Differential Requirement of the Extracellular Domain in Activation of Class B G Protein-coupled Receptors[*]

Li-Hua Zhao[‡§], Yanting Yin[§¶], Dehua Yang[‖], Bo Liu[§], Li Hou[§], Xiaoxi Wang[§], Kuntal Pal[¶], Yi Jiang[§], Yang Feng[‖], Xiaoqing Cai[‖], Antao Dai[‖], Mingyao Liu[‡], Ming-Wei Wang[‖‡‡], Karsten Melcher[¶1], and H. Eric Xu[§¶2]**

From the ‡Shanghai Key Laboratory of Regulatory Biology, Institute of Biomedical Sciences and School of Life Sciences, East China Normal University, Shanghai 200241, China, §VARI-SIMM Center for Structure and Function of Drug Targets and the CAS Key Laboratory of Receptor Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China, ¶Laboratory of Structural Sciences, Center for Structural Biology and Drug Discovery, Van Andel Research Institute, Grand Rapids, Michigan 49503, ‖The National Center for Drug Screening and the CAS Key Laboratory of Receptor Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China, **The Institute of Biosciences and Technology, Texas A&M University Health Science Center, Houston, Texas 77030, ‡‡School of Pharmacy, Fudan University, 826 Zhangheng Road, Shanghai 201203, China

G protein-coupled receptors (GPCRs) from the secretin-like (class B) family are key players in hormonal homeostasis and are important drug targets for the treatment of metabolic disorders and neuronal diseases. They consist of a large N-terminal extracellular domain (ECD) and a transmembrane domain (TMD) with the GPCR signature of seven transmembrane helices. Class B GPCRs are activated by peptide hormones with their C termini bound to the receptor ECD and their N termini bound to the TMD. It is thought that the ECD functions as an affinity trap to bind and localize the hormone to the receptor. This in turn would allow the hormone N terminus to insert into the TMD and induce conformational changes of the TMD to activate downstream signaling. In contrast to this prevailing model, we demonstrate that human class B GPCRs vary widely in their requirement of the ECD for activation. In one group, represented by corticotrophin-releasing factor receptor 1 (CRF$_1$R), parathyroid hormone receptor (PTH1R), and pituitary adenylate cyclase activating polypeptide type 1 receptor (PAC1R), the ECD requirement for high affinity hormone binding can be bypassed by induced proximity and mass action effects, whereas in the other group, represented by glucagon receptor (GCGR) and glucagon-like peptide-1 receptor (GLP-1R), the ECD is required for signaling even when the hormone is covalently linked to the TMD. Furthermore, the activation of GLP-1R by small molecules that interact with the intracellular side of the receptor is dependent on the presence of its ECD, suggesting a direct role of the ECD in GLP-1R activation.

The class B or secretin family of GPCRs consists of 15 receptors for peptide hormones that include glucagon, glucagon-like peptides, parathyroid hormone, and calcitonin (Fig. 1A). These receptors are important drug targets for many human diseases including diabetes, neurodegeneration, cardiovascular diseases, and psychiatric disorders. The full-length receptors consist of two modular domains: a globular extracellular domain (ECD)[3] defined by three conserved disulfide bonds and a TMD that contains seven transmembrane helices (1–4). The ECD is responsible for the high affinity and specificity of hormone binding, and the TMD is required for receptor activation and signal coupling to downstream G proteins and other signaling effectors (4). Peptide hormone binding is thought to proceed through fast binding of its C terminus to the ECD followed by a slower association of the peptide N terminus with the receptor TMD (5), which leads to conformational changes in the receptor TMD and the receptor activation. The activated receptors are coupled primarily to stimulatory G proteins, resulting in elevation of the intracellular cAMP level.

Structures of the ECDs of class B GPCRs in complex with their peptide ligands have been determined by x-ray crystallography (1, 6–9) and NMR (10) and have provided useful information about structural mechanisms of ligand recognition and selectivity (2, 11). Only recently, crystal structures of the iso-

3 The abbreviations used are: ECD, extracellular domain; TMD, transmembrane domain; GPCR, G protein-coupled receptors; GCG, glucagon; GCGR, GCG receptor; GLP-1, glucagon-like peptide-1; GLP-1R, GLP-1 receptor; PTH, parathyroid hormone; PTH1R, PTH1 receptor; CRF, corticotropin-releasing factor; CRF$_1$R, CRF receptor 1; PAC1R, pituitary adenylate cyclase activating polypeptide type 1 receptor; CRE, cAMP-response element; BETP, 4-(3-(benzyloxy)phenyl)-2-(ethylsulfinyl)-6-(trifluoromethyl)pyrimidine; ICL3, intracellular loop 3; RLU, renilla luciferase activity.
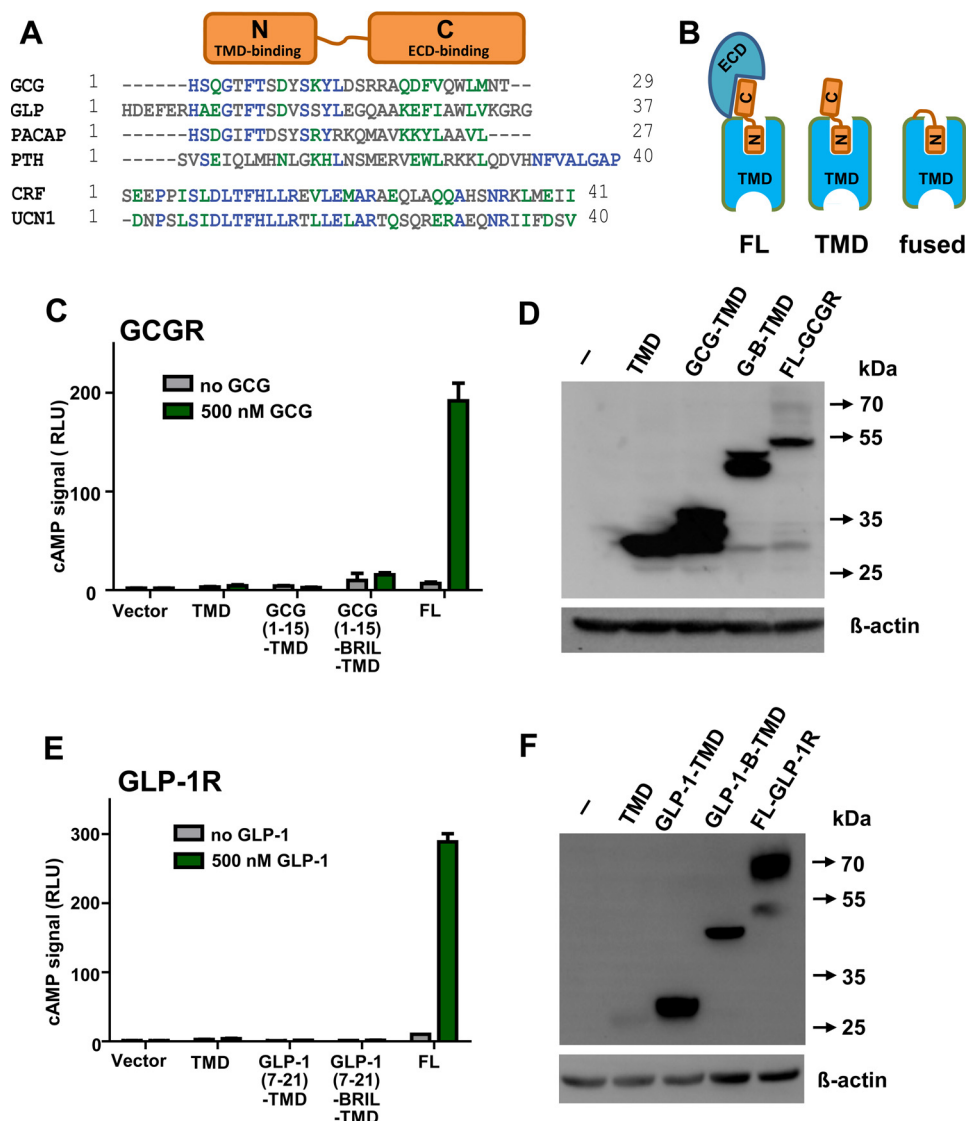
FIGURE 1. **High concentration of exogenous hormone and hormone tethering cannot bypass the ECD requirement for GCGR and GLP-1R.** *A*, alignment of peptide hormones GCG, GLP-1, PACAP, PTH CRF, and UCN1, marked with their N-terminal TMD binding regions and C-terminal ECD-binding regions. *B*, schematic representation of constructs. *FL*, full-length receptor; *fused*, fusion between peptide hormone N terminus and receptor TMD. *C* and *E*, receptor activation of GCGR and GLP-1R as measured by cAMP signals, which are relative luciferase activities of the indicated transfected constructs in the absence or presence of exogenous peptide hormone; *n* = 3, *error bars* = S.D. *D* and *F*, expression levels of proteins analyzed in *C* and *E* by immunoblotting using anti-FLAG antibodies. GCG-TMD is a fusion of GCG(1–15) with GCGR TMD, and *G-B-TMD* is a fusion of GCG(1–15) with BRIL and GCGR TMD. GLP-1-TMD is a fusion of GLP-1(7–21) with GLP-1R TMD, and *GLP-1-B-TMD* is a fusion of GLP-1(7–21) with BRIL and GLP-1R TMD. Note that the first six residues of GLP-1 are not present in the mature GLP-1 and are not required for receptor activation.

lated TMDs of two class B receptors in an inactive conformation have been published, *i.e.* the human glucagon receptor (GCGR) (12, 13) and human corticotropin-releasing factor receptor 1 (CRF_1R) (14). Although crystal structures of more than 20 class A GPCRs have been determined, currently no full-length structure of a class B receptor has been reported due to the inherent instability of these receptors and the limited availability of stabilizing high affinity small molecule agonists and antagonists.

