A unique hormonal recognition feature of the human glucagon-like peptide-2 receptor

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Glucagon-like peptides (GLP-1 and GLP-2) are two proglucagon-derived intestinal hormones that mediate distinct physiological functions through two related receptors (GLP-1R and GLP-2R) which are important drug targets for metabolic disorders and Crohn's disease, respectively. Despite great progress in GLP-1R structure determination, our understanding on the differences of peptide binding and signal transduction between these two receptors remains elusive. Here we report the electron microscopy structure of the human GLP-2R in complex with GLP-2 and a Gs heterotrimer. To accommodate GLP-2 rather than GLP-1, GLP-2R fine-tunes the conformations of the extracellular parts of transmembrane helices (TMs) 1, 5, 7 and extracellular loop 1 (ECL1). In contrast to GLP-1, the N-terminal histidine of GLP-2 penetrates into the receptor core with a unique orientation. The middle region of GLP-2 engages with TM1 and TM7 more extensively than with ECL2, and the GLP-2 C-terminus closely attaches to ECL1, which is the most protruded among 9 class B G protein-coupled receptors (GPCRs). Functional studies revealed that the above three segments of GLP-2 are essential for GLP-2 recognition and receptor activation, especially the middle region. These results provide new insights into the molecular basis of ligand specificity in class B GPCRs and may facilitate the development of more specific therapeutics.

INTRODUCTION

GLP-2R belongs to class B GPCR subfamily, which is mainly expressed in the gut, pancreas and brain. Its endogenous ligand is GLP-2 (GLP-2(1–33)), a member of the glucagon-like peptide family that also includes GLP-1 and glucagon. GLP-2 is encoded by the proglucagon gene and becomes active after post-translational processing by prohormone convertases. As a gastrointestinal hormone, it mainly regulates intestinal epithelial cell growth and functions that are crucial for digestion and absorption of nutrients. Similar to GLP-1, GLP-2 is rapidly inactivated by dipeptidyl peptidase 4 in vivo. Clinically, GLP-2 analogue, teduglutide, is used to treat short bowel syndrome and Crohn’s disease. Other therapeutic indications, including colitis, pediatric gastrointestinal disorders and inflammation of the intestinal mucosa, are currently under development. Recent studies in animal models have shown that GLP-2 also exerts beneficial effects on glucose homeostasis and spontaneous hypertension and depression. This prompts a renewed interest in examining the role of this peptide beyond the gastrointestinal tract.

The cryo-electron microscopy (cryo-EM) emerges as a primary methodology to determine the structure of GPCR–G protein complexes starting with the GLP-1R and the calcitonin receptor, followed by eight other class B GPCRs in complex with various G proteins. These structures suggest that class B GPCRs have a common activation pattern which basically coincides with the previously proposed two-domain binding model. Although class B receptors have roughly the same activation mode, the molecular details are receptor specific, especially in the ligand-binding region. Therefore, exploring the recognition mechanism between GLP-2 and GLP-2R is of interest for further understanding the mechanism of ligand recognition and receptor activation among class B GPCRs. In this study, we employed cryo-EM to determine the high-resolution structure of the human GLP-2R in complex with Gs protein. Based on our prior experience, NanoBiT strategy was used in this study to stabilize the GLP-2–GLP-2R–Gs complex and strengthen the interactions between GLP-2R and Gs, resulting in a cryo-EM structure at a global resolution of 3.0 Å (Supplementary information, Table S1). Our data provide a rational template to facilitate the design of better GLP-2 analogues for therapeutic use and expand our knowledge on the biology of this receptor family.

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RESULTS AND DISCUSSION

Structure determination

We used NanoBiT strategy22–24 to stabilize the complex, in which GLP-2R was directly attached to LgBiT and rat Gβ was linked to HiBiT with a 15-amino acid linker in between. Transiently transfected HEK 293T cells showed that the C-terminus-truncated GLP-2R(1–(1490)-LgBiT was a 6-fold stronger interaction with Gβ-SmBiT than the wild-type (WT) receptor(1–553)-LgBiT (Supplementary information, Fig. S1a, b). When SmBiT was replaced by HiBiT, the luminescence signal detected between GLP-2R and Gβ was 10-fold stronger (Supplementary information, Fig. S1c). A haemagglutinin (HA) signal peptide was added to the N-terminus of GLP-2R to increase the expression level. In addition to linking with HiBiT, a TEV protease site and an OMBP (optimized maltose binding protein)-MBP tag were attached to the C-terminus (Supplementary information, Fig. S2a). The OMP tag is codon-optimized on the basis of MBP to enhance affinity between the protein complex and amylose resin in order to obtain more complexes. Rat Gβ1 was attached to HiBiT with a 15-amino acid linker between them (Supplementary information, Fig. S2b). To form an active G protein-coupled complex, HA-GLP-2R(1–(1490))-LgBiT-TEV-OMBP-MBP was co-expressed with a dominant-negative bovine Gα (DNGα), rat Gβ1-HiBiT and bovine Gγ2 in Sf9 insect cells. Compared with the protein expressed without NanoBiT strategy, the yield decreased but the stability increased (Supplementary information, Fig. S1d, e). Negative-staining EM visualization displayed more particles with similar size (Supplementary information, Fig. S1f).

