The glucagon and glucagon-like peptide-1 (GLP-1) receptors are homologous family B seven-transmembrane (7TM) G protein-coupled receptors, and they selectively recognize the homologous peptide hormones glucagon (29 amino acids) and GLP-1 (30–31 amino acids), respectively. The amino-terminal extracellular domain of the glucagon and GLP-1 receptors (140–150 amino acids) determines specificity for the carboxyl terminus of glucagon and GLP-1, respectively. In addition, the glucagon receptor core domain (7TM helices and connecting loops) strongly determines specificity for the glucagon amino terminus. Only 4 of 15 residues are divergent in the glucagon and GLP-1 amino termini; Ser\(^2\), Gln\(^3\), Tyr\(^{10}\) and Lys\(^{12}\) in glucagon and the corresponding Ala\(^8\), Gln\(^9\), Val\(^{16}\), and Ser\(^{18}\) in GLP-1. In this study, individual substitution of these four residues of glucagon with the corresponding residues of GLP-1 decreased the affinity and potency at the glucagon receptor relative to glucagon. Substitution of distinct segments of the glucagon receptor core domain with the corresponding segments of the GLP-1 receptor rescued the affinity and potency of specific glucagon analogs. Site-directed mutagenesis identified the Asp\(^{385}\) → Glu glucagon receptor mutant that specifically rescued Ala\(^8\)-glucagon. The results show that three distinct epitopes of the glucagon receptor core domain determine specificity for the N terminus of glucagon. We suggest a glucagon receptor binding model in which the extracellular ends of TM2 and TM7 are close to and determine specificity for Gln\(^3\) and Ser\(^2\) of glucagon, respectively. Furthermore, the second extracellular loop and/or proximal segments of TM4 and/or TM5 are close to and determine specificity for Lys\(^{12}\) of glucagon.

Glucagon and GLP-1\(^1\) evolved from a common ancestor by a gene duplication in early vertebrate evolution, and human tissue-specific processing of their common precursor peptide, preproglucagon, generates glucagon in the pancreatic \(\alpha\)-cells and GLP-1 in the intestinal L-cells (1, 2). Activation of hepatic glucagon receptors (GluR) by glucagon stimulates glycogenolysis and gluconeogenesis. Activation of GLP-1 receptors (GLP-1R) on pancreatic \(\beta\)-cells by GLP-1 stimulates glucose-induced insulin secretion. Given their biological functions in glucose homeostasis, both receptors are promising targets for the treatment of type II diabetes.

GluR and GLP-1R belong to family B of the 7TM GPCRs, which includes the receptors for peptide hormones of the glucagon/PACAP superfamily: glucagon-like peptide-2 (GLP-2), glucagon-dependent insulinotropic polypeptide, pituitary adenylate cyclase-activating polypeptide (PACAP), vasoactive intestinal polypeptide (VIP), growth hormone-releasing hormone, and secretin and other peptide hormones such as calcitonin, corticotropin-releasing factor, and parathyroid hormone (PTH). The fingerprint of family B 7TM GPCRs is six conserved cysteines that form three disulfide bonds in the N-terminal extracellular domain (Nt-domain) (3). The structural constraints imposed by an identical disulfide bond pattern probably guide the Nt-domain of family B 7TM GPCRs into a common structural fold regardless of limited sequence identity (4, 5). The present knowledge about the structure and arrangement of the 7TM helices is based primarily on multiple sequence alignment analyses, although a functional coupling of conserved polar residues of the PTH receptor (PTH1R) suggests the existence of a cooperative helix-helix interface between TM2 and TM7 (6, 7).

