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Title

Structural and Functional Diversity among Agonist-Bound States of the GLP-1 Receptor

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Abstract

Recent advances in G protein-coupled receptor (GPCR) structural elucidation have strengthened previous hypotheses that multi-dimensional signal propagation mediated by these receptors depends, in part, on their conformational mobility. However, the relationship between receptor function and static structures is inherently uncertain. Here we examine the contribution of peptide agonist conformational plasticity to activation of the glucagon-like peptide-1 receptor (GLP-1R), an important clinical target. We employ variants of the peptides GLP-1 and exendin-4 to explore the interplay between helical propensity near the agonist N-terminus and the ability to bind to and activate the receptor. Cryo-EM analysis of a complex involving an exendin-4 analogue, the GLP-1R and G_s heterotrimer revealed two receptor conformers with distinct modes of peptide-receptor engagement. Our functional and structural data, along with molecular dynamics simulations,

suggest that receptor conformational dynamics associated with flexibility of the peptide N-terminal activation domain may be a key determinant of agonist efficacy.

Main text

Introduction

G protein-coupled receptors (GPCRs) are critical conduits for intercellular communication. Understanding mechanisms governing agonist activation of GPCRs is integral to interrogation of physiological processes controlled by these receptors and offers a basis for developing therapeutic agents. Recent advances have provided molecular-level snapshots of GPCR structure, including ligand-induced changes in GPCR structure, and of interactions between GPCRs and intracellular partner proteins.[1] However, static structures determined via x-ray crystallography or cryo-electron microscopy (cryo-EM) cannot always be extrapolated to understand receptor and transducer activation, which are dynamic processes.[2]

The GLP-1R is a class B1 peptide hormone GPCR that plays a critical role in glucose metabolism, and synthetic agonists of this receptor are used to treat type 2 diabetes.[3] The primary endogenous GLP-1R agonist is GLP-1(7-36)-NH_2.[3] Class B1 receptors feature a large extracellular domain (ECD) in addition to the ubiquitous heptahelical transmembrane domain (TMD). Initial agonist-receptor contact occurs between the C-terminal portion of the peptide and the ECD; the N-terminal portion of the agonist subsequently engages the TMD core, facilitating conformational changes that are registered by the G protein and other intracellular partners (Fig. 1a).[4] Most agonist C-terminal regions are α-helical when bound to class B1 receptor ECDs.[5] Insights into the structure of agonist N-termini embedded in receptor TMDs have emerged only recently. In cryo-EM structures of this receptor complexed to a heterotrimeric G protein and bound

to either GLP-1 or a synthetic peptide, ExP5,[6–8] α-helical secondary structure extends to the TMD-engaged N-terminus of the bound peptide. Comparable observations were reported for agonist peptides bound to several class B1 GPCRs,[9–12] but for other class B1 receptors, non-helical structure is observed near the agonist N-terminus.[10,13]

Here we describe an integrated chemical, pharmacological, structural and computational approach to elucidate mechanisms of signal transduction by the glucagon-like peptide-1 receptor (GLP-1R), based on comparisons involving two natural agonists, GLP-1 and exendin-4, and rationally designed analogues of these peptides. This work was motivated by previous but indirect evidence that GLP-1 activity might depend on adoption of a reverse turn near the peptide N-terminus.[14–17] For example, the Gly10→L-Ala analogue of GLP-1(7-36)-NH_2 was nearly 100-fold less potent than GLP-1 itself in terms of stimulating insulin release, while the Gly10→D-Ala diastereomer matched GLP-1 in potency.[18] Since replacing Gly with L-Ala stabilizes a right-handed α-helical conformation by up to 1 kcal/mol,[19–21] while replacing Gly with D-Ala *destabilizes* the α-helical conformation by up to 0.5 kcal/mol,[19] these observations raise the possibility that the N-terminal helical conformation in the cryo-EM structure of receptor-bound GLP-1[6] may not fully capture structural requirements for signaling. This consideration is important because efforts to engineer therapeutic GLP-1R agonists might be most effective if they target a conformation or conformational transition that is essential for signal propagation via the receptor.

Results

Potent agonists with D-Ala at the 4th position. We re-examined[18] the agonist activity of Gly10→L-Ala and Gly10→D-Ala variants of GLP-1(7-36)-NH_2 (Fig. 1b, 1c) in HEK293 cells transiently expressing the GLP-1R and stably expressing a cAMP biosensor. Intracellular cAMP production is typically monitored to detect GPCR-modulated activation of the stimulatory G

protein, $G_{\alpha S}$. Both Ala-containing diastereomers matched GLP-1 in terms of the maximum level of cAMP production. However, while the Gly10→D-Ala analogue was indistinguishable from the native hormone in terms of potency (EC_{50}), the Gly10→L-Ala was ~24-fold less potent (Fig. 1c; Table 1). This behavior is qualitatively consistent with earlier observations.[18,22] We explored the generality of these observations by evaluating Ala-containing derivatives of exendin-4 (exenatide) (Fig. 1b), a potent GLP-1R agonist isolated from a lizard venom that is used to treat type 2 diabetes.[23] Exendin-4 and GLP-1 are very similar over the first 11 residues, and Gly10 of GLP-1 corresponds to Gly4 of exendin-4 (Fig. 1b). The Gly4→D-Ala variant of exendin-4 was only slightly less potent than exendin-4 itself in terms of cAMP production, but the Gly4→L-Ala variant was ~30-fold less potent, which parallels the trend among GLP-1 analogues (Fig. 1d; Table 1). These data support the conclusion that an ability to access non-helical conformations, such as a reverse turn,[24] near the N-terminus correlates with higher GLP-1R agonist potency.

Probing the 4th position with cyclic β-residues. Non-traditional substitutions can yield GLP-1 analogues that show distinctive behavior, and such substitutions might provide insight into conformational requirements for agonist activity.[25–27] We explored a second set of GLP-1 and exendin-4 analogues in which the key Gly residue was replaced with a carefully selected β-amino acid residue. This experimental design was based on previous comparisons of the conformations and biological activities of conventional peptides (comprised entirely of α-amino acid residues) with the properties of analogues in which at least one α residue was replaced by a β-amino acid residue. Mixed-backbone peptides containing up to 25-33% β residues can adopt an α-helix-like secondary structure.[28] The constrained β residue derived from *trans*-(*S,S*)-2-aminocyclopentanecarboxylic acid ((*S,S*)-ACPC) is comparable to L-Ala in stabilizing a right-handed α-helix-like conformation (Fig. 1a).[29] We previously showed that GLP-1 analogues with

multiple (*S*,*S*)-ACPC substitutions in the C-terminal region, which is α-helical when bound to the ECD,[30] display substantial agonist activity.[31,32] In contrast, (*R,R*)-ACPC (Fig. 1a) destabilizes a right-handed α-helix-like conformation by >1 kcal/mol relative to (*S,S*)-ACPC or L-Ala.[29] As observed for D-Ala,[24] (*R*,*R*)-ACPC can replace Gly to stabilize turn segments.[33] These precedents led us to compare diastereomeric derivatives of GLP-1 and exendin-4 in which Gly10 or Gly4, respectively, was replaced by either (*S,S*)-ACPC or (*R,R*)-ACPC. Although the steric bulk of the $(CH_2)_3$ side chain might diminish activity relative to the natural GLP-1R agonists, these replacements should test the hypothesis that GLP-1R agonist activity is higher for ligands with a larger propensity to access non-helical conformations near the N-terminus, compared to those that are strongly disposed to maintain helicity. This hypothesis predicts that the Gly→(*R,R*)-ACPC analogues should be more active than the diastereomers containing (*S,S*)-ACPC.

The relative activities among ACPC-containing analogues of the two natural GLP-1R agonists were consistent with predictions of our hypothesis: the Gly10→(*R,R*)-ACPC analogue of GLP-1 was ~9-fold more potent than the (*S,S*)-ACPC diastereomer in eliciting cAMP production, and the Gly4→(*R,R*)-ACPC analogue of exendin-4 was ~5-fold more potent than the (*S,S*)-ACPC diastereomer (Fig. 1c, 1d). Moreover, both analogues containing (*S*,*S*)-ACPC had reduced maximum cAMP production compared to their diastereomers. In each case, even the more potent diastereomer was an inferior agonist relative to the natural all-α peptide, by ~45-fold in the GLP-1 series and ~220-fold in the exendin-4 series (Fig. 1c, 1d, Table 1). Despite this overall decline in potency, the patterns of relative activity among ACPC-containing analogues of GLP-1 and exendin-4 support the hypothesis that a propensity to sample non-helical conformations near the agonist N-terminus is important for GLP-1R activation, despite the observation of helical N-termini in available crystal and cryo-EM structures.

