Evolutionarily Conserved Residues at Glucagon-like Peptide-1 (GLP-1) Receptor Core Confer Ligand-induced Receptor Activation*§

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Received for publication, June 27, 2011, and in revised form, November 7, 2011 Published, JBC Papers in Press, November 21, 2011, DOI 10.1074/jbc.M111.276808

Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) play important roles in insulin secretion through their receptors, GLP1R and GIPR. Although GLP-1 and GIP are attractive candidates for treatment of type 2 diabetes and obesity, little is known regarding the molecular interaction of these peptides with the heptahelical core domain of their receptors. These core domains are important not only for specific ligand binding but also for ligand-induced receptor activation. Here, using chimeric and point-mutated GLP1R/GIPR, we determined that evolutionarily conserved amino acid residues such as Ile196 at transmembrane helix 2, Leu232 and Met233 at extracellular loop 1, and Asn302 at extracellular loop 2 of GLP1R are responsible for interaction with ligand and receptor activation. Application of chimeric GLP-1/GIP peptides together with molecular modeling suggests that His1 of GLP-1 interacts with Asn302 of GLP1R and that Thr7 of GLP-1 has close contact with Ile196, Leu232, and Met233 of GLP1R. This study may provide critical clues for the development of peptide and/or nonpeptide agonists acting at GLP1R.

Glucagon-like peptide-1 (GLP-1) is an important incretin hormone released from the intestinal L-cells. GLP-1 potentiates glucose-dependent insulin secretion and increases beta cell mass through regulation of cell proliferation and apoptosis (1, 2). Moreover, GLP-1 regulates the glucose level via slowing of gastric emptying and glucose-dependent inhibition of glucagon secretion (3). Another incretin, glucose-dependent insulinotropic polypeptide (GIP) is a 42-amino acid hormone that is produced by enteroendocrine K-cells (4). Like GLP-1, GIP stimulates insulin secretion in a glucose-dependent manner (5). GIP regulates lipid metabolism through direct actions on adipose tissues and is involved in bone formation through stimulation of osteoblast proliferation and inhibition of apoptosis (6). Because of their combined beneficial effects, both GLP-1 and GIP have gained attention as potential therapeutic agents for treatment of type 2 diabetes mellitus and obesity.

GLP-1 receptor (GLP1R) and GIP receptor (GIPR) belong to a class B (or secretin-like) G protein-coupled receptor (GPCR) family that features a relatively long N-terminal extracellular domain (ECD) of ~120 residues including several Cys residues that form a network of disulfide bridges (7). The ECD in this receptor family has been considered to be important for binding with peptide ligand (7–9). Indeed, various experimental approaches have demonstrated that the ECDs of GLP1R and GIPR are able to bind with their cognate peptide ligands, particularly the C-terminal portion of the peptide (10–15). This binding, however, may not fully account for ligand-induced receptor activation (7, 16–19). For instance, the N-terminally truncated exendin-4(9–39), a GLP-1 analog, is unable to activate GLP1R, whereas it binds to the receptor with high affinity (20). In contrast, exendin1–9, a short N-terminal fragment of exendin-4, is able to activate GLP1R with very low affinity (21). Likewise, a GIP fragment, GIP(7–30), is able to bind to GIPR with high affinity but fails to induce receptor activation, whereas the fragment GIP(1–14) exhibits a very low affinity toward the receptor but fully activates the receptor at a micro-molar concentration (22–24). Thus, the two-domain model explaining ligand binding followed by receptor activation has...
sequence similarity. The random coil structure is followed by the

pcDNA3 were kindly provided by Dr. Bernard Thorens, Uni-

versity of Lausanne, Lausanne, Switzerland. The CRE-luc vec-

tor containing four copies of CRE (TGACGTCA) was from

Construction of Chimeras and Mutants—For domain swap-

ping between GLP1R and GIPR, individual cDNA fragments of

interest were amplified through PCR using Pfu polymerase

ELPIS Biotech, Daejeon, Korea) and two specific primers, one

to the 5'- or the 3'-end of the receptor cDNAs and another

corresponding to the region of overlap between the two

receptors. The two fragments, one from GLP1R and the other

from GIPR, were subjected to a second round of PCR to generate

the chimeric cDNAs. All 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 then 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 medium

(DMEM) in the presence of 10% fetal bovine serum. For lucifer-

ase assays, cells were plated in 48-well plates 1 day before transfe-
tion 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 then harvested, and luciferase activity

in cell extracts was determined using a luciferase assay system

according to the standard methods for the Wallac 1420 VICTOR3

multilabel counter (PerkinElmer Life Sciences).