Structure-activity analyses of peptides of the glucagon/PACAP superfamily suggest that their entire length is required for optimal biological activity and that the C terminus is primarily involved in receptor binding, whereas the N terminus contains the residues involved in receptor activation (8). The N-terminally modified glucagon analog desHis\(^1\)Glu\(^9\)-glucagon is a potent GluR antagonist, which emphasizes the importance of His\(^1\) and Asp\(^9\) of glucagon in activation of GluR (9). The N termini of GLP-1 and exendin-4 are highly conserved, and the N terminus of exendin-4 is essential for activation of GLP-1R (10). Furthermore, His\(^7\), Gly\(^{10}\), Phe\(^{12}\), Thr\(^{13}\), and Asp\(^{15}\) of the GLP-1 N terminus are important for optimal binding and activation of GLP-1R, and they are all conserved in exendin-4 (11). NMR structures of GLP-1, glucagon, and PACAP-(1–38) in lipophilic solvents or dodecylphosphocholine micelles agree that the central and C-terminal parts are \(\alpha\)-helical, often with a central distortion of the helix geometry, whereas the N terminus is a flexible random coil (12–14). Interestingly, the N terminus of receptor-bound PACAP-(1–21) forms a specific \(\beta\)-coil structure, and the PACAP N terminus is important for receptor activation (8, 14). The N termini of glucagon and PACAP are highly conserved, and thus the glucagon N termini...
nus may form a PACAP-like structure upon binding to GluR. The isolated Nt-domain of family B TTM GPCRs is sufficient for low affinity ligand binding, and it is a critical determinant of ligand selectivity (3, 14, 15). The Nt-domain of GLP-1R binds exendin-4 (9–39) with high affinity, and therefore either the C-terminal extension of exendin-4 (the Trp cage) or divergent residues in exendin-4 and GLP-1 increase the affinity of exendin-4 at the Nt-domain of the GLP-1R relative to GLP-1 (16). Additional interactions with the extracellular loops (ECL) and the extracellular end of the 7TM helices may explain the high affinity ligand binding of intact receptors and provide additional determinants of ligand selectivity (17–20).

The molecular information about receptor-ligand complexes of family B TTM GPCRs is limited. A two-site binding model has been proposed for the ligand interaction with PTH1R, in which the PTH C terminus interacts with the PTH1R Nt-domain and the PTH N terminus interacts with the PTH1R core domain (21). Peptides of the glucagon/PACAP superfamily may follow a similar binding mechanism (14, 22). The conserved Asp198 in the boundary between TM2 and ECL1 of GLP-1R is important to maintain the binding site for the GLP-1 N terminus (23). Specifically, Asp198 in the N terminus of VIP and secretin interacts with positively charged residues in the extracellular end of TM2 of VIPAC1 (VIP receptor) and the secretin receptor, respectively (24, 25). The corresponding Gln3 in the N terminus of glucagon most likely interacts with the extracellular end of TM2 of GluR (26). In addition, p-benzoyl-L-phenylalanine in position 22 or 26 of the secretin C terminus cross-links to specific residues in the Nt-domain of the secretin receptor (27).

Family B TTM GPCRs selectively bind their natural ligands with high affinity, although they may bind homologous ligands with low affinity. GLP-1R binds GLP-1 with high affinity and glucagon with low affinity, and glucagon is a low potency full agonist of GLP-1R (22). The GLP-1R Nt-domain defines almost completely the glucagon/GLP-1 selectivity profile of GLP-1R by selective recognition of the GLP-1 C terminus, and the GLP-1R core domain is not important for glucagon/GLP-1 selectivity (22). In contrast, GluR has a very strong glucagon/GluL-1 selectivity profile and does not cross-react with GLP-1. The GluR Nt-domain selectively recognizes the glucagon C terminus, and the GluR core domain strongly determines specificity for the N terminus of glucagon.

EXPERIMENTAL PROCEDURES

Receptor Constructs—The cDNAs encoding the human GLP-1R and the human GluR were originally obtained from Dr. B. Thorens and Zymogenetics Inc., respectively, and subcloned into the mammalian expression vector pCDNA3.1/V5-His-TOPO® (Invitrogen) (10, 28). Chimeric glucagon/GLP-1 receptors were generated by overlap extension PCR, as previously described (22). Chimera A was composed of amino acid residues 1–144 of GluR and residues 148–463 of GLP-1R (22). Chimera receptor TM2 (ChTM2) was composed of residues 1–187 and 199–477 of GluR and residues 190–463 of GLP-1R. ChECL3 was composed of amino acid residues 1–359 and 385–477 of GluR and amino acid residues 362–387 of GLP-1R. Site-directed mutagenesis of GluR was done using QuikChange™ (Stratagene). Plasmid DNA was generated and sequenced as previously described (22).

