RESEARCH PAPER

N-terminally and C-terminally truncated forms of glucose-dependent insulinotropic polypeptide are high-affinity competitive antagonists of the human GIP receptor

L S Hansen[1,3,4], A H Sparre-Ulrich[1,3], M Christensen[4], F K Knop[3,4], B Hartmann[2,3], J J Holst[2,3] and M M Rosenkilde[1]

[1]Department of Neuroscience and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark, [2]NNF Center for Basic Metabolic Research, University of Copenhagen, Copenhagen, Denmark, [3]Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark, and [4]Center for Diabetes Research, Gentofte Hospital, University of Copenhagen, Copenhagen, Denmark

Correspondence
Mette M. Rosenkilde, Department of Neuroscience and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark.
E-mail: rosenkilde@sund.ku.dk

BACKGROUND AND PURPOSE

Glucose-dependent insulinotropic polypeptide (GIP) affects lipid, bone and glucose homeostasis. High-affinity ligands for the GIP receptor are needed to elucidate the physiological functions and pharmacological potential of GIP *in vivo*. GIP(1–30)NH$_2$ is a naturally occurring truncation of GIP(1–42). Here, we have characterized eight N-terminal truncations of human GIP(1–30)NH$_2$.

EXPERIMENTAL APPROACH

COS-7 cells were transiently transfected with human GIP receptors and assessed for cAMP accumulation upon ligand stimulation or competition binding with ^{125}I-labelled GIP(1–42), GIP(1–30)NH$_2$, GIP(2–30)NH$_2$ or GIP(3–30)NH$_2$.

KEY RESULTS

GIP(1–30)NH$_2$ displaced ^{125}I-GIP(1–42) as effectively as GIP(1–42) (K$_i$ 0.75 nM), whereas the eight truncations displayed lower affinities (K$_i$ 2.3–347 nM) with highest affinities for GIP(3–30)NH$_2$ and GIP(5–30)NH$_2$ (5–30)NH$_2$. Only GIP(1–30)NH$_2$ (E$_{max}$ 100% of GIP(1–42)) and GIP(2–30)NH$_2$ (E$_{max}$ 20%) were agonists. GIP(2- to 9–30)NH$_2$ displayed antagonism (IC$_{50}$ 12–450 nM) and Schild plot analyses identified GIP(3–30)NH$_2$ and GIP(5–30)NH$_2$ as competitive antagonists (K$_i$ 15 nM). GIP(3–30) NH$_2$ was a 26-fold more potent antagonist than GIP(3–42). Binding studies with agonist (^{125}I-GIP(1–30)NH$_2$), partial agonist (^{125}I-GIP(2–30)NH$_2$) and competitive antagonist (^{125}I-GIP(3–30)NH$_2$) revealed distinct receptor conformations for these three ligand classes.

CONCLUSIONS AND IMPLICATIONS

The N-terminus is crucial for GIP agonist activity. Removal of the C-terminus of the endogenous GIP(3–42) creates another naturally occurring, more potent, antagonist GIP(3–30)NH$_2$, which like GIP(5–30)NH$_2$, was a high-affinity competitive antagonist. These peptides may be suitable tools for basic GIP research and future pharmacological interventions.

Abbreviations

B$_{max}$, maximal binding capacity; DPP-4, dipeptidyl peptidase 4; DR, dose ratios; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; MW, molecular weight; 7TM, seven-transmembrane

Tables of Links

TARGETS
GPCRs
GIP receptor

LIGANDS
GIP, glucose-dependent insulinotropic polypeptide
GIP(6–30)NH$_2$
GIP(7–30)NH$_2$
GIP(1-30)NH$_2$
GIP(3-42)
GIP(3-30)
GIP(5-30)

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*., 2013).

Introduction

Glucose-dependent insulinotropic polypeptide (GIP) is a 42-amino acid peptide hormone secreted from the K cells in the proximal small intestine. It appears to influence lipid, bone and glucose homeostasis (Kreymann *et al.*, 1987; Baggio and Drucker, 2007). Together with glucagon-like peptide-1 (GLP-1), GIP is responsible for the incretin effect that accounts for about 60% of the insulin response after ingestion of glucose (Nauck *et al.*, 1986). Loss of the incretin effect is an early characteristic of type 2 diabetes (Holst *et al.*, 2011), and exploitation of GLP-1 physiology has led to the development of two novel drug classes (GLP-1 analogues and dipeptidyl peptidase 4, DPP-4, inhibitors) for the treatment of type 2 diabetes (Knop *et al.*, 2009). The GLP-1 receptor antagonist exendin(9–39) has been an essential research tool in the elucidation of GLP-1 biology both *in vivo* and *in vitro* (Thorens *et al.*, 1993; Al-Sabah *et al.*, 2014) and thereby also for the successful development of GLP-1-based pharmacological therapy.

Several antagonists of the GIP receptor have been reported with the majority of native GIP(1–42) truncations (Pederson and Brown, 1976; Moroder *et al.*, 1978; Maletti *et al.*, 1986; Sandberg *et al.*, 1986; Rossowski *et al.*, 1992; Gallwitz *et al.*, 1993; Wheeler *et al.*, 1995; Morrow *et al.*, 1996; Tseng *et al.*, 1996; Gelling *et al.*, 1997; Tseng *et al.*, 1999; Hinke *et al.*, 2001; Gault *et al.*, 2002a; Gault *et al.*, 2002b; Hinke *et al.*, 2004; Deacon *et al.*, 2006; Irwin *et al.*, 2006a; Irwin *et al.*, 2006b; Jorgensen *et al.*, 2007; Parthier *et al.*, 2007; Kerr *et al.*, 2011). An overview of the literature of GIP(1–42) truncations is presented in Supporting Information Table 1. Antibodies and a low MW compound with antagonistic properties for the GIP receptor have also been reported (Nakamura *et al.*, 2012; Ravn *et al.*, 2013). Importantly, none of these have been shown to act under human physiological conditions. Thus, an effective GIP receptor antagonist suitable for human studies remains to be found.

Like the GLP-1 receptor, the GIP receptor belongs to B1 family of seven-transmembrane (7TM) receptors, signalling through the Gα_s pathway (Brubaker and Drucker, 2002), but several other intracellular messengers have also been reported in relation to GIP receptor activation (Kubota *et al.*, 1997; Zhong *et al.*, 2000; Mentlein, 2009; Campbell and Drucker, 2013). The GIP receptor is found in the pancreatic islet cells,

osteocytes, cells in the gastrointestinal tract, adipocytes, and in the brain (Usdin *et al.*, 1993). GIP has glucose stabilizing effects, potentiating glucose-stimulated insulin secretion combined with glucagonotropic properties at low plasma glucose concentrations (Christensen *et al.*, 2011). In humans, GIP may have anabolic effects on adipose tissue under hyperinsulinemic and hyperglycemic conditions (Asmar *et al.*, 2014) and inhibits postprandial bone resorption (Nissen *et al.*, 2014). In agreement with these findings, GIP receptor knockout mice are resistant to diet-induced obesity, and crossing this mouse with the leptin mutant mouse, which is a known mouse model for obesity due to hyperphagia, led to reduction of weight gain by 23% (Miyawaki *et al.*, 2002). Furthermore, GIP receptor knockout mice showed decreased bone mineral density, decreased bone quality including strength and cortical thickness and increased osteoclast activity (Gaudin-Audrain *et al.*, 2013; Mieczkowska *et al.*, 2013).

