

Ligand Binding Pocket Formed by Evolutionarily Conserved Residues in the Glucagon-like Peptide-1 (GLP-1) Receptor Core Domain*

Received for publication, September 17, 2014, and in revised form, December 9, 2014. Published, JBC Papers in Press, January 5, 2015, DOI 10.1074/jbc.M114.612606

Mi Jin Moon^{†1}, Yoo-Na Lee[‡], Sumi Park[‡], Arfaxad Reyes-Alcaraz[‡], Jong-Ik Hwang[‡], Robert Peter Millar[§], Han Choe^{¶1,2}, and Jae Young Seong^{‡3}

From the [†]Graduate School of Medicine, Korea University, Seoul 136-705, Republic of Korea, [§]Mammal Research Institute, Department of Zoology and Entomology, University of Pretoria, Pretoria 0028, Medical Research Council Receptor Biology Unit, and University of Cape Town, Cape Town 7925, South Africa, and Centre for Integrative Physiology, University of Edinburgh, Edinburgh EH164TJ, Scotland, and [¶]Department of Physiology and Bio-Medical Institute of Technology, University of Ulsan College of Medicine, Seoul 138-736, Korea

Background: Little is known about the interaction between GLP-1 and the heptahelical core domain of GLP1R.

Results: GLP-1 Asp⁹ and Gly⁴ interact with the evolutionarily conserved residues in extracellular loop 3.

Conclusion: Ligand binding pocket formed by evolutionarily conserved residues in the GLP1R core domain.

Significance: This study highlights the mechanism underlying high affinity interaction between GLP-1 and the binding pocket of the receptor.

Glucagon-like peptide-1 (GLP-1) plays a pivotal role in glucose homeostasis through its receptor GLP1R. Due to its multiple beneficial effects, GLP-1 has gained great attention for treatment of type 2 diabetes and obesity. However, little is known about the molecular mechanism underlying the interaction of GLP-1 with the heptahelical core domain of GLP1R conferring high affinity ligand binding and ligand-induced receptor activation. Here, using chimeric and point-mutated GLP1R, we determined that the evolutionarily conserved amino acid residue Arg³⁸⁰ flanked by hydrophobic Leu³⁷⁹ and Phe³⁸¹ in extracellular loop 3 (ECL3) may have an interaction with Asp⁹ and Gly⁴ of the GLP-1 peptide. The molecular modeling study showed that Ile¹⁹⁶ at transmembrane helix 2, Met²³³ at ECL1, and Asn³⁰² at ECL2 of GLP1R have contacts with His¹ and Thr⁷ of GLP-1. This study may shed light on the mechanism underlying high affinity interaction between the ligand and the binding pocket that is formed by these conserved residues in the GLP1R core domain.

Glucagon-like peptide-1 (GLP-1)⁴ is generated by tissue-specific posttranslational processing of proglucagon in intestinal L-cells and plays a key role in glucose homeostasis (1). Activation of the GLP-1 receptor (GLP1R) in pancreatic β -cells by

GLP-1 potentiates glucose-dependent insulin secretion (2, 3). In addition to its insulinotropic effects, GLP-1 promotes growth, survival, and differentiation of β -cells (4, 5). Furthermore, GLP-1 slows down gastric emptying and promotes satiety. Thus, sustained activation of GLP1R results in weight loss (6, 7). Because of these multiple beneficial effects that regulate blood glucose concentration and body weight, GLP-1 is a promising therapeutic agent for the treatment of type 2 diabetes mellitus and obesity. However, circulating GLP-1 is rapidly degraded by dipeptidyl peptide-IV and cannot be administered orally due to its peptidergic chemical nature (8, 9). Thus, there is a great need to develop orally active small molecules that can act on GLP1R (10, 11). The delineation of high affinity ligand-receptor binding and receptor activation will contribute to the development of such molecules.

GLP1R is a member of the class B G protein-coupled receptor (GPCR) family, which includes the glucagon receptor (GCGR) subfamily consisting of five members GCGR, GLP1R, GLP2R, glucose-dependent insulinotropic polypeptide receptor (GIPR), and glucagon-related peptide receptor (GCRPR) (12–15). In addition, growth hormone-releasing hormone receptor (GHRHR), secretin receptor (SCTR), vasoactive intestinal peptide receptor 1 (VPAC1 receptor (VPAC1R)), VPAC2R, and pituitary adenylate cyclase-activating polypeptide receptor (PAC1 receptor, PAC1R) share amino acid sequence similarity with members of the GCGR subfamily (13, 16, 17). Class B GPCRs have a relatively long (~120 amino acids) N-terminal extracellular domain (ECD) with an α -helix at the N terminus and two antiparallel β sheets stabilized by three disulfide bonds and a salt bridge (18–21). The peptide ligands for this receptor family also share a common structure consisting of a random coiled N terminus followed by an α -helix (20, 22, 23).

According to the two-domain model for class B GPCR activation, the second half of the α -helix of the peptide binds to the

* This work was supported by grants from Basic Science Research Program (2013R1A1A2010481) of the National Research Foundation of Korea and Korea-South Africa Collaboration Program (2012K1A3A1A09033014) of the National Research Foundation of South Africa.

¹ Supported by a research fellowship grant from Korea University.

² To whom correspondence may be addressed. Tel.: 82-2-3010-4292; Fax: 82-2-3010-8148; E-mail: hchoe@ulsan.ac.kr.

³ To whom correspondence may be addressed. Tel.: 82-2-2286-1090; Fax: 82-2-921-4355; E-mail: jyseong@korea.ac.kr.

⁴ The abbreviations used are: GLP-1, glucagon-like peptide-1; GLP1R, GLP-1 receptor; GLP2R, GLP-2 receptor; ECD, extracellular domain; ECL, extracellular loop; GCGR, glucagon receptor; GCRPR, glucagon-related peptide (GCRP) receptor; GIP, glucose-dependent insulinotropic polypeptide; GIPR, GIP receptor; GPCR, G protein-coupled receptor; TMH, transmembrane helix; VPAC1R, VPAC1 receptor; PAC1R, PAC1 receptor.

TABLE 1

Amino acid sequences of modified GLP-1 peptides

Gly⁴ and Asp⁹ of GLP-1 were replaced with charged amino acids. Modified positions are shown in bold.

Peptides			
GLP-1	¹ HAEGTFTSDV	¹¹ SSYLEGQAAK	²¹ EFIAWLKGR
[Asp ⁴]GLP-1	¹ HAEDTFTSDV	¹¹ SSYLEGQAAK	²¹ EFIAWLKGR
[Glu ⁴]GLP-1	¹ HAEEFTSDV	¹¹ SSYLEGQAAK	²¹ EFIAWLKGR
[His ⁴]GLP-1	¹ HAHTFTSDV	¹¹ SSYLEGQAAK	²¹ EFIAWLKGR
[Lys ⁴]GLP-1	¹ HAKTFTSDV	¹¹ SSYLEGQAAK	²¹ EFIAWLKGR
[Arg ⁴]GLP-1	¹ HAERTFTSDV	¹¹ SSYLEGQAAK	²¹ EFIAWLKGR
[Glu ⁹]GLP-1	¹ HAEGTFTSEV	¹¹ SSYLEGQAAK	²¹ EFIAWLKGR
[Lys ⁹]GLP-1	¹ HAEGTFTSKV	¹¹ SSYLEGQAAK	²¹ EFIAWLKGR
[Arg ⁹]GLP-1	¹ HAEGTFTSRV	¹¹ SSYLEGQAAK	²¹ EFIAWLKGR

N-terminal ECD of the receptor (24–26). This induces a secondary interaction between the N-terminal moiety of the peptide and the receptor core domain consisting of transmembrane helices (TMHs) and extracellular loops (ECLs). This secondary interaction confers receptor activation and G protein coupling (27–29). The interactions between the N-terminal ECD of GLP1R and the second half of the α -helical part of GLP-1 and exendin-4 have been elucidated by x-ray crystallography (20, 22). However, a crystal structure for the ligand-bound receptor core domain is not yet available. This structure would provide useful information for understanding the mechanism of ligand-induced receptor activation.

Alanine scanning analysis revealed that His¹, Gly⁴, Thr⁷, and Asp⁹ in the N-terminal portion of GLP-1 are important for receptor binding and activation (30, 31). Recently, by using chimeric GLP1R/GIPR together with chimeric GLP-1/GIP peptides, we identified interactions of His¹ and Thr⁷ of GLP-1 with Ile¹⁹⁶/Lys¹⁹⁷ at TMH2, Met²³³ at ECL1, and Asn³⁰² at ECL2 of GLP1R (32). These results demonstrated the evolutionary pressure to conserve critical residues for ligand binding and activation of GLP1R (33). However, this study did not fully account for the ligand binding pocket of GLP1R, which may require additional residues, probably located at ECL3, for interaction with Gly⁴ and Asp⁹ of GLP-1.