Peptide Synthesis and Radiolabeling—Glucagon, GLP-1-(7–37), Ala8-glucagon, Glu9-glucagon, Val10-glucagon, Ser12-glucagon, Ser8, GLP-1, Gln9-GLP-1, Tyr16-GLP-1, and Lys18-GLP-1 were synthesized and characterized as previously described (22).

RESULTS

The Glucagon and GLP-1 Receptors—In GLP-1, Ala8, Glu9, Val10, and Ser12 were substituted with the corresponding residues of glucagon. The GLP-1 analogs were analyzed for their ability to bind and activate GLP-1R. In competition binding, GLP-1 displaced 125I-GLP-1 from GLP-1R with an IC50 value of 1.1 nM, and the IC50 values of the GLP-1 analogs were similar to that of GLP-1 (Table I). Whole cells transiently expressing the human GLP-1R gave a functional response with half-maximal stimulation (EC50) of the adenylyl cyclase at 12 pM GLP-1, and the GLP-1 analogs were full agonists and equipotent with GLP-1 (Table I). In glucagon, Ser2, Gln3, Tyr16, and

| Ligand Interactions of the Glucagon Receptor | TABLE I |
|---------------------------------------------|---------|
| GLP-1 receptor | Glucagon receptor | Chimera A |
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Lys<sup>12</sup> were substituted with the corresponding residues of GLP-1 (Fig. 1). The glucagon analogs were analyzed for their ability to bind and activate GluR. In competition binding, glucagon displaced <sup>125</sup>I-glucagon from GluR with an IC<sub>50</sub> value of 2.1 nM, and the glucagon analogs displaced <sup>125</sup>I-glucagon with significantly higher IC<sub>50</sub> values (Fig. 1 and Table I). Whole cells transiently expressing the human GluR, responded functionally with half-maximal stimulation at 11 pM of glucagon, and the potencies of the glucagon analogs were significantly lower (Fig. 1B and Table I). The results showed that the individual substitutions of Ala<sup>8</sup>, Glu<sup>9</sup>, Val<sup>16</sup>, and Ser<sup>18</sup> in the GLP-1 N terminus with the corresponding glucagon residues had only subtle effects on affinity or potency at GLP-1R relative to GLP-1. Conversely, the individual substitutions of Ser<sup>2</sup>, Gln<sup>3</sup>, Tyr<sup>10</sup>, and Ser<sup>12</sup> in the glucagon N terminus with the corresponding GLP-1 residues decreased the affinity and potency at GluR relative to glucagon.

Chimera A—The chimeric receptor, chimera A, consists of the GluR Nt-domain and the GLP-1R core domain (Fig. 1) (22). The single-substituted glucagon analogs were analyzed for their ability to bind and activate chimera A. In competition binding, glucagon displaced <sup>125</sup>I-glucagon from chimera A with an IC<sub>50</sub> value of 1.2 nM (Fig. 1C and Table I). The IC<sub>50</sub> values of Ala<sup>2</sup>-glucagon and Glu<sup>3</sup>-glucagon were lower than that of glucagon, whereas the IC<sub>50</sub> values of Val<sup>10</sup>-glucagon and Ser<sup>12</sup>-glucagon were similar to that of glucagon (Fig. 1C and Table I). In functional experiments with chimera A, all of the glucagon analogs were equipotent with glucagon (Fig. 1D and Table I). The results obtained with GluR and chimera A showed that substitution of the GluR core domain with the GLP-1R core domain increased the affinity and potency of Ala<sup>2</sup>-glucagon, Glu<sup>3</sup>-glucagon, Val<sup>10</sup>-glucagon, and Ser<sup>12</sup>-glucagon relative to glucagon.