Divergent trends in potency and affinity among agonists. An agonist's potency can be influenced by multiple factors including affinity for the receptor and the ability to shift the receptor into active conformations that transduce the signal via interaction with intracellular proteins. Agonist affinity for GPCRs has typically been measured via competition with a labelled probe ligand. We developed a competition assay based on detection of probe binding via bioluminescence resonance energy transfer (BRET). Key components for this assay were a version of the human GLP-1R with the bright, bioluminescent protein NanoLuc (NLuc) fused to the N-terminus, and a GLP-1(7-36) derivative bearing a tetramethylrhodamine moiety linked to a lysine side chain at position 36. This assay can be performed without washing, providing advantages relative to conventional binding assays.

Normalized IC_{50} values (relative to GLP-1) derived from the competition BRET assay at equilibrium, with metabolically poisoned but intact cells, show that all four modifications at Gly10 of GLP-1 and all four modifications at Gly4 of exendin-4 cause declines in affinity for the GLP-1R relative to the natural agonist. Effects of the substitutions on affinity, however, were distinct from the effects of the substitutions on agonist potency as assessed by stimulation of cAMP production. Thus, the synthetic analogues of each natural agonist displayed affinities that were similar to one another (~six-fold variation among the four GLP-1 analogues and ~two-fold variation among the four exendin-4 analogues), but potencies for cAMP production varied by two orders of magnitude in each set (Fig. 1e, 1f; Table 1). The Gly10→D-Ala analogue of GLP-1 was indistinguishable from GLP-1 itself in terms of EC_{50}, but this analogue showed a ~35-fold diminution in affinity. The D-Ala analogue was ~17-fold more potent than the L-Ala diastereomer but bound only ~3-fold more tightly to the GLP-1R (Fig. 2a, 2b).

Among the four GLP-1 analogues, Gly10→(*S,S*)-ACPC was the least efficacious agonist but had highest affinity for the receptor. Conversely, the Gly10→(*R,R*)-ACPC analogue showed the lowest affinity but induced the same maximal response as GLP-1. A similar pattern was evident among the four exendin-4 variants. Both (*S,S*)-ACPC-containing analogues were effective *antagonists* of GLP-1-induced cAMP production in HEK293GS22 cells. Indeed, the Gly4→(*S,S*)-ACPC derivative of exendin-4 proved to be an even more potent antagonist than exendin-(9-39), which is currently in clinical trials for the treatment of post-bariatric hypoglycemia (Fig. 2a and Supplementary Table 1).[34] The superior antagonist activity of the Gly4→(*S,S*)-ACPC analogue relative to exendin-(9-39) suggests that interactions between the GLP-1R and the eight N-terminal residues of the (*S,S*)-ACPC analogue are energetically favorable. Exendin-(9-39) cannot activate the GLP-1R because of the N-terminal truncation relative to exendin-4;[23] therefore, the ability of the Gly4→(*S,S*)-ACPC analogue of exendin-4 to stimulate cAMP production, albeit weakly, suggests that the N-terminus of this analogue can engage the TMD core in a manner comparable to that of exendin-4 (or GLP-1) in the agonist-receptor state that activates the G protein. This conclusion and the propensity of (*S,S*)-ACPC to promote a right-handed α-helix-like conformation[28] together suggest that in the G protein-activating state, helicity extends to the N-terminus of GLP-1R agonist peptides, as seen in recent structures. Why, then, are the peptides that contain (*S,S*)-ACPC at the fourth position such weak agonists?

The potency, affinity and antagonism data can be collectively explained if signal transduction via $G_{\alpha s}$ is facilitated by flexibility in the receptor-bound agonist, specifically, an ability to access a non-helical conformation near the N-terminus. Data from assays for recruitment of β-arrestin-1 or -2 to the GLP-1R[26] suggest that agonist N-terminal flexibility is important for this signaling channel as well (Fig. 2b, 2c and Supplementary Table 2). Further support for the

functional importance of non-helical conformations near the agonist N-terminus was obtained from a BRET-based assay that monitors receptor-mediated changes in G protein conformation upon binding to the ligand-occupied receptor. Conformational transitions in the G protein that allow for guanine nucleotide exchange contribute to the rate limiting steps in G protein activation.[7,35] The Gly4→D-Ala derivative of exendin-4 had similar potency to exendin-4 itself in inducing conformational changes in the heterotrimeric G protein, albeit with modestly lower maximal response, while the Gly4→(*S,S*)-ACPC, (*R,R*)-ACPC, and L-Ala derivatives of exendin-4 were markedly less potent (Fig. 2d, Extended Data Fig 1., and Supplementary Table 3). The lower maximal changes in the BRET response for the synthetic analogues suggest that these analogues induce different conformations of the G protein relative to GLP-1 itself.

Cryo-EM analysis of Ex4-D-Ala/GLP-1R reveals two conformers. Cryo-EM studies were undertaken to investigate the receptor-bound form of the potent exendin-4 analogue containing D-Ala in place of Gly4 (referred to below as Ex4-D-Ala). We co-expressed the human GLP-1R, dominant negative $G_{\alpha s}$, $G_{\beta 1}$, and $G_{\gamma 2}$ in *Trichoplusia ni* cells. Nanobody 35, excess peptide ligand (10 μM), and apyrase were added to form a complex, which was solubilized in lauryl maltose neopentyl glycol (LMNG)/cholesterol hemisuccinate (CHS) mixed-micelles as previously reported.[7] This complex was purified by sequential anti-FLAG affinity and size exclusion chromatography in the presence of saturating ligand (2.5 μM) to yield a monophasic peak on SEC containing each of the components of the complex, which was confirmed in negative stain TEM (Extended Data Fig. 2a-e). Although we were also able to form a GLP-1R/G-protein complex with the Gly4→(*S,S*)-ACPC analogue of exendin-4 (Extended Data Fig. 2f-j), yields were poor, and the sample was too heterogenous by size-exclusion chromatography (Extended Data Fig. 2g) and negative stain TEM (Extended Data Fig. 2j) to warrant imaging by cryo-EM.

The purified Ex4-D-Ala complex was vitrified, and single particles were imaged on a Titan Krios TEM. After 2D and 3D classification of particle images, the consensus structure was refined to 2.3 Å global resolution. Upon further, focused 3D classification, it became apparent that the particles could be separated into two distinct, high-resolution maps, which were generated from particles in a ~2:1 ratio (Extended Data Fig. 3). Structures were solved independently for each class of particles. The structure from the more abundant particle class is designated "conformer 1" below, and the other structure is "conformer 2."

Conformer 1 was refined to a nominal global resolution of 2.4 Å (Extended Data Fig. 4). Despite some orientation bias, high local resolution in the receptor core and G protein enabled modeling of most of the complex, including the N-terminus of the peptide within the receptor core (Fig. 3, 4, and Extended Data Fig. 4e, 5). The local resolution in the ECD was lower but allowed for fitting of the ECD backbone and modelling of side chains in the peptide vicinity. Poor resolution was observed for the $G_{\alpha s}$ alpha-helical domain and for ICL3 (residues 59-204 and 338-340, respectively); these segments were omitted from the atomic model.

Conformer 1 is very similar to our recently published high-resolution structure of GLP-1 bound to the human GLP-1R (Fig. 4d).[6] The agonist in conformer 1 adopted an α-helical conformation along its entire length, despite the presence of D-Ala near the N-terminus (Fig. 4f). The D-Ala residue displayed right-handed helical Φ and Ψ torsion angles, -61° and -50°, respectively. The methyl side chain of D-Ala was close to the side chains of two receptor residues, $M233^{3.36}$ and $Q234^{3.37}$ (Fig. 4a, 4b, and Extended Data Fig. 6a); these residues influence the affinity and potency of the natural agonist GLP-1.[36] Interactions of these receptor side chains with the agonist D-Ala side chain might compensate for well-established helix-destabilizing effects[19,21] of the D-Ala residue. Even though Ex4-D-Ala shares the same C-terminal residues as the G protein-

biased agonist exendin P5 (ExP5),[7,37] Ex4-D-Ala adopted a distinct position relative to the receptor from that displayed by ExP5 (Fig. 4f). In addition, Ex4-D-Ala induced an ECL1 conformation closer to that in the GLP-1-bound structure (Fig. 4e) than that in the ExP5-bound structure (Extended Data Fig. 6).

