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Molecules and Cells

Tyr[1] and Ile[7] of Glucose-Dependent Insulinotropic Polypeptide (GIP) Confer Differential Ligand Selectivity toward GIP and Glucagon-like Peptide-1 Receptors

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Glucagon like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are incretin hormones released in response to food intake and potentiate insulin secretion from pancreatic β cells through their distinct yet related G protein-coupled receptors, GLP1R and GIPR. While GLP-1 and GIP exhibit similarity in their N-terminal sequence and overall α-helical structure, GLP-1 does not bind to GIPR and vice versa. To determine which amino acid residues of these peptide ligands are responsible for specific interaction with their respective receptors, we generated mutant GIP in which several GLP-1-specific amino acid residues were substituted for the original amino acids. The potency of the mutant ligands was examined using HEK293 cells transfected with GLP1R or GIPR expression plasmids together with a cAMP-responsive element-driven luciferase (CRE-luc) reporter plasmid. A mutated GIP peptide in which Tyr[1], Ile[7], Asp[15], and His[18] were replaced by His, Thr, Glu, and Ala, respectively, was able to activate both GLP1R and GIPR with moderate potency. Replacing the original Tyr[1] and/or Ile[7] in the N-terminal moiety of this mutant peptide allowed full activation of GIPR but not of GLP1R. However, reintroducing Asp[15] and/or His[18] in the central α-helical region did not significantly alter the ligand potency. These results suggest that Tyr/His[1] and Ile/Thr[7] of GIP/GLP-1 peptides confer differential ligand selectivity toward GIPR and GLP1R.

INTRODUCTION

Glucagon like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are the major physiological incretins released in response to nutrient ingestion, and both peptides stimulate glucose-dependent insulin secretion (Fehmann et al., 1995). GLP-1 is a 30-amino acid peptide released from L cells of the small intestine following tissue-specific post-translational processing of proglucagon (Orskov et al., 1986), while GIP is a 42-amio acid peptide released from K cells of the small intestine (Gallwitz et al., 1996). Both GLP-1 and GIP are members of the secretin family of hormones, and exhibit similarity in amino acid sequence and three-dimensional structures. The first six or seven amino acid residues of both peptides form a random coil structure where they share approximately 70% amino acid sequence, followed by α-helical regions where the sequence similarity between GLP-1 and GIP is not pronounced. After this α-helix, the GIP peptide contains a random coil structure of 16 amino acids at the C-terminus (Neidigh et al., 2001; Parthier et al., 2007; Underwood et al., 2010).

GLP-1 and GIP exert their actions through the cognate G protein-coupled receptors (GPCRs), GLP-1 receptor (GLP1R) and GIP receptor (GIPR), respectively. Both receptors belong to the family of class 2 GPCRs, which possess relatively long N-terminal extracellular domains (ECD) of approximately 120 residues. These ECDs contain several Cys residues that form a network of disulfide bridges, and appear to be critical for binding with peptide ligands (Holtmann et al., 1995). Indeed, recent studies using X-ray crystallography demonstrate that the ECD of GLP1R and GIPRs bind to the central α-helical regions of their peptide ligands (Parthier et al., 2007; Underwood et al., 2010).

Both GLP1R and GIPR are expressed in the pancreatic β-cells, and activation of both receptors in response to ligand stimulation leads to accumulation of cAMP and influx of intracellular calcium, accelerating insulin release from secretory granules (Drucker et al., 1987; Fehmann et al., 1995). Thus, these receptors appear to share similar signaling pathway in the pancreatic β-cells, although they are stimulated by distinct ligands (Maida et al., 2009).

