Two distinct domains of the glucagon-like peptide-1 receptor control peptide-mediated biased agonism

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Running title: Structural insights into GLP-1R biased agonism

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Keywords: G protein-coupled receptor; glucagon-like peptide-1 receptor; GPCR structure-function; biased agonism; cell signaling; class B peptide hormone; extracellular signal-regulated kinase (ERK); arrestin; transmembrane domain

ABSTRACT

G protein-coupled receptors (GPCRs) can be differentially activated by ligands to generate multiple and distinct, downstream signaling profiles, a phenomenon termed biased agonism. The glucagon-like peptide-1 receptor (GLP-1R) is a class B GPCR and a key drug target for managing metabolic disorders; however, its peptide agonists display biased signaling that affect their relative efficacies. In this study, we combined mutagenesis experiments and mapping of surface mutations onto recently described GLP-1R structures, which revealed two major domains in the GLP-1/GLP-1R/Gs protein active structure that are differentially important for both receptor quiescence, and ligand-specific initiation and propagation of biased agonism. Changes to the conformation of transmembrane helix (TM) 5 and TM 6 and reordering of extracellular loop (ECL) 2 were essential for the propagation of signalling linked to cAMP formation and intracellular calcium mobilisation, whereas ordering and packing of residues in TMs 1 and 7 was critical for extracellular signal-regulated kinase 1/2 (pERK) activity. On the basis of these findings, we propose a model of distinct peptide–receptor interactions that selectively control how these different signaling pathways are engaged. This work provides important structural insight into class B GPCR activation and biased agonism.

GPCRs are ubiquitous integrators of extracellular signals for the control of cellular responses. As such they are key drug targets and approximately 40% of approved therapeutics act via this receptor class(1). Nonetheless, many potential drugs fail in late stage clinical trials due to lack of predicted efficacy, indicating gaps in our understanding of drug action and/or the specific contributions of signaling events to the control of diseases.

It has become increasingly evident that GPCRs are highly dynamic proteins that can adopt numerous ligand-specific conformational ensembles with distinct impact on signaling and regulatory profiles, even with ligands acting via a common binding pocket, a phenomenon termed biased agonism(2,3). This not only provides an unprecedented opportunity to sculpt biological responses for therapeutic benefit, but also creates increased challenges for drug discovery and development programs to identify spectra of ligand behaviour, and to elucidate structure-activity relationships linking observed behaviour to physiology and disease processes.
Class B peptide hormone GPCRs bind important physiological peptides of about 30-40 amino acids, including calcitonin, amylin, adrenomedullin, calcitonin gene-related peptide (CGRP), secretin, parathyroid hormones, vasoactive intestinal peptide, gastric inhibitory peptide, glucagon and the glucagon-like peptides(4,5). As such these receptors are crucial targets for treatment of chronic diseases, notably osteoporosis, migraine, obesity and type 2 diabetes. Class B GPCRs are pleiotropically coupled and biased agonism is commonly observed when signaling is studied across multiple pathways, creating novel therapeutic opportunities. However, optimally exploiting this property requires detailed mechanistic understanding of the drivers of bias(4).

The glucagon-like peptide-1 (GLP-1) receptor (GLP-1R) is a class B GPCR that is both critical to the natural incretin response of the body and a major target for treatment of metabolic disorders. It is among the best studied for biased agonism and such bias is readily observed for both naturally occurring and synthetic peptide mimetics(6-11). This biased agonism alters responses in pancreatic β-cells(11) and disease models in vivo(10). The prevailing view for class B GPCR peptide binding and receptor activation is a two-domain theory with initial binding of the peptide C-terminus to the receptor extracellular domain (ECD) that positions the peptide N-terminus relative to the receptor core to facilitate receptor activation(12). The prevalence of GLP-1R biased agonism and the therapeutic implications of this behaviour make understanding of the triggers for, and propagation of, bias important for rational drug design and development. We recently revealed that the extracellular loops (ECLs) of the GLP-1R play a crucial role in the biased agonism of exendin-4, oxyntomodulin and GLP-1(7-36)NH2 (GLP-1): the first clinically approved GLP-1 mimetic, a biased endogenous GLP-1R peptide and the most common circulating form of GLP-1, respectively(11,13). Nonetheless, interpretation of the data was limited by lack of experimentally determined structure for the GLP-1R core and, indeed, any full-length, active, class B GPCR structures. Recently, several near atomic resolution structures of the GLP-1R have been published that include structures of a stabilized, inhibitor-bound, human receptor transmembrane (TM) domain(14), a stabilized full-length human receptor bound to a modified, 11-mer peptide agonist(15), and GLP-1Rs in complex with heterotrimeric Gs protein, and either GLP-1(16) or a newly identified biased agonist, exendin-P5(17). In the meanwhile, the structure of a full-length glucagon receptor (GCGR) in complex with a weak partial agonist(18), and the full-length, active calcitonin receptor in complex with peptide agonist and heterotrimeric Gs were also solved(19). Collectively, this work has provided novel insights into gross conformational changes linked to the dynamics of class B GPCR activation, including marked kinking of transmembrane helix (TM) 6, outward movement of helices 6 and 7, and the interlinking ECL3, required for full activation, as well as manifest reorganization of other ECLs(16,17,19).

The ECD is structurally linked to the receptor core through extension of TM1, and there is accumulating evidence that the far N-terminus of the ECD may have a dynamic role in class B GPCR function, both with respect to maintenance of an inactive state, as has been suggested for the glucagon receptor (GCGR)(20), and in ligand-dependent signaling(21,22). Notably, the TM1 stalk domain is unstructured in complexes of class B receptors complexed to agonist and G protein, but maintains an extended α-helix in GCGR bound to a partial agonist, but without G protein(18). In the current study, we performed alanine scanning analysis of the GLP-1R surface of the far N-terminal 7 amino acids (residues 24-30, immediately post the receptor signal peptide), and the 21 amino acids that link TM1 and the ECD (residues 128-148), and assessed cell surface expression, peptide affinity, and peptide efficacy for activation of pathways linked to cAMP accumulation, intracellular calcium (iCa2+) mobilisation and phosphorylation of extracellular signal-regulated kinase 1/2 (pERK). Each of these pathways is of physiological relevance for GLP-1R signaling(4,23), and both these regions are dynamically involved in GLP-1R peptide affinity and signal transduction. Overlay of this new data and that from our prior mutagenic analysis of the ECLs(24) onto the recently solved GLP-1R
structures revealed that changes to the conformation of TMs 5 and 6 and reordering of ECL2 were essential for propagation of cAMP formation and Ca²⁺ mobilisation signaling, whereas ordering and packing of residues in TMs 1 and 7 was critical for pERK that is principally driven by Gi/Gβγ/arrestin interaction in the wild-type receptor.

RESULTS

The far N-terminal ECD (R24-V30) and TM1/ECD linker (E128-Y148) residues of GLP-1R were mutated to alanine by site-directed mutagenesis and stably expressed in CHO-FlpIn cells by isogenic recombination, with the exception of A28 that was mutated to glycine. The location of these amino acids within the GLP-1R extracellular surface is illustrated on inactive (TM1/ECD linker only) and active hGLP-1R models in Figure 1A.

Cell surface expression levels for WT and mutant GLP-1Rs in the CHO-FlpIn stable cell lines were measured through ELISA of anti-cMyc antibody binding to the N-terminal cMyc epitope. All receptors were expressed at the cell surface, however, the expression of G27A, T29A, E128A, K130A, S135A, E139A, Q140A, L144A and I146A mutants was significantly lower, and that of E138A markedly higher than WT GLP-1R. All the other mutant receptors were not significantly different from the WT (Figure 1B, Table 1).

Peptide Agonist Affinity

Heterologous whole cell competition binding with ¹²⁵I-exendin-4(9-39) was performed to determine peptide agonist affinity for the WT and mutant GLP-1Rs. As previously reported(25), at the WT receptor exendin-4 had the highest affinity (pKi≈9.31), followed by GLP-1 (pKi≈8.12), while oxyntomodulin had the lowest affinity (pKi≈7.52) (Table 1, Figure 2). No specific binding window could be established for the G132A mutant, despite good cell surface expression by ELISA, or for the low expressing mutants G27A, T29A, E128A and E139A; for all others, competitive binding isotherms were established for each of the peptides. Of the far N-terminal ECD residues that exhibited specific binding, only the A28G mutation modified affinity (<5-fold) for GLP-1 and exendin-4, with a similar trend for oxyntomodulin (Figure 2, Figure 3A, Table 1). Of the TM1/stalk residues, P137A, L141A and Y148A mutations reduced affinity for each of the agonist peptides, while the P137A and L141A mutations also reduced affinity of the antagonist exendin-4(9-39). Ligand-specific reductions in affinity were seen for Q140A (exendin-4) and L142A (oxyntomodulin). All other mutations were without significant effect on peptide affinity (Figure 2, Figure 3A, Table 1).

