Second Extracellular Loop of Human Glucagon-like Peptide-1 Receptor (GLP-1R) Has a Critical Role in GLP-1 Peptide Binding and Receptor Activation*§

Cassandra Koolé†, Denise Wootten‡, John Simms†, Laurence J. Miller†, Arthur Christopoulos†‡, and Patrick M. Sexton†‡

From the †Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences and Department of Pharmacology, Monash University, Parkville, Victoria 3052, Australia and the ‡Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Scottsdale, Arizona 85259

The glucagon-like peptide-1 receptor (GLP-1R) is a therapeutically important family B G protein-coupled receptor (GPCR) that is pleiotropically coupled to multiple signaling effectors and, with actions including regulation of insulin biosynthesis and secretion, is one of the key targets in the management of type II diabetes mellitus. However, there is limited understanding of the role of the receptor core in orthosteric ligand binding and biological activity. To assess involvement of the extracellular loop (ECL) 2 in ligand-receptor interactions and receptor activation, we performed alanine scanning mutagenesis of loop residues and assessed the impact on receptor expression and GLP-1 peptide agonist binding and activation of three physiologically relevant signaling pathways as follows: cAMP formation, intracellular calcium; (Ca^{2+}) mobilization, and phosphorylation of extracellular signal-regulated kinases 1 and 2 (pERK1/2). Although antagonist peptide binding was unaltered, almost all mutations affected GLP-1 peptide agonist binding and/or coupling efficacy, indicating an important role in receptor activation. However, mutation of several residues displayed distinct pathway responses with respect to wild type receptor, including Arg-299 and Tyr-305, where mutation significantly enhanced both GLP-1(1–36)-NH_{2} and GLP-1(7–36)-NH_{2} binding and activation of three pathologically important signaling pathways as follows: cAMP formation, intracellular calcium; (Ca^{2+}) mobilization, and phosphorylation of extracellular signal-regulated kinases 1 and 2 (pERK1/2). Although antagonist peptide binding was unaltered, almost all mutations affected GLP-1 peptide agonist binding and/or coupling efficacy, indicating an important role in receptor activation. However, mutation of several residues displayed distinct pathway responses with respect to wild type receptor, including Arg-299 and Tyr-305, where mutation significantly enhanced both GLP-1(1–36)-NH_{2} and GLP-1(7–36)-NH_{2} binding and activation of three pathologically important signaling pathways as follows: cAMP formation, intracellular calcium; (Ca^{2+}) mobilization, and phosphorylation of extracellular signal-regulated kinases 1 and 2 (pERK1/2). In addition, mutation of Cys-296, Trp-297, Asn-300, Asn-302, and Leu-307 significantly increased GLP-1(7–36)-NH_{2}-mediated signaling bias toward pERK1/2. Of all mutants studied, only mutation of Trp-306 to alanine abolished all biological activity. These data suggest a critical role of ECL2 of the GLP-1R in the activation transition(s) of the receptor and the importance of this region in the determination of both GLP-1 peptide- and pathway-specific effects.

**Background:** The ECL2 of family B GPCRs has been suggested to contribute to biological activity.

**Results:** Mutation of most ECL2 residues to alanine results in changes in binding and/or efficacy of GLP-1 peptide agonists.

**Conclusion:** The ECL2 of the GLP-1R is critical for GLP-1 peptide-mediated receptor activation and selective signaling.

**Significance:** This work reveals broad significance for ECL2 in maintaining receptor conformations driving selective signaling.

**GPCR**s are the largest family of transmembrane (TM)-spanning proteins, accounting for ~1% of the human genome, and are the leading target of marketed therapeutics (1, 2). Family B peptide hormone receptors are a small subfamily of GPCRs that include receptors for secretin, calcitonin, vasoactive intestinal polypeptide, pituitary adenylate cyclase-activating polypeptide, corticotrophin-releasing factor, parathyroid hormone, gastric inhibitory polypeptide, glucagon, and glucagon-like peptides (GLPs). Each receptor possesses a characteristically large and sequence-divergent extracellular N-terminal domain; however, there is conservation of key residues, including three disulfide bonds within this domain, that aids in stability and confers similarities in secondary structure (3, 4). The widely accepted peptide-receptor binding model for family B GPCRs is the two domain model, whereby the α-helical C terminus of the endogenous ligand interacts with the N-terminal domain of the receptor, and the N terminus of the peptide interacts with the core domain of the receptor, which includes both the extracellular loops and TM bundle (5–7). Generically, the N-terminal domain of the receptor is primarily responsible for ligand recognition and specificity, whereas the core of the receptor has a major influence in signaling specificity and transmission (8). Indeed, there is evidence through generation of both chimeric receptors and peptides to suggest that this is true for many family B receptors (9–16). However, there is also evidence that ligand recognition and affinity determination can rely on interaction with the receptor core (14, 17–24); this is particularly evident with the glucagon receptor, where changes in the N terminus of the glucagon peptide significantly alter its binding affinity at the glucagon receptor (17, 25, 26). In addition, sub-

*This work was supported in part by the National Health and Medical Research Council of Australia Project Grant 1002180 and Program Grant 519461 and by National Health and Medical Research Council Principal Research Fellowship (to P. M. S.) and a Senior Research Fellowship (to A. C.).

†This article contains supplemental Table S1 and Fig. S1.

‡Senior Research Fellow of the National Health and Medical Research Council.

§Principal Research Fellow of the National Health and Medical Research Council. To whom correspondence should be addressed: Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, 381 Royal Parade, Parkville, Victoria 3052, Australia. Tel.: 3-9903-9069; Fax: 3-9903-9581; E-mail: Patrick.Sexton@monash.edu.

The glucagon-like peptide-1 receptor (GLP-1R) is a therapeutically important family B G protein-coupled receptor (GPCR) that is pleiotropically coupled to multiple signaling effectors and, with actions including regulation of insulin biosynthesis and secretion, is one of the key targets in the management of type II diabetes mellitus. However, there is limited understanding of the role of the receptor core in orthosteric ligand binding and biological activity. To assess involvement of the extracellular loop (ECL) 2 in ligand-receptor interactions and receptor activation, we performed alanine scanning mutagenesis of loop residues and assessed the impact on receptor expression and GLP-1 peptide agonist binding and activation of three physiologically relevant signaling pathways as follows: cAMP formation, intracellular calcium; (Ca^{2+}) mobilization, and phosphorylation of extracellular signal-regulated kinases 1 and 2 (pERK1/2). Although antagonist peptide binding was unaltered, almost all mutations affected GLP-1 peptide agonist binding and/or coupling efficacy, indicating an important role in receptor activation. However, mutation of several residues displayed distinct pathway responses with respect to wild type receptor, including Arg-299 and Tyr-305, where mutation significantly enhanced both GLP-1(1–36)-NH_{2} and GLP-1(7–36)-NH_{2} binding and activation of three pathologically important signaling pathways as follows: cAMP formation, intracellular calcium; (Ca^{2+}) mobilization, and phosphorylation of extracellular signal-regulated kinases 1 and 2 (pERK1/2). Although antagonist peptide binding was unaltered, almost all mutations affected GLP-1 peptide agonist binding and/or coupling efficacy, indicating an important role in receptor activation. However, mutation of several residues displayed distinct pathway responses with respect to wild type receptor, including Arg-299 and Tyr-305, where mutation significantly enhanced both GLP-1(1–36)-NH_{2} and GLP-1(7–36)-NH_{2} binding and activation of three pathologically important signaling pathways as follows: cAMP formation, intracellular calcium; (Ca^{2+}) mobilization, and phosphorylation of extracellular signal-regulated kinases 1 and 2 (pERK1/2). In addition, mutation of Cys-296, Trp-297, Asn-300, Asn-302, and Leu-307 significantly increased GLP-1(7–36)-NH_{2}-mediated signaling bias toward pERK1/2. Of all mutants studied, only mutation of Trp-306 to alanine abolished all biological activity. These data suggest a critical role of ECL2 of the GLP-1R in the activation transition(s) of the receptor and the importance of this region in the determination of both GLP-1 peptide- and pathway-specific effects.

**GPCR**s are the largest family of transmembrane (TM)-spanning proteins, accounting for ~1% of the human genome, and are the leading target of marketed therapeutics (1, 2). Family B peptide hormone receptors are a small subfamily of GPCRs that include receptors for secretin, calcitonin, vasoactive intestinal polypeptide, pituitary adenylate cyclase-activating polypeptide, corticotrophin-releasing factor, parathyroid hormone, gastric inhibitory polypeptide, glucagon, and glucagon-like peptides (GLPs). Each receptor possesses a characteristically large and sequence-divergent extracellular N-terminal domain; however, there is conservation of key residues, including three disulfide bonds within this domain, that aids in stability and confers similarities in secondary structure (3, 4). The widely accepted peptide-receptor binding model for family B GPCRs is the two domain model, whereby the α-helical C terminus of the endogenous ligand interacts with the N-terminal domain of the receptor, and the N terminus of the peptide interacts with the core domain of the receptor, which includes both the extracellular loops and TM bundle (5–7). Generically, the N-terminal domain of the receptor is primarily responsible for ligand recognition and specificity, whereas the core of the receptor has a major influence in signaling specificity and transmission (8). Indeed, there is evidence through generation of both chimeric receptors and peptides to suggest that this is true for many family B receptors (9–16). However, there is also evidence that ligand recognition and affinity determination can rely on interaction with the receptor core (14, 17–24); this is particularly evident with the glucagon receptor, where changes in the N terminus of the glucagon peptide significantly alter its binding affinity at the glucagon receptor (17, 25, 26). In addition, sub-
GLP-1R ECL2 Is Critical for Receptor Activation

Substitution of many N-terminal residues of the GLP-1, vasoactive intestinal polypeptide, and secretin peptides for alanine significantly reduce binding affinity at the GLP-1R, VPAC receptors, and secretin receptor, respectively (21, 23, 24), illustrating that effective ligand recognition, binding, and subsequent biological activity require the entire length of peptide and involve multiple domains within the receptor.

Although crystal and NMR structures have been resolved for the isolated N terminus of several ligand-bound family B GPCRs, including the gastric inhibitory polypeptide receptor (27), corticotrophin-releasing factor receptors (28–30), parathyroid hormone 1 receptor (31), and GLP-1 R (32, 33), only limited mutagenesis and photoaffinity labeling data are available to aid in understanding the role of core domain residues. Nevertheless, the data available highlight the significance of the receptor core region in both peptide binding and receptor activation (34, 35), including residues within the putative ECL2 of the GLP-1R (36, 37), secretin receptor (18), corticotrophin-releasing factor receptors (38, 39), and parathyroid hormone receptors (16), suggesting this potentially forms a significant site of interaction for the N-terminal amino acids of the peptide ligands and/or plays an important role in stabilizing active state conformations in the presence of ligand.

Like most GPCRs, family B receptors are promiscuously coupled, including pathway coupling that leads to cAMP signaling, Ca2+, mobilization, and pERK1/2, each of which is linked to important physiological functions of the receptors (40–42). The relative activation of these signaling pathways may therefore be important for optimal development of therapeutics. Nonetheless, our mechanistic understanding of how family B receptors activate these distinct pathways is limited.