Binding Assay—GLP-1 and H4,T7,E15,A18]cGIP were radio-

odinated using the chloramine-T method and purified by

chromatography on a Sephadex G-25 column (Sigma-Aldrich)
in 0.01 M acetic acid and 0.1% BSA. Cells were transfected with

wild type or mutant receptor (300 ng of DNA/well in 12-well plates)

with Effectene (Qiagen). Forty-eight hours later, cells were

washed and incubated for 1 h with binding buffer (serum-

free DMEM with 0.1% BSA, pH 7.4) containing 100,000 cpm

125I-labeled ligand in the presence of various concentrations of

cold ligand. Cells were washed with ice-cold Dulbecco’s PBS
twice. Radioactivity of cell lysate resolved in 1% SDS and 0.2 M

NaOH was determined using the Wallac 1489 Wizard 3

g-counter (PerkinElmer Life Sciences).

Molecular Modeling—A homology model for GLP1R and

GLP-1 interaction was built on the basis of the crystal structure

(Protein Data Bank code 2RH1) of the human β2-adrenorecep-
tor (36), the crystal structure (Protein Data Bank code 3IOL) of

the ECD of the human GLP1R in complex with GLP-1 (32), and

the homology modeling program MODELLER 9v8 (37). The

sequence of GLP1R was manually aligned to the human β2-ad-

renoreceptor and other GPCRs with known structures based on

transmembrane helices predicted by TMHMM Server v.2.0

(38). During the homology modeling, a disulfide bond was

forced between Cys226 and Cys296. Distance restraints were

introduced between His3 of GLP-1 and Asn274 of GLP1R and

between Thr2 of GLP-1 and Ile196, Lys197, and Met302 of GLP1R.

All structural figures were prepared using PyMOL v1.4.1

(DeLano Scientific LLC, San Francisco, CA).

emerged: the central α-helical and C-terminal portions of the

peptide bind to the N-terminal ECD of the receptor (25–27)

followed by binding of the N-terminal moiety of the peptide

with the core domain including transmembrane helices

(TMHs) and extracellular loops (ECLs) of the receptor, allow-

ing receptor activation and G protein coupling (14, 28–30). The

former interaction has been clearly resolved by determination

of crystal structures of the ligand-bound ECDs of GLP1R and

GIPR (15, 31, 32). However, virtually no progress has been

made in the exploration of the ligand binding sites for the

receptors that discriminate between GLP-1 and GIP. Thus,

(33), indicating the presence of distinct amino acid residues in

the receptors that discriminate between GLP-1 and GIP. Thus,

the use of chimeric GLP1R/GIPRs is an excellent strategy to

explore the domain responsible for differential ligand affinity. In

the present study, using chimeric and point-mutated GLP1R/

GIPRs combined with chimeric GLP-1/GIP peptides, we

attempted to identify amino acid residues in the core domain of

GLP1R that confer ligand selectivity toward GLP-1. We found that

Asn302 of ECL2 of GLP1R likely interacts with His1 of GLP-1 and

that Ile196 of TMH2 and Leu232 and Met233 of ECL1 may form a

binding pocket for interaction with Thr7 of GLP-1.

EXPERIMENTAL PROCEDURES

Peptides—Wild type GLP-1, GIP, and chimeric GIPs (cGIPs)

were synthesized by AnyGen (Gwangju, Korea). All cGIPs pos-
sess a Ser residue at position 2 to protect from cleavage by
dipeptidyl peptidase IV (34) but do not contain the C-terminal

sequence (residues 31–42 of the full-length GIP), which is

unnecessary for the binding with GIPR and the subsequent acti-

vation of GIPR (22). In cGIPs, residues Tyr1, Ile7, Asp15, and/or

His18 were replaced by corresponding amino acid residues of

GIPRs combined with chimeric GLP-1/GIP peptides, we

attempted to identify amino acid residues in the core domain of

GLP1R that confer ligand selectivity toward GLP-1. We found that

Asn302 of ECL2 of GLP1R likely interacts with His1 of GLP-1 and

that Ile196 of TMH2 and Leu232 and Met233 of ECL1 may form a

binding pocket for interaction with Thr7 of GLP-1.