In the present study we constructed chimeric GLP1Rs in which the GLP1R ECL3 was replaced with the VPAC1R ECL3, which has a markedly different amino acid sequence. This chimeric receptor responded poorly to GLP-1, showing the importance of ECL3 for ligand-induced receptor activation. Additional experiments in which single amino acid mutations were introduced into GLP1R ECL3 revealed that the evolutionarily conserved basic residue Arg³⁸⁰ and the flanking hydrophobic residues Leu³⁷⁹ and Phe³⁸¹ were likely to mediate interactions with Gly⁴ and Asp⁹ of GLP-1. Based on this observation and our previous result (33), we propose that the ligand binding pocket of GLP1R is formed by evolutionarily conserved residues in TMH2, ECL1, ECL2, and ECL3.

EXPERIMENTAL PROCEDURES

Peptides—Wild-type GLP-1, glucagon, GCRP, GIP, GLP-2, and modified peptides were synthesized by AnyGen (Gwangju, Korea). The amino acid sequences of these peptides are shown in Table 1 and Fig. 7A.

Plasmids—The cDNA encoding human GLP1R was originally purchased from Benebiosis (Seoul, Korea) and subcloned into the mammalian expression vector pcDNA3 (Invitrogen).

Human VPAC1R, GLP2R, and GCGR in pcDNA3 were obtained from BRNscience Inc. (Seoul, Korea). Human GIPR was kindly provided by Dr. Bernard Thorens (Institute of Pharmacology and Toxicology, Switzerland). Chicken GCRPR was cloned from a White Leghorn hen brain cDNA library (15). The CRE-luc vector, which contains four copies of CRE (TGACGTCA), was obtained from Stratagene (La Jolla, CA).

Construction of Chimeras and Mutants—To swap domains between GLP1R and VPAC1R, individual cDNA fragments of interest were amplified by PCR with Pfu polymerase (ELPIS Biotech., Daejeon, Korea) and two specific primers. One primer corresponded to the 5' or 3' end of the receptor cDNAs, and the other primer corresponded to the region of overlap between the two receptors. One fragment was obtained from GLP1R, and the other was from VPAC1R. Both fragments were subjected to a second round of PCR to generate chimeric cDNAs. All of the chimeric constructs were cloned into the pcDNA3 expression vector at the HindIII and XhoI sites. The single and double mutants were constructed by PCR-based site-directed mutagenesis and cloned into pcDNA3 at the HindIII and XhoI sites. The DNA sequences of the chimeras and mutants were verified by automatic sequencing.

Cell Transfection and Luciferase Assays—HEK293T cells were maintained in Dulbecco's modified Eagle's media (DMEM) in the presence of 10% fetal bovine serum. For luciferase assays, cells (2.5×10^4) were plated in 48-well plates 1 day before transfection and transfected with Effectene reagent (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. Approximately 48 h after transfection, cells were treated with the respective ligands for 6 h. Cells were harvested 6 h after ligand treatment. Luciferase activities were determined in cell extracts with a luciferase assay system according to the standard methods for the Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT).

Binding Assay—GLP-1 and [Arg⁹]GLP-1 were radioiodinated by the chloramine-T method and purified by chromatography on a Sephadex G-25 column (Sigma) in 0.01 M acetic acid and 0.1% bovine serum albumin (BSA). Cells (1.2×10^5) were transfected with wild-type or mutant receptors (300 ng of DNA/well in 12-well plates) with Effectene (Qiagen). Forty-eight hours later, cells were washed and incubated for 1 h at 37 °C with binding buffer (serum-free DMEM with 0.1% BSA, pH 7.4) containing 100,000 cpm ¹²⁵I-labeled ligand (equivalent to ~30 nM) in the presence of various concentrations of cold ligand. Relative expression levels of receptors were determined using 500,000 cpm ¹²⁵I-labeled ligand (a concentration for submaximal binding toward the wild-type receptor) in the presence of 10 μ M cold ligand. Cells were washed twice with ice-cold Dulbecco's PBS. The radioactivity of the cell lysate resolved in 1% SDS and 0.2 M NaOH was determined on a Wallac 1489 Wizard 3 γ -counter (PerkinElmer Life Sciences).

Data Analysis—Data analysis was performed by nonlinear regression with a sigmoid dose-response curve. Agonist concentrations that induced half-maximal stimulation (EC₅₀) were calculated with GraphPad PRISM4 software (GraphPad Software Inc., San Diego, CA). All data are presented as the means \pm S.E. of at least three independent experiments. Group means were compared by Student's *t* test or one-way analysis of

Ligand Binding Pocket of the GLP-1 Receptor

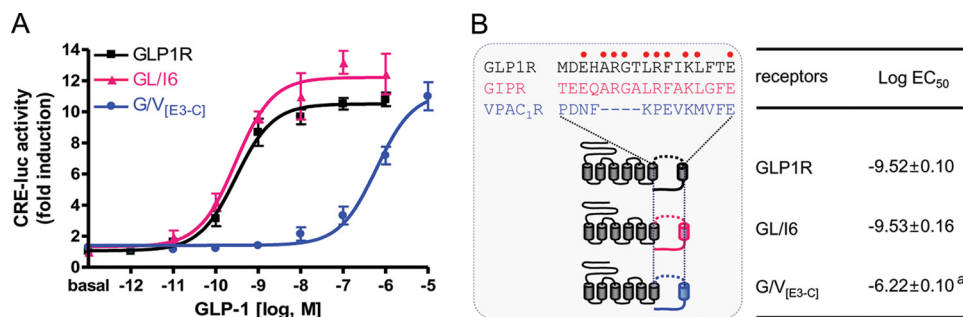


FIGURE 1. **GLP-1 potencies toward GLP1R chimeric receptors.** *A*, the GLP-1 potency of the wild-type and chimeric GLP1Rs with the sequence from ECL3 to C terminus of GIPR (*GL/I6*) or of VPAC1R (*G/V_[E3-C]*). *B*, schematic diagram for chimeric receptors and log EC₅₀ values of GLP-1. Amino acid sequences of ECL3 of GLP1R, GIPR, and VPAC1R were aligned above the receptor structures. The conserved residues between GLP1R and GIPR are indicated as *red dots*. The data on the sigmoidal curves and log EC₅₀ values represent the means ± S.E. of at least three independent experiments with triplicates. ^a, versus wild-type GLP1R ($p < 0.05$).

variance followed by the Bonferroni's multiple comparison test. $p < 0.05$ was considered to be statistically significant.

Molecular Modeling—A homology model for GLP1R and GLP-1 interaction was built with the homology modeling program MODELLER 9v11 (34) and was based on the crystal structures of two class B GPCRs (Protein Data Bank codes 4k5y, and 4l6r) and the ligand-bound ECD of human GLP1R and GIPR (Protein Data Bank codes 3iol and 2qkh). The sequence of GLP1R was manually aligned to the GPCRs of the crystal structures based on TMHs predicted by TMHMM Server v.2.0 (35) and evolutionarily conserved residues. During homology modeling, a disulfide bond was forced between Cys²²⁶ and Cys²⁹⁶ of GLP1R. The distance restraints between the following residue pairs were introduced: His¹ of GLP-1 ~ Asn³⁰² of GLP1R; Thr⁷ of GLP-1 ~ Ile¹⁹⁶, Lys¹⁹⁷, Met²³³, and Met³⁰² of GLP1R; Gly⁴ and Asp⁹ of GLP-1 ~ Arg³⁸⁰ of GLP1R. An α -helical secondary structure was enforced for each TMH. The variable target function method with the very thorough option and molecular dynamics optimization with the thorough option were applied during model building. All structural analyses and figure preparations were performed with ICM Version 3.7–3b (Molsoft, San Diego, CA) and Ligplot⁺ Version 1.4.3 (36). The electrostatic potential of the protein surface was calculated with the REBEL method, which solves the Poisson equation with the boundary element method (37). Structures for the mutants were generated by exchanging residues with other amino acids and minimizing energy locally.

RESULTS

The Second Half of GLP1R ECL3 Interacts with GLP-1—We previously exchanged TMH2, ECL1, and ECL2 of GLP1R and GIPR to create chimeric receptors. The peptide ligands for these receptors showed significantly altered potencies and affinities for the receptor chimera (32), demonstrating that residues in TMH2, ECL1, and ECL2 contribute to interactions between GLP-1 and GLP1R. However, exchanging ECL3 of GLP1R with that of GIPR did not affect the potency of GLP-1. This is likely due to a relatively high degree (47%) of sequence similarity between ECL3 of GLP1R and that of GIPR. Indeed, the ECL3 regions of GLP1R and its paralogous receptors GIPR, GLP2R, GCGR, and GCRPR exhibit considerable similarities in amino acid length and sequence (32). In our previous study with GLP1R/GIPR chimeric receptors, we were unable to identify

the residues in GLP1R ECL3 that interact with the peptide ligand. In this study we employed VPAC1R, which is phylogenetically closer to the GLP1R subfamily than other class B GPCR subfamilies, such as parathyroid hormone receptor, corticotropin-releasing hormone receptor, and calcitonin receptor subfamilies (13). Similarly, the amino acid sequence and secondary structure of the VIP peptide family are very similar to those of the GLP-1 peptide family (13).