Despite the similarities in N-terminal amino acid sequence and overall 3-dimensional structure between GLP-1 and GIP, GLP-1 does not bind to or activate GIPR and vice versa (Gallwitz et al., 1996). Extensive studies using alanine-substitution scanning of GLP-1 and GIP peptides have attempted to identify the amino acid residues critical for ligand-receptor interaction (Galwitz et al., 1994; Hinke et al., 2004). In addition, GLP-1/GIP chimeric peptides have been generated to explore the structural requirement

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Table 1. Amino acid sequences of GLP-1, GIP and GLP-1/GIP recombinant peptides

	1 10 20 30 42
GLP-1	HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR
rcGIP	**H**SEGTF**T**SDYTIAM**E**KI**A**QQDFVNWLLAQK
[Y^1]rcGIP	YSEGTF**T**SDYTIAM**E**KI**A**QQDFVNWLLAQK
[I^7]rcGIP	**H**SEGTF I SDYTIAM**E**KI**A**QQDFVNWLLAQK
[Y^1I^7]rcGIP	YSEGTF I SDYTIAM**E**KI**A**QQDFVNWLLAQK
[$D^{15}H^{18}$]rcGIP	**H**SEGTF**T**SDYTIAMDKIHQQDFVNWLLAQK
[Y^1]rcGIP-E4	YSEGTF**T**SDYTIAM**E**KI**A**QQDFVNWLLAQKGPSSGAPPPS
[Y^1I^7]rcGIP-E4	YSEGTF I SDYTIAM**E**KI**A**QQDFVNWLLAQKGPSSGAPPPS
GIP	YAEGTF I SDYSIAMDKIHQQDFVNWLLAQKGKKNDWKHNITQ

for specific ligand-receptor interaction either in RINm5F insulinoma cells expressing both GLP1R and GIPR (Galwitz et al., 1996) or in CHO-K1 cells transfected with GLP1R or GIPR plasmid (Hinke et al., 2004). However, neither the site-directed mutagenesis nor the chimeric peptide approach has identified the specific amino acid residues of GLP-1 or GIP responsible for discriminating between the two receptors.

In this study, we have defined the GLP-1- and GIP-specific amino acid residues that allow these peptides to bind specifically to their own receptors. Based on amino acid sequence comparison of GLP-1, GIP, and exendin-4 (a GLP1R agonist), we developed point-mutated recombinant GIP peptides where several GIP-specific amino acid residues were replaced with GLP-1-specific residues, and tested their ability to activate both GLP1R and GIPR. Our results demonstrate that the N-terminal moiety of each peptide, particularly the amino acid residues at positions 1 and 7, may be critical for this differential ligand selectivity.

MATERIALS AND METHODS

Recombinant GIP peptides

Wild type GLP-1, GIP, recombinant-GIP (rcGIP), [Y^1]rcGIP, [I^7]rcGIP, [Y^1, I^7]rcGIP, [D^{15}, H^{18}]rcGIP, [Y^1, I^7]rcGIP-E4, and [Y^1]rcGIP-E4 were synthesized from AnyGen (Korea). The amino acid sequences of these peptides are shown in Table 1.

Plasmid DNA constructs

The pcDNA3 expression vector was purchased from Invitrogen (USA). The CRE-luc vector which contains four copies of the cyclic AMP-responsive element (CRE; TGACGTCA) was purchased from Stratagene (USA). The cDNAs for rat GLP1R and human GIPR were kindly provided by Dr. Bernard Thorens (Institute of Pharmacology and Toxicology, Switzerland).

Cell culture, transfection and luciferase assay

HEK 293T cells were maintained at 37°C in DMEM (Dulbecco's modified Eagle's medium, Life Technologies, USA) with 10% heat-inactivated fetal bovine serum. Cells were plated in 48-well plates, and transfection was performed with Effectene reagent (QIAGEN, USA) the following day, according to the manufacturer's instructions. For each transfection, 200 ng of plasmid for rat GLP1R or human GIPR, and 200 ng of CRE-luc were used. About 48 h after transfection, cells were treated with the appropriate peptide ligand for 6 h. Cells were then harvested, and luciferase activity in the cell extracts was determined according to standard methods using a Multi-label counter (Perkin Elmer, USA). The luciferase activities were normalized using the β-galactosidase values.

Data analysis

All assays were performed in triplicate and repeated three times. Data analysis was performed using nonlinear regression with sigmoid dose-response curves. The peptide ligand concentrations inducing half-maximal stimulation (EC_{50}) or maximum-fold increase (E_{max}) was calculated using GraphPad PRISM4 software (GraphPad, USA). All data are presented as mean ± SEM. The data were analyzed by one-way ANOVA followed by Newman-Keuls post-hoc test. $P < 0.05$ was considered statistically significant.