TABLE 1

Amino acid sequences of GLP-1, GIP, and chimeric GIP peptides

|         | 1   | 10  | 20  | 30  | 42  |
|---------|-----|-----|-----|-----|-----|
| GLP-1   | HAE | GTFT | SD | VSVY | LEQOQA | K | E | A | K | L | V | K R |
| [H4,T7,E15,A18]cGIP | HSE | GTFT | SD | Y | T | A | E | K | Q | D | F | V | N | K | L | A | Q | K |
| [H4,T7]cGIP | YSE | GTFT | SD | Y | T | A | E | K | Q | D | F | V | N | K | L | A | Q | K |
| [H4,E15,A18]cGIP | HSE | GTFT | SD | Y | T | A | E | K | Q | D | F | V | N | K | L | A | Q | K |
| [H4,T7]cGIP | YSE | GTFT | SD | Y | T | A | E | K | Q | D | F | V | N | K | L | A | Q | K |
| GIP     | YAE | GTFT | S | DS | Y | A | I | M | D | K | H | Q | D | F | V | N | K | L | A | Q | K |

Binding Assay—A homology model for GLP1R and

GLP-1 interaction was built on the basis of the crystal structure

(Protein Data Bank code 2RH1) of the human β2-adrenorecep-
tor (36), the crystal structure (Protein Data Bank code 3IOL) of

the ECD of the human GLP1R in complex with GLP-1 (32), and

the homology modeling program MODELLER 9v8 (37). The

sequence of GLP1R was manually aligned to the human β2-ad-

renoreceptor and other GPCRs with known structures based on

transmembrane helices predicted by TMHMM Server v.2.0

(38). During the homology modeling, a disulfide bond was

forced between Cys226 and Cys296. Distance restraints were

introduced between His3 of GLP-1 and Asn274 of GLP1R and

between Thr2 of GLP-1 and Ile196, Lys197, and Met302 of GLP1R.

All structural figures were prepared using PyMOL v1.4.1

(DeLano Scientific LLC, San Francisco, CA).
Data Analysis—Data analysis was performed using nonlinear regression with a sigmoid dose response. The agonist concentrations that induced half-maximal stimulation (EC_{50}) or half-maximal inhibition of binding (IC_{50}) were calculated using GraphPad PRISM4 software (GraphPad Software Inc., San Diego, CA). All data are presented as mean ± S.E. of at least three independent experiments. Group means were compared using Student’s t test or one-way analysis of variance followed by Bonferroni’s multiple comparison test. p < 0.05 was accepted as significant.

RESULTS

Determination of Specific Core Regions of Receptor Conferring Differential Ligand Selectivity—To determine specific core regions of GLP1R and GIPR responsible for molecular interaction with their ligands, a series of GIPR/GLP1R (GI/L) chimeric receptors were constructed (Fig. 1A and Table 2). The reciprocal GLP1R/GIPR (GL/I) chimeras were also constructed (Fig. 1B and Table 3). HEK293T cells were transfected with the CRE-luc reporter vector. Cells were then treated with increasing concentrations of peptide ligand for 6 h, and luciferase activity was determined (35). Potencies of GLP-1 toward GI/L chimeric receptors were gradually decreased as the portion of GIPR was increased. Particulary, when the N-terminal ECD, TMH2, ECL1, and ECL2 were subsequently swapped with those of GIPR, the potency of GLP-1 was significantly decreased. Replacement of the region from the N terminus to ECL2 with that of GIPR (GI/L5) led to a loss of ability to respond to GLP-1 (Fig. 1A). This observation highly correlates with the finding that the chimeric GIPR hav-
ing the N terminus to ECL2 of GLP1R (GL/I5) responded to GLP-1 with high affinity similar to that of wild type GLP1R (Fig. 1B). Together, these findings suggest that the particular regions of GLP1R including the N-terminal ECD, TMH2, ECL1, and ECL2 form a core domain necessary for retaining full activity of the receptor in response to ligand stimulation.

The GLP1R chimera with the N-terminal ECD of GIPR (GI/L1) gained an ability to respond to GIP. The GLP1R chimera having the N terminus to ECL1 of GIPR (GI/L3) was fully activated by GIP, behaving like wild type GIPR (Fig. 1A). In accordance with this result, the GIPR chimera with the N-terminal ECD of GLP1R (GL/I1) lost the ability to bind with GIP, indicating that the N-terminal ECD and ECL1 of GIPR are critical for binding with GIP (Fig. 1B).

### Determination of Core Regions in GLP1R Interacting with His1 and Thr7 of GLP-1

Previously, using cGIPs in which Tyr1, Ile7, Asp15, and/or His18 were replaced by His, Thr, Glu, and Ala, respectively (Table 1), we demonstrated that Tyr/His1 and Ile/Thr7 of GIP/GLP-1 peptides confer differential ligand selectivity toward GIPR and GLP1R (35). In the present study, we applied these cGIPs to further determine the specific regions of GLP1R that potentially interact with Tyr/His1 and Ile/Thr7 of GIP/GLP-1 peptides (Fig. 2A).