To determine if ECL3 contributes to the interaction between GLP1R and GLP-1, the sequence from the start of ECL3 to the C terminus of GLP1R was replaced with that of VPAC1R to create the *G/V_[E3-C]* receptor (Fig. 1). Cells expressing the *G/V_[E3-C]* receptor were treated with increasing concentrations of GLP-1. This replacement greatly reduced the potency of GLP-1. In contrast, replacement with the GIPR ECL3 to C-terminal regions (*GL/I6*) (32) did not alter the potency of GLP-1 (Fig. 1). This result suggests that the sequence comprising ECL3 to TMH7 of GLP1R is important for GLP-1 interaction.

The GLP-1-interacting motifs in ECL3-TMH7 were further narrowed down by generating additional GLP1R/VPAC1R chimeric receptors. Chimeric receptors included the VPAC1R sequence from the second half (b) of ECL3 to the C terminus (*G/V_[E3b-C]*), from TMH7 to the C terminus of VPAC1R (*G/V_[T7-C]*), the entire VPAC1R ECL3 (*G/V_[E3ab]*), or the second half of VPAC1R ECL3 (*G/V_[E3b]*) (Fig. 2). The potency of GLP-1 for each of the chimeric receptors was measured. *G/V_[T7-C]*, which retained the second half of GLP1R ECL3, responded to GLP-1 in a similar manner to that of the wild-type GLP1R (Fig. 2). However, chimeric receptors *G/V_[E3b-C]*, *G/V_[E3ab]*, *G/V_[E3-C]*, and *G/V_[E3b]*, in which the second half of GLP1R ECL3 was absent, responded very poorly to GLP-1 (Fig. 2). These results suggest that the second half of ECL3 mediates the interaction of GLP1R with GLP-1.

Leu³⁷⁹, Arg³⁸⁰, and Phe³⁸¹ in ECL3 of GLP1R Are Involved in GLP-1 Interaction—The amino acid residues Leu³⁷⁹, Arg³⁸⁰, Phe³⁸¹, and Leu³⁸⁴ in the second half of GLP1R ECL3 are found in the corresponding positions of GIPR but not in VPAC1R, which has Lys, Pro, Glu, and Met in these positions, respectively (Fig. 1B). To investigate whether these amino acid residues were responsible for specific interactions with GLP-1, the residues were substituted for those in *G/V_[E3b]*, which had a marginal response to GLP-1. Single residue-substituted mutants,

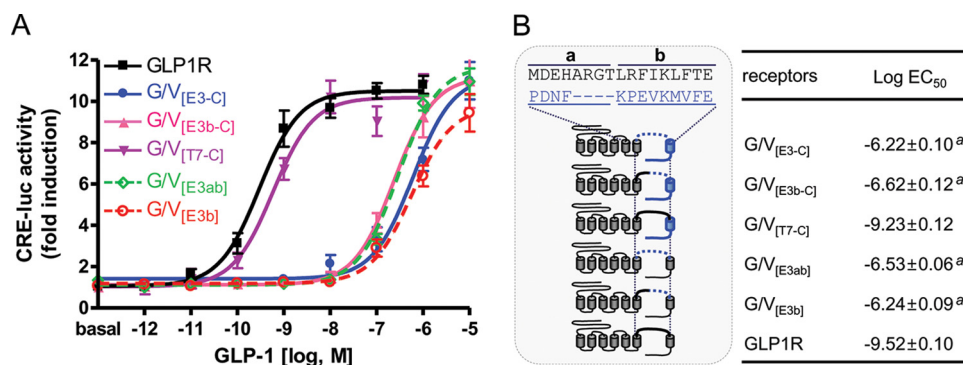


FIGURE 2. **GLP-1-interacting motif in ECL3 and TMH7 of GLP1R.** *A*, sigmoidal curves showing GLP-1 potency toward the wild-type or chimeric GLP1Rs, which have different parts of ECL3 and the C terminus of VPAC1R. *B*, schematic diagram for chimeric receptors and log EC₅₀ values of GLP-1. The VPAC1R ECL sequence was divided into a and b domains. The data on the sigmoidal curves and log EC₅₀ values are presented as the means ± S.E. of at least three independent experiments. ^a, versus wild-type GLP1R ($p < 0.05$).

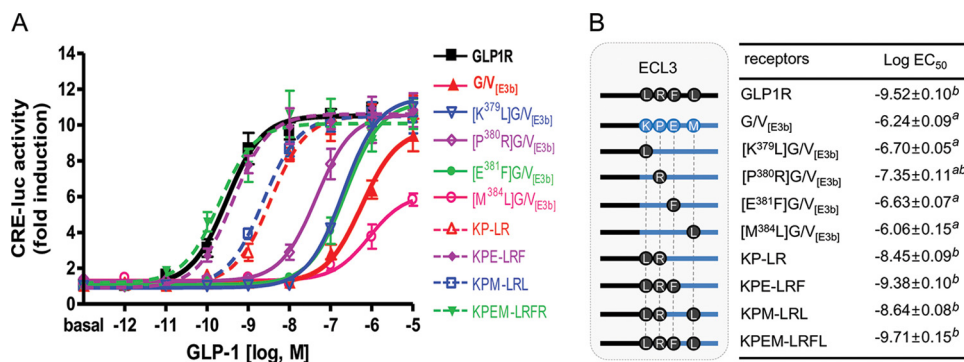


FIGURE 3. **Identification of GLP-1-interacting residues in the second half of GLP1R ECL3.** *A*, the potency of GLP-1 toward chimeric GLP1R, which has the second half of VPAC1R ECL3 (G/V_[E3b]), or mutant G/V_[E3b], into which residues from GLP1R ECL3 were introduced. *B*, schematic diagram for ECL3 mutations and log EC₅₀ values of GLP-1. The diagram shows that amino acid residues Leu³⁷⁹, Arg³⁸⁰, Phe³⁸¹, and/or Leu³⁸⁴ were reintroduced into G/V_[E3b]. The data on the sigmoidal curves and EC₅₀ values represent the means ± S.E. of at least three independent experiments. ^a, versus wild-type GLP1R ($p < 0.05$); ^b, versus G/V_[E3b] ($p < 0.05$).

such as K379L, P380R, and E381F in G/V_[E3b], increased the GLP-1 potency compared with that of G/V_[E3b]. The Arg³⁸⁰ substitution ([P380R]G/V_[E3b]) showed the greatest increase in GLP-1 potency out of all of the single residue mutations (Fig. 3). However, the M384L mutant did not increase the potency of GLP-1. Compared with [P380R]G/V_[E3b], the additional substitutions, K379L/P380R (KP-LR) and K379L/P380R/E381F (KPE-LRF), further increased the GLP-1 potency by 10- and 100-fold, respectively (Fig. 3). K379L/P380R/E381F exhibited ligand-responsive behavior similar to that of wild-type GLP1R. However, additional mutations of Met to Leu³⁸⁴ did not significantly augment the potency of GLP-1 (Fig. 3). These results suggest that the specific structure formed by basic Arg³⁸⁰ flanked by the two hydrophobic bulky residues, Leu³⁷⁹ and Phe³⁸¹, is likely critical for direct contact with the N-terminal moiety of the GLP-1 peptide.