Dissection of the Glucagon Receptor Core Domain—Small segments of the GluR core domain were substituted with the corresponding segments of GLP-1R. Ala<sup>2</sup>-glucagon, Glu<sup>3</sup>-glucagon, and Ser<sup>12</sup>-glucagon were analyzed for their ability to bind and activate three chimeric receptors: ChTM2, ChECL2, and ChECL3 (Fig. 1). In ChTM2, the extracellular end of TM2 of GluR was substituted with the corresponding segment of GLP-1R. In competition binding, glucagon displaced <sup>125</sup>I-glucagon from ChTM2 with an IC<sub>50</sub> value of 1.4 nM (Fig. 1E and Table II). The IC<sub>50</sub> value of Glu<sup>3</sup>-glucagon was lower than that of glucagon, whereas the IC<sub>50</sub> values of Val<sup>10</sup>-glucagon and Ser<sup>12</sup>-glucagon were higher than that of glucagon. On whole cells transiently expressing ChTM2, Glu<sup>3</sup>-glucagon was equipotent with...
glucagon, whereas Ala2-glucagon and Ser12-glucagon were less potent than glucagon (Fig. 1F and Table II). The results obtained with GluR and ChTM2 showed that substitution of the extracellular end of TM2 of GluR with the corresponding segment of GLP-1R increased the affinity and potency of Glu3-glucagon relative to glucagon, Ala2-glucagon, and Ser12-glucagon. In addition, the affinity but not the potency of Ala2-glucagon increased relative to glucagon and Ser12-glucagon.

In ChECL2, the second extracellular loop and the extracellular ends of TM4 and TM5 were substituted with the corresponding segments of GLP-1R. In competition binding, glucagon displaced 125I-glucagon from ChECL2 with an IC50 value of 3.1 nM (Fig. 1G and Table II). The IC50 value of Ser12-glucagon was similar to that of glucagon, whereas the IC50 values of Ala2-glucagon and Glu3-glucagon were higher than that of glucagon. On whole cells transiently expressing ChECL2, Ser12-glucagon was equipotent with glucagon, whereas Ala2-glucagon and Glu3-glucagon were less potent than glucagon (Fig. 1H and Table II). The results showed that substitution of the second extracellular loop and the extracellular ends of TM4 and TM5 with the corresponding segments of GLP-1R specifically increased the affinity and potency of Ser12-glucagon relative to glucagon, Ala2-glucagon, and Glu3-glucagon.

In ChECL3, the third extracellular loop and the extracellular ends of TM6 and TM7 were substituted with the corresponding segments of GLP-1R. In competition binding, glucagon displaced 125I-glucagon from ChECL3 with an IC50 value of 1.9 nM (Fig. 1I and Table II). The IC50 value of Ala2-glucagon was lower than that of glucagon, whereas the IC50 values of Glu3-glucagon and Ser12-glucagon were higher than that of glucagon. On whole cells transiently expressing ChECL3, Ala2-glucagon was equipotent with glucagon, whereas Glu3-glucagon and Ser12-glucagon were less potent than glucagon (Fig. 1J and Table II). The results showed that substitution of the third extracellular loop and the extracellular ends of TM6 and TM7 with the corresponding segments of GLP-1R specifically increased the affinity of Ala2-glucagon relative to glucagon, Glu3-glucagon, and Ser12-glucagon.

We were unable to generate a chimeric receptor that specifically increased the affinity and potency of Val10-glucagon; therefore, Val10-glucagon was not analyzed further.

**Point Mutations in the Glucagon Receptor**—The region defined by the GLP-1R segment of ChECL3 was investigated by site-directed mutagenesis of the corresponding segment of GluR. The divergent residues in this region were substituted individually to the corresponding residues of GLP-1R. Initially, the GluR point mutants were analyzed in binding and functional experiments with glucagon and Ala2-glucagon. In competition binding with the Asp385-Glu mutant, the affinity and potency of Ala2-glucagon were increased relative to glucagon, Ala2-glucagon, and Glu3-glucagon.
Ala<sup>2</sup>-glucagon was slightly higher than that of glucagon (Fig. 2C and Table II). In functional experiments with whole cells transiently expressing the Asp<sup>385</sup>-Glu mutant, the potency of Ala<sup>2</sup>-glucagon was slightly higher than that of glucagon (Fig. 2D and Table II). With the other GluR point mutants, the affinity and potency of Ala<sup>2</sup>-glucagon were lower than the affinity and potency of glucagon, in a manner similar to GluR (Table II). This is illustrated by the Ser<sup>379</sup>→Phe mutant (Fig. 2, A and B). Subsequently, the Asp<sup>385</sup>→Glu and Ser<sup>379</sup>→Phe mutants were analyzed in binding and functional experiments with Glu<sup>3</sup>-glucagon and Ser<sup>12</sup>-glucagon. At both mutants, the affinity and potency of Glu<sup>3</sup>-glucagon and Ser<sup>12</sup>-glucagon were significantly lower than the affinity and potency of glucagon. The results showed that the point mutation Asp<sup>385</sup>→Glu in the extracellular end of TM7 specifically increased the affinity and potency of Ala<sup>2</sup>-glucagon relative to glucagon, Glu<sup>3</sup>-glucagon, and Ser<sup>12</sup>-glucagon.