### Table 3

| Chimeric receptors | % wtGLP1R | EC50 [nM] |
|--------------------|-----------|-----------|
| wGIPR              | 81.69±1.49| >1000     |
| GL/I1              | 123.05±3.99| >1000     |
| GL/I2              | 53.88±21.65| >1000     |
| GL/I3              | 39.93±14.03| 20.63±8.88| >1000     |
| GL/I4              | 73.51±3.92 | 35.86±15.08| >1000     |
| GL/I5              | 37.15±9.09 | 0.37±0.17  | >1000     |
| GL/I6              | 96.87±18.74| 0.17±0.10  | >1000     |
| GL/I7              | 96.87±17.74| 0.20±0.09  | >1000     |
| wtGLP1R            | 100       | 0.18±0.10  | >1000     |
All cGIPs were able to activate the wild type GLP1R with lower potency than wild type GLP-1 but showed remarkably higher potency than wild type GIP (35). The potencies of all chimeric peptides toward GI/L1 were 20–100-fold higher than those toward the wild type GLP1R (Fig. 2). These increased potencies are likely due to a high affinity interaction between the GIPR ECD of this chimeric receptor and the C-terminal portion of the chimeric peptides. With regard to [H1,T7,E15,A18]cGIP and [H1,T7]cGIP, their increased potencies were gradually decreased as the GIPR portion of the chimeric receptors was increased. Compared with the potency of chimeric peptides toward GI/L1, there was a significant reduction in their potencies for GI/L2 that has TMH2 of GIPR, and a second significant decrease in potency was seen for GI/L5 containing ECL2 of GIPR (Fig. 2, B and C). Furthermore, there was a tendency for decreased potencies for GI/L3 harboring ECL1 of GIPR. This observation suggests that His1 and Thr7 in the chimeric peptide may interact with the amino acid residues in TMH2, ECL1, and ECL2 of GLP1R. Interestingly, the potency of His1-containing [H1,T7,E15,A18]cGIP was first decreased for GI/L5 (Fig. 2D), whereas the potency of Thr7-containing [T7,E15,A18]cGIP was decreased for GI/L2 (Fig. 2E). Thus, it is plausible that His1 of the peptide may bind with amino acid residues in ECL2 of GLP1R, and Thr7 may interact with amino acids in TMH2 of GLP1R. [E15,A18]cGIP exhibited a greatly increased potency toward GI/L1 compared with that toward the wild type GLP1R, and this potency was further increased as the portion of GIPR was increased in the chimeric receptors (Fig. 2F), behaving like wild type GIP. Thus, Glu15 and Ala18 residues of this peptide may marginally affect interaction of the ligand with the core region of GLP1R in good agreement with our previous study (35).

Identification of Specific Amino Acids within Core Regions of GLP1R Responsible for GLP-1 Binding and Receptor Activation—To further identify the specific amino acid residues of GLP1R conferring ligand binding and activation, the amino acid sequences of the regions comprising TMH2, ECL1, and ECL2 in several vertebrate GLP1Rs and human GIPR were analyzed. Amino acid sequence comparison of these regions revealed that Ile196 and Lys197 of TMH2, Leu232 and Met233 of ECL1, and Asn302 and Met303 of ECL2 in GLP1R are evolutionarily conserved across species but differ from the corresponding residues in GIPR. Other amino acid residues in these regions are conserved for both GLP1R and GIPR or highly variable across species (Fig. 3). Thus, the above mentioned residues are likely GLP1R-specific.

To investigate whether these amino acids are responsible for specific interactions with GLP-1, point-mutated GLP1Rs in which these conserved amino acids were replaced by corresponding amino acids of GIPR were generated (Fig. 4). All these mutant receptors responded less to GLP-1 than wild type GLP1R. The potencies of GLP-1 for GLP1R(L232V/M233T) and GLP1R(N302V/M303K) were 100- and 10-fold lower than that for wild type GLP1R, respectively (Fig. 4A). Particularly, GLP1R(I196S) did not respond to GLP-1. We then examined the binding affinity of these mutant receptors to GLP-1. Interestingly, the decrease in binding affinities of GLP-1 toward these mutant receptors was a bit smaller than the decrease in GLP-1 potencies to these receptors (Fig. 4B). GLP1R(L232V/M233T) and GLP1R(N302V/M303K) exhibited ~10- and 3-fold decreased affinity to GLP-1, respectively. Furthermore, binding affinity of GLP1R(I196S) to GLP-1 is not different from that of wild type GLP1R (Fig. 4B). According to the two-domain hypothesis (25–30), the primary binding residues reside in the N-terminal ECD of the receptor, which interacts with the C-terminal portion of the peptide. Indeed, the N-terminal ECD fragment is able to bind the C-terminal fragment of the peptide with high affinity. In contrast, binding between the core region of the receptor and N-terminal portion of the peptide is likely secondary, and this binding may be more important for inducing conformational change of the receptor, allowing recep-
tor activation. Thus, it is likely that despite subtle changes in binding affinity of the mutant receptors mutations at Ile196, Leu232 and Met233, and Asn302 and Met303 critically affect ligand-induced receptor activation.