Asp⁹ of GLP-1 May Interact with Arg³⁸⁰ of GLP1R—Substitutions at each of the residues in GLP-1 with Ala indicates that N-terminal residues His¹, Gly⁴, Thr⁷, and Asp⁹ are crucial for either maintaining the secondary structure of the peptide or for interaction with the receptor (30, 31). Our previous observation suggests that His¹ and Thr⁷ of GLP-1 may contact Asn³⁰² in ECL2 and Ile¹⁹⁶ in the upper half of THM2 of GLP1R (32). Interestingly, molecular modeling studies based on this biochemical observation suggested that Gly⁴ and Asp⁹ in GLP-1 interacted with residues in ECL3 of GLP1R. To address this

possibility, we generated mutant GLP1Rs in which the basic Arg³⁸⁰ was changed to acidic Asp (R380D) or neutral Gly (R380G), and hydrophobic Leu³⁷⁹ and Phe³⁸¹ were modified to either basic (L379R and F381R) or acidic (L379E and F381E) residues (Fig. 4). All of the mutants except for F381R exhibited decreased responsiveness and affinity to GLP-1 (Table 2). In particular, the GLP-1 potency of the R380D mutant receptor was reduced by >1000-fold compared with that of wild-type GLP1R (Fig. 4). In contrast, the basic Arg substitution in F381R GLP1R maintained a high affinity for GLP-1. To identify the amino acid residues in GLP-1 that interact with Arg³⁸⁰ of GLP1R, we generated modified GLP-1 peptides in which Gly⁴ was replaced with acidic (Asp and Glu) or basic (His, Lys, and Arg) residues and Asp⁹ was changed to a basic Lys or Arg residue or to an acidic Glu (Table 1). The potencies of the modified peptides were determined for wild-type and R380D mutant GLP1R (Table 2). All of the modified GLP-1 peptides except for [Glu⁴]GLP-1 exhibited substantially decreased potencies for wild-type GLP1R (Table 2 and Fig. 5A). These data indicate the importance of Gly⁴ and Asp⁹ in GLP-1 for receptor binding and activation. It is of interest to note that [Arg⁴]-, [Lys⁹]-, and [Arg⁹]GLP-1 had increased potencies for R380D GLP1R compared with wild-type GLP-1. Among all of the peptides, the potency of [Arg⁹]GLP-1 on R380D GLP1R was the greatest (Table 2 and Fig. 5B). We have confirmed these results using a pGlosensorTM-22F system that directly examines cAMP pro-

Ligand Binding Pocket of the GLP-1 Receptor

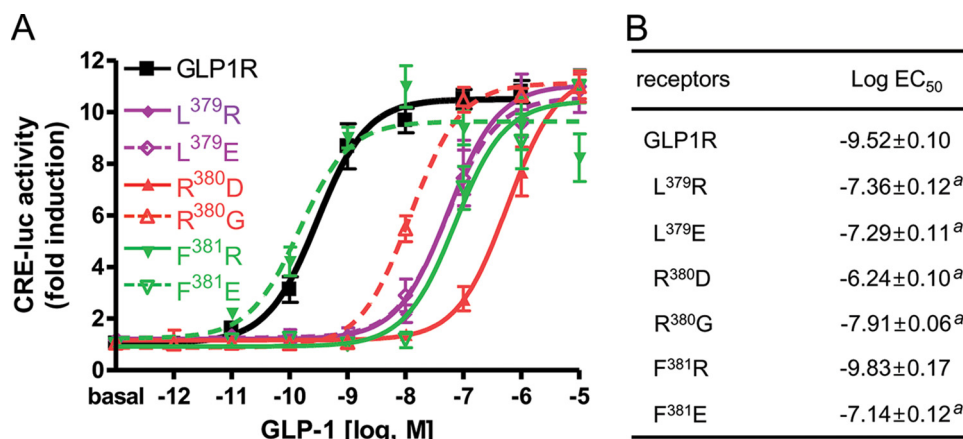


FIGURE 4. **Mutation of GLP-1-interacting residues in the second half of GLP1R ECL3.** A, sigmoidal curves of GLP-1-induced receptor activation. Amino acid residues Leu³⁷⁹, Arg³⁸⁰, Phe³⁸¹, or Leu³⁸⁴ in ECL3 were mutated to either basic or acidic residues. B, the log EC₅₀ values of mutant receptors. The data on the sigmoidal curves and EC₅₀ values are presented as the means ± S.E. of at least three independent experiments. ^a, versus wild-type GLP1R (*p* < 0.05).

duction in response to ligand stimulation (14). The results (data not shown) are similar in tendency to that of Fig. 5 such that the mutant peptides showed decreased potency and efficacy toward wild-type receptors while exhibiting higher potency or efficacy toward R380D GLP1R than wild-type peptide. These changes represent reciprocal mutations of charged residues in the ligand and receptor, and the result suggests a possible interaction of Asp⁹ of GLP-1 with Arg³⁸⁰ of GLP1R. To corroborate this, we performed a ligand-receptor binding assay. Iodinated [Arg⁹]GLP-1 exhibited significantly lower binding affinity for wild-type GLP1R but relatively high affinity for R380D, which had low affinity for wild-type GLP-1 (Fig. 5, C and D). We also examined the potency and affinity of the mutant GLP-1 peptides for the mutant receptors, L379R, L379E, F381R, and F381E (Table 2). However, none of the mutant peptides exhibited potencies and affinities higher than that of wild-type GLP-1 toward the mutant receptors. These results indicated that Leu³⁷⁹ and Phe³⁸¹ do not directly interact with the ligand but may be important for maintaining a receptor conformation that favors ligand binding. To determine the expression levels of wild-type and mutant receptors, we performed a binding assay using submaximal concentration of radiolabeled ligands (Table 2). Additionally, Western blot and confocal microscope for GFP-conjugated wild-type and mutant receptors reveals that all receptors seem to be stably expressed in the cells (data not shown).

Molecular Modeling Shows Direct Interaction between Asp⁹ of GLP-1 and Arg³⁸⁰ of GLP1R—The GLP-1-GLP1R interaction was examined on a three-dimensional atomic scale using a homology model. The crystal structures of the ligand-bound ECD of human GLP1R and human GIPR served as structural templates of the ECD modeling of GLP1R. The core domain structure was built based on the crystal structures of two class B GPCRs, human GCGR (38) and corticotropin-releasing factor 1 receptor (39). Although these two crystal structures represent inactive structures with antagonists, a lesson from the class A GPCR structures is that there is no significant structural difference in the ligand binding sites between agonist-bound and antagonist-bound structures, whereas there are large conformational changes in the G protein binding regions (40).

Sequence alignment between GLP1R and the GPCRs of the crystal structures was done manually with predicted TMHs and evolutionarily conserved residues. Seven distance restraints between GLP-1 and GLP1R were introduced during homology modeling as listed under “Experimental Procedures,” and extensive optimization was performed.

The final model provided interesting explanations for the effects of the GLP1R mutations. Initially, GLP1R Arg³⁸⁰ was located near Asp⁹ and Gly⁴ of GLP-1 (Fig. 6A). The interaction between the side chains of GLP1R Arg³⁸⁰ and GLP-1 Asp⁹ is likely due to an electrostatic attraction. Electrostatic surface charge analyses of wild-type and R380D GLP1R revealed the importance of the ionic charge at position 380 (Fig. 6, B and C). Arg³⁸⁰ is located at the entrance of the binding pocket formed by the ECLs and TMHs and conveys a positively charged electrostatic surface, permitting negatively charged Asp⁹ or Glu⁹ in GLP-1 to access this binding pocket for binding. The positive surface charge of the binding pocket may repel Lys⁹ or Arg⁹ residues of the mutant GLP-1 peptides. In contrast, mutation of Arg³⁸⁰ in GLP1R to Asp abolishes the positive surface charge of the area and creates a negative surface charge, repelling the acidic Asp⁹ or Glu⁹ in GLP-1 from the binding pocket. However, this acidic surface charge may permit [Arg⁹]GLP-1 or [Lys⁹]GLP-1 to interact with the mutant receptor (Fig. 5, B and D). Gly⁴ of GLP-1 is located close to Arg³⁸⁰ of GLP1R so that an introduction of a side chain by mutation of the residue would affect the binding of the ligand to the receptor. For instance, [Arg⁴]GLP-1 has a very low potency and affinity to the wild-type receptor, but it has a relatively high potency and affinity toward the R380G mutant receptor (Table 2). Thus, it can be postulated that a repulsion between GLP-1 Arg⁴ and GLP1R Arg³⁸⁰ results in the low affinity of [Arg⁴]GLP-1 toward the wild-type receptor, whereas the Arg⁴ mutation in the GLP-1 peptide may provide relatively high affinity to the R380G mutant receptor.

The hydrophobic residue Leu³⁷⁹ may not directly interact with the peptide ligand but may contribute to receptor conformation. For example, the side chain of Leu³⁷⁹ was within 6 Å of Phe³⁶⁷, Met³⁷¹, Asp³⁷², and Glu³⁷³ of TMH6, His³⁷⁴, Gly³⁷⁵, Gly³⁷⁷, Thr³⁷⁸, Arg³⁸⁰, and Phe³⁸¹ of ECL3, and Ile³⁸², Leu³⁸⁴,

TABLE 2

Ligand affinities and potencies of wild-type GLP1R and GLP1R mutants

Expression of receptors is shown as % maximal level of wild-type GLP1R. Binding affinity is presented as log IC₅₀. The potencies of the modified GLP-1 peptides are shown as log EC₅₀. The Emax values of each mutant in response to modified peptides were not significantly different from the Emax value of wild-type GLP1R to GLP-1.