The peptide secreted, GIP(1–42), is a substrate for the enzyme DPP-4, which is a protease involved in the inactivation of numerous bioactive peptides (Mentlein, 1999). DPP-4 cleaves the peptide bond following a proline or alanine at the penultimate position of the N-terminus of peptides and, thus, in the case of GIP(1–42), produces the metabolite GIP(3–42) (Mentlein *et al.*, 1993; Deacon, 2004). This degradation product is a GIP receptor antagonist in supraphysiological concentrations (Gault *et al.*, 2002c, Deacon *et al.*, 2006). Moreover, other truncated GIP variants have been reported to behave as antagonists including GIP (6–30)NH$_2$ and GIP(7–30)NH$_2$, both demonstrating high-affinity and potent inhibition *in vitro* and decreasing GIP-stimulated insulin secretion *in vivo* in rodents (Tseng *et al.*, 1996; Gelling *et al.*, 1997; Hinke *et al.*, 2001). DPP-4-resistant palmitoylated human GIP(3–30), with a C-terminal 9-amino acid extension originating from exendin(1–39), was recently presented as a GIP receptor antagonist in both *in vitro* and mouse studies resulting in weight loss and improved insulin sensitivity (Pathak *et al.*, 2015a). These findings are all consistent with the canonical paradigm for receptor activation in the 7TM B1 family, which includes the GIP receptor, demonstrating a pivotal role for the N-terminus of the ligands.

The C-terminally truncated and fully bioactive proGIP product, peptide GIP(1–30)NH$_2$, has been shown to be

formed in mice and in human intestine and pancreatic islets (Fujita *et al.*, 2010a; Fujita *et al.*, 2010b). Due to the lack of potent antagonists for the human GIP system and to the promising results in different species with truncated variants of GIP(1–42) (Pederson and Brown, 1976; Moroder *et al.*, 1978; Maletti *et al.*, 1986; Sandberg *et al.*, 1986; Rossowski *et al.*, 1992; Gallwitz *et al.*, 1993; Wheeler *et al.*, 1995; Morrow *et al.*, 1996; Tseng *et al.*, 1996; Gelling *et al.*, 1997; Tseng *et al.*, 1999; Hinke *et al.*, 2001; Gault *et al.*, 2002a; Hinke *et al.*, 2004; Deacon *et al.*, 2006; Irwin *et al.*, 2006a; Parthier *et al.*, 2007; Kerr *et al.*, 2011) summarized in Supporting Information Table 1, we decided to focus on N-terminally truncated forms of the backbone of the full agonist human GIP(1–30)NH$_2$. GIP(1–30)NH$_2$ and eight N-terminally truncated variants, including GIP(2–30)NH$_2$, GIP(3–30)NH$_2$, GIP(4–30)NH$_2$, GIP(5–30)NH$_2$, GIP(6–30)NH$_2$, GIP(7–30)NH$_2$, GIP(8–30)NH$_2$ and GIP(9–30)NH$_2$ (Figure 1A), were tested as agonists and antagonists with respect to cAMP accumulation and competitive binding. To further explore the role of the C-terminus, GIP(3–42) was characterized in parallel with GIP-(3–30)NH$_2$. Importantly, we found two novel, potent, competitive and high-affinity antagonists among the GIP(1–30)NH$_2$ truncations, and furthermore, we found that the absence of the C-terminus is important for potent GIP receptor antagonism.

Methods

Cell line and transfection
COS-7 cells were grown in 10% CO$_2$ and at 37°C in DMEM 1885 supplemented with 10% FBS, 2 mM glutamine, 180 units·mL^{-1} penicillin and 45 g·mL^{-1} streptomycin. Transfection of COS-7 cells was performed using the calcium phosphate precipitation method with chloroquine addition as previously described (Kissow *et al.*, 2012).

cAMP assay
COS-7 cells (30 000 cells per well) were seeded in 96-well plates 1 day before transfection with human GIP receptor cDNA. 2 days after transfection, the cells were washed once with HEPES buffered saline and incubated with HEPES buffered saline and 0.5 mM IBMX for 30 min at 37°C. The various truncated GIP variants were added to the cells and incubated for 30 min at 37°C in order to test for intrinsic activity. To test for antagonism of a given GIP variant, the cells were preincubated for 10 min at 37°C with the GIP analogue followed by 20 min of incubation with GIP(1–42). The potency of the antagonists was determined from dose–response curves of the antagonist in the presence of a constant concentration of the GIP(1–42) corresponding to 50–80% of the maximal cAMP accumulation response (E$_{max}$) of GIP(1–42). For Schild analysis, various antagonist concentrations were added 10 min prior to GIP(1–42) dose–response curves. After ligand incubation, the HitHunter™ cAMP XS assay (an enzyme fragment complementation-based assay; DiscoveRx, Birmingham, UK) was carried out according to the manufacturer's instructions. All experiments were made in duplicates and repeated at least 3 times. Luminescence was measured by PerkinElmer™ EnVision 2104 Multilabel Reader. In brief, the cells were lysed in the wells, the enzyme fragment-cAMP-antibody, an enzyme fragment, and the enzyme substrates were added followed by 1 h incubation at
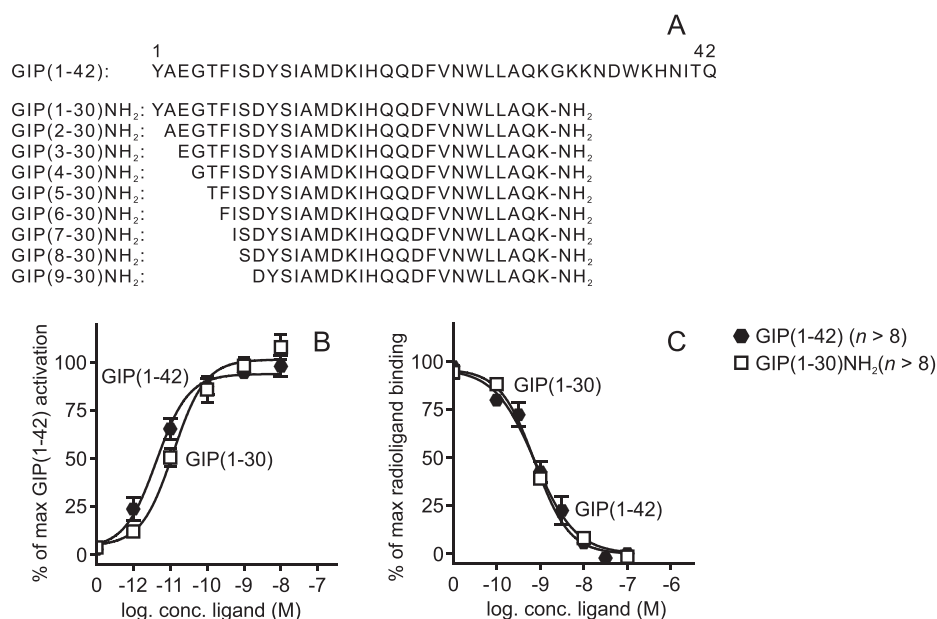

Figure 1
GIP(1–30)NH$_2$ is a high-affinity full agonist of the GIP receptor. (A) Alignment of the truncated GIP variants. Human native GIP(1–42) sequence was acquired from *National Center for Biotechnology Information* Protein Database. The GIP receptor was transiently transfected in COS-7 cells and used for functional (B) and binding studies (C). (B) cAMP accumulation assay with increased concentrations of native GIP(1–42) and GIP (1–30)NH$_2$, mean ± SEM, $n = 8$. (C) Competitive binding with the ^{125}I-GIP(1–42) radioligand displaced by GIP(1–42) and GIP(1–30)NH$_2$. Data shown are means ± SEM, $n = 13$.

room temperature on shaker tray. The other enzyme fragment was added to the wells and incubated for 4 h on shaker tray followed by measurements of luminescence. The ligand-induced cAMP competed with the binding of antibody to the first enzyme fragment and left the 2 fragments to fuse. The enzyme complex hydrolyzed the substrates and yielded luminescence. The number '*n*' refers to individual experiments with separate transfections although from same cell line.