A large-scale purification was subsequently performed using this approach. Cells were stimulated by 20 μM GLP-2 together with the camelid-derived nanobody Nb35 to stabilize the interface between Gα and Gβ.25 The complex was solubilized in lauryl maltose neopentyl glycol (LMNG) and cholesteryl hemi succinate (CHS), treated with TEV enzyme to remove tags from GLP-2R and then isolated by amylose resin. It was further purified by size-exclusion chromatography for cryo-EM specimen preparation (Supplementary information, Fig. S3a, b). The production of CAMP and ligand-binding ability of our modified hGLP-2R construct were reduced presumably caused by its lower expression level (~73% of WT; Supplementary information, Fig. S3c, d and Table S2).

Frozen-hydrated GLP-2–GLP-2R–Gα complexes were imaged using a Titan Krios microscope (Supplementary information, Fig. S4a). 2D classification showed clear secondary structure features and random distribution of particles in different directions enabling high-resolution cryo-EM map reconstruction (Supplementary information, Fig. S4b). A total of 284,669 particles were selected after consecutive 3D classifications, which yielded a cryo-EM density map with a global resolution of 3.0 Å (Supplementary information, Fig. S4c, d).

Overall architecture

In the cryo-EM structure of the GLP-2–GLP-2R–Gα complex, most of the receptor core, G protein subunits and the first 31 amino acids of GLP-2 were observed with high-resolution features, thereby allowing for accurate modeling of side-chain rotamers (Fig. 1; Supplementary information, Fig. S5). The receptor extracellular domain (ECD) was omitted from the final reported structure because of the relatively low resolution owning to its intrinsic flexibility, which is a general feature in most reported class B GPCR–Gα complex structures.

The overall structure of active GLP-2R–Gα complex is similar to other class B GPCR–Gα complexes such as GLP-1–GLP-1R–Gα13 ExP5–GLP-1R–Gα17 glucagon–GCR–Gα18 LA-PTH–PTh1R–Gα19 and GHRH–GHRHR–Gα20 with root mean square deviation (RMSD) values of 1.76, 1.05, 1.55, 1.09 and 0.95 Å for the whole complex, respectively. When pointing toward the glucagon receptor subfamily, notable conformational differences were observed in the extracellular half of GLP-2R where the peptide hormone binds, especially in TM1, ECL1 and TM7, indicating that GLP-2R utilizes a distinct peptide-recognition mode specific for GLP-2, but not for GLP-1, glucagon or GHRH.

Molecular recognition

The activated GLP-2R structure shows that GLP-2 is stably anchored in its position through an extensive network of interactions that involves TMs 1, 2, 3, 5 and 7, and ECLs 1, 2 and 3 (Fig. 2). The N-terminus of GLP-2, His19 (P indicates that the residue belongs to GLP-2), which is located above the conserved central polar network of class B GPCRs, is oriented toward TM3 and forms a hydrogen bond with H2681.30b (Wootten numbering in superscript27) and hydrophobic contacts with V2711.40b, W3403.36b and R3444.40b (Fig. 2a). Consistently, the truncated metabolite GLP-2(3–33) acts as a partial agonist with great reduction in binding affinity and potency.28,29 Meanwhile, mutants H268A, W340A and R344A decreased GLP-2 potency by 457–34- and 16-fold, respectively (Fig. 2d; Supplementary information, Table S3). Despite an identical N-terminal histidine in GLP-2, GLP-1 and glucagon, the side-chain orientations of histidine in the cognate receptors are different: the GLP-2 His19 is more distant from TM5 than that of GLP-1 and closer to TM3 than that of glucagon (Figs. 2, 3). Such difference may arise from the subtle changes in pocket residues at 3.37b (H268 for GLP-2R, Q234 for GLP-1R and Q232 for GCGR), 3.40b (W271 for GLP-2R, V237 for GLP-1R and I235 for GCGR) and ECL2 (N334 for GLP-2R, R299 for GLP-1R and N298 for GCGR). Another key residue in the N-terminus of GLP-2 is Asp29, whose side-chain forms a salt bridge with K2312.67b and a hydrogen bond with Y1861.47b. Alanine substitutions in Y1861.47b and K2312.67b had a moderate effect (4–16 fold) on GLP-2 potency (Fig. 2d).