RESULTS

The potency of GLP-1 and GIP for their receptors, GLP1R and GIPR

To investigate the ligand potency of GLP-1 and GIP, HEK293T cells were transfected with plasmid containing either GLP1R or GIPR, together with the CRE-luc reporter vector. Cells were then treated with increasing concentrations of native human GLP-1 or GIP. GLP-1 augmented CRE-luc reporter activity in a dose-dependent manner in cells harboring GLP1R (EC_{50}: 0.03 nM), but not in cells containing GIPR (Fig. 1A). Similarly, GIP activated GIPR with high potency (EC_{50}: 0.17 nM) (Fig. 1B). Although GIP was able to activate the GLP1R at a high concentration (1 μM), its potency against this receptor was extremely low compared to its activity against GIPR, and compared to the GLP-1-induced response of GLP1R. Together, these results demonstrated that GLP-1 and GIP bind to only their cognate receptors and do not cross-react with other receptors.

Amino acid sequence alignment of GIP/GLP-1 peptides

To identify residues specific to the function of GLP-1 and GIP, we compared the amino acid sequences of GLP-1, GIP and exendin-4, a closely-related 39 amino-acid peptide found in the venom of the Gila monster *Heloderma suspectum* (Göke et al., 1993). Exendin-4 is a potent GLP1R agonist, and shares approximately 50% sequence identity with GLP-1 (Göke et al., 1993). We used this information to help identify residues that are important for GLP-1 binding to GLP1R, yet distinct from corresponding residues in GIP.

To simplify the comparison of the three peptides, the residue-numbering scheme of GLP-1 was adapted to those of exendin-4 and GIP, such that the first residue of GLP-1, His^7 was renamed as His^1, Ala^8 of GLP-1 was renamed Ala^2 etc. (Fig. 2). The sequence alignment for human GLP-1, exendin-4, and human GIP reveals that the N-terminal regions (amino acid residues 1-7) of all three peptides exhibit a high degree of sequence similarity, while the central α-helical regions and C-

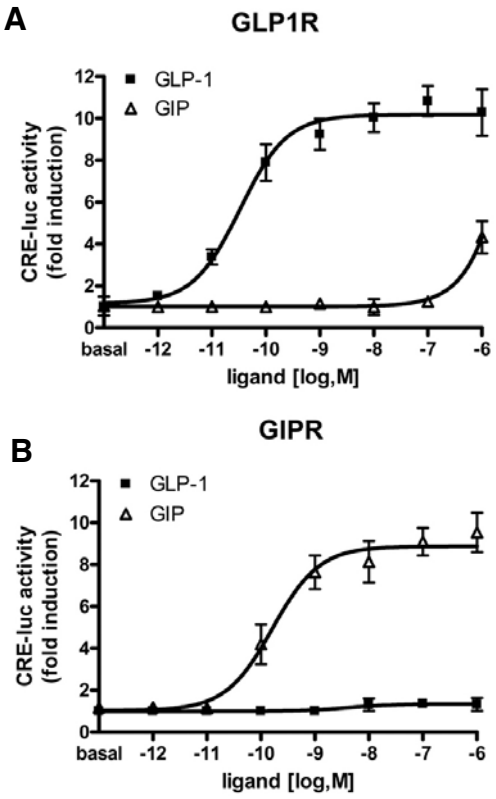

Fig. 1. Ligand selectivity of GLP1R and GIPR. Plasmids containing rat GLP1R (A) or human GIPR (B) cDNA were cotransfected with the CRE-luc reporter vector into HEK 293T cells. Forty-eight hours following transfection, cells were treated with increasing concentrations of GLP-1 (■) or GIP (△) peptides for 6 h, and luciferase activity was determined.

