STRUCTURE-FUNCTION STUDIES OF THE GASTRIC INHIBITORY POLYPEPTIDE/GLUCOSE DEPENDENT INSULINOTROPIC POLYPEPTIDE (GIP) RECEPTOR

by

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ABSTRACT

Incretins are hormones released from the gastrointestinal tract into the circulation during and after a meal that potentiate glucose-stimulated insulin secretion. Glucose dependent insulinotropic polypeptide (GIP) is now accepted as the most important incretin and, along with the glucagon-like peptide 1 (GLP-1 (7-36) amide) has therapeutic potential in Non-Insulin Dependent Diabetes Mellitus (NIDDM).

In the present study, a rat islet GIP receptor complementary (c)DNA (GIP-R1) was cloned and characterized. The islet cDNA was identical to that previously identified in a tumor cell line, except for a single nucleotide polymorphism resulting in one amino acid difference (Glu21→Gln21). When expressed transiently in COS-7 cells or stably in CHO-K1 cells the receptor displayed specific high affinity ^{125}I-GIP binding in both saturation (200-300 pM) and competition (IC_{50} 1-8 nM) binding studies, and GIP-dependent increases in cAMP production (EC_{50} 0.069-0.70 nM). Cells expressing GIP-R1 exhibited equivalent signaling in response to porcine and human GIP. In addition, COS-7 cells expressing the GIP-R1 cDNA displayed a biphasic increase in intracellular calcium in response to GIP.

Structure-function studies of GIP showed that the peptide could be truncated at its carboxy-terminal at residue 30 (GIP 1-30 amide) without affecting receptor affinity or efficacy. In contrast, amino-terminal truncation of GIP 1-30 resulted in fragments with reduced affinity and lacking receptor activation activity, that antagonized GIP-stimulated cAMP production. Importantly, GIP 6-30amide bound with nearly identical affinity to GIP but was a potent inhibitor of GIP action *in vitro*, suggesting that this region contains the binding core and that amino-terminal residues are important for receptor activation.

The latter finding is important given that GIP is metabolized by dipeptidyl-peptidase (DP) IV to biologically inactive GIP 3-42. The analogs Ppa^1-GIP 1-30 and D-Ala^2-GIP 1-30amide were shown to be resistant to DP IV degradation *in vitro*, but had slightly reduced affinity and efficacy at the GIP receptor. Such DP IV resistant analogs may be useful in NIDDM treatment.

Oligonucleotide-directed mutagenesis was used to examine regions important for ligand binding, receptor activation, and G-protein coupling. Studies of GIP/GLP-1 receptor chimeras indicated that the high affinity GIP binding domain lies within the extracellular amino-terminal of the GIP receptor, while the first transmembrane domain appears critical for GIP-specific receptor activation. A similar region of the GLP-1 receptor may be important for GLP-1 receptor activation.

The effect of truncating the carboxy-terminal-tail of the GIP receptor on ligand binding, second messenger coupling, and internalization was examined. Truncation by >37 amino acids greatly decreased expression, and a minimum carboxy-terminal tail length of 13 amino acids appears to be required for receptor expression. In contrast the carboxy-terminal-tail could be truncated by up to 50 amino acids without affecting receptor affinity, and with only small effects on G-protein coupling and receptor internalization.

These are the first detailed structure-function studies on GIP and its receptor in a cell system uncomplicated by factors inherent in whole animal preparations or immortalized β-cell lines. Further studies may lead to a better understanding of the apparent reduction of GIP receptors in NIDDM, and the development of GIP analogs that are useful in its treatment.

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ABBREVIATIONS:

Amino acid:	Three letter code	Single letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Try	W
Tyrosine	Tyr	Y
Valine	Val	V

Hormones and receptors*:

G-protein-coupled receptors = GPCRs
Gastric Inhibitory Polypeptide/Glucose dependent insulinotropic polypeptide = GIP
Glucagon-Like peptide-1 = GLP-1
Parathyroid hormone = PTH
Parathyroid related peptide = PTHrP
Glucagon = GLU
Gastrin releasing peptide = GRP
Gonadotropin-releasing hormone = GnRH
Neurokinin = NK
Luteinizing hormone = LH
Glicentin-related pancreatic peptide; GRPP
Angiotensin II type 1A receptor ($AT_{1A}R$)
Beta-adrenergic receptor = β-AR
Alpha-adrenergic receptor = α-AR
Muscarinic acetylcholine receptor = mAchR
Growth hormone-releasing hormone = GRH
Calcitonin = Cal

* receptors for hormones are designated by the hormones abbreviation followed by R

Miscellaneous:

Deoxyribonucleic acid = DNA
Ribonucleic acid = RNA
Cyclic-adenosine monophosphate = cAMP
Phosphotidylinositol 4,5,-bisphosphate = PIP_2
Phospholipase = PLC
Protein kinase C = PKC
cAMP-dependent protein kinase = PKA
Guanosine triphosphate (GTP) binding proteins = G-proteins
G-protein coupled receptor kinase = GRK
β-adrenergic receptor kinase = βARK
Non-insulin dependent diabetes = NIDDM
Single nucleotide polymorphism = SNP
High performance liquid chromatography = HPLC
Matrix-assisted laser desorption/ionization-time of flight mass spectrometry = MALDI-TOF MS
Carboxy-terminal = CT
Amino-terminal = NT
3-phenyl propionic acid/Des-amino-tyrosine = Ppa
Synthetic porcine = sp
Synthetic human = sh
Natural porcine = np
Wild type = wt

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Richard Wayne Gelling
13/8/98

CHAPTER 1

INTRODUCTION

1.1 OVERVIEW

Gastric inhibitory polypeptide/Glucose-dependent insulinotropic polypeptide (GIP) is a 42 amino acid hormone that is produced by the K-cells of the mammalian duodenal and jejunal mucosa, and is released in response to the ingestion of glucose, fat, and some amino acids (reviewed in Brown *et al.*, 1989; Pederson, 1993). GIP was initially identified and isolated on the basis of its ability to inhibit gastric acid secretion (enterogastrone action) (Brown *et al.*, 1970, 1989; Brown, 1971), and later was shown to be a potent stimulant of insulin secretion (incretin) in the presence of hyperglycemia (Dupre *et al.*, 1973). GIP is now widely acknowledged as being one of two established incretins involved in the enteroinsular axis in man (Brown *et al.*, 1989; Pederson, 1993; Fehmann *et al.*, 1995) and other species (Brown *et al.*, 1989; Pederson, 1993), the other being truncated glucagon-like peptide-1 [tGLP-1; GLP-1(7-36amide) and GLP-1(7-37)] (reviewed in Holst 1994, 1996; Drucker 1998). The latter incretin is now being investigated as a potential therapeutic agent in the treatment of non-insulin dependent diabetes mellitus (NIDDM) (Nauck *et al.*, 1989; Gutniak *et al.*, 1992, 1996; Byrne and Göke, 1996; Todd *et al.*, 1997; Nauck *et al.*, 1997a).