The middle region of GLP-2 forms both a polar network with several charged and polar amino acids in TM7 (K4143.35b and R4173.38b) and ECL2 (T333ECL2 and N334ECL2) via Gly41, Ser42, Asp39, Glu36 and Asn19 and a complementary nonpolar network with TM1 (R1711.32b, Y1721.33b, L1753.36b, L1763.39b and Q1794.40b) via Phe31, Met32, Ile33 and Leu44 (Fig. 2a). Intriguingly, we observed one water molecule bridging Ser77 and D2322.60b, which is absent in the GLP-1–GLP-1R structure.13 When comparing the active GLP-2R structure with the GLP-1-bound GLP-1R structure,13 the extracellular end of TM1 in GLP-2R is extended by six additional residues, thereby bending toward GLP-2 with the formation of additional hydrogen bond and hydrophobic contacts. These interactions further stabilize the peptide conformation in the bound state and may contribute to specific recognition of GLP-2. Partial unwinding of the short α-helix elevates ECL1 to an extended conformation that stands upwards in line with TM2 and TM3, thereby engaging the C-terminal half of GLP-2 through an extensive interface spanning from Leu146 to Trp255 (Figs. 2a, 3).

Specifically, Asp215 forms a salt bridge with R2421.41c, while Trp255 forms a hydrogen bond with D2441.41c and pi-pi stacking with W249ECL1, which was stabilized by Phe239. Besides, Leu146 and Ala149 pack against Y239ECL1, R2421.41c and Y252ECL1. These interactions strengthen the binding and function of GLP-2, consistent with the decreased potencies of R242A (by 5-fold), R242E (by 20-fold), W249A (by 1552-fold) and Y252A (by 5-fold) (Fig. 2d). Similar effects of mutations were observed with the binding of teduglutide20 (Table 1). Together, these results highlight the importance of ECL1 in GLP-2 recognition and receptor activation.

G protein coupling

In our model of the GLP-2R–Gα complex, G protein is anchored by the α5 helix of Gα5 (Gα5H5), thereby fitting to the cytoplasmic cavity of the transmembrane domain (TMD) involving TMs 2, 3, 5, 6, and 7 (Supplementary information, Fig. S6). Structural comparison of the GLP-2R–Gα complex with that of other class B

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GPCRs reveals substantial similarity in the G protein-binding interface, consistent with a common mechanism of Gs protein engagement. However, many receptor-specific interactions were observed. Y391\textsuperscript{αH5} is an essential residue in determining G protein coupling specificity by forming H-bond in class A GPCRs,\textsuperscript{30} however, this interaction was not observed among available class B GPCR–G protein complexes. Most strikingly, Y391\textsuperscript{αH5} formed a tight hydrogen bond with a water molecule, which connects to E281\textsuperscript{3.50b} and H214\textsuperscript{2.50b} in the GLP-2R–Gs complex (Supplementary information, Fig. S6), a phenomenon that was not observed among other class B GPCRs. Polar interactions also occurred between K368\textsuperscript{5.64b} and Q384\textsuperscript{GαH5}, H372\textsuperscript{ICL3} and R385\textsuperscript{αH5} as well as R382\textsuperscript{6.37b} and E392\textsuperscript{GαH5}. The ICL2 of GLP-2R formed additional hydrophobic interactions with GαH5. There were also limited interactions between GaH5 subunit and helix 8 of the receptor (E392\textsuperscript{GαH5}, N440\textsuperscript{8.47b} and G441\textsuperscript{8.48b}). These receptor-specific interactions may contribute to the G protein specificity of GLP-2R.

Ligand specificity

GLP-2 and GLP-1 share high sequence similarity (69.7%) and have almost identical residues in two regions: the N-terminus (first six residues in GLP-2) that penetrates into the TMD core, and the C-terminus (residues 18–33) that is recognized by the ECD and ECL1 of the receptor. On the contrary, the central region of the peptide mainly interacts with TMs 1, 2, 7, ECL1 and ECL2 of the receptor and differs to that of GLP-1 (Fig. 4; Supplementary information, Fig. S7a, b).

Structural comparison of GLP-2–GLP-2R–Gs and GLP-1–GLP-1R–Gs\textsuperscript{13} complexes reveals distinct features of their ligand-binding pockets (Figs. 3, 4; Supplementary information, Fig. S7c–e). Superimposing the TMD of GLP-2R with that of GLP-1R shows that the intracellular portions of both receptors, except ICL2 and ICL3, overlap very well, whereas the extracellular parts of TM1, TM5 and TM7 as well as all ECLs have different conformations and side-chain orientations (Fig. 4a; Supplementary information, Fig. S7c–e). Consistently, the N-terminal part of GLP-2 is reoriented relative to GLP-1. Furthermore, the rest of GLP-2 is elevated vertically (0.5 Å) and moves toward TM1–TM2 by 1–3 Å, allowing the extensive interaction with ECL1. Overall, the interface area measured by buried solvent-accessible surface of GLP-2–GLP-2R TMD is 3050 Å\textsuperscript{2}, significantly larger than that of GLP-1–GLP-1R TMD (2297 Å\textsuperscript{2}).