In this study, we explore the influence of individual ECL2 residues on human GLP-1R function. The GLP-1R is an important target in the development of therapeutics for type II diabetes mellitus, with actions including glucose-dependent increases in insulin biosynthesis and secretion, increasing β-cell mass, and decreasing body mass, all effects that address major symptoms of type II diabetes mellitus (43). Despite its therapeutic promise, relatively limited data are available on the contribution of domains in the receptor core on ligand binding and receptor activation. We have performed systematic substitution of each residue of ECL2 of the human GLP-1R by alanine and assessed the effects across a series of pharmacological outputs, which demonstrated critical residues for receptor activation that vary in an agonist peptide- or pathway-specific manner.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco’s modified Eagle’s medium (DMEM), hygromycin-B, and Fluo-4 acetoxymethyl ester were purchased from Invitrogen. Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific (Melbourne, Victoria, Australia). The QuikChange™ site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). AlphaScreen™ reagents, Bolton-Hunter reagent (125I), and 384-well ProxiPlates were purchased from PerkinElmer Life Sciences. SureFire™ ERK1/2 reagents were generously supplied by TGR Biosciences (Adelaide, South Australia, Australia). SigmaFast o-phenylene-diamine dihydrochloride tablets and antibodies were purchased from Sigma. GLP-1 peptides were purchased from American Peptide (Sunnyvale, CA). All other reagents were purchased from Sigma or Merck and were of an analytical grade.

**Receptor Mutagenesis**—To study the influence of specific amino acids of ECL2 on receptor function, the desired mutations were introduced to an N-terminally double c-Myc-labeled wild type human GLP-1R in the pEF5/FRT/V5-DEST destination vector (Invitrogen); this receptor had equivalent pharmacology to the untagged human GLP-1R (data not shown). Mutagenesis was carried out using oligonucleotides for site-directed mutagenesis from GeneWorks (Hindmarsh, South Australia, Australia) (supplemental Table S1) and the QuikChange™ site-directed mutagenesis kit (Stratagene). Sequences of receptor clones were confirmed by cycle sequencing as described previously (44). Mutated residues and their conservation across human family B peptide hormone receptors are illustrated in Fig. 1.

**Transfections and Cell Culture**—Wild type and mutant human GLP-1R were isogenically integrated into FlpIn-Chinese hamster ovary (FlpInCHO) cells (Invitrogen) and selection of receptor-expressing cells accomplished by treatment with 600 μg ml−1 hygromycin-B as described previously (44). Transfected and parental FlpInCHO cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and incubated in a humidified environment at 37 °C in 5% CO2.

**Radioligand Binding Assay**—FlpInCHO wild type and mutant human GLP-1R cells were seeded at a density of 3 × 10⁴ cells/well into 96-well culture plates and incubated overnight at 37 °C in 5% CO2, and radioligand binding was carried out as described previously (45). For each cell line in all experiments, total binding was defined by 0.5 nm 125I-exendin(9–39) alone, and nonspecific binding was defined by 1 μM exendin(9–39). For analysis, data are normalized to the B0 value for each individual experiment.

**cAMP Accumulation Assay**—FlpInCHO wild type and mutant human GLP-1R cells were seeded at a density of 3 × 10⁴ cells/well into 96-well culture plates and incubated overnight at 37 °C in 5% CO2, and cAMP detection was carried out as described previously (46). All values were converted to concentration of cAMP using a cAMP standard curve performed in parallel, and data were subsequently normalized to the response of 100 μM forskolin in each cell line.

**pERK1/2 Assay**—FlpInCHO wild type and mutant human GLP-1R cells were seeded at a density of 3 × 10⁴ cells/well into 96-well culture plates and incubated overnight at 37 °C in 5% CO2. Receptor-mediated pERK1/2 was determined using the AlphaScreen™ ERK1/2 SureFire™ protocol as described previously (44). Initial pERK1/2 time course experiments were performed over 1 h to determine the time at which agonist-mediated pERK1/2 was maximal. Subsequent experiments were then performed at the time required to generate a maximal pERK1/2 response (6 min). Data were normalized to the maximal response elicited by 10% FBS in each cell line, determined at 6 min (peak FBS response).

**Ca2+, Mobilization Assay**—FlpInCHO wild type and mutant human GLP-1R cells were seeded at a density of 3 × 10⁴ cells/
GLP-1R ECL2 Is Critical for Receptor Activation

well into 96-well culture plates and incubated overnight at 37 °C in 5% CO₂, and receptor-mediated Ca²⁺ mobilization was determined as described previously (47). Fluorescence was determined immediately after peptide addition, with an excitation wavelength set to 485 nm and an emission wavelength set to 520 nm, and readings were taken every 1.36 s for 120 s. Peak magnitude was calculated using five-point smoothing, followed by correction against basal fluorescence. The peak value was used to create concentration-response curves. Data were normalized to the maximal response elicited by 100 μM ATP.

Cell Surface Receptor Expression—FlpInCHO wild type and mutant human GLP-1R cells, with receptor DNA previously incorporated with an N-terminal double c-Myc epitope label, were seeded at a density of 25 × 10⁴ cells/well into 24-well culture plates and incubated overnight at 37 °C in 5% CO₂, washed three times in 1× PBS, and fixed with 3.7% paraformaldehyde at 4 °C for 15 min. Cell surface receptor detection was then performed as described previously (45). Data were normalized to the basal fluorescence detected in FlpInCHO parental cells. Specific ¹²⁵I-exendin(9–39) binding at each receptor mutant, as identification of functional receptors at the cell surface, was also determined (corrected for nonspecific binding using 1 μM exendin(9–39)).

Data Analysis—All data were analyzed using Prism 5.04 (GraphPad Software Inc., San Diego). For all analyses the data are unweighted, and each y value (mean of replicates for each individual experiment) is considered an individual point. Concentration response signaling data were analyzed using a three-parameter logistic equation as described previously (44) and as shown in Equation 1,

\[
Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{\log Ec50 - \log[A]}}, \quad \text{(Eq. 1)}
\]

where Bottom represents the y value in the absence of ligand(s); Top represents the maximal stimulation in the presence of ligand(s); [A] is the molar concentration of ligand, and Ec50 represents the molar concentration of ligand required to generate a response halfway between Top and Bottom. Similarly, Equation 1 was used in the analysis of inhibition binding data, instead replacing Ec50 with Ic50. In this case, Bottom defines the specific binding of the radioligand that is equivalent to non-specific ligand binding, whereas Top defines radioligand binding in the absence of a competing ligand, and the Ic50 value represents the molar concentration of ligand required to generate a response halfway between Top and Bottom. Ic50 values obtained were then corrected for radioligand occupancy as described previously (48) using the radioligand affinity (Kᵣ) experimentally determined for each mutant.

To quantify efficacy in the system, all data were fitted with an operational model of agonism (49) as shown in Equation 2,

\[
Y = \text{Bottom} + \frac{E_m - \text{Bottom}}{1 + (10^{\log K_A} + (10^{\log[A]}))(10^{\log Ec50} + \log[A])}, \quad \text{(Eq. 2)}
\]

where Bottom represents the y value in the absence of ligand(s); Eₘ represents the maximal stimulation of the system; Kₐ is the agonist-receptor dissociation constant, in molar concentration; [A] is the molar concentration of ligand, and τ is the operational measure of efficacy in the system, which incorporates signaling efficacy and receptor density. Constraints for this model were determined by fitting the operational model for a partial agonist to each of the peptides at the wild type receptor, with the most efficacious peptide fitted with Equation 3,

\[
Y = \text{Bottom} + \frac{E_m - \text{Bottom}}{1 + 10^{\log Ec50 - \log[A]}}, \quad \text{(Eq. 3)}
\]

and the less efficacious peptides were fitted with Equation 2, to obtain a value for the system maximum (Eₘ) at the wild type receptor. This value was then globally constrained in the operational model (Equation 2) when applied at each of the mutant receptors. All estimated τ values were then corrected to cell surface expression (τᵣ) as determined by specific ¹²⁵I-exendin(9–39) binding, and errors were propagated from both τ and cell surface expression. Changes in τᵣ with respect to wild type for each mutant were used to generate correlation plots, which were subsequently fitted with linear regression and outliers established at greater than three standard deviations from regression.

To quantify signaling bias, peptide agonist concentration-response curves were analyzed with nonlinear regression using an operational model of agonism (50), but modified to directly estimate the ratio of τᵣ/Kₐ, in a manner similar to that described by Figueroa et al. (51). For each pathway, as shown in Equation 4,

\[
Y = \frac{E_{max} \times (\tau/K_A)^n \times [A]^n}{[A]^n \times (\tau/K_A)^n + (1 + [A]/K_A)^n}, \quad \text{(Eq. 4)}
\]

the parameters are as defined for Equation 2. All estimated τᵣ/Kₐ ratios included propagation of error for both τᵣ and Kₐ. Changes in τᵣ/Kₐ ratios with respect to wild type of each mutant were used to quantify bias between signaling pathways. Accordingly, bias factors included propagation of error from τᵣ/Kₐ ratios of each pathway.

Data were also normalized to maximal agonist response at the wild type receptor in each signaling pathway, fitted with a three-parameter logistic equation, and equinomil concentrations of agonists in each pathway plotted against one other. In this way, the bias of any given agonist for one pathway over another can be visualized (50). In all cases, individual data sets were unweighted during the analyses.

Statistics—Changes in peptide affinity, potency, efficacy, and cell surface expression of ECL2 mutants in comparison with wild type control were statistically analyzed with one-way analysis of variance and Dunnett’s post test, and significance was accepted at p < 0.05.

RESULTS

Cell Surface Expression of Human GLP-1R ECL2 Alanine Mutants

Wild type c-Myc human GLP-1R and each of the human GLP-1R ECL2 alanine mutants (Fig. 1A) were isogenically integrated into FlpInCHO host cells by recombination, allowing the
comparison of cell surface expression by antibody detection of the N-terminal double c-Myc epitope label without complications arising from variations in gene transcription. In this study, we observed total abolishment of cell surface receptor expression of the W306A mutant (Fig. 2A and Table 1). Significant increases in cell surface receptor antibody labeling were observed for the N300A and M303A mutants, although decreases were observed for D293A, C296A, W297A, S301A, N304A, Y305A, and L307A. No other mutant deviated significantly in cell surface receptor antibody labeling in comparison with wild type, although there were occasional trends for increases or decreases. In most cases, the changes in cell surface expression identified through antibody detection of the epitope label were consistent with the pattern of specific binding of 125I-exendin(9–39) at each of the mutant receptors in comparison with wild type receptor, although generically the 125I-exendin(9–39) binding trended lower for the mutants relative to the wild type, when compared with receptor antibody labeling (Fig. 2B and Table 1). Notable exceptions to this were E294A and T298A that demonstrated increased 125I-exendin(9–39) binding but wild type levels of receptor antibody labeling and Y305A that displayed wild type levels of 125I-exendin(9–39) binding and reduced antibody labeling. At each of these receptor mutants, the affinity of exendin(9–39) (determined through homologous competition binding) was not significantly different from the wild type receptor (Table 1). As exendin(9–39) affinity was unaltered at all mutant receptors, reductions in 125I-exendin(9–39) binding but not antibody labeling may suggest a subpopulation of receptors for which the ligand binding domain of the receptor is misfolded, leading to loss of functional receptors at the cell surface. In these cases, however, antibody detection of the inserted epitope tag does not discriminate between different conformational states of the ligand binding domain and therefore has most likely detected all populations of receptor at the cell surface. The mechanism underlying high 125I-exendin(9–39) in the absence of changes to antibody labeling is less clear but may be due to altered conformation of the c-Myc epitope or an increase in the relative proportion of receptors in an inactive state.