Agonist Efficacy

Concentration-response curves for each of the peptides were established to determine agonist potency and maximal responses for canonical signaling endpoints, cAMP accumulation, iCa²⁺ mobilisation and pERK1/2 (Figures 3B-3D, Figures 4-6, Tables 2-4). These data were subject to operational modelling(26) to determine affinity-independent measures of efficacy (τ) for each pathway. The operational efficacy term "τ" is a measure of the number of receptors that need to be occupied to give a specified response. The τ values were normalized to receptor expression to derive a receptor expression-independent, quantitative measure of pathway activation “τc” for individual peptides, at each of the mutants(27,28).

cAMP Accumulation

Within the far N-terminus of the ECD, mutants G27A and T29A that had poor expression had very weak to no measurable cAMP response. Unlike binding affinity, which was unaltered, the R24A mutant had reduced efficacy for GLP-1 and exendin-4 (Figure 3B, Figure 4, Table 2). Within the TM1/ECD linker there was only limited correlation between effects on binding affinity and cAMP efficacy, and on cAMP efficacy between peptides. E128A that was poorly expressed responded very weakly to all peptides. The I147A mutant induced modest loss of efficacy, and the G132A mutant exhibited 10 to 30-fold decreased efficacy for all peptides. The L142A mutant had significantly attenuated efficacy for GLP-1, with similar fold decreases for exendin-4 and oxyntomodulin, although these latter effects did not achieve significance. The
E139A mutant that had undetectable $^{125}$I-exendin-4(9-39) binding, had reduced efficacy with exendin-4, and to a lesser extent oxyntomodulin, but not GLP-1. There were statistically significant increases in efficacy for oxyntomodulin at E133A, while K130A, S135A, Y145A and I146A caused selective increases in GLP-1 efficacy, and R131A induced a weak, selective decrease in GLP-1 efficacy (Figure 3B, Figure 4, Table 2).

**Intracellular Calcium (iCa$^{2+}$) Mobilization**

Due to relative weak iCa$^{2+}$ mobilization by oxyntomodulin, only a single high concentration (≈3 µM) was assessed. For GLP-1 and exendin-4, no measurable response was seen at the poorly expressed G27A, T29A and E128A mutants, while the E138A mutant exhibited increased efficacy for both peptides, even after correction for the higher cell surface expression (Figure 3C, Figure 5, Table 3). There was decreased efficacy for both peptides with the L142A mutant, while L144A abolished iCa$^{2+}$ mobilization, despite unaltered cAMP efficacy. There was selective loss of exendin-4 efficacy at the S135A and Y148A mutants, and of GLP-1 efficacy at the S136A, Q140A and I147A mutants, though these effects were relatively small (Figure 3C, Figure 5, Table 3). There was also a weak, selective increase in GLP-1 efficacy at the R24A mutant, while the opposite effect was seen for GLP-1 dependent cAMP efficacy (Figure 3C versus 3B, Tables 2 and 3). Of interest, though not quantitative, there was no measurable response for the single high concentration of oxyntomodulin at the P137A and Y148A mutants, despite approximately WT levels of cell surface receptor expression and limited (for P137A) or no (Y148A) effect of the mutation on oxyntomodulin affinity (Figure 3C, Table 1).

**ERK1/2 Phosphorylation**

As seen with the other pathways, the poorly expressed mutants G27A, T29A and E128A had no measurable pERK response to any of the peptides. For the far N-terminal ECD mutations, there was slightly increased efficacy for all peptides with the Q26A mutant, and a selective weak increase in efficacy for exendin-4 at the A28G and V30A mutants (Figure 3D, Figure 6, Table 4). For the TM1/ECD linker, there was increased efficacy for all 3 peptides at the K130A, E138A and I147A mutants, and decreased efficacy at the S136A mutant. A weak loss of efficacy for exendin-4 and oxyntomodulin, but not GLP-1, occurred with the G132A mutant, while the L144A mutant abolished pERK response to GLP-1 and oxyntomodulin but did not alter efficacy for exendin-4. Of the remaining mutants, only L141A (increased exendin-4 efficacy) and Q140A (weak loss of oxyntomodulin efficacy) had any significant effect (Figure 3D, Figure 6, Table 4).

**DISCUSSION**

**Structural Insights into GLP-1R Biased Agonism**

Our results indicate that the far N-terminus and linker region between TM1 and the ECD play discrete roles in receptor stability and expression, and in peptide-specific signaling. Recent advances in structure determination for class B GPCRs and particularly the GLP-1R provide an unprecedented opportunity to map key surface residues in 3-dimensional space relevant to both inactive and active structures. For the GLP-1R, four new structures have recently been solved, which include a modified human GLP-1R TM domain structure bound to negative allosteric modulators (NAMs) (14), a thermostabilized full-length human GLP-1R bound to a modified 11-mer agonist peptide (15), a fully active rabbit GLP-1R in complex with GLP-1 and the heterotrimeric Gs protein (16), and human GLP-1R in complex with the biased agonist, exendin-P5, and Gs protein (17). Details of structures, including differences from WT human GLP-1R, are described in Table 5. This work complements the previously published inactive structures of the related GCGR(29,30). The NAM-bound GLP-1R contains structural alterations, including an introduced cysteine bridge between TM helices 5 and 6, that disrupt key networks of the native inactive receptor. As such, we have used the inactive GCGR (PDB-4L6R) (29) as a template to model the inactive GLP-1R (Figure 1A) (28). The active rabbit GLP-1R complex has a global resolution of 4.1 Å, with limited side-chain resolution and ambiguity in potential modelling of ECLs, whereas the exendin-P5/GLP-1R complex has a global resolution of 3.3 Å with good side-chain resolution for most of the receptor; we have used this structure as the
principal template for comparative mapping of the effects of mutations between active and inactive states (Figure 1A). All full-length structures lack density for residues 24-28 of the far N-terminus, indicating that this segment is flexible upon ligand binding. However, the position of V30 that is resolved in the structures suggests that the far N-terminal residues likely make transient interactions with ECLs 2, 3 and/or the peptide agonist (Figure 7). The new data are considered holistically consistent with previously published ECL alanine scanning mutagenesis studies(11,13) to yield a comprehensive structure-function analysis of signal propagation networks in the GLP-1R. Nonetheless, as with all such structure-functional analyses our observations are influenced by the recombinant cellular environment, including the relative expression of transducer and regulatory proteins that will differ from endogenous sites of receptor expression.

Structural Reorganization Upon Receptor Activation

ECD-Receptor Core Interactions

There is accumulating evidence that interactions between the ECD and TM core of class B GPCRs contribute to receptor quiescence and peptide-mediated receptor activation(20,21,31). For the related GCGR, interactions of the far N-terminus and residues in ECL3 contribute to maintenance of a quiescent state(20), although ground state interactions at the intracellular face also play a key role(22,32,33). As noted above, the far N-terminus is dynamic in peptide bound states(15,16), making interpretation of mutations in the context of available structures difficult. The dramatic loss of cell surface expression for G27A and T29A is indicative of important roles of these amino acids in receptor stability, potentially via loss of stabilizing interaction between T29 and the receptor core that would be consistent with a role of the far N-terminus in maintaining receptor quiescence. Nevertheless, the GLP-1R is expressed at the cell surface when the full ECD is truncated(31), suggesting that loss of receptor expression is due to destabilizing interactions of the modified ECD. Glycines provide structural flexibility and G27 may be required for favourable positioning of T29. In the active, GLP-1- and exendin-P5-bound receptors, T29 is proximal to the peptide ligand, although not within hydrogen-bonding distance. In the structure of the modified 11-mer bound receptor, the position of the ECD is not constrained by peptide binding, where T29 interacts with the linker between TM1 and the ECD (Figure 7B).

