Polymorphism and Ligand Dependent Changes in Human Glucagon-Like Peptide-1 Receptor (GLP-1R) Function: Allosteric Rescue of Loss of Function Mutation[S]

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ABSTRACT

The glucagon-like peptide-1 receptor (GLP-1R) is a key physiological regulator of insulin secretion and a major therapeutic target for the treatment of type II diabetes. However, regulation of GLP-1R function is complex with multiple endogenous peptides that interact with the receptor, including full-length (1–37) and truncated (7–37) forms of GLP-1 that can exist in an amidated form (GLP-1(1–36)NH$_2$ and GLP-1(7–36)NH$_2$) and the related peptide oxyntomodulin. In addition, the GLP-1R possesses exogenous agonists, including exendin-4, and the allosteric modulator, compound 2 (6,7-dichloro-2-methylsulfonyl-3-tert-butylaminoquinoxaline). The complexity of this ligand-receptor system is further increased by the presence of several single nucleotide polymorphisms (SNPs) that are distributed across the receptor. We have investigated 10 GLP-1R SNPs, which were characterized in three physiologically relevant signaling pathways (cAMP accumulation, extracellular signal-regulated kinase 1/2 phosphorylation, and intracellular Ca^{2+} mobilization); ligand binding and cell surface receptor expression were also determined. We demonstrate both ligand- and pathway-specific effects for multiple SNPs, with the most dramatic effect observed for the Met^{149} receptor variant. At the Met^{149} variant, there was selective loss of peptide-induced responses across all pathways examined, but preservation of response to the small molecule compound 2. In contrast, at the Cys^{333} variant, peptide responses were preserved but there was attenuated response to compound 2. Strikingly, the loss of peptide function at the Met^{149} receptor variant could be allosterically rescued by compound 2, providing proof-of-principle evidence that allosteric drugs could be used to treat patients with this loss of function variant.

Introduction

The glucagon-like peptide-1 receptor (GLP-1R) is a key target in the development of treatments for type II diabetes mellitus, with actions including glucose-dependent increases in insulin synthesis and release; decreases in β-cell apoptosis, body mass, and gastric emptying; and a decrease in peripheral resistance to insulin that address fundamental symptoms associated with the condition (DeFronzo, 1992;

Drucker, 2006). The GLP-1R is a family B peptide hormone G protein-coupled receptor primarily expressed in pancreatic β-cells that responds to at least four distinct endogenous GLP-1 variants: two full-length GLP-1 peptides [GLP-1(1–36)NH$_2$ and GLP-1(1–37)] and two truncated and more prominent circulating forms [GLP-1(7–36)NH$_2$ and GLP-1(7–37)] (Baggio and Drucker, 2007). In addition, the related endogenous peptide oxyntomodulin and exogenous mimetic peptide exendin-4 act at the GLP-1R to increase the biosynthesis and secretion of insulin, decrease β-cell apoptosis and decrease gastric emptying in a similar manner to the endogenous GLP-1 peptides (Jarrousse et al., 1984; Jarrousse et al., 1985; Göke et al., 1993). Although levels of GLP-1 are reduced in patients with type II diabetes mellitus, the retention of its insulinotropic properties at the GLP-1R make it one of the most promising ligand-receptor systems to target in the development of treatments for type II diabetes (Nauck et al.,

ABBREVIATIONS: GLP-1R, glucagon-like peptide-1 receptor; SNP, single nucleotide polymorphism; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; FBS, Fetal bovine serum; CHO, Chinese hamster ovary; BSA, bovine serum albumin; compound 2, 6,7-dichloro-2-methylsulfonyl-3-tert-butylaminoquinoxaline.

1993; Toft-Nielsen et al., 2001). Recent medical developments to target this system include the GLP-1 mimetic liraglutide (Knudsen et al., 2000; Elbrønd et al., 2002) and dipeptidyl peptidase IV inhibitors that prolong the plasma half-life of endogenous GLP-1R peptides (Deacon et al., 1995a,b). The latter compounds fail to achieve the weight loss seen with peptide therapeutics, whereas the peptides have significant potential for reduced patient compliance due to the requirement for subcutaneous administration. To overcome this, small orally active drugs that can augment GLP-1R signaling are continually being pursued. One example, compound 2 (6,7-dichloro-2-methylsulfonyl-3-*tert*-butyl-aminoquinoxaline; Novo Nordisk A/S, Bagsværd, Denmark), allosterically enhances peptide binding affinity and subsequently influences insulin secretion (Knudsen et al., 2007; Koole et al., 2010) and provides an exemplar for understanding allosteric modulation of the receptor. However, developing allosteric therapeutics for a pleiotropically coupled receptor with multiple endogenous ligands poses a significant challenge (Koole et al., 2010). In addition, the presence of naturally occurring nonsynonymous single nucleotide polymorphisms (SNPs), add a further element of complexity in the development of these drugs for therapeutic application. The presence of SNPs may be linked to the rate of onset of disease or effectiveness of receptor targeted treatments. Although SNPs have been characterized in great detail for many G protein-coupled receptors, there is limited knowledge on the effects of SNPs at the GLP-1R. Several GLP-1R SNPs have been assessed previously in vitro, although not explored in a wide range of functional outputs (Beinborn et al., 2005; Fortin et al., 2010) and at least one has been reported to have a loose association with type II diabetes mellitus (Tokuyama et al., 2004). A better understanding of the role of these polymorphisms in receptor function is therefore required, not only to gain an insight into the effects they have on receptor function but also to understand their possible association with the onset of disease or effectiveness of drug therapies.

In this study, we found ligand- and pathway-dependent alteration in signaling via select polymorphisms of the GLP-1R. Furthermore, we demonstrate that the major loss of GLP-1R function to peptide agonists at the Met[149] polymorphic receptor variant can be restored via allosteric modulation of the receptor, providing potential therapeutic paths to the treatment of diabetic patients carrying this SNP.

Materials and Methods

Materials. Dulbecco's modified Eagle's medium (DMEM), hygromycin-B, and Fluo-4 acetoxymethyl ester were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific (Melbourne, VIC, Australia). The QuikChange site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). AlphaScreen reagents, Bolton-Hunter reagent (^{125}I), and 384-well ProxiPlates were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). SureFire extracellular signal-regulated kinases 1 and 2 (ERK1/2) reagents were generously provided by TGR Biosciences (Adelaide, SA, Australia). Sigma*Fast* o-phenylenediamine dihydrochloride tablets and antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Compound 2 was generated according to a method published previously (Teng et al., 2007), to a purity of >95%, and compound integrity was confirmed by NMR. GLP-1 and GLP-1 peptide analogs were purchased from American Peptide Co., Inc. (Sunnyvale, CA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) or BDH Merck (Melbourne, VIC, Australia) and were of an analytical grade.

Receptor Mutagenesis. Natural variants of the human GLP-1R with supporting nucleotide sequences reported to exist in the population were identified using the SwissProt database (http://www.uniprot.org/uniprot/P43220) and in a previous clinical report (Tokuyama et al., 2004) (Table 1; Fig. 1A). Each of these variants were introduced into a double c-myc-labeled wild-type human GLP-1R in the pEF5/FRT/V5-DEST destination vector (Invitrogen) using oligonucleotides for site-directed mutagenesis from GeneWorks (HindMarsh, SA, Australia) (Supplemental Table 1) and the QuikChange site-directed mutagenesis kit (Stratagene). The integrity of the subsequent receptor clones were confirmed by cycle sequencing as described previously (May et al., 2007). In this study, the wild-type

TABLE 1
Human GLP-1R single nucleotide polymorphisms

Single nucleotide polymorphisms were generated for this study based on those identified in the Swissprot database (www.uniprot.org/uniprot/P43220) in association with those identified to exist in a cohort of normal and diabetic subjects in Tokuyama et al., 2004. Frequency data is reported from the Swissprot database.

Residue	Amino Acid	Nucleotide Substitution	Frequency of Nucleotide Substitution		NCBI Identification Number	Reference in Addition to SwissProt
			Homozygous	Heterozygous		
7	Pro	CCG	0.60	0.29	rs10305420	Tokuyama et al., 2004
	Leu	CTG	0.11			
20	Arg	AGG	0.99	0.01	rs10305421	
	Lys	AAG	Unknown			
44	Arg	CGC	0.99	0.01	rs2295006	Tokuyama et al., 2004
	His	CAC	Unknown			
131	Arg	CGA	0.92	0.08	rs3765467	Tokuyama et al., 2004
	Gln	CAA	Unknown			
149	Thr	ACG	Unknown	Unknown	112198	Tokuyama et al., 2004
	Met	ATG	Unknown			
168	Gly	GGC	0.76	0.2	rs6923761	
	Ser	AGC	0.04			
260	Phe	TTC or TTT	0.31[a]	0.56	rs1042044	Tokuyama et al., 2004
	Leu	TTA	0.13			
316	Ala	GCC	0.98	0.02	rs10305492	
	Thr	ACC	Unknown			
333	Ser	TCC	0.99	0.01	rs10305493	
	Cys	TGC	Unknown			
421	Arg	CGG	0.99	0.01	rs10305510	
	Gln	CAG	Unknown			

[a] No global information for TTT nucleotide variant.

