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In depth analysis of the N-terminal bioactive domain of gastric inhibitory polypeptide

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Abstract

Gastric inhibitory polypeptide/glucose-dependent insulinotropic polypeptide (GIP) is an important gastrointestinal regulator of insulin release and glucose homeostasis following a meal. Strategies have been undertaken to delineate the bioactive domains of GIP with the intention of developing small molecular weight GIP mimetics. The molecular cloning of receptors for GIP and the related hormone GLP-1 (glucagon-like peptide-1) has allowed examination of the characteristics of incretin analogs in transfected cell models. The current report examines the N-terminal bioactive domain of GIP residing in residues 1–14 by alanine scanning mutagenesis and N-terminal substitution/modification. Further studies examined peptide chimeras of GIP and GLP-1 designed to localize bioactive determinants of the two hormones. The alanine scan of the GIP_{1-14} sequence established that the peptide was extremely sensitive to structural perturbations. Only replacement of amino acids 2 and 13 with those found in glucagon failed to dramatically reduce receptor binding and activation. Of four GIP_{1-14} peptides modified by the introduction of DP IV-resistant groups, a peptide with a reduced bond between Ala^2 and Glu^3 demonstrated improved receptor potency compared to native GIP_{1-14}. The peptide chimera studies supported recent results on the importance of a mid-region helix for bioactivity of GIP, and confirmed existence of two separable regions with independent intrinsic receptor binding and activation properties. Furthermore, peptide chimeras showed that binding of GLP-1 also involves both N- and C-terminal domains, but that it apparently contains only a single bioactive domain in its N-terminus. Together, these results

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should facilitate development of incretin based therapies using rational drug design for potential treatment of diabetes.
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Introduction

The incretin gut peptides, GIP and GLP-1, are responsible for at least 50% of the insulin response to a meal. As the hormonal components of the enteroinsular axis, the incretins act conditionally to augment pancreatic insulin release, only when circulating glucose concentrations are elevated. Both hormones are released from the small intestine in response to luminal nutrients, and act upon specific cell-surface receptors on endocrine β-cells resulting in activation of adenylyl cyclase and influx and/or mobilization of intracellular calcium, thus accelerating insulin release from secretory granules (Fehmann et al., 1995; Gelling et al., 1997; Xiao et al., 2000).

An understanding of the structure-activity relationships of the incretins has developed slowly, but their modes of action are beginning to be understood. The GIP molecule can be dissected into three bioactive domains. Both the N-terminal 14 amino acids and the mid-domain, comprising residues 19–30, are able to activate adenylyl cyclase in receptor transfected cells, stimulate insulin release in the perfused rat pancreas, and reduce glycemia in a rat bioassay. $GIP_{1-30NH2}$ exhibits equivalent insulinotropic activity to GIP_{1-42}, but lacks somatostatinotropic activity in the stomach, suggesting that the C-terminal 12 amino acids also contribute to the biological effects of this hormone, although not to the incretin effect (Hinke et al., 2001; Morrow et al., 1996). GLP-1 has not been subjected to as extensive fragment analysis as GIP, however, studies have shown that the N-terminal region is more important for receptor recognition than the C-terminal domain (Gallwitz et al., 1996; Hjorth et al., 1994). Furthermore, alanine scanning of the entire primary sequence of GLP-1 revealed positions 1, 4, 6, 7, 9, 22 and 23 as being particularly important either for maintenance of secondary structure or ligand-receptor interactions (Gallwitz et al., 1994; Adelhorst et al., 1994).

The current study extends the examination of the N-terminal bioactive domain of GIP_{1-14}. Synthetic peptides with singular residue replacements with alanine in each position of GIP_{1-14} were generated, to elucidate critical residues for receptor binding and activation. It is hypothesized that specific residues in GIP_{1-14} cannot be altered without negatively affecting efficacy or potency; the remaining residues which are tolerant to substitution may be modified in future studies to develop more potent low molecular weight GIP receptor ligands. Furthermore, as it is known that GIP is inactivated by dipeptidyl peptidase (DP) IV in vitro and in vivo (Hinke et al., 2002), several peptides based on GIP_{1-14} were designed to be resistant to proteolysis by this enzyme (Kühn-Wache et al., 2000), in order to render them more potent in vivo. Peptides were tested for stimulation of cyclic AMP production and binding affinity in radioligand displacement assays employing GIP receptor transfected Chinese hamster ovary (CHO-K1) cells. Additionally, a series of five peptide chimeras of GIP and GLP-1 were designed to delineate the binding and activation domains of GIP and GLP-1, in the perspective of the known bioactive domains of GIP. The latter set of peptides was tested on both GIP and GLP-1 receptor transfected CHO cells.