Receptor	GLP1R	L ³⁷⁹ R	L ³⁷⁹ E	R ³⁸⁰ D	R ³⁸⁰ G	F ³⁸¹ R	F ³⁸¹ E
% expression	100±15.0	83.3±3.0	80.5±2.8	95.0±2.9	202.6±20.6	128.2±10.3	83.6±3.6
EC₅₀ (nM)							
GLP-1	0.31±0.06	43.65±10.54	51.29±11.48	574.44±118.35	12.30±1.59	0.15±0.05	72.44±17.49
[D ⁴]GLP-1	190.55±52.51	>1000	>1000	>1000	575.44±74.25	302.00±93.07	>1000
[E ⁴]GLP-1	85.11±17.51	851.14±301.60	>1000	>1000	616.60±79.56	87.10±39.23	>1000
[H ⁴]GLP-1	45.71±8.56	>1000	>1000	>1000	141.25±23.76	24.55±12.24	>1000
[K ⁴]GLP-1	>1000	>1000	>1000	>1000	575.44±62.58	398.11±89.08	>1000
[R ⁴]GLP-1	123.03±23.02	>1000	>1000	64.57±10.57	16.60±1.80	125.89±38.80	>1000
[E ⁹]GLP-1	0.33±0.10	47.86±7.12	104.71±30.58	104.71±15.59	17.78±2.99	3.39±1.10	>1000
[K ⁹]GLP-1	52.48±8.83	186.21±60.32	>1000	12.88±1.92	19.05±3.92	34.67±10.13	>1000
[R ⁹]GLP-1	28.84±4.85	91.20±18.67	>1000	4.79±0.71	7.59±1.56	30.20±9.31	467.74±104.66
IC₅₀ (μM)							
GLP-1	0.05±0.01	0.59±0.18	0.54±0.17	1.05±0.22	0.21±0.04	0.06±0.01	>10
[D ⁴]GLP-1	1.00±0.22	>10	>10	>10	0.52±0.09	1.00±0.37	>10
[E ⁴]GLP-1	1.82±0.27	>10	>10	>10	0.65±0.14	1.00±0.26	>10
[H ⁴]GLP-1	1.29±0.26	>10	>10	>10	0.45±0.07	0.80±0.18	>10
[K ⁴]GLP-1	>10	>10	>10	>10	0.68±0.13	2.88±1.22	>10
[R ⁴]GLP-1	1.78±0.55	>10	>10	0.35±0.09	0.15±0.02	2.14±1.02	>10
[E ⁹]GLP-1	0.29±0.03	4.68±1.21	>10	0.41±0.08	0.27±0.05	0.47±0.14	>10
[K ⁹]GLP-1	1.41±0.12	1.02±0.32	>10	0.15±0.03	0.14±0.01	0.59±0.10	>10
[R ⁹]GLP-1	0.83±0.20	1.41±0.46	>10	0.19±0.02	0.23±0.05	1.32±0.27	>10

Phe³⁸⁵, and Leu³⁸⁸ of TMH7 (Fig. 6D). Thus, mutation of Leu³⁷⁹ to a bulky Arg may substantially alter receptor conformation and attenuate ligand binding (Fig. 4). Our model, however, suggests that the side chain of Phe³⁸¹ was orientated away from the binding pocket and interacted with only several residues of ECL3, enduring a substantial change to Arg³⁸¹ (Fig. 6E). The combined cationic nature of Arg at 380 and 381 is well tolerated but demonstrates preference of Asp⁹ relative to Glu⁹ of GLP-1. However, mutation to negatively charged residues at Phe³⁸¹ would affect the binding of the ligand significantly because the electrostatic attraction between the side chains of the receptor may disorient the side chain of Arg³⁸⁰.

Role of Arg³⁸⁰-corresponding Basic Residues in Related Receptors—The basic Arg³⁸⁰ of GLP1R is well conserved in the equivalent positions of the related paralogous receptors, GCGR (Arg³⁷⁸), GCRPR (Arg³⁷⁹), GLP2R (Lys⁴¹⁴), and GIPR (Arg³⁶²). Similarly, Gly at position 4 and acidic residue Asp or Glu at position 9 are common for GLP-1 and its related peptides, glucagon, GCRP, GLP-2, and GIP (Fig. 7A). This observation suggests that the GLP-1 peptide and receptor families may share a common mechanism of interaction between acidic Asp/Glu⁹ and/or Gly⁴ of the peptide and basic Arg³⁸⁰ in ECL3 of the corresponding receptor. To address this possibility, we generated mutant GCGR, GCRPR, GLP2R, and GIPR, in which the

Ligand Binding Pocket of the GLP-1 Receptor

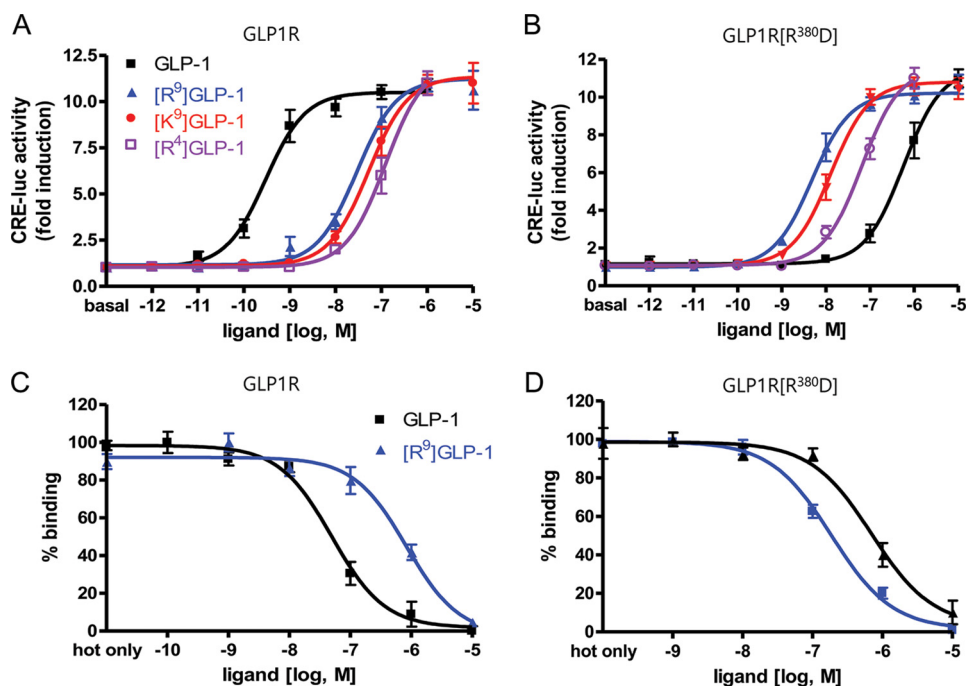


FIGURE 5. Effect of GLP-1 complementary mutation on the R380D GLP1R mutant. A, ligand potency of mutant GLP-1s in which Gly⁴ and Asp⁹ were replaced with basic residues, toward wild-type GLP1R. B, ligand potency of mutant GLP-1 peptides toward the R380D mutant. C, binding analysis of wild-type and [Arg⁹]GLP-1 peptides to GLP1R. D, ligand binding affinity of GLP-1 complementary mutation to mutant R380D. The results are presented as the means \pm S.E. of at least three independent experiments.