Competitive binding assay

COS-7 cells were seeded in 96-well plates 1 day after transfection with human GIP receptor cDNA. The number of cells seeded per well was selected to result in 5–10% specific binding of the added radioactive ligand (1000–5000 cells per well). Two days after transfection, cells were used for competition binding for 3 h at 4°C to inhibit receptor internalization using 6–10 pM per well of ^{125}I-GIP(1–42), ^{125}I-GIP(1–30)NH$_2$, ^{125}I-GIP(2–30)NH$_2$ or ^{125}I-GIP(3–30)NH$_2$ as well as relevant amounts of unlabelled ligands in 50 mM HEPES buffer and pH 7.4, supplemented with 0.5% (*w/v*) BSA. After incubation for 3 h at 4°C, the cells were washed twice in ice-cold binding buffer and lysed using 200 mM NaOH with 1% SDS for 30 min. Non-specific binding was determined as the binding of radioligand to untransfected cells. All determinations were made in duplicates and all experiments repeated at least 3 times. The samples were analysed for radioactivity using a Wallac Wizard 1470 Gamma Counter (GMI Inc., Ramsey, MN, USA). The number '*n*' refers to individual experiments with separate transfections although from same cell line.

Data analysis

IC$_{50}$, EC$_{50}$ and K$_d$/K$_i$ values were determined by nonlinear regression. These, as well as maximal binding capacity (B$_{max}$) values and Schild plot analysis, were carried out with the GraphPad Prism 6.0 software (GraphPad, San Diego, CA, USA) and Microsoft Excel™. Statistical analyses of two parameters (unpaired *t*-tests) and multiple comparisons (one-way ANOVAs) were also performed with GraphPad Prism 6.0. The calculations of B$_{max}$ and K$_i$ values were based on the formula for one class of binding sites in homologous competition binding studies and the Cheng–Prusoff formula respectively (DeBlasi *et al.*, 1989). K$_d$ is the *KD* determined by homologous receptor binding. Dose ratios (DR) for the Schild analyses were based on the potency shift of the GIP(1–42) dose–response curve in absence or presence of a fixed antagonist concentration (DR = EC$_{50}$ in presence of antagonist/EC$_{50}$ in absence of antagonist). Schild plots were performed with log (DR-1) (ordinate) and log(antagonist concentration) (abscissa) to estimate the slopes and K$_i$ values (Lazareno and Birdsall, 1993).

Materials

Wild-type human GIP receptor cDNA was purchased from Origene™, Rockville, MA, USA (SC110906), and cloned into the pCMV-Script vector. Human native GIP(1–42) was purchased from Bachem™, Bubendorf, Switzerland (H5645). All truncated GIP peptides were synthesized by Caslo™, Lyngby, Denmark, and based on the human GIP sequence. Porcine GIP(3–42) was custom synthesized by PolyPeptide

Laboratories (WolfenBüttel, Germany). The peptides had a purity of more than 95% by HPLC analyses and an MS controlled MW. ^{125}I-labelled native GIP(1–42) was purchased from PerkinElmer Life Sciences, Skovlunde, Denmark (NEX402025UC). Human GIP(1–30)NH$_2$, GIP(2–30)NH$_2$ and GIP(3–30)NH$_2$ were ^{125}I labelled using the standard stoichiometric chloramine T method as described previously (Holst and Bersani, 1991). The labelled peptides were purified by HPLC.

Results

GIP(1–30)NH$_2$ is a full GIP receptor agonist with high affinity equal to native GIP(1–42)

To establish the role of the C-terminus for agonism in the human GIP system, we first measured cAMP responses to human GIP(1–42) and human GIP(1–30)NH$_2$ in transiently transfected COS-7 cells expressing the human GIP receptor (Figure 1). GIP(1–30)NH$_2$ was a full agonist on the GIP receptor with a high potency (EC$_{50}$) of 11.2 pM [logEC$_{50}$ −10.95 ± 0.11], compared with the 6.0 pM [logEC$_{50}$ −11.21 ± 0.16] of GIP(1–42), and with the same efficacy as GIP(1–42), consistent with earlier studies (Fujita *et al.*, 2010a; Gault *et al.*, 2011). Binding studies were performed with ^{125}I-GIP(1–42) as the radioligand in the same cellular background. Truncation of the full length GIP(1–42) peptide at the 30-position did not change the affinity to the GIP receptor and, thus, resulted in affinities (IC$_{50}$) of 0.89 and 0.67 nM for GIP(1–30)NH$_2$ and GIP(1–42) respectively (Figure 1). Thus, GIP(1–30)NH$_2$ displayed the same potency, efficacy and affinity for the human GIP receptor as GIP(1–42).

The N-terminus is essential for high-affinity binding

To study the role of the N-terminus of human GIP(1–30) NH$_2$, the affinity of the eight N-terminally truncated peptides was compared with that of GIP(1–30)NH$_2$ in transiently transfected COS-7 cells using ^{125}I-GIP(1–42) as radioligand (Figure 2). Truncation resulted in decreased affinity with a tendency towards length dependency, with a span from 2.3-fold to 347-fold decrease in affinity compared with GIP(1–30)NH$_2$. GIP(3–30)NH$_2$ followed by GIP(5–30)NH$_2$ displayed the highest affinities, while GIP(9–30)NH$_2$ and GIP(6–30)NH$_2$ had more than 300-fold lower affinities compared with GIP(1–30)NH$_2$. Taken together, this emphasizes the importance of the N-terminus for receptor binding.

GIP(2–30)NH$_2$ is a partial agonist, and GIP(3- to 9–30)NH$_2$ are antagonists of the GIP receptor

We measured cAMP accumulation in COS-7 cells, transiently transfected with the human GIP receptor, after incubation with each of the GIP variants (Figure 3). Removal of the first amino acid from GIP(1–30)NH$_2$ created GIP(2–30)NH$_2$, which is a weak partial agonist with an efficacy of 20%
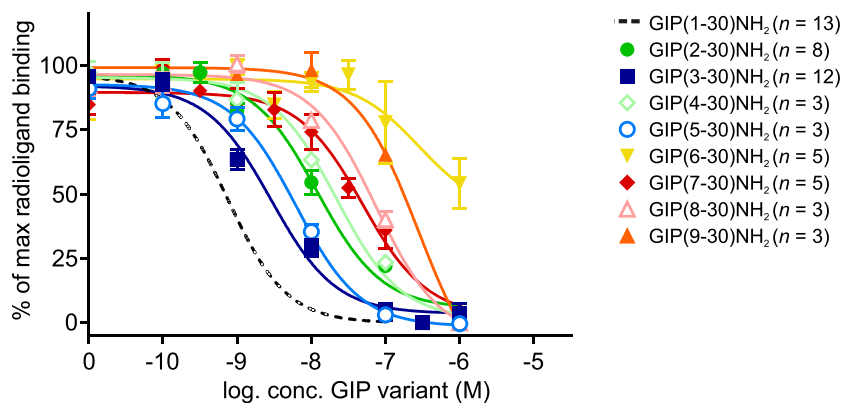
Figure 2

