Structural Determinants of Binding the Seven-transmembrane Domain of the Glucagon-like Peptide-1 Receptor (GLP-1R)

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The glucagon-like peptide-1 receptor (GLP-1R) belongs to the secretin-like (class B) family of G protein-coupled receptors. Members of the class B family are distinguished by their large extracellular domain, which works cooperatively with the canonical seven-transmembrane (7TM) helical domain to signal in response to binding of various peptide hormones. We have combined structure-based site-specific mutational studies with molecular dynamics simulations of a full-length model of GLP-1R bound to multiple peptide ligand variants. Despite the high sequence similarity between GLP-1R and its closest structural homologue, the glucagon receptor (GCGR), nearly half of the 62 stably expressed mutants affected GLP-1R in a different manner than the corresponding mutants in GCGR. The molecular dynamics simulations of wild-type and mutant GLP-1R-ligand complexes provided molecular insights into GLP-1R-specific recognition mechanisms for the N terminus of GLP-1 by residues in the 7TM pocket and explained how glucagon-mimicking GLP-1 mutants restored binding affinity for (GCGR-mimicking) GLP-1R mutants. Structural analysis of the simulations suggested that peptide ligand binding mode variations in the 7TM binding pocket are facilitated by movement of the extracellular domain relative to the 7TM bundle. These differences in binding modes may account for the pharmacological differences between GLP-1 peptide variants.

The glucagon-like peptide 1 receptor (GLP-1R)6 and the glucagon receptor (GCGR) are class B G protein-coupled receptors (GPCRs) involved in insulin release and glucose homeostasis, respectively. Accordingly, they have attracted extensive attention for their importance as targets for therapeutic intervention of type 2 diabetes (1, 2). Class B GPCRs consist of an N-terminal extracellular domain (ECD) linked to a seven-transmembrane helical (7TM) region. Studies examining truncated, chimeric, and mutated ligand and receptor variants together with multiple ligand-bound ECD crystal structures and two class B 7TM domain crystal structures are consistent with a “two-domain” binding mechanism for peptide hormone ligands to the secretin-like class B GPCRs (3–5). According to this peptide ligand binding mechanism, the C terminus of the peptide hormone forms an initial complex with the ECD, allowing the N terminus of the ligand to interact with the 7TM domain. It is the N-terminal interactions that ultimately result in activation of the receptor and stimulation of downstream coupling to G proteins and other effectors that mediate intra-

6 The abbreviations used are: GLP-1R, glucagon-like peptide-1 receptor; GPCR, G protein-coupled receptor; ECL, extracellular loop; ECD, extracellular domain; 7TM, seven-transmembrane; MD, molecular dynamics; GCGR, glucagon receptor; PDB, Protein Data Bank; COM, center of mass.

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cellular signaling processes. Crystal structures of peptide ligand-bound ECDs of 11 of the 15 secretin-like class B GPCRs have been solved, and these structures exhibit similar binding modes with respect to the C-terminal conformation of the peptide ligands (3, 5). Peptide ligand binding modes revealed by crystal structures of GLP-1- and exendin-(9–39)-bound GLP-1R ECD (6, 7) (supplemental Fig. S1) are consistent with GLP-1 and GLP-1R truncation, chimera, site-directed mutagenesis, and cross-linking studies (4, 7–10). In contrast, structural information on the 7TM domain of class B GPCRs is limited to GCGR (11) and corticotropin-releasing factor 1 receptor (12). So far, no full-length class B receptor structures have been determined resulting in a relative lack of information regarding the biochemical conformation of the N-terminal portion of the peptide hormones, as well as the role of the 7TM domain in peptide ligand recognition. NMR and x-ray crystallography studies indicate that the N-terminal region preceding the conserved α-helix of the class B peptide hormones (GLP-1 His7–Thr13, exendin-4 His8–Thr7, and glucagon His7–Thr13) is flexible in solution as well as in the ligand-bound state (6, 7, 13–15). The receptor-bound conformation of the N-terminal region of class B GPCR peptide ligands is proposed to be stabilized by an amino acid motif that is conserved in the class B GPCR peptide ligands (Thr11–Phe12–Thr13 in GLP-1) (16), and it may induce an N-capping conformation similar to that observed in the ECD-bound NMR structure of pituitary adenylate cyclase-activating polypeptide (PDB ID 1GEA) (17). Recently, NMR structures of an 11-mer GLP-1 analogue were solved in alternative conformations containing a C-terminal α-helix (PDB ID 2N08) and an N-terminal β-turn (PDB ID 2N09), and stabilization of these conformations by cyclization cross-links (PDB IDs 2N0N and 2N0I) differentially influenced GLP-1R binding affinity and agonist potency (18). The accumulated ligand and receptor structure-activity relationships suggest that a flexible conformation of the first seven residues allows the peptide ligands of class B GPCRs to interact with residues deep in the 7TM binding pocket (5) and that the ligand N terminus may adopt a more constrained conformation to activate the receptor as proposed for GLP-1 (18). A central polar hydrogen bond network has been identified in GLP-1R that plays a role in peptide ligand biased signaling (19, 20).

We have reported a full-length GCGR model that combines crystal structures of the GCGR 7TM domain (PDB ID 4L6R) (11), the antibody-bound GCGR ECD (PDB ID 4ERS) (21), and the GLP-1-bound GLP-1R ECD (PDB ID 3IOI) (7). This full-length GCGR model has been experimentally validated with extensive site-directed mutagenesis, electron microscopy (EM), hydrogen/deuterium exchange, and disulfide cross-linking studies (11, 22). Combination of the previously solved crystal structure of the homologous GCGR 7TM domain with systematic mutation studies has identified several residues in the transmembrane binding cavity of GCGR that play an important role in the recognition of glucagon (supplemental Table S1) (11). Detailed structural information on the molecular interactions of the flexible N terminus of GLP-1 with residues in the transmembrane helical binding pocket of GLP-1R is still limited. Photo-cross-linking studies have connected the C-terminal α-helix of GLP-1 to the ECD, TM1, and extracellular loop 2 (ECL2) and the flexible N-terminal region of GLP-1 to TM1 and ECL1 (supplemental Fig. S1) (10, 23, 24). Most site-directed mutagenesis studies of GLP-1R have focused on the ECD (7, 8), ECLs (25), intra-cellular loops (rat GLP-1R in particular) (26, 27), as well as the middle, intracellular, and membrane facing regions of transmembrane helices (19). To date, data for only 21 unique mutants over 18 positions in the helical 7TM binding pocket have been reported for GLP-1R (Y152D, R190K, K197H, D198H, W284F, K288M, Y305F, W306F, D0959, H363K, E364K, R3807, F3817, K383E, L3847, E3877, T3917, S3927, and S3927) (19, 20, 25, 28–30). In contrast, 70 unique mutants over 30 positions in the 7TM helical binding pocket of GCGR have been reported (5, 11) (Fig. 1 and supplemental Tables S1 and S2).