In an ensuing series of experiments, we further constructed point-mutated chimeric receptors in which these conserved amino acids of GLP1R were introduced into the corresponding sites of GIPR. We first examined the role of Ile196 and Lys197 at TMH2 in the ligand-induced receptor activation using the GI/L2 chimeric receptor. In chimeric receptor studies, the GLP-1 potency toward GI/L2 was almost 10-fold lower than that toward GI/L1 (Fig. 1A), proposing that specific amino acid residues, probably Ile196 and Lys197, at THM2 of GLP1R are likely responsible for this 10-fold decreased potency. Introducing both residues (Ser188 and Arg189 to Ile and Lys; GI[LK]/L2) or a single residue (Ser188 to Ile; GI[I]/L2) in GI/L2 restored the GLP-1 potency to a level as high as that toward GI/L1 (Fig. 5, A and B). To further determine Ile196/Lys197-interacting residues within the peptides, we applied chimeric peptides to cells expressing GI/L1, GI/L2, or GI[LK]/L2. All Thr7-containing peptides showed increased potencies to GI[LK]/L2 (Fig. 5C), indicating that Thr7 is primarily important for interaction with TMH2 Ile196/Lys197. We also determined the binding affinity of these mutant receptors to cGIP peptides using a radioligand displacement assay. Binding of125I-[H1,T7,E15,A18]cGIP to chimeric mutant receptors was displaced with various cGIP peptides. cGIP peptides having Thr7 exhibited a decreased affinity toward GI/L2 compared with that toward GI/L1 in contrast to His5-containing [H1,E15,A18]cGIP. [E15,A18]cGIP rather revealed an increased potency toward GI/L2 compared with GI/L1. Thr7-containing peptides showed increased potencies to GI[LK]/L2 (Fig. 5C), indicating that Thr7 is primarily important for interaction with TMH2 Ile196/Lys197. We also determined the binding affinity of these mutant receptors to cGIP peptides using a radioligand displacement assay. Binding of125I-[H1,T7,E15,A18]cGIP to chimeric mutant receptors was displaced with various cGIP peptides. cGIP peptides having Thr7 exhibited a decreased affinity toward GI/L2 compared with that toward GI/L1 and GI[LK]/L2 (Table 4) consistent with the results obtained using the receptor activation assay.
We then examined the function of Leu^{232} and Met^{233} in ECL1 of GLP1R using two chimeric receptors, GI/L3 and GL/I2. The GLP-1 potency toward GI/L3 was almost 10-fold lower than that toward GI/L2 (Fig. 1A). Substitution of Leu and Met for Val^{222} and Thr^{223} in ECL1 of GI/L3 generates GI/[LM]/L3. The GLP-1 potency toward this receptor was significantly increased compared with that toward GI/L3 (Fig. 6, A and B). However, the single amino acid substitution of Thr^{223} with Met did not increase the potency of GLP-1 (Fig. 6, A and B). We then introduced Leu and/or Met into the positions Val^{222} of GI/L2, GLP-1 potency toward GI/L2 and GI/L3 were similar (Fig. 2). Furthermore, none of the chimeric peptides were able to activate GL/I2 and GI/L3 (data not shown).

Finally, we investigated the effect of substitution of Asn and/or Met for the Val^{292} and Lys^{293} at ECL2. These substitutions in GI/L5 did not increase the GLP-1 potency compared with that toward GI/L4 (Fig. 7, A and B). As the GIPR portion in this chimera is too large, the two-amino acid substitution may not be enough to gain ability to respond to GLP-1. However, placing Asn alone (GI/[NM]/L4) or together with Met (GI/[NM]/L4) at positions of Val^{292} and Lys^{293} of GL/I4 greatly improved GLP-1 potency such that these receptors behaved like GL/I5 (Fig. 7, C and D). However, GI/[NM]/L4 led to a GLP-1 potency similar to that toward GI/L4. Then we added chimeric peptides to cells expressing GI/L4, GI/L5, and GI/[NM]/L5. cGIPs having His^1 had a decreased potency to GI/L5 compared with those toward GI/L4, and these decreased potencies were partly or completely restored in GI/[NM]/L5-expressing cells (Fig. 7E). There was no significant alteration in potency of His^1-lacking [T^7,E^15,A^18]cGIP and [E^15,A^18]cGIP to GI/L4, GI/L5, and GI/[NM]/L5. To further support these results, we performed a displacement assay using [^{125}I]-labeled [H^1,T^7,E^15,A^18]cGIP peptide. However, the affinity of [^{125}I]-labeled [H^1,T^7,E^15,A^18]cGIP peptide to chimeric receptors used in this series of experiments was too low to conduct the displacement assay. Thus, to increase binding affinity of these chimeric receptors to cGIPs, we generated IN-GL/I4, IN-GL/I5, and IN-GL/[NM]/I4 in which the N-terminal ECD of GIPR was introduced. In receptor activation and binding assays, His^1-containing cGIPs generally had lowered potencies and affinities to IN-GL/I4 compared with those toward IN-GL/I5. These decreased potencies and affinities were restored in IN-GL/[NM]/I4-expressing cells (Table 5). These results indicate that His^1 of the chimeric peptides likely interacts with Asn^{302} of GLP1R. Together, these observations suggest that the conserved amino acid residues