GLP-1R was defined as the form of the receptor comprised of the following residues at the sites of polymorphic variation: Pro[7], Arg[20], Arg[44], Arg[131], Thr[149], Gly[168], Phe[260], Ala[316], Ser[333], and Arg[421].

Transfections and Cell Culture. Wild-type and polymorphic 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 hygromycin B as described previously (May et al., 2007). 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% CO_2.

Radioligand Binding Assay. FlpInCHO wild-type and polymorphic human GLP-1R cells were seeded at a density of 3 × 10^{4} cells/well into 96-well culture plates and incubated overnight at 37°C in 5% CO_2. Growth media was replaced with binding buffer [DMEM containing 25 mM HEPES and 0.1% (w/v) BSA] containing 0.5 nM

[125]I-exendin(9–39) (K_i for the wild-type receptor, 12.5 nM) and increasing concentrations of unlabeled ligand. Cells were then incubated overnight at 4°C, followed by three washes in ice-cold 1× PBS to remove unbound radioligand. Cells were then solubilized in 0.1 M NaOH, and radioactivity determined by γ-counting. For interaction studies, competition of ^{125}I-exendin(9–39) binding by each orthosteric agonist was performed in the presence of 3 μM compound 2, added simultaneously. For all experiments, nonspecific binding was defined by 1 μM exendin(9–39).

cAMP Accumulation Assay. FlpInCHO wild-type and polymorphic human GLP-1R cells were seeded at a density of 3 × 10^{4} cells/well into 96-well culture plates and incubated overnight at 37°C in 5% CO_2, and cAMP detection carried out as described previously (Koole et al., 2010). For interaction studies, increasing concentrations of peptide ligand and 3 μM compound 2 were added simultaneously, and cAMP accumulation measured after 30 min of cell
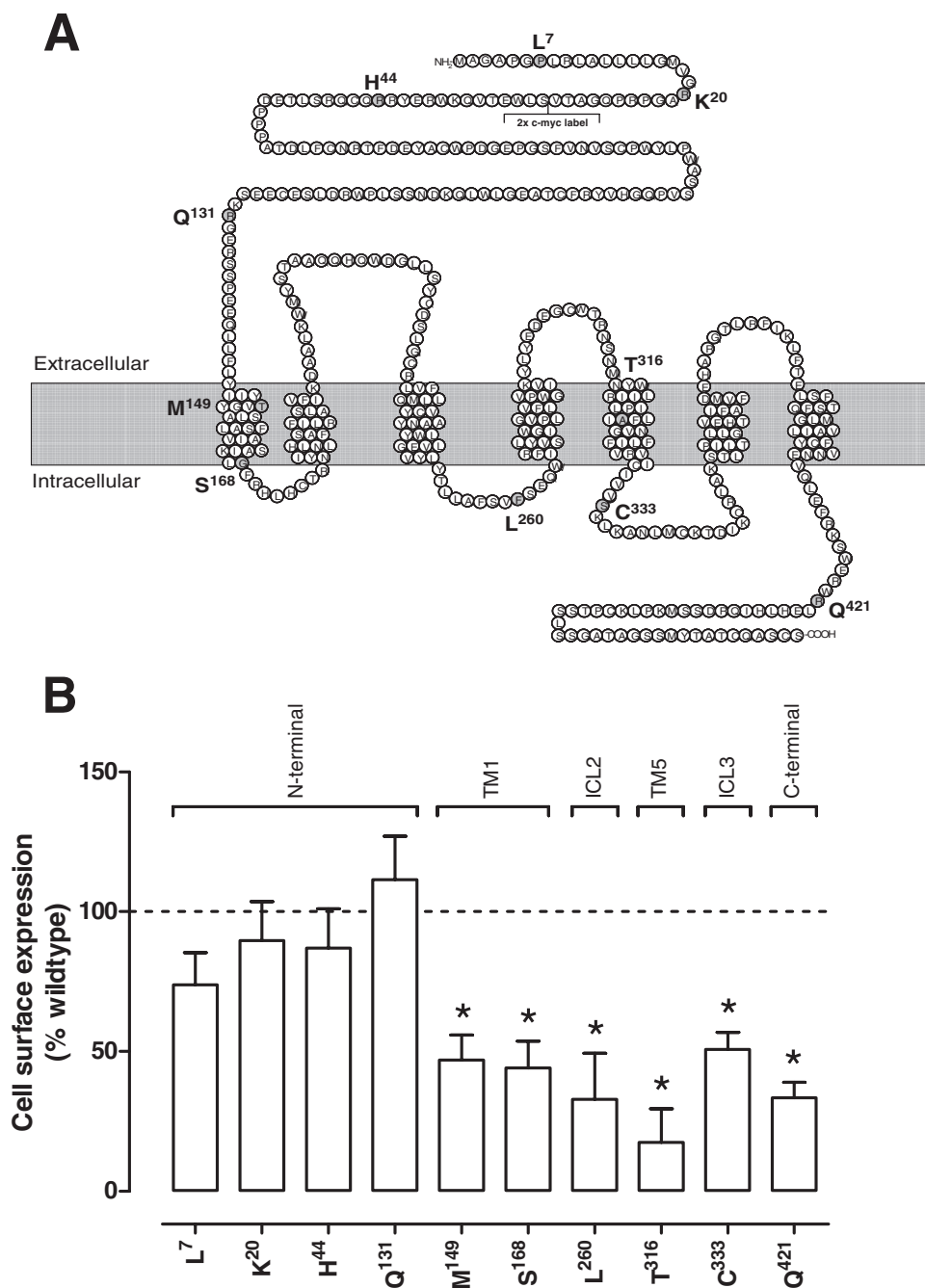
Fig. 1. A, schematic diagram of the human GLP-1R; location of residues subject to polymorphic variance is highlighted in gray, B, cell surface expression profiles of the human GLP-1R polymorphisms stably transfected into FlpInCHO cells as determined through antibody detection of the N-terminal c-*myc* epitope label. Statistical significance of changes in total cell surface expression compared with wild-type human GLP-1R expression (100%) were determined by one-way analysis of variance and Dunnett's post-test and are indicated with an asterisk (*, $p < 0.05$). All data are mean ± S.E.M. of five to seven independent experiments conducted in triplicate. TM, transmembrane; ICL, intracellular loop.

stimulation. 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 nM forskolin.

ERK1/2 Phosphorylation Assay. FlpInCHO wild-type and polymorphic human GLP-1R cells were seeded at a density of 3×10^4 cells/well into 96-well culture plates and incubated overnight at 37°C in 5% CO_2. Receptor-mediated ERK1/2 phosphorylation was determined by using the AlphaScreen ERK1/2 SureFire protocol as described previously (May et al., 2007). Initial ERK1/2 phosphorylation time-course experiments were performed over 1 h to determine the time at which ERK1/2 phosphorylation was maximal after stimulation by agonists. Subsequent experiments were then performed at the time required to generate a maximal ERK1/2 phosphorylation response (7 min). For interaction studies, increasing concentrations of peptide ligand and 3 μM compound 2 were added simultaneously, and ERK1/2 phosphorylation was measured after 7 min of cell stimulation. Data were normalized to the maximal response elicited by 10% FBS, determined at 7 min (peak FBS response).

Intracellular Ca^{2+} Mobilization Assay. FlpInCHO wild-type and polymorphic human GLP-1R cells were seeded at a density of 3×10^4 cells/well into 96-well culture plates and incubated overnight at 37°C in 5% CO_2, and receptor-mediated intracellular Ca^{2+} mobilization determined as described previously (Werry et al., 2005). 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 taken every 1.36 s for 120 s. For interaction studies, 3 μM compound 2 was added 30 min before peptide addition because of autofluorescence. Subsequent fluorescence was then determined immediately after peptide addition with the conditions detailed above. 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 polymorphic human GLP-1R cells, with receptor DNA previously N-terminally labeled with a double c-myc epitope label, were seeded at a density of 25×10^4 cells/well into 24-well culture plates and incubated overnight at 37°C in 5% CO_2, washed three times in 1× PBS and fixed with 3.7% paraformaldehyde at 4°C for 15 min. To determine the effects of compound 2 on cell surface receptor expression, adherent cells were treated for 4 or 18 h with 3 μM compound 2 and subsequently fixed as described above. Cell surface receptor detection was performed using a mouse monoclonal (9E10) primary antibody (1:2000) to detect the c-myc tag, and a mouse-raised IgG horseradish peroxidase-linked secondary antibody (1:2000), both diluted in blocking solution [1× PBS containing 2% (w/v) BSA and 0.05% (w/v) Tween 20]. Peroxidase activity was then measured using Sigma*Fast* o-phenylenediamine dihydrochloride tablets (Sigma) according to the manufacturer's instructions, and fluorescence detected at an emission wavelength of 492 nm. Data were normalized to the basal fluorescence detected in FlpInCHO parental cells.