Early ligand binding studies identified both high and low affinity GIP binding sites in islet-derived β-cell lines and a gastric tumor cell line (HGT-1) (reviewed in Brown *et al.* 1989; Pederson, 1993; McIntosh *et al.*, 1996). However, it was not clearly established that GIP receptors were expressed in pancreatic islets, although GIP was shown to increase both intracellular levels of cyclic (c)AMP (Amiranoff *et al.*, 1984,

Gallwitz *et al.*, 1993, Lu *et al.*, 1993a) and Ca^{2+} ($[Ca^{2+}]i$) in a tumor cell line (HIT-T15) (Lu *et al.*, 1993a) and isolated islets (Wahl *et al.*, 1992).

Usdin and colleagues (1993) recently isolated a complementary (c)DNA encoding a putative seven transmembrane receptor protein with a high degree of structural similarity to receptors in the VIP/secretin receptor family. Expression studies revealed that GIP was the only candidate peptide tested that elicited a high affinity cAMP response and increased $[Ca^{2+}]i$ in reporter cell lines (Usdin *et al.*, 1993). Interestingly, the presence of receptor mRNA transcripts was demonstrated in a number of extrapancreatic tissues by a reverse transcriptase polymerase chain reaction (RT-PCR)-based approach. This included the vasculature and brain, which had not previously been considered as GIP target tissues. Given this unexpected pattern of expression, and that the first partial cDNA was isolated from brain and the subsequent full length cDNA from a tumor β-cell line (RINm5F), it was initially questioned whether the cDNA encoded a pancreatic islet GIP receptor, or a closely related species.

In order to answer this question we have used RT-PCR to isolate a GIP receptor cDNA from isolated rat pancreatic islet mRNA. Once isolated the receptor cDNA was functionally expressed in the monkey kidney (COS-7) and Chinese hamster ovarian (CHO-K1) cell lines, and used to study a number of factors concerning receptor binding and activation, and the signal transduction pathways involved. These studies included the following:

1. The receptor binding and adenylyl cyclase stimulating activity of different synthetic preparations of porcine and human GIP were examined. In addition, the mode of

action of GIP in increasing [Ca^{2+}]i has been investigated. Furthermore, the stable GIP receptor expressing CHO-K1 cell-line (wtGIP-R1) was used to investigate the ability of truncated forms of GIP to activate or antagonize receptor activation, allowing the determination of regions of the polypeptide important for receptor binding and/or activation. These studies are important given controversy surrounding the efficacies of different synthetic GIP preparations (Nauck *et al.*, 1993a; Jia *et al.*, 1995), and the potential therapeutic value of the incretins for the treatment of NIDDM.

2. Site directed mutagenesis was used to generate chimeric GIP/GLP-1 receptors to examine which regions of the two highly related receptors were involved in ligand binding, ligand discrimination, and receptor activation.

3. Site directed mutagenesis was also used to truncate the GIP receptor carboxy-terminal (CT) tail, to examine its contribution to receptor expression, ligand binding affinity, G-protein coupling, receptor desensitization, and receptor uptake. These studies identified a five amino acid segment of the GIP receptor CT-tail region that appeared to be critical for functional receptor expression.

1.2 DISCOVERY OF GIP

The term enterogastrone was originally proposed by Kosaka and Lim (1930) to describe a blood borne gastric inhibitory chemical messenger released from the small intestine in response to fat. They showed that mucosal extracts, when administered in large doses (mg/Kg), inhibited meal and histamine stimulated acid secretion in dogs.

Later, two candidate intestinal hormones, secretin (Bayliss and Starling, 1902) identified on the basis of its ability to stimulate pancreatic secretion, and cholecystokinin (CCK) (Ivy and Oldberg, 1928) on its ability to stimulate gallbladder contraction, initially appeared to meet the requirements of an enterogastrone. Preparations of both secretin and CCK were shown to inhibit acid secretion in the denervated (Heidenhain) canine gastric pouch (Gillespie and Grossman, 1964). However early preparations of CCK were impure, and were demonstrated both to suppress histamine or gastrin stimulated acid secretion (Gillespie and Grossman, 1964; Brown and Magee, 1967) and stimulate acid secretion under fasting conditions (Magee and Nakamura, 1966). Brown and Pederson (1970) found that when 10% pure CCK-pancreozymin (CCK-PZ) was further purified to 40% the gastric inhibitory activity was reduced, while the stimulatory activity in the fasting state was enhanced. They reasoned that the gastric acid inhibitory activity was removed during further purification of CCK-PZ. Using the canine Bickel pouch as a bioassay Brown *et al.* (1969, 1970) purified the active substance from extracts of hog duodenal and jejunal mucosa and named it gastric inhibitory polypeptide, or GIP (Brown, 1971).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Human	Y	A	E	G	T	F	I	S	D	Y	S	I	A	M	D	K
Porcine																
Bovine																
Rat																
Mouse																

	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
Human	I	H	Q	Q	D	F	V	N	W	L	L	A	Q	K	G	K
Porcine		R														
Bovine		R														
Rat		R														
Mouse		R												R		

	33	34	35	36	37	38	39	40	41	42
Human	K	N	D	W	K	H	N	I	T	Q
Porcine		S								
Bovine		S			I					
Rat								L		
Mouse		S								

Fig. 1. Alignment of GIP Amino Acid Sequences from Several Species. Only variations from the human sequence are shown. Sequences were obtained from: human (Moody *et al.*, 1984), pig (Jörnvall *et al*, 1981), cow (Carlquist *et al*, 1984), rat (Higashimoto *et al*, 1992), and mouse (Schieldrop *et al.*, 1996).

1.2.1 GIP IS A MEMBER OF THE VIP/GLUCAGON/SECRETIN SUPERFAMILY

The complete 42 amino acid sequence of porcine GIP was first reported by Brown and Dryburgh (1971) and later corrected by Jörnvall *et al.* (1981). Comparison of GIP sequences from a number of different species indicates a high degree of conservation (>90%) at the amino acid level (Fig. 1). The human sequence differs at two amino acid positions from the porcine and rat sequences, and three positions from the bovine and mouse sequences. The highly conserved nature of different GIP species suggests that GIP has an important regulatory role. The structure of the preproGIP gene (Inagaki *et al*,

1989) indicates that it belongs to the glucagon gene family, and may have arisen from a common ancestral gene (Bell, 1986; Campbell and Scanes, 1992). The peptide products share both sequence similarities and some biological activities, and some of the more closely related peptide members are shown in Fig 2.

```
            1         10         20         30         40
GIP         YAEGTFISDYSIAMDKIHQQDFVNWLLAQKGKKNDWKHNITQ
Glucagon    HSQGTFTSDYSKYLDSRRAQDFVQWLMNT
GLP-1       HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR-NH2
            ···***·**·* ···     ··*··**
```

Fig. 2. Alignment of the Amino Acid Sequences of GIP with the Related Peptide Hormones Glucagon and Glucagon-Like Peptide-1 (7-36amide). Amino acids are presented as their single letter abbreviations. * = completely conserved, · = residues well conserved. Alignment was carried out using the PC Gene software package (IntelliGenetics, 1995).