Conformer 2 was refined to a nominal global resolution of 2.5 Å (Extended Data Fig. 4). Density for the ECD and ligand was very poorly resolved in this conformation, so we performed further refinement focused on the receptor alone (Extended Data Fig. 4 and 7). This additional refinement enabled visualization of density corresponding to the peptide ligand and extracellular domain in conformer 2. However, the resolution for these features was relatively low, and they were omitted from the atomic model. In addition to the ECD and ligand, ECL1 was omitted from the model for conformer 2, and sidechains were omitted for ECL3 because of low resolution. The poor resolution for these components suggests high mobility of the peptide, ECD, and ECL1 in conformer 2.

While poor local resolution disallowed *de novo* modelling of the ECD and bound peptide in conformer 2 at the atomic level, the ECD from the co-crystal structure of exendin-(9-39) and the GLP-1R ECD[5] could be confidently docked into the density for conformer 2 (Extended Data Fig. 7). The docked model revealed that the ECD position relative to the TMD was comparable for conformers 1 and 2 in our cryo-EM structures. The C-terminal portion of Ex4-D-Ala as observed in conformer 2 appeared helical and bound to the ECD in a fashion similar to that observed for the analogous portion of Ex4-D-Ala in conformer 1. Relative to conformer 1, however, the peptide as observed in conformer 2 was shifted outward from the TM-domain's central axis towards TM1, and there was a corresponding outward shift of TM1.

The ligand density in conformer 2 did not extend as deeply into the TMD as in conformer 1, and thus, the density does not support a helical conformation that extends to the N-terminus in conformer 2, in contrast to Ex4-D-Ala in conformer 1 (Extended Data Fig. 7d). Instead, the helix in Ex4-D-Ala of conformer 2 ended at Thr5, and continuous density for the remainder of the peptide projected towards ECL3. The resolution for the four N-terminal residues of Ex4-D-Ala in conformer 2 is very poor, which is consistent with high mobility of this segment (Fig. 4c). The features of the peptide in the conformer 2 structure support the hypothesis that the N-terminal segments of GLP-1R agonists occupying the orthosteric site can access non-helical conformations, which is consistent with our interpretation of functional data for the GLP-1 and exendin-4 analogues that are the focus of this study.

Comparison of the transmembrane portions of conformers 1 and 2 revealed that the orthosteric pocket of conformer 2 is more open than that of conformer 1 (Fig. 4d, 4e). This structural difference largely arises from outward motion of the top of TM6 and TM7 in conformer 2 and a more profound kink in the TM6 helix (~100° vs. ~71° for conformer 1 vs. 2), which together lead to a ~12 Å outward shift of ECL3 (measured Cα to Cα for D372^{ECL3}) in conformer 2 relative to conformer 1 (Fig. 4c-4e). The position and local conformation of ECL3 in conformer 2 are more similar to ECL3 in the structures reported for the GLP-1R bound to the small molecules TT-OAD2[38] and CHU-128[6], which do not contact this loop, than to ECL3 in the structure for the GLP-1R bound to GLP-1[6,39] or conformer 1 bound to Ex4-D-Ala (Extended Data Fig 6). In the case of peptide ligands, we hypothesize that an open ECL3 configuration, as observed in conformer 2, is required for the agonist N-terminal segment to explore non-helical conformations, because such N-terminal conformations would cause steric clashes with ECL3 positioned as in conformer 1.

The backbone of TM5 is similar in both conformers, but the R310$^{5.40}$ side chain adopts different rotamers in the two conformers. R310$^{5.40}$ is key for receptor activation,[40,41] and its side chain projects into the orthosteric binding pocket of the TMD in conformer 2. Conversely, the R310$^{5.40}$ side chain projects toward the ECL3 in conformer 1 (Fig. 4c). In conformer 1 and in the GLP-1-bound GLP-1R structure,[6] R310$^{5.40}$ forms a salt-bridge with E373^{ECL3}, but the outward shift of ECL3 in conformer 2 apparently prevents R310$^{5.40}$ from making a comparable contact. Overlaying the two conformers shows that the position of the R310$^{5.40}$ side chain guanidinium group in conformer 2 clashes with the agonist N-terminus in conformer 1 (Fig. 4c). Thus, unfavorable electrostatic and steric interactions make it impossible for conformer 2 to accommodate the positioning and N-terminal helicity of the agonist as observed in conformer 1. Beyond the orthosteric site in the TMD, the agonist, and the ECD, the structures of conformers 1 and 2 are largely similar.

Molecular dynamics simulations of receptor-bound Ex4-D-Ala. The detection of two conformers in the cryo-EM analysis and the varying local resolution in each of the conformers suggest greater structural dynamics of the GLP-1R:Ex4-D-Ala complex relative to other GLP-1R-peptide complexes recently analyzed via cryo-EM.[6] To gain further insight into the mobility of the receptor-Ex4-D-Ala complex, we undertook molecular dynamics (MD) simulations to probe the structural plasticity of the assembly.

Initial simulations involved Ex4-D-Ala, Ex4, and Ex4-L-Ala in aqueous solution (no receptor). Despite clear evidence from prior studies indicating the order of helix propensities to be L-Ala ~ (*S,S*)-ACPC > Gly > D-Ala > (*R,R*)-ACPC,[19,29,42] it was difficult to detect conformational differences among the GLP-1 analogues experimentally (circular dichroism or NMR; Extended Data Fig. 8), which presumably reflects fraying near helix termini.[43] In our peptide simulations,

residues near the N-terminus of each peptide were mostly unstructured, consistent with experimental observations.[15] The MD trajectories allowed us to calculate helix probability on a per-residue basis.[44] Ex4-D-Ala showed the lowest helix fraction, Ex4-L-Ala showed the highest helix fraction, and Ex4 itself showed an intermediate helix fraction across residues 3-5 (Extended Data Fig. 9). This trend is consistent with our experimental design hypothesis.

We performed simulations of a Ex4-D-Ala-receptor complex to evaluate possible interconversion between conformers 1 and 2. Such a substantial structural rearrangement is likely to occur on a timescale that cannot be readily assessed by classical MD simulations; therefore, we applied well-tempered metadynamics and supervised MD[9,45] to simulate partial unbinding and rebinding of the peptides to the TMD (Extended Data Fig. 10, Supplementary Table 4, and Videos S5-S7). Using a model derived from conformer 1 as a starting point, we found that the N-terminal section of Ex4-D-Ala can unbind and reengage the TMD either by returning to a conformer 1-like structure or by forming a structure similar to conformer 2. In the latter trajectory (Video S7), the peptide ligand is highly mobile, which is consistent with the low resolution observed for these components in EM reconstructions (Fig. 5a). Ex4-D-Ala in this trajectory features helicity that terminates at Thr5, while the N-terminal segment engages the receptor between TM5 and ECL3 in an extended conformation, favored by an opening of ECL3, as observed in conformer 2. The R310$^{5.40}$/E373^{ECL3} salt-bridge observed in conformer 1 is disrupted in this trajectory, and the two side chains are sterically blocked from interacting with one another by the peptide N-terminus. Parallel simulations performed with Ex4-L-Ala (VideoS5) and Ex4 (VideoS6) did not sample states attributable to conformer 2. We speculate that the simulations were not long enough for Ex-4 to sample short-lived states corresponding to conformer 2. This hypothesis is consistent with the

observation of only fully helical agonists (conformer 1-like states) in previously reported structures.

Collectively, the MD simulations are consistent with our experimental evidence, both structural and functional, and support the occurrence of non-helical conformations in the N-terminal segment of Ex4-D-Ala and, by extension, the native GLP-1R-bound agonists. Furthermore, the simulations are aligned with structural data in suggesting ligand-dependent plasticity of ECL3. This plasticity might be important for the role of this extracellular loop in signal transduction.[46]

Discussion

The combination of functional, structural and computational data presented here supports a new view of signal transduction via the GLP-1R in which two distinct states of the receptor-agonist complex play important roles in the transfer of information across the cell membrane. This possibility was raised by apparent inconsistencies between effects of targeted sequence changes in GLP-1 and exendin-4 analogues and recent structural findings. Our data are consistent with a model in which the completely helical agonist induces a GPCR conformation that activates an intracellular partner protein, but a distinct agonist conformation, lacking helicity in the N-terminal segment, is reversibly accessed to enable multiple rounds of partner protein activation from a single agonist-receptor engagement, which leads to high agonist efficacy.