Data Analysis. All data were analyzed in Prism 5.02 (GraphPad Software Inc., San Diego, CA). Concentration response signaling data were analyzed using a three-parameter logistic equation as described previously (May et al., 2007):

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{(\text{LogEC}_{50} - \log[A])}} \quad (1)$$

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 EC_{50} represents the molar concentration of ligand required to generate a response halfway between Top and Bottom. Likewise, this equation was used in the analysis of inhibition binding data, replacing EC_{50} with IC_{50}. In this case, Bottom defines the specific binding of the radioligand that is equivalent to nonspecific ligand binding, whereas Top defines radio-

ligand binding in the absence of a competing ligand, and the IC_{50} value represents the molar concentration of ligand required to generate a response halfway between Top and Bottom.

Statistics. Changes in peptide affinity, efficacy, potency, and cell surface expression of polymorphic variants compared with wild-type control were statistically analyzed with one-way analysis of variance and Dunnett's post test, and significance was accepted at $p < 0.05$. Differential modulation of oxyntomodulin potency by the allosteric ligand compound 2 at each of the polymorphic variants was assessed in comparison with an oxyntomodulin control using a paired t test, and statistical significance was accepted at $p < 0.05$.

Results

Cell Surface Expression of Human GLP-1R Polymorphic Variants. Each of the human GLP-1R polymorphisms was isogenically integrated into FlpInCHO host cells (Invitrogen) by recombination, allowing comparison of relative cell surface expression without complication from variation in gene transcription. In this study we observed an interesting trend in the cell surface expression profiles of the GLP-1R polymorphisms as assessed by antibody labeling of the N-terminally incorporated c-*myc* tag (Fig. 1B). Although the polymorphisms occurring in the N-terminal domain of the receptor seemed to have cell surface expression similar to that of the wild-type human GLP-1R, most other polymorphic variants distributed across the receptor had reduced cell surface expression, with greatest effect seen for the Thr[316] variant.

The Human GLP-1R Met[149] Polymorphic Variant Displays a Reduction in Orthosteric Agonist Affinity. To establish the binding profiles at each of the reported human GLP-1R polymorphisms, we performed equilibrium binding studies with the orthosteric GLP-1R agonists or the allosteric agonist compound 2, in competition with the radiolabeled orthosteric antagonist ^{125}I-exendin(9–39). There was no significant influence of the polymorphisms on orthosteric agonist potency for inhibition of the radiolabel, except for the Met[149] receptor variant, which had reductions in the affinities of GLP-1(7–36)NH_2, exendin-4, oxyntomodulin (Fig. 2; Table 2), and GLP-1(7–37) (data not shown)[1]. It is noteworthy that the extent of apparent affinity reduction differed between agonists, with a 251-fold shift for GLP-1(7–36)NH_2 and oxyntomodulin (Table 2) but only a 32-fold shift for exendin-4 (Table 2). Minimal effects were observed on GLP-1(1–36)NH_2, compound 2 (Fig. 2), and GLP-1(1–37) (data not shown)[2] binding within the concentration range measured, suggesting the affinity of these agonists is not greatly affected in any of the human GLP-1R variants compared with wild-type.

The Human GLP-1R Met[149] Polymorphic Variant Displays Reduced Orthosteric but Not Allosteric Agonist Potency in cAMP Accumulation. The cAMP accumulation profile of all orthosterically binding peptides at each of the GLP-1R polymorphic variants was similar to that of wild-type human GLP-1R, with the exception of the Met[149] polymorphism, where each peptide exhibited reduced potency, with observed decreases in potency of 158-fold for

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[1] The response to GLP1(7–36)NH_2 and GLP-1(7–37) are effectively equivalent; therefore, only a limited analysis was performed for the GLP-1(7–37) peptide with data limited to one or two experiments only.

[2] The response to GLP1(1–36)NH_2 and GLP-1(1–37) are effectively equivalent, therefore, only a limited analysis was performed for the GLP-1(1–37) peptide with data limited to one or two experiments only.

GLP-1(7–36)NH$_2$, 200-fold for exendin-4, and >500-fold for oxyntomodulin (Fig. 3; Table 3). Full concentration-response relationships for the lower potency agonists GLP-1(1–36)NH$_2$, GLP-1(1–37), oxyntomodulin, and compound 2 could not be determined; however, the effect appeared more profound for oxyntomodulin relative to the truncated GLP-1 peptides and exendin-4 (Fig. 3). In contrast, the potency and efficacy of the allosteric agonist compound 2 at the Met[149] polymorphic variant, was similar to that observed with the Thr[149] variant, with pEC$_{50}$ values of 5.5 ± 0.1 and 5.5 ± 0.1, respectively ($n = 4$) (Fig. 3; Table 3). However, at the Cys[333] polymorphism, where orthosteric peptide profiles were equivalent to those of wild-type, compound 2 displayed a reduced cAMP response (Fig. 3; Table 3).

Human GLP-1R Polymorphisms Have Variable Intracellular Ca^{2+} Mobilization Responses. In accord with previous studies (Koole et al., 2010), no Ca^{2+} response was seen for full-length GLP-1 peptides or compound 2 at the wild-type receptor, and this was true also for each of the polymorphic receptor variants (Fig. 4). In the case of oxyntomodulin, only a weak response was observed, and this was submaximal at 1 μM, even at the wild-type receptor. The weak response made interpretation of the effect of polymorphic variation difficult; however, a reduced response at 1 μM was seen for the Met[149] and Thr[316] variants (Fig. 4; Table 4).

No significant alteration in the potency of GLP-1(7–36)NH$_2$, GLP-1(7–37), or exendin-4 peptides was observed; however, marked reduction in peptide efficacy was found for selected receptor variants (Fig. 4; Table 4). For each of the higher affinity peptides, there was reduced efficacy at the Gln[131], Met[149], Ser[168], Thr[316], and Gln[421] variants, the greatest reduction in efficacy being seen at the Met[149] variant, which exhibited no measurable agonist-induced response (Fig. 4; Table 4). In addition, for exendin-4 only, there were reductions in efficacy at the Cys[333] and Lys[20] variants; for Lys[20], this occurred despite wild-type levels of cell surface

receptor expression. In systems with low receptor reserve, changes in receptor density can affect observed efficacy and potency. In many instances, there was evidence of reduced cell surface receptor expression, as assessed by antibody binding to the c-myc epitope (Fig. 1B), that probably contributed to the observed decrease in efficacy, because there is little reserve for coupling to this pathway. Intriguingly, the Leu[260] variant had essentially wild-type response to the peptides, despite significantly reduced cell surface receptor expression, suggesting that the Leu[260] substitution may actually favor coupling via Gα_q.

Effect of Receptor Polymorphisms on ERK1/2 Phosphorylation. Agonist-induced ERK1/2 phosphorylation was determined at 7 min for each of the human GLP-1R polymor-

TABLE 2
Effects of naturally occurring human GLP-1R polymorphisms on agonist binding

Data were analyzed using a three-parameter logistic equation as defined in eq. 1. pIC$_{50}$ values represent the negative logarithm of the concentration of agonist that inhibits binding of half the total concentration of radiolabeled antagonist ^{125}I-exendin(9–39). Data are normalized to maximum ^{125}I-exendin(9–39) binding of each individual data set, with nonspecific binding measured in the presence of 1 μM exendin(9–39). All values are mean ± S.E.M. of three to four independent experiments, conducted in duplicate. Data were analyzed with one-way analysis of variance and Dunnett's post test.