1.2.2 GIP GENE STRUCTURE AND POSTTRANSLATIONAL PROCESSING

The cDNAs encoding both rat (Higashimoto *et al.*, 1992; Tseng *et al.*, 1993) and human (Takeda *et al.*, 1987) GIP, as well as their genes (Higashimoto and Liddle, 1993; Inagaki, *et al.*, 1989) have been isolated. The human GIP gene has been mapped to the long arm of chromosome 17 (Inagaki, *et al.*, 1989). Both human and rat genes consist of 6 exons separated by 5 introns. The human gene exon 1 encodes most of the 5' untranslated (UT) region of the mRNA; exon 2 encodes the distal 5'UT, the signal peptide and a small portion of the amino-terminal cryptic peptide; exon 3 encodes the distal cryptic peptide along with the majority of the mature GIP peptide; exons 4 and 5 encode the remainder of the mature peptide and the carboxy-terminal peptide; and exon 6

encodes the 3'-UT region of the mRNA (Fig 3). This organization is conserved in the rat gene and with the genes of other related peptides suggesting a common ancestral gene (Campbell and Scanes, 1992). While the rat gene gives rise to a 144 amino acid prepro-hormone (Higashimoto and Liddle, 1993), the human gene encodes a 153 amino acid precursor (Inagaki, *et al.*, 1989). The differences in preprohormone size are due to an 8 amino acid deletion in the amino-terminal peptide, and a single amino acid deletion within the carboxy-terminal peptide in rat. Mature rat GIP is processed from the large precursor polypeptide by removal of a putative 21 amino acid signal peptide, and further cleavage resulting in the loss of the amino-terminal, and carboxy-terminal cryptic peptides, at single arginine residues 43 and 86, respectively. The human homolog is processed by removal of a 21 amino acid signal peptide, a 30 residue amino-cryptic peptide at position 50, and the carboxy-terminal cryptic peptide at residue 94. The GIP gene and its mRNA are depicted in Fig.3.

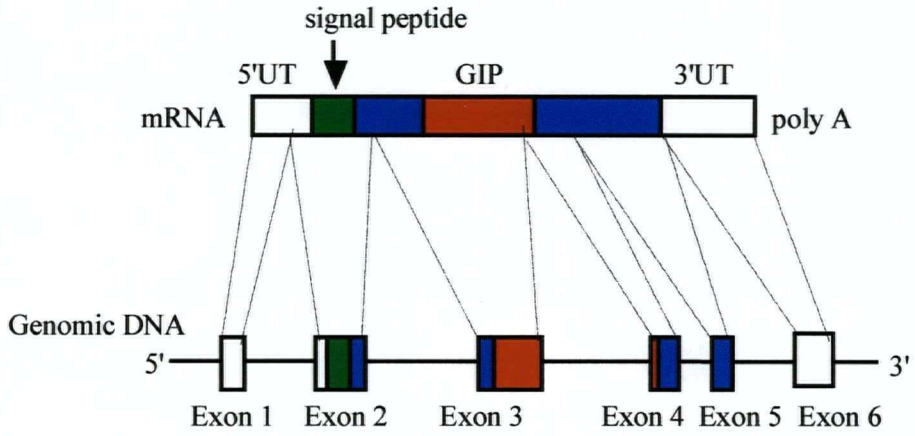


Fig. 3. Organization of the Human GIP Gene and Duodenal mRNA. Untranslated regions (UT) are represented as clear boxes, the signal peptide region is filled green, sequence encoding the mature peptide is fill red, and regions coding the pro-GIP amino- and carboxy-terminal peptides are blue. Adapted from Inagaki et al., 1989.

1.2.3 TISSUE DISTRIBUTION

The distribution of GIP was originally shown to be limited to specific endocrine cells (K cells) of the duodenum and jejunum in humans (Polak *et al.*, 1973; Buchan *et al.*, 1978, 1982), but to extend to the ileum in rat and dog (Buchan *et al*, 1982). Similar distributions of GIP mRNA have been described in human (Inagaki, *et al.*, 1989), and rat (Higashimoto *et al.*, 1992; Tseng *et al.*, 1993). A recent study also identified both GIP-like immunoreactivity and GIP mRNA within the submandibular salivary gland in rats (Tseng *et al.*, 1995). GIP mRNA was localized via *in situ* hybridization to the ductal cells of the submandibular gland (Tseng *et al.* 1995). However, the exact identity of submandibular-GIP and any physiological roles it may play remain to be clarified. It is also not known if submandibular -GIP exists in humans.

1.2.4 GIP RELEASE

GIP is released from the K-cells in response to nutrient ingestion. It is generally agreed that IR-GIP levels increase 5-6 fold following a mixed meal, however the absolute values measured vary from 12-92 pM during fasting to 35-235 pM after a meal (Alam and Buchanan, 1993). These discrepancies were most likely due to poor cross reactivity of antisera raised against natural porcine GIP for human and rat forms, and/or cross reactivity with as yet unidentified large molecular weight proteins (reviewed in Alam and Buchanan, 1993).

The sparse distribution of GIP cells has made it difficult to characterize the factors and molecular pathways involved in regulating GIP secretion. Carbohydrates stimulate

GIP release, which is appropriate for its role as an insulinotropic polypeptide. In human (Cataland *et al.*, 1974), dog (Pederson *et al*, 1975), and rat (Pederson *et al.*, 1982) an oral glucose load increased IR-GIP levels. Ingestion of carbohydrates (glucose, galactose, and sucrose) appears to be necessary as intravenous glucose failed to increase serum IR-GIP levels. While the exact mechanism remains to be determined, the sodium-dependent active transport of monosaccharides is a requirement for the carbohydrate stimulation of GIP secretion (Morgan *et al.*, 1979; Creuzfeldt and Ebert, 1977). Glucose has also been shown to stimulate GIP release *in vitro* from cultured isolated canine K cells (Kieffer *et al.*, 1994) and from an intestinal cell line (Kieffer *et al.*, 1995a), supporting the hypothesis that glucose affects K cells directly.