Select Mutants of the Human GLP-1R ECL2 Influence GLP-1(7–36)-NH2 Binding Affinity but Not Exendin(9–39) Affinity

To establish the binding profiles of each of the human GLP-1R ECL2 alanine mutants, equilibrium binding studies were performed with the endogenous peptide agonists GLP-1(1–36)-NH2 and GLP-1(7–36)-NH2 in competition with the radiolabeled orthosteric antagonist, 125I-exendin(9–39). There was no significant deviation in antagonist exendin(9–39) affinity at any of the mutated ECL2 residues, with the exception of the W306A mutant, where no value could be defined as it was undetectable at the cell surface (Table 1). Full inhibition curves for the GLP-1(1–36)-NH2 peptide could not be established over the concentration range tested; at the highest concentration assayed (1 μM), the level of binding inhibition for most of the mutants was not significantly different from wild type receptor (Fig. 3, A and B). However, no clear
effect on binding was observed for C296A, W297A, R299A, and Y305A (Fig. 3, A and B). This was likely a result of the poor radioligand binding window for these mutants arising from the low specific $^{125}$I-exendin(9–39) binding (Table 1).

There were no significant changes in affinity of GLP-1(7–36)-NH$_2$ in comparison with the wild type control for G285A, I286A, V287A, Y289A, L290A, Y291A, E294A, G295A, T298A, S301A, and N304A (Table 1). Decreases in binding affinity of GLP-1(7–36)-NH$_2$ in comparison with wild type were observed for K288A, E292A, D293A, R299A, N302A, M303A, Y305A, and L307A mutants, as highlighted by gray shading in Table 1 (Fig. 3, C and D, and Table 1).

**Effect of Human GLP-1R ECL2 Alanine Mutations on Peptide-mediated cAMP Accumulation**

**Binding Affinity-modified Mutants**—There was no measurable GLP-1(1–36)-NH$_2$-mediated cAMP response at the K288A, E292A, C296A, W297A or N300A mutants, and significant depression in $E_{max}$ was observed at the D293A, R299A, N300A, N302A, M303A, Y305A, and L307A mutants, as highlighted by gray shading in Table 1 (Fig. 3, C and D, and Table 1).

**Mutants with Unaltered Binding Affinity**—For mutants that were not affected at the level of peptide agonist binding, potency was mostly unaltered with two exceptions as follows: the V287A mutant, where decreased potency of GLP-1(7–36)-NH$_2$ was observed, and the T298A mutant that had increased potency of GLP-1(7–36)-NH$_2$ (Fig. 3). Application of the operational model indicated that each of these mutant receptors had a significantly reduced coupling efficiency for cAMP, after correction for functional cell surface receptor levels (Fig. 4A and Table 2). Throughout the results, the operational measure of efficacy ($\tau_\text{eff}$) is used as the principal measure of changes in efficacy for each pathway, as this accounts for both alterations in coupling efficiency and cell surface receptor expression (50).

All mutants displaying significantly decreased GLP-1(7–36)-NH$_2$ binding affinity (K288A, E292A, D293A, C296A, W297A, R299A, N300A, N302A, M303A, Y305A, and L307A) also exhibited reduced potency for cAMP in response to this peptide, although this was not significant for the M303A mutant (Fig. 4B and Table 2). Assessment of the effect on efficacy indicated that all mutants also had diminished coupling efficiency in addition to decreased affinity, although this effect was minimal for D293A, C296A, and Y305A, where statistical significance was not reached (Fig. 4, B and F, and Table 2). Not surprisingly, cells expressing the W306A mutant did not respond to peptide stimulation (Table 2).
TABLE 1

Effects of human GLP-1R ECL2 alanine mutants on peptide ligand binding and cell surface expression

| Binding (pIC_{50}) | Cell surface expression (%) | Specific binding (pIC_{50}) |
|------------------|-----------------------------|-----------------------------|
|                  | GLP-1(1–36)-NH$_2$         | Exendin(9–39)               | GLP-1(7–36)-NH$_2$         |
| Wildtype         | 8.7 ± 0.1                   | 7.7 ± 0.1                   | 100 ± 5                    |
| G295A            | 9.1 ± 0.1                   | 7.7 ± 0.1                   | 88 ± 3                     |
| G296A            | 8.8 ± 0.1                   | 7.7 ± 0.2                   | 79 ± 3                     |
| V287A            | 8.5 ± 0.1                   | 7.7 ± 0.1                   | 52 ± 4                     |
| K288A            | 6.8 ± 0.2                   | 8.0 ± 0.1                   | 88 ± 5                     |
| Y298A            | 8.9 ± 0.1                   | 7.6 ± 0.2                   | 64 ± 7                     |
| L290A            | 8.3 ± 0.1                   | 7.7 ± 0.1                   | 77 ± 7                     |
| Y291A            | 8.3 ± 0.1                   | 7.8 ± 0.2                   | 86 ± 7                     |
| E292A            | 6.7 ± 0.2                   | 7.7 ± 0.1                   | 103 ± 6                    |
| D293A            | 7.3 ± 0.1                   | 7.9 ± 0.1                   | 62 ± 5.5                   |
| E294A            | 8.8 ± 0.1                   | 8.0 ± 0.1                   | 81 ± 6                     |
| Q295A            | 8.7 ± 0.1                   | 7.7 ± 0.1                   | 90 ± 3                     |
| C296A            | 7.6 ± 0.4                   | 7.8 ± 0.2                   | 69 ± 5.9                   |
| M297A            | 5.9 ± 0.5                   | 7.9 ± 0.2                   | 60 ± 6                     |
| T298A            | 9.1 ± 0.1                   | 8.1 ± 0.1                   | 85 ± 6                     |
| R299A            | 7.2 ± 0.4                   | 7.4 ± 0.2                   | 94 ± 5                     |
| N300A            | 6.6 ± 0.2                   | 7.9 ± 0.1                   | 130 ± 6.9                  |
| S301A            | 8.8 ± 0.1                   | 8.1 ± 0.2                   | 63 ± 5                     |
| N302A            | 7.3 ± 0.1                   | 8.0 ± 0.1                   | 91 ± 6                     |
| M303A            | 8.1 ± 0.1                   | 8.1 ± 0.1                   | 125 ± 8.5                  |
| N304A            | 8.4 ± 0.3                   | 7.5 ± 0.2                   | 70 ± 2.7                   |
| Y305A            | 6.8 ± 0.2                   | 7.7 ± 0.1                   | 52 ± 5.5                   |
| W306A            | N.D.                        | N.D.                        | N.D.                       |
| L307A            | 7.6 ± 0.2                   | 8.0 ± 0.1                   | 47 ± 5.5                   |

* Data are statistically significant at p < 0.05, one-way analysis of variance, and Dunnett’s post test in comparison with wild type response.

** $R^2$ values for the curve fits were all >0.95, except for GLP-1(1–36)-NH$_2$ at the W297A ($R^2 = 0.42$) and R299A ($R^2 = 0.46$) mutants.

† GLP-1(1–36)-NH$_2$ binding profile deviates from wild type, but the pIC$_{50}$ value for this ligand was unable to be determined in the concentration range tested.

** R values for the curve fits were all >0.95, except for GLP-1(7–36)-NH$_2$ at the W297A ($R^2 = 0.42$) and R299A ($R^2 = 0.46$) mutants.

* Data are statistically significant at p < 0.05, one-way analysis of variance, and Dunnett’s post test in comparison with wild type response.

† GLP-1(1–36)-NH$_2$ binding profile deviates from wild type, but the pIC$_{50}$ value for this ligand was unable to be determined in the concentration range tested.

Effect of Human GLP-1R ECL2 Alanine Mutations on Peptide-mediated pERK1/2

Binding Affinity-modified Mutants—Peptide-induced pERK1/2 was determined at 6 min for each of the human GLP-1R ECL2 alanine mutants. Consistent with cAMP accumulation, there were no statistically significant differences in GLP-1(1–36)-NH$_2$ potency at these mutant receptors, although no measurable response was seen for either N300A or W306A (Fig. 5A and Table 3). In general, there was less impact of the ECL2 mutants on GLP-1(1–36)-NH$_2$-mediated pERK1/2 signaling relative to effects on cAMP response. Whereas the K288A, E292A, C296A, W297A, and N300A all lacked measurable cAMP responses in the presence of GLP-1(1–36)-NH$_2$, only the N300A mutant also failed to yield a pERK1/2 signal (Tables 2 and 3). Operational modeling of the effect of mutation on peptide efficacy failed to identify any significant changes for these mutants, although most trended toward lower efficacy (Fig. 5, A and E, and Table 3). The exception to this was R299A that trended higher in efficacy for pERK1/2 (Table 3).

As with the GLP-1(1–36)-NH$_2$ responses, there was generally less impact of ECL2 mutation on GLP-1(7–36)-NH$_2$-mediated pERK1/2 responses relative to the cAMP response (Fig. 5B and Tables 2 and 3). Potency was largely unaffected with only the K288A mutant exhibiting significantly lower potency, although there was a trend toward lower potency with some of the other affinity altered mutants, including E292A and C296A (Fig. 5, B and F, and Table 3). The greatest effects on GLP-1(7–36)-NH$_2$ efficacy were seen with E292A and N300A, the former being significantly lower (Fig. 5F and Table 3). K288A, D293A, W297A, N302A, Y305A, and L307A also trended toward lower efficacy. In contrast, C296A, R299A, and M303A displayed higher efficacy than the wild type receptor, although these effects did not reach significance. Interestingly, the effect on coupling efficiency of GLP-1(1–36)-NH$_2$ and GLP-1(7–36)-NH$_2$ was opposite for C296A and M303A suggesting that there are ligand-dependent effects imparted by these mutations (Table 3). Intriguingly, the R299A mutant displayed consistent effects for the two peptides but distinct effects in cAMP (decreased efficacy) versus pERK1/2 signaling (increased efficacy), suggesting a pathway selective role for this residue (Tables 2 and 3).

Mutants with Unaltered Binding Affinity—Overall, there was only limited impact of ECL2 mutants on the pERK1/2 responses for mutant receptors that had unaltered agonist binding affinity, and indeed, none of the effects achieved statistical significance for either GLP-1(1–36)-NH$_2$ or GLP-1(7–36)-NH$_2$ (Fig. 5, C and D, and Table 3). Nonetheless, there was further distinction in the pattern of pERK1/2 efficacy changes relative to Ca$^{2+}$, and cAMP signaling in that efficacy tended to increase for many of the mutants, including G285A, I286A, V287A, Y289A, G295A, and T298A in comparison with wild type (Fig. 5, C–F, and Table 3). The L290A and Y291A mutants tended to have decreased efficacy for the GLP-1(7–36)-NH$_2$ peptide but had little effect on GLP-1(1–36)-NH$_2$ responses. In contrast, N304A had an apparent increase in efficacy for GLP-1(1–36)-NH$_2$ but unaltered GLP-1(7–36)-NH$_2$ response (Table 3).