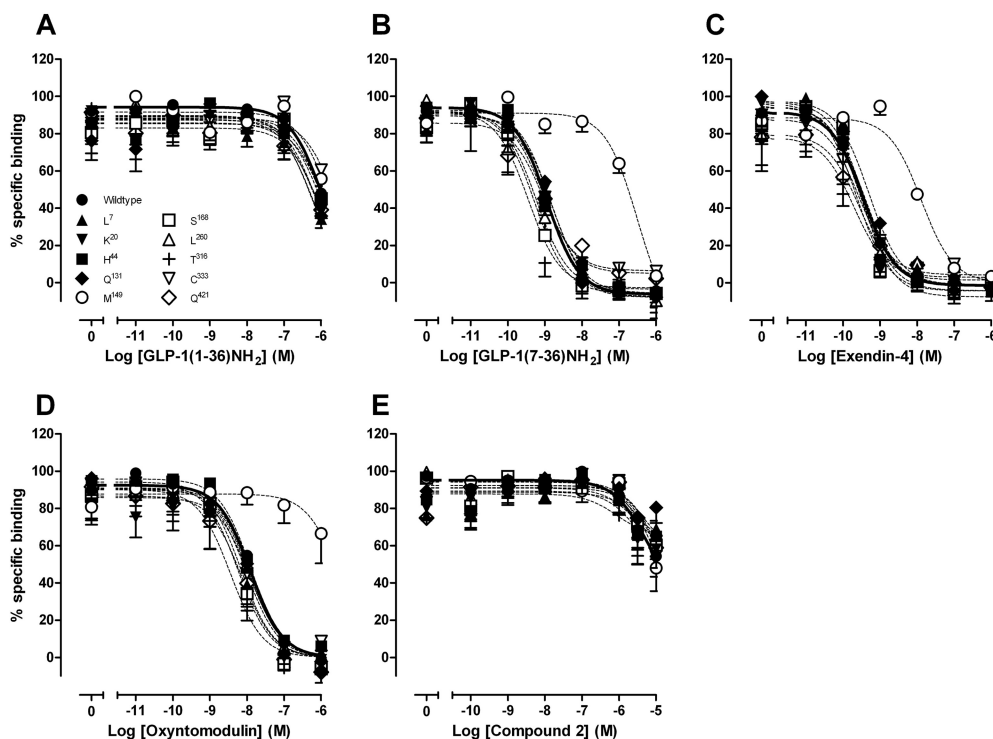|	pIC$_{50}$		
	GLP-1(7–36)NH$_2$	Exendin-4	Oxyntomodulin
Wild type	8.9 ± 0.1	9.4 ± 0.1	7.9 ± 0.1
Leu[7]	8.9 ± 0.2	9.5 ± 0.1	8.2 ± 0.1
Lys[20]	8.8 ± 0.1	9.6 ± 0.1	7.9 ± 0.2
His[44]	8.9 ± 0.1	9.5 ± 0.1	7.9 ± 0.1
Gln[131]	8.8 ± 0.1	9.3 ± 0.1	7.9 ± 0.1
Met[149]	6.5 ± 0.2*	7.9 ± 0.2*	5.5 ± 0.2*
Ser[168]	9.3 ± 0.1	9.5 ± 0.1	8.2 ± 0.1
Leu[260]	9.2 ± 0.2	9.4 ± 0.2	7.9 ± 0.1
Thr[316]	9.5 ± 0.2	9.7 ± 0.2	8.4 ± 0.2
Cys[333]	9.0 ± 0.1	9.6 ± 0.1	8.0 ± 0.1
Gln[421]	9.1 ± 0.2	9.6 ± 0.2	8.1 ± 0.2

* Statistically significant at $p < 0.05$.

Fig. 2. Characterization of the binding of GLP-1(1–36)NH$_2$ (A), GLP-1(7–36)NH$_2$ (B), exendin-4 (C), oxyntomodulin (D), and compound 2 (E) in competition with the radiolabeled antagonist ^{125}I-exendin(9–39) in whole FlpInCHO cells stably expressing each of the human GLP-1R polymorphisms or the wild-type GLP-1R. Data are normalized to the maximum ^{125}I-exendin(9–39) binding of each individual data set, with nonspecific binding measured in the presence of 1 μM exendin(9–39). Data are analyzed with a three-parameter logistic equation as defined in eq. 1. All values are mean ± S.E.M. of three to four independent experiments conducted in duplicate.

phic variants. Most receptor variants exhibited ERK1/2 responses similar to those of peptide agonists or compound 2 at the wild-type GLP-1R with the exception of the Met[149] variant (Fig. 5, Table 5). There was a decrease in the potency and/or efficacy of all orthosteric peptides with the Met[149] polymorphic variant, but this was not as pronounced as in the other pathways, suggesting alteration to signal bias of the receptor. The allosteric ligand compound 2 retained its weak agonism at this variant, sharing an ERK1/2 phosphorylation profile similar to that of compound 2 at the wild-type human GLP-1R (Fig. 5; Table 5).

Effect of Receptor Polymorphisms on the Allosteric Modulation of the cAMP Response to Oxyntomodulin by Compound 2. We have demonstrated previously that compound 2 positively modulates binding affinity and, as a

result cAMP potency, of the endogenous peptide agonists oxyntomodulin and, to a lesser extent, GLP-1(7–36)NH$_2$ and GLP-1(7–37) (Koole et al., 2010). Oxyntomodulin was the most robustly enhanced peptide and was used to explore whether allosteric regulation of the peptide response was retained at the polymorphic receptor variants. Compound 2 significantly enhanced the oxyntomodulin response at the wild-type receptor, Lys[20], and His[44] receptor variants (Table 6). At the Cys[333] variant, which had attenuated cAMP agonism to compound 2, the allosteric modulator failed to enhance the oxyntomodulin response. It is noteworthy that despite retaining cAMP agonism upon activation with compound 2, the oxyntomodulin response at the Leu[7] and Thr[316] variants was not significantly modified by compound 2, and although there was a trend toward enhancement with the Gln[131], Ser[168],
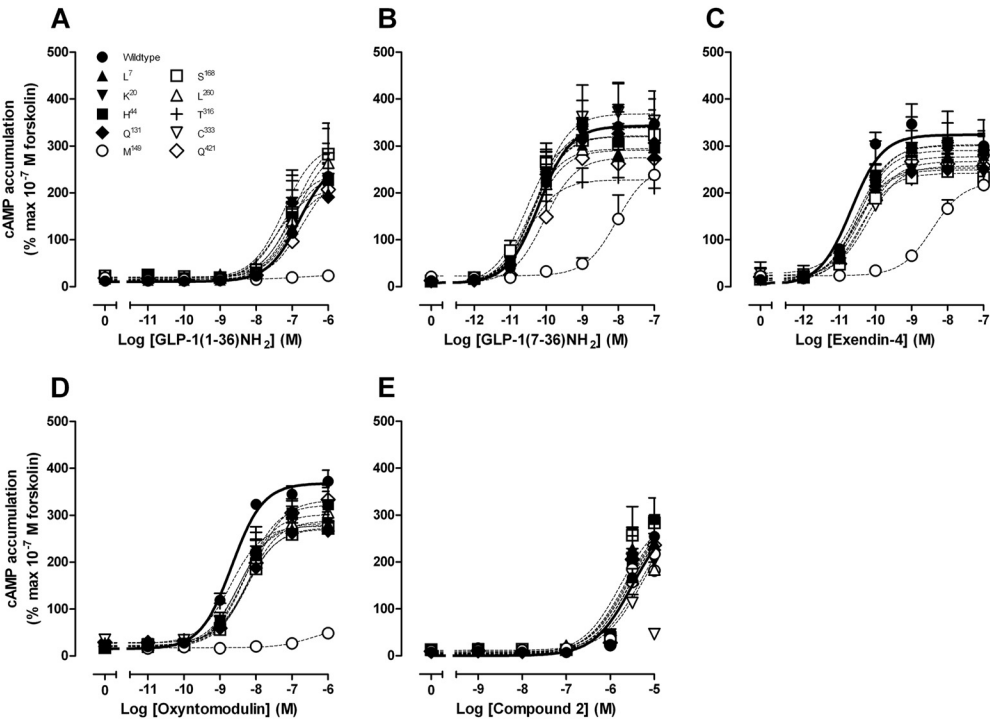
Fig. 3. Characterization of cAMP accumulation in the presence of GLP-1(1–36)NH$_2$ (A), GLP-1(7–36)NH$_2$ (B), exendin-4 (C), oxyntomodulin (D), and compound 2 (E) in FlpInCHO cells stably expressing each of the human GLP-1R polymorphisms or the wild-type GLP-1R. Data are normalized to the response elicited by 100 nM forskolin and analyzed with a three-parameter logistic equation as defined in eq. 1. All values are mean ± S.E.M. of 4 to 12 experiments conducted in duplicate.

TABLE 3
Effects of naturally occurring human GLP-1R polymorphisms on agonist signaling via cAMP
Data were analyzed using a three-parameter logistic equation as defined in eq. 1. pEC$_{50}$ values represent the negative logarithm of the concentration of agonist that produces half the maximal response. E_{max} represents the maximal response normalized to the response elicited by that of 100 nM forskolin. All values are mean ± S.E.M. of four to ten independent experiments, conducted in duplicate. Data were analyzed with one-way analysis of variance and Dunnett's post test.