Although the TMDs of class B GPCRs are highly homologous, homology of the ECDs is limited to the six disulfide-forming cysteines and only about a dozen other conserved residues. Most ECD structures have been obtained only in complex with ligands, indicating that ligand binding stabilizes these proteins and, therefore, favors crystallization of ECD-ligand complexes. In contrast to endogenous peptide hormones, small molecule antagonists, such as CP-376395 (a CRF_1R antagonist), which were discovered by high throughput screening, act allosterically within the TMD to block the binding and signaling of the peptide agonist ligands (14, 15).

In this report we demonstrate that the ECDs of some class B GPCRs, such as CRF_1R, PAC1R, and PTH1R, solely function as affinity traps, and hence, their requirement can be bypassed by mass action effects and hormone tethering, consistent with the prevailing model of peptide binding and activation of Class B GPCRs. In contrast to this prevailing model, activation of GCGR and GLP-1R is strictly dependent on the presence of their ECDs. We further provide comprehensive insights into the mechanism for the requirement of ECD in activation of GCGR and GLP-1R by small molecule ligands and by muta-
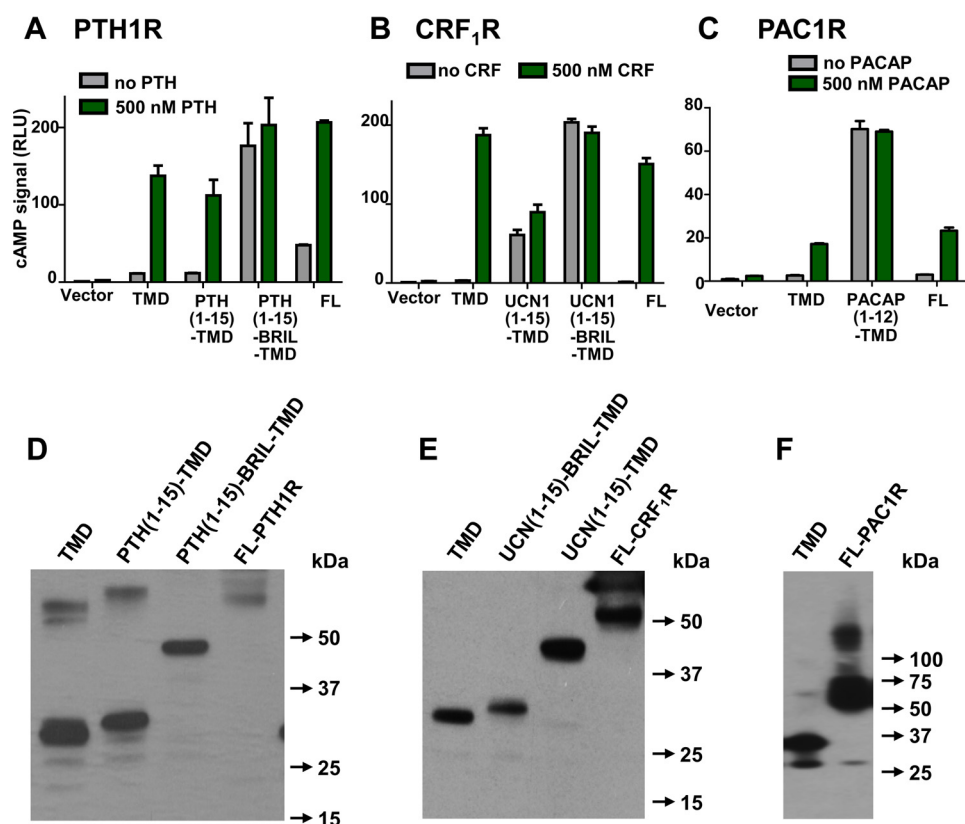
FIGURE 2. **High concentration of exogenous hormone and hormone tethering can bypass the ECD requirement for PTH1R, CRF₁R, and PAC1R.** *A–C*, receptor activation of PTH1R, CRF₁R, and PAC1R as measured by cAMP signals, which are relative luciferase activities of the indicated transfected constructs in the absence or presence of exogenous peptide hormone; $n = 3$, *error bars* = S.D. *D–F*, expression levels of PTH1R, CRF₁R, and PAC1R analyzed by immunoblotting using anti-FLAG antibodies. *FL*, full-length.
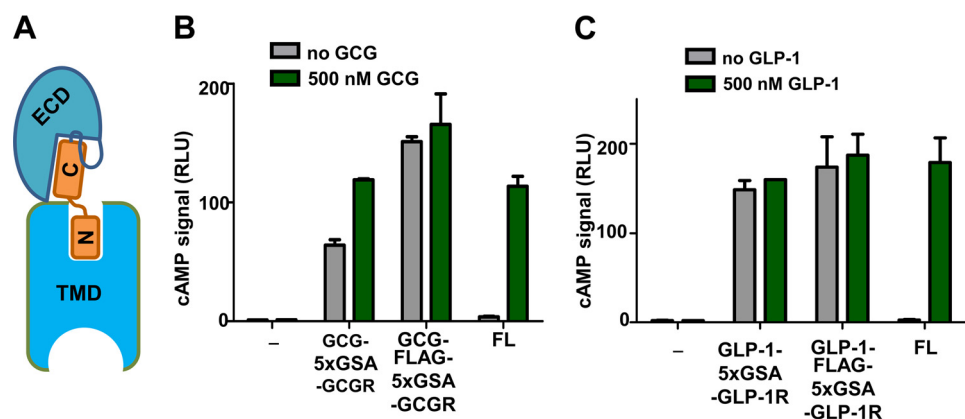

FIGURE 3. **Tethering of GCG and GLP-1 to their full-length receptors constitutively activates receptor signaling.** *A*, schematic depiction of the fusion constructs. *B* and *C*, fusion of full-length GCG (*B*) and GLP-1 (*C*) to their full-length (*FL*) receptors activates receptor signaling in the presence or absence of 500 nm exogenous peptides; $n = 3$, *error bars* = S.D.

tional studies. These results provide new mechanistic insights into activation of GCGR and GLP-1R, information that could be beneficial for the notoriously difficult development of high affinity small molecule agonists of these GPCRs and for the construct design for crystallizing the full-length receptors.

Results

Class B GPCRs Differ in Their ECD Requirements for Signaling—To examine the requirement of the GCGR ECD for signaling, we transiently transfected four different FLAG-tagged constructs into HEK293 cells: the full-length receptor,

TMD, TMD-interacting N terminus of glucagon (1–15) fused to the TMD, and a fusion in which glucagon (1–15) and the TMD are separated by a thermostabilized cytochrome b562 variant, BRIL, which has been used for facilitating GPCR crystallization (16) (Fig. 1, *B–D*). If the ECD only functions as an affinity trap to increase the local concentration of the glucagon N terminus, we reasoned that a high concentration of exogenous glucagon may bypass the requirement for the ECD. We, therefore, studied the activation of a cAMP-responsive CRE-luciferase reporter to monitor GCGR signaling in the presence and absence of 500 nm glucagon whose physiological concen-
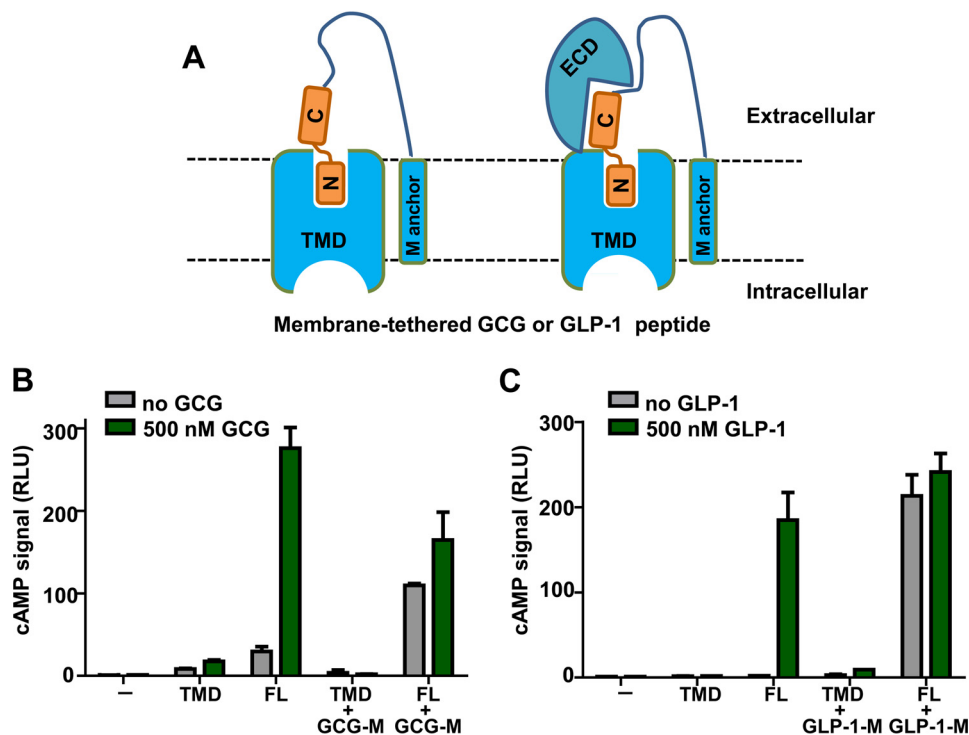
FIGURE 4. **Membrane-anchored GCG and GLP-1 constitutively activate their full-length receptors but not their TMD.** *A*, schematic depiction of the experiment. *B* and *C*, fusion of the C termini of GCG (*B*) and GLP-1 (*C*) to a single membrane-spanning helix (*GCG-M* and *GLP-1-M*) constitutively activate their corresponding full-length receptors but not their corresponding TMDs; $n = 3$, *error bars* = S.D.
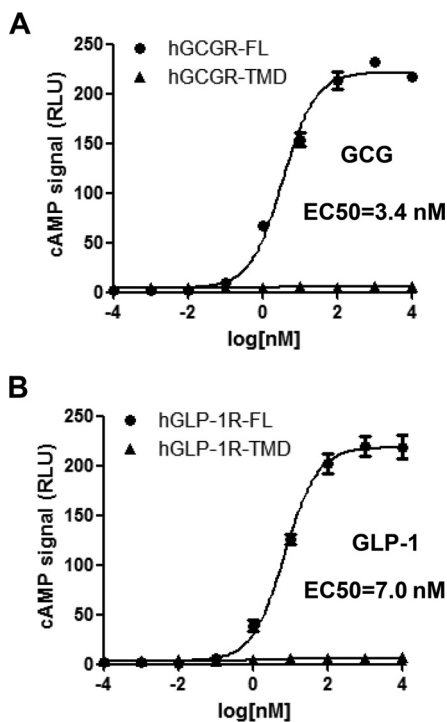
FIGURE 5. **Concentration curves of GCG (*A*) and GLP-1 (*B*) in activation of their full-length and TMD receptors.** For the full-length (*FL*) receptor, the EC_{50} value for GCG is 3.4 nM and the EC_{50} value for GLP-1 is 7.0 nM. For TMD, there is no activation up to 10 μM GCG or GLP-1. All data are presented as cAMP signal (RLU); $n = 3$, *error bars* = S.D.

