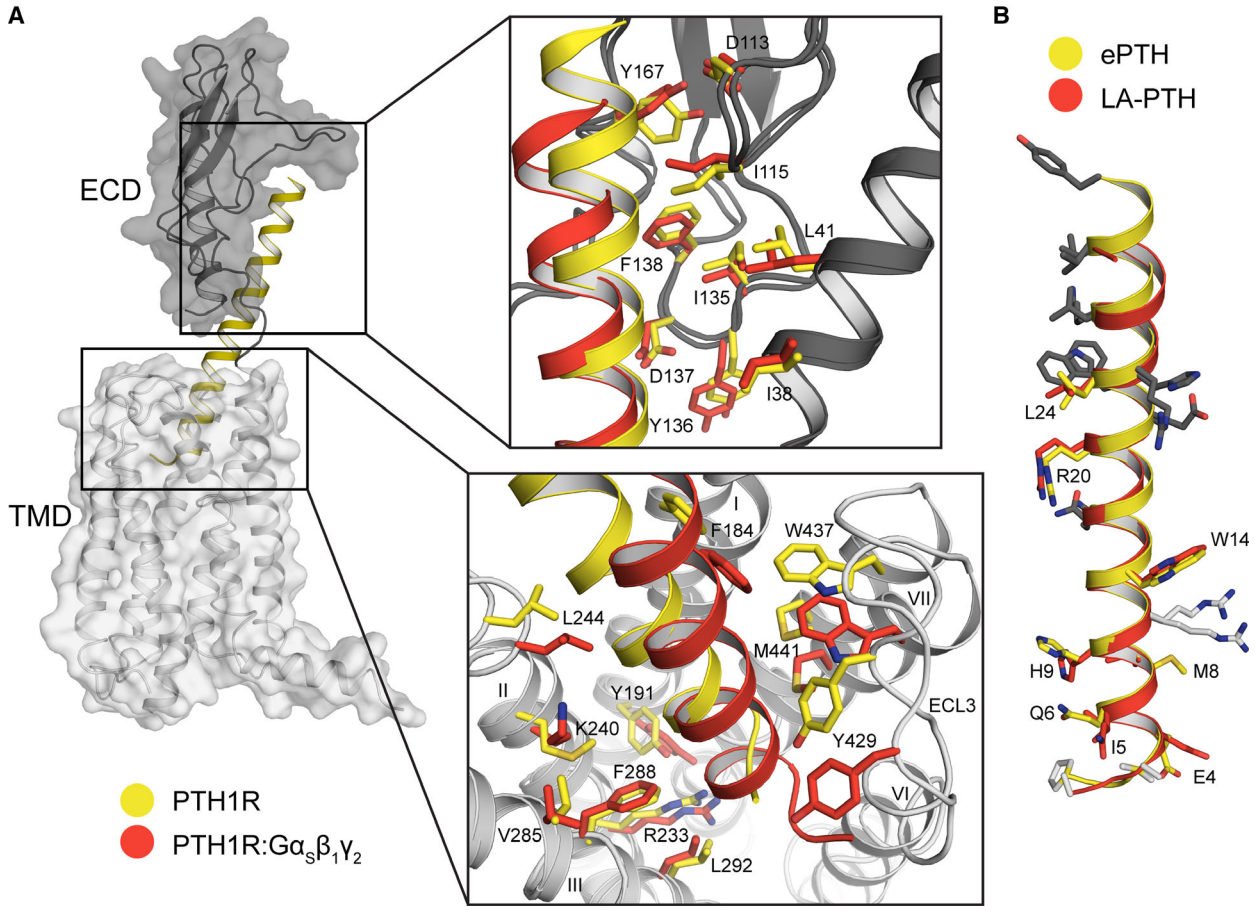


**Fig. 2.** Comparison of peptide agonist binding in PTH1R:ePTH and PTH1R:LA-PTH:G$\alpha_s\beta_1\gamma_2$. (A) Overview of PTH1R:ePTH with close-up views on superpositions with PTH1R:LA-PTH:G$\alpha_s\beta_1\gamma_2$ of the ligand binding site of the ECD and the orthosteric ligand binding pocket of the TMD colored in dark and light gray, respectively. The ligand and the receptor residues interacting with the ligand are highlighted in yellow and red for PTH1R:ePTH and PTH1R:LA-PTH:G$\alpha_s\beta_1\gamma_2$, respectively. Note that the G protein is not shown in the overview structure on the left. (B) Superposition of the peptide agonists ePTH and LA-PTH, colored as in A. Ligand side chains interacting with the ECD and the TMD are shown in dark and light gray, respectively. Side chains, which are identical in ePTH and LA-PTH, are highlighted in the respective color of the ligand.