According to our model, conformer 1 in our cryo-EM structure of the Ex4-D-Ala-GLP-1R complex approximates the form of the agonist-bound receptor required for G protein activation. Adoption of this receptor conformation is presumably favored under conditions used to form a complex that is sufficiently stable to be imaged (inclusion of dominant negative G protein and

nanobody 35, apyrase treatment). However, agonist efficacy might be compromised if conformer 1 were too long-lived. Dynamics of TMD engagement and release could impact the number of G protein activation cycles that result from a single agonist-binding event. If the agonist can partially disengage from the TM core but retain contact with the ECD, then the receptor could release the activated G protein and be ready to activate a newly recruited G protein. Conformer 2 in our cryo-EM structure might represent a partially ligand-bound state of the GLP-1R, which would presumably occur on the energy surface of the agonist-receptor complex at a position between the completely dissociated and fully bound states (Fig. 5e).

The stabilities of partially bound and fully bound states and the height of the intervening energy barrier could all be affected by alterations at Gly10 of GLP-1 or Gly4 of exendin-4 (Fig. 5), and changes in these factors might explain the functional variations observed among the peptides studied here. We propose that poor efficacy of peptides containing (*S,S*)-ACPC arises because this residue stabilizes helicity near the N-terminus, relative to the native Gly, and thereby raises the energy barrier between the partially and fully bound states. Hindered exchange between these states might prevent the activation of multiple G proteins after a single agonist-receptor association event. For the analogues containing D-Ala, on the other hand, disfavoring helicity near the N-terminus might lower the barrier for interconversion between the partially and fully bound states and therefore enhance the likelihood that multiple G protein activation cycles ensue from a single agonist-receptor association. In this case, the diminished affinities of the D-Ala analogues relative to the natural agonists could be compensated by an increase in average number of G proteins activated, leading to the observed similarity in receptor activation potency of the D-Ala analogues relative to GLP-1 and exendin-4.

We cannot rule out the possibility that either of our cryo-EM-derived conformers, alone, represents the signal-transducing form of the agonist-receptor complex, and that the other conformer lacks functional significance. However, in addition to being supported by data presented above, our two-state GLP-1R activation hypothesis is consistent with previous studies that support a role for ligand mobility in activation of other GPCRs.[47–49] Our findings are distinct from these precedents, however, in suggesting that at least two distinct states of an agonist-receptor complex play important and complementary roles in the signal transduction mechanism.

Our conclusions are consistent with emerging evidence that conformational mobility in agonist and receptor can be functionally important in signal-transducing states of other GPCR-peptide complexes.[47–49] The mode of agonist mobility highlighted in this work may be evolutionarily conserved among peptide agonists of related GPCRs; Gly at the fourth position from the N-terminus is found in glucagon, GLP-2 and several other hormones.[16] Other sites of essential mobility may be present in more distantly related hormones, such as parathyroid hormone.[50]

Understanding the role of structural dynamics in the propagation of molecular information across the cell membrane is important in terms of elucidating GPCR function and developing improved therapeutic agents. A dynamics-based approach to drug design would represent a departure from traditional approaches, which focus on promoting a specific conformation rather than retaining or enhancing particular modes of conformational mobility that might contribute to efficacy by mechanisms other than high-affinity binding. A deeper understanding of the conformational possibilities available to GPCRs bound to flexible agonists, and of relationships among conformational states and signal transduction, will enhance prospects for elucidating signal-propagating mechanisms at the molecular level and optimizing therapeutic performance.

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Author Contributions Statement

B.P.C. designed the project, synthesized peptides, generated the nanoluc-GLP-1R construct, and expressed and purified protein complex. G.D. performed and analyzed molecular dynamics simulations. B.P.C., P.Z., and T.T.T. conducted in vitro assays. B.P.C., S.J.P., and M.J.B. processed the cryo-EM data, built the model, and performed refinement. S.J.P. and M.J.B. performed multivariate analysis, assisted in data interpretation, and assisted in figure preparation. X.L. performed NMR measurements and analyzed spectra. R.D. prepared the cryo-EM sample and collected EM data. P.M.S., D.W., and S.H.G. supervised the project. B.P.C., P.M.S., D.W., and

S.H.G. interpreted data, generated figures, and wrote the manuscript. All authors reviewed and edited the manuscript.

Competing Interests Statement

S.H.G. is a cofounder of Longevity Biotech, Inc., which is pursuing biomedical applications for α/ß-peptides. P.M.S. receives research funding from Laboratoires Servier in the area of GPCR drug discovery. The current study is 100% independent from all academic or commercial collaborations with industry.

Tables

	cAMP Production				Whole-Cell Affinity		
	pEC_{50}	EC_{50} (nM)	% Max	$EC_{50\ rel.}$	pIC_{50}	IC_{50} (nM)	IC_{50} rel.
GLP-1[a]	10.5 ± 0.1	0.031	95 ± 4	1	8.27 ± 0.07	5.4	1
GLP-1-D-Ala	10.4 ± 0.09	0.044	102 ± 4	1.4	6.73 ± 0.05	187	35
GLP-1-*R*,*R*-X	8.87 ± 0.1	1.4	99 ± 5	45	6.19 ± 0.06	650	120
GLP-1-L-Ala	9.12 ± 0.1	0.76	99 ± 5	24	6.31 ± 0.06	500	93
GLP-1-*S*,*S*-X	7.91 ± 0.2	12	60 ± 10	400	6.91 ± 0.08	120	22
Ex-4	10.6 ± 0.1	0.026	99 ± 4	0.8	7.79 ± 0.04	16	3
Ex-4-D-Ala	10.0 ± 0.1	0.093	110 ± 4	3	6.88 ± 0.03	134	25
Ex-4-*R*,*R*-X	8.25 ± 0.1	5.6	87 ± 5	180	6.69 ± 0.03	200	37
Ex-4-L-Ala	8.62 ± 0.08	2.4	96 ± 4	77	6.84 ± 0.04	143	26
Ex-4-*S*,*S*-X	7.54 ± 0.1	29	16 ± 2	940	7.10 ± 0.04	80	15

Table 1 | EC_{50} values, maximal responses, and IC_{50} values from 3-parameter sigmoidal fits for concentration-response data in Fig 1. EC_{50} rel. indicates cAMP production potency relative to GLP-1 by the quotient (peptide EC_{50}) / (GLP-1 EC_{50}). IC_{50} rel. indicates the affinity relative to GLP-1 by the quotient (peptide IC_{50}) / (GLP-1 IC_{50}) [a] GLP-1 was averaged over six sets of independent measurements. Uncertainties are expressed as standard error of the mean.

Figure Legends/Captions (for main text figures)

Fig. 1 | **Probing the relationship between agonist N-terminal conformational propensity and receptor activation with single substitutions. a,** Left: Cartoon depiction of an agonist peptide (purple) bound to a class-B GPCR. The extracellular domain (ECD) and transmembrane domain (TMD) are labeled. Right: Amino acid residues used to replace Gly10 of GLP-1 or Gly4 of exendin-4. **b**, Sequences of exendin-4, GLP-1, and analogues. Lowercase ‘a’ represents D-Ala, uppercase ‘X’ represents the β-amino acid residue (*S*,*S*)-X, and lowercase ‘x’ represents (*R*,*R*)-ACPC. **c-d**, Activation of GLP-1R-FLAG by GLP-1, exendin-4, or an analogue as measured by cAMP production. Data points represent the mean of three independent experiments. **e-f**, Competition binding assay for Nluc-GLP-1R, detected via BRET, under equilibrium conditions, performed with intact, NaN_3-treated HEK293GS22 cells. Data points represent the mean of either three or four independent experiments, for **f** and **e** respectively. Error bars represent standard error.