tration in blood is ~25 pM (17, 18). As shown in Fig. 1, *C* and *D*, the TMD was well expressed but remained inactive under either condition. As expected, full-length GCGR was active in
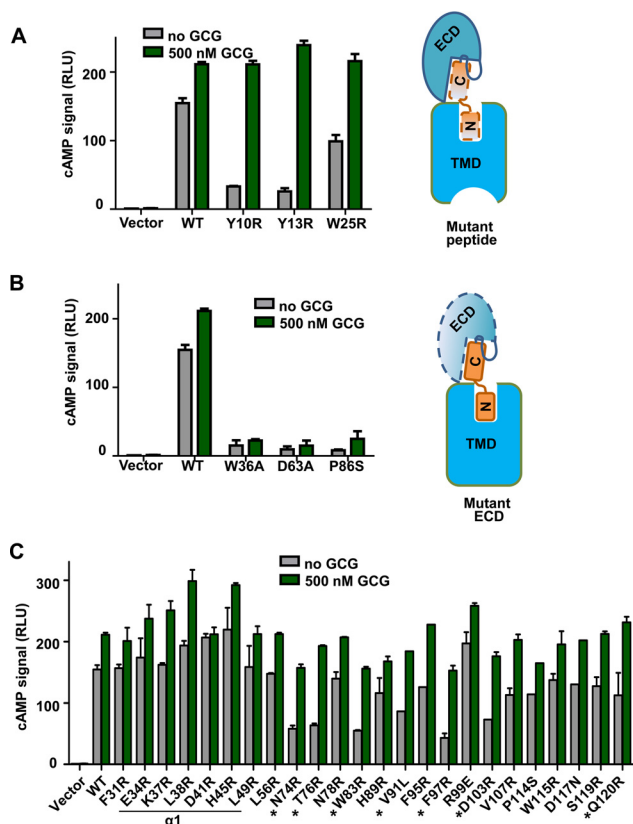
FIGURE 6. **Mutational analysis of the GCG-GCGR fusion protein.** cAMP reporter activity of transfected expression constructs that have mutations within the GCG part (*A*) or the GCGR part (*B* and *C*) of the fusion protein; $n = 3$, *error bars* = S.D. Residues substituted with alanine in *B* are known GCG-interacting residues; residues labeled by an *asterisk* are located on a discrete surface shown in Fig. 8.

FIGURE 7. **Expression levels of GCG-GCGR fusion protein determined by FACS (*A*) and by immunoblotting (*B*) using anti-FLAG antibody.**

the presence, but not the absence, of the hormone. Although pioneering work by the Vale laboratory demonstrated that directly fusing CRF to the CRF$_1$RΔECD generated a constitutively active receptor (19), a chimeric fusion between glucagon and the GCGR TMD with or without BRIL linker was inactive (Fig. 1*C*).
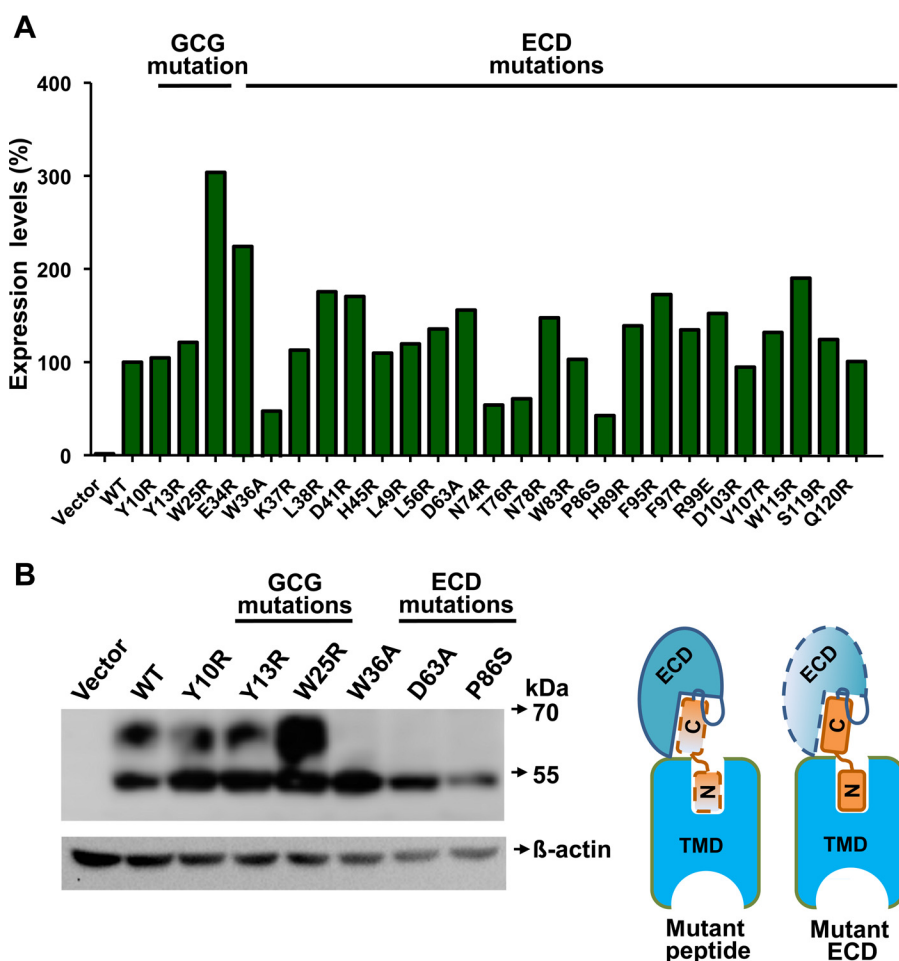
Next, we extended this analysis to four other members of class B GPCRs; GLP-1R, PTH1R, CRF$_1$R, and PAC1R. GLP-1R behaved in a manner similar to GCGR in that activity required both the full-length receptor and free peptide hormone (Fig. 1*E*), even though the GLP-1R TMD and GLP-1-TMD fusions were well expressed (Fig. 1*F*). In contrast, the ECDs for PTH1R, CRF$_1$R, and PAC1R were not required in the presence of high concentrations of their respective hormones, and their requirements could also be bypassed by directly fusing the N-terminal, TMD-interacting hormone fragments to their respective TMDs (Fig. 2), resulting in constitutive receptor activation. The results of the chimeric receptor experiments are in agreement with previous reports on CRF$_1$R (19) and PTH1R (20), consistent with the prevailing model that the ECD is only required for high affinity binding but not for activation of these receptors. Fusion of glucagon and GLP-1 to their full-length receptors, separated by 5 repeats of a Gly-Ser-Ala linker (15 amino acid linker = GSA$_5$), constitutively activated the CRE-luciferase reporter (Fig. 3), indicating that fused glucagon and GLP-1 in

the presence of their respective ECD can productively interact with the TMD.