Class B GPCRs, including the GLP-1R, exhibit an extracellular-oriented V-shape cavity within the TM bundle that provides domain separation of the external facing segments of TMs 1, 7 and 6, and TMs 2, 3, 4 and 5, respectively (Figure 8A). Nonetheless, in the inactive model, there are key hydrogen-bonded interactions between D198 of TM2 and Y145, Y148 and T149 of TM1 that coordinate Y145 and Y148 away from TM7 (Figure 9A), and this facilitates tight packing of TMs 1 and 7. Consistent with this, D198A mutation leads to marked loss of receptor expression and/or GLP-1-stimulated cAMP signaling(34-36). In the active, exendin-P5-bound structure, this hydrogen bond network is weakened, with loss of interactions between D198 and both Y148 and T149, facilitating the movement of TM1 towards TM7, while the kink in TM1 is stabilized by hydrogen bonding of the side chain of T149 with the backbone oxygen of Y145 (Figure 9B). A similar pattern of interaction is observed in the 11-mer bound structure (Figure 9E). While the position of the kink in TM1 of the GLP-1/GLP-1R structure is likely conserved(17) (although modelled differently in 5VAI), there is further loss of the interaction between Y145 and D198 and this leads to an anti-clockwise rotation of the upper end of TM1, with a parallel rotation of TM7 that is not seen in the exendin-P5 structure (Figures 9B, C). This likely contributes to the distinct conformation of ECL3 between the GLP-1- and exendin-P5-bound structures that has been linked to efficacy differences of the two peptides(17).

Both Y148 and T149(37,38) play important roles in peptide agonist affinity, but do not make direct interactions with GLP-1 in the active structure, and the same is true for P137A and L141A in the TM1 stalk. Thus, these residues contribute to the reorganization of TM1 and packing with TM7. These changes are also likely to impact the position of E139. Mutation of this
amino acid causes loss of exendin-4binding. In the GLP-1 bound active structure, the side-chain of E139 is directed towards the GLP-1 peptide, suggesting a direct interaction that may be more prevalent for the truncated exendin peptide (and, indeed, this is observed in the exendin-P5/GLP-1R/Gs structure(17)). This would be consistent with the lack of effect of the E139A mutant on GLP-1 signaling, and only limited attenuation of oxyntomodulin and exendin-4 mediated cAMP production (Figure 3).

Of the TM1 stalk residues, only mutations to T149 also translate into major impact on signaling(37,38), implying that its role in structural reorganization is also critical to activation transition and effector binding. This loss can be recovered by allosteric modulator binding at the intracellular face of the receptor that is predicted to destabilize ground state interactions at the base of the receptor(14,37,38).

In the active and Gs protein-complexed receptor structures, the linker region between the ECD and the core is poorly resolved, suggesting a high degree of flexibility even when the peptide is bound. In the inactive GCGR (PDB 4L6R)(29), the TM1 stalk is present as an extended $\alpha$-helix, and while this may be partially due to crystal-packing artefacts, an extended $\alpha$-helix is also present in the structure of the full-length GCGR bound to a partial agonist peptide, NNC1702(18) (Figure 7). This suggests that order to disorder transition of the TM1 stalk may be required for full receptor activation. Indeed, this would be required to accommodate the movement of TM1 towards TM7, seen in the active and active-like structures. An important role for the TM1 stalk is supported by our current mutagenesis data (Figure 3). Polar residues in this region, particularly S136, K130 and E138, had effects on peptide signaling in a pathway and peptide-specific manner, indicating that formation and disruption of interactions formed by these amino acids contribute to conformational transition for activation. Similar behavior was seen for the G132A mutant, suggesting that backbone flexibility plays a role in these effects. Somewhat surprisingly, E128A had a profound effect on receptor expression, presumably via destabilization of the receptor protein, in a similar manner to that observed for the T29A mutant. Although speculative, it is possible that these amino acids are in close proximity in the inactive receptor and form part of an important network that stabilizes this state. In studies where E128A is further overexpressed by transient expression, there is <3-fold loss of GLP-1 potency and ~5-fold loss of exendin-4 potency for cAMP production(39), consistent with a limited role of this residue for peptide binding.

In the inactive homology model, the deeper binding cavity occupied by agonist peptides is capped by a series of large aromatic residues, particularly W297 and Y305 (Figures 8B, C vs. D, E), whose position is predicted to be stabilized by H-bonding. In the active structure, these amino acids undergo large-scale movements associated with reorganization of ECL2 that reorients W297 away from the binding pocket, where it forms interactions with other ECL2 residues, accompanied by small outward movements of R299 and N300 that directly contact the peptide in the active structure. Release of ground state interactions in the inactive ECL2 loop structure enables unwinding of the top of TM6 with an ~180-degree rotation of Y305 accompanied by an ~9 Å displacement of the $\gamma$ carbon. Previous molecular dynamics (MD) simulations on the inactive GLP-1R model suggested that this aromatic cap provided a significant energy barrier to deeper entry of the GLP-1 N-terminus, with entry facilitated by Glu9 of the peptide that forms a salt-bridge with R190 of the receptor(11). Intriguingly, mutation of W297 and the adjacent C296 (that is covalently linked to C226 in TM3) markedly attenuated GLP-1 and exendin-4 affinity, but did not impact oxyntomodulin affinity. Oxyntomodulin contains an uncharged Gln, positionally equivalent to GLP-1 Glu9, and does not interact with R190 in the receptor core(11). It is possible, therefore, that oxyntomodulin binds in a shallower orientation. A significant and selective decrease in oxyntomodulin affinity for F381A, L142A and K202A mutants and lack of an effect for the R380A mutant are consistent with this hypothesis (Figure 10).

**Peptide-mediated Signalling and Bias**

Comparison of the position of mutated residues that affect GLP-1 mediated cAMP...
formation between fully active and inactive models reveals two major networks involved in GLP-1R function (Figures 10, 11). The first includes residues of ECL2 and the membrane proximal TM regions and the proximal segment of ECL3. K288A impacted ligand binding and was critical for propagation of cAMP signaling\(^\text{(13,40,41)}\). It stabilizes the center of the ECL2 network and may coordinate interactions between the ECL2 residues. Both exendin-4 and oxyntomodulin were similarly affected by mutations to ECL2 indicating a general role in propagation of cAMP signaling (Figures 10, 11). Despite lack of effect of W297A on oxyntomodulin binding, it was crucial to signaling of all three peptides. In the active, agonist-bound calcitonin receptor (CTR), there is a high degree of overlap in the structural organization of ECL2 in spite of considerable sequence variation; R281 that is positionally equivalent to K288 of the GLP-1R appears to play a similar coordinating role in maintenance of this structure \(^\text{(19,42)}\). The organization of the ECL2 network is also required for calcium signaling, though it does not appear as important for peptide-mediated pERK (Figures 10, 11).

The second network involves residues in TM1 and the TM7 proximal residues of ECL3 (Figures 10, 11). This network exhibits a higher degree of peptide-specific effects that are likely related to both differences in the peptide sequences and orientation of the peptides in the active structures. As noted above, this region of the receptor is involved in coordination of TM1 in the inactive structure and the reorganization and packing of TM1 with TM7 in the active structures (Figure 9). Unlike GLP-1 and exendin-4, oxyntomodulin-mediated cAMP production is also weakly attenuated by mutation to amino acids in ECL1 that sit within the short \(\alpha\)-helix formed in the active structure that extends to the ECL2 network. This is likely due to the predicted distinct positioning of oxyntomodulin when bound to the receptor (Figure 10). The boundary of ECL1 at the top of TM3 is covalently linked to ECL2 by disulphide linkage of C226 (TM3) and C296 (ECL2), while R227 in inactive/partially active structures may also stabilize ECL2. R227A mutation decreases affinity of all three peptides, though with minimal impact on signaling efficacy\(^\text{(32)}\). As such, the extent to which the oxyntomodulin-specific effects are due to unique direct interactions with ECL1 vs. potential differences in C296 and W297 is not clear.

Overall, the pattern of effect of mutation was similar for calcium and cAMP signaling across GLP-1 and exendin-4 where efficacy effects could be quantified, though there was generally a greater magnitude of effect on calcium signaling (Figures 10, 11). Previous pharmacological inhibitor studies revealed that both these pathways were regulated by G protein interaction at the wild-type receptor in the CHO-FlpIn cell background, although Gi and Gq interactions had more prominent roles in calcium mobilization\(^\text{(11)}\); this is indicative of broad similarities in changes required to enable G protein coupling. Exendin-4 mediated signaling is also generally more sensitive to mutation than that mediated by GLP-1, and the required ECL2 network extends to the top of TM4 for this peptide (Figure 10).