**DISCUSSION**

The glucagon analogs bound and activated GluR with lower affinity and potency than glucagon. Substitution of the entire GluR core domain with the GLP-1R core domain rescued the affinity and potency of all of the glucagon analogs relative to glucagon. In addition, the corresponding GLP-1 analogs bound and activated GLP-1R with the same affinity and potency as GLP-1. Apparently, the GluR core domain selectively recognized Ser<sup>2</sup>, Gln<sup>3</sup>, Tyr<sup>10</sup>, and Ser<sup>12</sup> of the glucagon N terminus and discriminated the corresponding residues of the GLP-1 N terminus. In contrast, the GLP-1R core domain (GLP-1R and chimera A) potently accommodated both the GLP-1 and glucagon N termini, although the substituted residues in the GLP-1 and glucagon analogs may interact differently with the GLP-1R core domain than the corresponding residues of native GLP-1 and glucagon.

Dissection of the GluR core domain identified three distinct epitopes of the GLP-1R core domain that rescued the affinity and potency of specific glucagon analogs. The extracellular end of TM2 (ChTM2) rescued Gln<sup>3</sup>-glucagon, ECL2 and the proximal segments of TM4 and TM5 (ChECL2) rescued Ser<sup>12</sup>-glucagon, and ECL3 and the proximal segments of TM6 and TM7 (ChECL3) rescued Ala<sup>2</sup>-glucagon. ChTM2, ChECL2, and ChECL3 did not compromise glucagon binding or potency, which confirmed the structural integrity of the chimeric receptors. Two nonexclusive explanations may account for these results: 1) the GLP-1R segments of ChTM2, ChECL2, and ChECL3 interact directly with Glu<sup>3</sup>, Ser<sup>12</sup>, and Ala<sup>2</sup>, respectively, and/or 2) the GLP-1R segments of ChTM2, ChECL2, and ChECL3 are required to maintain a local binding site conformation that accommodates the interaction with Glu<sup>3</sup>, Ser<sup>12</sup>, and Ala<sup>2</sup>, respectively. Nevertheless, it is difficult to explain the results without considering the proximity of the GLP-1R segments in ChTM2, ChECL2, and ChECL3 with Glu<sup>3</sup>, Ser<sup>12</sup>, and Ala<sup>2</sup> of the glucagon analogs, respectively. In addition, the corresponding segments of GluR define the strong glucagon/GLP-1 selectivity profile of the GluR core domain by selective recognition of the glucagon N terminus. Most family B 7TM GPCRs have two positively charged residues in TM2, whereas GluR has only one and a neutral hydrophobic residue in place of the other (Lys<sup>187</sup> and Ile<sup>194</sup> in human GluR). Furthermore, the glucagon/PACAP superfamily peptides have either Asp or Glu in position 3 except glucagon, which has a Gln. The K187R/I194K GluR mutant rescued both affinity and potency of Asp<sup>3</sup>-glucagon relative to glucagon (26). The additional positive charge of the K187R/I194K GluR mutant and the chimeric receptor ChTM2 probably accommodates the extra negative charge of Asp<sup>3</sup>-glucagon and Glu<sup>3</sup>-glucagon, respectively. In fact, Glu<sup>3</sup>-glucagon bound ChTM2 with higher affinity than glucagon but was equipotent with glucagon in functional experiments with ChTM2. The small discrepancy in affinity *versus* potency suggests that Glu<sup>3</sup> of Glu<sup>3</sup>-glucagon provides a binding determinant for interaction with ChTM2 that is not equally favorable for activation of ChTM2. However, the discrepancy is small compared with the total rescue of Glu<sup>3</sup>-glucagon by ChTM2. The corresponding Asp<sup>3</sup> of secretin and VPAC<sub>1</sub> receptor, respectively, and the interaction with Arg in the center of TM2 is important for receptor activation (24, 25). Site-directed mutagenesis of a highly conserved His in the cytoplasmic end of TM2 leads to constitutive activity of several family B 7TM GPCRs (30–32). Collectively, it appears that TM2 is important for agonist binding and activation of family B 7TM GPCRs. Ile<sup>194</sup> in TM2 of GluR may serve as a selectivity determinant that prevents access of homologous peptides to the activation site of GluR.