Membrane tethering of glucagon and GLP-1 has been shown as another way to engineer proximity between receptor and peptide hormone and to bypass the requirement for exogenous hormone (19). In this approach proximity occurs by membrane co-localization, *i.e.* without directly fusing hormone and receptor and thereby avoiding potential steric constraints impeding the interaction between covalently linked binding partners. In these experiments, we fused glucagon and GLP-1 to a long flexible linker and the single membrane-spanning helix of CD8 (20) and co-expressed these chimeric peptides with both the GCGR and GLP-1R TMDs as well as full-length receptors. Consistent with the previous report (20), membrane-anchored glucagon and GLP-1 constitutively activated their full-length receptors (Fig. 4). However, they failed to stimulate signaling through GCGR and GLP-1R lacking respective ECDs (Fig. 4). Collectively, we conclude that class B GPCRs vary in the extent of their ECD requirements for receptor activation and that the receptors can be divided into two functionally distinct subgroups: one group including PTH1R, CRF$_1$R, and PAC1R, in which the ECD only functions as affinity trap and is, therefore, dispensable when the affinity requirement is bypassed, and the other group including GCGR and GLP-1R that requires the ECD for receptor activation. This conclusion is further supported by
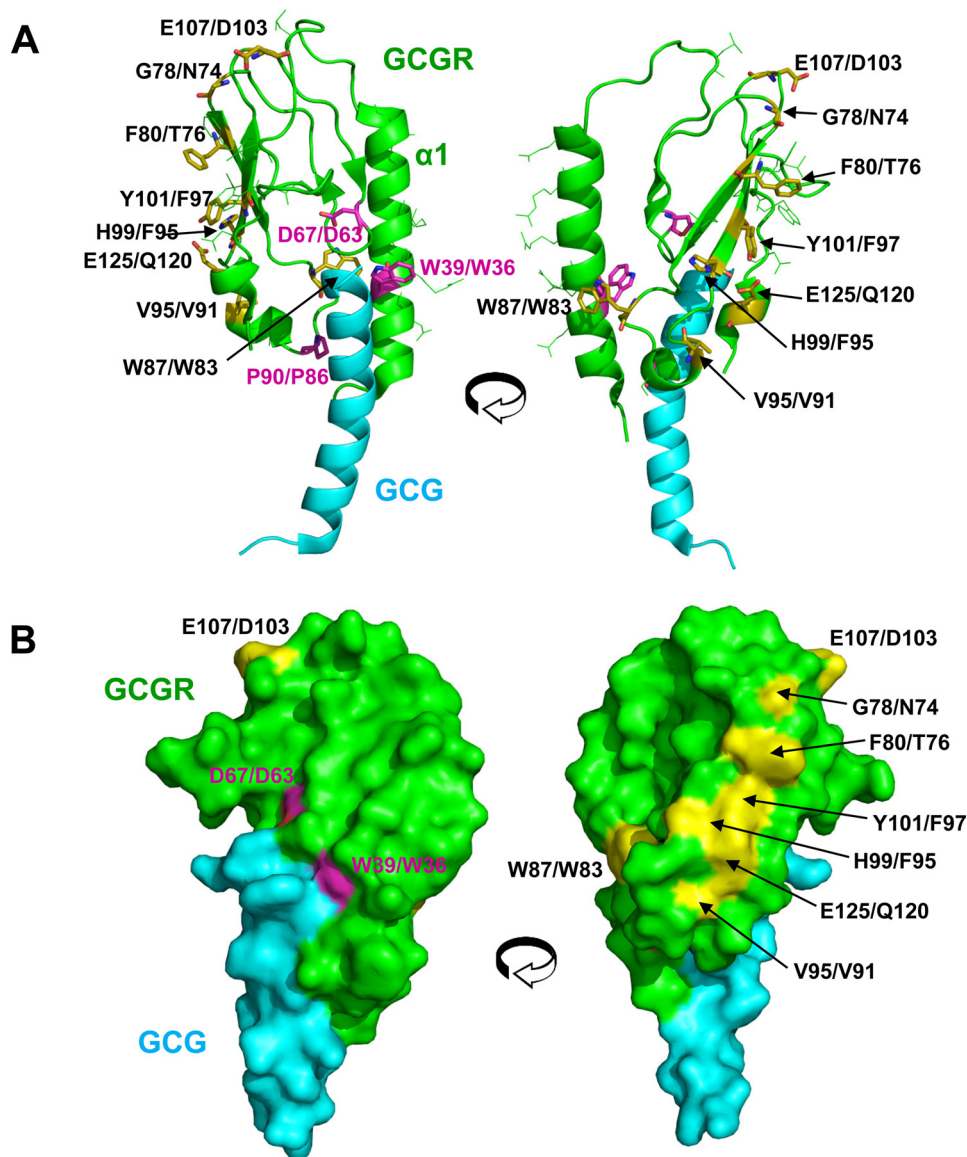
FIGURE 8. **Mapping of GCGR ECD mutations onto the structure of the GLP-1R-GLP-1 complex.** Schematic (*A*) and surface depictions (*B*) of the GLP-1R ECD/GLP-1 structure (PDB code 3IOL) are shown. Residues that correspond to mutationally sensitive GCGR residues identified in Fig. 6 are highlighted and labeled by their GLP-1R residue number followed by the residue numbers of the corresponding amino acids in GCGR. Residues that are critical for GCG binding are shown in *magenta*, residues whose mutations decreased reporter gene activity by ≥50% are shown as *yellow stick models* (*A*) or *yellow surface patches* (*B*), and all other mutated residues are shown as *green line models*.

full-concentration curves of GCG and GLP-1 to their respective receptors (Fig. 5). Both GCG and GLP-1 activate their respective full-length receptor at low nM concentrations, but they cannot activate their TMDs lacking the ECD even at 10 μM of peptides.

Activity-compromising Mutations in the GCGR ECD Cluster at a Surface Distant from Hormone Binding—To understand the ECD requirement for activation of GCGR, we carried out mutational studies of the ECD and the fused hormone peptide in the context of the GCG-GSA$_5$-GCGR fusion protein. We first mutated residues in the glucagon part of the protein. Tyr-10 and Tyr-13 in the N terminus of glucagon have been predicted to make key interactions with the GCGR TMD (12). Their replacements with arginine residues (Y10R and Y13R) strongly reduced activity of the hybrid receptor, but activity could be fully recovered in the presence of 500 nM exogenous

glucagon (Fig. 6A). This indicates that the intramolecular interaction with the fused mutant peptide can be efficiently displaced by exogenous native peptide, suggesting that the glucagon-ECD interaction is an important contributor for the overall affinity of glucagon-GCGR binding and activation. Replacement of Trp-25 in the ECD-interacting C terminus of the fused glucagon had a partial signaling defect even though the fusion positions the hormone in the vicinity of the TMD independent of an ECD interaction.

We subsequently introduced 27 single amino acid alterations into the fusion proteins that collectively span the entire length of the ECD: W36A, D63A, P86S, F31R, E34R, K37R, L38R, D41R, H45R, L49R, L56R, N74R, T76R, N78R, W83R, H89R, V91L, F95R, F97R, R99E, D103R, V107R, P114R, W115R, D117N, S119R, and Q120R (Fig. 6, *B* and *C*). Three groups of mutant proteins stood out. The first group of mutations
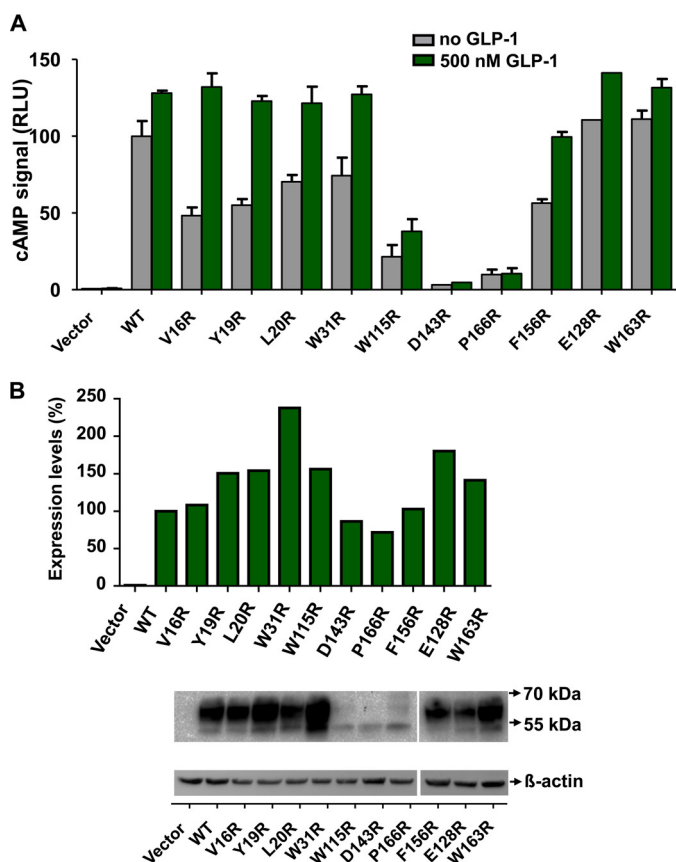
FIGURE 9. **Activity and expression levels of GLP-1R ECD mutant proteins.**
A, cAMP activity of GLP-1(7–36)-FLAG-GSA$_5$-GLP-1R fusion receptors; $n = 3$, *error bars* = S.D. *B*, expression levels of the fusion receptors determined by FACS (*top*) and by immunoblotting (*bottom*), both with anti-FLAG antibody.

included Trp-36, Asp-63, and Pro-86, which were previously identified as glucagon binding residues in a structure model of the full-length glucagon-GCGR complex (12), and their mutations abrogated glucagon binding (12, 21). The corresponding fusion proteins were expressed at levels comparable with those of the wild type fusion protein (Fig. 7) but almost abolished receptor activation both in the presence or absence of exogenously added glucagon (Fig. 6*B*). Second, seven well expressed mutant proteins (N74R, T76R, W83R, V91L, F95R, F97R, D103R Q120R; marked with *asterisks* in Fig. 6*C*) had activities that were reduced by ≥2-fold compared with the wild type yet whose activity defect could be rescued by 500 nM exogenous glucagon. We mapped the corresponding residues on the crystal structure of the structurally highly homologous GLP-1R ECD in complex with GLP-1 (6) (PDB code 3IOL; highlighted in *yellow* in Fig. 8, *A* and *B*). Surprisingly, none of these residues was in bonding distance to the peptide hormone, and none was on the long α1-helix, which is thought to interact with the TMD under basal conditions but not in the presence of glucagon (22, 23). Instead, all of them were exposed on the third accessible surface of the complex (the other two surfaces are formed by the hormone and the α1-helix). Therefore, a substantial set of residues required for GCGR activity did not appear to be involved in ligand binding but likely formed an inter- or intramolecular interaction surface. Third, six mutations mapped to the α1-helix (E34R, K37R, L38R, D41R, H45R, and L49R; Figs.