The orientation of the N-terminal histidine between GLP-1 and GLP-2 is rather different (Fig. 2b; Supplementary...
Fig. 2 Molecular recognition of GLP-2 by GLP-2R. a Extensive contacts between GLP-2R (sticks with orange red carbons) and GLP-2 (sticks with forest green carbons), showing that the N-terminal residue His1 can interact with H2683.37b, V2713.40b and W3405.36b, together with other interactions between agonist and receptor (left). The interactions focus on the ECL1 of GLP-2R and GLP-2 (right). b, c Structural comparison of the TMD binding pockets among GLP-2–GLP-2R–Gs, GLP-1–GLP-1R–Gs,13 and glucagon–GCGR–Gs.19 GLP-2 in forest green, GLP-2R in orange red; GLP-1 in blue, GLP-1R in light blue; glucagon in magenta and GCGR in light magenta. Receptor ECD and G protein are omitted for clarity. d Effects of mutation in the peptide-binding pocket on cAMP accumulation. cAMP levels were measured in WT receptor and alanine-mutated TMs and ECLs. Data shown are means ± SEM of at least three independent experiments.
information, Fig. S7c). GLP-1 faces toward TM5 to form cation-pi stacking and hydrogen bond with the rotated down side-chain of R2995.36b, while that of W3065.36b and R3105.40b switch out from the receptor core to accommodate the approaching of histidine. However, by replacing arginine with threonine, the ECL2 of GLP-2R is no longer able to dip into the receptor core and becomes distant from the N-terminal histidine of GLP-2. Therefore, W3405.36b and R3445.40b of GLP-2R rotate inwards. Together with the inward movement of the extracellular part of TM5, they push the N-terminus of GLP-2 away from TM5 to reside closely with TM3, where H2683.37b contributes a hydrogen bond to stabilize the conformation (Supplementary information, Fig. S7c). Compared to GLP-1, the middle region of GLP-2 has more extensive contacts with the ligand-binding pocket (Fig. 4b).

The interface areas between TMD and GLP-2 contributed by TM1 and TM7 are 864 Å² and 432 Å², respectively, markedly larger than those between TMD and GLP-1 (330 Å² and 303 Å², respectively). Specifically, in the extracellular half of GLP-2R, TM1 forms at least one additional helical turn, thereby moving toward TM2 (5 Å, measured by Cα of the residue at 1.33b) and bending down toward GLP-2. This ultimately leads to a much larger interaction pattern for GLP-2 (ranging from Asp3P to Asn16P) than for GLP-1, where only two residues of GLP-1 interact with TM1.

Meanwhile, side-chain orientations of TM1 residues are reorganized (Supplementary information, Fig. S7d). For example, Y1821.43b is reoriented ~90° from an outside facing position to a position pointing to TM2 and engages T-shaped stacking with the ligand-binding pocket of GLP-2R. The binding cavity of GLP-2R is compared with that of GLP-1-bound GLP-1R (PDB code: 5VAI),13 ExP5-bound GLP-1R (PDB code: 6B3J),17 glucagon-bound GCGR (PDB code: 6LMK)16 and LA-PTH-bound PTH1R (PDB code: 6NBF).19

Table 1. Binding of GLP-2 and teduglutide with GLP-2R mutants.

| Receptor mutant | Human GLP-2 (1–33) | Teduglutide |
|----------------|-------------------|-------------|
|                | pIC₅₀            | % Binding of WT   | pIC₅₀            | % Binding of WT   |
| WT (1–553)     | 8.5 ± 0.07       | 99.2 ± 2.3     | 8.8 ± 0.05       | 99.7 ± 1.9        |
| Y1821.43A      | 9.1 ± 1.2        | 3.4 ± 1.0***   | 9.2 ± 0.8        | 3.2 ± 1.3***      |
| Y1861.47A      | N.B.             | N.B.          | N.B.             | N.B.              |
| K2312.67A      | N.B.             | N.B.          | N.B.             | N.B.              |
| R242ECL1E      | N.B.             | N.B.          | N.B.             | N.B.              |
| R242ECL1A      | 8.0 ± 0.5        | 4.2 ± 0.6***   | 8.7 ± 0.8        | 5.1 ± 1.5***      |
| D244ECL1A      | 8.6 ± 0.2        | 69.1 ± 4.9***  | 9.0 ± 0.2        | 77.5 ± 5.7***     |
| N247ECL1A      | 8.2 ± 0.1        | 130.7 ± 4.5*** | 8.6 ± 0.1        | 135.2 ± 4.4***    |
| W249ECL1A      | 9.0 ± 1.2        | 2.2 ± 1.2***   | 8.8 ± 1.7        | 2.9 ± 2.0***      |
| Y252ECL1A      | 8.4 ± 0.8        | 8.3 ± 1.8***   | 9.0 ± 0.6        | 6.3 ± 1.7***      |
| H2683.37A      | 8.0 ± 1.4        | 2.1 ± 0.8***   | 9.7 ± 3.2        | 0.7 ± 1.8***      |
| N334ECL2A      | 9.5 ± 1.4        | 2.4 ± 1.3***   | 9.7 ± 2.7        | 2.0 ± 2.1***      |
| W3405.36A      | 7.1 ± 0.4        | 7.3 ± 0.6***   | 8.8 ± 0.7        | 8.6 ± 1.8***      |
| R3445.40A      | 8.6 ± 0.5        | 5.0 ± 0.8***   | 9.3 ± 0.6        | 3.5 ± 0.8***      |
| K4147.35A      | 8.5 ± 0.2        | 15.2 ± 1.0***  | 8.7 ± 0.2        | 13.9 ± 0.8***     |
| ECL1 (GLP-1R)d | 8.9 ± 1.7        | 3.4 ± 1.3***   | 7.8 ± 0.7        | 1.2 ± 0.5***      |
| ECL1 (poly-alanine)d | 8.2 ± 1.3 | 3.0 ± 1.1*** | N.B. | N.B. |