Although GLP-1R and GCGR share over 50% sequence identity, they have opposing physiological roles; GCGR is activated by glucagon during fasting and mobilizes glucose into the blood, whereas GLP-1R functions postprandially by stimulating insulin secretion to control circulating glucose levels (1, 2). It is thus necessary to understand the ligand selectivity and recognition mechanism of the GLP-1R/GCGR axis to design specific drugs directed at either receptor. Chimeric constructs of GLP-1 in combination with glucagon have been used to identify structural determinants of ligand selectivity for GLP-1R and GCGR (31–33). Substitution of C-terminal GLP-1 residues with corresponding glucagon residues decreases GLP-1R binding and maintains low binding affinity for GCGR. The affinity of this GLP-1/glucagon chimera is however rescued by substituting the 7TM domain of GCGR with that of GLP-1R, suggesting that the N-terminal region of GLP-1 (His7–Leu20) is selectively recognized by the 7TM domain of GLP-1R (31–33). Chimeric peptide ligands consisting of the N-terminal part of glucagon combined with the C-terminal part of GLP-1 have high affinity for both GCGR and GLP-1R (31). These GLP-1/glucagon chimera experiments demonstrate that the ECD and 7TM domains of GLP-1R both play a role in GLP-1/glucagon selectivity but do not provide detailed structural insights into the molecular mechanism that drives selective recognition of the N-terminal region of GLP-1 by the 7TM domain of GLP-1R. The objectives of this study were therefore to identify the structural determinants of GLP-1 binding in the 7TM domain of GLP-1R and to elucidate the molecular mechanism of selective recognition of GLP-1.

Experimental Procedures

Construction of GLP-1R and GCGR Point Mutants—To study the influence of specific residues on receptor function, the desired mutations were introduced to wild-type GCGR (11) and N-terminally FLAG® tag-labeled wild-type human GLP-1R in the pcDNA3.1/V5-His-TOPO vector (Invitrogen); this GLP-1R receptor construct had equivalent pharmacology to GLP-1R (Y152D, R190K, K197H, D198H, W284F, K288M, Y305F, W306F, D0959, H363K, E364K, R3807, F3817, K383E, L3847, E3877, T3917, S3927, and S3927) (19, 20, 25, 28–30). In contrast, 70 unique mutants over 30 positions in the 7TM helical binding pocket of GCGR have been reported (5, 11) (Fig. 1 and supplemental Tables S1 and S2).

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Cell Culture and Transfection—CHO-K1 or HEK-293T cells were seeded onto 96-well poly-d-lysine or fibronectin-treated cell culture plates (PerkinElmer Life Sciences) at a density of 2.7 × 10^4 per well. After overnight culture, the cells were transiently transfected with wild-type or mutant GLP-1R or GCGR DNA using Lipofectamine 2000 transfection reagent (Invitrogen).

Whole Cell Binding Assay—Cells were harvested 24 h after transfection, washed twice with the blocking buffer (F12 supplemented with 33 mM HEPES and 0.1% bovine serum albumin (BSA), pH 7.4), and incubated with blocking buffer for 2 h at 37 °C. For homogeneous binding, the cells were incubated in binding buffer (PBS with 10% BSA, pH 7.4) with a constant concentration of ^125I-GLP-1, ^125I-exendin(9–39), or ^125I-glucagon (40 pm) and different concentrations of unlabeled GLP-1 (3.57 pm to 1 pm), exendin-4 (3.57 pm to 1 pm), exendin-(9–39) (3.57 pm to 1 pm), or truncated GLP-1 (GLP-1(7–36), GLP-1(9–36), and GLP-1(15–36) (17.86 pm to 5 pm) at room temperature for 3 h. Cells were washed three times with ice-cold PBS and lysed by 50 μl of lysis buffer (PBS supplemented with 20 mM Tris-HCl, 1% Triton X-100, pH 7.4). The plates were subsequently counted for radioactivity (counts/min (cpm)) in a scintillation counter (PerkinElmer Life Sciences).

cAMP Assay—cAMP accumulation was measured using HTRF-cAMP dynamic kit (Cisbio International, Gif sur Yvette Cedex, France) according to the manufacturer’s instructions. Briefly, HEK 293T cells were transfected with plasmids bearing wild-type and mutant GLP-1R. Twenty four h post-transfection, cells were collected and used to seed white poly-D-lysine-coated 384-well plates at a density of 8000 cells per well. Cells were incubated for a further 24 h at 37 °C. They were then incubated for 30 min in assay buffer (DMEM, 1 mM 3-isobutyl-1-methylxanthine) with different concentrations of peptides at 37 °C. The cells were then washed three times with ice-cold PBS and lysed by 50 μl of lysis buffer (PBS supplemented with 20 mM Tris-HCl, 1% Triton X-100, pH 7.4). The plates were subsequently counted for radioactivity (counts/min (cpm)) in a scintillation counter (PerkinElmer Life Sciences) using a scintillation mixture (OptiPhase SuperMix, PerkinElmer Life Sciences).