6*C* and 8) and showed moderately higher activity than the wild type fusion protein, except for L49R, which displayed the same activity as the wild type. This observation is consistent with the proposed inhibitory effect of the α1-helix on basal GCGR activity (22, 23). To explore functional conservation, we also analyzed the effects of 10 corresponding GLP-1/GLP-1R mutants in the context of the GLP-1/GLP-1R fusion protein. As seen in Fig. 9, these mutations had similar, but less pronounced effects, especially in the absence of exogenous GLP-1.

Small Molecule GLP-1R Modulators Mimic Endogenous Peptide Hormones or Modify the GLP-1R Intracellular Loop 3— Several non-peptidic GLP-1R agonists were identified by high throughput screening and subsequent structural optimization (24–26). Interestingly, none of them appears to simply bind the GLP-1R TMD. One set of compounds, exemplified by the large substituted cyclobutanes Boc5 (27), S4P (27), and WB4−24 (28, 29) (Fig. 10*A*) competed with the ECD binding antagonist exendin(9−39) (an N-terminally truncated, non-TMD interacting version of the Gila Monster GLP-1R agonist extendin-4; Ref. 30) indicating that they directly bind the ECD. In addition, Boc5 and S4P largely, but not completely, displaced labeled GLP-1 from GLP-1R and may, therefore, also interact with the TMD (27). In contrast, the pyrimidine BETP (31) was not competed by exendin(9−39) or GLP-1 and is, therefore, thought to allosterically activate GLP-1R (31−34). Recent work has demonstrated that BETP functions by forming covalent adducts with Cys-347 in the intracellular loop 3 (ICL3) of GLP-1R, which may mimic a physiological covalent modification (35).

We tested these compounds for their ability to stimulate either full-length GLP-1R or the GLP-1R TMD. All compounds stimulated cAMP production in cells transfected with full-length GLP-1R, and no compound, even up to 100 μM concentration, could activate signaling in the absence of the ECD (Figs. 10*B* and 11). Moreover, when we mutated the GLP-1R residue that becomes covalently modified by BETP, Cys-347, to alanine, BETP almost completely lost its ability to activate the full-length GLP-1R, whereas activation by GLP-1 was unaffected (Fig. 10*C*). Collectively, this shows that BETP activates GLP-1R by a mechanism that is both markedly different and independent of activation by the native peptide and that may involve binding and covalent modification of ICL3. Despite these distinct mechanisms, BETP activation of GLP-1R is still dependent on the intact GLP-1R ECD.

Discussion

Biochemical, mutational, and structural experiments have provided evidence for a two-domain, two-step model of class B GPCR activation (4). This model posits that the C termini of peptide hormones bind the receptor ECDs to bring the peptide N termini into the vicinity of the receptor TMDs, which then allows the N termini to bind a pocket inside of the TMD. The latter is thought to induce conformational changes that are propagated to the intracellular side of the receptor TMD to allow coupling to heterotrimeric G proteins to stimulate the intracellular signaling cascade. In the simplest version of the model, the ECD would only function as affinity trap, and its
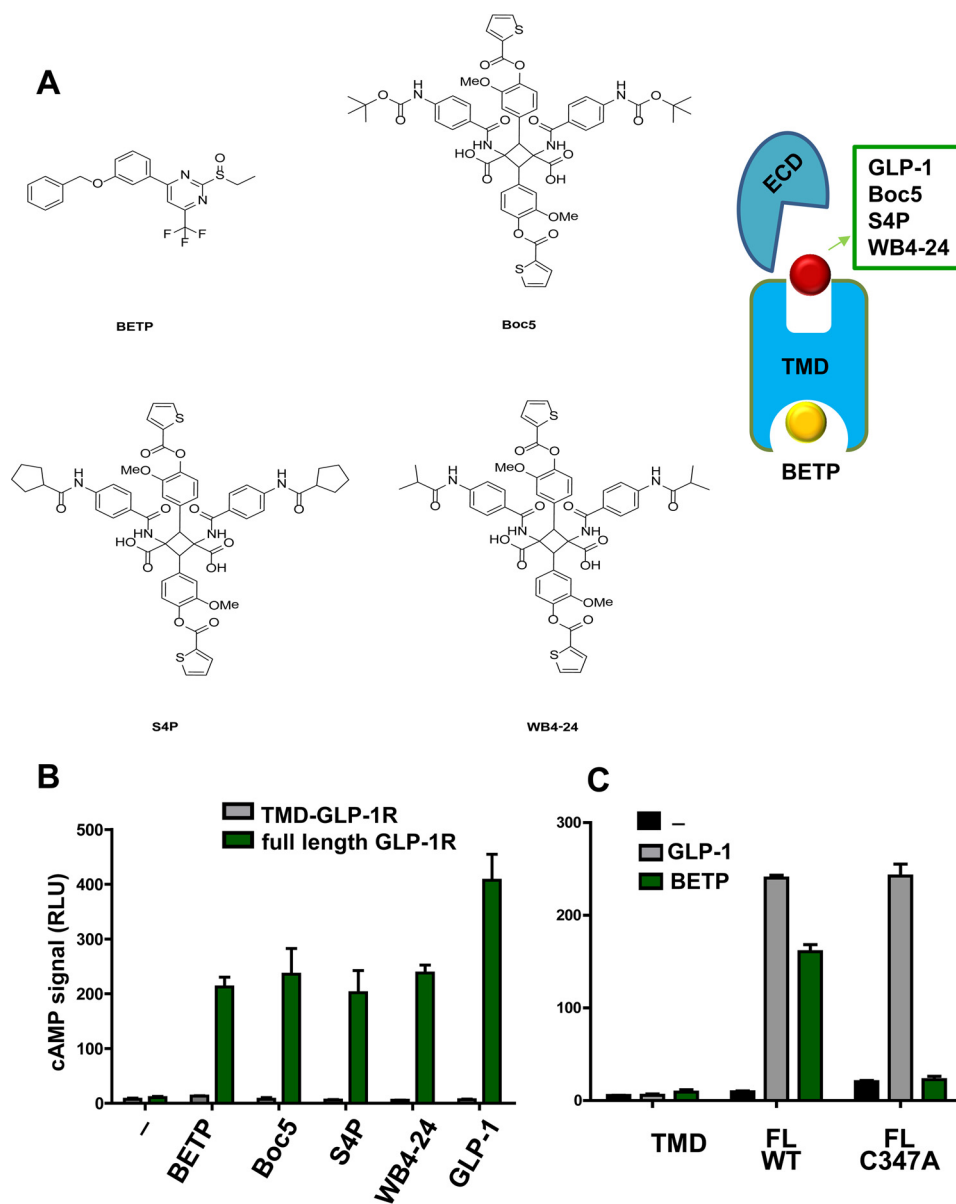
FIGURE 10. **Differential stimulation of human GLP-1R and GLP-1R TMD signaling by small molecule GLP-1R activators.** *A*, structures of BETP, Boc5, S4P, and WB4−24. *B*, cAMP reporter activities of the indicated transfected expression constructs in the absence or presence of 1 μM GLP-1 (control) or 10 μM concentrations of the indicated small molecule activators; $n = 3$, *error bars*, S.D. C. Activation of wild type (*WT*) and C347A GLP-1R was by BETP and GLP1; $n = 3$, *error bars* = S.D.

requirement for receptor activation could thus be bypassed by mass action or tethering.

In this study we have demonstrated that class B GPCRs differ in their requirement for the ECD in receptor activation. As shown in Fig. 12, one group of receptors, represented by CRF_1R, PAC1R, and PTH1R, can be activated without ECD when peptide is presented in high concentrations (Fig. 12*A*) or fused to the TMD (Fig. 12*B*). In contrast, the other group, represented by GCGR and GLP-1R, requires the ECD for activation even in the presence of a very high concentration of native peptide or when the peptide hormones were artificially tethered to the TMD or to the membrane (Figs. 1, 3, and 4). It is unlikely that this requirement is due to an inherent lack of hormone affinity for the GCGR and GLP-1R TMDs. Rather, our mutational analysis of glucagon in this study and quantitative binding assays of

GLP-1/glucagon chimeric hormones in a previous study (36) indicate that both hormones bind the TMDs of their respective receptors.