Ingestion of fat has also been shown to be a potent stimulator of GIP release (Brown *et al.*, 1975; Cleator and Gourlay, 1975; Falko *et al*, 1982). In man, fat is more potent and results in a more prolonged elevation of circulating IR-GIP levels than glucose (Morgan, 1996). However, as would be expected, the increase in IR-GIP in the absence of elevated glucose levels does not increase insulin secretion (Cleator and Gourlay, 1975; Falko *et al*, 1982). The chain length of the fatty acids (FA), as well as the degree of saturation, affects the ability of ingested fats to stimulate GIP release. Whereas longer chain (O'Dorisio *et al.*, 1976; Ross and Shaffer, 1981) more highly saturated (Lardinois *et al.*, 1988) FA are potent secretagogues, short and medium chain fatty acids do not stimulate IR-GIP release. The exact nature of the differences in potency is unclear, however it has been suggested that the selective esterification of only the long chain FA and their incorporation into chylomicrons may play a role (Kwasowski *et al.* 1985).

It is unclear if protein or protein-digestion products stimulate GIP release physiologically. While meat or meat extracts were shown not to increase IR-GIP levels (Cleator and Gourlay, 1975), Thomas *et al* (1976) showed that intraduodenal administration of a mixture of basic amino acids, but not a mixture of aromatic amino acids, resulted in IR-GIP release. Enhancement of sodium-dependent amino acid transport, by increasing Na^+ K^+ ATPase activity with corticosteroid or alloxan, increased IR-GIP release (Schulz *et al.*, 1982), suggesting that in common with carbohydrates, active transport of amino acids is coupled to GIP release.

GIP release appears to be inhibited by hyperinsulinaemia in both the rat (Bryer-Ash *et al.*, 1994) and human (Takahashi *et al.*, 1991). However some species differences may exist, as inhibition of GIP release was attenuated in the rat, but not in human under hyperinsulinaemic, hyperglycaemic conditions (Bryer-Ash *et al.*, 1994; Takahashi *et al.*, 1991). Regulation of GIP release by the autonomic nervous system is not well understood, with studies indicating that both sympathetic and parasympathetic nerves can stimulate, inhibit or have no effects on GIP release, depending on the experimental conditions (reviewed in McIntosh, 1991).

It has been demonstrated recently that both GIP and GLP-1 are metabolized by circulating dipeptidyl peptidase IV (DP IV) (Mentlein *et al.*, 1993b), both *in vivo* (Kieffer *et al.*, 1995b; Deacon *et al.*, 1995) and *in vitro* (Mentlein *et al.*, 1993b; Kieffer *et al.*, 1995b; Pauly *et al.*, 1996) to non-insulinotropic forms of GIP3-42 and GLP-1 (9-36amide) (Brown *et al.*, 1981; Schmidt *et al.*, 1986a; Suzuki *et al.*, 1989; Gefel *et al.*, 1990). Most GIP radioimmunoassays (RIAs) recognize carboxy-terminal epitopes and measure both biologically active and inactive peptide forms, suggesting that previously

reported GIP levels are overestimations of the biologically active hormone concentration. The use of RIAs with amino-terminal directed antisera is required to determine the biologically relevant levels of GIP that are reached in the basal and stimulated states.

1.2.5 REGULATION OF GIP GENE EXPRESSION

Few studies have examined the regulation of GIP gene expression. Rat GIP mRNA levels in the small intestine and submandibular salivary gland have been shown to increase in response to glucose (Tseng *et al.*, 1994, 1995; Higashimoto *et al.*, 1995) and fat (Tseng *et al.*, 1993; Higashimoto *et al.*, 1995) administration, while only glucose appeared to increase tissue IR-GIP levels (Higashimoto *et al.*, 1995). Increases in mRNA were greatest for long term fat administration (Higashimoto *et al.*, 1995). Schieldrop *et al.* (1996) recently reported that GIP mRNA expression in the intestinal tumor STC_{6-14} cell line increased 3 fold in response to increasing culture media glucose concentrations from 5 mM to 25 mM. Interestingly, while Higashimoto *et al.* (1995) noted a decrease in both GIP mRNA and tissue IR-GIP levels in response to food deprivation, that was rapidly reversed (within 1 day) by reinstitution of feeding, Sharma *et al.*, (1992) observed a 2 fold increase in GIP mRNA levels in response to food deprivation. It appears, as is the case with secretion, that GIP gene expression is regulated by dietary nutrient content. Given that both the rat and human 5' flanking regions contain a putative cAMP response element, and AP 1 and AP 2 sites (Higashimoto and Liddle, 1994; Inagaki *et al.*, 1989), recognition sites for transcription factors that are regulated by cAMP/protein kinase (PK)A and PKC pathways, it seems likely that other factors are involved in the regulation of GIP gene expression. A very recent study has identified both a distal and proximal

promoter, the former containing a GATA related sequence between positions –193 and –182 that promotes cell-line specific expression of a luciferase reporter construct (Boylan *et al.*, 1997).

1.3 BIOLOGICAL EFFECTS

1.3.1 ENTEROGASTRONE ACTIVITIES OF GIP

As stated above, GIP was first isolated on the basis of its gastric inhibitory actions in the denervated stomach (Brown *et al.*, 1969, 1970; Brown, 1971). However, studies in both man (Maxwell *et al.*, 1980) and dog (Soon-Shiong *et al.*, 1979) showed that GIP had only week inhibitory effects on the innervated stomach. Yamagisihi and Debas (1980) reported that, while intra-duodenal administration of oleic acid completely inhibited acid secretion in response to a meal of liver extract, GIP infusion resulted in only 40% inhibition of the acid secretion. In a comparative study, Soon-Shiong *et al.* (1984) measured the inhibitory effects of GIP on pentagastrin stimulated acid secretion from both the denervated Heidenhain pouch, and the innervated stomach within the same animal. While GIP inhibited acid secretion from the denervated Heidenhain pouch by 80%, only 30% inhibition occurred in the innervated stomach. Further, it was observed that GIP had no effect on acid secretion in the Heidenhain pouch if it was administered against a background of Bethanechol, suggesting that the enterogastrone effect of GIP might not be direct, but mediated via a second humoral agent under cholinergic control. McIntosh *et al.* (1981a) demonstrated that GIP was able to stimulate immunoreactive-somatostatin (IR-SS) release from the isolated perfused rat stomach, and release of IR-SS was inhibited by vagal stimulation, or acetylcholine administration. This suggested that

the enterogastrone activity of GIP is mediated by stimulation of the release of neuroendocrine somatostatin from the D-cells of the stomach. Additional studies have indicated a role for the sympathetic nervous system in modulating this pathway (McIntosh *et al.*, 1981b). While GIP appears to display some of the characteristics of an enterogastrone, it is obviously not the only one, and it likely acts in concert with a number of neuronal and/or humoral agents to achieve the full acid inhibitory action observed *in vivo*.