Materials and methods

Peptide synthesis

Reagents were from Novabiochem, Roth, Merck, Fluka and Bachem (Germany). Peptides were synthesized using the automated Symphony peptide synthesizer (Rainin Instrument Co., Woburn, USA), using a modified solid phase Fmoc-protocol, precisely as previously described (Hinke et al., 2001; Kühn-Wache et al., 2000; Manhart et al., 2003). Synthetic peptides were subjected to preparative and analytical HPLC (high performance liquid chromatography) using a LiChrograph HPLC apparatus (Merck-Hitachi, Darmstadt, Germany), and identity and purity were assessed by mass spectrometry (MALDI-TOF MS). Alanine scanning substitution was performed on GIP_{1-14OH} (^{1}YAEGTFISDYSIAM^{14}OH); endogenous Ala^2 and Ala^{13} were replaced with Ser^2 and Tyr^{13}, the corresponding amino acids in the glucagon primary sequence. Additional GIP_{1-14OH} peptides were synthesized with a reduced scissile bond ([Tyr^1-Ala^2-ψ(CH_2NH)]GIP_{1-14OH}), reversed chirality of residue 2 ([D-Ala^2]GIP_{1-14OH}) or proline^3 substitution ([Pro^3]GIP_{1-14OH}), all modifications which have been shown to confer resistance to DP IV cleavage (Kühn-Wache et al., 2000), as well as a further peptide containing a synthetic β-turn mimetic (β-turn dipeptide; BTD), $GIP_{1-11\text{-}BTD\text{-}12-14OH}$. Five peptide chimeras were synthesized based on the known primary sequences of $GIP_{1-30NH2}$ (^{1}YAEGTFISDYSIAMDKIHQQDFVNWLLAQK^{30}NH2) and GLP-$1_{[7-36]NH2}$ (^{7}HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR^{36}NH2); for the purposes of this article, subscript amino acid designations for GLP-1 have been numbered according to the primary sequence of the processed hormone (i.e. GLP-$1_{[7-36]NH2}$=GLP-$1_{[1-30]NH2}$). Chimeric peptides synthesized were GLP-$1_{[1-14]}$/$GIP_{[15-30]NH2}$(CH1), $GIP_{[1-14]}$/GLP-$1_{[15-30]NH2}$(CH2), GLP-$1_{[1-11]}$/$GIP_{[12-30]NH2}$(CH3), $GIP_{[1-11]}$/GLP-$1_{[12-18]}$/$GIP_{[19-30]NH2}$(CH4), and $GIP_{[1-14]}$/GLP-$1_{[15-18]}$/$GIP_{[19-30]NH2}$(CH5). Preparations of GIP_{1-42} and $GIP_{1-30NH2}$ were also synthesized in the Halle laboratory (described previously (Hinke et al., 2001)), but GLP-$1_{[7-36]NH2}$(GLP-$1_{[1-30]NH2}$) was purchased from Bachem (Torrance, USA).

Peptide iodination

GIP_{1-42} and GLP-$1_{[7-36]NH2}$ were radioactively labeled with 125-iodine using the Chloramine-T method (Hinke et al., 2002). Reacted peptides were separated from free iodine by gel filtration (Sephadex G-15; Pharmacia, Canada), and then subjected to reverse phase HPLC to obtain mono-component bioactive iodinated peptides. Use of this method in our laboratory routinely generates iodinated compounds of specific radioactivities in the range of 45 to 65 MBq/nmol, as determined by antibody displacement protocols.

Cell culture

Chinese hamster ovary (CHO-K1) cells stably transfected with eucaryotic expression plasmids (pcDNA3; Invitrogen, Carlsbad, USA) containing cDNA sequences for the open reading frames of the wild type human GLP-1 receptor (wtGLP-1R cells) or rat GIP receptor (wtGIPR cells) have been previously described (Gelling et al., 1997). Cells were grown in DMEM/F12 (1:1 Dulbecco-modified Eagle's medium and Ham's F12) nutrient mixture supplemented with 10% newborn bovine serum, antibiotics and 0.8 mg/ml G418 as the selection agent (all cell culture reagents were from Gibco Life

Sciences, Canada). Cells were harvested by trypsin/EDTA treatment, and dispensed at 50,000 cells per well in 24 well plates; after two days of culture, each well contained $2-4 \times 10^5$ cells to be used in cyclic AMP stimulation and binding competition experiments.

Cyclic AMP and competitive binding studies

Experiments were carried out as previously described (Gelling et al., 1997; Hinke et al., 2001; Hinke et al., 2002). For cAMP stimulation experiments, cells were washed twice in 37 °C assay buffer (15 mM HEPES-buffered DMEM/F12 with 0.1%BSA; HEPES (N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]) and BSA (bovine serum albumin) from Sigma Chemical Co., USA), and allowed to pre-incubate for one hour in this solution. Stimulation proceeded for 30 min in the same buffer additionally supplemented with 0.5 mM IBMX (isobutylmethylxanthine; Research Biochemicals Intl., USA) and 100 Kallikrein inactivating units (KIU) aprotinin (Trasylol; Bayer, Canada), and peptide at the concentrations indicated in the figures. Intracellular cAMP was extracted in ice cold 70% ethanol and after removal of cell debris and drying, cAMP was measured by radioimmunoassay (Biomedical Technologies Inc., USA).

Whole cell binding studies began by washing cells twice with ice cold assay buffer, followed by addition of the same media with aprotinin, 50,000 cpm ^{125}I-GIP or ^{125}I-GLP-1, and appropriate peptide concentrations. Equilibrium binding was achieved during overnight incubation (12–16 h, 4 °C), then cells were washed twice with assay buffer, solubilized in 0.2 M NaOH, and transferred to test tubes for counting cell associated radioactivity.

Data analysis

Each independent experiment consisted of triplicate determinations, and a minimum of three independent experiments (cyclic AMP stimulation and binding study) were conducted for each peptide; data represent the mean ± S.E.M. of the three experiments. Cyclic AMP data are presented as fmol/1000 cells, or normalized either to basal cAMP levels or to the maximal cAMP level obtained by stimulation with native hormone for each experiment. Binding data are presented as a percentage of binding in absence of competitor (%B/B_0), and non-specific binding was estimated as the binding in the presence of 1 μM GIP or GLP-1, where appropriate. Significance of differences was assessed by analysis of variance (ANOVA) and Dunett's t-test, with 5% significance level.