Effect of Human GLP-1R ECL2 Alanine Mutations on Peptide-mediated Ca$^{2+}$, Mobilization

Binding Affinity-modified Mutants—Consistent with previous data (46), there was no Ca$^{2+}$ response with full-length full-length or truncated peptide. Overall, similar effects on efficacy were observed for GLP-1(1–36)-NH$_2$ and GLP-1(7–36)-NH$_2$ across the ECL2 mutants (Fig. 4, E and F, and Table 2). G285A, I286A, V287A, and Y289A, located at the top of TM4/proximal ECL2, had minimal effects on the efficacy of both peptides. Similarly, there was little effect on cAMP efficacy with the G295A mutant. All other mutants displayed loss of efficacy for both peptides, although the effect was not always significant for both peptides. For example, there was a greater effect on GLP-1(1–36)-NH$_2$ efficacy with the L290A mutant and a greater effect on GLP-1(7–36)-NH$_2$ efficacy for the E294A, T298A, and S301A mutants. For both peptides, the greatest decrease in efficacy was seen with the N304A mutant (Table 2).
GLP-1 at either the wild type receptor or any of the alanine mutants (data not shown).

Mutants that displayed reduced GLP-1(7–36)-NH₂ binding affinity (K288A, E292A, D293A, C296A, W297A, R299A, N300A, N302A, M303A, Y305A, and L307A) displayed either significantly reduced Ca²⁺/H⁺i signaling or complete loss of signaling (Fig. 6A and Table 4). Notably, receptors with abolished Ca²⁺/H⁺i signaling (K288A, E292A, C296A, W297A, and N300A) were those that had the weakest ability to couple to the cAMP pathway, with the exception of C296A (Table 2). In addition, these mutants also had weak coupling to the pERK1/2 pathway, with the exception of C296A and W297A (Table 3). Dramatic reductions in GLP-1(7–36)-NH₂ efficacy were observed at the remaining affinity-affected mutant receptors D293A, R299A, N302A, M303A, Y305A, and L307A (Fig. 6, A and C, and Table 4). These decreases in ability to couple to the Ca²⁺, pathway were mostly reflected in cAMP responses, although the extent of reductions in efficacy was in some cases inconsistent; D293A and Y305A were the most affected mutants in Ca²⁺, but the least affected in cAMP coupling in the presence of GLP-1(7–36)-NH₂ (Tables 2 and 4). Generically, the decreases in Ca²⁺, coupling were also reflected in pERK1/2 pathway coupling, with the exception of R299A and M303A (Tables 3 and 4).

Mutants with Unaltered Binding Affinity—All mutants had reduced GLP-1(1–36)-NH₂ efficacy relative to the wild type receptor, although the effect was not significant for G285A, I286A, V287A, L290A, G295A, and S301A that were among the least affected with respect to cAMP response (Fig. 6, B and C, and Table 4). For nonbinding affected mutants, the greatest effect on efficacy was observed with the N304A mutant, again consistent with the magnitude of effect on cAMP signaling (Tables 2 and 4).

Effect of Human GLP-1R ECL2 Alanine Mutations on Peptide-mediated Signal Bias

As noted previously (46), GLP-1(1–36)-NH₂ exhibits distinct signal bias to that evoked by GLP-1(7–36)-NH₂. GLP-1(7–36)-NH₂ is strongly biased toward cAMP formation relative to either pERK1/2 or Ca²⁺, mobilization (supplemental Fig. S1, B and C). In
contrast, GLP-1(1–36)-NH₂ has equivalent preference for cAMP and pERK1/2 (supplemental Fig. S1A). GLP-1(7–36)-NH₂, however, has only weak bias for the pERK1/2 pathway relative to Ca²⁺/H₁ mobilization (supplemental Fig. S1D). As the GLP-1(1–36)-NH₂ peptide did not elicit an Ca²⁺/H₁ response, relative bias against this pathway could not be determined. Nonetheless, the absence of response is indicative of bias toward cAMP and pERK1/2.

Bias plots provide a convenient visual representation of relative pathway response that is independent of absolute potency (50), although bias factors are a quantitative measure of changes to pathway bias, relative to the wild type receptor (50).
Comparison of all mutants in this manner indicates that most ECL2 mutants engender at least subtle changes to receptor bias, although residues Gly-285 and Ile-286 at the top of TM4 are exceptions to this (supplemental Fig. S1). The G285A mutation is interesting in that it engenders efficacy improvements for cAMP and pERK1/2 responses and was minimally altered with respect to Ca²⁺/H₁₁₀₀₁ᵢ signaling. The mutants with the most dramatic effects on relative signal bias were also those that were altered in binding affinity of GLP-1(7–36)-NH₂, with the exception of N304A, which consistently had a large differential effect across pathways, and the T298A mutant that was among those mutants with greatest effect on pERK1/2/cAMP for GLP-1(1–36)-NH₂ and for Ca²⁺/H₁₁₀₀₁ᵢ/pERK1/2 for GLP-1(7–36)-NH₂ (supplemental Fig. S1, A and D), although the effect of these mutants was not significant in bias factor calculations (Table 5).

Bias of cAMP or pERK1/2 relative to Ca²⁺/H₁₁₀₀₁ᵢ was only moderately affected by most mutants (supplemental Fig. S1, C and D); the principal effect for Ca²⁺/H₁₁₀₀₁ᵢ for GLP-1(7–36)-NH₂ was a loss of efficacy for cAMP production rather than alteration to bias per se, and this is reflected in the generally small changes to bias factors for these pathways (Table 5). This is probably not surprising as Ca²⁺/H₁₁₀₀₁ᵢ is the weakest coupled pathway at the wild type receptor, as discussed above. The greatest effects on bias were seen with specific mutants on pERK1/2/cAMP profiles (supplemental Fig. S1, A and B; Table 5). This was evident as preservation of pERK1/2 response with near abolition of cAMP response for R299A, N302A, N304A, and Y305A for GLP-1(1–36)-NH₂ and a complete reversal of bias for GLP-1(7–36)-NH₂ such that pERK1/2 was favored over cAMP for N300A, W297A, C296A, and R299A. There was no clear bias for E292A, N302A, N304A, and L307A, indicating loss of the cAMP bias seen with the wild type receptor.

The effect of mutations to both increase and decrease efficacy of ERK1/2 activation likely reflects the divergent pathways that link receptor activation to pERK1/2, including both G protein-independent (52–55) and G protein-dependent mechanisms (56). It is interesting to note that the most profound differential effects on signaling occur with mutation to residues distal to, or including, the conserved Cys-296/Trp-297 motif. This segment of ECL2 is also the most important with respect to binding of small molecule ligands into the TM region of family A GPCRs (57–61).

### Table 2: Effects of Human GLP-1R ECL2 Alanine Mutants on Peptide Agonist Signaling via cAMP

|                  | pEC₅₀ | Eₘₐₓ | log₁₀(τₐ) | pEC₅₀ | Eₘₐₓ | log₁₀(τₐ) |
|------------------|-------|------|-----------|-------|------|-----------|
| Wildtype         | 7.3 ± 0.1 | 166.7 ± 5.3 | 0.65 ± 0.06 (4.47) | 9.0 ± 0.1 | 167.4 ± 4.6 | 0.82 ± 0.06 (6.61) |
| G285A            | 7.7 ± 0.2 | 139.1 ± 9.7⁺ | 0.78 ± 0.09 (6.03) | 9.1 ± 0.1 | 155.2 ± 4.9 | 0.96 ± 0.08 (8.12) |
| I286A            | 7.6 ± 0.2 | 127.7 ± 9.2⁺ | 0.53 ± 0.09 (3.39) | 8.5 ± 0.2 | 156.8 ± 10.2 | 0.72 ± 0.08 (5.25) |
| V287A            | 7.2 ± 0.2 | 134.4 ± 10.4⁺ | 0.54 ± 0.12 (3.47) | 8.4 ± 0.1⁺ | 137.3 ± 7.0⁺ | 0.49 ± 0.12 (3.09) |
| K288A            | N.D.     | N.D.   | N.D.      | N.D.   | N.D.   | N.D.      |
| Y289A            | 7.6 ± 0.3 | 119.3 ± 12.9⁺ | 0.64 ± 0.09 (4.37) | 8.0 ± 0.2 | 110.9 ± 6.9⁺ | 0.94 ± 0.08 (3.09) |
| L290A            | 7.3 ± 0.1 | 102.8 ± 5.1⁺ | 0.20 ± 0.10 (1.58) | 8.7 ± 0.1 | 134.1 ± 7.1⁺ | 0.48 ± 0.08 (3.02) |
| Y291A            | 7.2 ± 0.1 | 119.3 ± 6.8⁺ | 0.24 ± 0.11 (1.74) | 8.6 ± 0.1 | 126.9 ± 5.9⁺ | 0.31 ± 0.10 (2.04) |
| E292A            | N.D.     | N.D.   | N.D.      | N.D.   | 6.9 ± 0.1⁺ | 94.9 ± 5.4⁺ |
| C293A            | 6.8 ± 0.3 | 64.1 ± 6.7⁺ | 0.14 ± 0.07 (0.72) | 7.8 ± 0.1⁺ | 118.0 ± 3.8⁺ | 0.53 ± 0.09 (3.39) |
| E294A            | 7.4 ± 0.1 | 144.3 ± 7.3 | 0.40 ± 0.17 (2.51) | 9.1 ± 0.1 | 122.5 ± 4.5⁺ | 0.31 ± 0.17 (2.04) |
| G295A            | 7.4 ± 0.1 | 145.7 ± 5.1 | 0.65 ± 0.08 (4.47) | 9.1 ± 0.0 | 113.6 ± 1.9⁺ | 0.47 ± 0.07 (2.95) |
| C296A            | N.D.     | N.D.   | N.D.      | N.D.   | 6.9 ± 0.0⁺ | 99.4 ± 2.7⁺ |
| W297A            | N.D.     | N.D.   | N.D.      | N.D.   | 6.5 ± 0.1⁺ | 45.4 ± 3.6⁺ |
| T298A            | 7.8 ± 0.1 | 126.9 ± 5.3⁺ | 0.41 ± 0.12 (2.57) | 9.7 ± 0.1⁺ | 116.2 ± 5.3⁺ | 0.41 ± 0.12 (2.57) |
| R299A            | 7.2 ± 0.5 | 15.6 ± 2.8⁺ | -0.42 ± 0.10 (0.38)⁺ | 7.6 ± 0.1⁺ | 92.4 ± 4.1⁺ | 0.36 ± 0.07 (2.29)⁺ |
| N300A            | N.D.     | N.D.   | N.D.      | N.D.   | 6.3 ± 0.1⁺ | 94.5 ± 6.1⁺ |
| S301A            | 7.5 ± 0.2 | 102.9 ± 6.5⁺ | 0.30 ± 0.08 (2.00) | 9.0 ± 0.2 | 105.9 ± 6.5⁺ | 0.36 ± 0.07 (2.29) |
| N302A            | 7.0 ± 0.3 | 52.4 ± 6.4⁺ | -0.56 ± 0.18 (0.28)⁺ | 7.8 ± 0.1⁺ | 120.3 ± 5.9⁺ | 0.29 ± 0.17 (1.95)⁺ |
| M303A            | 7.3 ± 0.2 | 100.3 ± 6.4⁺ | 0.13 ± 0.09 (1.35)⁺ | 8.5 ± 0.2 | 119.1 ± 7.5⁺ | 0.38 ± 0.09 (2.40)⁺ |
| N304A            | 7.4 ± 0.3 | 69.6 ± 6.0⁺ | 0.09 ± 0.13 (1.23)⁺ | 8.5 ± 0.2 | 71.2 ± 4.8⁺ | 0.08 ± 0.12 (1.20)⁺ |
| Y305A            | 6.9 ± 0.2 | 44.9 ± 3.6⁺ | -0.57 ± 0.14 (0.27)⁺ | 7.4 ± 0.2⁺ | 136.0 ± 11.8⁺ | 0.62 ± 0.15 (4.17)⁺ |
| W306A            | N.D.     | N.D.   | N.D.      | N.D.   | N.D.   | N.D.      |
| L307A            | 7.0 ± 0.2 | 52.4 ± 3.9⁺ | -0.27 ± 0.09 (0.54)⁺ | 7.6 ± 0.1⁺ | 101.0 ± 4.9⁺ | 0.33 ± 0.08 (2.14)⁺ |

* Data were statistically significant at p < 0.05, one-way analysis of variance and Dunnett’s post test in comparison with the wild type response.
DISCUSSION

It is widely accepted that the ECLs of family A GPCRs, particularly ECL2, are an important component for receptor function, with roles that include facilitating ligand binding, receptor trafficking, communication of ligand signal, and/or stabilization of an active ligand-receptor complex. Indeed, there is a plethora of evidence for many of these features (62–66), including the activation of rhodopsin relying on the displacement of the β-hairpin forming ECL2 via TM movement to allow ligand binding (67), the design of antibodies against ECL2 of several GPCRs that initiate a functional response (68, 69), and the substitution of several domains in the ECL2 of the human gonadotropin-releasing hormone receptor converting the activity of an antagonist to an agonist (70).