GIP(3–30)NH$_2$ and GIP(5–30)NH$_2$ display the highest affinity among the eight truncated GIP variants. The binding of ^{125}I-GIP(1–42) to transiently transfected COS-7 cells with the GIP receptor was tested in the presence of increasing amounts of GIP(1–30)NH$_2$, GIP(3–30)NH$_2$, GIP(5–30)NH$_2$, GIP(2–30)NH$_2$, GIP(4–30)NH$_2$, GIP(7–30)NH$_2$, GIP(8–30)NH$_2$, GIP(9–30)NH$_2$ or GIP(6–30)NH$_2$. Data shown are means ± SEM.
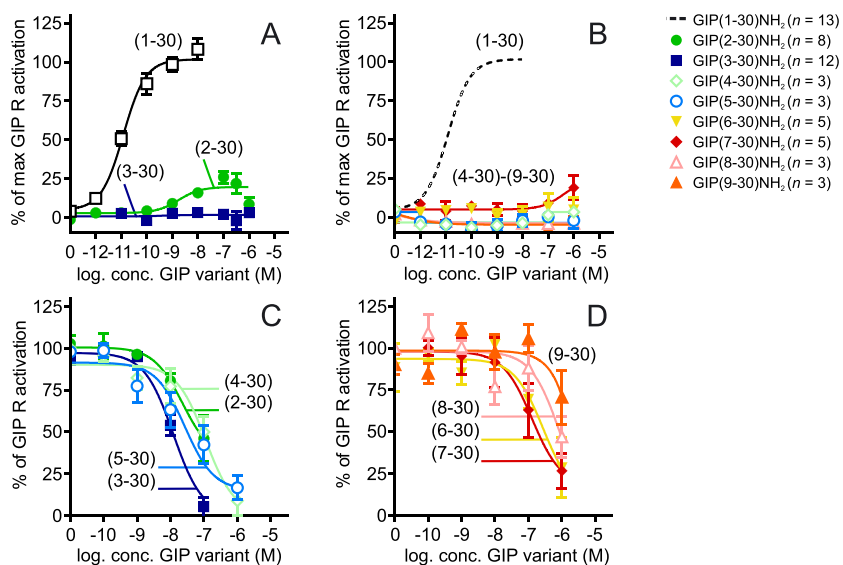

Figure 3

GIP(3–30) and GIP(5–30) are the most potent GIP receptor antagonists. cAMP accumulation in transiently transfected COS-7 cells with GIP receptor. (A, B) Ligand dose–response stimulated cAMP accumulation. Data shown are means ± SEM. (C, D) Dose–response curves of antagonists inhibited a constant amount of native GIP(1–42) corresponding to 50–80% of max receptor activation. Data shown are means ± SEM.

compared with GIP(1–30)NH$_2$ and a potency of 3.7 nM [logEC$_{50}$ −8.43 ± 0.33, $n = 8$], which is >3000-fold lower than GIP(1–30)NH$_2$. Removal of the second amino acid completely eliminated intrinsic activity (Figure 3A), a pattern that was also seen for the remaining truncations (Figure 3B). To determine whether the inactive forms had antagonistic properties, increasing concentrations of the GIP variants were added to a submaximal (50–80%) activation by GIP(1–42). All were able to inhibit the cAMP response induced by GIP(1–42) (Figure 3C and D). The most potent antagonists were GIP (3–30)NH$_2$ and GIP(5–30)NH$_2$ with IC$_{50}$ of 11.8 and 11.9 nM, respectively (Table 1), in agreement with their high binding affinities. Similar to the binding studies, the shortest

GIP variant, GIP(9–30)NH$_2$, had the lowest antagonistic potency with a 38-fold right shift compared with GIP-(3–30)NH$_2$.

GIP(3–30)NH$_2$ and GIP(5–30)NH$_2$ are competitive antagonists

A Schild analysis was performed for the four most potent antagonists, in addition to the previously described antagonists GIP(6–30)NH$_2$ and GIP(7–30)NH$_2$ (Gelling *et al.*, 1997; Tseng *et al.*, 1999; Hinke *et al.*, 2001). This analysis determines whether an antagonist acts competitively and is illustrated by the Schild plot. A straight line with a Hill slope of 1.0 indicates competitive antagonism. The antagonists

Table 1

Affinity and inhibitory potencies of the GIP variants

	Competitive binding				cAMP accumulation		
	$logIC_{50} \pm SEM$	K_i (nM)	Fold	n	$logIC_{50} \pm SEM$	IC_{50} (nM)	n
GIP(1–30)NH$_2$	−9.05 ± 0.02	0.89	1.0	13	—	—	—
GIP(2–30)NH$_2$	−7.85 ± 0.04	14.3	16	10	−7.66 ± 0.1	21.7	4
GIP(3–30)NH$_2$	−8.63 ± 0.04	2.3	2.6	12	−7.93 ± 0.04	11.8	6
GIP(4–30)NH$_2$	−7.67 ± 0.02	21.5	24	3	−6.97 ± 0.4	108	4
GIP(5–30)NH$_2$	−8.23 ± 0.05	5.9	6.6	3	−7.92 ± 0.4	11.9	4
GIP(6–30)NH$_2$	−6.46 ± 0.09	347	391	10	−6.47 ± 0.6	342	4
GIP(7–30)NH$_2$	−7.58 ± 0.08	26	30	9	−6.86 ± 0.4	137	7
GIP(8–30)NH$_2$	−7.10 ± 0.04	79	89	3	−6.88 ± 0.5	133	5
GIP(9–30)NH$_2$	−6.51 ± 0.08	307	345	3	−6.35 ± 0.6	450	4

The data shown are the IC$_{50}$ values from the binding studies (Figure 2) with the fold change relative to the affinity of GIP(1-30)NH$_2$ and the cAMP accumulation studies (Figure 3) with antagonist properties.

were added in various constant concentrations to the dose–response curves of GIP(1–42) (Figure 4). All six antagonists were able to right shift the GIP(1–42) dose–response curve with no changes in efficacy. However, only GIP(3–30)NH$_2$ and GIP(5–30)NH$_2$ act as pure competitive antagonists

judged by a straight line with a slope of 1 (inserts in Figure 4). These 2 ligands displayed slopes of 0.93 ± 0.02 and 1.1 ± 0.04, respectively, while the slopes for GIP(2–30)NH$_2$, GIP(4–30)NH$_2$, GIP(6–30)NH$_2$ and GIP(7–30)NH$_2$ were 0.49 ± 0.14, 0.75 ± 0.02, 0.38 ± 0.13 and 0.17 ± 0.03 respectively (Figure 4).
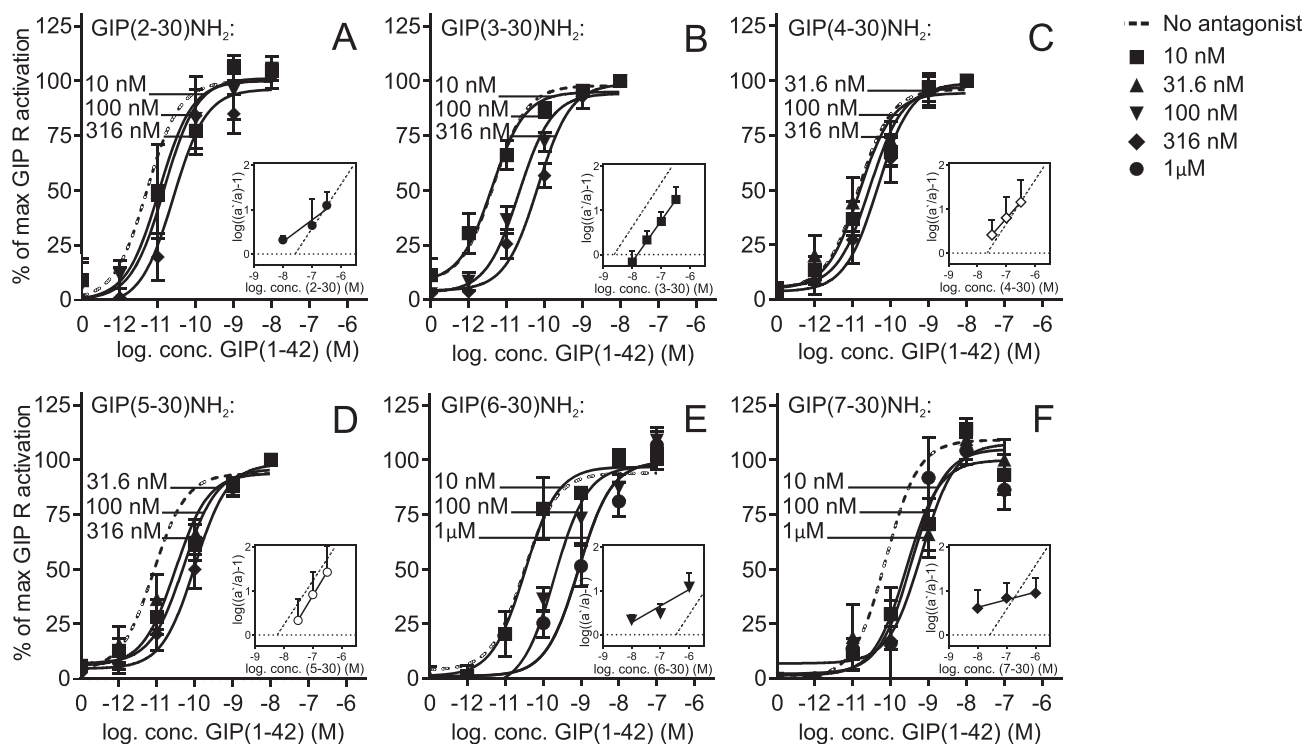
Figure 4