***P < 0.001.
aAll data were fitted with a three-parameter logistic curve to obtain pIC₅₀ values. Data represent means ± SEM of at least three independent experiments performed in duplicate. One-way ANOVA and Dunnett’s post hoc tests were used to determine statistical differences.
bWT wild-type.
cN.B. no binding of the radioligand.
dGLP-2R mutants with ECL1 (residues 236–257) substituted by the corresponding segment of GLP-1R or poly-alanine are labeled as ECL1 (GLP-1R) and ECL1 (poly-alanine), respectively.
Phe6P of GLP-2. Accompanying TM1 movement to TM2, the extracellular tip of TM7 moves toward TM1 and the side-chain of K4147.35b points to GLP-2 with formation of a salt bridge with Glu9P. In the C-terminus of GLP-2, we observed that it forms a more extensive interaction with the ECL1 of GLP-2R compared to the GLP-1–GLP-1R ECL1 interaction (interface area = 791 Å² vs 424 Å²). Different from a 2.5-turn α-helix of ECL1 in both activated GLP-1R and GCGR, the ECL1 of GLP-2R partially unwinds vertically in line with TM2 and TM3, providing additional interactions not seen in its counterparts (Figs. 3, 4a; Supplementary information, Fig. S7e). Alternatively, the α1-helix of GLP-1R ECD is in close contact with the C-terminus of GLP-1. Because the ECD structure...
of GLP-2R is unavailable, its impact on the structure and dynamics remains elusive.

Considering the uneven sequence conservation and distinct binding modes of GLP-2 and GLP-1, we designed chimeric GLP-2/GLP-1 peptides to explore the molecular basis of ligand specificity. As shown in Fig. 4c, replacement of the first six residues of GLP-2 by those of GLP-1 (i.e., double mutant D3PE and S5PT) decreased cAMP accumulation by 12-fold compared to the native peptide, suggesting that subtle substitution may directly alter receptor activation. In addition, chimeric GLP-2 whose C-terminal fragment (residues 20–33) was replaced with that of GLP-1 displayed a 17-fold potency reduction in GLP-2R signaling but successfully elicited GLP-1R-mediated cAMP accumulation (Fig. 4c). Replacement of the GLP-2 middle region (residues 7–19) that mainly interacts with TM1, TM7 and ECL1 by the corresponding part of GLP-1, almost abolished GLP-2R-mediated cAMP accumulation but fully rescued that induced by GLP-1R (Fig. 4c), indicative of a fundamental role of this region in determining the specificity for ligand recognition.

Conformational changes

Triggered by GLP-2 binding and G protein coupling, GLP-2R would presumably undergo significant conformational changes. It shares a high degree of sequence similarity in the TMD region with GLP-1R (61%) that has been studied extensively both structurally and functionally. Thus, inactive, intermediate and active GLP-1R structures published previously provide good references for the present study. Superimposing the TMD of GLP-2R with that of GLP-1R reveals that activated GLP-2R shows a similar conformation to that of GLP-1-bound or ExP5-bound GLP-1R–Gβγ complexes (Ca RMSD = 1.41 and 0.91 Å, respectively), distinct from inactive GLP-1R (Ca RMSD = 1.79 Å). Further comparison demonstrates that diversified peptide–receptor interactions converge to induce conserved and extensive conformational transition of the TMD of GLP-2R and GLP-1R (Fig. 5).