FIGURE 5. pERK1/2 profiles of human GLP-1R ECL2 alanine mutants. Characterization of pERK1/2 in the presence of GLP-1(1–36)-NH₂ (A and C) and GLP-1(7–36)-NH₂ (B and D) in FlpInCHO cells stably expressing the wild type human GLP-1R or each of the human GLP-1R ECL2 alanine mutants that effect peptide binding affinity (A and B) or has no significant effect on peptide binding affinity (C and D) is shown. Data are normalized to the maximal response elicited by 10% FBS and analyzed with an operational model of agonism as defined in Equation 2. All values are means ± S.E. of five to seven independent experiments, conducted in duplicate. Visual representation of ERK1/2 coupling efficacy (log c) in the presence of GLP-1(1–36)-NH₂ (E) and GLP-1(7–36)-NH₂ (F). Statistical significance of changes in coupling efficacy in comparison with wild type human GLP-1R was determined by one-way analysis of variance and Dunnett’s post-test and is indicated with an asterisk (*, p < 0.05). All values are log c ± S.E. five to seven independent experiments, conducted in duplicate.
In this study we have used alanine scanning of ECL2 and adjacent residues to probe the function of this domain of the human GLP-1R. Virtually all mutations impacted on receptor function, in particular on ligand efficacy, although the nature and extent of effect varied considerably depending on the pathway, the ligand, and the mutation. Collectively, the data are indicative of a critical role for the GLP-1R ECL2 in the activation transition of the receptor. A global role for this domain in activation transition is supported by analysis of efficacy changes to mutants across the different pathways (Fig. 7). Although there are exceptions (discussed below), there was generally a good correlation between the magnitude of efficacy change for the different pathways for individual mutants, although the direction of change in the case of pERK1/2 was not always in the same direction as the other two pathways (that were almost uniformly negative) (Fig. 7). The correlations suggest that the mutations alter the ensemble of conformations formed/sampled by the receptor in response to agonists; for a subset of mutants there is greater propensity to form conformations linked to activation of pERK1/2, but this is gained at the expense of conformations linked to either cAMP formation or Ca\textsuperscript{2+} mobilization. The almost uniform loss of Ca\textsuperscript{2+} efficacy with individual mutations suggests that there is a high energy barrier for formation of conformations allowing coupling to this pathway, and this is consistent with the inability of GLP-1(1–36)-NH\textsubscript{2} to activate this pathway.

The recent solution of the crystal structure of the agonist-bound \(\beta_\text{2}\)-adrenergic receptor in complex with Go\(\alpha\) (71) has provided novel insight into the structural changes in the receptor that accompany activation transition. This receptor undergoes two major rearrangements on its intracellular face as follows: an outward displacement of TM6 by \(\sim 14\) Å and an \(\alpha\)-helical extension of TM5. These changes open up the receptor for interaction with the G protein. In the activated structure,

### TABLE 3

Effects of human GLP-1R ECL2 alanine mutants on peptide agonist signaling via pERK1/2

|             | GLP-1(1-36)NH\textsubscript{2} | GLP-1(7-36)NH\textsubscript{2} |
|-------------|----------------------|----------------------|
| Effector    | pEC\textsubscript{50} | E\textsubscript{max} | log \(E_{\text{max}}\) | pEC\textsubscript{50} | E\textsubscript{max} | log \(E_{\text{max}}\) |
| Wildtype    | 7.2 ± 0.2            | 5.7 ± 0.5            | 0.38 ± 0.10 (2.40) | 7.8 ± 0.2            | 5.3 ± 0.4            | 0.19 ± 0.07 (1.55) |
| G285A       | 7.1 ± 0.1            | 8.3 ± 0.6            | 0.98 ± 0.13 (9.55) | 8.1 ± 0.2            | 4.6 ± 0.4            | 0.30 ± 0.09 (2.00) |
| I286A       | 7.2 ± 0.2            | 6.5 ± 0.8            | 0.65 ± 0.11 (4.47) | 7.8 ± 0.2            | 5.7 ± 0.4            | 0.37 ± 0.09 (2.34) |
| V287A       | 6.9 ± 0.2            | 8.6 ± 0.7            | 0.61 ± 0.14 (4.07) | 7.6 ± 0.3            | 5.3 ± 0.6            | 0.33 ± 0.13 (2.14) |
| K288A       | 6.6 ± 0.6            | 3.3 ± 1.1            | -0.01 ± 0.38 (0.98) | \textit{6.7 ± 0.3}\* | 2.9 ± 0.5\*         | -0.09 ± 0.28 (0.81) |
| Y289A       | 7.3 ± 0.4            | 4.9 ± 0.8            | 0.55 ± 0.11 (3.55) | 7.4 ± 0.3            | 5.0 ± 0.6            | 0.31 ± 0.09 (2.04) |
| L290A       | 7.0 ± 0.2            | 5.5 ± 0.6            | 0.40 ± 0.11 (2.51) | 7.9 ± 0.3            | 3.4 ± 0.4\*         | -0.03 ± 0.10 (0.93) |
| Y291A       | 6.9 ± 0.3            | 5.7 ± 1.0            | 0.32 ± 0.12 (2.09) | 7.8 ± 0.3            | 4.1 ± 0.4            | 0.02 ± 0.11 (1.05) |
| E292A       | 6.7 ± 0.3            | 3.9 ± 0.7            | 0.08 ± 0.20 (1.20) | 7.2 ± 0.2            | 2.0 ± 0.2\*         | -0.37 ± 0.19 (0.43) |
| D293A       | 7.2 ± 0.3            | 4.0 ± 0.5            | 0.10 ± 0.14 (1.26) | 7.5 ± 0.3            | 2.7 ± 0.3\*         | -0.18 ± 0.14 (0.66) |
| E294A       | 7.1 ± 0.2            | 6.0 ± 0.5            | 0.28 ± 0.19 (1.91) | 7.8 ± 0.2            | 4.0 ± 0.3            | -0.18 ± 0.18 (0.66) |
| G295A       | 6.9 ± 0.2            | 8.1 ± 0.9            | 0.79 ± 0.11 (6.17) | 7.8 ± 0.2            | 5.9 ± 0.5            | 0.45 ± 0.09 (2.82) |
| C296A       | 7.8 ± 0.4            | 2.0 ± 0.4\*         | -0.23 ± 0.21 (0.59) | 6.9 ± 0.3            | 4.7 ± 0.7            | 0.47 ± 0.14 (2.95) |
| W297A       | 7.9 ± 0.5            | 2.2 ± 0.4\*         | -0.21 ± 0.16 (0.62) | 7.3 ± 0.4            | 3.0 ± 0.5\*         | 0.05 ± 0.14 (1.12) |
| T298A       | 7.8 ± 0.2            | 6.9 ± 0.5            | 0.91 ± 0.16 (8.13) | 8.0 ± 0.2            | 6.1 ± 0.5            | 0.27 ± 0.13 (1.86) |
| R299A       | 7.5 ± 0.3            | 5.4 ± 0.8            | 0.55 ± 0.13 (3.55) | 7.5 ± 0.2            | 4.2 ± 0.4            | 0.32 ± 0.10 (2.09) |
| N300A       | \textit{N.D.}        | \textit{N.D.}        | \textit{N.D.}        | 7.6 ± 0.3            | 3.1 ± 0.4\*         | -0.30 ± 0.14 (0.50) |
| S301A       | 7.3 ± 0.2            | 4.8 ± 0.5            | 0.38 ± 0.10 (2.40) | 7.8 ± 0.2            | 4.5 ± 0.4            | 0.18 ± 0.08 (1.51) |
| N302A       | 7.0 ± 0.3            | 5.4 ± 1.0            | -0.02 ± 0.21 (0.95) | 7.5 ± 0.3            | 4.5 ± 0.5            | -0.03 ± 0.19 (0.93) |
| M303A       | 7.2 ± 0.3            | 5.5 ± 0.8            | 0.29 ± 0.16 (1.95) | 7.8 ± 0.2            | 5.5 ± 0.4            | 0.32 ± 0.10 (2.09) |
| N304A       | 7.5 ± 0.2            | 5.8 ± 0.5            | 0.88 ± 0.15 (7.59) | 7.9 ± 0.3            | 3.2 ± 0.4\*         | 0.12 ± 0.13 (1.32) |
| Y305A       | 7.5 ± 0.2            | 3.4 ± 0.3            | -0.19 ± 0.14 (0.65) | 7.5 ± 0.3            | 3.3 ± 0.3\*         | -0.20 ± 0.12 (0.63) |
| W306A       | \textit{N.D.}        | \textit{N.D.}        | \textit{N.D.}        | \textit{N.D.}        | \textit{N.D.}        | \textit{N.D.}        |
| L307A       | 7.4 ± 0.2            | 2.8 ± 0.2\*         | -0.16 ± 0.15 (0.69) | 7.6 ± 0.2            | 3.1 ± 0.3\*         | -0.07 ± 0.10 (0.85) |

* Data were statistically significant at \(p < 0.05\), one-way analysis of variance, and Dunnett’s post test in comparison with wild type response.
GLP-1R ECL2 Is Critical for Receptor Activation

The α5 helix of Gα forms a network of polar interactions with TM5, and therefore, the transmission of conformational change to TM5 is likely to be very important for at least Gα interactions. Our data are indicative of ECL2 playing a critical role in the activation transition of the GLP-1R, potentially via effects on the conformation of TM5. Consistent with this, there was generally greater impact of mutation to residues that were distal to the conserved Cys (Cys-296) that resides near the top of TM5.

For all mutants expressed at the cell surface, binding of the antagonist peptide exendin(9–39) was unaltered, and binding affinity changes were restricted to the high affinity agonist peptide GLP-1(7–36)-NH2 for a subset of mutants. The interpretation of binding affinity changes for agonist peptides can be difficult. Although agonists enable the transition of the receptor to facilitate G protein binding, the ternary complex of the receptor with the G protein (or indeed with other regulatory proteins) provides thermodynamically reciprocal (allosteric) regulation of agonist binding (72). This has been empirically demonstrated for GPCRs using purified proteins, and for the β2-adrenoreceptor, it has been demonstrated that the full conformational alteration to the receptor requires both agonist and G protein to be bound (73, 74). As such, major effects on receptor-G protein interactions will manifest as selective loss of high affinity agonist binding, such as those observed in this study, and distinguishing direct effects on peptide binding from indirect effects is problematic.