Peptide-mediated pERK was least sensitive to GLP-1R surface mutations, being principally confined to mutations of the distal ECL3/TM7 boundary, and residues of TM1 and the linker extension that provides physical connection to the ECD, which were revealed in the current study (Figures 10, 11). This was particularly evident for exendin-4, that was least affected by mutation (Figure 11). Intriguingly, there was effectively no involvement of the ECL2 residues that were absolutely required for cAMP and calcium signaling. Inhibitor studies at the wild-type receptor indicated that Gs and Gq have limited contribution to exendin-4 mediated pERK, with signaling principally driven by Gi, G\(\beta\gamma\) and arrestin interaction, though those are likely to be at least partly interdependent\(^\text{(11)}\). For GLP-1, and more prominently oxyntomodulin, selective mutations in ECL2 also impacted pERK and this could relate to greater contribution of Gq (GLP-1) and Gs (oxyntomodulin) in pERK at the wild-type receptor. Also of interest, for TM1 and ECL3/TM7, the effects of individual mutation were highly peptide specific (Figure 10). The data are consistent with a model whereby selective and peptide-specific interactions alter the TM1/TM7 interface linked to Gi/G\(\beta\gamma\)/arrestin coupling to...
Moreover, our mutational data suggests these changes can occur relatively independent of the reorganization of TMs 5 and 6 that are critical for cAMP formation and Ca\(^{2+}\) mobilisation; events that are Gs/Gq dependent at the wild-type receptor. Exendin-P5 is a G protein-biased peptide agonist that exhibits bias towards cAMP relative to arrestin interaction, compared to the related exendin-4 peptide and GLP-1(17,43). It has distinct actions in vivo compared to exendin-4(43). As noted above, comparison of the exendin-P5- and GLP-1-bound active GLP-1R structures reveals major differences in ECL3 and the upper TM boundaries of TMs 6 and 7 that are linked to distinct rotational differences in the upper region of TM1. Mutation in these domains has confirmed peptide specific differences in the engagement of GLP-1 vs. exendin-P5 with amino acids in TM1, as well as the TM6 proximal region of ECL3(17), thereby providing additional structural evidence for distinctions in the role of these domains in propagation of signaling. An important caveat for extrapolation of our observations to more proximal measures of signaling is that they are based on inferences from wild-type receptor signaling. Direct measurement of proximal transducer engagement for mutant receptors will be required to validate hypotheses.

The novel structures for the GLP-1R are enabling us to begin to unravel the complexities associated with receptor activation and biased agonism. Combining new data from the current study with our previous work on GLP-1R ECLs in the context of inactive and active structures has advanced our understanding of receptor domains that control signaling. Importantly, the work provides evidence for two, at least partially independent, structural domains linked to signaling. One involves the interface between TMs 5 and 6 and is linked to reorganization of ECL2 into a structured network that is required for propagation of signaling linked to Gs and Gq dependent pathways at the wild-type receptor. The second is the interface between TMs 1 and 7 that, while important for at least Gs-dependent, cAMP signaling, may be independently linked to Gi/Gβγ/arrestin-mediated signaling that is the key driver of pERK at the wild-type receptor. Our data support a model where distinct peptide-receptor interactions can provide selective control of how these different networks are engaged.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis**

Desired mutations were introduced to N-terminally double cMyc labeled human GLP-1R in pDONR201 (Invitrogen) via MutadirectTM kit (Beijing SBS Genetech Co., Ltd., China), then LR recombination reactions were conducted to transfer the N-terminally double c-Myc labeled human GLP-1R gene into the pEF5/FRT/V5-DEST destination vector using Gateway Technology (Invitrogen). The oligonucleotides for mutagenesis were purchased from GeneWorks (Thebarton, SA, Australia) and mutants were confirmed by automated DNA sequencing.

**Stable Cell Line Generation and Cell Culture**

The mutant or wild-type (WT) receptor genes were integrated into the FlpIn-Chinese hamster ovary (FlpIn-CHO) cells, passage 4 (Invitrogen) using the Flp-InTM system. Stable Flp-In expression cell lines were generated through polyclonal selection, screening and maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 10% (v/v) FBS, μg600/ml hygromycin B at 37 °C in 5% CO\(_2\). The WT receptor is expressed at ~170,000 receptors/cell. Cell lines were routinely tested for mycoplasma and were mycoplasma free. Stable cells were frozen at passage 14 and all assays were performed with cells between passage 14 and 25.

**Heterologous Whole Cell Competitive Binding Assay**

Competition of \(^{125}\text{I}-\text{exendin-4}_{(9-39)}\) binding to hGLP-1R was performed as previously described(13) on whole cells in 96-well plates using the radiolabeled antagonist \(^{125}\text{I}-\text{exendin-4}_{(0.39)}\) (~0.1 nM), and increasing concentrations of unlabeled peptide. Non-specific binding was defined by co-incubation with 1 μM unlabeled exendin-4_{(0,39)}. Following overnight incubation, non-bound ligand was removed and radioactivity was determined using a gamma counter.

**Cell Surface Expression by Enzyme Linked Immunosorbent Assay**

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1.5x10^5 cells/well were seeded into 24-well culture plates and incubated overnight. Expression was determined through detection of N-terminally double cMyc of GLP-1R by ELISA as previously described (13). Data were normalized to WT GLP-1R and Flp-In CHO parental cell lines.

Calcium Mobilization
3x10^4 cells/well were seeded into 96-well culture plates and incubated overnight. Cells were incubated with Fluo4-AM for 45 min, stimulated with different concentrations of peptides and fluorescence was determined in a Flexstation plate reader every 1.36 s for 120 s after ligand addition as previously described (13). Data were normalized to the maximal response elicited by 100 µM ATP.

cAMP Accumulation
3x10^4 cells/well were seeded into 96-well culture plates and incubated overnight. Cells were stimulated with increasing concentrations of ligands for 30 min in the presence of IBMX. The liquid was discarded, changed to absolute ethanol and volatilized to dry in room temperature. cAMP was detected using PerkinElmer LANCE kit as previously described (11). Data were normalized to the response of 100 µM forskolin.

ERK1/2 phosphorylation
3x10^4 cells/well were seeded into 96-well culture plates and incubated overnight. Initially, pERK1/2 time-course experiments were performed over 1 h to identify the time point when the pERK1/2 response is maximal. Subsequently, dose responses for different agonists were determined at this peak time point with stimulation performed after serum starvation overnight. pERK1/2 was detected using an Alphascreen assay as previously described (13). Data were normalized to the maximal response elicited by 10% FBS determined at 6 min.

Data Analysis
IC_{50} values were estimated from competitive inhibition of ^{125}I-exendin binding using a 3-parameter logistic equation \text{[log(inhibitor vs response)]}\text{[log(inhibitor vs response)]} in Prism (v7; Graphpad). In all cases, the concentration of the radioligand was ≤1% of the K_d. Under these conditions the IC_{50} approximates K_i and such data are reported as pK_i. E_{max} and EC_{50} were estimated from concentration-response curves using with a 3-parameter logistic equation in Prism (v7). These values are a composite of affinity, efficacy and stimulus response coupling. The Black and Leff operational model of agonism (26) was applied to separate effects on pathway-specific signaling from those that modify ligand affinity. Derived values (τ) were normalized to experimentally determined levels of cell surface expression to provide a measure of efficacy (τ_e) that is independent of affinity and altered cell surface receptor expression (11). Log τ_e values for mutant receptors were statistically compared to those of the WT receptor using a one-way analysis of variance (ANOVA) and Dunnett’s post-test. Significance was accepted at P< 0.05.

Molecular Modeling and Mapping of Mutational Effects
A homology model of the inactive GLP-1R TM domain was built using the minimally modified GCGR (PDB-4L6R) (29), as previously described (28); the first amino acid in this model is Arg134. The thermostabilized and full-length human GLP-1R bound to modified 11-mer peptide agonist (PDB-5NX2) (15), the full-length and GLP-1 bound rabbit GLP-1R in complex with Gs (PDB-5VA1) (16) and the full-length and exendin-P5 (ExP5) bound human GLP-1R in complex with Gs (PDB-6B3J) (16) were used as deposited; the first amino acids in these structures are Thr29, Thr29 and Val30, respectively.

ACKNOWLEDGEMENTS
This work was supported by National Health and Medical Research Council of Australia (NHMRC) project grants (1061044, 1065410 and 1126857), NHMRC program grant (1055134), Shanghai Science and Technology Development Fund (15DZ2291600), National Natural Science Foundation of China (81573479), Strategic Priority Research Program of the Chinese Academy of Sciences (CAS; XDA12020347 and XDA12040308). SL received the Postgraduate Overseas Study Fellowship from CAS, DW is a NHMRC Career Development Fellow, CK is a NHMRC CJ Martin Fellow, PMS is a NHMRC Principal
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CONFLICT OF INTEREST STATEMENT
The authors declare no conflict of interest.

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Participated in research design: Lei, Yang, Wang, Wootten, Sexton
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**FIGURE LEGENDS**

Figure 1. Location of the TM/N-terminal ECD interface in active and inactive models of the human GLP-1R and expression of GLP-1R constructs in stable cell lines.