In addition to the endogenous peptide hormones, the small molecule GLP-1R agonists Boc5, S4P, and WB4−24 also directly bind the receptor ECD (27, 28) and require the ECD for signaling (Fig. 10), consistent with a critical role of the ECD in mediating conformational changes in the TMD and intracellular loops. In contrast, BETP, a small molecule GLP-1R modulator that does not bind the ECD, signals through a distinct mechanism that appears to function in parallel to GLP-1 (Refs. 32, 34, and 35) and that induces a distinct ("biased") downstream signaling response (34). Surprisingly, the ability of BETP but not GLP-1 to stimulate GLP-1R signaling depended on Cys-347 in ICL3 (Fig. 10*C*), the same residue that has been shown to
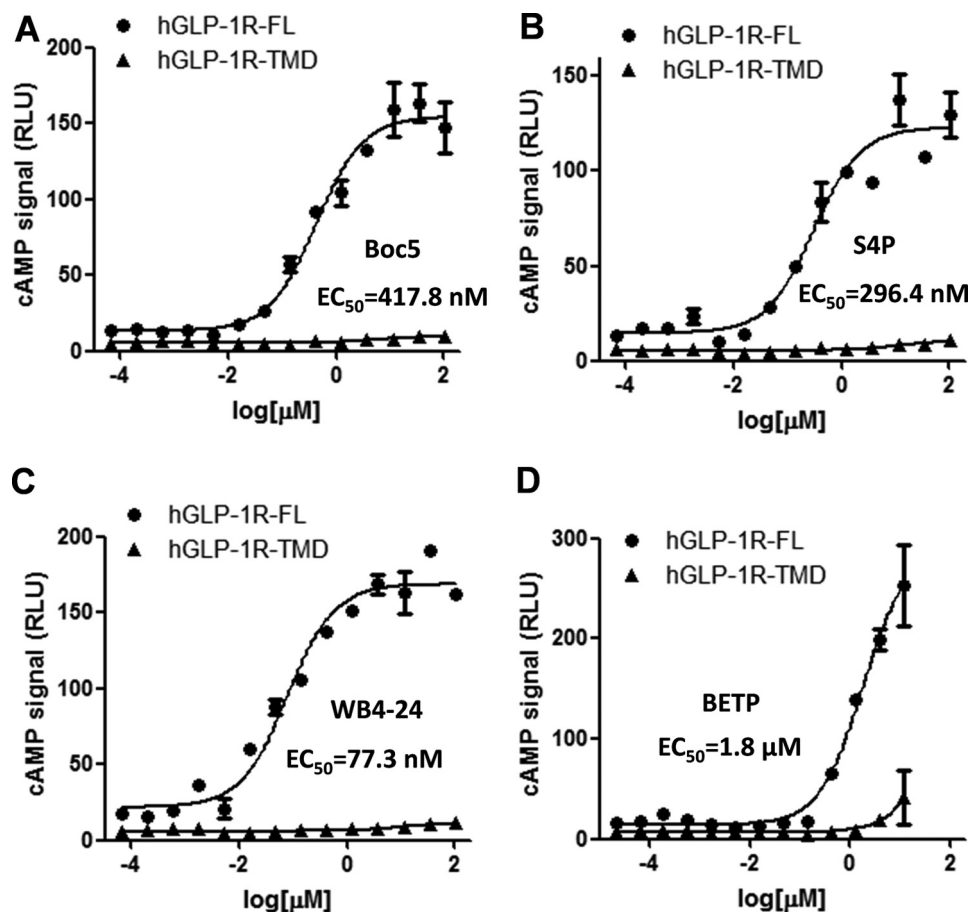
FIGURE 11. **Concentration curves of small molecules in activation of full-length human GLP-1R and TMD.** All data are presented as cAMP signal (*RLU*); *n* = 3, *error bars* = S.D. The EC$_{50}$ values for the compounds are noted in the figures. The large deviation of BETP at the 30 μM data point is caused by the toxicity of this compound at high concentrations. *FL*, full-length.

be covalently modified by BETP. These results suggest that the ECD played a direct role in the activation of GLP-1R, even by the small molecule agonists that act on the intracellular side of the receptor.

The inability to bypass the tethering function of the GCGR and GLP-1R ECDs implies a mechanism that hormone binding to the ECDs of GCGR and GLP-1R directly affects the ECD-TMD interaction (Fig. 12*C*), which could contribute to conformational changes and activation of the receptors. Strong evidence has been presented for an intramolecular ECD-TMD interaction in GCGR that is regulated by glucagon (22, 23). It has been proposed that the α1-helix of the ECD interacts with the extended first transmembrane helix (the TM1 stalk) and the extracellular loops of the TMD to auto-inhibit GCGR activity under basal conditions and that this interaction is disrupted by glucagon binding (22, 23). Consistent with this model, we observed that mutations in α1 residues mildly increase GCGR activity (Fig. 6*C*). However, we unexpectedly found that activity-compromising mutations in residues that are not involved in ligand binding and that do not affect the level of membrane-bound receptor all localize to a contiguous surface of the ECD. This surface faces away from hormone and TMD and thus may form an interface for a currently unidentified interaction that is important for receptor activation. We speculate that this proposed ECD-TMD interface (Fig. 12*C*) could serve as a possible

mechanism for the requirement of the ECD for activation of GCGR and GLP-1R. In addition, our results presented in this paper provide a basis for re-classifying class B GPCRs based on their differential requirement of the ECDs for receptor activation.

Experimental Procedures

Reagents—Restriction enzymes were purchased from Fermentas. DNA polymerase was obtained from New England Bio-Labs. DMEM/high glucose and fetal bovine serum (FBS) were from HyClone and Gibco. Trypsin-EDTA, phosphate-buffered saline (PBS), GlutaMAX, HEPES, sodium pyruvate, and sodium butyrate were procured from Gibco. Doxycycline was from Sigma.

Peptide Synthesis—Human PTH(1–34), glucagon(1–29), GLP-1(7–36), PACAP(1–27), and CRF(1–41) peptides for cAMP assays were custom-synthesized and HPLC-purified by GL Biochem (Shanghai) Ltd. Peptides were dissolved and diluted in H$_2$O.

Small Molecule GLP-1R Modulators—Boc5, S4P, WB4–24 and pyrimidine BETP were synthesized in-house with purities >95% using published procedures (37, 38). These compounds were dissolved in 100% DMSO and diluted in buffer (final DMSO concentration was 1%) and added at 10 μM final com-
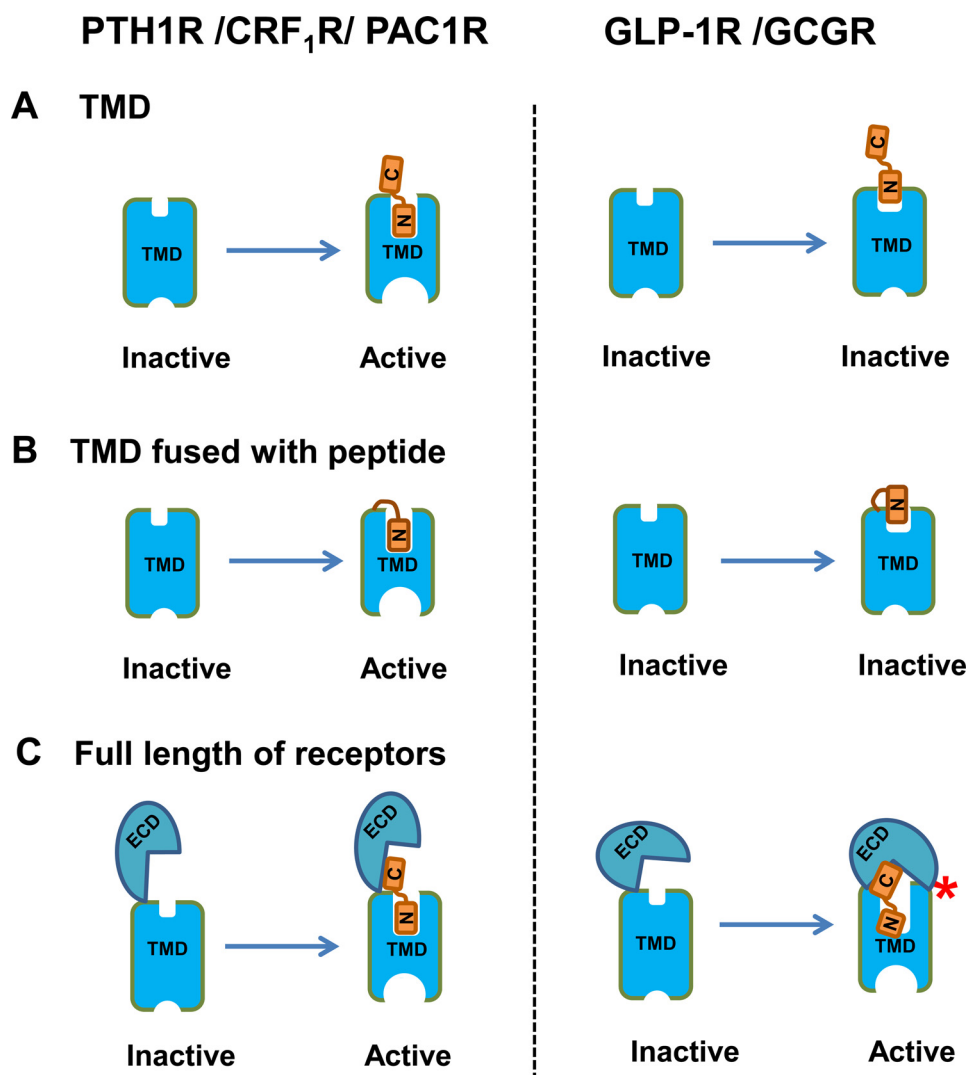
PTH1R /CRF₁R/ PAC1R
GLP-1R /GCGR

FIGURE 12. **A working model for the differential requirement of ECD for activation of Class B GPCRs.** *A*, the TMDs of PTH1R, CRF₁R, and PAC1R are activated by exogenous peptides, whereas the TMDs of GLP-1R and GCGR cannot be activated by exogenous peptide. *B*, the TMDs of PTH1R, CRF₁R, and PAC1R can be activated by the fused peptide, but the TMDs of GLP-1R and GCGR cannot be activated by the fused peptide. *C*, all full-length receptors of class B GPCR can be activated by the peptide. The requirement of the ECD for activation of GCGR/GLP-1R indicates a possible ECD-TMD contact that is marked with a *red star*.

pound concentration. Equivalent DMSO concentration was added to control samples.