terminal regions are highly variable (Fig. 2). In the N-terminal region, the first and seventh residues of GIP (Tyr^1 and Ile^7) differ from those in the corresponding positions of GLP-1 and exendin-4. In the central α-helical regions, Ser^{11}, Phe^{22}, and Trp^{25} are conserved among all three peptides, while Glu^{15} and Ala^{18} are conserved between GLP-1 and exendin-4, but not in GIP. All five of these residues are linearly localized on the surface of the α-helical structure of the peptides, which is known to be important for ligand-receptor interaction (Al-Sabah and Donnelly, 2003). In GIP, the presence of Asp^{15} and His^{18}, instead of the Glu^{15} and Ala^{18} found in the helical region of the other two peptides, and the GIP/GLP-1 replacements of Tyr/His^1 and Ile/Thr^7 in the N-terminal region, suggested to us that these residues may be responsible for the differential selectivity of these ligands toward GIPR and GLP1R. Thus, we generated a recombinant GIP (rcGIP) where Tyr^1, Ile^7, Asp^{15}, His^{18} of GIP were replaced with those corresponding amino acid residues of GLP-1 (Table 1). In addition, this chimeric peptide possesses a Ser residue at the position 2 to protect cleavage by dipeptidyl peptidase IV (Hinke et al., 2002), but does not contain the C-terminal sequence (residues 31 to 42 of the full-length GIP), which is unnecessary for the binding of GIP and the subsequent activation of GIPR (Hinke et al., 2001).

Potencies of recombinant GIP peptides

The potencies of mutated GIP peptides (Table 2) toward GIPR and GLP1R were examined by luciferase assay in HEK 293T cells expressing either GIPR or GLP1R. The rcGIP peptide, in which Tyr^1, Ile^7, Asp^{15}, and His^{18} of GIP were replaced with the corresponding His, Thr, Glu, and Ala of GLP-1, activated both GLP1R and GIPR with similar, moderate potency (Table 2 and Fig. 3). This result shows that substitution of these residues into GIP greatly enhanced its binding affinity for GLP1R, yet allowed rcGIP to retain some ability to activate GIPR. Thus, although it has gained new activity towards GLP1R, this peptide seems unable to distinguish between GLP1R and GIPR.

To further define the amino acid residues of GIP/GLP-1 peptides conferring receptor selectivity, we generated additional peptides where one or two amino acids in rcGIP were restored to those of GIP. Restoration of Tyr^1 in rcGIP ([Y^1]rcGIP) greatly enhanced the potency to activate GIPR, restoring it to that of wild type GIP. [Y^1]rcGIP, however, did not significantly reduce the ability to activate GLP1R (Fig. 4 and Table 2). This result indicates that Tyr^1 of GIP is the most critical for binding to and activation of GIPR, and that GLP1R is relatively tolerant of the Tyr^1 substitution in the peptide ligand. Placing Ile^7 in rcGIP ([I^7]rcGIP) increased its potency against GIPR by 5-fold, while decreasing its ability to activate GLP1R by 10-fold, indicating that Ile/Thr^7 of the peptides is, at least in part, responsible for differential ligand selectivity.

It is interesting that [Y^1, I^7]rcGIP behaves like wild type GIP such that the potency of this peptide toward GIPR is as high as wild type GIP, and its potency to activate GLP1R is as low as wild type GLP-1. Restoration of both Asp^{15} and His^{18} in rcGIP ([D^{15}, H^{18}]rcGIP), compared to rcGIP, slightly increased the ability to activate GIPR but decreased the potency to activate GLP1R (Fig. 4 and Table 2), suggesting that Tyr/His^1 and Ile/Thr^7 of the GIP/GLP-1 peptides are the most critical residues conferring differential ligand selectivity to GIPR and GLP1R while Asp/Glu^{15} and His/Ala^{18} residues have a minor effect.

Addition of the exendin-4 tail to the rcGIP peptides

It is known that the exendin-4 C-terminal tail sequence enhances the binding affinity of exendin-4 to the ECD of GLP1R

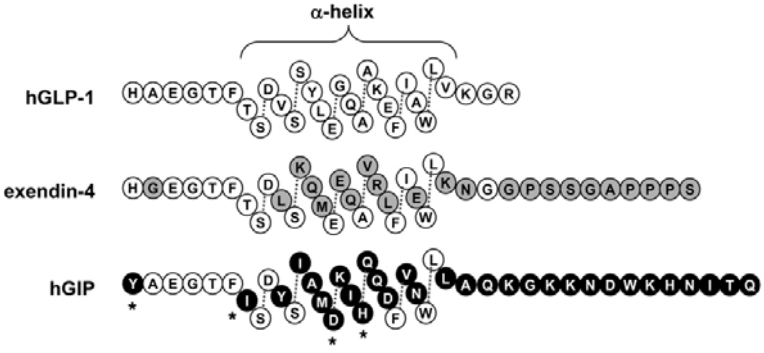

Fig. 2. Amino acid sequences of human (h) GLP-1, exendin-4 and hGIP. The central α-helical regions of the peptides are indicated. The hGLP-1 sequence and conserved amino acids among the three peptides are shown as open circles. The exendin-4-specific amino acids are indicated in gray, and the hGIP-specific amino acids are shown in black. The positions of GLP-1- and GIP-specific residues are marked with asterisks.