	cAMP Accumulation									
	pEC$_{50}$					E_{max}				
	GLP-1 (1–36)NH$_2$	GLP-1 (7–36)NH$_2$	Exendin-4	Oxyntomodulin	Compound 2[a]	GLP-1 (1–36)NH$_2$	GLP-1 (7–36)NH$_2$	Exendin-4	Oxyntomodulin	Compound 2[b]
Wild type	6.8 ± 0.2	10.2 ± 0.2	10.7 ± 0.1	8.7 ± 0.1	5.5 ± 0.1	270 ± 36	343 ± 21	299 ± 9	368 ± 9	254 ± 44
Leu[7]	7.1 ± 0.2	10.5 ± 0.2	10.4 ± 0.2	8.5 ± 0.1	5.6 ± 0.1	217 ± 18	294 ± 18	277 ± 14	283 ± 13*	242 ± 50
Lys[20]	7.3 ± 0.2	10.3 ± 0.2	10.6 ± 0.2	8.5 ± 0.2	5.6 ± 0.1	244 ± 26	339 ± 19	249 ± 12	279 ± 18*	200 ± 26
His[44]	7.1 ± 0.2	10.4 ± 0.2	10.4 ± 0.2	8.3 ± 0.1	5.7 ± 0.1	244 ± 27	321 ± 18	302 ± 16	322 ± 17	291 ± 46
Gln[131]	7.4 ± 0.3	10.4 ± 0.2	10.6 ± 0.2	8.3 ± 0.2	5.6 ± 0.1	209 ± 31	322 ± 21	253 ± 15	271 ± 14*	225 ± 35
Met[149]	N.D.	8.0 ± 0.3*	8.4 ± 0.1*	N.D.	5.5 ± 0.1	N.D.	N.D.	223 ± 13	N.D.	217 ± 24
Ser[168]	6.9 ± 0.2	10.5 ± 0.2	10.4 ± 0.2	8.3 ± 0.2	5.8 ± 0.1	314 ± 33	320 ± 17	242 ± 11	273 ± 14*	283 ± 13
Leu[260]	6.9 ± 0.2	10.4 ± 0.3	10.5 ± 0.2	8.4 ± 0.1	5.3 ± 0.1	297 ± 28	291 ± 21	290 ± 13	302 ± 12	184 ± 30
Thr[316]	7.2 ± 0.2	10.5 ± 0.3	10.5 ± 0.4	8.7 ± 0.3	5.3 ± 0.1	304 ± 33	228 ± 16*	301 ± 30	277 ± 22*	182 ± 18
Cys[333]	6.9 ± 0.2	10.2 ± 0.3	10.2 ± 0.2	8.2 ± 0.2	N.D.	259 ± 24	331 ± 26	257 ± 12	288 ± 16	46 ± 8*
Gln[421]	6.7 ± 0.2	10.1 ± 0.2	10.3 ± 0.2	8.1 ± 0.1	5.6 ± 0.1	242 ± 23	275 ± 15	267 ± 13	332 ± 15	236 ± 45

N.D., data unable to be experimentally defined or with incomplete curves.
[a] For estimation of pEC$_{50}$ values, E_{max} was set to ≤300% of the 100 nM forskolin response.
[b] Compound 2 response at 10^{-5} M.
* Statistically significant at $p < 0.05$.

Leu[260], and Gln[421] variants, this was attenuated relative to the wild-type receptor.

Allosteric Rescue of GLP-1R Function at the Met[149] Polymorphic Variant. Compound 2 exhibits differential modulation of individual peptide agonists at the "wild-type" GLP-1R, a behavior termed "probe dependence" (Keov et al., 2011), with greatest modulation of the oxyntomodulin response relative to that of the more potent peptides, GLP-1(7–36)NH_2, GLP-1(7–37), and exendin-4 and indeed has minimal effect on exendin-4-mediated cAMP responses (Koole et al., 2010). Remarkably, compound 2 rescued the binding and

cAMP signal of most peptide agonists at the Met[149] receptor variant (excluding full-length GLP-1 peptides) (Figs. 6 and 7; Tables 7 and 8), with binding affinity and potency of truncated GLP-1 and exendin-4 recovered to within 8-fold of the wild-type receptor (Figs. 6 and 7; Tables 7 and 8). Because agonist functional potency is a composite of efficacy and affinity, this parameter can be influenced by receptor expression and receptor reserve. Consequently, we assessed the role of compound 2 on cell surface expression of the Met[149] variant of the receptor. Compound 2 had no significant effect on the level of cell surface expression of Met[149] over the time
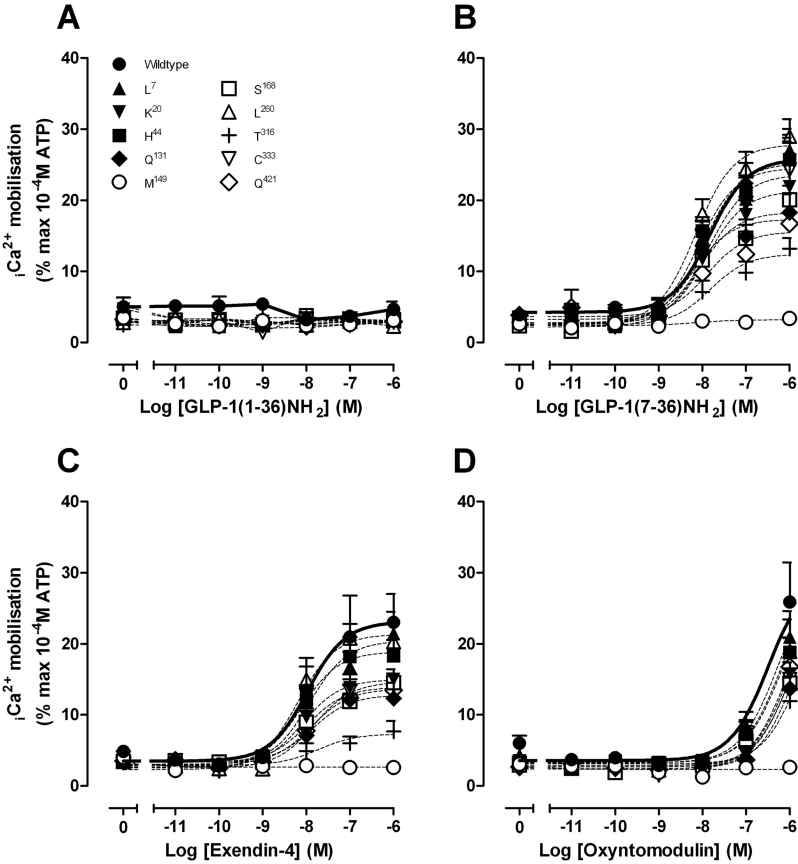

Fig. 4. Characterization of intracellular Ca^{2+} mobilization in the presence of GLP-1(1–36)NH_2 (A), GLP-1(7–36)NH_2 (B), exendin-4 (C), and oxyntomodulin (D) in FlpInCHO cells stably expressing each of the human GLP-1R polymorphisms or the wild-type GLP-1R. Data are normalized to the maximal response elicited by 100 μM ATP and analyzed with a three-parameter logistic equation as defined in eq. 1. All values are mean $\pm$ S.E.M. of five to nine experiments conducted in duplicate.

TABLE 4

Effects of naturally occurring human GLP-1R polymorphisms on agonist signaling via intracellular Ca^{2+} mobilization

Data were analyzed using a three-parameter logistic equation as defined in eq. 1. pEC_{50} values represent the negative logarithm of the concentration of agonist that produces half the maximal response. E_{max} represents the maximal response normalized to the response elicited by that of 10^{-4} M ATP. All values are mean $\pm$ S.E.M. of 5 to 10 independent experiments, conducted in duplicate. Data were analyzed with one-way analysis of variance and Dunnett's post test.

	Intracellular Ca^{2+} Mobilization					
	pEC_{50}			E_{max}		
	GLP-1(7–36)NH_2	Exendin-4	Oxyntomodulin	GLP-1(7–36)NH_2	Exendin-4	Oxyntomodulin[a]
Wild type	7.8 $\pm$ 0.2	8.0 $\pm$ 0.3	N.D.	25.8 $\pm$ 1.5	23.1 $\pm$ 2.5	25.9 $\pm$ 5.5
Leu[7]	8.0 $\pm$ 0.2	7.9 $\pm$ 0.2	N.D.	25.2 $\pm$ 1.4	20.4 $\pm$ 1.4	21.0 $\pm$ 2.5
Lys[20]	7.9 $\pm$ 0.2	8.1 $\pm$ 0.1	N.D.	21.3 $\pm$ 1.4	15.0 $\pm$ 0.7*	15.7 $\pm$ 1.6
His[44]	8.1 $\pm$ 0.2	8.1 $\pm$ 0.2	N.D.	24.5 $\pm$ 1.4	18.9 $\pm$ 1.3	18.8 $\pm$ 2.1
Gln[131]	8.2 $\pm$ 0.2	7.9 $\pm$ 0.3	N.D.	17.3 $\pm$ 1.4*	12.8 $\pm$ 1.1*	13.7 $\pm$ 1.5
Met[149]	N.D.	N.D.	N.D.	N.D.	N.D.	2.6 $\pm$ 0.4*
Ser[168]	8.1 $\pm$ 0.2	8.0 $\pm$ 0.2	N.D.	18.3 $\pm$ 1.0*	13.9 $\pm$ 0.8*	14.4 $\pm$ 0.9
Leu[260]	8.2 $\pm$ 0.1	8.2 $\pm$ 0.1	N.D.	27.8 $\pm$ 1.2	21.3 $\pm$ 1.0	19.0 $\pm$ 1.9
Thr[316]	7.8 $\pm$ 0.2	7.7 $\pm$ 0.5	N.D.	12.4 $\pm$ 1.0*	7.3 $\pm$ 0.9*	11.9 $\pm$ 1.9*
Cys[333]	7.8 $\pm$ 0.3	7.8 $\pm$ 0.2	N.D.	23.6 $\pm$ 2.4	14.6 $\pm$ 1.1*	15.0 $\pm$ 2.4
Gln[421]	7.9 $\pm$ 0.3	7.9 $\pm$ 0.3	N.D.	15.6 $\pm$ 1.7*	13.6 $\pm$ 1.3*	16.8 $\pm$ 7.8

N.D., data unable to be experimentally defined or with incomplete curves.