1.3.2 INCRETINS AND THE ENTEROINSULAR AXIS CONCEPT

The possible existence of a substance released from the duodenum and capable of modulating carbohydrate disposal was first investigated, without success, by Bayliss and Starling (1902). Moore *et al.* (1906) proposed that the duodenum produced a "chemical excitant", the absence of which caused diabetes. Their treatment of diabetics with the acid extracts of hog duodenum, although initially promising, was ultimately unsuccessful. Studies using an intestinal extract free of secretin activity, which caused hypoglycemia in dogs (Zunz and La Barre, 1929; La Barre and Still, 1930), led La Barre (reviewed in Brown et al., 1989) in 1932 to propose the term incretin to describe humoral activity from the gut that enhances glucose deposition. However later studies by Loew *et al* (1940) failed to demonstrate any effect of several different intestinal extracts on blood glucose levels, and interest in possible insulinotropic intestinal factors declined over the following years.

It was the advent of the radioimmunoassay technique, allowing the direct monitoring of insulin levels, which led to renewed interest in the existence of a possible

incretin. As oral glucose was far more potent a stimulator of insulin release than intravenous glucose in humans (Elrick *et al.*, 1964; McIntyre *et al.*, 1964), it was concluded that an additional stimulus for insulin release existed, and that it appeared to originate from the small intestine (McIntyre *et al.*, 1965). Unger and Eisentraut (1969) proposed the term "enteroinsular axis" to describe the hormonal link between the gut and the pancreatic islets. However, it was known that other factors such as neural connections and nutrients had direct effects on the insulin secreting cells of the islet. Creutzfeldt (1979) therefore suggested that the term "enteroinsular axis" be used to encompass nutrient, neural, and hormonal signals from the gut to all of the islet cells secreting insulin (β-cells), glucagon (α-cells), somatostatin (δ-cells), and pancreatic polypeptide (PP-cells). The term 'incretin' should be used to refer more specifically to a hormone of the enteroinsular axis released in response to nutrients, particularly carbohydrates, that stimulates insulin secretion at physiological levels in the presence of glucose (Creuzfeldt, 1979).

1.3.3 EVIDENCE FOR GIP AS AN INCRETIN

Evidence of GIP's incretin activity in the enteroinsular axis was first provided by Dupré and Beck (1966) when they demonstrated that impure preparations of CCK exhibited insulinotropic properties. In subsequent studies, Rabinovitch and Dupré (1972) showed that this insulinotropic activity was diminished as the purity of the CCK preparation increased, similar to the loss of gastric inhibitory activity observed by Brown and Pederson (1970). Dupre *et al.* (1973) demonstrated in man that a purified preparation of GIP, when infused in the presence of elevated glucose, stimulated insulin release.

Furthermore, it was shown that GIP had no insulinotropic properties in the euglycemic state, which suggested that GIP was only insulinotropic in the presence of elevated circulating glucose. GIP has since been shown to be insulinotropic in a number of species including human (Elahi *et al.*, 1979) and dog (Pederson *et al.*, 1975), and in vitro with the isolated perfused rat pancreas (Pederson and Brown, 1976, 1978), isolated islets (Siegal and Creutzfeldt, 1985; Shima *et al.*, 1988), and β-cell lines (Kieffer *et al.*, 1993; Lu *et al.*, 1993a,b).

1.3.4 GIP EFFECTS ON OTHER ISLET CELL TYPES

GIP has also been shown to be a stimulator of glucagon release from the islet α-cell. Studies in the perfused rat pancreas (Pederson and Brown, 1978) showed that GIP stimulated glucagon release when glucose levels were below 5.5 mM, but responses were suppressed at higher glucose concentrations. In humans, GIP was unable to reverse either mild or moderate glucose-suppressed glucagon release (Elahi *et al.*, 1979). In contrast GIP was able to reverse glucose-suppressed glucagon release at glucose levels as high as 11 mM in the mouse (Opara and Go, 1991), suggesting species differences may exist. It is unclear if GIP stimulates somatostatin release from islet δ-cells in a physiologically relevant manner, as only weak stimulation in the perfused rat pancreas has been reported (Schmid *et al.*, 1990).

1.3.5 EXTRAPANCREATIC EFFECTS OF GIP

The extrapancreatic effect of GIP on lipid metabolism has received the most attention in recent years (reviewed in Morgan, 1996). Treatment of rat adipocytes with

GIP in the nM range, increased glucose uptake both in the presence of insulin (Starich *et al.*, 1985), and when administered alone (Hauner *et al.*, 1988). The demonstration that 1-100 nM GIP also stimulated lipogenesis in adipocytes (Hauner *et al.*, 1988), and rat adipose tissue explants (Oben *et al.*, 1991) supported a positive regulatory role for GIP in *de novo* triglyceride synthesis. It was also shown that, while GIP alone displayed low lipolytic activity, high concentrations inhibited glucagon- and isoproterenol-stimulated lypolysis (Hauner *et al.*, 1988; Dupre *et al.*, 1976). There is also evidence that GIP can positively regulate lipid deposition from dietary sources. It was demonstrated that increasing dietary fat intake in rats resulted in increased basal GIP and insulin secretion, as well as increasing lipoprotein lipase (LPL) sensitivity to the two hormones (Morgan, 1996). GIP has also been shown to increase LPL activity in fat pad explants (Morgan, 1996) and mouse 3T3-L1 preadipocytes (Eckel *et al.*, 1979), and to increase clearance of plasma trigycerides in the rat (Ebert *et al.*, 1991).

Another extrapancreatic effect of GIP that has been examined is the inhibition of glucagon-stimulated hepatic glucose production in rat and man (Hartmann *et al.*, 1986; Elahi *et al.*, 1986). Given that recent studies have failed to identify GIP receptor mRNA in liver, the mechanism by which this occurs is unknown, and it may be an indirect effect (Morgan, 1996).

1.4 EVIDENCE FOR FURTHER INCRETIN(S)

Initial studies suggested that GIP alone could account for the incretin response to glucose ingestion (Elahi *et al.*, 1979). However, infusion of GIP antisera in rats was only shown to reduce, and not ablate, insulin responses to oral (Lauritsen *et al.*, 1981) or

intraduodenal (Ebert and Creutzfeldt, 1982) glucose, or to intraduodenal acid accompanied by an intravenous glucose infusion (Ebert *et al.*, 1979b). The degree of suppression (20%-50%) was dependent upon the antibody infusion protocol utilized. These studies suggested the existence of other incretin(s).