Fig. 2 | **Diverse measures of GLP-1R engagement. a**, Inhibition of GLP-1-stimulated cAMP production in HEK293GS22 cells expressing hGLP-1R. Cells were preincubated for 15 min with increasing concentrations of the GLP-1 or exendin-4 containing (*S,S*)-ACPC or Ex (9-39) followed by stimulation with 0.25 nM GLP-1. Grey symbols with dotted connecting lines represent the cAMP accumulation in response to the (*S,S*)-ACPC derivative of GLP-1 (*S*,*S*-X) or exendin-4 before addition of GLP-1. **b**, β-arrestin-1 recruitment to GLP-1R-Rluc8, detected via BRET, for GLP-1 and analogues. **c**, β-arrestin-2 (R939E, R395E) recruitment to GLP-1R-Rluc8, detected via BRET, for GLP-1 and analogues. **d**, G protein conformational rearrangement as measured by BRET between Gαs–nanoluc and $G\beta_1\gamma_2$–venus at a terminal timepoint (12 min) for exendin-4 and analogues. The P-values compare the fitted maximal responses of Ex4-D-Ala and GLP-1 or Ex4. The P values were determined by one-way ANOVA with Bonferroni’s post-test. Data points for **a-c** represent the mean of three independent experiments, while for **d** n = 3, 7, 7, 3, 4, and 3 independent replicates for GLP-1, Ex4, Ex4-D-Ala, Ex4-R,R-X, Ex4-L-Ala, and Ex4-S,S-X, respectively. Error bars represent standard error.

Fig. 3 | **Cryo-EM structure of Ex4-D-Ala bound to the GLP-1R in complex with the heterotrimeric G-protein and nanobody 35. a,** The models of the two conformers are shown within the cryo-EM-derived density maps, which are depicted as a transparent surface. GLP-1R in conformer 1 is colored blue, while GLP-1R in conformer 2 is colored orange. The number of particles used in the reconstruction indicated an approximately 2:1 ratio of conformer 1 to conformer 2. Dominant negative Gαs, Gβ1, Gγ, and nanobody 35 are colored yellow, aqua, purple, and gray, respectively. **b**, The orthosteric binding pocket of GLP-1R in conformer 1 is shown with the ECD and ECL3 removed for clarity. Ligand density is shown in red. **c**, he orthosteric binding pocket of GLP-1R in conformer 2 is shown with the ECD and ECL3 removed for clarity. The ligand density is colored red within 2 Å of the fitted ligand (see Extended Data Fig. 7 for the fit of Ex4-D-Ala for conformer 2).

Fig. 4 | **Comparisons of conformers 1 and 2 for Ex4-D-Ala bound to the GLP-1R. a**, A close-up, side-view of the orthosteric binding pocket of conformer 1. TM6, ECL3, and TM7 were removed for clarity. **b**, A close-up, side-view of the orthosteric binding pocket of conformer 1 rotated relative to the view in **a**. TM4, ECL3, and TM5 were removed for clarity. **c**, An overlay of conformer 1 and conformer 2, with the GLP-1R shown in blue and orange, respectively. The ligand in conformer 1 is shown in grey; density assigned to the ligand in conformer 2 is shown in red. **d**, A side-view comparison of GLP-1 bound the GLP-1R (PDB 6X18), the GLP-1R without agonist (PDB 6LN2) or G-protein bound, and Ex4-D-Ala bound to the GLP-1R in conformers 1 and 2 (colored blue and orange, respectively); no ligands are shown. **e**, An extracellular view of the structures compared in Fig. 4d, but with the extracellular domain removed for clarity. The boxes indicate movements of specific helices or loops relative to the GLP-1R as seen in the agonist-free crystal structure (PDB 6LN2). **f,** A comparison of positioning and conformation of three receptor-bound agonists (GLP-1 in green (PDB 6X18), ExP5 in teal (PDB 6B3J), and Ex4-D-Ala as observed in conformer 1 in red) when the receptor-G-protein complexes are aligned.

Fig. 5 | **MD simulations of Ex4-D-Ala bound to the GLP-1R, and a proposed, simplified energy landscape for the interaction of the GLP-1R with peptide agonists. a,** Comparison of the conformer 2 model (orange) with MD frames after simulated rebinding. Snapshots were taken every 25 ns from 50 ns to 200 ns. **b,** Proposed, simplified energy landscape for interaction of the GLP-1R with peptide agonists. Dissociated agonist (colored purple) and the GLP-1R are represented as a high energy state at the left. The GLP-1R with fully engaged GLP-1 is represented as the deep energy well at the right. Because the Ex4-D-Ala binds to the GLP-1R with lower affinity than does GLP-1, the energy well for the fully engaged state for Ex4-D-Ala (shown in red) is shallower. The central energy well reflects a bound state of intermediate stability that features significant internal motion; we hypothesize that conformer 2 represents this intermediate state.

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Methods

Cell culture. HEK293-GS22 cells were maintained in 75 cm^2, culture-treated, vented flasks (Corning) in a humidified atmosphere at 37°C with 5% CO_2. Cell medium was 0.22 μm-filtered DMEM supplemented with 10% (v/v) FBS and penicillin/streptomycin. Cells were subcultured every 4-5 days at confluency. HEK293FT cells were maintained under similar conditions except their medium was supplemented with 100-fold diluted 100X L-glutamine, 100X sodium pyruvate and, 100X MEM NEAA to working concentrations of 1X. After receipt of cells, cultures were not tested for mycoplasma contamination.

cAMP-Glosensor luminescence receptor activation assays. HEK293 cells stably expressing the Glosensor-22F (Promega) luminescent cAMP-sensing protein[51] were grown to confluence, harvested, and then 1/3 of the collected cells were plated onto a 10 cm tissue-treated dish with 10 mL 10% FBS in DMEM without penicillin/streptomycin. The cells were then incubated at 37°C with 5% CO_2 overnight. After the overnight incubation, the medium was aspirated, 4.5 mL McCoy's 5A modified medium with 10% FBS was added, and the cells were incubated at 37°C with 5% CO_2. During this incubation, 5 μg of GLP-1R

receptor plasmid (as a solution in endotoxin-free TE buffer (Qiagen); the construct contained a C-terminal FLAG tag and a His_6 tag immediately upstream of the FLAG tag) and 15 µL FuGENE HD transfection reagent was added to 1 mL of opti-MEM. After 20 min, 4.5 mL of DMEM with 10% FBS was added to the cells, and 1 mL of the transfection mixture was gently pipetted into the medium. The cells were then returned for incubation overnight. The next day, the cells were washed with Dulbecco's phosphate buffered saline (DPBS), harvested with 0.05% Trypsin-EDTA, and resuspended into 4 mL 10% FBS in DMEM without penicillin/streptomycin. The cell suspension was diluted to approximately 500,000 cells/mL in medium, and 100 µL of cell suspension was pipetted into each well (providing ~50,000 cells/well) of a white-bottom, white-walled, 96-well plate. The cells in the 96-well plate were incubated at 37°C with 5% CO_2 overnight. After 24 h, the medium was removed by inverting and gently flicking the plate. DPBS with D-luciferin (500 µM) was quickly added to the plate (90 µL/well). The plate containing cells was allowed to sit for approximately 20 min at room temperature before addition of peptide (as 10 µL/well diluted in DPBS). Pipette tips were changed between serial dilutions. The plate was then transferred to a BioTek Synergy 2 plate reader with no optical filter ("hole"), 1 mm vertical probe offset, and read with a sensitivity value of 200.

Data analysis. Curves were generated from luminescence values observed between 10 and 20 min. Reported EC_{50} and %Max values were a result of normalizing, averaging, and then fitting data to three-parameter sigmoidal curves in GraphPad Prism 6. The bottom of the curves was constrained to 0%. Normalization was performed with 100% representing the top of GLP-1's curve for individual experiments and 0% representing the luminescence value in the absence of peptide.