Table 2. Ligand sensitivities of GLP1R and GIPR

Peptide	GLP1R		GIPR	
	EC_{50} [log, *M*]	E*max* Fold induction	EC_{50} [log, *M*]	E*max* Fold induction
GLP-1	-10.48 ± 0.14	10.18 ± 0.33	no response	no response
GIP	> -6[a]	no response	-9.79 ± 0.16	8.87 ± 0.39
rcGIP	-8.59 ± 0.21[a]	10.34 ± 0.65	-8.01± 0.20[c]	9.54 ± 0.69
[Y^1]rcGIP	-8.05 ± 0.14[a,b]	9.28 ± 0.48	-9.58 ± 0.20[d]	8.90 ± 0.45
[I^7]rcGIP	-7.19 ± 0.09[a,b]	11.61 ± 0.53	-8.68 ± 0.18[c,d]	8.61 ± 0.42
[Y^1I^7]rcGIP	> -6[a, b]	no response	-9.76 ± 0.18[c,d]	9.91 ± 0.43
[$D^{15}H^{18}$]rcGIP	-8.22 ± 0.13[a,b]	10.48 ± 0.47	-8.33 ± 0.14[c,d]	9.93 ± 0.14
[Y^1]rcGIP-E4	-8.03 ± 0.08[a,b]	9.17 ± 0.27	-10.08 ± 0.12[c,d]	9.03 ± 0.25
[Y^1I^7]rcGIP-E4	> -6[a,b]	no response	-9.93 ± 0.16[c,d]	9.90 ± 0.38

[a]Versus GLP-1 toward GLP1R ($p < 0.05$)
[b]Versus rcGIP toward GLP1R ($p < 0.05$)
[c]Versus GIP toward GIPR ($p < 0.05$)
[d]Versus rcGIP toward GIPR ($p < 0.05$)

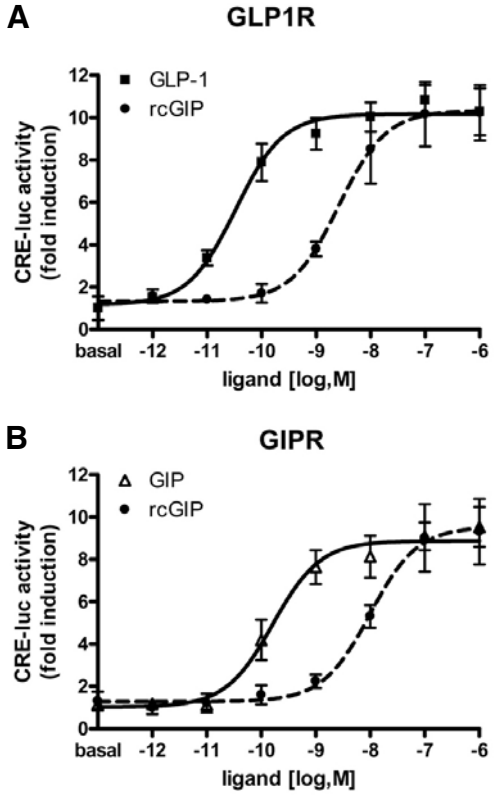

Fig. 3. Ligand potency of recombinant (rc) GIP toward GLP1R and GIPR. Plasmids containing GLP1R (A) or GIPR (B) cDNA were cotransfected with the CRE-luc reporter vector into HEK 293T cells. Forty-eight hours following transfection, cells were treated with graded concentrations of rcGIP (dashed lines, ●) peptide for 6 h, and luciferase activity was determined.