Quantification of Cellular GLP-1R Construct Expression Levels by Flow Cytometry—Approximately 1 × 10^5 transfected cells were blocked with PBS containing 5% BSA at room temperature for 15 min and incubated with 1:1000 diluted primary antibody (anti-FLAG, Invitrogen) at room temperature for 1 h. The cells were then washed three times with PBS containing 1% BSA followed by 1-h incubation with anti-rabbit Alexa-488-conjugated secondary antibody (1:300, Invitrogen) at 4 °C in the dark. After the washes, the cells were resuspended in 200 μl of PBS containing 1% BSA for detection in a flow cytometer (Accuri™ C6, BD Biosciences) utilizing laser excitation and emission wavelengths of 488 and 519 nm, respectively. For each assay point, ~20,000 cellular events were collected, and the total fluorescence intensity of positive expression cell population was calculated.

Residue Numbering—Peptide ligand residue numbers are annotated as three-letter amino acid residues with residue number as superscript (e.g. His^7, histidine at position 7), and the receptor residue numbers are annotated as single letter amino acid, Uniprot number, and Wootten numbering for class B GPCRs as superscript (19), according to IUPHAR guidelines (34) and GPCR residue numbering guidelines (35), respectively. GLP-1 and glucagon peptide ligands start with amino acid residue 7 (His^7) due to post-translational processing.

Construction of a Full-length GLP-1R Model—A full-length GLP-1R model was constructed based on an experimentally validated (11, 22) full-length GCGR-glucagon complex model combining crystal structures of the GCGR 7TM domain (PDB ID 4L6R) (11), the antibody-bound GCGR ECD (PDB ID 4ERS) (21), and the GLP-1-bound GLP-1R ECD (PDB ID 3IOL) (7). The GLP-1-bound full-length GLP-1R model satisfies spatial constraints defined by GLP-1R cross-linking studies (10, 23, 24) connecting the following: (i) Ala^24 in the C-terminal part of the α-helix of GLP-1 to E133^{31b} in TM1 stalk; (ii) Leu^20 in the N-terminal part of the α-helix of GLP-1 to W297^{ECL2} in ECL2 between TM4 and TM5 in GLP-1R; and (iii) Phe^12 and Val^16 in the flexible N-terminal region of GLP-1 to Y145^{3.43b} and L141^{3.90b} in TM1 of GLP-1R, respectively. A photo-labile probe at position 6 (one position before His^7) in GLP-1 may target Y205^{ECL1} between TM2 and TM3 in GLP-1R in an intermediate conformation in which the N terminus of GLP-1 does not fully protrude in the 7TM binding pocket of GLP-1R, consistent with a significant decrease of affinity and potency of this [Bpa^6,Arg^26,34]GLP-1(7–36) ligand compared with GLP-1 (23).

Molecular Dynamics (MD) Simulations—The full-length GLP-1-bound GLP-1R model was used to prepare mutant receptors and peptides as the starting structure of eight independent MD simulations: wild-type GLP-1 R bound to the following: (i) wild-type; (ii) R190^{2.60b}K mutant; (iii) Q234^{3.37b}E mutant; (iv) E364^{5.53b}Q; (v) E387^{4.42b}D mutant GLP-1R; (vi) Ala^{3}Ser mutant GLP-1 bound to E387^{7.42b}D mutant GLP-1R; (vii) Glu^{6}Gln mutant GLP-1 bound to R190^{2.60b}K mutant; and (viii) Q234^{3.37b}E mutant GLP-1R. To set up the simulation systems, the eight starting structures were embedded separately in a 90 × 90 Å^2 palmitoyloleoylphosphatidylcholine bilayer generated with VMD (Version 1.9.2) (36). In each system, lipids located within 1 Å of the complex models were removed. Subsequently, each system was solvated by TIP3P water molecules with 0.15 M NaCl and included ~114,160 atoms (90 × 90 × 140 Å^3). MD simulations were performed using the GROMACS 4.6.1 package (37) with isothermal-isobaric (NPT) ensemble and periodic boundary condition. The CHARMM36-CAMP force field (38) was applied. For each system, energy minimizations were first performed to relieve unfavorable contacts, followed by equilibration steps of 50 ns in total to equilibrate the lipid bilayer and the solvent, with restraints to the main chain of the protein and the peptide ligand. The temperature of each system was maintained at 310 K using the v-rescale method (39) with a coupling time of 0.1 ps. The pressure was kept at 1 bar using the Berendsen barostat (40) with τ_p = 1.0 ps and a compressibility of 4.5 × 10^-5 bar^-1. SETTLE (41) constraints and LINCS (42) constraints were applied on the hydrogen-involved covalent bonds in water molecules and in other molecules, respectively, and the time step was set to 2 fs. The long range electrostatic interactions were computed using the Particle-
Mesh Ewald algorithm (43) with a real space cutoff of 1.4 nm. For each system, one 1000-ns production run was performed. Analysis of Protein-Peptide Ligand Interactions—For each snapshot extracted at 100-ps intervals, we calculated the H-bond interactions formed by the main-chain atoms as well as the side-chain atoms of the first three N-terminal residues of peptides with specific receptor residues using the g_hbond program in the GROMACS 4.6.1 package (37), based on a distance cutoff of 3.5 Å and an H-bond interaction angle of 150°–210°. Ionic interactions were monitored using the g_mindist program, based on a 4-Å distance cutoff between two heavy atoms with opposite formal charge. Apolar contacts were also monitored using the g_mindist tool, based on a 5-Å minimum distance cutoff between carbon atoms.

Analysis of Protein Conformations—To describe the motions between the ECD and 7TM domain of different GLP-1R/Ligand complexes, we constructed a Cartesian coordinate system in a similar way as described previously for full-length GCGR (22). As the whole ECD and residues preceding I147 in TM1 are very dynamic in most simulations, we took the C92 atom of I147 in TM1 as the origin (designated as point O), the outward membrane normal as its z axis, the plane parallel to the membrane surface as the xy plane, and the plane defined by the z axis and the center of mass (COM) of the 7TM domain as the xz plane. Of all systems, the distance d between the COMs of the ECD and 7TM domain is monitored, as well as the polar angle and the azimuthal angle of vector OC (linking the origin and the COM of ECD) describing the swing and rotation motions of the ECD in the simulations.