Based on structural superpositions with the inactive state GCGR:antagonist complex and the G$\alpha_s$-bound PTH1R:agonist complex (Fig. 3A), the PTH1R:ePTH complex captures an intermediate conformation on the transition of the receptor toward the G protein-bound state. The extracellular receptor portion is already found in an essentially active conformation with the ECD adopting an orientation relative to the TMD that is already in transition between the inactive GCGR:NNC1702 structure and the PTH1R:LA-PTH:G$\alpha_s$ structure. Yet, the intracellular part of the receptor still displays the hallmarks of an inactive conformation. After the initial, fast binding of the ligand to the ECD (step 1), the receptor has to translate from an inactive state to the active, G protein-bound state. The PTH1R:ePTH complex captures a conformation where the peptide agonist already has induced the conformational rearrangements in the orthosteric pocket, but no G protein is bound yet to stabilize the conformational changes on the intracellular receptor portion. Thus, the PTH1R:ePTH complex for the first time provides structural insight into how the intermediate state on the receptor activation pathway may look like after the agonist has already bound.

Importantly, although the crystallized receptor:ligand complex has been engineered and stabilized in the absence of G protein, in the PTH1R:ePTH crystal structure as well as in the PTH1R:LA-PTH:G$\alpha_s\beta_1\gamma_2$ structure obtained by cryo-EM subsequent to our structure, the agonists are involved in the same set of interactions with the receptor. The interactions between the ECD and the respective peptide ligand are