The most dramatic effects on receptor function were seen with those mutants that also had reduced affinity for GLP-1(7–36)-NH2. For the mutants with greatest loss of apparent affinity, K288A, E292A, C296A, W297A, and N300A, there was a corresponding major loss of receptor function, although the loss of affinity alone was insufficient to account for the extent of functional loss. For the GLP-1(7–36)-NH2 peptide, these mutations led to abolition of the Ca2+ response and marked decrease in potency for cAMP formation. For most mutants, there was also a parallel loss in cAMP efficacy, and the exception to this was C296A, where efficacy was preserved (Tables 1–4). For GLP-1(1–36)-NH2, there was total loss of response in cAMP formation. The effect on pERK1/2 was more complex. Lys-288, Cys-296, and Trp-297 are each very highly conserved across the B subfamily of peptide hormone GPCRs and this may indicate a structural role for these residues (Fig. 1A). This is supported by the loss of functional cell surface receptors seen with alanine mutants of these residues (Table 1 and Fig. 2). For Cys-296, a structural role is clearly evident with this amino acid forming a disulfide bond with Cys-226 at the top of TM3; this structural motif is highly conserved across the superfamily of GPCRs and is evident in solved crystal structures for family A GPCRs (57–61, 75, 76). In the rat GLP-1R, double mutation of Cys-226 and Cys-296 to alanine restored the loss of GLP-1(7–36)-NH2 binding affinity and cAMP signaling seen with individual mutation of these residues, indicating that the disulfide link itself is not required for efficient activation of the receptor (77).

Recent photoaffinity cross-linking work has demonstrated that Leu-20 of GLP-1(7–36)-NH2 is proximal to Trp-297 of the receptor, with subsequent molecular modeling indicating that it could form a direct interaction that may contribute to the loss of function observed with mutation of this residue (36). Interestingly, Leu-20 is distal to the segment of the GLP-1 peptide (and indeed other related peptide hormones) that is linked to agonistic activity. Dipeptidyl peptidase IV cleavage of the two N-terminal residues markedly attenuates the activity of GLP-1(7–36)-NH2 and truncation by six amino acids is sufficient to abolish agonist activity (78). In this study binding affinity of the antagonist peptide exendin(9–39) was unaltered by any of the mutations, and thus it seems unlikely that the extent of loss of function seen with the W297A is due to loss of a direct interaction. It has been speculated that low binding affinity for GLP-1(7–36)-NH2 for the isolated N-terminal extracellular domain of the GLP-1R is due to decreased capacity of GLP-1(7–36)-
NH₂ to form an extended α-helix in the absence of the receptor core (79). It is possible that one role of Trp-297 is to help stabilize peptide secondary structure. Nonetheless, Trp-297 is completely conserved across all B family members suggesting that it is structurally important.

Mutation of Lys-288 to alanine in the rat GLP-1R leads to a similar decrease in GLP-1(7–36)-NH₂ affinity to that observed in this study, and although a cAMP response via the rat GLP-1R is detectable, there is a marked decrease in potency. At the equivalent position in other family B receptors, this amino acid is highly conserved with an invariant basic residue (Arg or Lys) present (Fig. 1A). Al-Sabah and Donnelly (37) have speculated that Lys-288 likely resides at the border between TM4 and ECL2 and that a basic residue in this position is potentially required for important interactions with neighboring TM residues. Nonetheless, such basic amino acids may also reside at the hydrophobic face of TM helices and undergo what is termed “snorkeling,” where the side chain is oriented parallel to the membrane helix leading to stabilization of the top of the TM helix (80), and this may be important in receptor function.

Glu-292 and Asn-300 are partially conserved across B family receptors, being homologous with more closely related members of the family (Fig. 1A). These receptors also have the greatest degree of homology with the N-terminal sequences of their activating peptides (Fig. 1B), and this may suggest more direct importance for interaction between the receptors and peptides. Although among the most deleterious of mutations for all pathways, their effect on relative efficacy across pathways is consistent with most other ECL2 mutations (Fig. 7). Nonetheless, the N300A mutant, like C296A and W297A, reversed GLP-1(7–36)-NH₂-mediated signal bias between pERK1/2 and cAMP formation (supplemental Fig. S1B).

Of all the mutants studied, only W306A was not expressed at the cell surface, indicating that the mutation leads to misfolding of the receptor. Curiously, double mutation of Tyr-305 and Trp-306 to alanine in the rat GLP-1R resulted in a population of receptors that were cell surface-expressed and responded to peptide agonists, albeit with marked effects on potency (77), suggesting that either interspecies differences in receptor sequence provide for greater stability of rat receptor structure or that the additional mutation compensated for some of the detrimental interactions arising from individual mutation of Trp-306.

All of the other mutations that altered the affinity of GLP-1(7–36)-NH₂, D293A, R299A, N302A, M303A, Y305A, and TABLE 5

| Residue | Wildtype | G285A | I296A | V287A | K298A | Y289A | L290A | Y291A | E292A | D293A | E294A | G295A | C296A | W297A | T298A | R299A | N300A | S301A | S302A | M303A | N304A | Y305A | W306A | L307A |
|---------|----------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| pEC₅₀  | 7.5 ± 0.1| 7.3 ± 0.2| 8.0 ± 0.3| 7.3 ± 0.2| N.D. | 7.4 ± 0.3| 7.5 ± 0.2| 7.2 ± 0.2| N.D. | 7.5 ± 0.4| 7.6 ± 0.3| 7.3 ± 0.2| N.D. | N.D. | 7.8 ± 0.1| 7.3 ± 0.5| N.D. | 7.6 ± 0.2| 6.6 ± 0.4| 7.2 ± 0.1| 7.3 ± 0.3| 7.2 ± 0.4| 7.0 ± 0.4| 2.4 ± 0.5*|
| Eₜₐₙ | 12.2 ± 0.8| 8.7 ± 0.9*| 5.9 ± 0.6*| 7.0 ± 0.7*| N.D.| 4.8 ± 0.6*| 6.8 ± 0.7*| 6.7 ± 0.7*| N.D.| 2.0 ± 0.3*| 6.6 ± 0.8*| 8.3 ± 0.7*| N.D.| N.D.| 7.0 ± 0.4*| 2.7 ± 0.6*| N.D.| 6.7 ± 0.5*| 5.9 ± 1.4*| 6.1 ± 0.4*| 2.9 ± 0.4*| 1.7 ± 0.3*| 2.4 ± 0.5*| -0.64 ± 0.17 (0.23)*|

* Data were statistically significant at p < 0.05, one-way analysis of variance, and Dunnnett’s post test in comparison with wild type response.

| Bias Factors | GLP-1(1–36)NH₂ | GLP-1(7–36)NH₂ | cAMP-ERK | cAMP-ERK | cAMP-cA¹ | ERK-cA¹ |
|--------------|----------------|----------------|----------|----------|----------|--------|
| Wildtype     | 0.00 ± 0.22    | 0.00 ± 0.19    | 0.00 ± 0.17 | 0.00 ± 0.21 |
| G285A        | -0.39 ± 0.23   | 0.02 ± 0.10    | 0.42 ± 0.23 | 0.40 ± 0.22 |
| I296A        | -0.34 ± 0.27   | -0.19 ± 0.22   | 0.41 ± 0.19 | 0.58 ± 0.22 |
| V287A        | -0.44 ± 0.24   | -0.29 ± 0.24   | 0.15 ± 0.25 | 0.44 ± 0.29 |
| K298A        | N.D.           | N.D.           | N.D.      | N.D.     |
| Y289A        | 0.03 ± 0.25    | -0.33 ± 0.21   | 0.19 ± 0.36 | 0.52 ± 0.34 |
| L290A        | -0.45 ± 0.32   | -0.43 ± 0.35   | -0.07 ± 0.29 | 0.35 ± 0.40 |
| Y291A        | -0.36 ± 0.30   | -0.60 ± 0.30   | 0.96 ± 0.29 | 0.65 ± 0.33 |
| E292A        | N.D.           | N.D.           | N.D.      | N.D.     |
| D293A        | -1.17 ± 0.35   | -0.90 ± 0.33   | -0.45 ± 0.80 | 0.45 ± 0.68 |
| E294A        | -0.22 ± 0.26   | -0.03 ± 0.30   | 0.22 ± 0.27 | 0.25 ± 0.34 |
| G295A        | -0.50 ± 0.23   | -0.55 ± 0.25   | 0.05 ± 0.20 | 0.60 ± 0.25 |
| C296A        | N.D.           | -1.61 ± 0.19*  | N.D.      | N.D.     |
| W297A        | N.D.           | -2.92 ± 0.38*  | N.D.      | N.D.     |
| T298A        | -1.79 ± 0.23   | -0.94 ± 0.22   | 0.25 ± 0.18 | 0.80 ± 0.20 |
| R299A        | -1.53 ± 0.43*  | -1.36 ± 0.31*  | -0.72 ± 0.43 | 0.64 ± 0.48 |
| N300A        | N.D.           | -2.64 ± 0.99*  | N.D.      | N.D.     |
| S301A        | -0.36 ± 0.22   | -0.62 ± 0.27   | -0.21 ± 0.23 | 0.31 ± 0.30 |
| S302A        | -1.17 ± 0.33   | -1.13 ± 0.24*  | -0.11 ± 0.22 | 1.02 ± 0.30 |
| M303A        | -0.45 ± 0.25   | -0.77 ± 0.27   | 0.05 ± 0.24 | 0.92 ± 0.30 |
| N304A        | -1.07 ± 0.23   | -0.60 ± 0.33   | -0.62 ± 0.55 | 0.67 ± 0.48 |
| Y305A        | -1.76 ± 0.46*  | -1.32 ± 0.19*  | -0.47 ± 0.63 | 0.95 ± 0.64 |
| W306A        | N.D.           | N.D.           | N.D.      | N.D.     |
| L307A        | -1.16 ± 0.47   | -1.28 ± 0.31*  | -0.32 ± 0.56 | 0.93 ± 0.61 |

* Data were statistically significant at p < 0.05, one-way analysis of variance, and Dunnnett’s post test in comparison with wild type response.
L307A were among those residues that conferred the largest
differential effects on either signal bias (supplemental Fig. S1)
or magnitude of change to efficacy across pathways (Fig. 7). Of
the other mutants within ECL2, which did not alter GLP-1(7–36)-NH₂ affinity, only N304A that is near the apex of TM5
caued significant differential effects across signaling pathways.

These data are indicative of a novel and important role for ECL2
in conferring the distinct conformations that underlie pathway
selective signaling. Whether this occurs purely through effects
on the conformation of TM5 or whether additional interactions/of ECL2 residues with other loop regions are involved is
unclear.