Of the six antagonists, only GIP(3–30)NH$_2$ and GIP(5–30)NH$_2$ are the competitive antagonists. GIP(1–42)-mediated cAMP accumulation assayed for transiently transfected COS-7 cells with the GIP receptor in the absence of and with increasing concentrations of either GIP(2–30)NH$_2$, GIP(3–30)NH$_2$, GIP(4–30)NH$_2$, GIP(5–30)NH$_2$, GIP(6–30)NH$_2$ or GIP(7–30)NH$_2$. The corresponding Schild plot is presented with a comparison with a linear regression with a slope of 1.0 and the X-intercept of K$_i$ for the antagonist. GIP(2–30)NH$_2$ (A, $n = 4$), GIP(3–30)NH$_2$ (B, $n = 6$), GIP(4–30)NH$_2$ (C, $n = 3$), GIP(5–30)NH$_2$ (D, $n = 4$), GIP(6–30)NH$_2$ (E, $n = 3$) and GIP(7–30)NH$_2$ (F, $n = 4$). Data shown are means ± SEM.

The lack of ability to compete equally with the agonist could indicate an allosteric component in the antagonistic properties of these ligands. The X-intercept or pA_2-value of the Schild plot corresponds to the affinity constant of the antagonist if the Hill slope equals 1. For the 2 competitive antagonists $GIP(3–30)NH_2$ and $GIP(5–30)NH_2$, the pA_2-values were 14.9 and 15.2 nM, respectively, thus in the same range as the K_i determined from the binding studies (2.3 and 5.9 nM respectively). In summary, this analysis identified $GIP(3–30)NH_2$ and $GIP(5–30)NH_2$ as high-affinity competitive GIP receptor antagonists.

The functionalities of the ligands reflect the binding properties

The N-terminal truncations of $GIP(1–30)NH_2$ had a span in affinities (Ki) from 1 to 350 nM (Figure 2 and Table 1) and, concomitantly, displayed different pharmacodynamics with both competitive and non-competitive antagonistic properties (Figures 3, 4). To further analyse the receptor interaction of these variants, we performed homologous competitive binding studies with ^{125}I-GIP(1–30)NH$_2$, ^{125}I-GIP(2–30)NH$_2$ and ^{125}I-GIP(3–30)NH$_2$ as radioligands (representing a full agonist, a partial agonist and a competitive antagonist

respectively). The K_d values for $GIP(1–30)NH_2$, $GIP(2–30)NH_2$ and $GIP(3–30)NH_2$ obtained from the homologous binding experiments (Figure 5 and Table 2) were in the same range as the K_i values obtained in the heterologous binding experiments using ^{125}I-GIP(1–42) as radioligand (Table 1). However, minor, yet significant, changes were observed upon a closer look at the affinities, as higher affinities were observed when $GIP(1–30)NH_2$ and $GIP(2–30)NH_2$ competed with their own iodinated versions (homologous binding), compared with when they competed with ^{125}I-GIP(1–42) (heterologous binding) ($P = 0.012$ and $P = 0.0031$, respectively; Figure 5). Thus, the lack of C-terminus decreased the ability of GIP $(1–30)NH_2$ and $GIP-(2–30)NH_2$ to compete with the full-length agonist $GIP(1–42)$ for the GIP receptor. In contrast, the N-terminally truncated antagonist $GIP(3–30)NH_2$ was able to displace the homologous radioligand with the same affinity as the full agonist ^{125}I-GIP-(1–42) radioligands ($P = 0.45$; Figure 5). The B_{max} was calculated from the homologous binding studies (DeBlasi et al., 1989) and uncovered significantly more binding sites for the antagonists compared with the two agonists (Figure 5), which illustrates the general property of antagonists to stabilize several inactive receptor confirmations, while agonists preferentially bind to the active confirmation(s) (Rosenkilde et al., 1994).
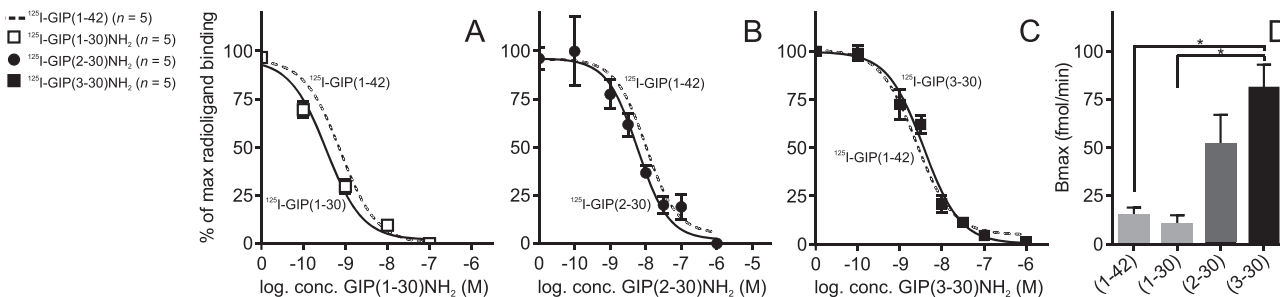

Figure 5

The homologous binding curves are equivalent to the heterologous binding studies with native ^{125}I-GIP(1–42) radioligand. (A–C) Transiently transfected COS-7 cells with the GIP receptor were used in homolog competitive binding studies with ^{125}I-GIP(1–30)NH$_2$, ^{125}I-GIP(2–30)NH$_2$ and ^{125}I-GIP(3–30)NH$_2$ and heterologous binding studies with ^{125}I-GIP(1–42), mean ± SEM. (D) B_{max} values calculated from the homologous binding curves for GIP(1–42), GIP(1–30)NH$_2$, GIP(2–30)NH$_2$ and GIP(3–30)NH$_2$. Data shown are means ± SEM, $n = 5$. Significance determined by multiple comparisons (one-way ANOVA).