* Statistically significant at $p < 0.05$.

[a] Response at 10^{-6} M oxyntomodulin.

scale of the assay (data not shown). We also performed interaction studies between compound 2 and GLP-1(7–36)NH₂, oxyntomodulin, or exendin-4 for ERK1/2 phosphorylation and intracellular Ca²⁺ mobilization, but no modulation of these responses was observed at this receptor variant (data not shown). This indicates that compound 2 retains the pathway-specific modulation of response that we had previously observed at the wild-type human GLP-1R (Koole et al., 2010). No modulation of GLP-1(1–36)NH₂ at the Met[149] variant was observed (not shown).

Discussion

There is a paucity of information examining either the prevalence or influence of GLP-1R polymorphisms in, and on,

susceptibility to diseases such as type II diabetes and obesity. Indeed, only a single study identified the Met[149] polymorphism in a patient with type II diabetes (Tokuyama et al., 2004), and study of other polymorphisms in vitro has been very limited (Beinborn et al., 2005; Fortin et al., 2010). Greater understanding of the potential pharmacological impact of GLP-1R polymorphisms is thus required to drive clinical research in this area. In this study, we have identified major pharmacological differences in the signaling profile or allosteric modulation of multiple human GLP-1R polymorphic variants and, importantly, that the loss of function associated with the most detrimental substitution, Met[149], can be allosterically rescued with a small molecule modulator.

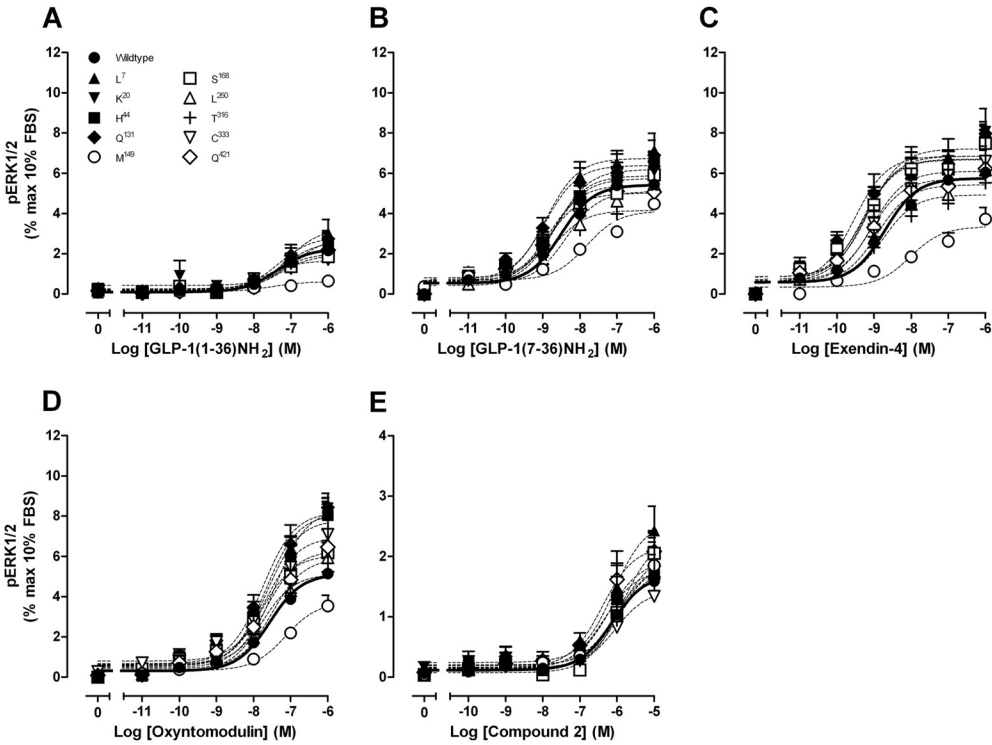The GLP-1R is pleiotropically coupled to signaling path-

Fig. 5. Characterization of ERK1/2 phosphorylation in the presence of GLP-1(1–36)NH₂ (A), GLP-1(7–36)NH₂ (B), exendin-4 (C), oxyntomodulin (D), and compound 2 (E), in FlpInCHO cells stably expressing each of the human GLP-1R polymorphisms or the wild-type GLP-1R. Data are normalized to the maximal response elicited by 10% FBS and analyzed with a three-parameter logistic equation as defined in eq. 1. All values are mean ± S.E.M. of four to five independent experiments conducted in duplicate.

TABLE 5
Effects of naturally occurring human GLP-1R polymorphisms on agonist signaling via ERK1/2 phosphorylation

Data were analyzed using a three-parameter logistic equation as defined in eq. 1. pEC_{50} values represent the negative logarithm of the concentration of agonist that produces half the maximal response. E_{max} represents the maximal response normalized to the response elicited by that of 10% FBS. All values are mean ± S.E.M. of four to five independent experiments, conducted in duplicate. Data were analyzed with one-way analysis of variance and Dunnett's post test.

	ERK1/2 Phosphorylation									
	pEC_{50}					E_{max}				
	GLP-1 (1–36)NH₂	GLP-1 (7–36)NH₂	Exendin-4	Oxyntomodulin	Compound 2	GLP-1 (1–36)NH₂	GLP-1 (7–36)NH₂	Exendin-4	Oxyntomodulin	Compound 2
Wild type	7.4 ± 0.4	8.5 ± 0.3	8.7 ± 0.2	7.6 ± 0.1	6.1 ± 0.3	2.3 ± 0.4	5.4 ± 0.4	6.6 ± 0.4	6.4 ± 0.3	1.7 ± 0.3
Leu[7]	7.1 ± 0.2	8.8 ± 0.2	9.2 ± 0.2	7.7 ± 0.1	6.1 ± 0.2	3.3 ± 0.4	7.1 ± 0.4	7.2 ± 0.4	8.1 ± 0.3	2.6 ± 0.3
Lys[20]	6.9 ± 0.2	8.7 ± 0.1	9.3 ± 0.2	7.4 ± 0.1	5.9 ± 0.2	2.8 ± 0.5	5.7 ± 0.2	6.9 ± 0.4	8.4 ± 0.4	1.9 ± 0.4
His[44]	7.2 ± 0.2	8.6 ± 0.2	9.3 ± 0.2	7.6 ± 0.2	6.3 ± 0.3	2.6 ± 0.3	6.2 ± 0.3	6.7 ± 0.4	7.8 ± 0.6	1.8 ± 0.2
Gln[131]	7.3 ± 0.2	8.9 ± 0.2	9.5 ± 0.2	7.8 ± 0.2	6.2 ± 0.3	2.8 ± 0.2	6.4 ± 0.3	6.9 ± 0.4	8.2 ± 0.5	1.9 ± 0.3
Met[149]	N.D.	7.8 ± 0.3	8.0 ± 0.2	7.1 ± 0.2	5.9 ± 0.3	0.6 ± 0.1	4.1 ± 0.5	3.4 ± 0.3*	3.7 ± 0.3*	2.0 ± 0.4
Ser[168]	7.3 ± 0.2	8.8 ± 0.2	9.3 ± 0.3	7.8 ± 0.1	5.9 ± 0.2	2.0 ± 0.2	5.4 ± 0.3	6.7 ± 0.5	6.1 ± 0.3	2.3 ± 0.3
Leu[260]	7.3 ± 0.4	8.4 ± 0.4	8.9 ± 0.4	7.5 ± 0.3	6.0 ± 0.3	2.2 ± 0.3	5.1 ± 0.6	5.4 ± 0.7	5.9 ± 0.7	1.9 ± 0.3
Thr[316]	7.3 ± 0.4	8.8 ± 0.3	8.8 ± 0.3	7.8 ± 0.3	6.0 ± 0.3	2.0 ± 0.3	4.2 ± 0.4	4.9 ± 0.5	5.1 ± 0.6	1.8 ± 0.3
Cys[333]	7.7 ± 0.2	8.7 ± 0.2	9.1 ± 0.3	7.7 ± 0.2	6.0 ± 0.2	1.7 ± 0.2	5.9 ± 0.4	6.1 ± 0.5	7.0 ± 0.5	1.4 ± 0.2
Gln[421]	7.2 ± 0.4	8.9 ± 0.3	9.1 ± 0.2	7.7 ± 0.4	6.1 ± 0.3	2.3 ± 0.4	5.0 ± 0.5	5.7 ± 0.4	6.3 ± 0.9	2.9 ± 0.4

N.D., data unable to be experimentally defined or with incomplete curves.
* Statistically significant at $p < 0.05$.

ways, and the physiological response to peptides represents a convergence of all pathways activated. In this study, we chose three well characterized second messenger pathways, each linked to physiological outcomes from the receptor, to assess the functional impact of the GLP-1R polymorphisms. Both cAMP production and intracellular Ca^{2+} mobilization are critical to the incretin response (Baggio and Drucker, 2007), whereas ERK1/2 signaling is involved in pancreatic β-cell growth and survival (Klinger et al., 2008; Quoyer et al., 2010). Furthermore, the GLP-1R can respond to multiple endogenous peptides, as well as mimetics such as exendin-4 that are used clinically (Göke et al., 1993), requiring detailed assessment to infer the impact of polymorphic receptor variance clinically. Our analysis has revealed major new findings on the pharmacology of GLP-1R polymorphisms that have implications both physiologically and for development of therapeutic interventions for treatment of patients carrying these receptor variants.