Results

Transfected cell binding and activation characteristics

Peptides were purified to >93% purity by HPLC, and masses measured by mass spectrometry matched predicted molecular weights (Table 1). Each peptide was characterized using cyclic AMP stimulation and radioligand displacement studies on transfected CHO-K1 cells, denoted wtGIPR and wtGLP-1R. GIP_{1-14OH} based analogs were only tested on wtGIPR cells, as GIP_{1-14OH} does not stimulate cAMP production in non-transfected CHO cells or wtGLP-1R cells (Hinke et al., 2001).

Table 1
Predicted and expected molecular masses of synthetic peptides

Peptide	Predicted Mass	Measured Mass
GIP(1–42OH)	4984.3	4984.7
GIP(1–14OH)	1567.8	1569.3
[Ala^{1}]GIP(1–14OH)	1475.7	1475.2
[Ser^{2}]GIP(1–14OH)	1583.8	1583.2
[Ala^{3}]GIP(1–14OH)	1509.7	1511.4
[Ala^{4}]GIP(1–14OH)	1581.8	1586.8
[Ala^{5}]GIP(1–14OH)	1537.7	1533.2
[Ala^{6}]GIP(1–14OH)	1491.7	1487.7
[Ala^{7}]GIP(1–14OH)	1525.7	1526.1
[Ala^{8}]GIP(1–14OH)	1551.8	1546.6
[Ala^{9}]GIP(1–14OH)	1523.7	1523.3
[Ala^{10}]GIP(1–14OH)	1475.7	1477.8
[Ala^{11}]GIP(1–14OH)	1551.8	1553.6
[Ala^{12}]GIP(1–14OH)	1525.7	1525.4
[Tyr^{13}]GIP(1–14OH)	1659.9	1648.2
[Ala^{14}]GIP(1–14OH)	1507.7	1506.0
[D-Ala^{2}]GIP(1–14OH)	1567.8	1570.6
[Pro^{3}]GIP(1–14OH)	1535.8	1536.0
[Y^{1}A$^{2}\psi$(CH$_2$NH)]GIP(1–14OH)	1552.9	1554.2
GIP(1–11-[BTD]-12–14OH)	1766.8	1769.5
GIP$_{1\text{-}30NH2}$	3552.0	3553.3
GLP-1$_{[7-36]NH2}$	3297.5	ND*
CH1	3497.0	3502.2
CH2	3352.0	3360.5
CH3	3449.0	3451.0
CH4	3472.0	3476.3
CH5	3424.0	3426.3

*Not determined (purchased from Bachem, Torrance, CA).

Under the described conditions, wtGIPR cells normally produced 2.01 ± 0.07 fmol/1000 cells of cyclic AMP in the basal state, and were maximally stimulated by GIP (at 10 nM) to produce 328.4 ± 25.1 fmol/1000 cells. Similarly, wtGLP-1R cells had a basal cAMP level of 2.74 ± 0.32 fmol/1000 cells, and GLP-1 stimulated production to a maximum of 126.2 ± 14.1 fmol/1000 cells. The difference in maximal cAMP production does not reflect greater potency of GIP than GLP-1 or different receptor expression levels of these cells. These stable cell lines have been well characterized, and while basal cAMP and EC_{50} values are relatively constant, maximal cAMP generally varies between 100 and 400 fmol/1000 cells, but is consistent for several weeks to months from passages of a given freezer stock (Gelling, 1998). Studies presented here examine cAMP production because it is by this signaling pathway that GIP is thought to exert it's positive effects on the pancreatic beta cell (Kashima et al., 2001; McIntosh et al., 1996), however, it should be emphasized that GIP may activate additional cAMP independent pathways to mediate its physiological effects in vivo. In binding studies, approximately 8% of ^{125}I-GIP bound to wtGIPR cells and an equivalent percentage of ^{125}I-GLP-1 bound to wtGLP-1R cells. Non-specific binding values for the two cell lines were 1.2% and 1.7% respectively.

Binding displacement of ^{125}I-GIP by synthetic GIP_{1-14} analogs

In order to elucidate key residues contained within amino acids 1–14 of GIP conferring biological activity, an alanine scan was performed. Also because DP IV degradation inactivates native GIP (Hinke et al., 2002), and cleaves GIP_{1-14} to GIP_{3-14} (K. Kühn-Wache, 1999, unpublished data), three DP IV-resistant analogues were generated; DP IV resistance was confirmed by mass spectrometry (K. Kühn-Wache, 2001, unpublished data). Binding displacement curves were performed for all peptides, and full curves are presented for selected peptides in Fig. 1A. Substitution of any residue of the 1–14 primary sequence resulted in significantly reduced binding affinity/ability to displace ^{125}I-GIP_{1-42}, with the notable exception of [Tyr^{13}]GIP_{1-14OH} (N.B. GIP normally has alanines in position 2 and 13, thus these