![FIGURE 6. Role of Leu^{232} and Met^{233} at ECL1 of GLP1R in ligand potency.](image)

**TABLE 4** Binding affinities of cGIP peptides toward GIPR/GLP1R chimeric receptors

| Chimeric peptides | IC_{50} | GI/L1 | GI/L2 | GI/[LM]/L3 | GI/[NM]/L3 |
|-------------------|--------|-------|-------|-------------|-------------|
| [H^1,T^7,E^15,A^18]cGIP | 47.08 ± 5.40 | 223.48 ± 32.42 | 35.08 ± 0.40 | a Versus GI/L1 (p < 0.05). |
| [H^1,T^7]cGIP | 48.42 ± 0.56 | 245.46 ± 145.68 | 35.72 ± 4.09 | a Versus GI/L2 (p < 0.05). |
| [H^1,E^15,A^18]cGIP | 580.15 ± 298.68 | 36.35 ± 1.67 | 192.89 ± 41.53 | |
| [T^7,E^15,A^18]cGIP | 102.40 ± 28.99 | 240.68 ± 41.16 | 29.12 ± 4.00 | a Versus GI/[LM]/L3 (p < 0.05). |
| [T,E^15,A^18]cGIP | 5.40 ± 2.23 | 223.48 ± 32.42 | 35.08 ± 0.40 | a Versus GI/[NM]/L3 (p < 0.05). |

**FIGURE 6.** Role of Leu^{232} and Met^{233} at ECL1 of GLP1R in ligand potency. The construction of chimeric receptors and point mutations at these receptors are shown (A and C). Chimeric and point-mutated receptor cDNAs were cotransfected with the CRE-luc reporter vector into HEK293T cells. Forty-eight hours following transfection, cells were treated with increasing concentrations of GLP-1 (B and D) for 6 h, and luciferase activity was measured. Results are presented as mean ± S.E. of at least three independent experiments. a, versus GI/L2 (p < 0.05); b, versus GI/L3 (p < 0.05); c, versus GI/L3 (p < 0.05); d, versus GI/L2 (p < 0.05).
such as Ile196 of TMH2, Leu232 and Met233 of ECL1, and Asn302 of ECL2 of GLP1R may confer specific GLP-1 binding and receptor activation through interaction with His1 and Thr7 of GLP-1.

Molecular Modeling—To determine whether the interaction of GLP-1 and GLP1R suggested from the experiments is feasible or not at the atomic level and to get more structural insights from the experiments, we carried out a homology modeling of the ligand and the receptor. Although we added some experimental data during the modeling, the modeling result is highly consistent with the experiments (Fig. 8). GLP-1 colored in yellow binds in a highly kinked \(H\)-helical conformation in a surface groove of the N-terminal ECD of GLP1R (15, 32). The N-terminal seven amino acids of GLP-1 with no secondary structure interact with transmembrane domains and extracellular loops.
of GLP1R. Particularly, His\textsuperscript{1} of GLP-1 was in close contact with Asn\textsuperscript{302} of GLP1R. It is possible that two nitrogen atoms at side chains of His\textsuperscript{1} of GLP-1 may electrostatically attract the oxygen atom at the side chain of Asn\textsuperscript{302} of GLP1R. Thr\textsuperscript{7} of GLP-1 was also in close contact with Ile\textsuperscript{196}, Leu\textsuperscript{232}, and Met\textsuperscript{233} of GLP1R. The oxygen atom of Thr\textsuperscript{7} of GLP-1 was close enough to make a hydrogen bond to a carbon atom of Ile\textsuperscript{196} of GLP1R. The sulfur atom of Met\textsuperscript{233} of GLP1R may also make a hydrogen bond interaction with the carbon atom at the side chain of Thr\textsuperscript{7} of GLP-1. However, point mutation at the chimeric receptor revealed that Ile\textsuperscript{196} in TMH2 alone may account for interaction with Thr\textsuperscript{7} of GLP-1. Thus, this model is in an agreement with the experimental results.