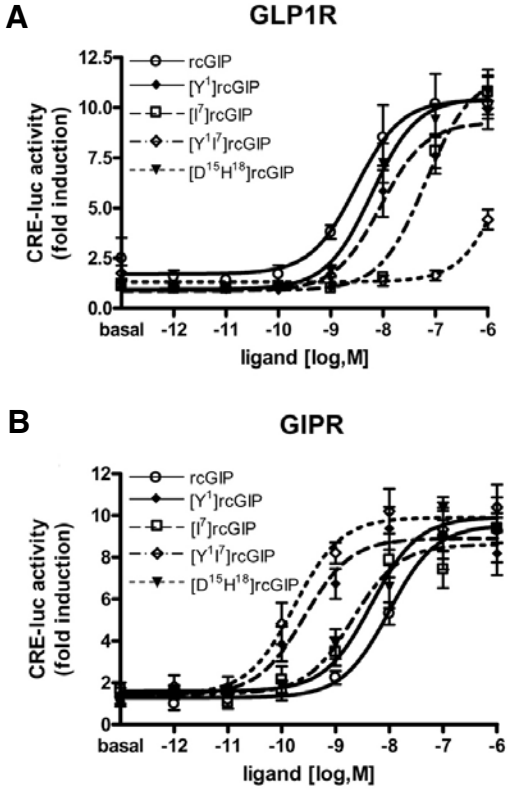

Fig. 4. Ligand potency of amino acid substituted rcGIP toward GLP-1R and GIPR. Plasmids containing GLP1R (A) or GIPR (B) cDNA were cotransfected with the CRE-luc reporter vector into HEK 293T cells. Forty-eight hours following transfection, cells were treated with different concentrations of amino acid substituted rcGIP peptides (○ for rcGIP; ● for [Y^1]rcGIP; □ for [I^7]rcGIP; ◇ for [Y^1I^7]rcGIP and ▽ for [$D^{15}H^{18}$]rcGIP) for 6 h, and luciferase activity was measured.

(Al-Sabah and Donnelly, 2003). To examine whether this sequence affects the potency of our mutant peptides toward GLP1R and GIPR, we added this sequence to [Y^1]rcGIP and [Y^1, I^7]rcGIP, generating [Y^1]rcGIP-E4 and [Y^1, I^7]rcGIP-E4. However, addition of the exendin-4 tail sequence failed to improve the potency to GLP1R (Fig. 5A) and did not alter potency to GIPR (Fig. 5B).

DISCUSSION

GLP-1 and GIP share common actions on islet β-cells, acting through the structurally distinct yet related receptors GLP1R and GIPR. Their incretin effect is strictly glucose-dependent and is essential for the maintenance of glucose homeostasis.

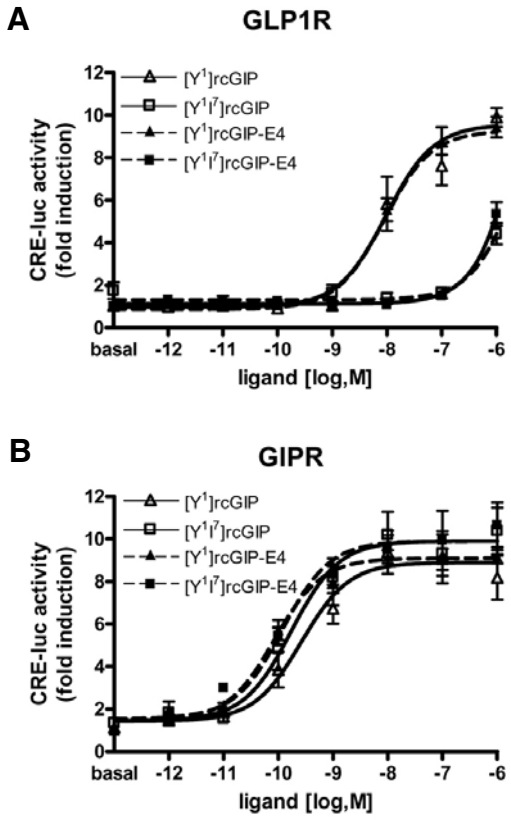

Fig. 5. Potency of rcGIP-E4 peptides toward GLP-1R and GIPR. Plasmids containing GLP1R (A) or GIPR (B) cDNA were cotransfected with the CRE-luc reporter vector into HEK 293T cells. Forty-eight hours following transfection, cells were treated with increasing concentrations of rcGIP peptides (△ for [Y^1]rcGIP and □ for [Y^1I^7]rcGIP) and rcGIP-E4 peptides (▲ for [Y^1]rcGIP-E4 and ■ for [Y^1I^7]rcGIP-E4, dashed line) for 6 h, and luciferase activity was determined.