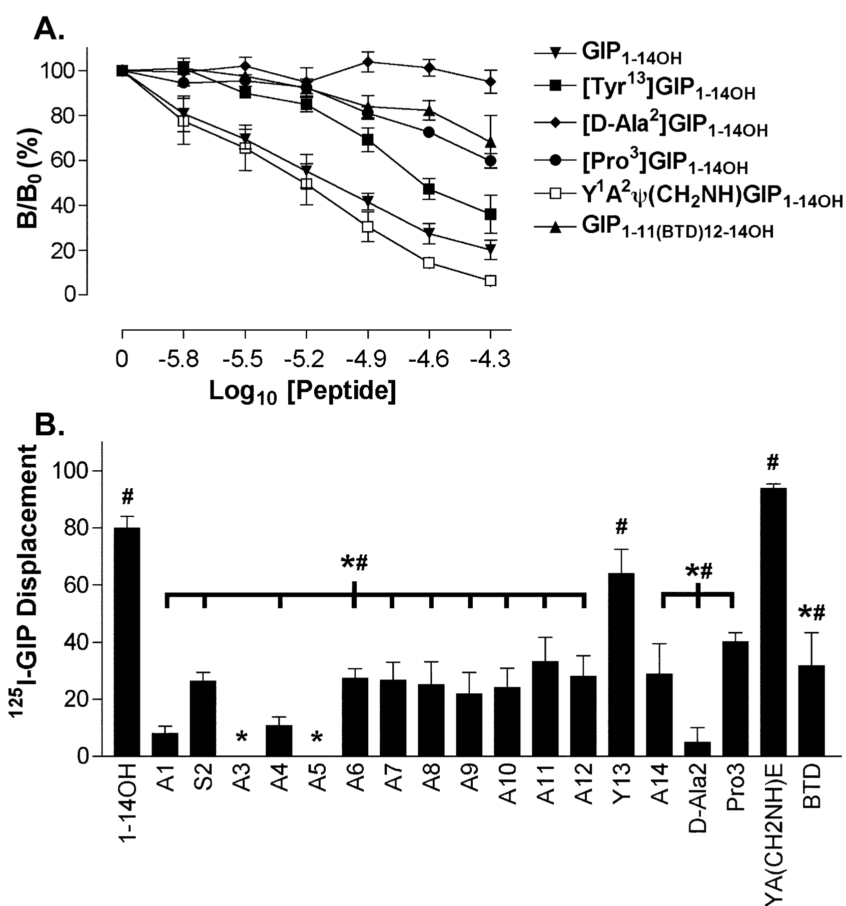

Fig. 1. Competition-binding of modified GIP_{1-14} analogues on wtGIPR cells. (A) Concentration-dependent displacement of ^{125}I-GIP_{1-42} binding by GIP_{1-14OH} and substituted peptides. (B) Displacement of GIP tracer by 50 μM peptide. Peptides are a series of GIP analogues based on amino acids 1–14 of the primary sequence with single amino acid substitutions (to Ala {A}, Ser {S} or Tyr {Y}, where indicated), modified/substituted N-terminal amino acids (D-Ala^2, Pro^3), a reduced scissile bond between amino acids 2 (A) and 3 (E; Glu), or introduction of a synthetic β-turn mimetic (BTD; beta-turn dipeptide) beween residues 11 and 12. Data represent the mean ± S.E.M. of ≥3 experiments (# = significantly non-zero, * = significantly different from GIP_{1-14OH}; $P < 0.05$).

residues were replaced with those found in glucagon). Residues which may be particularly important for favoring structure for binding or forming contacts with residues of the GIP receptor are suggested by the complete loss of binding potency for GIP_{1-14OH} analogues with Ala^1, Ala^3, Ala^4 or Ala^5 substitutions (Fig. 1B). [D-Ala^2] substitution of GIP_{1-14OH} was not well tolerated, whereas reduction of the scissile bond between residues 2 and 3 ([Tyr^1-$Ala^2\psi(CH_2NH)$]GIP_{1-14OH} gave a similar degree of displacement compared to native sequence, and [L-Pro^3]GIP_{1-14OH} and $GIP_{1-11(BTD)12-14OH}$ were somewhat less potent (Fig. 1).

Activation of the GIP receptor by synthetic GIP_{1-14} analogs

Results in Fig. 2A show that GIP_{1-14OH} was indeed a full agonist of the GIP receptor (EC_{50} = 780 ± 190 nM) although exhibiting significantly lower potency than full length GIP (EC_{50} = 239 ± 13 pM), while still producing similar maximal cAMP accumulation in wtGIPR cells (GIP_{1-42}: 312 ± 18 fmol/1000 cells; GIP_{1-14OH}: 323 ± 50 fmol/1000 cells; Fig. 2). DP IV-resistant GIP_{1-14OH} analogues,