Results

Comparative Structure-based Mutagenesis of GLP-1R and GCGR—We constructed a full-length GLP-1-bound GLP-1R model to guide the systematic GLP-1R mutagenesis studies probing the peptide-binding site. The structural model was based on a 4-Å distance cutoff between two heavy atoms with opposite formal charge. Apolar contacts were also monitored using the g_mindist tool, based on a 5-Å minimum distance cutoff between carbon atoms.

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based on an experimentally validated full-length GCGR-glucagon complex model that combined the crystal structures of the GCGR 7TM domain (11), the antibody-bound GCGR ECD (21), and the GLP-1-bound GLP-1R ECD (7). These two models are consistent with the results from mutation studies of GCGR, GLP-1R, and other class B GPCRs and photo-cross-linking studies connecting residues of GLP-1R and GLP-1, as described under “Experimental Procedures” and summarized in Table 1, Fig. 1, and supplemental Fig. S1 (5, 11). Based on this model, a total of 66 mutants were created and tested for expression (complementing the 76 human GLP-1R mutants from supplemental Table S1) (7, 8, 19, 20, 25, 28–30, 32). Of these mutations, 64 covering 40 different positions had expression levels greater than 30% of wild-type allowing further characterization. Of these expressing mutants, 40 covering 23 positions in GLP-1R had more than 4-fold reduction in GLP-1 binding (IC50 values) relative to wild-type (Fig. 1). GLP-1R mutants generally had similar effects on GLP-1 binding affinity and agonist potency (supplemental Table S3). The R1902.60bA, R1902.60bK, K1972.67bA, D1982.68bA, K2884.64bA, E292ECL2A, and W297ECL2A GLP-1R mutations analyzed in this study showed similar negative effects on GLP-1 binding as reported previously (19, 25, 28, 30), whereas H212ECL1A (rat GLP-1R) and N304ECL2A exhibited no significant effect on GLP-1 binding, similar to the corresponding mutants in GCGR (Table 1). In contrast, 28 mutants (containing 21 unique residue positions) exerted differential effects on GLP-1R compared with the corresponding mutants in GCGR. For example, Y1521.47bH, V1942.64bA, M2333.36bF, Q2343.37bN, E3645.36bA, K3656.56bQ, L3887.43bF showed no significant effect on GLP-1 binding, whereas the corresponding mutants in GCGR all had a marked effect (in most cases negative) on glucagon binding (Table 1). In contrast,
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The effects of GLP-1R mutations on full-length and truncated GLP-1 and exendin-4 ligand binding

| GLP-1R construct | GLP-1 (IC50 mM)* | GLP-1-(7–36) (IC50 mM)* | GLP-1-(9–36) (IC50 mM)* | Exendin-4 (IC50 mM)* | Exendin-(9–39) (IC50 mM)* |
|------------------|------------------|-------------------------|--------------------------|---------------------|-------------------------|
| wild type        | 15 ± 1           | 6.1 ± 3.2               | 234 ± 62                 | 104 ± 26            | 12 ± 4                  | 26 ± 7                  |
| Y148F            | 129 ± 72         | 72.2 ± 46               | 169 ± 46                 | 70 ± 3              | 87 ± 11                 | 6.1 ± 0.7               |
| R190K            | 154 ± 68         | 175 ± 29               | 220 ± 27                 | 110 ± 15            | 64 ± 9                  | 12 ± 3                  |
| K197I            | 415 ± 139        | 502 ± 119               | 390 ± 155                | 266 ± 52            | 78 ± 12                 | 27 ± 4                  |
| D198A            | 663 ± 124        | 355 ± 36               | 154 ± 87                 | 211 ± 1             | 141 ± 51                | 24 ± 4                  |
| M204E            | 253 ± 59         | 134 ± 9                | 220 ± 23                 | 120 ± 1             | 46 ± 11                 | 11 ± 4                  |
| Y220D            | 211 ± 15         | 88 ± 8                 | 268 ± 45                 | 139 ± 3             | 30 ± 5                  | 6.1 ± 0.3               |
| R299S            | 0.3 ± 0.04       | 0.3 ± 0.05             | 22 ± 6                   | 21 ± 9              | 0.7 ± 0.04              | 13 ± 6                  |
| M233A            | 1.8 ± 0.3        | 1.7 ± 0.3              | 10 ± 2                   | 9.1 ± 1             | 12 ± 2                  | 69 ± 11                 |
| L384A            | 718 ± 231        | 910 ± 30               | 209 ± 83                 | 97 ± 10             | 66 ± 14                 | 8.4 ± 2.7               |
| E364Q            | 178 ± 28         | 90 ± 27                | 149 ± 46                 | 81 ± 28             | 43 ± 7                  | 23 ± 2                  |

* IC50 values (means ± S.E. of at least three independent experiments) are based on 125I-exendin-(9–39) displacement studies in CHO cells expressing GLP-1R. Values shown are means ± S.E. of at least three independent experiments.

TABLE 2

Y148F, M233A, Q234E, F364Q, and L384A mutations in GLP-1R decreased GLP-1 binding, although the same mutations of the corresponding residues in GCGR had no significant impact on glucagon affinity. The GCGR-mimicking GLP-1R E387D mutant had a 3-fold greater negative impact on GLP-1 binding than the inverse GCGR D385E mutant on glucagon binding. The GCGR-mimicking GLP-1R mutants R190K, M204E, Y220D, and R299S significantly reduced GLP-1 binding affinity (Table 1), whereas the reverse GLP-1R-mimicking GCGR mutants K187R, R201M, D218Y, and S297R did not show any marked effect on glucagon binding.