1.4.1 ISOLATION, PROHORMONE PROCESSING, AND TISSUE DISTRIBUTION OF GLP-1

Unlike GIP which was isolated using traditional biochemical techniques, the existence of glucagon like peptides was not determined until the isolation of the proglucagon (PG) cDNA from anglerfish (Lund *et al.*, 1982) and hamster (Bell *et al.*, 1983). Lund and coworkers (1982) identified the glucagon sequence plus one 34 amino acid glucagon-like peptide (GLP) sequence in the carboxy-terminal of PG, while Bell *et al.* (1983) identified two carboxy-terminal glucagon-like sequences in the hamster cDNA, which were referred to as GLP-1 and GLP-2 respectively. Based on data from the hamster cDNA and the human gene (Bell *et al.*, 1983) it was predicted that the preprohormone corresponds to a 20 amino acid signal peptide and a 160 amino acid prohormone (PG1-160). GLP-1, unlike GIP, has a wide tissue distribution, and is found in L-cells of the small intestine (the majority within the ileum), colon and rectum, pancreatic α-cells, and brain neurons (reviewed in Fehmann *et al.*, 1995). The gene for PG1-160 is expressed in both intestine and pancreas (Novac *et al*, 1987), but the propeptide is differentially processed in the two tissues (reviewed in Ørskov, 1992 and Fehmann *et al.*, 1995). In the islet α-cell, PG1-30 (glicentin-related pancreatic peptide; GRPP), glucagon (PG33-61), PG64-69 and PG72-158, the major pancreatic proglucagon

fragment (MPGF) corresponding to GLP-1, GLP-2 and an intervening peptide II (IP II), are the main prohormone processing products (Fig. 4). This large unprocessed carboxy-terminal peptide can be further processed to small amounts of PG72-108 [GLP-1(1-37)] in the pig, and PG72-107 [GLP-1 (7-36amide)] in humans (Holst *et al.*, 1994). Intestinal glucagon cells or L-cells process PG1-160 to glicentin (PG1-69), oxyntomodulin (PG33-69), intervening peptide 2 (IP-2), GLP-1 (1-37) and GLP-2 (Fig. 4). GLP-1 (1-37) is cleaved after position 6 from the amino-terminus yielding GLP-1 (7-37), the majority of which is further processed and amidated to give GLP-1 (7-36amide) [1] (reviewed in Ørskov, 1992 and Fehmann *et al.*, 1995). The latter, mature, peptide form accounts for 80% of the circulating immunoreactive GLP-1/tGLP-1 in man (Ørskov, 1992 and Fehmann *et al.*, 1995). While it has been shown recently that GLP-2 influences hexose transport in intestinal basolateral membranes (Cheeseman and Tsang, 1996), and stimulates intestinal epithelial proliferation (Drucker *et al.*, 1996), the biological relevance of many of the products of proglucagon (MPGF, oxyntomodulin, glicentin) remains unknown and requires further investigation.

[1] For the sake of brevity GLP-1 will be used to refer to GLP-1 (7-36amide). Abbreviations for other forms and fragments will be noted in the text.

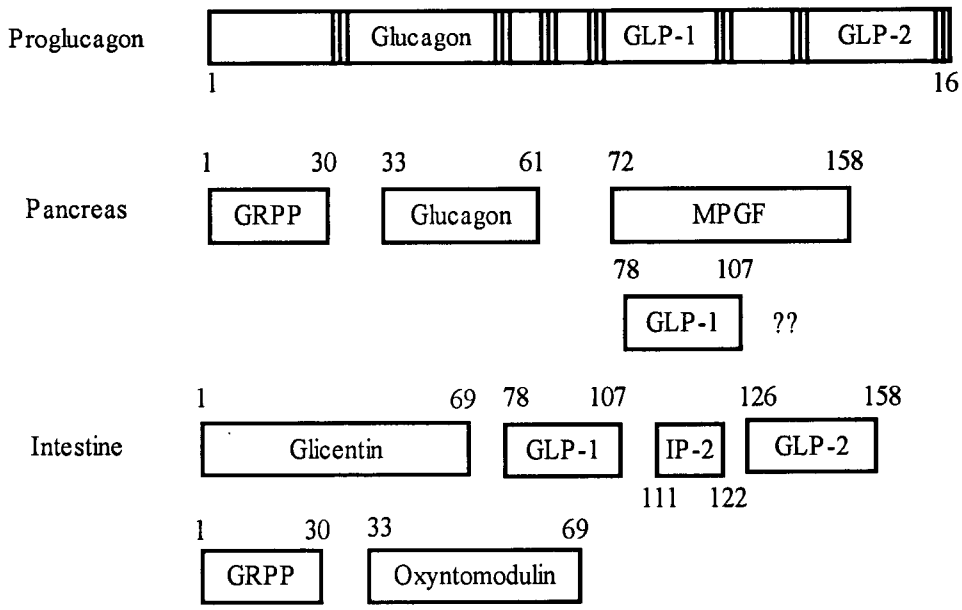


Fig. 4. Processing of Proglucagon in the Pancreas and Intestine. Glucagon is the main peptide product with known biological activity resulting from prohormone processing in the islet α-cell. Processing in the intestine gives rise to GLP-1 and GLP-2. The biological function of the other proglucagon fragments are unknown. Greater than 80% of the circulating GLP-1 levels in humans correspond to GLP-1(7-37amide). Adapted from Fehmann et al. (1995).

1.4.2 REGULATION OF GLP-1 RELEASE

The pathways involved in the regulation of GLP-1 secretion are unclear. It has been shown that oral ingestion of nutrients stimulates GLP-1 release even though the majority of L-cells exist within the ileum and are therefore unlikely to be directly exposed to high concentrations of nutrients. It has been suggested that GLP-1 secretion may be triggered by neuronal, as well as nutrient, stimuli (Roberge and Brubaker, 1993). Cholinergic stimulation of GLP-1 release has been demonstrated in isolated rat ileum (Reviewed in Fehmann *et al.*, 1995) and, through extrapolation from pharmacological studies in the intestinal cell line STC-1 (Abello *et al.*, 1994), this is thought to be mediated via activation of muscarinic (predominately M_3) receptors. A hormonal feed-forward loop, in which nutrients in the duodenum cause release of GIP, which in turn stimulates GLP-1 release, has been postulated to be operational in rats (Roberge and Brubaker, 1993), however studies in humans have failed to show any effect of GIP on GLP-1 levels (Nauck *et al.*, 1993a). Unlike GIP secretion in humans, GLP-1 release appears to be inhibited by hyperglycemia, but not hyperinsulinaemia (Takahashi *et al.*, 1991).

1.4.3 GLP-1 ACTION ON INSULIN RELEASE

The main biological action of GLP-1 is thought to be the potentiation of glucose stimulated insulin release. Initial studies found that high concentrations of GLP-1 (1-37), but not GLP-2, stimulated insulin release from perfused rat pancreas (Schmidt *et al.*, 1985). The close sequence homology to GIP and glucagon led some investigators to

speculate that truncated GLP-1 would be more insulinotropic than GLP(1-37) (Crueutzfeldt *et al.*, 1993). The potent insulinotropic activity of GLP-1 was first demonstrated in the isolated perfused pancreas from pig (Holst *et al.*, 1987) and rat (Mojsov *et al.* 1987). Numerous studies that followed demonstrated that the insulinotropic activity of GLP-1 was greatest in the presence of elevated glucose (reviewed in Creutzfeldt and Ebert, 1993). Of interest was the finding that the amidated and glycine extended forms, GLP-1 and GLP-1(7-37) respectively, did not differ in their biological activity or apparent biological half-lives (Ørskov *et al.*, 1993), raising questions as to why GLP-1(7-37) is further processed. The insulinotropic activity of GLP-1 has been confirmed in a number of β-cell lines (Lu *et al.*, 1993a; Montrose-Rafizadeh *et al.*, 1994), isolated islets (D'Alessio *et al.*, 1989; Siegel *et al.*, 1992; Suzuki *et al*, 1992), and in humans (Kreymann *et al.*, 1987; Nauck *et al.*, 1993a,b).