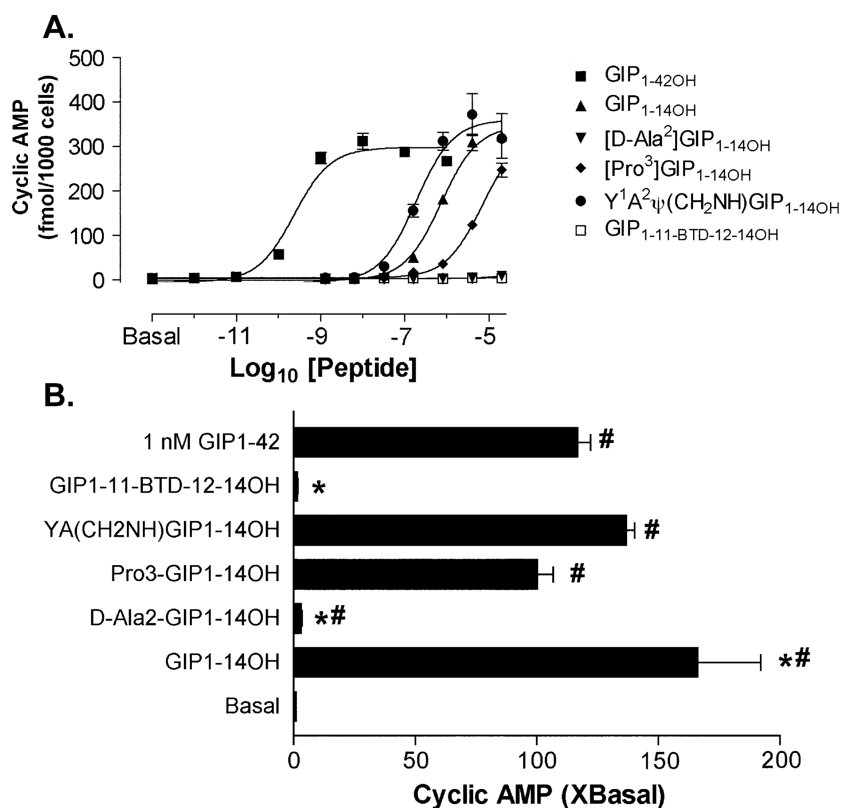

Fig. 2. Concentration-response curves of intracellular cyclic AMP production in wtGIPR cells by N-terminally modified GIP_{1-14} peptides. (A) Full concentration-response curve for N-terminally modified analogues. (B) Fold-basal responses for maximal cAMP production values measured. Data represent mean ± S.E.M. of at least 3 independent experiments (# = significantly greater than basal, * = significantly different than 1 nM GIP_{1-42}, $P < 0.05$).

[Pro^3]GIP_{1-14OH} and [Tyr^1-$Ala^2\psi(CH_2NH)$]GIP_{1-14OH} were also able to stimulate maximal cAMP production, the former being less and the latter more efficacious compared to GIP_{1-14OH} (EC_{50} values: [Pro^3]GIP_{1-14OH}, 7.01 ± 0.75 μM {$p > 0.05$}, and [Tyr^1-$Ala^2\psi(CH_2NH)$]GIP_{1-14OH}, 190 ± 25 nM {$p< 0.05$}; Fig. 2A). Reversing chirality of position 2 or inducing a β-turn completely ablated the ability to activate the GIP receptor. A concentration of 20 μM was the highest tested for alanine scanning mutants of GIP_{1-14OH} (Fig. 3). At this concentration, almost all residues appeared to be important for signaling, although this may partially reflect binding displacement, which was weak for many of the peptides tested as high as 50 μM (Fig. 1). Only Ala^1, Ser^2, Ala^3, Ala^4 and Tyr^{13} substituted peptides were able to increase cAMP levels above basal ($P < 0.05$), although Ala^1 and Ala^3 were extremely weak. 20 μM native GIP_{1-14OH}, as well as Ser^2 and Tyr^{13} substituted analogues were more potent than 1 nM GIP_{1-42} when examining cAMP production in wtGIPR cells (Fig. 3).

GIP and GLP-1 peptide chimeras delineate receptor binding and activation domains

Chimeric peptides were used to distinguish the roles of GIP_{1-14} and GIP_{19-30} for receptor binding and activation, when replaced by or linked with a region of the GLP-1 sequence. When GIP's N-terminal and mid-region (C-terminal) bioactive domains were linked by small intervening sequences of GLP-1 (CH4 and CH5), IC_{50} values were only shifted 16- to 27-fold compared to the binding of native GIP to the GIP receptor (Fig. 4). In contrast, when only the C-terminus (CH1 and CH3) or N-terminus (CH2) of GIP was present, IC_{50} values were in the low μM range (Fig. 4, Table 2), comparable to IC_{50} values obtained for N- and C-terminal fragments in isolation (Hinke et al., 2001). GIP receptor stimulated cyclic AMP data also corroborated prior results from GIP fragment analysis (Hinke et al., 2001), CH2 yielded similar cAMP production to GIP_{1-14}, CH1 and CH3 also stimulated cAMP production to a level similar to that with GIP_{19-30}. Hence, the added GLP-1 sequence in these chimeras did not contribute to the binding affinity or bioactivity of these peptides at the GIP receptor. The bioactivity of the two domains of GIP appears to be additive, since activation by CH4 or CH5

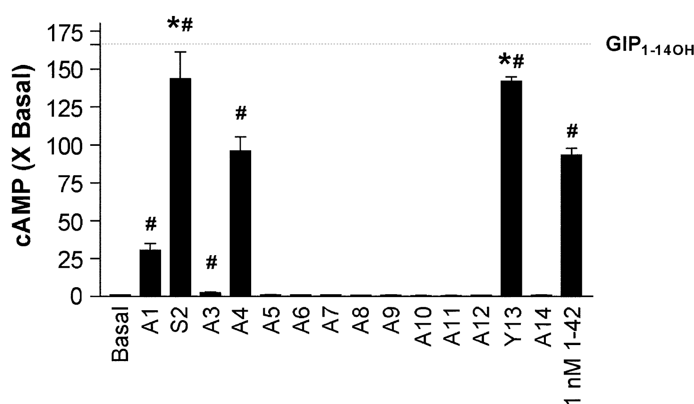

Fig. 3. Cyclic AMP production by 20 μM of substituted GIP_{1-14} peptides. An alanine scan of GIP_{1-14OH} was performed (where endogenous alanine residues were found, they were substituted with residues found at the corresponding positions of glucagon). Peptides were tested over a concentration range between 0 and 20 μM. Only the highest concentration is shown. (# = significantly greater than basal, * = significantly greater than 1 nM GIP_{1-42}, $P < 0.05$).