Table 2

Homologous and heterologous binding studies

	^{125}I-GIP(1–30)NH$_2$					^{125}I-GIP(2–30)NH$_2$					^{125}I-GIP(3–30)NH$_2$				
	log (IC$_{50}$)	±SEM	IC$_{50}$ (nM)	Fold change GIP(1–30)NH$_2$	n	log (IC$_{50}$)	±SEM	IC$_{50}$ (nM)	Fold change GIP(1–30)NH$_2$	n	log (IC$_{50}$)	±SEM	IC$_{50}$ (nM)	Fold change GIP(1–30)NH$_2$	n
GIP(1–42)NH$_2$	−9.24	0.19	0.58	1.9	3	−9.36	0.087	0.43	0.9	3	−8.97	0.0015	1.07	0.6	3
GIP(1–30)NH$_2$	−9.52	0.16	0.30	1.0	5	−9.32	0.482	0.48	1.0	3	−8.78	0.063	1.7	1.0	3
GIP(2–30)NH$_2$	−7.59	0.18	26	84.3	4	−8.57	0.28	2.7	10.5	5	−8.11	0.065	7.7	4.6	4
GIP(3–30)NH$_2$	−8.35	0.071	4.4	14.5	4	−9.12	0.20	0.76	1.6	3	−8.47	0.12	3.4	2.0	5
GIP(6–30)NH$_2$	−5.97	0.066	1065	3502	5	−6.47	0.28	340	707	4	−6.43	0.26	370	223	4
GIP(7–30)NH$_2$	−7.43	0.25	37	120.9	5	−7.54	0.23	29	60.6	5	−7.68	0.16	21	12.7	5

The data shown are the IC50 values from homologous and heterologous binding studies (Figure 5, 6) with the fold change relative to the affinity of GIP(1-30)NH$_2$.

The binding properties were further elucidated through heterologous binding studies with ^{125}I-GIP(1–30)NH$_2$, ^{125}I-GIP(2–30)NH$_2$ and ^{125}I-GIP(3–30)NH$_2$ displaced by GIP (1–42), GIP(1–30)NH$_2$, GIP(2–30)NH$_2$ and GIP(3–30)NH$_2$ and the previously described GIP(6–30)NH$_2$ and GIP(7–30)NH$_2$ (Supporting Information Figure 1 and Table 2). Again, the agonists GIP(1–30)NH$_2$ and GIP(1–42) displaced the agonist radioligand ^{125}I-GIP(1–30)NH$_2$ most efficiently, while their affinities decreased in competition with the radiolabeled antagonists. The opposite was observed for the antagonists that displaced the partial agonist ^{125}I-GIP(2–30)NH$_2$ and the antagonist ^{125}I-GIP(3–30)NH$_2$ radioligand with highest affinities. Thereby, when looking at apparent affinities, the largest effects of increased truncation of GIP(1–30)NH$_2$ were observed with the agonist as radioligand with >3000-fold decrease in affinity of GIP(7–30)NH$_2$ compared with GIP (1–30)NH$_2$ measured with agonist radioligand and only 223-fold decrease when measured with ^{125}I-GIP(3–30)NH$_2$ as radioligand. This pattern was observed for all four antagonists (Table 2).

The C-terminal part of GIP acts as a negative regulator of the antagonist action of GIP(3–42)

The identification of GIP(3–30)NH$_2$ as the most potent antagonist prompted us to compare it with GIP(3–42) in order to directly determine the impact of the C-terminal amino acids 31 through 42. We also included the porcine GIP(3–42), representing a low-potent antagonist on the human GIP receptor *in vitro*, with no ability to antagonize porcine GIP (1–42)-mediated insulin secretion in pigs at physiological concentrations (Deacon *et al.*, 2006). Porcine GIP(3–42) has arginine in position 18 and serine in position 34, whereas the human sequence has histidine and asparagine respectively (Figure 6). Like GIP(3–30)NH$_2$ (Figure 3), neither of the GIP(3–42) variants had any intrinsic agonistic activity

in cAMP accumulation assay (data not shown, $n = 3$), but both were able to antagonize submaximal (50–80%) human GIP(1–42)-induced activation (Figure 6). Importantly, human GIP(3–42) was remarkably less potent than human GIP(3–30) NH$_2$ (26-fold lower potency; Figure 6), and 1 μM of this resulted in only 4.9-fold shift in the dose–response curve of human GIP(1–42) compared with 247-fold for human GIP-(3–30)NH$_2$ (Figure 6). The porcine variant displayed higher potency compared with human GIP(3–42), yet not as high as human GIP(3–30)NH$_2$. Thus, the C-terminus has a functional role as its absences improve the antagonistic properties in GIP(3–30)NH$_2$ compared with GIP(3–42).

Discussion and conclusion

In this pharmacological study of truncated human GIP variants, we evaluated GIP(2- to 9–30)NH$_2$ as antagonists of the human GIP receptor. GIP(1–30)NH$_2$ was a full agonist with affinity equal to the full-length native GIP(1–42), and GIP(2–30)NH$_2$ acted as a low-potent partial agonist and an antagonist. Both the affinities and the antagonistic properties appeared to decrease by the truncation length. GIP(3–30)NH$_2$ and GIP(5–30)NH$_2$ were high-affinity competitive antagonists of the human GIP receptor, whereas the rest displayed lower affinities and more complex antagonism. Additionally, when comparing human GIP(3–30)NH$_2$ with GIP(3–42), the C-terminus (position 31–42) was found to dampen the antagonist properties of GIP(3–42).

Many truncated GIP variants have been presented, but GIP(3–30)NH$_2$ and GIP(5–30) NH$_2$ seem superior

GIP has been truncated into numerous variants and characterized in various species (Figure 7 and Supporting Information Table 1).
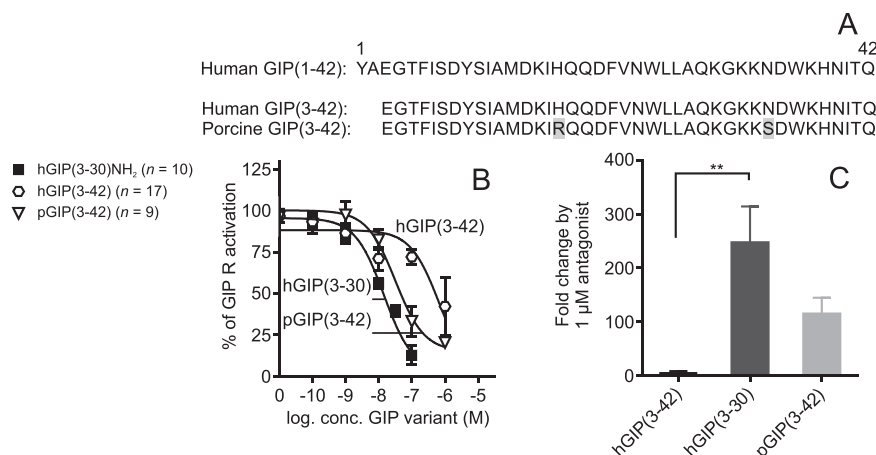