**A** Surface residues of the GLP-1R. Left hand panel, inactive model of the hGLP-1R TM domain (residues 134-422) based on the inactive glucagon receptor (4L6R). Right hand panel, active full-length exendin-P5 (ExP5)/hGLP-1R/Gs complex (6B3J). The far N-terminal ECD (residues 24-30) and the TM1/ECD stalk (residues 128-148) are illustrated in grey. ECL residues mutated in previous analyses (24) are colored by ECL. ECL1 (residues 201-223; light blue), ECL2 (residues 285-307; dark blue), ECL3 (residues 372-387; light purple). The position of ExP5 is shown as red ribbon representation.

**B** Cell surface expression was determined by ELISA to the N-terminal cMyc epitope tag on the hGLP-1R and mutant hGLP-1R isogenically expressed in CHO-FlpIn cells. Data are normalized to the wild-type receptor. All values are mean ± S.E.M of four to six independent experiments, conducted in duplicate. One-way ANOVA analysis of variance and Dunnett’s post-test were performed to determined statistical differences (*P<0.05).

Figure 2. Competitive inhibition of $^{125}$I-exendin(9-39) binding by peptide agonists for Ala mutants of the hGLP-1R N-terminal ECD and TM1 and linker region. Binding affinity data are expressed as a percentage of measured bound versus bound in the absence of peptide, each corrected for non-specific binding (measured in the presence of 1µM unlabeled exendin(9-39)). Inhibition curves of wildtype and mutant receptors stimulated by GLP-1(7-36)NH$_2$ (upper panels), exendin-4 (middle panels) or oxyntomodulin (lower panels) in CHO-FlpIn cells stably expression wildtype or mutant receptors. Data are fitted with a three-parameter logistic equation. All values are means ± S.E.M of four to six independent experiments, conducted in duplicate.

Figure 3. Changes in affinity (A) and efficacy (B-D) of the agonists GLP-1, exendin-4, and oxyntomodulin at mutant GLP-1Rs.

**A** $pK_a$ values for the agonist peptides were derived from competition of $^{125}$I-exendin-4(9,39) binding. Data are plotted as differences in $pIC_{50}$ of the alanine mutants compared to the wild-type (WT) hGLP-1R for GLP-1 (left panel), exendin-4 (middle panel), and oxyntomodulin (right panel). All $pIC_{50}$ values were mean ± S.E.M of three independent experiments performed in triplicate. **B-D** $\Delta\log EC_n$ values are the difference in the coupling efficacy (log$_e$) for (B) cAMP accumulation, (C) iCa$^{2+}$ mobilization, and (D) ERK phosphorylation (pERK) of the alanine mutant GLP-1Rs compared to the WT receptor for GLP-1 (left panels), exendin-4 (middle panels), and oxyntomodulin (right panels). All functional values are mean ± S.E.M of four to six independent experiments, conducted in duplicate. One-way ANOVA and Dunnett’s post-test were performed to determined statistical differences (*P<0.05) and the bars were colored according to the fold change between WT and mutant receptors (yellow, 2-5 fold decrease; light orange, 5-10 fold decrease; dark orange, 10-30 fold decrease; red, >30-fold decrease; green, increased affinity (A) or efficacy (B-D)). N.D. not defined. One-way ANOVA analysis of variance and Dunnett’s post-test were performed to determined statistical differences (*P<0.05).

Figure 4. cAMP concentration response curves for Ala mutants of the hGLP-1R N-terminal ECD and TM1 and linker region. Concentration response curves for cAMP accumulation of wildtype and mutant receptors stimulated by GLP-1(7-36)NH$_2$ (upper panels), exendin-4 (middle panels) or
oxyntomodulin (lower panels) in CHO-FlpIn cells stably expression wildtype or mutant receptors. Data are normalized to the response elicited by the wildtype and analyzed with an operational model of agonism. All values are means ± S.E.M of four to six independent experiments, conducted in duplicate.

Figure 5. iCa$^{2+}$ mobilization concentration response curves for Ala mutants of the hGLP-1R N-terminal ECD and TM1 and linker region. Concentration response curves or iCa$^{2+}$ mobilization of wildtype and mutant receptors stimulated by GLP-1(7-36)NH$_2$ (upper panels), exendin-4 (middle panels) or oxyntomodulin (lower panels) in CHO-FlpIn cells stably expression wildtype or mutant receptors. Data are normalized to the response elicited by the wildtype and analyzed with an operational model of agonism. All values are means ± S.E.M of four to six independent experiments, conducted in duplicate.

Figure 6. pERK1/2 concentration response curves for Ala mutants of the hGLP-1R N-terminal ECD and TM1 and linker region. Concentration response curves or pERK of wildtype and mutant receptors stimulated by GLP-1(7-36)NH$_2$ (upper panels), exendin-4 (middle panels) or oxyntomodulin (lower panels) in CHO-FlpIn cells stably expression wildtype or mutant receptors. Data are normalized to the response elicited by the wildtype and analyzed with an operational model of agonism. All values are means ± S.E.M of four to six independent experiments, conducted in duplicate.

Figure 7. Peptide bound, full-length structures of GLP-1R and GCGR. (A) Full-length structures illustrating the relative position of the N-terminal ECD to the receptor core. (B) Zoom-in of the resolved far N-terminal residue(s) and TM1/ECD stalk (highlighted in dark grey). The backbones of the peptide agonists are illustrated in ribbon [GLP-1, exendin-P5 (ExP5) and NNC1702] or X-stick (11-mer).

Figure 8. Peptide binding to the GLP-1R requires reorganization of aromatic/hydrophobic residues in the receptor core. (A) Side view of the inactive GLP-1R model (grey) or exendin-P5 (ExP5)-bound GLP-1R structure (blue), with the ExP5 peptide illustrated in purple. Residues that occupy the core of the receptor in the inactive model are shown as space fill. (B-E) Top view of the structures where the ECD has been omitted for clarity. (B, C) Inactive GLP-1R model. (D, E) ExP5-bound GLP-1R structure.

Figure 9. Polar residues in the GLP-1R TM1 are coordinated by D198 (TM2) in the inactive model, orienting key side chains away from TM7 and facilitating tight packing of TM1/TM7. View facing TM1/TM2 boundary. Key TM1 side-chains interacting with D198 are depicted in X-stick representation and labelled. (A) Inactive GLP-1R model. (B) exendin-P5 (ExP5)/GLP-1R structure. (C) GLP-1/GLP-1R structure. (D) NNC1702/GCGR structure. (E) 11-mer agonist/GLP-1R structure. TM helices are labelled in roman numerals.

Figure 10. Peptide selective effects on agonist affinity, cAMP accumulation, iCa$^{2+}$ mobilization and pERK. Mutated amino acids with similar effects across peptides are not highlighted. Maps for affinity, cAMP and pERK include all three peptides. The map for iCa$^{2+}$ includes only GLP-1 and exendin-4. In the “affinity” panel, the exendin-P5 peptide is displayed in magenta.

Figure 11. Reorganization of the extracellular surface of the GLP-1R is critical to propagation of signaling. Amino acids involved in efficacy across cAMP (upper panels), iCa$^{2+}$ (middle panels) and pERK (lower panels) for GLP-1 (left hand panels) and exendin-4 (right hand panels) mapped onto the inactive GLP-1R model and fully active, exendin-P5 (ExP5)-bound, GLP-1R structure. Displayed in surface representation are mutated amino acids that affect efficacy; yellow (2-5 fold reduction in affinity), light orange (5-10 fold reduction in affinity), red (>30 fold reduction in affinity), or green (increased affinity). Mutated residues not affected are displayed as grey (inactive receptor) or blue (active receptor).
Table 1. Effects of human GLP-1R TM1/N-terminal mutants on peptide ligand binding and cell surface expression.