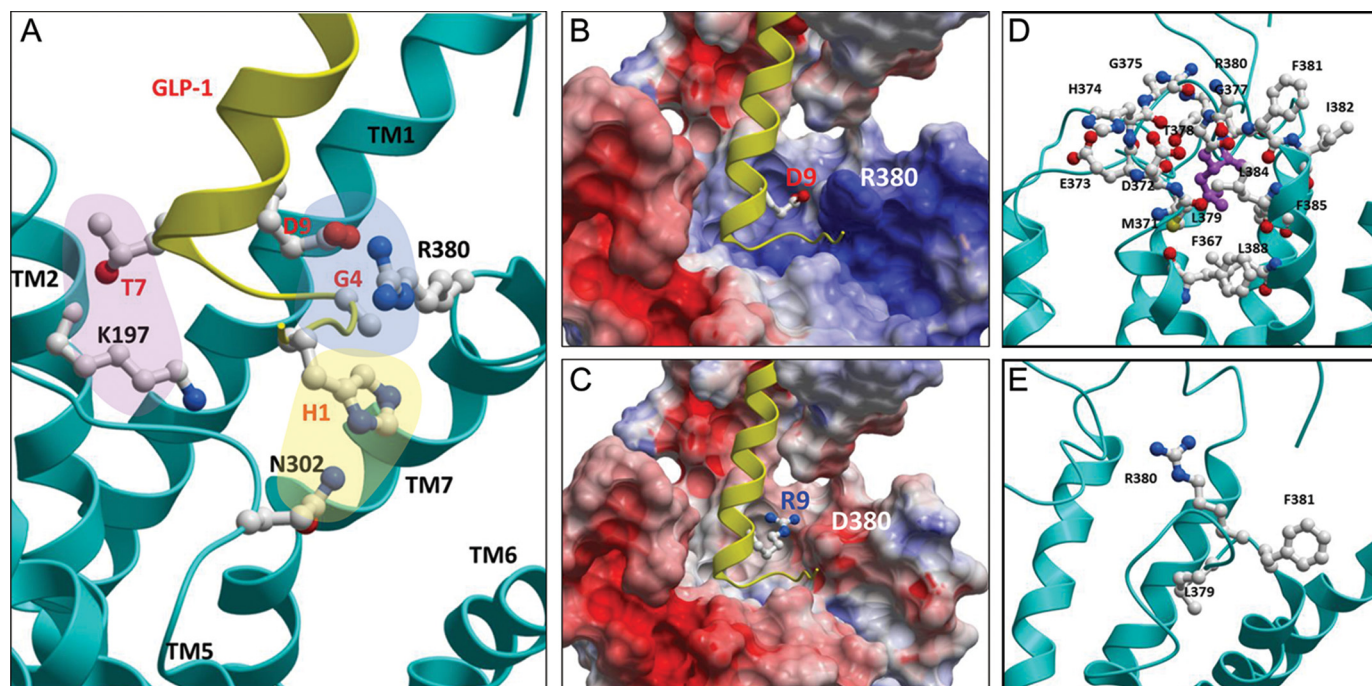


FIGURE 6. Molecular model showing the interaction between GLP-1 and the core of GLP1R. A homology model of the GLP1R and GLP-1 interaction was built based on the crystal structures of class B GPCR cores and the ECD of human GLP1R and GiPR in complex with GLP-1 and GiPR, respectively. The model is consistent with the experimental results in this study. A, the enlarged view shows the residues that are important for interactions between GLP-1 (yellow) and GLP1R (cyan). The carbon, nitrogen, and oxygen atoms of the residues are colored gray, blue, and red, respectively. The orientation of each TMH (TM1 to TM7) is indicated. The electrostatic potentials of GLP1R wild-type (B) and R380D mutant (C) are shown with the binding pockets formed by ECLs and TMHs at the center. The positive and negative electrostatic values are colored blue and red, respectively. The atoms of Asp⁹ and Arg⁹ of GLP-1 are shown in a ball-and-chain diagram in B and C, respectively. Residue 380 is located at the entrance of the binding pocket and interacts with Asp⁹ of GLP-1. The atoms that are located within 6 Å from the side chains of Leu³⁷⁹ (in violet) are shown in a ball-and-chain model to show the extensive interaction network of the residue (D). Leu³⁷⁹ has contacts with many neighboring residues in TMH6, ECL3, and TMH7 such that mutation of Leu³⁷⁹ would result in a structural change that affects the binding of GLP-1. In contrast, the side chain of Phe³⁸¹ protrudes away from the binding pocket so that mutation of the residue would have little structural change (E).

Ligand Binding Pocket of the GLP-1 Receptor

tial approximation of the residues in the peptide and receptor that interact with each other. Phe⁶ of GLP-1 is in close proximity to Tyr¹⁴⁵ of GLP1R according to that study (46). Recent results from another study that used the same method showed discrepancies compared with crystallography experiments. For example, although GLP-1 Ala¹⁸ was proposed to be located close to Glu¹³³ of the ECD of GLP1R by photoaffinity labeling (45), the ligand-bound crystal structure of the GLP1R ECD shows a hydrophobic interaction between GLP-1 Ala¹⁸ and Leu³² of the ECD (20, 21). Molecular docking studies with biochemical analyses have suggested possible ligand binding pockets in the GLP1R core domain (47, 50, 51). However, these modeling approaches failed to provide a common consensus for the ligand binding pocket. Instead, these approaches differed in their identification of residues that contact the ligand. Thus, stricter biochemical analyses with an appropriate strategy may be helpful for constructing a more reliable ligand-bound receptor model.

Although GLP-1 and its related peptides share a high degree of sequence identity and structural similarity, they generally exhibit specific binding to their own receptors with some cross-reactivity toward paralogous receptors (32, 52). This observation suggests that there are distinct amino acid residues among paralogs of the peptide and receptor that mediate selective interactions with their own partners. Thus, our study is based on the concept of coevolution of the peptide ligand and receptor family. Evolutionary pressure preserves the amino acid residues that are essential for the basic protein structure and for primary interactions between ligand and receptor family members. Specific changes in the amino acid sequence permit selective interaction between a ligand-receptor pair (53–57). In the former case, residues are conserved across paralogous members. However, in latter case, residues are conserved within orthologous members and differ from those of paralogs (33). This hypothesis is supported by recent crystal structures for the ligand-bound ECD of GLP1R and GIPR (18, 20, 21) and by molecular docking models for the ligand-bound ECD of GCGR and GLP2R (58, 59). The hydrophobic face of the peptides is formed by the conserved residues, Phe²², Ile/Val²³, Trp²⁵, and Leu²⁶, and points toward the hydrophobic binding cavity of the ECD. The ECD hydrophobic binding cavity is formed by conserved hydrophobic residues, which are localized primarily in the N-terminal α -helix, at the end of the β 2 strand, and in the loop between the β 3 and β 4 strands. These evolutionarily conserved residues may contribute to primary interactions across these peptide and receptor family members (27, 52). In contrast, the residues located at positions 16–20 vary across paralogous peptides but are conserved among orthologs of each paralog (33). These conserved residues may account for the selectivity and strong interaction between each peptide-receptor pair (18, 21).

In the N-terminal portion of GLP-1, His¹, Gly⁴, Thr⁷, and Asp⁹ are important for receptor binding and activation (30, 31). His¹ and Thr⁷ are proposed to interact with evolutionarily conserved residues in GLP1R, which are Ile¹⁹⁶/Lys¹⁹⁷ in TMH2, Met²³³ in ECL1, and Asn³⁰² in ECL2 (32). The current study suggests that Gly⁴ and Asp⁹ are likely to have close contacts with Arg³⁸⁰ flanked by hydrophobic Leu³⁷⁹ and Phe³⁸¹ in ECL3.

According to the model, Arg³⁸⁰ provides the basic surface of the binding pocket, which allows the acidic Asp⁹ of GLP-1 to enter the pocket. In contrast, mutation of Arg³⁸⁰ to Asp acidifies the binding pocket, which interferes with binding of GLP-1 Asp⁹ but permits interaction with [Arg⁹]GLP-1. Leu³⁷⁹ and Phe³⁸¹ may affect either the receptor conformation or surface charge of the binding pocket to promote ligand binding.

Interestingly, His¹, Gly⁴, Thr⁷, and Asp⁹ of GLP-1 are highly conserved across the related peptides, glucagon, GCRP, and GLP-2, although GIP has Tyr¹ and Ile⁷. Thus, similarities in the ligand binding pocket structures of GLP1R, GCGR, GCRPR, and GLP2R may accommodate the conserved residues in the N-terminal moieties of the peptides. Indeed, mutation of Arg³⁷⁸ to Asp in GCGR greatly reduced glucagon potency. This mutation effect was partially compensated by glucagon containing an Arg⁹ residue. Likewise, mutation of Arg³⁷⁹ to Asp in GCRPR greatly reduced the potency of wild-type GCRP. However, this mutation was tolerant to GCRP that contained an Arg⁹ residue. Furthermore, GLP-1 and GCRP can cross-interact with their evolutionary related receptors, although the affinity or potency of cross-interaction is lower than that of the natural cognate interaction (14). These observations may support the concept that GLP1R, GCGR, and GCRPR have similar ligand binding pockets formed by evolutionarily conserved residues in the core domain. However, these receptors may also have distinct structures formed by ortholog-specific residues in the core domain that interact with ortholog-specific residues in the corresponding peptide. For example, in addition to the highly common residues Gly⁴, Phe⁶, Thr⁷, and Asp/Glu⁹, glucagon-specific residues, Ser², Gln³, Tyr¹⁰, and Lys¹², are also important for receptor binding and activation (60, 61). Indeed, a recent glucagon-docked GCGR modeling based on the antagonist-bound GCGR crystal structure revealed putative interactions of these glucagon-specific residues with GCGR (38). However, this model did not show direct interaction between Asp⁹ of glucagon and Arg³⁷⁹ of GCRPR. This is likely due to the distance restraints between glucagon and GCGR being based on photo-cross-linking studies between GLP-1 and GLP1R (49).