The combined analyses of GluR mutants and single-substituted glucagon analogs demonstrated the functional significance of the correlated substitution of residues in glucagon (Gln<sup>3</sup>) and GluR (Ile<sup>194</sup>). Glucagon is highly conserved during evolution, and specifically position 3 is occupied by Gln in vertebrates except bony fish, where position 3 is occupied by either Gln, Asp, or Glu. In bony fish, glucagon and GLP-1 have overlapping biological activities, and GLP-1 acquired the incretin function after the divergence of bony fish and mammals (33). Specificity of receptor-ligand pairs most likely evolved to ensure distinct physiological functions, and therefore the change of selection pressure on position 3 of glucagon may reflect the divergence of the biological activities of glucagon and GLP-1.

Eight divergent residues in the region defined by the GLP-1R segment of ChECL3 was investigated by site-directed mutagenesis of GluR. Only the substitution of Asp<sup>385</sup> with the corresponding Glu of GLP-1R rescued the affinity and potency of Ala<sup>2</sup>-glucagon without disturbing the pharmacological profile of the other glucagon analogs relative to glucagon. It is difficult to explain the rescue of affinity of Ala<sup>2</sup>-glucagon by a direct interaction with Glu<sup>385</sup> in the Asp<sup>385</sup>→Glu GluR mutants. It seems more likely that the Asp<sup>385</sup>→Glu mutation stabilized a local binding site conformation that preferably interacted with Ala<sup>2</sup>-glucagon. Nevertheless the results strongly suggest proximity between Ala<sup>2</sup> of Ala<sup>2</sup>-glucagon and the extracellular end of TM7 of the GluR mutant Asp<sup>385</sup>→Glu.

The dissection of the GluR core domain provides three constraints that orient the glucagon N terminus with respect to the structural elements of the GluR core domain. Given the structural integrity of the GluR mutants in this study, we suggest a binding model in which Ser<sup>379</sup> of glucagon is close to Asp<sup>385</sup> of TM7, Gln<sup>3</sup> of glucagon is close to Ile<sup>194</sup> on TM2, and...
Lys^{12} is close to ECL2 and/or proximal segments of TM4 and/or TM5 (Fig. 3). The proximity of Ser^{7} with TM7 and Gln^{3} with TM2 is consistent with the potential helix-helix interface between TM2 and TM7 of PTH1R. Accordingly, the high level of amino acid identity of the predicted 7TM helices of family B 7TM GPCRs probably reflects an arrangement into a conserved helical bundle structure in which TM2 is close to TM7. Proximity of TM2 and TM7 is consistent with a rhodopsin-like arrangement of the 7TM helices, although the orientation may be either clockwise or counterclockwise (34).

The position of ECL2 relative to the extracellular end of TM3 is probably constrained by a conserved disulfide bond of many family A and B 7TM GPCRs, and ECL2 is involved in peptide-agonist binding of family A 7TM GPCRs (34–38). ECL2 of the family A and B 7TM GPCRs, and ECL2 is involved in peptide-agonist binding, and specifically four residues in the N-terminal half of Lys^{12} is close to ECL2 and/or proximal segments of TM4 and/or TM5 of GluR that determines specificity for Lys^{12} of glucagon.

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