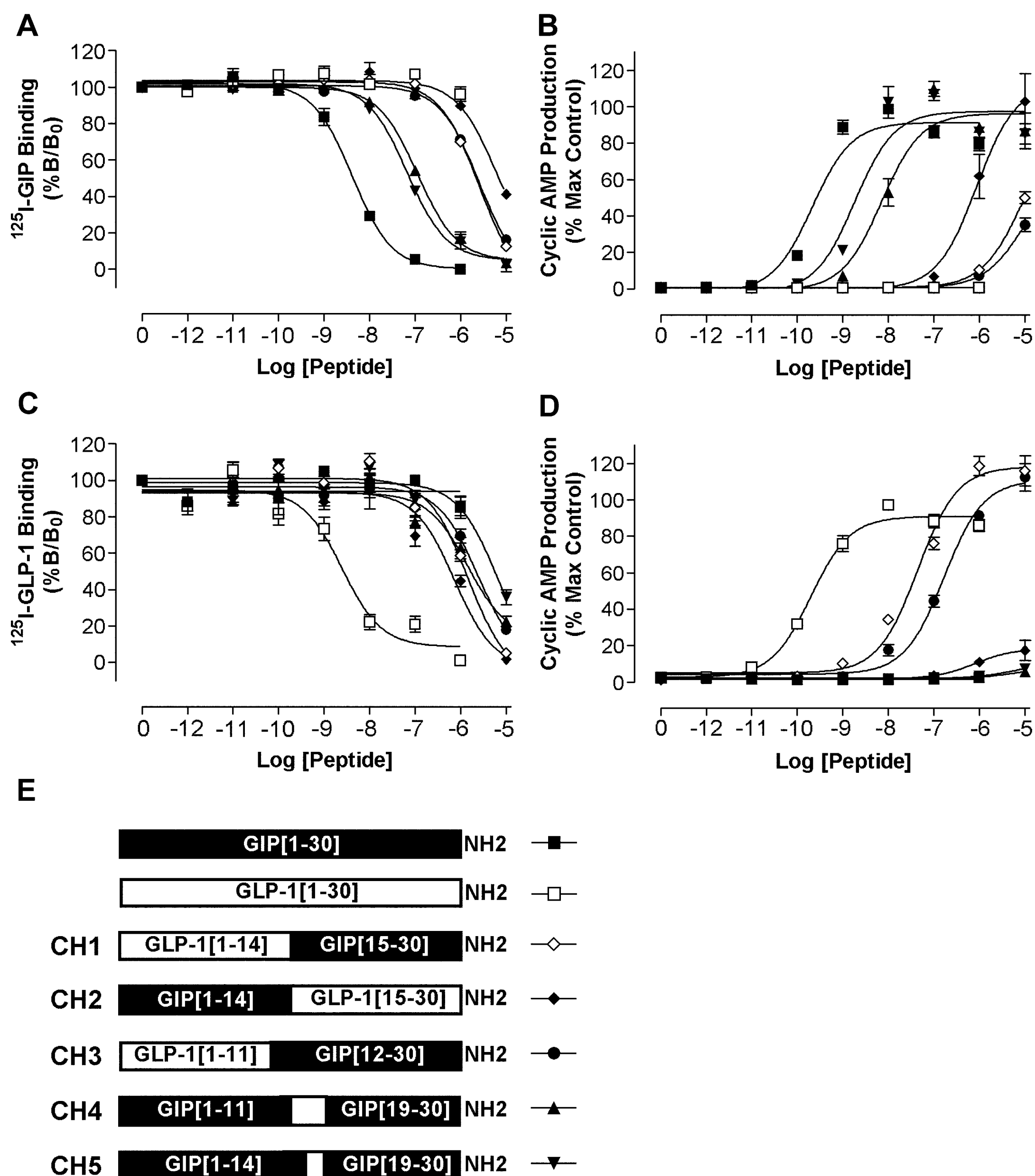

Fig. 4. Comparison of GIP/GLP-1 chimeras on wtGIPR and wtGLP-1R cells. (A) Binding displacement of ^{125}I-GIP and (B) cyclic AMP production by GIP (■), GLP-1 (□) and GIP/GLP-1 chimeras (◇, CH1; ♦, CH2; , CH3; ▲, CH4; ▼, CH5) using wtGIPR cells. (C) Binding displacement of ^{125}I-GLP-1 and (D) cyclic AMP production by GIP, GLP-1 and GIP/GLP-1 chimeras using wtGLP-1R cells. Data represent mean ± S.E.M. of at least three independent experiments. See text for methodological details and Table 2 for IC_{50} and EC_{50} values. (E) A diagrammatic representation of peptide chimeras (see methods for precise amino acid sequences).

Table 2
Bioactivity and binding parameters of synthetic peptides

GIP Receptor

Peptide	Cyclic AMP EC_{50}	Max cAMP (%)	Binding IC_{50}	Max Displacement
GIP	222 ± 24 pM	100	4.14 ± 0.48 nM	100
GLP-1	–	0.61 ± 0.08[a]	ND	ND
CH1	10.6 ± 7.7 μM	50.0 ± 3.3	2.50 ± 0.12 μM	87.3 ± 1.0
CH2	988 ± 149 nM	103 ± 15	6.16 ± 0.75 μM	58.8 ± 1.0
CH3	9.34 ± 0.94 μM	35.1 ± 3.7	2.58 ± 0.14 μM	83.8 ± 1.5
CH4	7.70 ± 1.72 nM	110 ± 4	114 ± 10 nM	96.4 ± 1.2
CH5	1.86 ± 0.27 nM	106 ± 3	66.4 ± 2.6 nM	97.9 ± 3.4

GLP-1 Receptor

Peptide	Cyclic AMP EC_{50}	Max. cAMP (%)	Binding IC_{50}	Max. Displacement
GIP	–	2.37 ± 0.61[a]	ND	ND
GLP-1	223 ± 41 pM	100	3.02 ± 0.73 nM	100
CH1	45.1 ± 5.3 nM	119 ± 5	1.20 ± 0.22 μM	94.7 ± 1.3
CH2	745 ± 200 nM	17.3 ± 5.6	594 ± 179 nM	98.3 ± 1.7
CH3	ND	112 ± 7	4.94 ± 2.60 μM	82.1 ± 2.5
CH4	ND	5.72 ± 0.48	1.07 ± 0.32 μM	77.3 ± 2.9
CH5	ND	7.41 ± 0.40	376 ± 201 nM	64.2 ± 4.1

ND, Not Determined.