**DISCUSSION**

GLP-1 and GIP are physiologically and clinically important because of their multiple functions in the control of glucose homeostasis and obesity. Because of their combined beneficial effects, both GLP-1 and GIP have been reported as potential therapeutic agents for the treatment of type 2 diabetes mellitus and obesity. Exploring the domains or amino acid residues of the receptors that confer ligand binding and receptor activation is ultimately important for the design of molecular models for the ligand-receptor complex, facilitating the development of potent peptide as well as nonpeptide agonists regulating GLP1R and GIPR activities. Although the ligand binding residues in the N-terminal ECD of GLP1R and GIPR have been determined recently by x-ray crystallography of the ligand-bound ECD (15, 31, 32), ligand-interacting residues in the core of the receptors that also confer receptor activation have been poorly understood. The present study demonstrated for the first time specific amino acid residues in the core of GLP1R that potentially interact with the N-terminal moiety, in particular His\textsuperscript{1} and Thr\textsuperscript{7} of GLP-1.

As the binding domains for the N-terminal moiety of GLP-1/GIP are important for both receptor binding and activation, many approaches such as alanine scanning, photoaffinity labeling, and molecular modeling-based approaches have been taken (19, 28, 39–43). The alanine scanning approaches revealed that the residues including Lys\textsuperscript{197}, Asp\textsuperscript{198}, Lys\textsuperscript{202}, Met\textsuperscript{204}, Tyr\textsuperscript{205}, Asp\textsuperscript{215}, and Arg\textsuperscript{227} at TMH1 and ECL1 are likely important for binding to the N-terminal moiety of GLP-1 as mutation at these residues led to a significant decrease in ligand affinity (28, 40). However, these observations did not delineate the individual residues in the N-terminal moiety responsible for binding with these residues. Furthermore, it is also possible that mutations of these residues to Ala can alter the receptor conformation, interfering with binding to the ligand. Recently, using a photoaffinity labeling technique, Chen et al. (42) observed that Tyr\textsuperscript{205} in ECL1 is located in close proximity to p-benzoyl-L-phenylalanine at position 6 of GLP-1. However, the mutation of Tyr\textsuperscript{205} to Ala in GLP1R did not alter either receptor activity or binding to ligand, indicating that Tyr\textsuperscript{205} is not the direct binding site for the N-terminal moiety of GLP-1. Thus, this approach is likely only useful for determining
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the close proximity of ligand binding sites, not direct binding sites. Furthermore, because photolabile \( p \)-benzoyl-L-phenylalanine should be incorporated at an appropriate position of the ligand that will not alter bioactivity of the peptide, photolabile \( p \)-benzoyl-L-phenylalanine cannot be incorporated at positions such as positions 1 (His) and 7 (Thr) that are critical for receptor binding or activation. Thus, there is a limitation in the use of this approach to explore the critical residues for ligand binding and receptor activation. The modeling approach obviously requires the biochemical analysis because a proper molecular model for the ligand-bound crystal structure of the class B receptor has not been made.

Our approach is based on the evolutionary pressure to conserve critical residues for ligand binding and receptor activation in class B GPCRs across vertebrate species. GLP1R, GLP2R, GCGR, and GIPR exhibit a high degree (37–44%) of amino acid sequence identity with one another, and their peptide ligands exhibit ~70% amino acid sequence identity at the N-terminal moiety, suggesting that these receptors possess a similar receptor topology to build a ligand binding pocket. However, their exact ligand interaction motifs are different. Indeed, a gain of function mutation such as substitution of GLP1R-specific amino acids for those in the GIPR portion of the chimeric receptors greatly improved the GLP-1 potency in this study, supporting the above hypothesis. The use of chimeric peptides to define the exact binding site for individual amino acid residues of the N-terminal moiety is quite reliable when chimeric receptors or point-mutated receptors are applied. Thus, our strategy can be extended to explore to exact binding site of other class B GPCRs.