Binding data was analyzed using a three-parameter logistic equation and normalized to the maximal binding of the radiolabelled antagonist $^{125}$I-exendin-4(9-39) and the non-specific binding in the presence of 1 µM exendin-4(9-39). pKi is the negative logarithm of peptide affinity. All the values for binding are mean ± S.E.M. of three independent experiments, conducted in triplicate. Cell surface expression was accessed through ELISA detecting the N-terminal cMyc epitope label on the receptor. Mutant data is compared to the wild-type human GLP-1R expression and shown as percentage. The data for cell surface expression are mean ± S.E.M. of four to six independent experiments, conducted in duplicate. One-way ANOVA and Dunnett’s post-test were used to determine statistical differences (*P<0.05).

| Mutant  | GLP-1(7-36)NH₂ | Exendin-4 | Oxyntomodulin | Exendin(9-39) | Cell surface expression (% Wild-type) |
|---------|----------------|-----------|----------------|--------------|-------------------------------------|
| Wild-type | 8.12±0.06 | 9.31±0.06 | 7.52±0.08 | 7.85±0.05 | 100±7 |
| R24A | 8.10±0.08 | 9.54±0.13 | 7.69±0.08 | 7.74±0.06 | 130±8 |
| P25A | 8.17±0.09 | 9.56±0.12 | 7.80±0.08 | 7.71±0.06 | 107±15 |
| Q26A | 7.96±0.09 | 9.23±0.11 | 7.56±0.10 | 7.72±0.07 | 115±13 |
| G27A | ND | ND | ND | ND | 28±8* |
| A28G | 7.67±0.08* | 8.89±0.11* | 7.07±0.08* | 7.61±0.06 | 106±15 |
| T29A | ND | ND | ND | ND | 16±5* |
| V30A | 8.16±0.09 | 9.31±0.11 | 7.72±0.08 | 7.77±0.08 | 108±8 |
| E128A | ND | ND | ND | ND | 6±2* |
| S129A | 8.10±0.11 | 9.94±0.10 | 7.71±0.15 | 7.79±0.10 | 83±6 |
| K130A | 8.12±0.08 | 9.25±0.07 | 7.52±0.12 | 7.64±0.09 | 51±8* |
| R131A | 8.28±0.12 | 9.37±0.09 | 7.72±0.14 | 7.78±0.09 | 114±15 |
| G132A | ND | ND | ND | ND | 80±6 |
| E133A | 8.16±0.07 | 9.45±0.10 | 7.70±0.09 | 7.84±0.10 | 97±12 |
| R134A | 8.12±0.09 | 9.14±0.08 | 7.60±0.12 | 7.65±0.11 | 118±4 |
| S135A | 8.35±0.09 | 9.40±0.10 | 7.79±0.09 | 7.82±0.09 | 60±14* |
| S136A | 7.84±0.11 | 9.29±0.07 | 7.39±0.13 | 7.59±0.15 | 123±15 |
| P137A | 7.18±0.13* | 8.54±0.09* | 6.94±0.12* | 6.98±0.18* | 102±14 |
| E138A | 8.22±0.07 | 9.19±0.10 | 7.21±0.07 | 7.87±0.07 | 164±13* |
| E139A | ND | ND | ND | ND | 37±8* |
| Q140A | 7.84±0.15 | 8.65±0.13* | 7.68±0.21 | 8.15±0.16 | 37±6* |
| L141A | 7.50±0.06 | 8.53±0.06* | 6.17±0.28* | 7.17±0.06* | 136±8 |
| L142A | 7.89±0.07 | 9.23±0.07 | 6.89±0.10* | 7.84±0.07 | 94±11 |
| F143A | 8.19±0.06 | 9.33±0.09 | 7.35±0.09 | 7.65±0.06 | 102±11 |
| L144A | 7.94±0.15 | 9.41±0.17 | 7.15±0.19 | 8.03±0.15 | 25±6* |
|       | Y145A     | Y146A     | Y147A     | Y148A     |
|-------|-----------|-----------|-----------|-----------|
| Value | 8.25±0.09 | 9.51±0.09 | 7.38±0.10 | 7.92±0.07 |
|       | 8.07±0.09 | 9.32±0.12 | 7.57±0.14 | 7.91±0.09 |
|       | 7.78±0.05 | 9.00±0.08 | 7.26±0.08 | 7.67±0.08 |
|       | 6.79±0.15*| 8.06±0.06*| 6.26±0.25*| 8.09±0.08 |
|       |           |           |           | 67±8      |
Table 2. Effects of human GLP-1R TM1/N-terminal mutants on agonists-mediated cAMP accumulation.

cAMP accumulation data were analyzed using a three-parameter logistic equation to determine $pE_{50}$ and $E_{max}$ values. $pE_{50}$ is the negative logarithm of the molar concentration of agonist that induced half the maximal response. $E_{max}$ for mutants is expressed as a percentage of wild-type. $Log_{max}$ is the operational efficacy value (determined via the Black and Leff operational model (25)), corrected for cell surface expression of GLP-1R. All values for cAMP accumulation are mean ± S.E.M. of four to six independent experiments, conducted in duplicate. One-way ANOVA and Dunnett’s post-test were used to determine statistical differences (*P<0.05).

| GLP-1$_{7-36}$NH$_2$ | Exendin-4 | Oxyntomodulin |
|---------------------|-----------|----------------|
|                      | $pE_{50}$ | $E_{max}$ | $Log_{max}$ | $pE_{50}$ | $E_{max}$ | $Log_{max}$ |
| Wild-type           | 9.84±0.04 | 97.77±1.13 | 0.77±0.07 | 10.43±0.03 | 99.32±0.90 | 0.81±0.09 |
| R24A                | 10.55±0.14* | 77.09±2.92* | 0.18±0.05* | 10.83±0.16 | 74.16±3.10* | 0.13±0.06* |
| P25A                | 10.33±0.08 | 97.02±2.21 | 0.67±0.10 | 10.67±0.18 | 97.12±4.65 | 0.67±0.10 |
| Q26A                | 10.20±0.09 | 92.56±2.61 | 0.53±0.07 | 10.56±0.08 | 97.69±2.24 | 0.66±0.11 |
| G27A                | 9.36±0.49 | 6.91±0.99* | -0.70±0.16* | 10.39±0.57 | 6.62±0.94* | -0.70±0.16* |
| A28G                | 10.14±0.10 | 102.20±3.14 | 0.84±0.15 | 10.51±0.12 | 110.00±3.61 | 1.22±0.19 |
| T29A                | ND | ND | ND | ND | ND | ND |
| V30A                | 9.93±0.16 | 108.20±5.48 | 1.09±0.14 | 10.50±0.10 | 110.50±3.01 | 1.24±0.22 |
| E128A               | 9.87±0.39 | 4.59±0.42* | -0.25±0.14* | 10.62±0.72 | 4.63±0.48* | -0.22±0.25 |
| S129A               | 9.85±0.10 | 98.92±3.30 | 0.83±0.12 | 10.44±0.09 | 111.50±2.93 | 1.45±0.31 |
| K130A               | 9.35±0.09 | 116.20±3.83* | 1.47±0.20* | 10.05±0.18 | 77.74±4.44* | 0.59±0.05 |
| R131A               | 10.15±0.21 | 76.34±4.84* | 0.22±0.05* | 10.78±0.15 | 101.40±4.12 | 0.78±0.14 |
| G132A               | 9.83±0.16 | 33.04±1.67* | -0.31±0.06* | 10.58±0.25 | 35.90±2.36* | -0.28±0.06* |
| E133A               | 9.74±0.17 | 87.76±9.42 | 0.55±0.07 | 10.09±0.16 | 74.40±5.28 | 1.01±0.38 |
| R134A               | 9.86±0.12 | 102.80±4.06 | 0.80±0.15 | 10.28±0.17 | 89.67±4.44 | 0.48±0.08 |
| S135A               | 9.72±0.15 | 114.00±5.30* | 1.24±0.11 | 10.09±0.09 | 99.42±2.79 | 1.11±0.15 |
| S136A               | 9.42±0.23 | 96.37±7.34 | 0.67±0.10 | 10.00±0.10 | 81.16±2.54* | 0.29±0.05 |
| P137A               | 9.64±0.12 | 101.30±3.90 | 0.82±0.13 | 10.45±0.29 | 88.74±7.37 | 0.50±0.07 |
| E138A               | 9.63±0.15 | 94.25±4.49 | 0.41±0.08 | 10.46±0.14 | 112.30±4.24* | 1.22±0.18 |
| E139A               | 10.29±0.19 | 58.81±3.12* | 0.43±0.06 | 10.90±0.51 | 28.03±3.75* | -0.08±0.07* |
| Q140A               | 7.91±0.15* | 59.59±5.48* | 0.52±0.08 | 8.86±0.14* | 59.75±3.60* | 0.45±0.07 |
| L141A               | 9.18±0.18 | 95.20±5.60 | 0.51±0.09 | 10.43±0.12 | 113.20±3.68* | 1.42±0.23 |
| L142A               | 9.48±0.19 | 72.12±4.84* | 0.24±0.08* | 10.79±0.27 | 76.65±5.75* | 0.21±0.05 |
| F143A               | 10.18±0.13 | 91.47±3.55 | 0.56±0.11 | 10.78±0.21 | 84.00±4.91* | 0.41±0.10 |
| L144A               | 8.03±0.18* | 53.89±5.51* | 0.57±0.07 | 8.90±0.14* | 68.52±3.71* | 0.75±0.09 |