Statistics: The EC_{50} and IC_{50} values for all peptide chimeras were significantly different relative to native hormone controls ($p < 0.05$). CH1, CH2, and CH3 were unable to displace 100% of ^{125}I-GIP binding on wtGIPR cells at 10 μM; CH3, CH4 and CH5 were unable to displace 100% of ^{125}I-GLP-1 binding on wtGLP-1R cells at 10 μM ($p < 0.05$). At 10 μM, CH1 and CH3 were unable to stimulate maximal cAMP production on wtGIPR cells, nor could CH2, CH4, or CH5 on wtGLP-1R cells ($p < 0.05$). Neither GIP nor GLP-1 were able to stimulate significant cAMP above basal or displace appropriate tracer on non-cognate receptors.

[a] Basal cAMP was 0.70 ± 0.05 %Max in GIPR cells and 2.30 ± 0.72% Max in GLP-1R cells.

resulted in greater cyclic AMP production in wtGIPR cells than other chimeras or bioactive peptide fragments.

Discussion

Glucose-dependent insulinotropic polypeptide has been considered as a potential future therapeutic option for type II diabetes mellitus treatment based on positive results in diabetic rodent models. The main drawbacks of such a therapy are that this peptide hormone is rapidly inactivated in vivo by serum proteases and the cost and difficulty in synthesizing the bioactive molecule (30–42 amino acids in length). Several studies have suggested that the first drawback can be overcome by modification of the N-terminus of GIP to render the molecule resistant to cleavage, and this confers greater in vivo potency. So far, human studies investigating effects of DPIV resistant GIP analogs on glucose homeostasis have been extremely limited, and therefore the actual benefit of this approach cannot be fully ascertained (reviewed in (Hinke et al., 2003a,b; Gault et al., 2003)). Fragment analysis has sought to minimize the bioactive domains of GIP, simplifying the synthesis of the molecule, improving yield, and reducing cost

(Hinke et al., 2001; Morrow et al., 1996). Although smaller bioactive GIP fragments have been identified (12−14 amino acids in length), at present, their biological effects are much weaker than native full length hormone. The current report is the first study to attempt to improve the efficacy of small molecular weight ligands of the GIP receptor through identification of residues within this sequence which are tolerant or intolerant to substitution. Alanine scanning of GIP_{1-14} aimed to identify these residues, and additional analogs with N-terminal modifications were tested to circumvent proteolytic inactivation by serum enzymes.

Sequential alanine substitution of the GIP_{1-14} amino acid sequence established the sensitivity of this peptide to structural perturbations (Figs. 1 and 3). Only replacement of amino acids 2 (Ala^2Ser) and 13 ($Ala^{13}Tyr$) with those found in glucagon failed to produce dramatic reductions in receptor binding and activation. Given the high selectivity of glucagon receptors for glucagon, and GIP receptors for GIP, the results indicate that residues 2 and 13 are unlikely to be important ligand-receptor contacts, unless the domain responsible for receptor selectivity resides in the C-termini of the peptide hormones. While this may be partially true, GIP_{1-14} was not able to activate transfected glucagon or GLP-1 receptors (S. Hinke, 2000, unpublished results), suggesting intrinsic selectivity of this hormone fragment. There is some evidence allowing discrimination of residues important for receptor binding from those for activation. $[Tyr^{13}]GIP_{1-14}$ bound and activated the GIP receptor with similar efficacy to native GIP_{1-14}, however, $[Ser^2]GIP_{1-14}$ displayed comparatively lower displacement of ^{125}I-GIP, with similar receptor activation. Similarly, the only other peptides from the alanine scanning experiment to show significant cAMP stimulation were those substituted in position 1 and 3, although these peptides also showed little radioligand displacement, compared to peptides which were unable to activate the receptor (Figs. 1 and 3).

Alanine scanning of the bioactive PTH_{1-14} N-terminus identified residues 3, 10, 11, 12, 13 and 14 as being tolerant to substitution (i.e. identity not important for receptor activation) (Luck et al., 1999). Comparison of Fig. 3 to the data of Luck et al (1999), clearly indicates that although both GIP_{1-14} and PTH_{1-14} are bioactive, it is the N-terminus of GIP_{1-14} which is more tolerant to substitution, whereas the C-terminus of PTH_{1-14} which may be altered without negatively affecting receptor activation. Although GIP_{1-14} and PTH_{1-14} share no conserved sequence, their receptors are structurally related, and conserved residues are within their putative hormone binding domains (as well as in their transmembrane segments) (Harmar, 2001). Presently, little is known regarding the molecular mechanisms by which GIP binds to the GIP receptor, other than that the primary binding domain of the receptor is confined to its large extracellular N-terminal domain, and that residues necessary for ligand binding are divided between GIP_{1-14} and GIP_{19-30} (Gelling et al., 1997; Hinke et al., 2001; Morrow et al., 1996). Future studies should clarify precise amino acid contact sites via computational and experimental means. Alanine scanning of the entire primary sequence of GLP-1 revealed specific residues, which if replaced, dramatically reduced receptor binding and activation (Gallwitz et al., 1994; Adelhorst et al., 1994); the data for GLP-1 show clear dissimilarities to the data presented here for GIP_{1-14}, however, as these reports examined full length hormone, it is likely that single site modification would only reveal critical residues, as it is presumed that GLP-1 also interacts with its receptor via multiple contact sites (thus identified residues may likely be biased towards those needed for secondary structure rather than direct receptor interaction). Alanine scanning of the entire sequence of GIP_{1-42} or GIP_{1-30} would serve the same purpose, however it is unclear if these results would aid the development of small molecular weight ligands.