1.4.4 GLP-1 EFFECTS ON OTHER ISLET CELL TYPES

GLP-1 has been shown to inhibit pancreatic glucagon secretion, and this could enhance the hormone's glucose lowering effects. A potent glucoganostatic action has been demonstrated *in vivo* in humans (Nauck et al., 1993a; Schirra et al., 1998), and *in vitro* in the isolated perfused rat (Matsuyama, *et al.*, 1988) and dog (Kawai *et al.*, 1989) pancreas, and isolated human islets (Fehman *et al.*, 1995). GLP-1 has also been shown to be a potent stimulator of somatostatin release from islet δ-cells. In both the perfused rat and dog pancreas GLP-1 is somatostatinotropic (Komatsu, *et al.*, 1989; Kawai *et al.*, 1989) and, additionally, GLP-1 has been demonstrated to stimulate somatostatin release in isolated human islets (Fehmann et al., 1995). Therefore it is unclear if the inhibitory

action of GLP-1 on glucagon secretion is mediated directly on the α-cell, or via the release of islet somatostatin, which then inhibits glucagon release. However recent studies have demonstrated, using autoradiography with ^{125}I-GLP-1 (Heller and Aponte, 1995), immunocytochemistry, and single cell RT-PCR, the presence and expression of GLP-1 receptors on a subpopulation (~20%) of dispersed rat α-cells (Heller *et al.*, 1997). This is in contrast to an earlier study, in which Moens *et al.* (1996), failed to detect GLP-1 receptors or receptor mRNA in fluorescence-activated cell sorted (FACS)-sorted rat α-cells, using Western and Northern analysis. Interestingly, Heller and Aponte (1995) have identified releasable IR-GLP-1 within some α-cells, the secretion of which is inhibited by high glucose levels, suggesting that GLP-1 may play an intra-islet regulatory role when lower glucose levels exist.

1.4.5 CONTRIBUTION OF GIP AND GLP-1 TO THE INCRETIN EFFECT

Although GIP and GLP-1 are widely considered to be the two most important incretins (reviewed in Brown *et al.*, 1989; Pederson, 1993; Holst, 1994), it is presently unclear as to their relative contribution to the overall incretin response to a meal. As mentioned above, infusion of GIP antisera in rats reduced insulin responses by 20-50%, depending upon the antibody infusion protocol utilized. Subsequently, it was reported that administration of the GLP-1 receptor antagonist exendin (9-39) to rats resulted in an estimated 60% inhibition of insulin responses to intraduodenal glucose (Kölligs *et al.*, 1995) and 48% inhibition of responses to a meal (Wang *et al.*, 1995b). Since immunoneutralization suffers from the drawback of limited accessibility of antibody to

the target cells, it is clearly important to develop a GIP antagonist to enable similar *in vivo* investigations on the role of this peptide.

While the investigations described herein were in progress, a GIP fragment, GIP7-30amide, was shown to inhibit GIP-stimulated cAMP production *in vitro* and, at very high concentrations, to blunt GIP stimulated insulin secretion by approximately 75% in both anaesthetized and conscious rats (Tseng *et al.*, 1996a). Importantly, while GIP7-36amide did not affect GLP-1-stimulated insulin release in the anesthetized rat, there was a 75% decrease in post-prandial insulin levels in antagonist treated rats, although no significant change in glucose handling was observed. Since similar reductions in post-prandial insulin release were observed with the GLP-1 specific antagonist exendin (9-39) in conscious rats (Wang *et al.*, 1995), it seems that the two incretins together can account totally for the potentiation of insulin release resulting from the ingestion of nutrients. Given the conflicting results, it is still unclear which incretin, if either, is the more important, or if they are redundant systems that co-evolved to ensure blood glucose levels are well maintained.

There has been controversy concerning the relative potency of GIP from different animal species. The majority of studies using porcine GIP have shown it to be potently insulinotropic in rats (Pederson and Brown, 1976), humans (Elahi *et al.*, 1979), and other species (reviewed in Brown *et al.*, 1989; Pederson, 1993). However, while earlier studies showed that synthetic human (sh) GIP increased circulating insulin to levels similar to those observed with the natural porcine (np) GIP peptide (Nauck *et al.*, 1989), poor responses to shGIP were subsequently reported in both normal controls and individuals with NIDDM (Nauck *et al.*, 1993a), although these investigators did not comment on this

discrepancy. *In vitro* results suggesting that GLP-1 was insulinotropic at much lower peptide concentrations in rat islets (Siegel *et al.*, 1992) and the perfused rat pancreas (Shima *et al.*, 1988) led Jia *et al.* (1995) to examine a number of different peptide preparations in the isolated perfused rat pancreas. They observed a greatly reduced insulin response to a synthetic human preparation when compared to np and synthetic porcine (sp) GIP preparations, and suggested that different responses previously reported may in fact have been due to differences in the quality of the synthetic preparations, since the sequence differences between the peptides were relatively minor. Furthermore they found similar glucose and concentration thresholds (~20 pM) for spGIP and GLP-1 (Jia *et al.*, 1995). In a recent study it was found that both GIP and GLP-1 levels correlate with plasma insulin levels throughout the day, strongly supporting their incretin roles in glucose homeostasis (Ørskov *et al.*, 1996). However the exact overall and temporal contributions of these two incretins requires further examination.

1.5 GIP BINDING SITES

There have been relatively few studies on receptors for GIP, mainly due to the problem of producing biologically active ^{125}I-GIP peptide and the apparent low expression of such receptors. The presence of GIP receptors was first demonstrated in transplantable hamster insulinoma cells (Maletti *et al.*, 1984) and the insulin secreting hamster β-cell line, In 111 (Amiranoff *et al.*, 1984, 1985). GIP receptors have since been described on a number of other neoplastic cells, including human insulinomas (Maletti, *et al.*, 1987) and β-TC3 cells (Kieffer *et al.*, 1993). These early studies described high affinity binding sites, ranging from 0.2-7 nM, and most did not detect any cross reactivity

in binding with other members of the glucagon/secretin peptide superfamily, although Kieffer *et al.* (1993) observed that 1 μM glucagon displaced 20% of the ^{125}I-GIP binding to β-TC3 cells. Several studies identified both high and low affinity binding sites (Amiranoff *et al.*, 1984, 1985; Maletti *et al.*, 1984, 1987) using Scatchard analysis.