$E_{max}$ is the negative logarithm of the molar concentration of agonist that induced half the maximal response. $E_{max}$ for mutants is expressed as a percentage of wild-type. $Log_{max}$ is the operational efficacy value (determined via the Black and Leff operational model (25)), corrected for cell surface expression of GLP-1R. All values for cAMP accumulation are mean ± S.E.M. of four to six independent experiments, conducted in duplicate. One-way ANOVA and Dunnett’s post-test were used to determine statistical differences (*P<0.05).
| Residue | Y145A | Y146A | Y147A | Y148A |
|---------|-------|-------|-------|-------|
|         | 9.36±0.13 | 9.41±0.16 | 9.34±0.20 | 8.45±0.12* |
|         | 116.40±5.51 | 108.30±6.35 | 80.68±5.54* | 106.20±6.02 |
|         | 1.44±0.21* | 1.43±0.18 | 0.32±0.05* | 1.08±0.07* |
|         | 100.00±0.09 | 102.30±5.28 | 109.00±5.12 | 106.20±6.02 |
|         | 1.25±0.25 | 0.78±0.10 | 0.32±0.05* | 0.14±0.07* |
|         | 7.57±0.11 | 7.79±0.27 | 7.74±0.35 | 7.74±0.36 |
|         | 103.00±4.88 | 103.90±4.88 | 103.90±4.88 | 103.00±4.88 |
|         | 0.80±0.22 | 0.80±0.22 | 0.80±0.22 | 0.80±0.22 |
|         | 0.98±0.07 | 0.98±0.07 | 0.98±0.07 | 0.98±0.07 |

* Denotes values from biophysical experiments.
Table 3. Effects of human GLP-1R TM1/N-terminal mutants on agonists-mediated iCa\textsuperscript{2+} mobilization.

Ca\textsuperscript{2+} mobilization data were analyzed using a three-parameter logistic equation to determine $p\text{EC}_{50}$ and $E_{\text{max}}$ values. $p\text{EC}_{50}$ is the negative logarithm of the molar concentration of agonist that induced half the maximal response. $E_{\text{max}}$ for mutants is expressed as a percentage of wild-type. Log $\tau$ is the operational efficacy value (determined via the Black and Leff operational model\textsuperscript{(24)}), corrected for cell surface expression of GLP-1R. All values for cAMP accumulation are mean ± S.E.M. of four to six independent experiments, conducted in duplicate. One-way ANOVA and Dunnett’s post-test were used to determine statistical differences (*P<0.05).

| GLP-1(7-36)NH\textsubscript{2} | Exendin-4 |
|-------------------------------|------------|
| pEC\textsubscript{50} | E\textsubscript{max} | Log $\tau$ | pEC\textsubscript{50} | E\textsubscript{max} | Log $\tau$ |
| Wild-type | 7.83±0.04 | 100.00±1.11 | -0.22±0.03 | 8.45±0.04 | 100.00±1.35 | -0.22±0.02 |
| R24A | 7.60±0.27* | 179.60±20.27* | 0.19±0.10* | 8.25±0.12 | 130.40±5.76* | -0.12±0.05 |
| P25A | 7.89±0.17 | 151.30±11.07 | 0.09±0.09 | 8.07±0.14 | 120.50±6.80 | -0.11±0.05 |
| Q26A | 8.14±0.29 | 179.60±20.27* | 0.02±0.09 | 8.31±0.12 | 110.40±4.87 | -0.24±0.05 |
| G27A | ND | 16.77±4.36* | ND | ND | ND | ND |
| A28G | 7.86±0.14 | 96.66±4.36* | -0.27±0.10 | 8.45±0.19 | 165.90±11.23* | 0.21±0.09 |
| T29A | ND | ND | ND | ND | ND | ND |
| V30A | 7.93±0.24 | 70.75±7.07* | 0.17±0.07 | 8.31±0.24 | 111.50±9.64 | -0.17±0.06 |
| E128A | ND | ND | ND | ND | ND | ND |
| S129A | 7.83±0.12 | 101.30±5.03 | -0.13±0.07 | 8.47±0.19 | 80.73±5.52 | -0.27±0.06 |
| K310A | 7.83±0.18 | 52.81±4.09* | -0.32±0.13 | 7.89±0.17 | 59.33±4.30* | -0.25±0.11 |
| R311A | 7.76±0.25 | 133.80±14.52* | -0.05±0.11 | 8.50±0.24 | 98.00±8.13 | -0.28±0.07 |
| G132A | 7.79±0.20 | 58.45±4.75* | -0.47±0.13 | 8.14±0.23 | 76.96±7.21* | -0.29±0.07 |
| E133A | 7.89±0.18 | 89.51±6.77 | -0.28±0.10 | 8.30±0.21 | 84.37±6.62 | -0.31±0.06 |
| R134A | 8.15±0.32 | 79.99±9.90 | -0.44±0.09 | 8.36±0.19 | 84.59±5.60 | -0.40±0.06 |
| S135A | 7.99±0.20 | 48.35±3.85* | -0.44±0.15 | 8.71±0.22 | 34.19±2.48* | -0.61±0.11* |
| S136A | 7.81±0.18 | 61.68±4.72* | -0.61±0.12* | 8.56±0.16 | 106.70±5.75 | -0.26±0.06 |
| P137A | 7.35±0.25 | 612.00±14.18 | 0.08±0.12 | 8.13±0.10 | 126.00±5.21 | -0.04±0.08 |
| E138A | 7.56±0.13 | 102.90±11.92* | 0.35±0.10* | 8.27±0.20 | 253.40±18.09* | 1.13±0.27* |
| E139A | 7.94±0.20 | 42.50±3.51* | -0.30±0.11 | 7.94±0.21* | 58.47±4.87* | -0.12±0.08 |
| Q140A | 8.89±0.30* | 20.06±2.38* | -0.83±0.14* | 7.49±0.42* | 34.38±4.54* | -0.41±0.17 |
| L141A | 7.49±0.25 | 101.00±11.56 | -0.34±0.11 | 7.79±0.18 | 80.66±6.34 | -0.49±0.09 |
| L142A | 8.74±0.32* | 25.97±2.71* | -0.95±0.17* | 8.77±0.23 | 33.66±2.54* | -0.82±0.11* |
| F143A | 7.65±0.12 | 127.20±7.06 | -0.05±0.11 | 8.48±0.08 | 140.70±3.98* | 0.05±0.05 |
| L144A | ND | ND | ND | ND | ND | ND |
| Y145A | 6.76±0.20* | 108.50±10.35 | -0.12±0.12 | 8.10±0.25 | 94.96±9.30 | -0.19±0.09 |
|            |    |             |          |            |    |             |          |            |
|------------|----|-------------|----------|------------|----|-------------|----------|------------|
|            | I146A | 7.97±0.19 | 60.52±4.60* | -0.23±0.10 | 8.80±0.28 | 52.91±5.09* | -0.30±0.07 |
|            | I147A | 7.60±0.22 | 44.28±4.42* | -0.73±0.15* | 8.50±0.28 | 66.87±6.33* | -0.51±0.07 |
|            | Y148A | 7.99±0.25 | 40.42±3.79* | -0.59±0.14 | 8.30±0.21 | 18.91±1.50* | -0.95±0.22* |
Table 4. Effects of human GLP-1R TM1/N-terminal mutants on agonists-mediated pERK1/2. pERK1/2 phosphorylation data were analyzed using a three-parameter logistic equation to determine $pEC_{50}$ and $Emax$ values. $pEC_{50}$ is the negative logarithm of the molar concentration of agonist that induced half the maximal response. $Emax$ for mutants is expressed as a percentage of wild-type. $Log_e$ is the operational efficacy value (determined via the Black and Leff operational model[2]), corrected for cell surface expression of GLP-1R. All values for cAMP accumulation are mean ± S.E.M. of four to six independent experiments, conducted in duplicate. One-way ANOVA and Dunnett’s post-test were used to determine statistical differences (*P<0.05).