Plasmid Construction—Codon-optimized cDNAs of human class B GPCRs lacking the predicted signal peptide coding region were synthesized by Genewiz (CRF₁R (residues 23–384), PAC1R (residues 22–422), PTH1R (residues 27–486), GCGR (residues 26–431), and GLP-1R (residues 24–463)). The encoded fragments were digested with BamHI and NotI restriction endonucleases and inserted into a modified pcDNA6 expression vector to encode a fusion protein consisting of an N-terminal human IgG leader (MGWSCIILFL-VATATGVHSE) for targeting into the cell membrane and a FLAG tag (DYKDDDD) at the C terminus for detection by immunoblotting. In addition to the full-length receptors, we generated the same set of constructs with (i) the TMDs of GCGR (residues 123–431), GLP-1R (residues 140–463), PTH1R (residues 182–484), CRF₁R (residues 110–384), and PAC1R (residues 148–421), (ii) fusions between the N-terminal peptide hormones (glucagon (1–15), GLP-1(7–21), PTH(1–

15), UNC(1–15), PACAP(1–12)) and their corresponding TMDs, (iii) fusions of the same N-terminal peptide fragments as above to the BRIL-TMD constructs, and (iv) fusions of the full-length peptide hormones to the N terminus of their corresponding full-length receptors with five copies of Gly-Ser-Ala (GSA₅) linker. All these constructs contain the same IgG leader as above. The TMD and full-length PAC1R constructs were N-terminally tagged with two copies of maltose-binding protein, which was used for affinity tag purification.

Mutagenesis—Thirty single amino acid mutations were made in the GCG-GCGR fusion receptor, including W36A, D63A, P86S, F31R, E34R, K37R, L38R, D41R, H45R, L49R, L56R, N74R, T76R, N78R, W83R, H89R, V91L, F95R, F97R, R99E, D103R, V107R, P114R, W115R, D117N, S119R, and Q120R, which collectively span the entire length of the ECD, and Y10R, Y13R, and W25R, which are residues in the fused glucagon. Site-directed mutagenesis experiments were carried out using the QuikChange method (Agilent). Mutations and all plasmid constructs were confirmed by DNA sequencing.

Cell Culture—A HEK293 suspension cell line was cultured in DMEM/high glucose medium with L-glutamine (Hyclone) supplemented with 10% FBS (Gibco). Cells were incubated at 37 °C and supplemented with 5% CO_2 in a humidified chamber. When cells reached 80–90% confluence they were detached by trypsinization and reseeded by 4–6-fold dilution into fresh medium. They were also seeded in 24-well plates when needed, and plasmids were transfected into the cells. Protein expression was induced by adding doxycycline and sodium butyrate at 1 μg/ml and 10 mM, respectively. After induction of protein expression, cells were incubated for 18–24 h until collected for further use.

Transfection and Luciferase Assays—Cells were plated at a density of 5×10^4 per well in a 24-well plate and incubated overnight at 37 °C. Cells were then transiently transfected using Lipofectamine reagent (Life Technologies) with expression plasmids of a CRE-driven fly luciferase reporter (200 ng) and a TK promoter-driven renilla luciferase (10 ng), which was used as an internal transfection control. Three hours after transfection, the cells were treated with 1 μM doxycycline for 24 h, then peptides or ligands were added to cells for a 4-h incubation.

After 4-h incubation with peptides or small molecule GLP-1R modulators, cells were harvested and lysed in Passive Lysis Buffer (Promega). The cAMP concentration in lysate was determined using the dual-luciferase reporter assay system from Promega according to the manufacturer's instructions with an EnVision plate reader (PerkinElmer Life Sciences). Renilla luciferase was used for internal transfection controls. We assessed GPCR activity by the cAMP signal, which is the activity of CRE-driven fly luciferase reporter relative to renilla luciferase activity. The first measurement (firefly luciferase (RLU) and the second measurement (Renilla luciferase) were measured sequentially form a single sample. All experiments were performed in triplicated transfection experiments.

Membrane-tethered Ligands—Codon-optimized sequences encoding glucagon(1–29) and GLP-1(7–36), were synthesized by GENEWIZ. These sequences were cloned in a modified pcDNA6 expression vector that contains an IgG leader (MGWSCIILFLVATATGVHSE) followed by the peptide hormone, a MYC tag (EQKLISEEDL), a NG5 linker (GNGNGNGNGNGNGNGNGNG), a single pass TMD (ALCWVGLGVLAAGVLVVTAIVYV) with a 3×FLAG tag at the C terminus of the fusion constructs. These constructs followed the same design as the published membrane-anchored PTH (19). IgG leader is the signal peptide that guides the secretory protein into the cell membrane. Constructs were transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen).

Western Blots—Cells were harvested by centrifugation, and their pellets were solubilized in cell lysis reagent (CelLytic™ M, Sigma) supplemented with 1 mM PMSF and centrifuged at $16,100 \times g$ for 30 min. The supernatants were subjected to reducing and non-reducing SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% milk in TBST (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) and incubated with anti-FLAG M2 antibodies produced in mouse (Sigma) or monoclonal anti-β-actin antibody produced in mouse clone AC-15 (Sigma) followed by HRP-conjugated anti-mouse antibodies in 5% milk dissolved in TBST. Protein bands were detected by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific), and images were collected using an ChemiDocTM XRS+ imager (Bio-Rad).

FACS Analysis—To prepare samples for FACS, the cells were washed twice with PBS resuspended in PBS containing 0.1% BSA and incubated for 15 min on ice with monoclonal anti-FLAG® M2-FITC antibody produced in mouse (Sigma) (1 μg per 10^6 cells), and samples were analyzed using a BD Accuri™ C6 Flow Cytometer instrument using Accuri C6 software (BD Biosciences).

Statistical Analysis—GraphPad Prism software version 5.0 (GraphPad Software Inc., San Diego, CA) was used to fit data to a 3-parameter dose-response curve. All data are presented as the mean ± S.D.

References

1. Pal, K., Swaminathan, K., Xu, H. E., and Pioszak, A. A. (2010) Structural basis for hormone recognition by the Human CRFR2α G protein-coupled receptor. *J. Biol. Chem.* **285,** 40351–40361
2. Parthier, C., Reedtz-Runge, S., Rudolph, R., and Stubbs, M. T. (2009) Passing the baton in class B GPCRs: peptide hormone activation via helix induction? *Trends Biochem. Sci.* **34,** 303–310
3. Archbold, J. K., Flanagan, J. U., Watkins, H. A., Gingell, J. J., and Hay, D. L. (2011) Structural insights into RAMP modification of secretin family G protein-coupled receptors: implications for drug development. *Trends Pharmacol. Sci.* **32,** 591–600
4. Hoare, S. R. (2005) Mechanisms of peptide and nonpeptide ligand binding to Class B G-protein-coupled receptors. *Drug Discov. Today* **10,** 417–427
5. Castro, M., Nikolaev, V. O., Palm, D., Lohse, M. J., and Vilardaga, 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
6. 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 in complex with the extracellular domain of the glucagon-like peptide-1 receptor. *J. Biol. Chem.* **285,** 723–730
7. Kumar, S., Pioszak, A., Zhang, C., Swaminathan, K., and Xu, H. E. (2011) Crystal structure of the PAC1R extracellular domain unifies a consensus fold for hormone recognition by class B G-protein-coupled receptors. *PLoS ONE* **6,** e19682
8. 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
9. 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
10. Grace, C. R., Perrin, M. H., Gulyas, J., Rivier, J. E., Vale, W. W., and Riek, R. (2010) NMR structure of the first extracellular domain of corticotropin-releasing factor receptor 1 (ECD1-CRF-R1) complexed with a high affinity agonist. *J. Biol. Chem.* **285,** 38580–38589
11. Pal, K., Melcher, K., and Xu, H. E. (2012) Structure and mechanism for recognition of peptide hormones by Class B G-protein-coupled receptors. *Acta Pharmacol. Sin.* **33,** 300–311

12. Siu, F. Y., He, M., de Graaf, C., Han, G. W., Yang, D., Zhang, Z., Zhou, C., Xu, Q., Wacker, D., Joseph, J. S., Liu, W., Lau, J., Cherezov, V., Katritch, V., Wang, M. W., and Stevens, R. C. (2013) Structure of the human glucagon class B G-protein-coupled receptor. *Nature* **499,** 444–449