Extensive molecular characterization of PTH_{1-14} by multi-site substitution and modification suggested it was possible to produce small peptide fragments with improved potency (Shimizu et al., 2000; Shimizu et al., 2001a; Shimizu et al., 2001b; Carter and Gardella, 2001). Four modified GIP_{1-14} peptides were generated for the current study (Figs. 1 and 2). Introduction of a reduced peptide bond between Ala^2 and Glu^3 resulted in a peptide with improved receptor potency compared to GIP_{1-14}, while at the same time this modification conferred DP IV resistance. Hence, systematic screening of modified GIP_{1-14} peptides will likely produce potent peptide ligands with increased agonistic or antagonistic activity in vivo. Notably, substitution of [D-Ala] in position 2, a modification conferring enzyme resistance to DP IV, was found to be well tolerated in the context of longer peptides based on GIP_{1-30} or GIP_{1-42} (Hinke et al., 2002; Kühn-Wache et al., 2000), whereas it resulted in a peptide with poor potency when introduced to GIP_{1-14}. Conversely, DP IV resistant $[Pro^3]GIP_{1-30}$ and $[Tyr^1\text{-}Ala^2\Psi(CH_2NH)]GIP_{1-30}$ were shown to be weak partial agonists of the GIP receptor with high binding affinity (Kühn-Wache et al., 2000; Hinke et al., 2003a,b), thus allowing them to act as receptor antagonists under certain conditions, yet similar analogs based on GIP_{1-14} displayed similar in vitro cAMP stimulating ability to unmodified GIP_{1-14} (Fig. 2). Hence, due to the potential interaction between the N- and C-termini of GIP which affects biological activity (Hinke et al., 2001), it may not be possible to predict potency of smaller GIP mimetics where this interaction is not present, and thus empirical testing must be conducted.

Previous studies employing chimeric GIP/GLP-1 peptides used RINm5F insulinoma cells, which express both GIP and GLP-1 receptors (Gallwitz et al., 1996; Gallwitz et al., 1995). Hence, only receptor binding data from these experiments are useful, since ^{125}I-GIP and ^{125}I-GLP-1 bind specifically to their respective receptors. The current bioactivity studies clearly show that the peptide chimeras are capable of cross-reacting between receptors when studied independently from one another. The studies of Gallwitz and co-workers on the binding of $GIP_{[1-11]}$/GLP-$1_{[12-22]}$/$GIP_{[23-31]}$ indicated that a domain conferring high affinity binding to the GLP-1R resided in the core residues 12–22, since only marginally higher binding affinity was exhibited with GLP-$1_{[1-22]}$/$GIP_{[23-31]}$ (Gallwitz et al., 1996). However, since CH1 and CH2 were both able to displace greater than 94% of ^{125}I-GLP-1 binding (Fig. 4, Table 2), it can be concluded that binding of GLP-1 also involves both N- and C-terminal domains. Fragment analysis of GLP-1 indicated that the N-terminus of the molecule was important for receptor recognition and activation using RINm5F insulinoma cells (Gallwitz et al., 1990). Corroborative results using N- and C-terminally substituted GLP-1 analogs and peptide chimeras with the N-termini of members of the secretin/glucagon/VIP superfamily combined with the C-terminus of GLP-1 were demonstrated by Xiao et al. (2001) using GLP-1R/CHO (R7) cells and mouse bioassay. The current studies also demonstrate the importance of the N-terminus of GLP-1, but additionally conclude that GLP-1 has only a single bioactive domain (in contrast to GIP, which contains two), since only CH1 and CH3, peptides bearing 14 and 11 N-terminal GLP-1 amino acids (respectively) demonstrated potent cAMP stimulation on the GLP-1 receptor (Fig. 4, Table 2). Results shown here on chimeric peptides are in support of recently published structure-activity relationships of GIP, whereby analogs of N- and C-terminal bioactive domains of GIP separated by helical linkers retained biological potency (Manhart et al., 2003).

In conclusion, structure-activity relationships among the peptide hormones of the glucagon/secretin family demonstrate both parallels and differences. Some hormones can be shown to have short N-terminal domains, such as GIP_{1-14}, $parathormone_{1-14}$ and $secretin_{1-13}$ that can exhibit independent bioactivity as short peptides while GLP-$1_{[7-20NH2]}$ and $glucagon_{1-14}$ are inactive at their cloned receptors (Hinke et al., 2001). However, the bioactive domains of the latter two hormones appear to be

primarily confined to the N-termini in their full length moieties, even if they are inactive as short peptides. Unique to GIP and glucagon, is a second bioactive domain contained in their C-terminal 19–30 or 19–29 sequences, respectively. In the case of GIP, it appears that this C-terminal bioactive domain acts in an additive fashion with the N-terminal domain, whereas for glucagon, "mini-glucagon$_{19-29}$", an endogenously occurring molecule, appears to have contrasting biological effects to the full length hormone (Dalle et al., 1999). The data presented in the current report should aid the development of small molecular weight ligands of the GIP receptor and possibly potent hybrid peptides capable of activating both of the incretin receptors, perhaps resulting in peptide mimetics with greater glucose-lowering actions in vivo.

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