### Agonist-mediated ERK1/2 phosphorylation

| GLP-1$\text{[7-36]NH}_2$ | Wildtype | R24A | P25A | Q26A | G27A | A28G | T29A | V30A | E128A | S129A | K130A | R131A | G132A | E133A | R134A | S135A | S136A | P137A | E138A | E139A | Q140A | L141A | L142A | F143A | L144A | Y145A | I146A |
|------------------------|----------|------|------|------|------|------|------|------|------|-------|-------|-------|-------|-------|-------|-------|------|------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| $pEC_{50}$            | 8.07±0.05 | 8.21±0.19 | 8.51±0.13 | 8.57±0.16 | ND   | 8.25±0.24 | ND   | 8.16±0.12 | ND   | 8.42±0.12 | 7.93±0.08 | 7.93±0.16 | 8.19±0.17 | 8.37±0.13 | 8.09±0.13 | 8.06±0.18 | 7.81±0.16 | 7.64±0.15 | 8.29±0.21 | 7.95±0.20 | 7.02±0.21 | 7.71±0.11 | 8.00±0.16 | 8.22±0.16 | ND   | 7.78±0.41 | 7.14±0.34 |
| $Emax$                | 100.00±1.87 | 108.50±7.64 | 125.30±5.34 | 157.30±7.93* | 76.61±6.48 | 131.40±11.84* | 120.80±5.95 | 120.80±5.95 | 120.80±5.95 | 104.10±5.95 | 121.00±5.95 | 110.80±6.22 | 101.80±5.00 | 104.10±4.55 | 120.80±5.95 | 120.80±5.95 | 120.80±5.95 | 107.80±5.45 | 110.10±5.22 | 52.17±4.96* | 137.10±6.84* | 160.40±6.71 | 116.70±6.85 | ND   | 86.36±13.92 | 68.81±13.41* |
| $Log_e$               | 8.77±0.05 | 8.33±0.18 | 8.95±0.19 | 8.68±0.16 | 8.14±0.30 | 8.71±0.29 | 8.96±0.23 | 8.90±0.15 | 9.00±0.17 | 9.08±0.21 | 8.88±0.13 | 9.03±0.29 | 9.08±0.15 | 9.36±0.21 | 9.19±0.09 | 8.26±0.16 | 7.80±0.25 | 8.82±0.13 | 7.98±0.24 | 7.70±0.31 | 8.52±0.13 | 8.05±0.17 | 8.24±0.28 | 7.57±0.36 | 8.13±0.28* | 8.30±0.22 |

### pERK1/2 phosphorylation data

|        | Exendin-4 | Oxyntomodulin |
|--------|------------|--------------|
| $pEC_{50}$ | $Emax$ | $Log_e$ | $pEC_{50}$ | $Emax$ | $Log_e$ |
| 7.72±0.04 | 101.40±1.64 | 0.01±0.03 | 7.53±0.04 | 100.40±4.85 | -0.07±0.07 |
| 7.82±0.11 | 110.80±6.22 | 0.09±0.07 | 7.73±0.14 | 126.10±12.12 | 0.40±0.12* |
| 7.66±0.22 | 108.20±9.56 | 0.11±0.08 | 7.66±0.20 | 110.20±9.56 | 0.11±0.08 |
| 7.45±0.11 | 107.80±5.45 | 0.07±0.08 | 7.45±0.15 | 86.46±5.89 | -0.04±0.08 |
| 7.51±0.12 | 122.50±6.66 | 0.55±0.09* | 7.50±0.16 | 104.10±7.36 | 0.00±0.08 |
| 7.45±0.24 | 35.06±4.55* | -0.51±0.15* | 7.67±0.17 | 72.90±5.38 | -0.19±0.08 |
| 7.63±0.16 | 79.86±5.78 | -0.20±0.08 | 7.57±0.14 | 48.80±3.35* | -0.22±0.10 |
| 6.73±0.25* | 32.54±6.85* | -0.73±0.16* | 7.17±0.16 | 112.30±9.51 | 0.13±0.09 |
| 7.30±0.16 | 258.30±20.51* | 0.88±0.18* | 7.30±0.16 | 110.10±5.22 | 0.13±0.09 |
| 7.90±0.18 | 51.16±4.23* | 0.01±0.08 | 7.68±0.26* | 27.40±5.10* | -0.39±0.19* |
| 7.30±0.18 | 126.10±11.22 | 0.13±0.10 | 7.03±0.19 | 97.49±11.29 | 0.06±0.10 |
| 7.50±0.22 | 104.80±10.36 | 0.06±0.08 | 7.50±0.22 | 108.20±9.56 | 0.11±0.08 |
| ND       | ND         | ND          | ND       | ND         | ND          |
| 7.32±0.46 | 106.00±24.78 | 0.15±0.09 | 7.38±0.18 | 89.57±8.13 | 0.26±0.08 |
|       | I147A     | Y148A    |
|-------|-----------|----------|
| Value | 7.77±0.16 | 7.11±0.21* |
| Value | 144.9±9.75* | 93.4±11.28 |
| Value | 0.45±0.11* | 0.16±0.11 |
| Value | 8.21±0.12 | 8.01±0.20* |
| Value | 140.8±6.46* | 105.6±8.68 |
| Value | 0.38±0.09* | 0.25±0.07 |
| Value | 7.5±0.17 | 7.12±0.17 |
| Value | 149.1±11.18* | 78.4±7.84 |
| Value | 0.51±0.11* | 0.03±0.10 |
Table 5. Sequence variations in published structures.

| PDB ID | Description                                                                 | Structure Fragment       | Mutations/differences from hGLP-1R                                                                 | Missing residues in the structure                        |
|--------|------------------------------------------------------------------------------|--------------------------|--------------------------------------------------------------------------------------------------|----------------------------------------------------------|
| 5VEW   | Crystal structure of human GLP-1R transmembrane domain in complex with negative allosteric modulator PF-06372222 | Residues 128–431.       | S193C, I196F, Y205G, ΔT207, ΔA208, ΔA209, ΔQ210, ΔQ211, AH212, ΔQ213, W214G, S225A, M233X, S271A, I317C, G318I, K346A, C347F, G361C | E128, S129, K130, R131, G132, E133, R134, S135, M204, D215, E373, H374, A375, R376, G377, T378, L379, E423, H424, L425, H426, I427, Q428, R429, D430, S431 |
| 5NX2   | Crystal structure of human GLP-1 receptor bound to the 11-mer agonist Peptide 5 | Residues 24-432          | T207E, Q211A, D215R, L232F, L260F, G295A, T298A, C329A, P358A, G361A, H363V, V405A                      | R24, P25, Q26, G27, A28, E418, R419, W420, R421, L422, E423, H424, L425, H426, I427, Q428, R429, D430, S431, S432   |
| 5VAI   | Cryo-EM structure of active rabbit GLP-1 receptor in complex with GLP-1 and Gs protein | Residues 24-422          | T106A, H112P, Q140R                                                                                | R24, P25, Q26, G27, A28, S129, R130, R131, G132, E133, K134, L422                                       |
| 6B3J   | Cryo-EM structure of active human GLP-1 receptor in complex with exendin-P5 and Gs protein | Residues 23-466          | None                                                                                               | A23-T29, S129-S136, N338-T342, H424-G466                                                           |
Figure 1

A

Inactive hGLP-1R model:

ExP5/GLP-1R/Gs structure

Exendin-P5

B

Expression (% WT)

60-79% 40-59% 20-39% 0-19%

>140%

Figure 1
Figure 2
Figure 3

A: Affinity
B: cAMP
C: iCa²⁺
D: pERK

-2.0  -1.5  -1.0  -0.5  0.0  0.5  1.0  1.5  2.0

Log (τ) Δ WT

Reduced function
Enhanced function
2-5 fold
5-10 fold
10-30 fold
>30 fold

Unstructured in fully active GLP-1R/agonist/Gs complexes
Figure 4
Figure 5
Figure 6
Figure 7

A

GLP-1/GLP-1R
ExP5/GLP-1R
11mer/GLP-1R
NNC1702/GCGR

B

GLP-1/GLP-1R
ExP5/GLP-1R
11mer/GLP-1R
NNC1702/GCGR

11mer peptide

Figure 7
Figure 8
Figure 9

ExP5/GLP-1R

GLP-1/GLP-1R

11mer/GLP-1R

NNC1702/GCGR

Inactive GLP-1R model
GLP-1

cAMP efficacy

Exendin-4

iCa\textsuperscript{2+} efficacy

ECL1
ECL2
ECL3
TM1/ECD stalk

ECL1
ECL2
ECL3
TM1/ECD stalk

pERK efficacy

Figure 11
Two distinct domains of the glucagon-like peptide-1 receptor control peptide-mediated biased agonism

Saifei Lei, Lachlan Clydesdale, Antao Dai, Xiaqing Cai, Yang Feng, Dehua Yang, Yi-Lynn Liang, Cassandra Koole, Peishen Zhao, Thomas Coudrat, Arthur Christopoulos, Ming-Wei Wang, Denise Wootten and Patrick M Sexton

J. Biol. Chem. published online May 1, 2018

Access the most updated version of this article at doi: 10.1074/jbc.RA118.003278

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