All polymorphic receptors were well expressed at the cell surface although total expression was reduced with individual variants, relative to "wild-type" receptor. The greatest effect was observed with the Thr[316] substitution, located at the second extracellular loop/transmembrane 5 interface. The majority of the polymorphic variants had minimal impact on receptor interaction with agonists and consequent signaling. However, with respect to intracellular Ca^{2+} mobilization, the loss in Ca^{2+} signaling was largely paralleled by a decrease in cell surface receptor expression, particularly for the Thr[316] variant, which exhibited the lowest level of cell surface expression and a weak Ca^{2+} response to each of the agonists. For this variant, there was also a parallel loss in efficacy of the low-potency peptide agonist oxyntomodulin. In the current study, receptor constructs were isogenically integrated into the host cell genome and thus the loss of cell surface receptor expression is probably linked to changes in protein stability or trafficking. Although the prevalence of the Thr[316] polymorphism is relatively rare (Table 1), homozygote expression in people could potentially lead to impaired GLP-1 responses.

The greatest impact on Ca^{2+} signaling occurred with the

Met[149] variant where responses to all peptides were effectively abolished. There was also greater impact of polymorphic variation on exendin-4 Ca^{2+} signaling relative to the GLP-1(7–36)NH_2 peptide. In particular, there was significant attenuation of exendin-4 signaling with the Lys[20] and Cys[333] variants that had no effect on GLP-1(7–36)NH_2 response. Although the mechanistic basis for this is unclear, it nonetheless provides additional evidence for a differential mode of receptor activation by exendin-4, relative to the GLP-1 peptides, that is not affinity driven. It is interesting to note that one of these variants, Cys[333], also displayed selectively reduced cAMP signaling by compound 2.
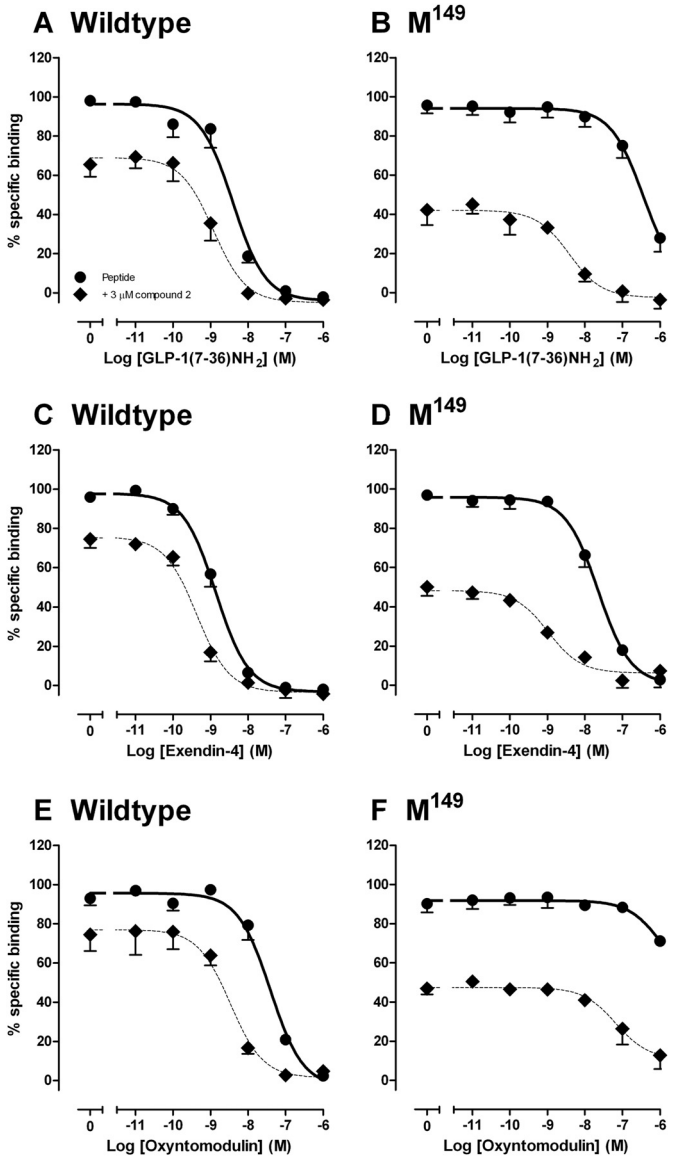
TABLE 6
Differential modulation of oxyntomodulin at naturally occurring human GLP-1R polymorphisms in cAMP accumulation

Data were analyzed using a three-parameter logistic equation as defined in eq. 1. pEC_{50} values represent the negative logarithm of the concentration of agonist that produces half the maximal response. All data are normalized to the response elicited by that of 10^{-7}M forskolin, and are mean ± S.E.M. of three to five independent experiments, conducted in duplicate.

	pEC50	
	Oxyntomodulin	+ 3 μM Compound 2
Wild type	8.7 ± 0.1	10.2 ± 0.7*
Leu[7]	8.7 ± 0.1	9.1 ± 0.7
Lys[20]	8.6 ± 0.2	9.5 ± 0.5*
His[44]	8.4 ± 0.1	10.2 ± 1.2*
Gln[131]	8.7 ± 0.1	9.2 ± 0.4
Met[149]	N.D.	7.7 ± 1.5*
Ser[168]	8.9 ± 0.1	9.4 ± 0.4
Leu[260]	8.6 ± 0.2	9.2 ± 0.5
Thr[316]	8.6 ± 0.1	8.7 ± 0.6
Cys[333]	8.2 ± 0.1	8.3 ± 0.2
Gln[421]	8.4 ± 0.1	9.1 ± 0.6

N.D., data unable to be experimentally defined or with incomplete curves.
* Statistically significant, $P < 0.05$ compared with oxyntomodulin control, paired t test.

Fig. 6. Characterization of the binding of GLP-1(7–36)NH_2 (A and B), exendin-4 (C and D), and oxyntomodulin (E and F) in whole FlpInCHO cells stably expressing the human GLP-1R wild-type (A, C, and E) or human GLP-1R Met[149] variant (B, D, and F) in the presence (♦) or absence (●) of 3 μM compound 2 and in competition with the radiolabeled antagonist ^{125}I-exendin(9–39). Data are normalized to the maximum ^{125}I-exendin(9–39) binding of each individual data set, with nonspecific binding measured in the presence of 1 μM exendin(9–39). Data are analyzed with a three-parameter logistic equation as defined in eq. 1. All values are mean ± S.E.M. of three to four independent experiments conducted in duplicate.

Assessment of ligand binding revealed minimal effect of GLP-1R polymorphic variants on potency, with the exception of the Met[149] variant. It is noteworthy that the loss of peptide potency at this variant was limited to GLP-1(7–36)NH$_2$/GLP-1(7–37), exendin-4, and oxyntomodulin, with no observed effect on the antagonist exendin(9–39) [Beinborn et al., 2005; pIC$_{50}$ values of 8.0 and 8.1, $n = 1$ for the wild-type and Met[149] variant, respectively (data not shown)], full-length GLP-1 peptides, or compound 2 binding. These data are consistent with the critical involvement of amino acid 149 in activation transition of the receptor by peptide agonists, with the higher relative affinity of the truncated GLP-1 peptides, exendin-4, and oxyntomodulin linked to their ability to interact/induce an activated state of the receptor.

Consistent with previous data, the most detrimental of all polymorphisms that we studied was the Met[149] polymorphic