Probing GLP-1R Mutants with Truncated Peptide Ligands—To assess whether the mutational effects in the 7TM-binding site resulted from interactions with the N terminus of peptide ligands, nine GLP-1R mutants were investigated in 125I-ligand displacement studies with six different full-length and truncated peptides ligands (GLP-1, GLP-1-(7–36), GLP-1-(9–36), GLP-1-(15–36), exendin-4, and exendin-(9–39)) (Table 2). The GLP-1R mutant set included three GCGR-mimicking GLP-1R mutants (R190K, K197I, and E387D), four mutants that abolished GLP-1 binding, although the corresponding/reverse GCGR mutants did not affect glucagon binding (Y148F, M233A, Q234E, and F364Q), and two mutants that diminished GLP-1 binding in a similar way as corresponding GCGR mutants that affected glucagon binding (D198A and L384A), and one mutant that increased GLP-1 binding, although the corresponding GCGR mutant decreased glucagon binding (E364Q) (Table 1 and Fig. 2). With the exception of the F364Q and E364Q, all of the other seven mutants led to reduced binding of GLP-1 and GLP-1-(7–36), whereas binding of all the truncated peptide ligands GLP-1-(9–36), GLP-1-(15–36), exendin-(9–39) was not affected compared with wild-type GLP-1R (Table 2). These results demonstrate that the effects of these mutations on GLP-1R binding result from interactions between these residues located in the 7TM domain of the receptor and the N-terminal region of the ligand. GLP-1R (1–36) lacks the seven first residues of GLP-1 and GLP-1-(7–36) that are proposed to target the 7TM-binding site in our peptide ligand-bound GLP-1R structural models (Fig. 3A). GLP-1R (1–36) lacks the first two residues that are required to form a tight interaction with the 7TM domain of GLP-1R, reflected by the decreased affinity of this truncated analogue compared with GLP-1 (Table 2). Most of the mutants affected both GLP-1 and exendin-4 binding (Table 2), suggesting that the N-terminal regions of GLP-1 and exendin-4 share similar binding modes in general. The M233A, Q234E, and E387D mutants, however, only decreased GLP-1 binding but did not significantly affect exendin-4 affinity (Table 1 and Fig. 3), indicating that interactions and compatibility with these three residues are more important for GLP-1 binding. These observations are in line with previous NMR, x-ray crystallography, and truncated and chimeric GLP-1R and peptide ligand studies showing that GLP-1 binding is largely determined by interactions with the ECD, but also require interactions with the 7TM domain of GLP-1R, whereas exendin-4 binding affinity is mainly deter-
mined by interactions with the ECD of GLP-1R and is less dependent on interactions with the 7TM domain than GLP-1 (4, 6, 7, 9, 32, 33). The E364.53bQ mutant, located deep in the transmembrane bundle, has increased affinity for both full-length and truncated GLP-1 and exendin-4 (Table 2), implying that this mutation stabilizes GLP-1R in a conformation with high affinity for peptide ligands.

Comparison of GLP-1R-GLP-1 and GCGR-Glucagon Binding Modes—The GLP-1-bound GLP-1R model was subjected to MD simulations to evaluate it and to further investigate the molecular details of GLP-1R-ligand interactions aimed at understanding the observed differential mutational effects between GLP-1R and GCGR. GLP-1R residues shown to be important in mutation studies are involved in GLP-1 interactions in the MD simulations (Table 1 and Figs. 1, 2, and 4). MD simulations of wild-type GLP-1R and the high affinity E364.53bQ mutant of GLP-1R displayed frequent ionic interactions between the terminal amino group of GLP-1 and E387.42b in combination with tight (E364.53Q) and transient (WT) ionic H-bond interactions between K197.26b and Glu9, consistent with the negative effect of the GCGR-mimicking K197.26bI mutant (Table 1, Fig. 4, and supplemental Fig. S2). The simultaneous ionic interactions of GLP-1 with K197.26b and E387.42b offer an explanation for the positive effect of the E364.53Q mutant on GLP-1 binding (Table 2). The position of the flexible GLP-1R-specific K197.26b residue is stabilized by an H-bond interaction network with D198.26b and Y148.147b, which may explain the relatively negative effects of D198.26bA, D198.26bN, and Y148.147bF mutants on ligand binding compared with that of corresponding mutants in GCGR (Table 1 and Fig. 4). When Glu9 forms an ionic H-bond interaction with K197.26b, the amine group of His7 forms ionic H-bond interactions with E364.53b exclusively (Fig. 4D), but when GLP-1 adopts a more constrained binding conformation stabilized by intra-molecular ionic interactions between His7 and Glu9, the N terminus of GLP-1 forms ionic H-bond interactions with E387.42b exclusively (Fig. 4E). The model is consistent with the observation that mutations of single E364.53bN and E387.42bQ mutants do not affect GLP-1 binding affinity or signaling potency, although the double E364.53bN/E387.42bQ mutant completely abolishes GLP-1 binding (span <10%).

Analysis of MD simulations of glucagon-bound GCGR, consistent with previously reported hydrogen/deuterium exchange data (15), showed that the N-terminal His7 residue of glucagon forms a stable ionic H-bond interaction with D385.7.42b and a transient H-bond or ionic interaction with E362.6b (Fig. 4, A–C). In GLP-1-bound GLP-1R, the R190.6b residue forms a tight intra-helical H-bond with E364.53b at the bottom of the
7TM-binding site, whereas in glucagon-bound GCGR the ionic interaction between K187.60b and E364.53b is more transient (Fig. 4, D–F). This difference between GLP-1R and GCGR may explain why the R190.60bK mutant diminishes GLP-1 binding to GLP-1R, while the inverse K187.60bR does not affect glucagon binding to GCGR (Table 1). In addition to this tight ionic interaction network deep in the transmembrane helical bundle, our MD simulations support an earlier proposed (29) ionic interaction between Asp15 and R380.7.35b (Fig. 4) and predict a previously untested electrostatic interaction between Glu21 and R299.ECL2. This predicted interaction is supported by the mutation of these residues to Gln and Ser (mimicking GCGR). The GCGR mutation data and glucagon-bound GCGR model suggest that R378.7.35b plays a similar role in binding Asp15 of glucagon and explain why the reverse S299.ECL2R mutant in GCGR did not affect glucagon affinity, as the homologous glucagon Asp21 formed an intra-helical H-bond with Arg24 (Fig. 4, A–C, and supplemental Fig. S1). In contrast, the previously reported GCGR model (11, 22) suggested that D208.ECL1 forms an ionic interaction with the glucagon-specific Arg23 residue, explaining the negative effect of the GCGR D208.ECL1Q mutant on glucagon binding (Figs. 1 and 2 and supplemental Table S1). These predictions were indeed confirmed by our mutation experiments (Table 3). Systematic microsecond MD simulation studies of different wild-type/mutant GLP-1R/GLP-1 complexes provided insight into subtle GLP-1R-specific recognition mechanisms of the N terminus of GLP-1 by GLP-1R-specific hot spots in the 7TM domain described below (Fig. 5 and supplemental Fig. S3). The H-bond/ionic interaction between E387.7.42b and the N-terminal amine group of His7 is disrupted in the GCGR-mimicking E387.7.42bD mutant, resulting in expulsion of the N terminus of GLP-1 from the 7TM