FIGURE 7. Correlation plots of pathway efficacy (logₑ) of human GLP-1R ECL2 alanine mutants. Correlation plots of changes in pathway coupling efficacy (logₑ) of human GLP-1R ECL2 alanine mutants with respect to wild type receptor are shown. A, cAMP versus pERK1/2 for GLP-1(1–36)-NH₂; B, cAMP versus pERK1/2 for GLP-1(7–36)-NH₂; C, Ca²⁺, versus cAMP for GLP-1(7–36)-NH₂; D, Ca²⁺, versus cAMP for GLP-1(7–36)-NH₂ after exclusion of D293A and Y305A; and E, pERK1/2 versus Ca²⁺ for GLP-1(7–36)-NH₂. Data were fit by linear regression. The line of regression and 99% confidence intervals are displayed.
GLP-1R ECL2 Is Critical for Receptor Activation

The two GLP-1 peptides used in this study differ only by a six-amino acid extension to the GLP-1(7–36)-NH₂ sequence. Not surprisingly, most mutations had similar effects on responses to both peptides. Nonetheless, GLP-1(1–36)-NH₂ exhibits distinct signal bias to that demonstrated by GLP-1(7–36)-NH₂, indicating that it produces a different ensemble of receptor conformations to that of the truncated peptide. Consistent with the effects of the different peptides in altering receptor bias, C296A, R299A, N302A, N304A, and Y305A had differential effects on the truncated and full-length GLP-1 peptides. Differential effects were also seen with the G285A mutant. Both peptides displayed increased efficacy at this mutant receptor; however, greater improvement in pEKR1/2 efficacy was seen for GLP-1(1–36)-NH₂ relative to GLP-1(7–36)-NH₂. Alanine at this position may thus provide some conformational restriction that allows the lower affinity full-length peptide to more readily activate the pEKR1/2 pathway compared with the wild-type receptor.

In conclusion, we have demonstrated a critical role for ECL2 in activation of the GLP-1R. However, the precise molecular mechanisms driving these effects remain elusive in the absence of high resolution structural information for family B GPCRs. Our data point to a major role for ECL2 in activation transition and that changes to structure in this receptor region can alter pathway bias of the receptor that can be manifested in a ligand-specific manner.

REFERENCES
1. Harmar, A. J., Hills, R. A., Rosser, E. M., Jones, M., Buneman, O. P., Dunbar, D. R., Greenhill, S. D., Hale, V. A., Sharman, J. L., Bonner, T. I., Catterall, W. A., Davenport, A. P., Delargrange, P., Dollery, C. T., Foord, S. M., Gutman, G. A., Lauert, V., Neubig, R. R., Ohlstein, E. H., Olsen, R. W., Peters, J., Pin, J. P., Ruffolo, R. R., Sears, D. B., Wright, M. W., and Spedding, M. (2009) IUPHAR-DB. The IUPHAR database of G protein-coupled receptors and ion channels. Nucleic Acids Res. 37, D680–D685
2. Overington, J. P., Al-Lazikani, B., and Hopkins, A. L. (2006) How many drug targets are there? Nat. Rev. Drug Discov. 5, 993–996
3. Bazarsuren, A., Grauschopf, U., Wozny, M., Reusch, D., Hoffmann, E., Schaefer, W., Panzner, S., and Rudolph, R. (2002) In vitro folding, functional characterization, and disulfide pattern of the extracellular domain of human GLP-1 receptor. Biophys. Chem. 96, 305–318
4. Grauschopf, U., Lilie, H., Honold, K., Wozny, M., Reusch, D., Esswein, A., Schäfer, W., Rücknagel, K. P., and Rudolph, R. (2000) The N-terminal fragment of human parathyroid hormone receptor 1 constitutes a hormone binding domain and reveals a distinct disulfide pattern. Biochemistry 39, 8878–8887
5. Bisello, A., Adams, A. E., Mierke, D. F., Pellegrini, M., Rosenblatt, M., Suva, L. J., and Cherev, M. (1998) Parathyroid hormone-receptor interactions identified directly by photocross-linking and molecular modeling studies. J. Biol. Chem. 273, 22498–22505
6. Dong, M., Li, Z., Pinon, D. I., Lybrand, T. P., and Miller, L. J. (2004) Spatial approximation between the amino terminus of a peptide agonist and the top of the sixth transmembrane segment of the secretin receptor. J. Biol. Chem. 279, 2894–2903
7. Dong, M., Pinon, D. I., Cox, R. F., and Miller, L. J. (2004) Molecular approximation between a residue in the amino-terminal region of calcitonin and the third extracellular loop of the class B G protein-coupled calcitonin receptor. J. Biol. Chem. 279, 31177–31182
8. Hoare, S. R. (2005) Mechanisms of peptide and nonpeptide ligand binding to Class B G protein-coupled receptors. Drug Discov. Today 10, 417–427
9. Bergwitz, C., Gardella, T. J., Flannery, M. R., Potts, J. T., Jr., Kronenberg, H. M., Goldring, S. R., and Jüppner, H. (1996) Full activation of chimeric receptors by hybrids between parathyroid hormone and calcitonin. Evidence for a common pattern of ligand-receptor interaction. J. Biol. Chem. 271, 26469–26472
10. Graziano, M. P., Hey, P. J., and Strader, C. D. (1996) The amino-terminal domain of the glucagon-like peptide-1 receptor is a critical determinant of subtype specificity. Receptors Channels 4, 9–17
11. Holtmann, M. H., Hadac, E. M., and Miller, L. J. (1995) Critical contributions of amino-terminal extracellular domains in agonist binding and activation of secretin and vasoactive intestinal polypeptide receptors. Studies of chimeric receptors. J. Biol. Chem. 270, 14394–14398
12. Wälmen, A., Göke, B., and Göke, R. (1996) The isolated N-terminal extracellular domain of the glucagon-like peptide-1 (GLP-1) receptor has intrinsic binding activity. FEBS Lett. 398, 43–47
13. Wälmen, A., Van Eyll, B., Göke, B., and Göke, R. (1997) Five out of six tryptophan residues in the N-terminal extracellular domain of the rat GLP-1 receptor are essential for its ability to bind GLP-1. Peptides 18, 301–305
14. Gelling, R. W., Wheeler, M. B., Xue, J., Gromorey, S., Nian, C., Pederson, R. A., and McIntosh, C. H. (1997) Localization of the domains involved in ligand binding and activation of the glucagon-dependent insulinotropic polypeptide receptor. Endocrinology 138, 2640–2643
15. Castro, M., Nikolaev, V. O., Palm, D., Lohse, M. J., and Vilardejo, J. P. (2005) Turn-on switch in parathyroid hormone receptor by a two-step parathyroid hormone binding mechanism. Proc. Natl. Acad. Sci. U.S.A. 102, 16084–16089
16. Bergwitz, C., Jusseaume, S. A., Luck, M. D., Jüppner, H., and Gardella, T. J. (1997) Residues in the membrane-spanning and extracellular loop regions of the parathyroid hormone (PTH)-2 receptor determine signaling selectivity for PTH and PTH-related peptide. J. Biol. Chem. 272, 28861–28868
17. Hjorth, S. A., Adelhorst, K., Pedersen, B. B., Kirk, O., and Schwartz, T. W. (1994) Glucagon and glucagon-like peptide 1. Selective receptor recognition via distinct peptide epitopes. J. Biol. Chem. 269, 30121–30124
18. Holtmann, M. H., Ganguli, S., Hadac, E. M., Dolu, V., and Miller, L. J. (1996) Multiple extracellular loop domains contribute critical determinants for agonist binding and activation of the secretin receptor. J. Biol. Chem. 271, 14944–14949
19. Buggy, J. J., Livingston, J. N., Rabin, D. U., and Yoo-Warren, H. (1995) Glucagon-glucagon-like peptide 1 receptor chimeras reveal domains that determine specificity of glucagon binding. J. Biol. Chem. 270, 7474–7478
20. Xiao, Q., Jeng, W., and Wheeler, M. B. (2000) Characterization of glucagon-like peptide-1 receptor-binding determinants. J. Mol. Endocrinol. 25, 321–335
21. Adelhorst, K., Hedegaard, B. B., Knudsen, L. B., and Kirk, O. (1994) Structure-activity studies of glucagon-like peptide-1. J. Biol. Chem. 269, 6275–6278
22. Mojsos, S. (1992) Structural requirements for biological activity of glucagon-like peptide-1. Int. J. Pept. Protein Res. 40, 333–343
23. Nicole, P., Lins, L., Rouyer-Fessard, C., Drouet, C., Fulcrand, P., Thomas, A., Couvigneau, A., Martinez, J., Brasseur, R., and Laburde, M. (2000) Identification of key residues for interaction of vasoactive intestinal peptide with human VPAC1 and VPAC2 receptors and development of a highly selective VPAC1 receptor agonist. Alamine scanning and molecular modeling of the peptide. J. Biol. Chem. 275, 24003–24012
24. Dong, M., Le, A., Te, J. A., Pinon, D. I., Bordin, A. J., and Miller, L. J. (2011) Importance of each residue within secretin for receptor binding and biological activity. Biochemistry 50, 2983–2993
25. Runge, S., Wulf, B. S., Madsen, K., Bräuner-Osborne, H., and Knudsen, L. B. (2003) Different domains of the glucagon and glucagon-like peptide-1 receptors provide the critical determinants of ligand selectivity. Br. J. Pharmacol. 138, 778–794
26. Runge, S., Gram, C., Bräuner-Osborne, H., Madsen, K., Knudsen, L. B., and Wulf, B. S. (2003) Three distinct epitopes on the extracellular face of the glucagon receptor determine specificity for the glucagon amino terminus. J. Biol. Chem. 278, 28005–28010
27. Partther, C., Kleinschmidt, M., Neumann, P., Rudolph, R., Manhart, S., Schlenzig, D., Fanghanel, J., Rahfeld, J. U., Demuth, H. U., and Stubbs, M. T. (2003) Crystal structure of the incretin-bound extracellular domain of a G protein-coupled receptor. Proc. Natl. Acad. Sci. U.S.A. 104, 13942–13947
GLP-1R ECL2 Is Critical for Receptor Activation

28. Grace, C. R., Perrin, M. H., DiGruccio, M. R., Miller, C. L., Rivier, J. E., Vale, W. W., and Riek, R. (2004) NMR structure and peptide hormone-binding site of the first extracellular domain of a type B1 G protein-coupled receptor. Proc. Natl. Acad. Sci. U.S.A. 101, 12836–12841.

29. Grace, C. R., Perrin, M. H., Gulyas, J., Digruccio, M. R., Cantle, J. P., Rivier, J. E., Vale, W. W., and Riek, R. (2007) Structure of the N-terminal domain of a type B1 G protein-coupled receptor in complex with a peptide ligand. Proc. Natl. Acad. Sci. U.S.A. 104, 4858–4863.

30. Pioszak, A. A., Parker, N. R., Suino-Powell, K., and Xu, H. E. (2008) Molecular recognition of corticotropin-releasing factor by its G protein-coupled receptor CRFR1. J. Biol. Chem. 283, 32900–32912.

31. Pioszak, A. A., and Xu, H. E. (2008) Molecular recognition of parathyroid hormone by its G protein-coupled receptor. Proc. Natl. Acad. Sci. U.S.A. 105, 5034–5039.

32. Runge, S., Thøgersen, H., Madsen, K., Lau, J., and Rudolph, R. (2008) Crystal structure of the ligand-bound glucagon-like peptide-1 receptor extracellular domain. J. Biol. Chem. 283, 11340–11347.

33. Underwood, C. R., Garibay, P., Knudsen, L. B., Hastrup, S., Peters, G. H., Rudolph, R., and Reedtz-Runge, S. (2010) Crystal structure of glucagon-like peptide-1 complex with the extracellular domain of the glucagon-like peptide-1 receptor. J. Biol. Chem. 285, 723–730.