variant (Beinborn et al., 2005). This variant has previously been associated with a case of type II diabetes (Tokuyama et al., 2004) and a loss of binding affinity and cAMP potency in the presence of GLP-1(7–36)NH$_2$ and exendin-4 only (Beinborn et al., 2005). By performing similar binding and cAMP accumulation assays in the presence of all peptide agonists of the GLP-1R, we observed that the effect of the Met[149] polymorphic variant is ligand-dependent, with a greater effect on oxyntomodulin (251-fold decrease in binding affinity, >500-fold decrease in cAMP potency) relative to GLP-1(7–36)NH$_2$ (decreases of 251- and 158-fold in binding affinity and cAMP potency, respectively) or exendin-4 (decreases of 32- and 200-fold in binding affinity and cAMP potency, respectively). We also found that effects on signaling at this polymorphic variant are pathway-dependent, in that the loss of function was more pronounced in cAMP accumulation and Ca^{2+} mobilization than ERK1/2 phosphorylation. Intriguingly, both the binding and signaling responses to compound 2 were unaltered, providing supporting evidence for a distinct molecular mechanism for receptor activation from that of orthosteric-acting peptide agonists. In parallel, and perhaps most importantly, compound 2 retained its ability to modulate peptide-mediated binding and cAMP responses at the Met[149] polymorphic variant, indicating that there is potential for developing therapeutics to treat patients possessing polymorphic receptor variants that are refractory to peptide-mimetic therapy. The data from the Met[149] polymorphic variant is also informative, suggesting that the principal allosteric
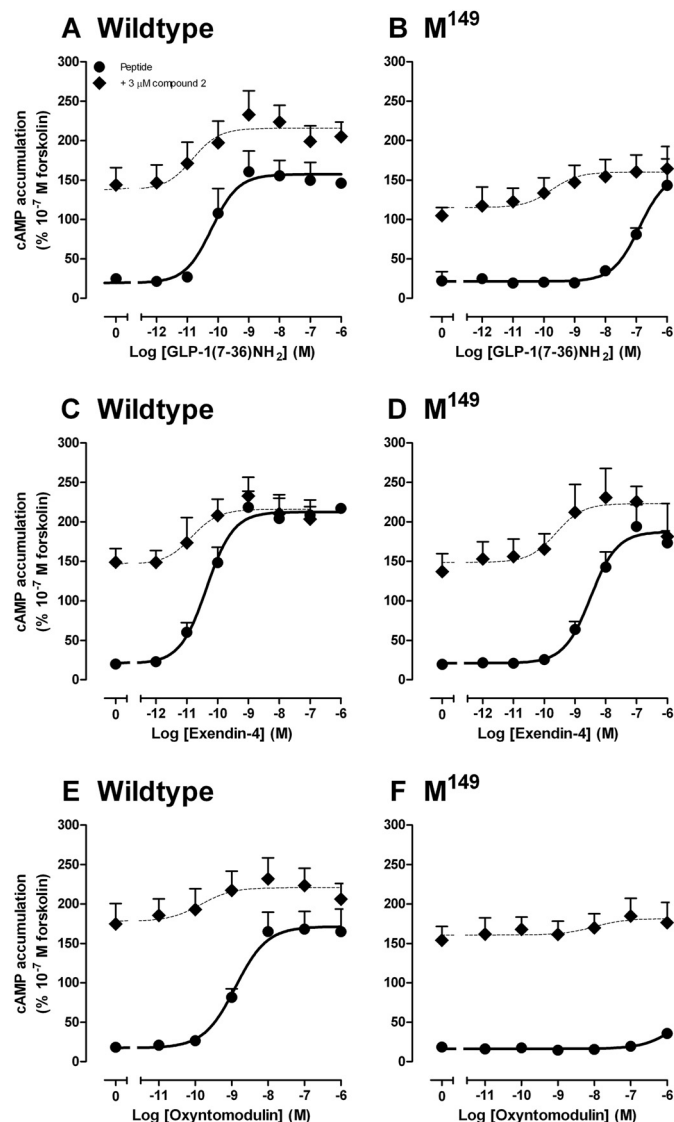

Fig. 7. Characterization of cAMP accumulation generated by GLP-1(7–36)NH$_2$ (A and B), exendin-4 (C and D), and oxyntomodulin (E and F) in FlpInCHO cells stably expressing the human GLP-1R wild-type (A, C, and E) or human GLP-1R Met[149] variant (B, D, and F) in the presence (♦) or absence (●) of 3 μM compound 2. Data are normalized to the response elicited by 100 nM forskolin and analyzed with a three-parameter logistic equation as defined in eq. 1. All values are mean ± S.E.M. of three to five independent experiments conducted in duplicate.

TABLE 7

Differential modulation of agonist binding at the human GLP-1R Met[149] receptor variant by compound 2

Data were analyzed using a three-parameter logistic equation as defined in eq. 1. pIC$_{50}$ values represent the negative logarithm of the concentration of agonist that inhibits binding of half the total concentration of radiolabeled antagonist, ^{125}I-exendin(9–39). All data are normalized to the maximum ^{125}I-exendin(9–39) binding in each individual data set, with non-specific binding measured in the presence of 1 μM exendin(9–39). All values are mean ± S.E.M. of three to four independent experiments, conducted in duplicate.

	pIC$_{50}$			
	Wild Type		Met[149]	
	Peptide	+ 3 μM Compound 2	Peptide	+ 3 μM Compound 2
GLP-1(7–36)NH2	8.4 ± 0.1	8.9 ± 0.1*	6.4 ± 0.2	8.4 ± 0.2*
Exendin-4	8.9 ± 0.1	9.4 ± 0.1	7.6 ± 0.1	9.0 ± 0.2*
Oxyntomodulin	7.4 ± 0.1	8.5 ± 0.2*	6.0 ± 1.0	7.2 ± 0.3

* Statistically significant, $P < 0.05$ compared with peptide control, paired t test.

TABLE 8

Differential modulation of agonist peptides at the human GLP-1R Met[149] receptor variant by compound 2 in cAMP accumulation

Data were analyzed using a three-parameter logistic equation as defined in eq. 1. pEC$_{50}$ values represent the negative logarithm of the concentration of agonist that produces half the maximal response. All data are normalized to the response elicited by 10^{-7} M forskolin and are mean ± S.E.M. of three to five independent experiments, conducted in duplicate.

	pEC$_{50}$			
	Wild Type		Met[149]	
	Peptide	+ 3 μM Compound 2	Peptide	+ 3 μM Compound 2
GLP-1(7–36)NH2	10.2 ± 0.3	10.8 ± 0.6	7.0 ± 0.2	9.7 ± 0.8*
Exendin-4	10.4 ± 0.2	10.8 ± 0.6	8.5 ± 0.2	9.6 ± 0.7†
Oxyntomodulin	8.9 ± 0.2	9.8 ± 1.0†	N.D.	N.D.

N.D., data unable to be experimentally defined or with incomplete curves.
* Statistically significant, $P < 0.05$ compared with peptide control, paired t test.
† $P = 0.05$ compared with peptide control, paired t test.

effect of compound 2 is to lower the energy barrier for activation transition, in particular with respect to the receptor conformation(s) linked to $G\alpha_s$ coupling. At the "wild-type" receptor, GLP-1(7–36)NH_2, GLP-1(7–37), and exendin-4 are all highly potent and efficacious peptide ligands, and addition of compound 2 has a limited effect on receptor binding and signaling output because the receptor is already in a highly active state. The weaker agonist, oxyntomodulin, which has a lower response in the system, however, was positively modulated to a greater extent by compound 2. At the Met[149] variant, our results suggest that there is a higher barrier to establish an active state transition as engaged by the peptide agonists. The addition of compound 2, which modifies the receptor in a manner that is insensitive to the Met[149] substitution, lowers the barrier to allow the recovery of the response, so that an active conformation can more readily be achieved. As also previously noted by Beinborn et al. (2005), the Met[149] polymorphic variant does not alter antagonist [exendin(9–39)] binding to the receptor. This is consistent with the involvement of residue 149 in an activation transition rather than directly disrupting peptide binding interactions.

The recovery of GLP-1(7–36)NH_2 and exendin-4 potency at the Met[149] polymorphic variant in cAMP accumulation to within 8-fold of the GLP-1R wild-type range provides an excellent example of the potential to rescue detrimental receptor polymorphisms with the application of allosteric ligands. Nonetheless, broad assessment of the impact of different GLP-1R polymorphisms on the allosteric potentiation of oxyntomodulin cAMP response indicated that multiple receptor variants had attenuated responses. Not surprisingly, the C[333] variant had minimal allosteric response because this substitution also led to attenuation of cAMP signaling by compound 2, suggesting that it interferes with the ability of compound 2 to promote a $G\alpha_s$-interacting conformation of the receptor; previous work has demonstrated a correlation between allosteric potentiation and the ability of compounds to promote an active state of the receptor (Leach et al., 2010; Keov et al., 2011). Intriguingly, the Leu[7], Gln[131], Ser[168], Leu[260], Thr[316], and Gln[421] also exhibited attenuated allosteric enhancement of oxyntomodulin cAMP signaling in the presence of compound 2. The mechanistic basis for the loss of effect is unclear, but it highlights the potential complexity of the allosteric interaction between peptides and small molecule ligands. Although this effect is likely to be chemotype-dependent, it nonetheless highlights the need for careful consideration in clinical trial design where potential allosteric drugs are being assessed. In the case of the GLP-1R, at least one of the variants with loss of oxyntomodulin modulation, Leu[7], is reported in the SwissProt database to occur in 40% of assessed populations in either homozygous or heterozygous form.

In conclusion, we have demonstrated important pharmacological effects arising from polymorphisms of the GLP-1R. For the Met[149] variant, this is likely to be clinically relevant, albeit for a small percentage of patients. Importantly, we have demonstrated that loss of function arising from polymorphisms such as the Met[149] substitution can be rescued by allosteric modulation of the receptor, with small molecule compounds providing scope for therapeutic intervention for patients whose disease is linked to such polymorphic variation.

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Correction to "Polymorphism and Ligand Dependent Changes in Human Glucagon-Like Peptide-1 Receptor (GLP-1R) Function: Allosteric Rescue of Loss of Function Mutation"

The authors regret this error and apologize for any confusion and inconvenience it may have caused.

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