Their biological effects on the stimulation of insulin secretion are additive, accounting for 50-70% of postprandial insulin secretion (Nauck et al., 1993). In addition to its incretin effect, GIP has been implicated in lipid metabolism and the development of obesity via direct actions on adipose tissue, and in bone formation via stimulation of osteoblast proliferation and inhibition of apoptosis (Baggio and Drucker, 2007). On the other hand, GLP-1 alternatively regulates glucose levels through the slowing of gastric emptying and glucose-dependent inhibition of glucagon secretion. Further, GLP-1 promotes satiety, and sustained activation of GLP1R promotes weight loss (Baggio and Drucker, 2007). Due to their combined beneficial effects, both GLP-1 and GIP have been identified as potential therapeutic agents for the treatment of diabetes mellitus and obesity. Therefore, increasing our understanding of the mechanisms through which these similar peptides recognize their own receptors is of great interest in the design of new molecules for the treatment of these diseases.

Studies using alanine scanning mutagenesis and N-terminal substitution/modification of GLP-1 and GIP have explored the bioactive domains of GLP-1 and GIP, yet have been unable to identify individual residues that are required for specific functions. For instance, the results of alanine scanning of the entire primary sequence of GLP-1 suggest that positions 1, 4, 6, 7, 9, 22, and 23 are crucial for either maintaining secondary structure of the peptide or for interaction with the receptor (Adelhorst et al., 1994; Gallwitz et al., 1994), but were unable to define the residues responsible for distinguishing between GLP1R and GIPR. For GIP, many of residues in the N-terminal moiety were found to be required for receptor binding and activation, because their replacement with alanine abolished these functions (Hinke et al., 2004). Additionally, chimeric GIP/GLP-1 peptides in which large segments of the peptides were swapped each other have been employed to determine the structural requirements for interaction with the receptors (Gallwitz et al., 1996; Hinke et al., 2004). In these studies, however, the role of individual residues could not be determined. Thus, neither previous point-mutations nor the chimeric peptide approach has identified the specific amino acid residues of the peptides responsible for differential ligand selectivity.

Based on sequence alignment among GLP-1, exendin-4, and GIP, we were able to define four GLP-1/GIP-specific amino acid residues likely to be functionally important, His/Tyr^1, Thr/Ile^7, Glu/Asp^{15}, and Ala/His^{18}. We generated a recombinant rcGIP peptide in which Tyr^1, Ile^7, Asp^{15}, and His^{18} were replaced with the corresponding residues of GLP-1, and found that this substitution greatly enhanced the potency of rcGIP toward GLP1R compared to the wild type GIP. In addition, the potency toward GIPR was reduced to approximately the same as for GLP1R, suggesting that these residues allow rcGIP to interact with GLP1R and GIPR with equivalent, though moderate affinity.

Returning Tyr^1 to rcGIP restored its ability to activate GIPR to that of wild type GIP, but this substitution slightly decreased the potency for GLP1R. Substitution of Ile^7 for Thr in rcGIP caused a 5-fold increase in potency toward the GIPR, but a 10-fold decrease in potency toward GLP1R. The rcGIP peptide with Tyr^1 and/or Ile^7 behaved like wild type GIP such that it fully activated GIPR but lost potency against GLP1R. However, the recombinant peptide with Asp^{15} and His^{18} slightly altered ligand affinity for either GIPR or GLP1R, and therefore is still equally active against GLP1R and GIPR. Thus, it is likely that the His/Tyr^1 and Thr/Ile^7 residues in the N-terminal moiety of these peptides is particularly important for differential ligand selectivity while Glu/Asp^{15} and Ala/His^{18} in the central α-helical region are less critical for discriminating between GIPR and GLP1R.