In the extracellular half of GLP-2R, accompanying the unwinding of the last two turns of TM6, TM7 moves outward and bends toward TM6 via a conserved pivot point G4297.50b, which expands the TMD-binding pocket to accommodate peptide entrance and...
connection. Meanwhile, TM1 moves inwards and folds down toward GLP-2, which contributes both polar and hydrophobic contacts to stabilize the binding. Furthermore, different from the ordered α-helical ECL1 in inactive GLP-1R and β-hairpin in inactive GCGR that cover the binding pocket and probably block peptide insertion, ECL1 of active GLP-2R, GLP-1R and GCGR simultaneously move outward and stand upwards in line with TM2 and TM3. Together with inward movement of α-helical extended TM2, raised ECL1 of GLP-2R forms extensive interaction with the C-terminal half of GLP-2 (Fig. 4a, b) and was shown to be essential for peptide binding and receptor activation (Fig. 4c). As far as the intracellular half is concerned, the positions of TM1–2–3 of active GLP-2R resemble that of active GLP-1R (Fig. 5). The most profound conformational change is the sharp kink in the middle of TM6, leading to an outward movement of the intracellular portion of TM6 as measured by Cα of Lyv (19.0 Å, similar to that of other Gα-coupled class B receptors). In contrast, in the absence of G protein coupling, the binding of peptide 5 promotes a rigidly outward shift of the intracellular tip of TM6 of GLP-1R by 12.1 Å without a sharp kink, thereby creating an intracellular crevice for G protein coupling. For PTH1R and GCGR (whose crystal structures were determined with PGS domain fusion in ICL3 and T4L fusion at ICL2, respectively), agonist binding failed to promote the outward movement of the intracellular half of TM6 as seen in the inactive state, probably due to the fusion. With more structural and dynamics studies on class B GPCRs, the activation process from inactive to active states via an intermediate state, i.e., allosteric communication between extracellular peptide binding and intracellular G protein coupling, will be better understood.

Role of ECL1
Relative to ECL2 and ECL3, ECL1 in class B GPCRs is the most divergent extracellular loop with flexible lengths, ranging from 8 (CRF2R) to 27 (PTH1R) residues. Inspired by the intriguing ECL1 conformation of GLP-2R, we performed structural comparison of ECL1 across class B GPCRs to unveil its functionality (Fig. 6). Among 20 agonist-bound structures from 9 receptors, we calculated the height of ECL1 (defined as the maximum vertical distance of Cα carbon relative to the membrane layer) and the ECL1–agonist interface area. We found that GLP-2R has the most protruded ECL1 with a height of 22.6 Å and this ECL1 closely attaches to GLP-2 with the largest interface area of 791 Å². The ECL1 of all activated GLP-1R and GCGR structures consistently adopt α-helical conformation bridging extended TM2 and TM3 helices by two short linkers to form extensive interactions with bound agonists. Remarkably, significant conformational changes of ECL1 upon receptor activation were observed relative to their inactive conformations: (i) in the inactive GCGR, ECL1 forms a β-hairpin conformation and further compacts β-sheet with the stalk region; and (ii) in the inactive GLP-1R, ECL1 moves toward TM1 and connects ECD in its closed TMD-interacting conformation. As far as CRF1R, CRF2R, AM1R, AM2R and CLR are concerned, their ECL1 are relatively short (consisting of 8–11 residues) and can only form one-turn α-helix to connect the adjacent transmembrane helices TM2 and TM3, contributing limited contacts with the peptides. PTH1R has the longest ECL1, but it is unstructured in both LA-PTH–PTH1R–Gαs and ePTH–PTH1R structures, probably owing to the great mobility of PTH1R’s ECD and specific binding mode, consistent with the mutagenesis studies. To date, four PAC1R structures were reported, namely, PACAP38–PAC1R–Gαs (3.0 Å), PACAP–PAC1R–mini-Gαs (3.9 Å), PACAP38–PAC1R–Gαs (3.5 Å) and maxadilan–PAC1R–Gαs (3.6 Å). The ECL1 was not resolved in the first two structures, and the third map showed that the ECL1 closely interacted with PACAP38 (pituitary adenylate cyclase activating polypeptide-38) without detailed residue contact information due to limited resolution. Interestingly, maxadilan, a peptidic PAC1R agonist, reorganized the ECD orientation and reshaped the ligand-binding pocket conformation, including ECL1 (Fig. 6b). Collectively, these data demonstrate the diversity and flexibility of ECL1 among class B GPCRs.

To further explore the functional importance of ECL1 in ligand binding and receptor activation, we performed mutagenesis studies on this part of the receptor (Table 1). Replacement of GLP-2R ECL1 by poly-alanine abolished the ability of GLP-2 to elicit cAMP response, while replacement by that of GLP-1R diminished its potency by 196-fold. Meanwhile, single-point mutations on deeply buried residues (R242, W249 and Y252) in the interface between GLP-2 and ECL1 also demonstrated negative impact on ligand binding (Supplementary information, Table S5).

In conclusion, the structure of the GLP-2–GLP-2R–Gαs complex delineates detailed interactions between GLP-2 and GLP-2R that account for the peptide binding specificity, and provides new insights into the structural reorganization of class B GPCRs upon...
activation. Combined with previous research on the GLP-1R, our results reveal that subtle differences in the sequence of TMD binding pocket and the consequent diversified conformational changes of the extracellular half accommodate peptide binding and empower the receptor to be specifically triggered by the cognate hormone. This complex structure points to an essential role of ECL1 in peptide recognition and receptor activation, in addition to that of ECD. This work also highlights the applicability of the NanoBiT tethering strategy to stabilize GPCR–G protein complexes for structural studies.