Figure 6

Human GIP(3–42) is a low-potent antagonist on the human GIP receptor compared with human GIP(3–30)NH$_2$ and porcine GIP(3–42). (A) Alignment of the truncated GIP variants. Human and porcine GIP(1–42) sequence was acquired from *National Center for Biotechnology Information Protein Database*. The human GIP receptor transiently transfected in COS-7 cells was used in cAMP accumulation assay (B and C). (B) Dose–response curves of antagonists inhibited a constant amount of native GIP(1–42) corresponding to 50–80% of max receptor activation. Data shown are means ± SEM. (C) Fold change in potency of human GIP(1–42) by 1 μM antagonist. The bars display the mean fold change ± SEM, $n = 4$. Significance determined by multiple comparisons (one-way ANOVA).
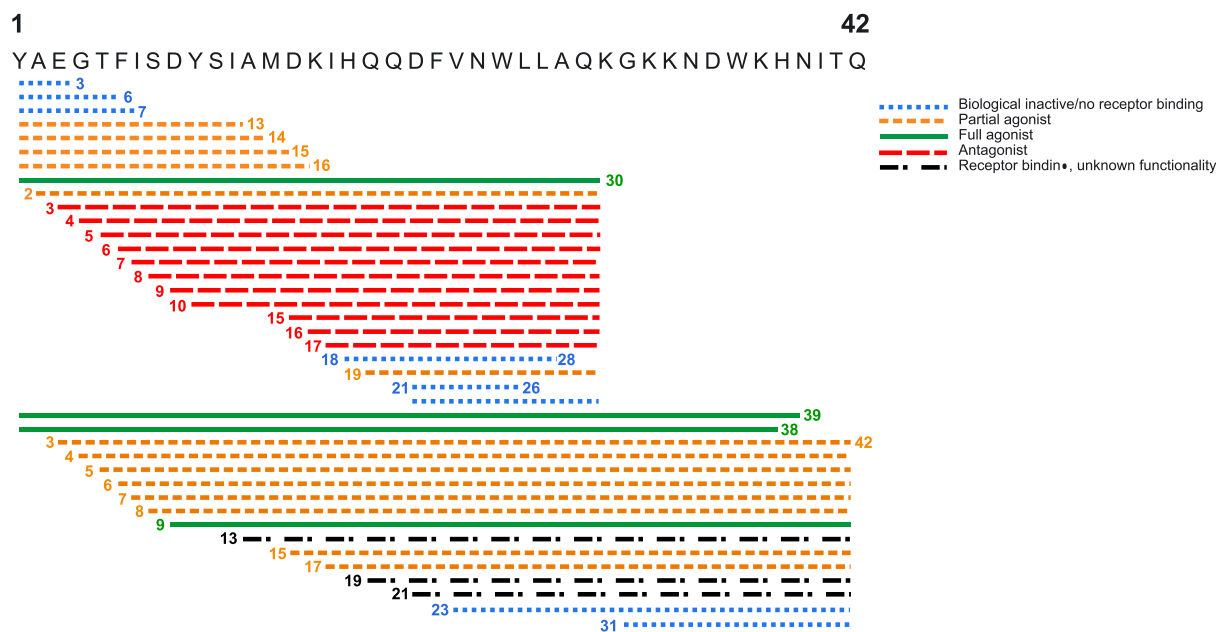
Figure 7

Overview of truncated GIP variants studied *in vitro* or *in vivo* in rodents. The truncated GIP variants of various species are biological inactive/no receptor binding, partial agonist, full agonist, antagonist or receptor binding but unknown functionality. This figure is based on the studies referred in supplementary Table 1.

None of the previous studies have focused on the pure human system, and recently, the species differences have been shown to influence the pharmacology fundamentally (Sparre-Ulrich *et al.*, 2015). Several studies have established GIP(1–30)NH_2 as a full agonist with equal potency to GIP(1–42) primarily using the rat system (Rossowski *et al.*, 1992; Gallwitz *et al.*, 1993; Morrow *et al.*, 1996; Tseng *et al.*, 1996; Hinke *et al.*, 2001). In line with this, we find GIP(1–30)NH_2 to be a high-affinity full agonist of the human GIP receptor. Eight N-terminally truncated GIP(1–30)NH_2 variants have previously been published: GIP(6–30)NH_2, GIP(7–30)NH_2, GIP(10–30), GIP(15–30)NH_2, GIP(16–30)NH_2, GIP(17–30)-NH_2, GIP(19–30)NH_2 and GIP(21–30)NH_2 (Maletti *et al.*, 1986; Wheeler *et al.*, 1995; Morrow *et al.*, 1996; Tseng *et al.*, 1996; Gelling *et al.*, 1997; Tseng *et al.*, 1999; Hinke *et al.*, 2001; Gault *et al.*, 2002a). The porcine GIP(6–30)NH_2 has been presented as a high-affinity and potent rat GIP receptor antagonist [IC_{50} of 3.1 nM in competition with porcine ^{125}I-GIP(1–42)] with 58% reduction in human GIP(1–42)-induced cAMP response by 100 nM porcine GIP(6–30)NH_2 (Gelling *et al.*, 1997). In a recent study, GIP(6–30)Cex-K^{40}[Pal] inhibited human GIP(1–42)-induced cAMP production from the human GIP receptor (IC_{50} 3.1 nM) and insulin release (IC_{50} 49 pM) in a rat beta cell line and enhanced insulin sensitivity and improved glucose tolerance in diabetic mice (Pathak *et al.*, 2015b). On the human GIP receptor, we observed GIP(6–30)NH_2 to have the lowest affinity of the truncated GIP(1–30)NH_2 variants and poor antagonistic properties. This is not in line with previous studies, but the pharmacological differences between rodent and human GIP systems are not yet clarified (Sparre-Ulrich *et al.*, 2015). In contrast to the findings for GIP(6–30)NH_2, our results of the human GIP(7–30)NH_2 are in line with previous studies using the porcine GIP(7–30)NH_2 on the rat GIP receptor (Tseng *et al.*, 1996; Gelling *et al.*, 1997; Tseng

et al., 1999; Hinke *et al.*, 2001). We found GIP(3–30)NH_2 and GIP(5–30)NH_2 to be superior to GIP(6–30)NH_2 and GIP-(7–30)NH_2 and to be the only competitive antagonists. Recently, palmitoylated human GIP(3–30)NH_2 extended with the nine last amino acids from exendin(1–39) was published as a GIP receptor antagonist in mice with significant effects on weight loss and improved glycemic control and insulin sensitivity (Pathak *et al.*, 2015a). Our results from the human receptor are consistent with this. Our systematic truncation approach revealed that the potency and affinity of the antagonists decreased with the length of the truncations (Figures 2, 3, 1, 4, 8) and that amino acids 2–5 in human GIP(1–30)NH_2 are important for potent inhibition. A possible explanation for the decreased antagonist properties of GIP(6- to 9–30)NH_2 could be found in changes of the secondary structure (Figure 8). GIP(1–42) has an α-helix initiated by amino acid Thr-5 as the helix-capping residue (Parthier *et al.*, 2007). Removal of this threonine in GIP(6–30)NH_2 decreases the antagonism (IC_{50}) by 29-fold and the affinity (K_i) by 59-fold, which could be due to an impaired ability to attain this important secondary structure. The fact that GIP(6–30)-NH_2 displays the lowest affinity and potency indicates that not only the disruption of the α-helix initiation by the lack of position 5 [which is also missing in the truncations GIP(7- to 9–30)NH_2] but also the exact position of the truncation and N-terminally exposed amino acids is essential for proper receptor binding.

The N-terminus has a pivotal role in GIP receptor activation

We have previously demonstrated that porcine GIP(3–42) was unable to induce cAMP accumulation (Deacon *et al.*, 2006), underlining the necessity of the first two amino acids of
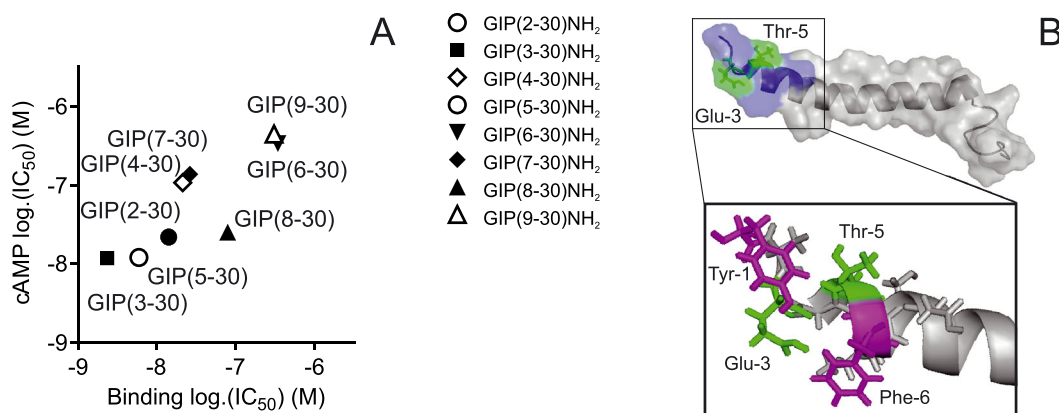
Figure 8

Correlation of affinity and antagonistic potency (A) and structure of the N-terminus of GIP (B). (A) The correlation of calculated affinities (binding log.IC50) and antagonistic potencies (cAMP log.IC$_{50}$) plotted for the eight GIP receptor antagonists (data from Table 1). (B) The published structure (Parthier *et al.*, 2007) of the native GIP(1–42) peptide with amino acids 1–9 in blue, Glu[3] and Thr[5] in green and Tyr[1] and Phe[6] in pink.