13. Jazayeri, A., Doré, A. S., Lamb, D., Krishnamurthy, H., Southall, S. M., Baig, A. H., Bortolato, A., Koglin, M., Robertson, N. J., Errey, J. C., Andrews, S. P., Teobald, I., Brown, A. J., Cooke, R. M., Weir, M., and Marshall, F. H. (2016) Extra-helical binding site of a glucagon receptor antagonist. *Nature* **533,** 274–277

14. Hollenstein, K., Kean, J., Bortolato, A., Cheng, R. K., Doré, A. S., Jazayeri, A., Cooke, R. M., Weir, M., and Marshall, F. H. (2013) Structure of class B GPCR corticotropin-releasing factor receptor 1. *Nature* **499,** 438–443

15. Bortolato, A., Doré, A. S., Hollenstein, K., Tehan, B. G., Mason, J. S., and Marshall, F. H. (2014) Structure of Class B GPCRs: new horizons for drug discovery. *Br J. Pharmacol.* **171,** 3132–3145

16. Chun, E., Thompson, A. A., Liu, W., Roth, C. B., Griffith, M. T., Katritch, V., Kunken, J., Xu, F., Cherezov, V., Hanson, M. A., and Stevens, R. C. (2012) Fusion partner toolchest for the stabilization and crystallization of G protein-coupled receptors. *Structure* **20,** 967–976

17. Ruiter, M., La Fleur, S. E., van Heijningen, C., van der Vliet, J., Kalsbeek, A., and Buijs, R. M. (2003) The daily rhythm in plasma glucagon concentrations in the rat is modulated by the biological clock and by feeding behavior. *Diabetes* **52,** 1709–1715

18. Gravholt, C. H., Møller, N., Jensen, M. D., Christiansen, J. S., and Schmitz, O. (2001) Physiological levels of glucagon do not influence lipolysis in abdominal adipose tissue as assessed by microdialysis. *J. Clin. Endocrinol. Metab* **86,** 2085–2089

19. Nielsen, S. M., Nielsen, L. Z., Hjorth, S. A., Perrin, M. H., and Vale, W. W. (2000) Constitutive activation of tethered-peptide/corticotropin-releasing factor receptor chimeras. *Proc. Natl. Acad. Sci. U.S.A.* **97,** 10277–10281

20. Fortin, J. P., Zhu, Y., Choi, C., Beinborn, M., Nitabach, M. N., and Kopin, A. S. (2009) Membrane-tethered ligands are effective probes for exploring class B1 G protein-coupled receptor function. *Proc. Natl. Acad. Sci. U.S.A.* **106,** 8049–8054

21. Zhou, C., Dhall, D., Nissen, N. N., Chen, C. R., and Yu, R. (2009) Homozygous P86S mutation of the human glucagon receptor is associated with hyperglucagonemia, alpha cell hyperplasia, and islet cell tumor. *Pancreas* **38,** 941–946

22. Koth, C. M., Murray, J. M., Mukund, S., Madjidi, A., Minn, A., Clarke, H. J., Wong, T., Chiang, V., Luis, E., Estevez, A., Rondon, J., Zhang, Y., Hötzel, I., and Allan, B. B. (2012) Molecular basis for negative regulation of the glucagon receptor. *Proc. Natl. Acad. Sci. U.S.A.* **109,** 14393–14398

23. Yang, L., Yang, D., de Graaf, C., Moeller, A., West, G. M., Dharmarajan, V., Wang, C., Siu, F. Y., Song, G., Reedtz-Runge, S., Pascal, B. D., Wu, B., Potter, C. S., Zhou, H., Griffin, P. R., Carragher, B., Yang, H., Wang, M. W., Stevens, R. C., and Jiang, H. (2015) Conformational states of the full-length glucagon receptor. *Nat. Commun.* **6,** 7859

24. Wang, M. W., Liu, Q., and Zhou, C. H. (2010) Non-peptidic glucose-like peptide-1 receptor agonists: aftermath of a serendipitous discovery. *Acta Pharmacol. Sin.* **31,** 1026–1030

25. Willard, F. S., Bueno, A. B., and Sloop, K. W. (2012) Small molecule drug discovery at the glucagon-like peptide-1 receptor. *Exp. Diabetes Res.* **2012,** 709893

26. Wootten, D., Savage, E. E., Willard, F. S., Bueno, A. B., Sloop, K. W., Christopoulos, A., and Sexton, P. M. (2013) Differential activation and modulation of the glucagon-like peptide-1 receptor by small molecule ligands. *Mol. Pharmacol.* **83,** 822–834

27. Chen, D., Liao, J., Li, N., Zhou, C., Liu, Q., Wang, G., Zhang, R., Zhang, S., Lin, L., Chen, K., Xie, X., Nan, F., Young, A. A., and Wang, M. W. (2007) A nonpeptidic agonist of glucagon-like peptide 1 receptors with efficacy in diabetic db/db mice. *Proc. Natl. Acad. Sci. U.S.A.* **104,** 943–948

28. Liu, Q., Li, N., Yuan, Y., Lu, H., Wu, X., Zhou, C., He, M., Su, H., Zhang, M., Wang, J., Wang, B., Wang, Y., Ma, D., Ye, Y., Weiss, H. C., Gesing, E. R., Liao, J., and Wang, M. W. (2012) Cyclobutane derivatives as novel nonpeptidic small molecule agonists of glucagon-like peptide-1 receptor. *J. Med. Chem.* **55,** 250–267

29. Fan, H., Gong, N., Li, T. F., Ma, A. N., Wu, X. Y., Wang, M. W., and Wang, Y. X. (2015) The non-peptide GLP-1 receptor agonist WB4–24 blocks inflammatory nociception by stimulating beta-endorphin release from spinal microglia. *Br J. Pharmacol.* **172,** 64–79

30. Thorens, B., Porret, A., Bühler, L., Deng, S. P., Morel, P., and Widmann, C. (1993) Cloning and functional expression of the human islet GLP-1 receptor: demonstration that exendin-4 is an agonist and exendin-(9–39) an antagonist of the receptor. *Diabetes* **42,** 1678–1682

31. Sloop, K. W., Willard, F. S., Brenner, M. B., Ficorilli, J., Valasek, K., Showalter, A. D., Farb, T. B., Cao, J. X., Cox, A. L., Michael, M. D., Gutierrez Sanfeliciano, S. M., Tebbe, M. J., and Coghlan, M. J. (2010) Novel small molecule glucagon-like peptide-1 receptor agonist stimulates insulin secretion in rodents and from human islets. *Diabetes* **59,** 3099–3107

32. Knudsen, L. B., Kiel, D., Teng, M., Behrens, C., Bhumralkar, D., Kodra, J. T., Holst, J. J., Jeppesen, C. B., Johnson, M. D., de Jong, J. C., Jorgensen, A. S., Kercher, T., Kostrowicki, J., Madsen, P., Olesen, P. H., Petersen, J. S., Poulsen, F., Sidelmann, U. G., Sturis, J., Truesdale, L., May, J., and Lau, J. (2007) Small-molecule agonists for the glucagon-like peptide 1 receptor. *Proc. Natl. Acad. Sci. U.S.A.* **104,** 937–942

33. Koole, C., Wootten, D., Simms, J., Valant, C., Sridhar, R., Woodman, O. L., Miller, L. J., Summers, R. J., Christopoulos, A., and Sexton, P. M. (2010) Allosteric ligands of the glucagon-like peptide 1 receptor (GLP-1R) differentially modulate endogenous and exogenous peptide responses in a pathway-selective manner: implications for drug screening. *Mol. Pharmacol.* **78,** 456–465

34. Wootten, D., Savage, E. E., Valant, C., May, L. T., Sloop, K. W., Ficorilli, J., Showalter, A. D., Willard, F. S., Christopoulos, A., and Sexton, P. M. (2012) Allosteric modulation of endogenous metabolites as an avenue for drug discovery. *Mol. Pharmacol.* **82,** 281–290

35. Nolte, W. M., Fortin, J. P., Stevens, B. D., Aspnes, G. E., Griffith, D. A., Hoth, L. R., Ruggeri, R. B., Mathiowetz, A. M., Limberakis, C., Hepworth, D., and Carpino, P. A. (2014) A potentiator of orthosteric ligand activity at GLP-1R acts via covalent modification. *Nat. Chem. Biol.* **10,** 629–631

36. Runge, S., Wulff, 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,** 787–794

37. He, M., Guan, N., Gao, W. W., Liu, Q., Wu, X. Y., Ma, D. W., Zhong, D. F., Ge, G. B., Li, C., Chen, X. Y., Yang, L., Liao, J. Y., and Wang, M. W. (2012) A continued saga of Boc5, the first non-peptidic glucagon-like peptide-1 receptor agonist with in vivo activities. *Acta Pharmacol. Sin.* **33,** 148–154

38. Eng, H., Sharma, R., McDonald, T. S., Edmonds, D. J., Fortin, J. P., Li, X., Stevens, B. D., Griffith, D. A., Limberakis, C., Nolte, W. M., Price, D. A., Jackson, M., and Kalgutkar, A. S. (2013) Demonstration of the innate electrophilicity of 4-(3-(benzyloxy)phenyl)-2-(ethylsulfinyl)-6-(trifluoromethyl)pyrimidine (BETP), a small-molecule positive allosteric modulator of the glucagon-like peptide-1 receptor. *Drug Metab. Dispos.* **41,** 1470–1479