GLP-1 and GIP belong to the evolutionarily-related peptide family comprising secretin, GLP-2, glucagon, pituitary adenylyl cyclase activating peptide (PACAP), vasointestinal peptide (VIP) and growth hormone-releasing hormone (GHRH). Genes for this family are thought to result from the duplication of exons, genes, or chromosomes in a common ancestor (Sherwood et al., 2000). The high degree of conservation in the N-terminal moiety of these peptides supports this hypothesis. The N-terminus of each peptide starts with either His^1 (for PACAP, VIP, glucagon, GLP-1, GLP2, and secretin) or Tyr^1 (for GHRH and GIP). A recent study using computer-aided molecular modeling of GIP bound to its receptor, combined with site-directed mutagenesis studies, has revealed that the N-terminal moiety of receptor-bound GIP is predicted to be surrounded by transmembrane helices (TMH) 2, 3, 5 and 6 of the receptor. Particularly, Tyr^1 may interact with Gln^{224} in TMH3, Arg^{300} in TMH5, and Phe^{357} in TMH6 of the receptor (Yaqub et al., 2010). It is interesting to note that these predicted Tyr^1-interacting residues in GIPR are also conserved in GLP1R. Since [Y^1]rcGIP greatly enhanced the potency of rcGIP toward GIPR while retaining its potency toward GLP1R, GLP1R appears relatively tolerant for substitution of Tyr^1 for His in rcGIP peptide.

Most of the peptides in this family contain Thr at position seven (including PACAP, VIP, glucagon, GLP-1, GHRH, and

secretin), or Ser (GLP-2); only GIP contains Ile at this position. As the substitution of Ile^7 for Thr, either alone or together with Tyr^1 substitution, greatly reduced ligand potency toward GLP1R, Thr/Ile^7 appears to be more critical for ligand-receptor selectivity between these peptides and their receptors. Elucidation of the interactions between the Thr/Ile^7 residues of the ligand and individual amino acid residues in the receptor molecules would further enhance the knowledge of how GIP and GLP-1 discriminate between their own receptor.

Recent X-ray crystallography and computer-aided molecular modeling of the GIP-GIPR complex have revealed that the α-helical region of the peptide mainly interacts with ECD of GIPR (Parthier et al., 2007; Yaqub et al., 2010). Likewise, the α-helical region of GLP-1 binds to ECD of GLP1R, but N-terminal moiety of GLP-1 likely interacts with TMH of the receptor (Al-Sabah and Donnelly, 2003; Underwood et al., 2010). Our amino acid sequence alignment reveals that Glu/Asp^{15} and Ala/His^{18} are GLP-1- and GIP-specific residues in the α-helical region of the peptide. As both rcGIP and [D^{15}, H^{18}]rcGIP were neutral for GLP1R and GIPR, Glu/Asp^{15} and Ala/His^{18} in the central α-helical regions of GLP-1 and GIP may minimally confer specific binding to ECD of their own receptor, while these residues may contribute to interaction with the receptor to some extent (Adelhorst et al., 1994; Gallwitz et al., 1994). That is, either of [D^{15}, H^{18}]GIP or [E^{15}, A^{18}]GLP-1 may be able to bind to the ECD of the opposite receptors. In addition, the C-terminal tail of exendin-4 did not affect ligand selectivity for GLP1R and GIPR. In contrast, His/Tyr^1 and Thr/Ile^7 in the N-terminal moiety are likely the critical for discriminating own receptor from the other.

Peptide hormones and their GPCRs have become diversified through evolutionary mechanisms such as gene duplication and chromosome duplication, followed by gene modifications, generating families of related yet distinct peptides and receptors (Cho et al., 2007; Lee et al., 2009). Our study is the first to demonstrate that, in the GIP/GLP-1 family, the specific amino acids at positions 1 and 7 in the N-terminal region confer differential ligand selectivity toward their own receptors, and are more critical for this function than those at the central α-helical region and C-terminal domains. Further, this study may aid the development of potent hybrid peptides capable of activating both of GLP1R and GIPR. Indeed, [Y^1]rcGIP is able to activate both receptors with high potency for GIPR and moderate potency for GLP1R. However, its efficacy in the control of glucose homeostasis in vivo needs to be further examined.

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