GIP(1–42) for *agonistic* properties. Many 7TM receptors of family A (for instance, the chemokine and C5a receptors) and most B1 receptors are thought to be activated in (at least) two steps. Firstly, the ligand binds to the extracellular domain of the receptor, and secondly, the N-terminus of the ligand interacts with transmembrane domains, resulting in receptor activation (Clark-Lewis *et al.*, 1995; Hjorth and Schwartz, 1996; Vilardaga *et al.*, 2011; Thiele and Rosenkilde, 2014). Previously, alanine screening of human GIP identified Tyr[1] to be crucial for GIP receptor-mediated insulin secretion *in vitro* (Alaña *et al.*, 2006). In line with this, NMR and molecular modelling described the interaction of Tyr[1] with multiple amino acids of the transmembrane domains of the GIP receptor (Malde *et al.*, 2007; Yaqub *et al.*, 2010). Ala[2] is thought to both interact with residues of TM3 and participate in hydrogen bonding with Thr[5], which is thought to be important for receptor activation (Yaqub *et al.*, 2010). Accordingly, our study demonstrates that Tyr[1] and Ala[2] are pivotal for GIP receptor activation by the binding to and stabilization of the active conformations as seen by the low efficacy of GIP-(2–30)NH$_2$ and no intrinsic activity of GIP(3–30)NH$_2$ (and of subsequent truncations). The differences in B$_{max}$ values and the radioligand-dependent affinities (agonists compete best with agonists and antagonists with antagonists; Supporting Information Figure 1) underline the structural alterations in the recognition and conformational constraining of active and inactive GIP receptor conformations. A similar phenomenon with changes in apparent affinities determined by the choice of radioligand has previously been described, for example, in the neurokinin system (Rosenkilde *et al.*, 1994) and in the chemokine system (Jensen *et al.*, 2008). Apart from a surprising finding for GIP(19–30)NH$_2$, which seems to be a weak partial agonist *in vivo* and *in vitro* (Figure 7) (Maletti *et al.*, 1986; Morrow *et al.*, 1996; Hinke *et al.*, 2001), the N-terminal truncations of GIP(1–30)NH$_2$ beyond the first two amino acids do not activate the GIP receptor, emphasizing the essential role of the Tyr[1] and Ala[2] for GIP receptor activation in the GIP(1–30)NH$_2$ scaffold (absence of amino acid 31 to 42).

The effects of the C-terminus for receptor activation are unmasked by N-terminal GIP truncations

In a previous study, consecutive N-terminal truncations of human GIP(1–42) were characterized *in vitro* in the rat GIP receptor and in leptin mutant mice (Kerr *et al.*, 2011). In contrast to our findings for the similar truncations in the human GIP(1–30)NH$_2$ scaffold, GIP(3- to 8–42) displayed partial agonism, and GIP(9–42) acted as a full GIP receptor agonist *in vitro*. Only GIP(8–42), with a E$_{max}$ of 10% in terms of cAMP release, was able to antagonize the action of GIP(1–42) *in vivo* with decreased insulin secretion and increased plasma glucose in an acute setting. In line with our results with GIP (5–30)NH$_2$, GIP(5–42) was the most potent antagonist *in vitro*; however, the length-dependent functional changes described here for the human GIP(1–30)NH$_2$ variants were not observed in the truncated GIP(1–42) variants (Kerr *et al.*, 2011). All together, this indicates that the C-terminus is important for intrinsic activity, and even though the activities of GIP(1–30)NH$_2$ and GIP(1–42) are similar, the N-terminally truncated GIP variants reveal that the C-terminus (position 31–42) improves intrinsic activity. It is, however, also possible that species differences may play a role as the rat system was used for the GIP(1–42) truncations and we used the human system for the GIP(1–30)NH$_2$ truncations.

Improved antagonistic properties in the absence of the C-terminus of GIP

Our study demonstrates that although GIP(1–30)NH$_2$ and GIP (1–42) were equally potent and efficient agonists, GIP(1–30) NH$_2$ displayed a threefold lower apparent affinity in competition with ^{125}I-GIP(1–42) as compared with ^{125}I-GIP(1–30) NH$_2$. In contrast, GIP(1–42) displaced both radioligands with the same affinity, which together with the same amount of binding sites (B$_{max}$) for the two agonists indicate largely overlapping binding sites. Interestingly, in terms of (antagonistic) functionality of the N-terminal truncated variants, the

C-terminus had a huge negative impact, as GIP(3–30)NH$_2$ was a 26-fold more potent antagonist compared with GIP(3–42). Taken together, our study combined with previous studies (Gallwitz *et al.*, 1993; Wheeler *et al.*, 1995; Morrow *et al.*, 1996; Tseng *et al.*, 1996; Hinke *et al.*, 2001; Kerr *et al.*, 2011) indicates that the C-terminus (position 31–42) has only a minute impact on the binding and no impact on the agonistic properties of GIP (with preserved N-terminus), whereas it affects the functionality of the N-terminally truncated GIP variants. Thus, the presence of the C-terminus enhances the intrinsic activity (confers agonism) to GIP(3- to 8–42) (Kerr *et al.*, 2011), while the absence of it in our GIP(3- to 9–30)NH$_2$ variants improves the antagonism. In other words, the best antagonism was obtained in the absence of the C-terminus.

GIP(3–30)NH$_2$ may be a suitable research tool for the human GIP system

We identified GIP(3–30)NH$_2$ and GIP(5–30)NH$_2$ as the most potent and efficacious antagonists for the human GIP receptor (Figure 7). They were also the only truncated GIP variants with true competitive properties. As GIP(1–30)NH$_2$ is expressed in human pancreatic α-cells and in specific enteroendocrine cells (Fujita *et al.*, 2010b), the presence of GIP(1–30)NH$_2$ in human plasma is highly likely. Due to the efficient DPP-4 degradation of N-terminally intact GIP, GIP(3–30)NH$_2$ should therefore also be present, similar to what has been observed for GIP(3–42) (Deacon *et al.*, 2006). Given the high and competitive antagonistic potency of GIP(3–30)NH$_2$ and its putative presence in the body, which would decrease the risk of immune reactions, GIP(3–30)NH$_2$ has the potential to be a safe and efficient GIP receptor antagonist suitable for human studies. Our discovery could therefore contribute to a better elucidation of the human GIP system including the determination of GIP's contribution to the human incretin effect and extrapancreatic functions of GIP. Ultimately, the promising characteristics of GIP(3–30)NH$_2$ and GIP(5–30)NH$_2$ can lead to the development of GIP receptor antagonists as a future therapeutic possibility.

Author contributions

L. S. H. contributed to the study design, conducted the experiments and wrote and edited the manuscript. A. H. S.-U. conducted the experiments and reviewed and edited the manuscript. M. C., F. K. K., B. H. and J. J. H. contributed to the study design and reviewed and edited the manuscript. M. M. R. contributed to the study design and wrote and edited the manuscript.

Conflict of interest

Authors declare that they have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.13384

Figure S1 The functionality of the N- and C-terminal truncated ligands reflects the binding properties.

Table S1 The table displays the in vitro and in vivo references to figure 8, overview of truncated GIP variants. Percentages compared to full agonist GIP(1-42) or GIP(1-30) results. Affinity data is in italic. GIP = species sequence of ligand, RL = GIP(1-42) radioligand, GIPR = GIP receptor, B = bovine, P = porcine, R = rat, M = mouse, Ha = hamster, Hu = human, US = unknown species, UC = unknown concentration.