GLP2R and GIPR are expected to have binding pocket structures that are distinct from that of GLP1R. GLP2R has different residues, Val²³⁰ and Leu²⁶⁷, at the corresponding positions of GLP1R Ile¹⁹⁶ and Met²³³. In addition, GLP2R has Ala at the equivalent position of Leu³⁷⁹ of GLP1R. According to our modeling data, the hydrophobic bulky side chain of Leu³⁷⁹ contacts TMH6 and TMH7, contributing to the receptor conformation that favors ligand binding. Thus, replacing Leu³⁷⁹ in GLP2R with an Ala, which has a shorter side chain, may affect the structure of the binding pocket. Alanine scanning of GLP-2 showed that the residues at positions 2, 5, 6, and 17 are important for receptor activation (62), indicating that receptor-interacting residues in GLP-2 differ from those of GLP-1. Because GIP contains Tyr¹ and Ile⁷ and GIPR possesses Ser, Thr, and Val at the corresponding positions of Ile¹⁹⁶, Met²³³, and Asn³⁰² in GLP1R, the binding pocket structure of GIPR will be markedly different from that of GLP1R. Thus, mutations at Lys⁴¹⁴ in GLP2R and Arg³⁶² in GIPR did not critically affect ligand binding.

In summary, our study suggests that residues Gly⁴ and Asp⁹ in the GLP-1 N terminus and Asp³⁸⁰ in ECL3 of GLP1R confer ligand-induced receptor activation. Furthermore, this interaction appears to be conserved in glucagon/GCGR and GCRP/GCRPR pairs. Together with our previous study demonstrating interactions between His¹ and Thr⁷ in GLP-1 and Ile¹⁹⁶, Met²³³, and Asn³⁰² in GLP1R (32, 32), this study sheds light on the mechanism underlying the high affinity interaction between the GLP-1 peptide family and the binding pocket in the receptor that is formed by evolutionarily conserved residues. Identifying the structure of the ligand-binding pocket is important for *in silico* virtual screening of small molecules that activate GLP1R, which could be further developed by medicinal chemistry in treatments for diabetes and obesity.

REFERENCES

- Mojsov, S., Heinrich, G., Wilson, I. B., Ravazzola, M., Orci, L., and Habener, J. F. (1986) Preproglucagon gene expression in pancreas and intestine diversifies at the level of post-translational processing. *J. Biol. Chem.* **261**, 11880–11889
- Fehmann, H. C., Göke, R., and Göke, B. (1995) Cell and molecular biology of the incretin hormones glucagon-like peptide-I and glucose-dependent insulin releasing polypeptide. *Endocr. Rev.* **16**, 390–410
- Moon, M. J., Kim, H. Y., Park, S., Kim, D. K., Cho, E. B., Hwang, J. I., and Seong, J. Y. (2011) Insulin contributes to fine-tuning of the pancreatic β -cell response to glucagon-like peptide-1. *Mol. Cells* **32**, 389–395
- Drucker, D. J. (2003) Glucagon-like peptides: regulators of cell proliferation, differentiation, and apoptosis. *Mol. Endocrinol.* **17**, 161–171
- List, J. F., and Habener, J. F. (2004) Glucagon-like peptide 1 agonists and the development and growth of pancreatic β -cells. *Am. J. Physiol. Endocrinol. Metab.* **286**, E875–E881
- Nauck, M. A., Heimesaat, M. M., Behle, K., Holst, J. J., Nauck, M. S., Ritzel, R., Hübner, M., and Schmiegel, W. H. (2002) Effects of glucagon-like peptide 1 on counterregulatory hormone responses, cognitive functions, and insulin secretion during hyperinsulinemic, stepped hypoglycemic clamp experiments in healthy volunteers. *J. Clin. Endocrinol. Metab.* **87**, 1239–1246
- Zander, M., Madsbad, S., Madsen, J. L., and Holst, J. J. (2002) Effect of 6-week course of glucagon-like peptide 1 on glycaemic control, insulin sensitivity, and β -cell function in type 2 diabetes: a parallel-group study. *Lancet* **359**, 824–830
- Mentlein, R., Gallwitz, B., and Schmidt, W. E. (1993) Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1(7–36)amide, peptide histidine methionine and is responsible for their degradation in human serum. *Eur. J. Biochem.* **214**, 829–835
- Kieffer, T. J., McIntosh, C. H., and Pederson, R. A. (1995) Degradation of glucose-dependent insulinotropic polypeptide and truncated glucagon-like peptide 1 *in vitro* and *in vivo* by dipeptidyl peptidase IV. *Endocrinology* **136**, 3585–3596
- 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
- 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
- Wang, Y., Meng, F., Zhong, Y., Huang, G., and Li, J. (2012) Discovery of a novel glucagon-like peptide (GCGL) and its receptor (GCGLR) in chickens: evidence for the existence of GCGL and GCGLR genes in nonmammalian vertebrates. *Endocrinology* **153**, 5247–5260
- Hwang, J. I., Moon, M. J., Park, S., Kim, D. K., Cho, E. B., Ha, N., Son, G. H., Kim, K., Vaudry, H., and Seong, J. Y. (2013) Expansion of secretin-like G protein-coupled receptors and their peptide ligands via local duplications before and after two rounds of whole-genome duplication. *Mol. Biol. Evol.* **30**, 1119–1130
- Park, C. R., Moon, M. J., Park, S., Kim, D. K., Cho, E. B., Millar, R. P., Hwang, J. I., and Seong, J. Y. (2013) A novel glucagon-related peptide (GCRP) and its receptor GCRPR account for coevolution of their family members in vertebrates. *PLoS ONE* **8**, e65420
- Hwang, J. I., Yun, S., Moon, M. J., Park, C. R., and Seong, J. Y. (2014) Molecular evolution of GPCRs: GLP1/GLP1 receptors. *J. Mol. Endocrinol.* **52**, T15–T27
- Harmar, A. J. (2001) Family-B G-protein-coupled receptors. *Genome Biol.* **2**, REVIEWS3013
- Oh, D. Y., Kim, K., Kwon, H. B., and Seong, J. Y. (2006) Cellular and molecular biology of orphan G protein-coupled receptors. *Int. Rev. Cytol.* **252**, 163–218
- Parthier, C., Kleinschmidt, M., Neumann, P., Rudolph, R., Manhart, S., Schlenzig, D., Fanghänel, J., Rahfeld, J. U., Demuth, H. U., and Stubbs, M. T. (2007) Crystal structure of the incretin-bound extracellular domain of a G protein-coupled receptor. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 13942–13947
- Tan, Y. V., Couvineau, A., Murail, S., Ceraudo, E., Neumann, J. M., Lacapère, J. J., and Laburthe, M. (2006) Peptide agonist docking in the N-terminal ectodomain of a class II G protein-coupled receptor, the VPAC1 receptor. Photoaffinity, NMR, and molecular modeling. *J. Biol. Chem.* **281**, 12792–12798
- Runge, S., Thøgersen, H., Madsen, K., Lau, J., and Rudolph, R. (2008) Crystal structure of the ligand-bound glucagon-like peptide-1 receptor extracellular domain. *J. Biol. Chem.* **283**, 11340–11347
- 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
- Neidigh, J. W., Fesinmeyer, R. M., Prickett, K. S., and Andersen, N. H. (2001) Exendin-4 and glucagon-like-peptide-1: NMR structural comparisons in the solution and micelle-associated states. *Biochemistry* **40**, 13188–13200
- Alaña, I., Malthouse, J. P., O'Harte, F. P., and Hewage, C. M. (2007) The bioactive conformation of glucose-dependent insulinotropic polypeptide by NMR and CD spectroscopy. *Proteins* **68**, 92–99
- Al-Sabah, S., and Donnelly, D. (2003) A model for receptor-peptide binding at the glucagon-like peptide-1 (GLP-1) receptor through the analysis of truncated ligands and receptors. *Br. J. Pharmacol.* **140**, 339–346
- López de Maturana, R., Willshaw, A., Kuntzsch, A., Rudolph, R., and Donnelly, D. (2003) The isolated N-terminal domain of the glucagon-like peptide-1 (GLP-1) receptor binds exendin peptides with much higher affinity than GLP-1. *J. Biol. Chem.* **278**, 10195–10200
- Dong, M., Li, Z., Zang, M., Pinon, D. I., Lybrand, T. P., and Miller, L. J. (2003) Spatial approximation between two residues in the mid-region of secretin and the amino terminus of its receptor. Incorporation of seven sets of such constraints into a three-dimensional model of the agonist-bound secretin receptor. *J. Biol. Chem.* **278**, 48300–48312
- Runge, S., Wulff, B. S., Madsen, K., Bräuner-Osborne, H., and Knudsen, L. B. (2003a) Different domains of the glucagon and glucagon-like peptide-1 receptors provide the critical determinants of ligand selectivity. *Br. J. Pharmacol.* **138**, 787–794
- López de Maturana, R., Treece-Birch, J., Abidi, F., Findlay, J. B., and Donnelly, D. (2004) Met-204 and Tyr-205 are together important for binding GLP-1 receptor agonists but not their N-terminally truncated analogues. *Protein Pept. Lett.* **11**, 15–22
- 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
- Adelhorst, K., Hedegaard, B. B., Knudsen, L. B., and Kirk, O. (1994) Structure-activity studies of glucagon-like peptide-1. *J. Biol. Chem.* **269**, 6275–6278
- Gallwitz, B., Witt, M., Paetzold, G., Morys-Wortmann, C., Zimmermann,