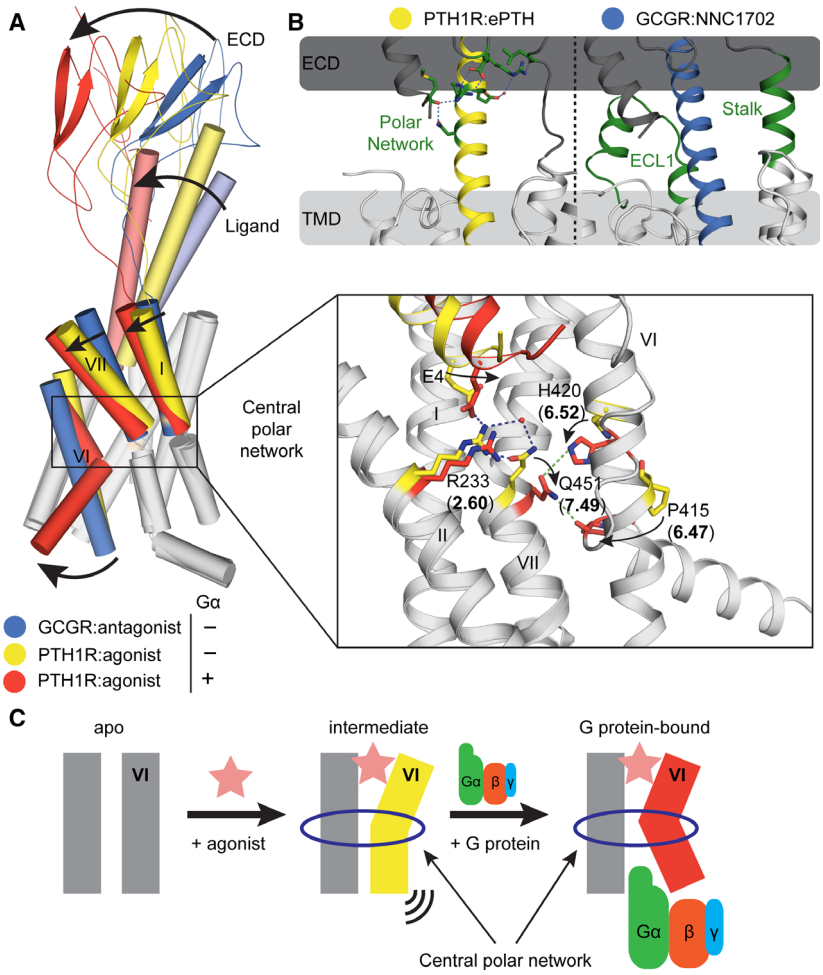


**Fig. 3.** Conformational changes during receptor activation. (A) Superposition of PTH1R:ePTH (PDB ID: 6FJ3), PTH1R:LA-PTH:$G\alpha_s\beta_1\gamma_2$ (PDB ID: 6NBF), and GCGR:NNC1702 (PDB ID: 5YQZ) with close-up view on the central polar network of PTH1R, colored as in Fig. 2. For clarity, ligands are colored in a lighter shade in the overview. Conformational changes are indicated by arrows. (B) Conformation-stabilizing interactions at the ECD–TMD junction of PTH1R and GCGR are highlighted in green with the ligand colored in yellow and blue, respectively. (C) Schematic representation of the receptor activation pathway. Left: inactive, ligand-free state (apo); middle: agonist-bound, intermediate state with extracellularly active receptor portion and intracellularly flexible helix VI; right: active, G protein-bound receptor.

identical in the various structures. Due to variances in the transmembrane helix conformation, resulting from the altered global helical bundle conformation (Fig. 2A,B), mainly the positions in space (but not the orientation) of some amino acid side chains in the orthosteric pocket of the TMD differ. This shows that the agonist binds to the receptor essentially in a very similar manner in the absence or presence of G protein and that the intermediate conformation of the PTH1R:ePTH complex is thus mainly determined by the absence of an intracellularly bound G protein, as previously reported for other GPCRs, such as the $\beta_2$ adrenergic receptor [29,30].

In both PTH1R structures, the agonistic peptides adopt an upright position (Fig. 3A), in contrast to the inactive state structure of GCGR, where the peptide antagonist is located in the orthosteric pocket in a transversal position. We find that upon receptor activation the C-terminal tip of the peptide ligand (which is bound to the ECD) is moved by ~15 Å toward an upright position, accompanied by a ~24 Å movement of the ECD itself (measured from the N-terminal end of the β3 strand of the ECD) when comparing the inactive GCGR and the G protein-bound PTH1R structure. As there is potentially a high-energy barrier for the displacement and unwinding of helix VI during receptor activation, mechanistically, the ECD might even act as a lever pushing the peptide down along helix II into the TMD, thereby enabling the large rearrangements in transmembrane helices I, VI, and VII.

The importance of the displacement and unwinding of the extracellular tip of helix VI for receptor activation is further evidenced by the distinct position of the N terminus of ePTH in the intermediate state, compared to the N terminus of LA-PTH in the $G\alpha_s$-bound receptor. The N terminus of ePTH is located farther away from helix VI and slightly shifted toward helices I and II, possibly enabled by the two stabilizing mutations $Y191^{1.43}C$ and $K240^{2.67}M$ (superscript indicates Wootten numbering scheme for class B GPCRs [31]), which replace bulky residues at the lower part of the binding pocket. Consequently, the crystallized PTH1R

construct cannot adopt the full helical shifts necessary for G protein binding and signal transduction, but is rather captured in an intermediate conformation, where it has been stabilized (in the presence of the agonist but in the absence of G protein). Apparently, the shift of the agonist N terminus away from helix VI through mutation of the distal surface of the orthosteric pocket can have an effect that is related to N-terminal truncations of class B peptide agonists which transform these ligands into antagonists (e.g., by omitting $H_1$ of glucagon in NNC1702). Thus, the crystal structure of the PTH1R:ePTH complex potentially reveals an intriguing alternative strategy for the design of high-affinity peptide antagonists through careful reengineering of the ligand surface distal to helix VI in the orthosteric binding pocket.

## Rearrangement of a polar interaction network in the receptor core

In contrast to class A GPCRs, where receptor activation is mediated by a contraction of the orthosteric binding pocket at the extracellular portion of the TMD, which translates into the outward movement of the N-terminal end of helix VI at the intracellular side of the receptor, activation of class B GPCRs is characterized by an opening of the orthosteric binding pocket, while the intracellular side still needs to open to accommodate the G protein. This hourglass-like simultaneous opening of both the extracellular and intracellular portions of the TMD requires the heptahelical bundle of secretin-type receptors to be tightly tethered in the mid-helical kink region. The first agonist-bound and G protein-bound class B GPCR structures of the calcitonin receptor and GLP1R have revealed the presence of a previously predicted conserved central polar network in the kink region of the TMD helices, located just one helical turn below the bottom of the orthosteric pocket [27,32]. The high resolution of the PTH1R:ePTH complex crystal structure not only allowed us to observe several ordered waters within the polar network but also enabled the identification of several additional residues, which are involved in stabilizing the receptor core, thus detecting the extended dimensions of the central polar network. Importantly, the comparison of the agonist-bound intermediate-state PTH1R:ePTH complex with the $G\alpha_s$-bound PTH1R structure now for the first time enables us to precisely delineate the amino acid side-chain rearrangements of conserved key residues upon G protein binding during class B receptor activation.