Bioluminescence resonance energy transfer (BRET) whole-cell competition ligand binding assay:

HEK293 cells stably expressing the Glosensor-22F (Promega) luminescent cAMP-sensing protein were harvested, transfected, and transferred to 96-well plates identically as above (cAMP-activation assay) except 1.2 µg of nanoluc-fused[52,53] GLP-1R plasmid (nluc-GLP-1R, as a solution in endotoxin-free TE buffer) and 5 µL FuGENE HD reagent were used for the transfection step. Forty-eight hours after transfection, the medium was removed by inverting and gently flicking the plate. A solution of DPBS, 0.1% BSA, and 0.02% NaN_3 was added to the wells (90 µL/well), and the plate was transferred to a cold room (4 °C) for 15 min. Dilutions of unlabeled, competitor peptides were made in DPBS in a polypropylene 96-well plate. The energy acceptor, GLP-1(7-35)-Lys((5)-6-tetramethylrhodamine)-NH_2, was added (10 µL, 2 µM in DPBS) to each solution of diluted competitor peptide and mixed thoroughly. The resulting mixtures of competitor peptides and energy acceptor peptide (final concentration of 20 nM) were pipetted to the wells containing cells. The 96-well plate was covered with aluminum foil and allowed to sit at 4°C. After 16-24 h, the plate was removed from the cold room and allowed to equilibrate at room temperature for 5 min. A solution of H-Coelenterazine (2 mL DPBS + 100 µL of 1.4 mM H-Coelenterazine in ethanol) was prepared and added to the 96-well plate (10 µL solution/well). The plate was then transferred to a BioTek Synergy 2 plate reader and read with a 1 s integration time over the course of 1 h with 460 nm (40 nm bandwidth) and 590 nm (35 nm bandwidth) optical filters with a sensitivity value of 170.

Data analysis. For individual experiments, the calculated BRET data (maximum I_{590nm}/I_{460nm} values) were fit to 3-three-parameter sigmoidal curves with shared top and bottom values. Those top and bottom values were applied to normalize the data to 100% and 0%, respectively. Then, the normalized data were averaged among the biological replicates and once again fit to a 3-three-parameter sigmoidal curves with top and bottom constraints of 100% and 0%, respectively.

GLP-1R cAMP inhibition assays. HEK293 cells stably expressing the Glosensor-22F (Promega) luminescent cAMP-sensing protein were harvested, transfected, and transferred to 96 well plates identically as above (cAMP-activation assay) except using 10 µg of hGLP-1R plasmid (as a solution in endotoxin-free TE buffer (Qiagen), pCMV6-XL5 [hGLP1-R]) and 30 µL FuGENE HD reagent were used for the transfection step. Forty-eight hours after transfection, the medium was removed from the plates by inverting and gently flicking the plate. DPBS with D-luciferin (500 µM) was quickly added to the plate (90 µL/well). The plate containing cells was allowed to sit for approximately 5 min at room temperature before addition of peptides (as 10 µL/well diluted in DPBS). Pipette tips were changed between serial dilutions. The plate was then transferred to a BioTek Synergy 2 plate reader with no optical filter ("hole"), 1 mm vertical probe offset, and read with a sensitivity value of 200. After 15 min, GLP-1 (7-36)-NH_2 was added to each well to a final concentration of 250 pM, and the plate was re-read for 30 min. Inhibition curves were generated from luminescence values observed between 10 and 20 min.

Data analysis. For individual experiments, data from the selected timepoints were fit to 3-three-parameter sigmoidal curves with shared top values. Bottom values were constrained for GLP-1-*S,S*-X and Ex-4-*S,S*-X based on their maximal luminescence in the absence of GLP-1. Ex9-39 was constrained with a bottom value of 0%. Top and bottom values were applied to normalize the data to 100% and 0%, respectively. Then, the normalized data were averaged among the biological replicates and once again fit to a 3-three-parameter sigmoidal curve.

Bioluminescence resonance energy transfer (BRET) β-arrestin recruitment assay. HEK293FT cells were grown confluence, harvested, and then 1/3 of the collected cells were plated onto a 10 cm tissue-treated dish with 10 mL 10% FBS in DMEM (with NEAA, L-glutamine, and sodium pyruvate, see above) without penicillin/streptomycin. The cells were then incubated at 37°C with 5% CO_2 overnight. After 24 h, a transfection mixture was made with 1:1 polyethylenimine (PEI, 1 mg/mL in water, pH 7.0):DNA in 1 mL opti-MEM. Either GFP^2-β-arrestin-1 (14 µg) or GFP^2-β-arrestin-2(R393E, R395E) (14 µg) along with GRK5 (250 ng) and GLP-1R-Rluc8 (250 ng for β-arrestin-1 experiments or 130 ng for β-arrestin-2 experiments) was added to the PEI/Opti-MEM mixture, and the resulting transfection mixture was incubated for 20 min at room temperature. The cell medium was then aspirated, replaced with 4.5 mL of DMEM without FBS, and the transfection mixture was gently pipetted into the medium. Six hours after transfection (with incubation at 37°C with 5% CO_2), 4.5 mL of DMEM supplemented with 20% FBS was added to the dish. Twenty-four hours after transfection (with incubation at 37°C with 5% CO_2), cells were washed with DPBS, harvested with 0.05% Trypsin-EDTA, and resuspended into 4 mL 10% FBS in DMEM (with NEAA, L-glutamine, and sodium pyruvate, see above). The cell suspension was diluted to approximately 1,000,000 cells/mL in medium, and 100 µL of cell suspension was pipetted to each well (providing ~100,000 cells/well) of a white-bottom, white-walled, 96-well plate. The cells in the 96-well plate were incubated at 37°C with 5% CO_2 overnight. Twenty-four hours after adding cells to the 96-well plate, the medium was removed by pipette, the cells were washed twice with DPBS (with glucose, 100 µL/well), and 100 µL of DPBS (with glucose) was added to each well. The cells were incubated at 37°C with 5% CO_2 for 45 min to 1 h before addition of peptide (as 10 µL dilutions in DPBS). After addition of peptides, the cells were allowed to sit at room temperature for 20 min before addition of the Rluc8 substrate, DeepBlueC (10 µL/well of 60 µM DeepBlueC in 2:1 DPBS:ethanol). The 96-well plate was transferred to a BioTek Synergy 2 plate reader with 400 nm (20 nm bandwidth) and 528 nm (30 nm bandwidth) optical filters, 1 mm vertical probe offset, 1-second integration time and read at maximum (200) sensitivity. Concentration-response curves were generated with I_{528nm}/I_{400nm} values taken between 15 and 45 min after initial read, as signal variability was found to be relatively high at earlier timepoints.

Data analysis. Reported EC_{50} and %Max values were a result of normalizing, averaging, and fitting data to three-parameter sigmoidal curves in GraphPad Prism 6. The bottom of the curves was constrained to 0%. Normalization was performed with 100% representing the top of GLP-1's curve for individual experiments and 0% representing the bottom value if the curves were fit with raw data and constrained to have a shared minimum.

Bioluminescence resonance energy transfer (BRET) G protein conformation assay. HEK293A cells that stably express hGLP-1R were transiently transfected with Gαs-Nluc, Gβ1, and Gγ2-Venus at 1:1:1 ratio (20 μg total DNA/T175 flask). 24 hours post transfection, cells were harvested and homogenized with a polytron homogenizer at 4°C in membrane buffer (20mM BisTris pH 7.4, 50 mM NaCl, 1 mM $MgCl_2$, 1×P8340 (protease inhibitor cocktail, Sigma). Cell homogenate was applied to a stepped sucrose gradient (60%, 40%, homogenate) and centrifuged at 22500 rpm for 2.5 hours at 4°C. The layer between 40% and homogenate were collected and diluted in membrane preparation buffer and centrifuged at 30,000 rpm for 20 min at 4°C. The final pellet was resuspend in 100 μL membrane buffer, aliquoted and stored in -80°C. Total protein concentration was determined using a nanodrop spectrophotometer. 5 μg per well of cell membrane was incubated with furimazine (1:1,000 dilution from stock) in assay buffer (1× HBSS, 10 mM HEPES, 0.1% (w/v) BSA, 1× P8340 protease inhibitor cocktail, 1 mM DTT and 0.1 mM PMSF, pH 7.4). The GLP-1R-induced BRET signal between $G\alpha_s$-Nluc and Gγ-Venus was measured at 30 °C using a PHERAstar (BMG LabTech with Emission 1: 475-30nm, and Emission 2: 535-30nm). Baseline BRET measurements were taken for 2 min at 15 second intervals before addition of vehicle or increasing concentration of the ligands, and the measurement was continued for a further 10 minutes before GTP was added (30 μM) to induce G protein dissociation. Data were corrected for baseline and vehicle treated samples.