The chimeric GLP1R/GIPR study suggests that for GLP1R the N-terminal ECD, TMH2, ECL1, and ECL2 are critical for ligand binding and receptor activation, whereas for GIPR, the N-terminal ECD and ECL1 are important for binding with GIP. Based on sequence comparison of vertebrate GLP1Rs and human GIPR, we were able to find GLP1R-specific amino acid residues, Ile\(^{196}\) and Lys\(^{197}\) of TMH2, Leu\(^{232}\) and Met\(^{233}\) of ECL1, and Asn\(^{302}\) and Met\(^{303}\) of ECL2. Indeed, the point mutation of these residues in the chimeric receptors and wild type GLP1R greatly affected responsiveness to GLP-1. Of these residues, Xiao et al. (19) already determined that Lys\(^{197}\) of GLP1R is important for GLP-1 binding. The amino acid sequence alignment of class B GPCRs such as receptors for glucagon-like peptide-2, glucagon, vasoactive intestinal polypeptide, pituitary adenylate cyclase-activating polypeptide, growth hormone-releasing hormone, and secretin revealed that Lys\(^{197}\) is highly conserved in the majority of these receptors. In fact, a single substitution with alanine in place of Lys\(^{197}\) (equivalent to Lys\(^{197}\) of GLP1R) of the secretin receptor affects binding to its ligand (44). These data suggest that this residue is important for ligand-receptor interactions in this receptor family. In contrast, Ile\(^{196}\), Leu\(^{232}\), Met\(^{233}\), and Asn\(^{302}\) are unique to GLP1R and are therefore likely to contribute more to selective binding to GLP-1.

Previously, we demonstrated that the N-terminal moiety, particularly Tyr\(^{1}/\text{His}^{1}\) and Ile\(^{7}/\text{Thr}^{7}\), of GIP/GLP-1 peptides may confer differential ligand selectivity toward GIPR and GLP1R (35). Application of these chimeric peptides to cells expressing the GLP1R/GIPR chimeric receptors revealed that His\(^{1}\) of GLP-1 may interact with ECL2 of GLP1R. Because Asn\(^{302}\) in ECL2 is GLP1R-specific and involved in selective interaction with GLP-1, it is presumably that His\(^{1}\) of GLP-1 is closely localized to Asn\(^{302}\) of GLP1R when this ligand-receptor pair bind each other. Interestingly, Asn\(^{302}\) is also highly conserved at the corresponding position of glucagon receptors (Asn\(^{300}\) for human) and GLP-2 receptors (Asn\(^{356}\) for human) across vertebrate species. These receptors and GLP1R have peptide ligands with a His residue at the first position. Thus, it may be postulated that His\(^{1}\) of GLP-1, glucagon, and GLP-2 likely interact with this conserved Asn in ECL2 of their cognate receptors. In contrast, Thr\(^{7}\) of GLP-1 likely interacts with amino acid residues in TMH2 and ECL1. As the GLP1R-specific amino acid residue in TMH2 is Ile\(^{196}\), Thr\(^{7}\) of GLP-1 may have a close contact with this residue. In addition, Thr\(^{7}\) of GLP-1 may be involved in interaction with Leu\(^{232}\) and Met\(^{233}\) at ECL1.

With regard to GIP and GIPR interaction, Tyr\(^{1}\) and Ile\(^{7}\) of GIP likely interact with the amino acid residues in the upper half of TMH2. The sequence comparison of various vertebrate GIPR and GLP1R revealed that Ser\(^{147}\) and Leu\(^{196}\) in the TMH1 and Ile\(^{186}\), Leu\(^{189}\), Ser\(^{190}\), and Arg\(^{391}\) in TMH2 are highly conserved in GIPRs, but these residues are not observed in the corresponding position of GLP1R. Thus, these residues may contribute to the formation of a binding pocket allowing GIP-specific binding. However, this possibility remains to be investigated further. In contrast to our proposal, recently, a molecular modeling-based approach suggests that Tyr\(^{1}\) of GIP interacts with a binding pocket formed by Gln\(^{224}\) in TMH3, Arg\(^{300}\) in TMH5, and Phe\(^{357}\) in TMH6. Point mutation of these residues to Ala in GIPR led to a marked decrease in ligand potency (39). However, all these residues are highly conserved at the corresponding positions of most class B family GPCRs across vertebrate species, indicating that these residues may be commonly important for maintaining receptor conformation conferring receptor activation. Thus, the reductions in ligand potency toward Ala-substituted GIPRs are likely due to modification of the structure related to receptor activation rather than reduced interaction of these residues with Thr\(^{7}\) of GIP.

In summary, one major bottleneck for the development of novel GLP-1 and GIP analogs is the lack of information regarding the molecular structure of ligand-bound GLP1R and GIPR. The present study first demonstrated molecular determinants in the core of GLP1R and GIPR responsible for interaction with the N-terminal moiety, particular positions 1 and 7 of their cognate peptide ligands. Receptor sites participating in the interaction with other residues of the N-terminal moiety of the peptide need to be further investigated to refine the structure of ligand-bound receptor. Because the ligand binding site within the TMH core can be considered as a target of nonpeptide ligands (45, 46), our study may facilitate molecular modeling of the ligand-bound receptor structure followed by extensive medicinal chemistry.

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