At the base of the orthosteric pocket, peptide recognition is relayed into the receptor core through interaction of $E_4$ of the ligand with $R233^{2.60}$ of the central polar network. The importance of this key interaction is highlighted by the fact that acidic amino acids at the equivalent position of $E_4$ in PTH are highly conserved across the endogenous hormones that activate class B receptors [24]. In the absence of a heterotrimeric G protein, $R233^{2.60}$ hydrogen-bonds to and thus stabilizes the orientation of $Q451^{7.49}$, a conserved residue within the central polar network that appears to act as a molecular switch between receptor activation states. Upon full activation of the receptor (when the G protein is bound), the hydrogen bond network axis, defined by hormone position 4-$R233^{2.60}$-$Q451^{7.49}$, is rearranged, and $Q451^{7.49}$ becomes free to reorient downward into the receptor core, thereby establishing new hydrogen bonds with the inward-rotated side chain of $H420^{6.52}$ and the backbone of $P415^{6.47}$ of the class B conserved $P^{6.47}$-x-x-$G^{6.50}$ motif. These pronounced and potentially highly conserved rearrangements within the central polar network enable the stabilization of the distinct kink in helix VI that appears to be a hallmark of class B receptor activation and is mediating the simultaneous opening of the intracellular and extracellular sides. Although the exact sequence of these events cannot be determined alone by static snapshots from the receptor's conformational space and certainly demands the integration of additional dynamic and kinetic experimental data together with molecular modeling, the described side-chain rearrangements are clearly interlinked with the large conformational changes during class B GPCR activation.

## Perspectives

Structural insights into agonist complexes of class B GPCRs have been held up due to low expression levels and inherent instability of these receptors, as well as the required presence of the ECD for full-length peptide binding. For PTH1R, we have applied a combined approach of directed evolution followed by screening for thermostabilizing mutations on the TMD and rational engineering of the ECD and the peptide agonist. This separate engineering of a multidomain protein and its ligand is in principle broadly applicable and, in the case of PTH1R, enabled us to obtain a receptor: agonist complex crystal structure at 2.5 Å resolution. The observed polar network at the ECD–TMD junction and the absence of direct interactions between the ligand and ECL1 highlight a different mode of stabilization of the relative orientation of ECD and TMD and contradict the proposed general mechanism of ligand

binding via ECL1 and the stalk, which was based solely on observations of two closely related receptors (GCGR and GLP1R). As to date structures of agonist complexes with resolved ECD and TMD are available only for four of the 15 class B receptors, likely more interaction modes are to be discovered, and a more diverse set of models for the activation mechanism of secretin-like GPCRs has to be developed. Furthermore, the molecular extensions through ordered waters and additional interacting residues of the central polar network, revealed in the receptor core of the PTH1R crystal structure, contribute important additions to our current understanding of class B GPCR function.

Due to the large and solvent-exposed nature of the orthosteric binding pocket and the significant conformational rearrangements associated with receptor activation in the respective receptor regions, structure-based design of orally available small-molecule drugs has remained challenging for class B GPCRs. Therefore, the precise delineation of the orthosteric binding pocket, including water-mediated interactions, will hopefully facilitate and accelerate the development of such molecules. While remaining challenging, we believe that for the PTH1R, the development of such improved therapeutics for the treatment of osteoporosis and other dysregulations of calcium metabolism has now become feasible.

## Acknowledgments

This work was supported by Schweizerischer Nationalfonds Grants 31003A_153143 and 31003A_182334 and KTI Grant 18022.1 PFLS-LS, all to A.P.; C.K. is the recipient of a fellowship of the German Academy of Sciences Leopoldina (LPDS 2009-48) and a Marie Curie fellowship of the European Commission (FP7-PEOPLE-2011-IEF #299208).

## Conflict of interest

The authors declare no conflict of interest.

## Author contributions

JE and JS wrote the initial version of the manuscript and prepared the figures. AP and CK contributed to the revision and discussion of the manuscript.

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