34. Lopez de Maturana, R., and Donnelly, D. (2002) The glucagon-like peptide-1 receptor binding site for the N-terminus of GLP-1 requires polarity at Asp 198 rather than negative charge. FEBS Lett. 530, 244–248.

35. Lopez de Maturana, R., Treece-Birch, J., Abidi, F., Findlay, J. B., and Donnelly, D. (2004) Met-204 and Tyr-205 are together important for binding GLP-1 receptor agonists but not their N-terminally truncated analogues. Protein Pept. Lett. 11, 15–22.

36. Miller, L. J., Chen, Q., Lam, P. C., Pinon, D. I., Sexton, P. M., Abagyan, R., and Dong, M. (2011) Refinement of glucagon-like peptide 1 docking to its intact receptor using mid-region photolabile probes and molecular modeling. J. Biol. Chem. 286, 15895–15907.

37. Al-Sabah, S., and Donnelly, D. (2003) The positive charge at Lys-288 of the glucagon-like peptide-1 (GLP-1) receptor is important for binding the N-terminus of peptide agonists. FEBS Lett. 553, 342–346.

38. Assil-Kishawi, I., and Abou-Samra, M. A. (2002) Sauvagine cross-links to the second extracellular loop of the corticotropin-releasing factor type 1 receptor. J. Biol. Chem. 277, 32558–32561.

39. Liaw, C. W., Grigorieidis, D. E., Lovenberg, T. W., De Souza, E. B., and Maki, R. A. (1997) Localization of ligand-binding domains of human corticotropin-releasing factor receptor. A chimeric receptor approach. Mol. Endocrinol. 11, 980–985.

40. Baggio, L. L., and Drucker, D. J. (2007) Biology of incretins. GLP-1 and GIP. Gastroenterology 132, 2131–2157.

41. Quoyer, J., Longuet, C., Broca, C., Linck, N., Costes, S., Varin, E., Bockaert, J., Bertrand, G., and Dalle, S. (2010) GLP-1 mediates antiapoptotic effect of salbutamol by phosphorylating Bad through a BH3-only domain of the BH3-interacting-domain-death-protein. Proc. Natl. Acad. Sci. U.S.A. 107, 15895–15907.

42. DeFea, K. A., Zalesky, J., Thoma, M. S., Déry, O., Mullins, R. D., and Bunnett, N. W. (2000) β-Arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. J. Cell Biol. 148, 1267–1281.

43. Sheny, S. K., Drake, M. T., Nelson, C. D., Houtz, D. A., Xiao, K., Mabushhi, S., Reiter, E., Premont, R. T., Lichtarge, O., and Lefkowitz, R. J. (2006) β-Arrestin-dependent, G protein-independent ERK1/2 activation by the β2-adrenergic receptor. J. Biol. Chem. 281, 1261–1273.

44. Pearson, G., Robinson, F., Beers Gibson, T., Xu, B. E., Karandikar, M., Berman, K., and Cobb, M. H. (2001) Mitogen-activated protein (MAP) kinase pathways. Regulation and physiological functions. Endocr. Rev. 22, 153–183.

45. Warne, T., Serrano-Vega, M. J., Baker, J. G., Moukhmetzianov, R., Edwards, P. C., Henderson, R., Leslie, A. G., Tate, C. G., and Schertler, G. F. (2005) Crystal structure of a β2-adrenergic G-protein-coupled receptor. Nature 434, 486–491.

46. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Crystal structure of rhodopsin. A G protein-coupled receptor. Science 289, 739–745.

47. Cherezov, V., Rosenbaum, D. M., Hanson, M. A., Rasmussen, S. G., Thian, F. S., Kobilka, T. S., Choi, H. I., Kuhn, P., Weiss, W. I., Kobilka, B. K., and Stevens, R. C. (2007) High resolution crystal structure of an engineered human β2-adrenergic G-protein-coupled receptor. Science 318, 1258–1265.

48. Shien, Y. J., Liu, W., Zhao, Q., Katritch, V., Han, G. W., Hanson, M. A., Shi, L., Newman, A. H., Javitch, J. A., Cherezov, V., and Stevens, R. C. (2010) Structure of the human dopamine D3 receptor in complex with a D2/L3 selective antagonist. Science 330, 1091–1095.

49. Jakobs, V. P., Griffith, M. T., Hanson, M. A., Cherezov, V., Chien, E. Y., Lane, J. E., Ijzermans, A. P., and Stevens, R. C. (2008) The 2.6 Å crystal structure of a human A2A adenosine receptor bound to an antagonist. Science 322, 1211–1217.

50. Ahn, K. H., Rettelovitz, A. C., Mierke, D. F., and Kendall, D. A. (2009) Dual role of the second extracellular loop of the cannabinoid receptor 1. Ligand binding and receptor localization. Mol. Pharmacol. 76, 833–842.

51. Avlani, V. A., Gregory, K. J., Morton, C. J., Parker, M. W., Sexton, P. M., and Christopoulos, A. (2007) Critical role for the second extracellular loop in the binding of both orthosteric and allosteric G protein-coupled receptor ligands. J. Biol. Chem. 282, 25677–25686.

52. Conner, M., Hawtin, S. R., Simms, J., Wootten, D., Lawson, Z., Conner, A. C., Parlow, R. A., and Wheatley, M. (2007) Systematic analysis of the entire second extracellular loop of the V(1a) vasopressin receptor. Key
residues, conserved throughout a G-protein-coupled receptor family, identified. J. Biol. Chem. 282, 17405–17412

65. Scarselli, M., Li, B., Kim, S. K., and Wess, J. (2007) Multiple residues in the second extracellular loop are critical for M3 muscarinic acetylcholine receptor activation. J. Biol. Chem. 282, 7385–7396

66. Shi, L., and Javitch, J. A. (2004) The second extracellular loop of the dopamine D2 receptor lines the binding-site crevice. Proc. Natl. Acad. Sci. U.S.A. 101, 440–445

67. Ahuja, S., Hornak, V., Yan, E. C., Goncalves, J. A., Syrett, N., Gnicek, M., Sakmar, T. P., Reeves, P. J., Smith, S. O., and Eilers, M. (2009) Helix movement is coupled to displacement of the second extracellular loop in rhodopsin activation. Nat. Struct. Mol. Biol. 16, 168–175

68. Abu All, S., Quitterer, U., Grigoriev, S., Hämmer, M., Haasemann, M., Jarnagin, K., and Müller-Esterl, W. (1996) Extracellular domains of the bradykinin B2 receptor involved in ligand binding and agonist sensing defined by anti-peptide antibodies. J. Biol. Chem. 271, 1748–1755

69. Lebesgue, D., Wallukat, G., Mijares, A., Granier, C., Argibay, J., and Hobebeke, J. (1998) An agonist-like monoclonal antibody against the human H2 receptor. Eur. J. Pharmacol. 348, 123–133

70. Ott, T. R., Troskie, B. E., Roeske, R. W., Illing, N., Flanagan, C. A., and Millar, R. P. (2002) Two mutations in extracellular loop 2 of the human GnRH receptor convert an antagonist to an agonist. Mol. Endocrinol. 16, 1079–1088

71. Rasmussen, S. G., Devree, B. T., Zou, Y., Kruse, A. C., Kobilka, T. S., Thian, F. S., Chae, H. J., Pardon, E., Calinski, D., Mathiesen, J. M., Shah, T. S., Lyons, J. A., Caffrey, M., Gellman, S. H., Steyaert, J., Skiniotis, G., Weis, W. I., Sunahara, R. K., and Kobilka, B. K. (2011) Crystal structure of the β2 adrenergic receptor-Gs protein complex. Nature 477, 549–555

72. Christopoulos, A., and Kenakin, T. (2002) G protein-coupled receptor allosterism and complexing. Pharmacol. Rev. 54, 323–374

73. Rosenbaum, D. M., Zhang, C., Lyons, J. A., Holl, R., Aragao, D., Arolwa, D. H., Rasmussen, S. G., Choi, H. J., Devree, B. T., Sunahara, R. K., Chae, P. S., Gellman, S. H., Dorr, R. O., Shaw, D. E., Weis, W. I., Caffrey, M., Gmeiner, P., and Kobilka, B. K. (2011) Structure and function of an irreversible agonist-β(2) adrenoceptor complex. Nature 469, 236–240

74. Rasmussen, S. G., Choi, H. J., Pardon, E., Casarosa, P., Chae, P. S., Devree, B. T., Rosenbaum, D. M., Thian, F. S., Kobilka, T. S., Schnapp, A., Konetzki, I., Sunahara, R. K., Gellman, S. H., Pautsch, A., Steyaert, J., Weis, W. I., and Kobilka, B. K. (2011) Structure of a nanobody-stabilized active state of the β(2) adrenoceptor. Nature 469, 175–180

75. Shimamura, T., Shirotishi, M., Weyand, S., Tsujimoto, H., Winter, G., Katritch, V., Abagyan, R., Cherezov, V., Liu, W., Han, G. W., Kobayashi, T., Stevens, R. C., and Iwata, S. (2011) Structure of the human histamine H1 receptor complex with doxepin. Nature 475, 65–70

76. Wu, B., Chien, E. Y., Mol, C. D., Fenalti, G., Liu, W., Katritch, V., Abagyan, R., Brooun, A., Wells, B., Bi, F. C., Hamel, D. J., Kuhn, P., Handel, T. M., Cherezov, V., and Stevens, R. C. (2010) Structures of the CXCR4 chemokine GPCR with small molecule and cyclic peptide antagonists. Science 330, 1066–1071

77. Mann, R. J., Al-Sabah, S., de Maturana, R. L., Sinfield, J. K., and Donnelly, D. (2010) Functional coupling of Cys-226 and Cys-296 in the glucagon-like peptide-1 (GLP-1) receptor indicates a disulfide bond that is close to the activation pocket. Peptides 31, 2289–2293

78. Montrose-Rafizadeh, C., Yang, H., Rodgers, B. D., Beday, A., Pritchette, L. A., and Eng, J. (1997) High potency antagonists of the pancreatic glucagon-like peptide-1 receptor. J. Biol. Chem. 272, 21201–21206

79. Mann, R. J., Nasr, N. E., Sinfield, J. K., Paci, E., and Donnelly, D. (2010) The major determinant of exendin-4/glucagon-like peptide 1 differential affinity at the rat glucagon-like peptide 1 receptor N-terminal domain is a hydrogen bond from SER-32 of exendin-4. Br. J. Pharmacol. 160, 1973–1984

80. Segrest, J. P., De Loof, H., Dohlman, J. G., Brouillette, C. G., and Anantharamia, G. M. (1990) Amphipathic helix motif. Classes and properties. Proteins 8, 103–117
Second Extracellular Loop of Human Glucagon-like Peptide-1 Receptor (GLP-1R) Has a Critical Role in GLP-1 Peptide Binding and Receptor Activation
Cassandra Koole, Denise Wootten, John Simms, Laurence J. Miller, Arthur Christopoulos and Patrick M. Sexton

J. Biol. Chem. 2012, 287:3642-3658.
doi: 10.1074/jbc.M111.309328 originally published online December 6, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M111.309328

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2011/12/06/M111.309328.DC1

This article cites 80 references, 47 of which can be accessed free at
http://www.jbc.org/content/287/6/3642.full.html#ref-list-1