MATERIALS AND METHODS

Constructs

WT human GLP-2R was modified to include a HA signal peptide at the N-terminus. LgBiT and an OMPB-MBP tag were added to the C-terminus of the receptor with a TEV site between them. A bovine Gαs was used with G226A and A366S mutations to stabilize the complex; at GB1 was attached to HiBiT with a 15-amino acid linker between them; bovine Gγ2 was also used in cloning. All of the above constructs were generated in pFastBac and pcDNA3.1 vectors for protein expression in insect cells and functional assays in mammalian cells, respectively.

Protein expression

The complex was co-expressed in Sf9 cells (Expression System, Davis, USA) grown in ESF-921 serum-free medium (Expression System) at 27 °C, 120 rpm. Baculovirus was added at a proportion of 1:1:1:1 for HA-GLP-2R(1–490)-LgBiT-TEV-OMBP-MBP, Gαs, Gβ5, HiBiT and Gγ when the cell density reached 3 × 10^6/mL. After 48 h incubation, the pellet was harvested by PBS using centrifugation at 2000 rpm for 20 min.

Complex purification

Sf9 cells were suspended in 20 mM HEPES, pH 7.4, 100 mM NaCl and 10% (v/v) glycerol in the presence of Protease Inhibitor Cocktail (Topscience, Shanghai, China). Complex was formed by adding 10 mM CaCl₂, 10 mM MgCl₂, 1 mM MnCl₂, 50 mM/mL apyrase (Sigma-Aldrich, Darmstadt, Germany), 20 μM GLP-2, 100 μM TCEP (Sigma-Aldrich) and 10 μg/μL Nb35 into the cell lysate and incubated at room temperature (RT) for 1.5 h. Cell membranes were solubilized by 0.5% (v/v) TCEP (Anatrace, Maumee, USA) supplemented with 0.1% (w/v) CHS (Anatrace) with 5 μM GLP-2 and Protease Inhibitor Cocktail (Topscience) at 4 °C for 2 h, followed by centrifugation at 65,000×g for 30 min at 4 °C. The supernatant was then treated with an amylase-resin (NEB, Ipswich, USA) for 2 h at 4 °C. After packing, the column was washed with buffer containing 20 mM HEPES, pH 7.4, 100 mM NaCl, 10% (v/v) glycerol, 5 μM GLP-2, 25 μM TCEP, 5 mM MgCl₂, 1 mM MnCl₂, 0.1% (w/v) TCEP and 0.02% (w/v) CHS first (10 column volumes), and then with decreased concentrations of detergents 0.03% (w/v) TCEP, 0.01% (w/v) TCEP, 0.001% (w/v) TCEP and 0.0008% (w/v) CHS (20 column volumes). TEV enzyme was added to the resin and kept at 4 °C overnight to remove OMPB-MBP tag. The complex was eluted from the resin and concentrated to 500 μL using a 100 kDa MWCO Amicon Ultra Centrifugal Filter (Merck Millipore, Darmstadt, Germany). Size-exclusion chromatography was carried out by loading the protein sample to Superdex 200 Increase 10/300 GL (GE Healthcare, Boston, USA) to obtain the monomer complex. The column was pre-equilibrated with 20 mM HEPES, pH 7.4, 100 mM NaCl, 5 μM GLP-2, 100 μM TCEP, 2 mM MgCl₂, 0.00075% (w/v) TCEP, 0.00025% (w/v) CHS and 0.0002% (w/v) GDN. Nb35 was purified by nickel affinity chromatography as previously described,25 followed by size-exclusion chromatography using a HiLoad 16/600 Superdex 75 column (GE Healthcare). The column was pre-equilibrated with 20 mM HEPES, pH 7.4 and 100 mM NaCl. Glycerin 30% (v/v) was added to collect Nb35 which was frozen in liquid nitrogen and stored at −80 °C.

Negative-staining electron microscopy

Purified GLP-2R−Gγ complex was diluted to 0.012 mg/mL with 20 mM HEPES, pH 7.4, 100 mM NaCl, 5 μM GLP-2, 100 μM TCEP, 2 mM MgCl₂, 0.00075% (w/v) TCEP, 0.00025% (w/v) GDN and 0.0002% (w/v) CHS. Eight microliters of protein complex and 8 μL uranifomate were applied to a glow-discharged 300-mesh-mesh cooper grid (EMCN, Beijing, China) for 1 min and then sucked with filter paper. After grilling the grid with a lamp, the samples were imaged at RT with a Talos L120C electron microscope (FEI, Hillsboro, USA) operated at 120 kV. Images were recorded at magnification of 57,000× and a defocus value of −1 μm.