**FIGURE 4.** Analysis of receptor-ligand interactions in GLP-1-bound GLP-1R and glucagon-bound GCGR. Comparison of 1000-ns MD simulations of GLP-1 (green) in GLP-1R (A–C) and glucagon (light green) in GCGR (D–F). Based on the analysis of receptor-ligand interaction patterns in GLP-1R (C) and GCGR (F), the structures of two representative snapshots at similar time frames (MD snapshots 1 and 2, indicated by green arrows and lines) of GLP-1R (A and B) and GCGR (D and E) MD simulations are shown. The analyses of H-bond interactions between positively and negatively charged groups (red), other H-bond interactions in which backbone groups (black) or only side-chain groups (gray) are involved, and apolar contacts (yellow) of specific residues in GLP-1/GLP-1R (C) and between specific residues in glucagon/GCGR (F) are shown.

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TABLE 3

Combined GLP-1 and GLP-1R mutation studies

The IC₅₀ values of GLP-1 mutants that show <4-fold change (blue), 4–10-fold increase (orange), >10-fold increase (red), and >40-fold decrease (cyan) compared with IC₅₀ values for wild-type GLP-1R are color-coded according to the snake plot in Fig. 1. Radioligand displacement curves of representative mutants are shown in Fig. 5. Expression and span levels are reported in supplemental Table S3 and supplemental Table S5, respectively.

| GLP-1R construct | GLP-1* | GLP-I/Ala(Ser)* | GLP-I/Glu(Gln)* |
|------------------|--------|----------------|----------------|
| Wild-type        | 15 ± 1 | 61 ± 9         | 25 ± 4         |
| R1902.60bK       | 47 ± 1 | 109 ± 21       | 25 ± 4         |
| Q2343.37bE       | 244 ± 15 | 118 ± 15       | 81 ± 16        |
| E3877.42bD       | 128 ± 24 | 23 ± 10        | 92 ± 18        |

* IC₅₀ values (means ± S.E. of at least three independent experiments) are based on [¹²⁵I]-exendin-(9–39) displacement studies in CHO cells expressing GLP-1R. Values shown are means ± S.E. of at least three independent experiments.

Binding pocket of GLP-1R in MD simulations and diminished GLP-1 binding in radioligand displacement studies (Fig. 5, A–C, and supplemental Fig. S3). MD simulations suggest that this H-bond interaction network is re-established by the glucagon-mimicking Ala⁸Ser GLP-1 mutant via formation of an additional stabilizing H-bond with the carboxylic group of D387.43b with the hydroxyl group of the Ser⁸ side chain, leading to restored binding affinity equal to the wild-type GLP-1R (wild-type complex, Fig. 5, A–C, and supplemental Fig. S3). The ionlock between R1902.60b and E3646.53b is broken in the R1902.60bK mutant, resulting in an increased distance between TM2 and TM7 and destabilization of the ionic interaction network between the N-terminus of GLP-1 and the GLP-1R-binding site (Fig. 5, D–F, and supplemental Fig. S3). The GLP-1 Glu³Gln mutant lacks strong ionic intra-molecular interaction between Glu³ and the N-terminal amino group of His⁷ and can therefore adopt an extended conformation that allows the N-terminus of GLP-1 to form ionic interactions with E3646.53b and E387.42b deep in the GLP-1R-binding pocket. In the MD simulations, this binding mode stabilizes an ionic interaction network between K197.67b, E3646.53b, and His⁷, whereas Gln⁹ forms a transient H-bond with K197.67b, restoring the receptor’s binding affinity (Fig. 5, D–F, and supplemental Fig. S3). The Q2343.37bE mutation causes electrostatic repulsion with Glu³ leading to a binding orientation of the N-terminus of GLP-1 that does not allow the formation of intermolecular ionic interactions with E387.42b (Fig. 5G). The MD simulations indicate the neutral Gln⁹ residue in the GLP-1 Glu³Gln mutant is compatible with the negatively charged E2343.37b residue in the GLP-1R Q2343.37bE mutant and allows the N-terminus of GLP-1 to penetrate deeper into the 7TM binding pocket of GLP-1R and interact with E387.42b, resulting in a restored receptor binding affinity (Figs. 4 and 5, G–I, and supplemental Fig. S3).

Discussion

Molecular Mechanism of Class B GPCR Peptide Ligand Selectivity in the 7TM Domain—By combining systematic receptor and peptide ligand site-directed mutagenesis studies with extensive MD simulations, we have identified conserved and receptor-specific peptide interaction hot spots in the 7TM domains of GLP-1R and GCGR. Mutations of homologous residues in the 7TM domain of other class B GPCRs have been shown to affect peptide ligand binding as well (5), including human gastric inhibitory polypeptide or glucose-dependent insulinotropic peptide (GIP) (R183.26b, R190.26b, Q224.36b, R300.40b, and F377.53b) (44, 45), human vasoactive intestinal polypeptide (VIP) (1R1882.60b, K195.67b, and L375.43b) (46, 47), and rat secretin (Y124.43b, Y128.47b, R176.40b, K173.43b, D174.42b, F201.36b, W274.53b, F337.56b, and L353.43b) (48–50) receptors. Our comparative GLP-1R and GCGR studies show differential effects for almost half of the 62 stably expressed mutants of different residue positions in the 7TM domain, demonstrating that binding of homologous peptide ligands of similar size by homologous class B GPCRs is determined by receptor-specific structural features in the 7TM domain (Table 1 and Figs. 1 and 2). The negative effects of GLP-1R mutants located deep in the 7TM pocket were not observed for truncated peptide ligands of GLP-1R, indicating that these differential mutation effects result from GLP-1R-specific interactions between the 7TM domain and the N-terminal region of the ligand (Table 2 and Fig. 3).