Complex Purification. Insect cell-pellets overexpressing FLAG-GLP-1R-His, DNGαs,[54] Gβ1 and Gγ2 from 1.25 L of culture (~30 g) were thawed and suspended in 80 mL of 30 mM HEPES, 50 mM NaCl, 2 mM $MgCl_2$ and 5 mM $CaCl_2$ (pH 7.4) supplemented with 2 μL benzonase and 2 x cOmplete Protease Inhibitor Cocktail tablets. GLP-1R ligand (peptide as 2.5 mM stocks in H_2O) was added to a final concentration of 10 μM. The mixture was stirred for 30 min. at room temperature. To the mixture was added apyrase (10 μL) and ~1 mg nanobody 35. The mixture was stirred for another 30 min. at room temperature. After stirring, 20 mL of detergent solution (5% LMNG and 0.3% CHS w/v in ddH_2O) and 1.4 mL 5 M NaCl was added to the cell suspension. The suspension was Dounce homogenized 5-times with a tight pestle. Then, 80 mL of 30 mM HEPES, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM $CaCl_2$ (pH 7.4) and 0.8 mL of 5 M NaCl were added. The resulting mixture was stirred for 1 h at 4 °C. Insoluble debris were removed by centrifugation (30,000 g for 15 min.), and the supernatant was filtered with a glass fiber prefilter. Anti-FLAG affinity gel (~3 mL) was equilibrated in 30 mM HEPES, 50 mM NaCl, 2 mM $MgCl_2$ and 5 mM $CaCl_2$ (pH 7.4) and added to the filtered supernatant. The resulting mixture was placed on a rotator for 2 h at room temperature. The resin was then transferred to a glass column and washed with 100 mL wash buffer (20 mM HEPES, 100 mM NaCl, 2 mM $MgCl_2$, 5 mM $CaCl_2$, 2.5 μM GLP-1R ligand, and 0.01% LMNG + 0.0006% CHS). Crude complex was eluted with 25 mL elution buffer (20 mM HEPES, 100 mM NaCl, 2 mM $MgCl_2$, 0.2 mg/mL FLAG peptide, 2.5 μM GLP-1R ligand, 10 mM EGTA, and 0.01% LMNG + 0.0006% CHS, pH 7.4). TCEP (0.5 M) was added to the elution volume to a final concentration of 0.1 mM. The elution mixture was concentrated to a volume of ~0.5 mL with a 100 kDa MWCO centrifugal concentrator, and this solution was filtered with a 0.22 μm centrifugal filter before purification by size exclusion chromatography (SEC). The complex was resolved with on a Superdex 200 Increase 1G/300 GL column with 0.9 mL/min SEC running buffer (20 mM HEPES, 100 mM NaCl, 2 mM $MgCl_2$, 2.5 μM GLP-1R ligand, 0.1 mM TCEP and 0.01% LMNG + 0.0006% CHS, pH 7.4) SEC fractions containing complex were collected, pooled, concentrated to ~4 mg/mL, flash frozen in liquid nitrogen, and

stored at -80 °C for further use. Small aliquots of SEC fractions were directly flash frozen for negative stain electron microscopy.

SDS-PAGE and Western Blotting. Samples were prepared for SDS-PAGE and western blotting with a 1:1:1 mixture of sample, 10% (w/v) sodium dodecyl sulfate (aq.), and Laemmli loading buffer containing 2-mercaptoethanol. Sample mixtures were not heated before loading onto gels. SDS-PAGE samples (10 µL) were loaded and run on Mini-PROTEAN TGX Precast 4-15% gels. SDS-PAGE gels were stained with InstantBlue Coomassie stain (Abcam). For western blots, proteins were transferred (20 V, 12 h, 4 °C) onto PVDF membranes and blocked with 5% bovine serum albumin. Antibody solutions were applied for 1 h at room temperature.

Negative-stain transmission electron microscopy. Samples collected and flash frozen directly from SEC fractions were thawed and diluted to ~0.01-0.03 mg/mL with SEC buffer (without detergent). Immediately after dilution, 4 µL of sample was spotted on freshly glow-discharged (positive polarity, air chamber, 10 mA, 30 s) EM grids (carbon film on Cu, 300 mesh). After 60 s, excess sample was blotted away with Whatman filter paper. Uranyl formate (10 µL, 0.77% w/v aqueous solution) was applied to the grid and blotted three times. On the third application of uranyl formate, the solution was allowed to sit on the grid for 30 s before blotting. The grid was dried and loaded into a Talos L120C microscope. Micrographs were collected at 120 kV accelerating voltage, 73,000 magnification, and approximately -0.5 to -1.0 µm defocus values. Data were imported into and processed with RELION 3.1.

Cryo-electron microscopy. CryoEM was performed as previously described[55] with minor modifications. Samples (3 µL) were applied to acetone-prewashed, glow-discharged Ultrafoil R1.2/1.3 Au 300 mesh grids (Quantifoil GmbH, Großlöbichau, Germany) and flash frozen in liquid ethane using a Vitrobot Mark IV (Thermo Fisher Scientific, Waltham, MA, USA). Blot force was set to 19 and blot time set to 10 s. The Vitrobot sample chamber was set to 100% humidity and 4°C. Data were collected on a Titan Krios G3i microscope (Thermo Fisher Scientific, Waltham, MA, USA) operated at an accelerating voltage of 300 kV with a 100 µm objective aperture at an indicated magnification of 105 000× in nanoprobe EFTEM mode and a spot size of 5. A Gatan K3 direct electron detector positioned post a Gatan Quantum energy filter (Gatan, Pleasanton, CA, USA), operated in a CDS mode with a slit width of 25 eV was used to acquire dose-fractionated images. Movies were recorded as compressed TIFFs in normal-resolution mode yielding a physical pixel size of 0.83 Å/pixel with an exposure time of 5.011 s amounting to a total exposure of 49.8 $e^{-}/Å^{2}$ for at an exposure rate of 9.94 $e^{-}/ Å^{2}$/second that was fractionated into 71 subframes. The target defocus was set to −1.5 µm with 0.1 µm increments between holes. Beam-image shift was used to acquire data from 9 surrounding holes after which the stage was moved to the next collection area.

Cryo-electron microscopy processing: All processing was performed in RELION 3.1 beta[56] unless specified otherwise. 5805 movies were imported and motion-corrected with MotionCor2.[57] CTF estimation was performed with GCTF v1.06.[58] Particle picking was performed with crYOLO[59] which yielded 3,450,481 initial particle projections. Reference free 2D classification was performed and classes were manually selected providing 1,284,047 projections. Initial 3D classification with alignment was performed with a lowpass filtered (16 Å) reference map derived from a GLP-1R/G-protein structure. Two favorable classes were manually selected providing 671,599 particle projections. These particles were subjected to Bayesian polishing, CTF refinement, and 3D-auto refinement. This process provided a map with a global resolution of 2.32 Å (0.143 FSC, with detergent micelle and α-helical domain masked) corresponding to the consensus map. A mask excluding the density for the G protein, detergent micelle, and the distal region of the ECD was used to perform 3D classification without alignment (30 iterations, regularization parameter τ of 10) on the particles comprising the consensus refinement. Two classes were manually selected, and particles (totaling 414,673 images) from these classes were refined in RELION 3.1 providing the density

map for “conformer 1” with a global resolution of 2.41 Å (0.143 FSC, with detergent micelle and α-helical domain masked). Another class was manually selected with 221,955 particles. These particles were subjected to refinement in RELION 3.1 providing a map with a global resolution of 2.51 Å (0.143 FSC, with detergent micelle and α-helical domain masked) corresponding to “conformer 2.” Additional refinement focused on the receptor was performed on both sets of particles comprising conformer 1 and conformer 2.

Atomic Modelling: The structure of GLP-1/GLP-1R/Gs (PDB: 5VAI)[8] was used as a template and rigidly fit into the cryo-EM density. COOT[60] 0.9.4.1 EL and ISOLDE 1.1.0[61] (implemented in UCSF ChimeraX 1.1[62]) were used to manually fit and refine the models. The structure of the PF-06882961/GLP-1R/Gs (PDB: 6X1A)[6] was used as a template for the Gs α-helical domain. Automated real-space refinement and validation were performed with the PHENIX v1.19 software package.[63]

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Circular dichroism spectroscopy: Lyophilized peptide powder was reconstituted in either ultrapure water or 30% 2,2,2-trifluoroethanol (% v/v) in ultrapure water to 25 µM (unless otherwise specified). Solutions were pipetted into capillary tubes (0.5 mm pathlength, Helix Biomedical Accessories, # CAP-100Q), sealed with Cha-seal™ Tube Sealing Compound (# 510), and loaded into a Jasco capillary cell adaptor. Circular dichroism spectra were acquired with a Jasco J-1500 spectrophotometer at 25° C from 340 nm to 180 nm in 0.1 nm wavelength increments with a scan speed of 100 nm/min. Spectra were baseline corrected by subtracting the mean CD signal of 280 nm to 340 nm. The data represent the mean of three scans of each sample.