Cryo-EM

Three microliters of the purified GLP-2−GLP-2R−Gγ complex at about 20 mg/mL was applied on a glow-discharged holey carbon grid (Quantifoil R1/2/1/3), blotted, and subsequently sample-coated grids were vitrified by plunging into liquid ethane using a Vitrobot Mark IV (ThermoFisher Scientific, Waltham, USA). Automatic data collection was performed on a Titan Krios equipped with a Gatan K2 Summit direct electron detector in the Center of Cryo-Electron Microscopy, Zhejiang University (Hangzhou, China). The microscope was operated at 300 kV accelerating voltage, at a nominal magnification of 29,000× in counting mode, corresponding to a pixel size of 1.014 Å. A total of 2296 movies were obtained at a dose rate of about 8 electrons per Å² per second with a defocus ranging from −0.5 to −2.5 μm. The total exposure time was 8 s and intermediate frames were recorded in 0.2 s intervals, resulting in an accumulated dose of 64 electrons per Å² and a total of 40 frames per micrograph.

Data processing and 3D reconstructions

Dose-fractionated image stacks were subjected to beam-induced motion correction using MotionCor2.44 A sum of all frames, filtered according to the exposure dose, in each image stack was used for further processing. Contrast transfer function parameters for each micrograph were determined by Gctf v1.06.45 Particle selection, 2D and 3D classifications were performed on a binned dataset with a pixel size of 2.028 Å using RELION-3.0-beta2.46 Auto-picking yielded 1,498,893 particle projections were subjected to reference-free 2D classification to discard false positive particles or particles categorized in poorly defined classes, producing 485,548 particle projections for further processing. This subset of particle projections was subjected to a round of maximum-likelihood-based three dimensional classifications with a pixel size of 2.028 Å, resulting in one well-defined subsets with 162,932 projections. Further 3D classifications with mask on the complex produced two good subsets accounting for 332,946 particles, which were subsequently subjected to a round of 3D classifications with mask on the receptor. A selected subset containing 284,669 projections was then subjected to 3D refinement and Bayesian polishing with a pixel size of 1.014 Å. After last round of refinement, the final map has an indicated global resolution of 3.0 Å at a Fourier shell correlation (FSC) of 0.143. Local resolution was determined using the Bsoft package with half maps as input maps.

Model building

The initial template of the TMDs in GLP-2R was generated using SWISS-MODEL.47 The models of G, protein and Nb35 from
transiently transfected with different GLP-2R constructs or pCDNA3.1 vector as previously described. Following 24-h incubation, the cells were transferred to Costa® microtiter plates (150,000 cells/well, PerkinElmer) to achieve a 5%–10% specific binding. Forty-eight hours after transfection, the cells were washed twice in binding buffer (50 mM HEPES, pH 7.4, supplemented with 1 mM CaCl₂, 5 mM MgCl₂ and 0.5% (v/v) BSA) and incubated for 15 min at 4°C. An increasing concentration of unlabeled GLP-2(1–33) or teduglutide (ranging from 0.1 nM to 1 µM) followed by a fixed concentration of [¹²⁵I]GLP-2(1–33, M10Y) (10–15 pM) was added to the cells and incubated for 4 h at 4°C. The cells were then washed twice in binding buffer (4°C), lysed and counted for radioactivity using a Wallac Gamma Counter (PerkinElmer).

Structural analysis
The interface area was calculated by FreeSASA using the Sharpe-Rupley algorithm with a probe radius of 1.2 Å. Structural figures were prepared using UCSF Chimera and PyMOL (The PyMOL Molecular Graphics System v2.1). RMSD analysis was performed in PyMOL.

**DATA AVAILABILITY**
The atomic coordinates and the electron microscopy maps have been deposited in the Protein Data Bank (PDB) under accession number 7D68 and Electron Microscopy Data Bank (EMDB) under accession number EMD-30590, respectively. All relevant data are available from the authors upon request and/or included in the manuscript or Supplementary information.

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**AUTHOR CONTRIBUTIONS**
W.S., Z.C., F. Zhao and F. Zhou designed the expression constructs, purified the GLP-2–GLP-2R–G complex, prepared the final samples for negative staining/data collection towards the structure and participated in manuscript preparation; L.N.C. and D.D.S. performed specimen screening, cryo-EM grid preparation/data collection, map calculation, structure analysis, bulk figure preparation and participated in manuscript writing; Q.Z. performed structural analysis, figure preparation, and participated in manuscript writing; S.G., W.J.C.v.d.V., X.C., Y.C. and Y. Zhou conducted ligand binding and signaling experiments; L.H.Z. supervised construct preparation, protein expression and purification; S.Z. and H.Z. assisted in structural analysis; Y.J. participated in data analysis and manuscript editing; M.M.R. directed receptor binding studies and edited the manuscript; D.Y. supervised mutagenesis and signaling experiments, participated in data analysis and manuscript preparation; H.E. X., Y. Zhang and M.W.W. initiated the project, supervised the studies, analyzed the data and wrote the manuscript with inputs from all co-authors.
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