The MD simulations of wild-type and mutant GLP-1R-ligand complexes provide molecular insights into GLP-1R-specific recognition mechanisms for the N terminus of GLP-1 by residues in the 7TM domain, and they explain how glucagon-mimicking GLP-1 mutants restore binding affinity for (GCGR mimicking) GLP-1R mutants (Figs. 4 and 5). The combined mutation and molecular dynamics investigations suggest that the negatively charged Glu³ residue of GLP-1 does not allow the N-terminus of GLP-1 to adopt to changes in the 7TM-binding site as follows: (i) by attractive and repulsive electrostatic interactions with the positively charged (K197.67b) and negatively charged (Q2343.37bE mutant, E387.42b) residues in the receptor; and (ii) by constraining the ligand conformation via intra-molecular ionic interactions with the peptide N-terminal His⁷ (Figs. 4 and 5). Although the intra-molecular H-bond-stabilized, bend conformation of residues 8–11 in GLP-1 in the MD simulations (Fig. 4B) differs from the disulfide cross-link stabilized β-turn conformation of homologous residues 2–5 of an 11-mer GLP-1 analogue observed in NMR structures (PDB ID 2N09) (18), both studies suggest that GLP-1 may adopt a constrained conformation in the 7TM-binding site of GLP-1R. The neutral Gln⁹ residue in glucagon allows the peptide ligand to adopt a more extended binding mode deeper in the 7TM binding pocket of GCGR in which the N-terminal amine of glucagon (His⁷) forms an ionic interaction with E⁵.53b, observed in our GCGR-glucagon MD simulation runs and mimicked by the binding modes of GLP-1 Glu³Gln mutants (Figs. 4, D–F, and 5). Comparison of the GLP-1R and GCGR models explains why mutation of E⁶.53b into neutral (Asn, Gln) residues has a negative effect on GCGR-glucagon binding and not on GLP-1R-GCGR binding (Fig. 1 and supplemental Table S1). Comparison of MD simulations of complexes of GLP-1 with GLP-1R (Fig. 4, A–C), glucagon with GCGR (Fig. 4, D–F), and the glucagon- and GCGR-mimicking GLP-1 (Glu³Gln) with GLP-1R (R190.26bK) (Fig. 5E) indicates that the extended, deeper peptide ligand binding mode of glucagon/GLP-1 (Glu³Gln) is facilitated by the weaker ionic interaction network between K².60b and E⁶.53b in GCGR and the GLP-1R R190.26bK mutant compared with the tighter ionic lock between R190.26bK and E364.53b in wild-type GLP-1R. These observations are in
line with the results of recent mutagenesis and modeling studies, including alanine mutants R190<sup>2.60b</sup>A and E364<sup>6.53b</sup>A, which suggested that an ionic lock between R190<sup>2.60b</sup> and E364<sup>6.53b</sup> plays a role in GLP-1R ligand-biased signaling (20), emphasizing the important role of this polar H-bond network in both functional activity and ligand recognition by GLP-1R.

### FIGURE 5. MD simulations explain combined GLP-1R and GLP-1 mutation studies

GLP-1 binding to GLP-1R mutants R190<sup>2.60b</sup>K(A), E387<sup>7.42b</sup>D(D), and Q234<sup>3.37b</sup>E(G) is restored by combination with glucagon-mimicking GLP-1 mutants Ala<sup>8</sup>Ser(B) and Glu<sup>9</sup>Gln(E and H), is determined by<sup>125</sup>I-exendin-(9–39) displacement studies(C, F, and I), and is consistent with representative snapshots(A, B, D, E, G, and H) and interaction analyses(C, F, and I) of MD simulations studies(supplemental Fig. S3). Data in<sup>125</sup>I-exendin-(9–39) displacement curves(C, F, and I) are expressed as a percentage of specific<sup>125</sup>I-exendin-(9–39) binding in the presence of 3.57 pmol unlabeled peptide. Each point(± S.E.) represents the mean value of at least three independent experiments done in triplicate(IC<sub>50</sub> data presented in Table 3 and supplemental Table S5).
GIP receptor shares R\(^2.60b\), K\(^2.67b\), and E\(^6.53b\) residues with GLP-1R, suggesting that the structurally aligned Glu\(^3\) residue of GIP may play a similar role in determining the receptor-ligand binding mode as Glu\(^9\) in GLP-1. The GLP-1R binding mode model may also serve as a useful template for other class B GPCRs such as vasoactive intestinal peptide receptor type 1 (VPAC1), pituitary adenylate cyclase-activating polypeptide (PACAP) receptor (PAC1), and secretin receptor that all contain R\(^2.60b\), K\(^2.67b\)/R\(^2.67b\), and E\(^7.42b\) residues (but no E\(^6.53b\)) and that bind natural peptide ligands with conserved Ser and Asp residues structurally aligned to Ser\(^8\) and Glu\(^9\) of GLP-1. Previously reported combined receptor and ligand mutation studies have indeed suggested that homologous residues Gln\(^9\) of glucagon, Asp\(^1\) of secretin, and Asp\(^3\) of VIP may be located within the same vicinity in the 7TM domain of GCGR (K1872.60b and I1942.67b) (51, 52), rat secretin receptor (Y1281.47b, R1662.60b, Lys1732.67b, and D1742.68b) (48, 49), and VPAC1 (R1882.60b and K1952.67b) (46), respectively, consistent with our mutation studies and structural model.