Ligand Binding Pocket of the GLP-1 Receptor

- B., Eckart, K., Fölsch, U. R., and Schmidt, W. E. (1994) Structure/activity characterization of glucagon-like peptide-1. *Eur. J. Biochem.* **225**, 1151–1156
32. Moon, M. J., Kim, H. Y., Park, S., Kim, D. K., Cho, E. B., Park, C. R., You, D. J., Hwang, J. I., Kim, K., Choe, H., and Seong, J. Y. (2012) Evolutionarily conserved residues at glucagon-like peptide-1 (GLP-1) receptor core confer ligand-induced receptor activation. *J. Biol. Chem.* **287**, 3873–3884
33. Moon, M. J., Park, S., Kim, D. K., Cho, E. B., Hwang, J. I., Vaudry, H., and Seong, J. Y. (2012) Structural and molecular conservation of glucagon-like Peptide-1 and its receptor confers selective ligand-receptor interaction. *Front. Endocrinol. (Lausanne)* **3**, 141
34. Sali, A., and Blundell, T. L. (1993) Comparative protein modelling by satisfaction of spatial restraints. *J. Mol. Biol.* **234**, 779–815
35. Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E. L. (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J. Mol. Biol.* **305**, 567–580
36. Laskowski, R. A., and Swindells, M. B. (2011) LigPlot+: multiple ligand-protein interaction diagrams for drug discovery. *J. Chem. Inf. Model* **51**, 2778–2786
37. Totrov, M., and Abagyan, R. (2001) Rapid boundary element solvation electrostatics calculations in folding simulations: successful folding of a 23-residue peptide. *Biopolymers* **60**, 124–133
38. 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
39. 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
40. Katritch, V., Cherezov, V., and Stevens, R. C. (2012) Diversity and modularity of G protein-coupled receptor structures. *Trends Pharmacol. Sci.* **33**, 17–27
41. 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
42. Montrose-Rafizadeh, C., Yang, H., Rodgers, B. D., Beday, A., Pritchette, L. A., and Eng, J. (1997) High potency antagonists of the pancreatic glucagon-like peptide-1 receptor. *J. Biol. Chem.* **272**, 21201–21206
43. Xiao, Q., Jeng, W., and Wheeler, M. B. (2000) Characterization of glucagon-like peptide-1 receptor-binding determinants. *J. Mol. Endocrinol.* **25**, 321–335
44. López de Maturana, R., and Donnelly, D. (2002) The glucagon-like peptide-1 receptor binding site for the N terminus of GLP-1 requires polarity at Asp198 rather than negative charge. *FEBS Lett.* **530**, 244–248
45. Chen, Q., Pinon, D. I., Miller, L. J., and Dong, M. (2009) Molecular basis of glucagon-like peptide 1 docking to its intact receptor studied with carboxyl-terminal photolabile probes. *J. Biol. Chem.* **284**, 34135–34144
46. Chen, Q., Pinon, D. I., Miller, L. J., and Dong, M. (2010) Spatial approximations between residues 6 and 12 in the amino-terminal region of glucagon-like peptide 1 and its receptor: a region critical for biological activity. *J. Biol. Chem.* **285**, 24508–24518
47. Lin, F., and Wang, R. (2009) Molecular modeling of the three-dimensional structure of GLP-1R and its interactions with several agonists. *J. Mol. Model* **15**, 53–65
48. Koole, C., Wootten, D., Simms, J., Miller, L. J., Christopoulos, A., and Sexton, P. M. (2012) Second extracellular loop of human glucagon-like peptide-1 receptor (GLP-1R) has a critical role in GLP-1 peptide binding and receptor activation. *J. Biol. Chem.* **287**, 3642–3658
49. Miller, L. J., Chen, Q., Lam, P. C., Pinon, D. I., Sexton, P. M., Abagyan, R., and Dong, M. (2011) Refinement of glucagon-like peptide 1 docking to its intact receptor using mid-region photolabile probes and molecular modeling. *J. Biol. Chem.* **286**, 15895–15907
50. Coopman, K., Wallis, R., Robb, G., Brown, A. J., Wilkinson, G. F., Timms, D., and Willars, G. B. (2011) Residues within the transmembrane domain of the glucagon-like peptide-1 receptor involved in ligand binding and receptor activation: modelling the ligand-bound receptor. *Mol. Endocrinol.* **25**, 1804–1818
51. Kirkpatrick, A., Heo, J., Abrol, R., and Goddard, W. A., 3rd. (2012) Predicted structure of agonist-bound glucagon-like peptide 1 receptor, a class B G protein-coupled receptor. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 19988–19993
52. Moon, M. J., Kim, H. Y., Kim, S. G., Park, J., Choi, D. S., Hwang, J. I., and Seong, J. Y. (2010) Tyr1 and Ile7 of glucose-dependent insulintropic polypeptide (GIP) confer differential ligand selectivity toward GIP and glucagon-like peptide-1 receptors. *Mol. Cells* **30**, 149–154
53. Acharjee, S., Do-Rego, J. L., Oh, D. Y., Oh da, Y., Ahn, R. S., Choe, H., Vaudry, H., Kim, K., Seong, J. Y., and Kwon, H. B. (2004) Identification of amino acid residues that direct differential ligand selectivity of mammalian and nonmammalian V1a type receptors for arginine vasopressin and vasotocin. Insights into molecular coevolution of V1a type receptors and their ligands. *J. Biol. Chem.* **279**, 54445–54453
54. Wang, C., Yun, O., Maiti, K., Oh, D. Y., Oh da, Y., Kim, K. K., Chae, C. H., Lee, C. J., Seong, J. Y., and Kwon, H. B. (2004) Position of Pro and Ser near Glu7.32 in the extracellular loop 3 of mammalian and nonmammalian gonadotropin-releasing hormone (GnRH) receptors is a critical determinant for differential ligand selectivity for mammalian GnRH and chicken GnRH-II. *Mol. Endocrinol.* **18**, 105–116
55. Li, J. H., Choe, H., Wang, A. F., Maiti, K., Wang, C., Salam, A., Chun, S. Y., Lee, W. K., Kim, K., Kwon, H. B., and Seong, J. Y. (2005) Extracellular loop 3 (EL3) and EL3-proximal transmembrane helix 7 of the mammalian type I and type II gonadotropin-releasing hormone (GnRH) receptors determine differential ligand selectivity to GnRH-I and GnRH-II. *Mol. Pharmacol.* **67**, 1099–1110
56. Cho, H. J., Acharjee, S., Moon, M. J., Oh, D. Y., Vaudry, H., Kwon, H. B., and Seong, J. Y. (2007) Molecular evolution of neuropeptide receptors with regard to maintaining high affinity to their authentic ligands. *Gen. Comp. Endocrinol.* **153**, 98–107
57. Lee, Y. R., Tsunekawa, K., Moon, M. J., Um, H. N., Hwang, J. I., Osugi, T., Otaki, N., Sunakawa, Y., Kim, K., Vaudry, H., Kwon, H. B., Seong, J. Y., and Tsutsui, K. (2009) Molecular evolution of multiple forms of kisspeptins and GPR54 receptors in vertebrates. *Endocrinology* **150**, 2837–2846
58. Venneti, K. C., and Hewage, C. M. (2011) Conformational and molecular interaction studies of glucagon-like peptide-2 with its N-terminal extracellular receptor domain. *FEBS Lett.* **585**, 346–352
59. 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
60. Perret, J., Van Craenenbroeck, M., Langer, I., Vertongen, P., Gregoire, F., Robberecht, P., and Waelbroeck, M. (2002) Mutational analysis of the glucagon receptor: similarities with the vasoactive intestinal peptide (VIP)/pituitary adenylate cyclase-activating peptide (PACAP)/secretin receptors for recognition of the ligand's third residue. *Biochem. J.* **362**, 389–394
61. Runge, S., Gram, C., Brauner-Osborne, H., Madsen, K., Knudsen, L. B., and Wulff, B. S. (2003) Three distinct epitopes on the extracellular face of the glucagon receptor determine specificity for the glucagon amino terminus. *J. Biol. Chem.* **278**, 28005–28010
62. DaCampa, M. P., Yusta, B., Sumner-Smith, M., Crivici, A., Drucker, D. J., and Brubaker, P. L. (2000) Structural determinants for activity of glucagon-like peptide-2. *Biochemistry* **39**, 8888–8894