Molecular dynamics. *Ex4-D-Ala, Ex4-L-Ala, and Ex4 preparation for partial unbinding-binding simulations.* The missing stalk region of GLP-1R (residues 129-137) was added to models derived from GLP-1R:Ex4-D-Ala:Gs (conformer 1) using rabbit GLP-1R sequence (PDB: 5VAI)[8] by superposition on the structure of GLP-1R:Ex4:Gs from previous work.[49] The Gs protein was removed, with the exception of Gα helix h5 (residues 370-394), which was retained to keep GLP-1R in fully active conformation during the simulations (in line with our previous work[9]). The resulting simplified GLP-1R:Ex4-D-Ala:Gα(h5) system was prepared for simulations with the CHARMM36[64] force field using VMD[65] and in-house python HTMD[66] and Tcl (Tool Command Language) scripts. Pdb2pqr[67] and PROPKA[68] software were used to add hydrogen atoms appropriate for a simulated pH of 7.0. The structure was superimposed on the secretin receptor structure PDB 6WI9 from the OPM database[69] to orient the receptor before insertion into a rectangular pre-built 100 Å x 100 Å 1-palmitoyl-2-oleyl-sn-glycerol-3-phosphocholine (POPC) bilayer; lipid molecules overlapping the receptor were removed. TIP3P water molecules were added to the 100 Å x 100 Å x 155 Å simulation box using the VMD Solvate plugin 1.5 (Solvate Plugin, Version 1.5. at <http://www.ks.uiuc.edu/Research/vmd/plugins/solvate/). Overall charge neutrality was maintained by adding Na+ and Cl- counter ions to a final ionic concentration of 150 mM using the VMD Autoionize plugin 1.3 (Autoionize Plugin, Version 1.3. at <http://www.ks.uiuc.edu/Research/vmd/plugins/autoionize/). The complex GLP-1R:Ex4-L-Ala:Gα(h5) was modelled from GLP-1R:Ex4-D-Ala:Gα(h5) by mutating Ex4-D-Ala in position 4 to L-Ala, with VMD psfgen plugin. The natural form of Ex4 (Gly in position 4) in complex with GLP-1R was simulated from the full-length complex GLP-1R:Ex4:Gs from our previous work,[49] removing the whole Gs complex except Gαs h5.

Systems equilibration and MD settings. AceMD3[70] (which is based on OpenMM[71]) was used for both equilibration and MD productive simulations. Isothermal-isobaric conditions (Langevin thermostat[72] with a target temperature of 300 K and damping of 0.1 ps-1 and Berendsen barostat[73] with a target pressure 1 atm) were employed to equilibrate the systems through a multi-stage procedure (integration time step of 2 fs). Initial steric clashes between lipid atoms were reduced through 1500 conjugate-gradient minimization steps. A restraint of 1 kcal mol^{-1} $Å^{-2}$, applied to protein atoms and lipid phosphorus atoms, was gradually released over 100 ns of MD simulations (first 4 ns for lipid phosphorus atoms, 80 ns for the protein atoms other than α carbon). Productive trajectories were collected in the canonical ensemble (NVT) at 310 K, using a thermostat damping of 0.1 ps^{-1} with an integration time step of 4 fs (through hydrogen mass repartitioning13) and the M-SHAKE algorithm14 to constrain the bond lengths involving hydrogen atoms. The cut-off distance for electrostatic interactions was set at 9 Å, with a switching function applied beyond 7.5 Å. Long-range Coulomb interactions were handled using the particle mesh Ewald summation method (PME)[74] by setting the mesh spacing to 1.0 Å. Trajectory frames were written every 50 ps of simulations.

Non-equilibrium protocol for partial unbinding-binding simulations. The equilibrated complexes GLP-1R:Ex4-D-Ala:Gα(h5), GLP-1R:Ex4-L-Ala:Gα(h5), and GLP-1R:Ex4:Gα(h5) underwent the same unbinding/binding protocol (Table S5), in analogy with previous reports.[9] Briefly, well-tempered metadynamic[75] was employed on the distance between the centroid of the peptide residues comprised between T5 and E15, and the TMD of GLP-1R (residues E138-V405) to dissociate the agonists N-terminal from the TMD up to 20 Å (five replicas for each system, Table 1). Successively, three supervised MD (SuMD)[76,77] binding simulations were run from one of the final configurations obtained during the partial unbinding (chosen according to the fewer contacts between the peptide N-terminus and GLP-1R) until the distance between the centroids did not change over 30 ns of SuMD simulation. For each replica, an additional 300 ns of classic MD were performed starting from the final SuMD states.

Ex4-D-Ala, Ex4-L-Ala, and Ex4 simulations in water. Agonist peptides Ex4-D-Ala, Ex4-L-Ala, and Ex4 from the complexes GLP-1R:Ex4-D-Ala:Gα(h5), GLP-1R:Ex4-L-Ala:Gα(h5), and GLP-1R:Ex4:Gα(h5) respectively were centered in a simulation box with a padding of 20 Å by adding TIP3P water molecules using the VMD Solvate plugin 1.5 (Solvate Plugin, Version 1.5. at <http://www.ks.uiuc.edu/Research/vmd/plugins/solvate/). Overall charge neutrality was maintained by adding Na+ and Cl- counter ions to a final ionic concentration of 150 mM using the VMD Autoionize plugin 1.3 (Autoionize Plugin, Version 1.3. at <http://www.ks.uiuc.edu/Research/vmd/plugins/autoionize/). The three resulting systems were first minimized with AceMD through 500 conjugate-gradient minimization steps and then equilibrated over 1 ns in the NPT ensemble. Three independent 2 µs-long replicas were performed in NVT (310K) for each peptide (Table S5).

MD Analysis. The DSSP (dictionary of secondary structure of proteins) analysis[44] was performed using AmberTools19.[78] Contacts between peptides position 4 and GLP1-R were computed for the partial unbinding replicas using GetContacts (at https://getcontacts.github.io/), with a distance set to 3.5 Å. Videos were generated using VMD and avconv (at https://libav.org/avconv.html).

NMR Experiments. Peptides were analyzed on a Bruker Avance III HD 600 MHz spectrometer equipped with a 1.7 mm triple resonance indirect detection cryogenic probe. Each peptide was dissolved in 600 µL of 30% TFE-d^3 in 9:1 H_2O:D_2O at 1 mM with a trace amount of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as internal reference. All spectra were acquired at 25°C. The following standard Avance pulse programs were employed: 1D with solvent suppression using excitation sculpting (zgesgp), phase-sensitive 2D TOCSY with excitation sculpting (180 water-selective pulse-ES element) using DIPSI-2 (dipsi2esgpph), phase-sensitive 2D TOCSY with excitation sculpting using W5 (cosygpphppw5), and

phase-sensitive 2D NOESY with excitation sculpting using 180 water-selective pulse (ES element) (noesyesgpph). TOCSY experiments used a mixing time of 60 ms. NOESY experiments used a mixing time of 200 ms. Data were processed using TopSpin 3.6.1. Data were analyzed using MestReNova and NMRFAM Sparky,[79] with employment of sequential assignment procedures to assign chemical shifts of protons.

Statistical analysis. Quantitative data are reported as arithmetic means and errors are reported as standard errors unless otherwise stated. Errors in fitted parameters (EC_{50}, IC_{50}, Maximal response, and bias factors) were calculated using Graphpad Prism 6.0. Sample sizes and statistical analyses are provided in figure and table legends.

Code availability. No new code was used in this study. A list of software used is available in the methods section and reporting summary.

Data Availability. Sequencing data for nLuc-GLP-1R is available at Addgene (ID 124831). Atomic coordinates and cryo-EM density maps for Ex4-D-Ala-bound GLP-1R/Gs in conformer 1 and conformer 2 have been deposited in the Protein Data Bank under accession numbers 7S1M and 7S3I and EMDB entries EMD-24805 and EMD-24825, respectively. Source data are provided with this paper.