The observation that the glucagon-mimicking Ala\(^8\)Ser mutant of GLP-1 restores binding of the GCGR-mimicking E3877.42bD mutant of GLP-1R is consistent with previous mutation studies showing that the affinity of the reciprocal Ser\(^8\)Ala mutant of glucagon restores binding of the GLP-1R-
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mimicking D3857.42bE mutant of GCGR (52). Our MD simulations indicate that Ala8 is compatible with the longer E7.42b side chain, whereas Ser9 stabilizes the ionic interaction with D7.42b by forming an additional H-bond with the shorter D7.42b side chain (Figs. 4 and 5). These proposed subtle steric requirements for the formation of (alternative) H-bond interaction networks between the 7TM domain of class B GPCRs and the N-terminal region of their peptide ligands motivate detailed combined investigation of both receptor and ligand structure-activity and structure-selectivity relationships.

Motions between the ECD and 7TM Domain of GLP-1R Accommodate Different GLP-1 Binding Modes—In our recent hybrid structural biology studies combining x-ray crystallography, electron microscopy, hydrogen/deuterium exchange, and cross-linking studies, we demonstrated how peptide ligand binding determines the relative orientation of the ECD and 7TM domains of the homologous GCGR (22). In the current study, we investigated how the relative motions between the ECD and 7TM domain of wild-type and mutant GLP-1R can accommodate different binding modes of GLP-1 and GLP-1 mutants (Fig. 6). In 1-μs MD simulations, GLP-1-bound wild-type GLP-1R adopts a similar open conformational state as glucagon-bound GCGR, as reported previously (22), with similar distances and swing and rotation motions of the ECD relative to the 7TM domain (supplemental Fig. S4). The relatively smaller swing motion (2-fold smaller polar angle θ) and larger rotational motion (2-fold higher Azimuthal angle ϕ) of the ECD of the E3877.42b GLP-1R mutant allows the GLP-1 Ala8Ser mutant to bind closer to TM7 to form a tight H-bond network with the mutated D3877.42b residue (Figs. 5A and 6A). The larger swing motion of the ECD of the R1907.67b GLP-1R mutant (supplemental Fig. S4) enables the GLP-1 Glu9Gln mutant to position its helical region closer to the helix to allow its N-terminal region to bind in an extended conformation in the 7TM pocket and form an ionizing interaction network with E364.53b and E3877.42b simultaneously (Figs. 5B and 6B). The relatively smaller swing motion and larger rotational motion of the ECD of the Q234.37b GLP-1R mutant allows the GLP-1 Glu9Gln mutant to interact and form an H-bond interaction network with the mutated E234.37b residue and simultaneously interact with E364.53b and E3877.42b (Figs. 5A and 6A).

The comparative mutation and modeling studies suggest that binding of GLP-1 to GLP-1R is controlled by a similar conformational selection mechanism as proposed previously for the binding of glucagon to GCGR (22). Although chimeric receptor and peptide/protein ligand studies have indicated that the first binding step with the ECD provides the largest contribution to ligand binding affinity (3, 9), previous (5) and current site-directed mutagenesis studies demonstrate that structural and electrostatic compatibility with the 7TM domain plays an equally important role in the molecular recognition of peptide ligands by GPCRs. Integration of the recent hybrid structural biology studies of the conformational states of full-length class B GPCRs (11, 22) with previous (3, 5) and current structure-based molecular pharmacology investigations suggests that a combination of optimal interaction networks between the peptide ligand and (i) ECD and (ii) 7TM domains and (iii) relative flexibility of the ECD and 7TM domains and (iv) the peptide ligand determines GPCR-peptide ligand selectivity. The importance of these different factors is likely to be receptor-ligand complex-specific and may for example explain pharmacology differences observed between GLP-1 peptide variants. The differential point mutation effects on GLP-1 compared with exendin-4 in the 7TM-binding site, for example (Fig. 2 and Table 2), are in line with previous truncated and chimeric GLP-1R and peptide ligand studies and NMR and crystal structures showing that exendin-4 has increased binding affinity for the ECD of GLP-1R because it has a more structured α-helix than GLP-1 and forms a tighter ionic interaction network with the ECD of GLP-1R (7, 14, 33). Several N-terminally truncated forms of GLP-1 (53, 54), glucagon (55), GIP (56), parathyroid hormone (PTH) (57), and corticotrophin-releasing hormone (CRH) (58) peptides are competitive antagonists for their corresponding receptor, whereas several C-terminally truncated ligands remain active (albeit with lower binding affinity), indicating that interactions with peptide ligands in the 7TM domain of class B GPCRs are required for receptor activation. Differences in the binding modes of the N termini of GLP-1 variants may therefore be important determinants of their functional activity at GLP-1R and control ligand-biased signaling (20).

In conclusion, our combined mutation and MD simulation studies suggest that diminished binding affinity of GLP-1R mutants can be rescued by subtle changes in the peptide ligand, facilitated by a variety of structural mechanisms, determined by ligand peptide conformation, electrostatic side-chain interactions/compatibility, as well as movements of the ECD relative to the 7TM domain. This demonstrates the complexity of peptide ligand recognition by class B GPCRs, and illustrates how full-length receptor models complemented by extensive mutagenesis and simulations of the conformational dynamics of receptor-ligand complexes can be used to investigate class B GPCR structure-activity and structure-selectivity relationships.

Author Contributions—C. dG. and D. Y. designed the site-directed mutagenesis experiments. D. Y., A. D., X. C., and Y. F. performed the site-directed mutagenesis experiments. L. Y., C. dG., and H. Y. constructed the full-length GLP-1R model and performed and analyzed the MD simulations. C. dG., H. J., R. C. S., and M.-W. W. were responsible for the overall project strategy and management. C. dG., D. Y., L. Y., H. Y., G. S., M. A. H., R. C. S., and M.-W. W. prepared the manuscript with discussions and improvements from A. D., X. C., Y. F., S. R. R., H. Y., and H. J.

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Note Added in Proof—During the preparation of the proofs a new crystal structure of the 7TM domain of GCGR was reported (PDB ID 5E7F, Jazayeri et al. (59)). In this structure a small molecule antagonist is bound to an extrahelical allosteric binding site that is distinct from the orthosteric peptide ligand binding site, and the receptor adopts a similar conformation as observed in the previously solved GCGR crystal structure (PDB ID 4LR6, Siu et al. (11)) that was used
as one of the templates to model the full-length GCGR and GLP-1R structures described in the current article.

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