As a result of chemical (Couvineau *et al.*, 1984), or ultraviolet irradiation (Amiranoff *et al.*, 1986) cross-linking experiments, a 64 kDa ^{125}I-GIP-labelled protein was identified in membranes from β-cell tumors, suggesting a molecular weight of 59 kDa for the receptor. Treatment of these membranes, with the reducing agent dithiothreitol, decreased the receptors electrophoretic mobility indicating that the receptor contained intrachain disulfide bond(s). However these linkages did not appear necessary for binding of ^{125}I-GIP, as pretreatment of membranes did not interfere with labeling of the receptor (Amiranoff *et al.*, 1986). The GIP receptor was also shown to be a glycoprotein containing N-acetylglucosamine, mannose and possibly sialic acid (Amiranoff *et al.*, 1986).

1.5.1 ISOLATION OF THE GIP RECEPTOR cDNA AND GENE

In 1993 Usdin *et al.* isolated a partial cDNA from rat cerebral cortex, and a full length cDNA from a rat tumor cell line (RINm5F), encoding a putative seven transmembrane G-protein coupled receptor protein of 455 amino acids, with a high degree of similarity (25-45% identity) to other receptors in the VIP/glucagon/secretin receptor family. Receptors within this sub-family display little sequence identity to other G-protein receptors, such as the β-adrenergic receptors, have large amino-terminal extracellular domains and, as with the VIP/glucagon/secretin superfamily, appear to share

a common ancestral gene (Fehmann *et al.*, 1995; Ulrich et al., 1998). Analysis of the predicted amino acid sequence indicated that the receptor shared the greatest identity with the glucagon (44%) and GLP-1 (40%) receptors (Usdin *et al.*, 1993). The region of greatest divergence between these receptors was in the carboxy-terminal tail region, while the amino terminus-extracellular domain was well conserved, with 35% and 39% identity to the GLP-1 and glucagon receptors respectively. The receptor had a predicted molecular weight of 50,063 assuming cleavage of a putative 18 amino acid signal peptide. The presence of three potential glycosylation sites in the amino-terminal region may account for the discrepancy in size observed (~59 kDa) in previous cross-linking experiments (Couvineau *et al.*, 1984; Amiranoff *et al.*, 1986). Expression studies revealed that GIP was the only candidate peptide tested that elicited a high affinity cAMP response and increased intracellular calcium ($[Ca^{2+}]i$) in reporter cell lines (Usdin *et al.*, 1993).

One of the objectives of work described in this Thesis was to isolate and determine if the cDNA isolated from RIN cells (Usdin *et al.*, 1993) was also expressed in primary rat islets, to allow a more complete characterization of the receptor than has been possible with primary tissues and cell lines.

1.5.2 GIP RECEPTOR GENE EXPRESSION AND TISSUE DISTRIBUTION

Studies using Northern blot analysis, RT-PCR, and *in situ* hybridization in the rat have shown there is widespread tissue distribution of the GIP receptor mRNA, with significant levels in pancreas, stomach, intestine, adipose tissue, adrenal cortex, heart, lung and endothelium of major blood vessels, but not in the spleen or liver (Usdin *et al.*,

1993; Yasuda *et al.*, 1994). Several of these regions have not been considered as target tissues for GIP suggesting as yet unknown functions exist. Most surprising was the widespread distribution of the GIP receptor mRNA found in the brain (Usdin *et al.*, 1993). *In situ* analysis with GIP receptor specific oligonucleotides identified mRNA in the telencephalon, diencephalon, brain stem, cerebellum and pituitary (Usdin *et al.*, 1993). Autoradiographic studies using ^{125}I-GIP for the most part supported the regional localization of GIP mRNA in the brain (Kaplan and Vigna, 1994). For example, binding was observed in several areas of the telencephalon (motor, somatosensory, and auditory), forebrain (anterior olfactory nucleus, lateral septal nucleus, subiculum) and midbrain regions (inferior colliculus). In contrast, although GIP mRNA was detected in the pituitary by RT-PCR (Usdin *et al.*, 1993), and GIP was shown to affect anterior pituitary release of follicle-stimulating hormone (FSH) and growth hormone (GH) in ovarectomized rats (Ottlecz *et al.*, 1985), no binding sites were observed in either the pituitary or hypothalamus (Kaplan and Vigna, 1994). The distribution in regions of the brain which are inaccessible to blood borne peptides is especially confusing, in that neither GIP mRNA (Higasimoto *et al.*, 1992; Tseng *et al.*, 1993) nor IR-GIP (Buchan *et al.*, 1982) has been demonstrated in the brain. It is possible that the GIP mRNA is too rare too detect, or a homologous brain peptide exists. Preliminary studies by Usdin *et al.* (1993) on E12, E17 and E19 day rat embryos did not detect any GIP mRNA in the central nervous system of rats, suggesting that the peptide is not expressed during early development. It may also be that the receptor is expressed in the brain but does not serve a function.

Identification of the GIP receptor mRNA in the pancreas, stomach, and adipose tissue supports a direct action of the peptide on these tissues as discussed in section 1.3. The expression of GIP, GLP-1, and glucagon receptors has been examined in FACS purified α- and β-cells, and non-β-cell populations (Moens *et al.*, 1996). Northern blot analysis indicated that all three receptors were expressed at high levels in purified β-cells (≥90% pure), whereas only the GIP receptor appeared to be expressed at significant levels in the non-β-cell fraction (≥80% α-cells, ≤ 10% β-cells). Comparison of total RNA from a transplantable insulinoma (MSL-G2-IN) and co-derived glucagonoma (MSL-GAN) supported the selective expression of the GIP receptor mRNA on glucagon producing cells (Moens *et al.*, 1996). The lack, or low level expression, of the GLP-1 receptor on the alpha cell was confirmed with Western blots using a GLP-1 receptor specific antibody. These findings support a direct effect of GIP on the α-cell, as was suggested by studies in the perfused rat pancreas (Pederson and Brown, 1978), and strongly support its role in direct regulation of islet cell function.

Surprisingly, apart from the localization of the GIP receptor to islet cells of humans (Gremlich *et al.*, 1995), the only other studies to date on receptor expression in other tissues are those on the "ectopic" expression in adrenal cells from a group of patients with Cushing's syndrome (Lacroix *et al.*, 1992; Reznik *et al.*, 1992) and adrenal hyperplasia. Given that Usdin *et al.* (1993) identified GIP receptor mRNA in the rat adrenal, the food dependent hyper-GIP sensitivity and resulting cortisol hypersecretion, may be due to overexpression of the GIP receptor or a defect in the pathway normally regulating GIP sensitivity, rather than aberrant expression.

1.6 GLP-1 RECEPTOR BINDING STUDIES

As with the receptors for GIP, those for GLP-1 were first demonstrated on β-cell lines (Göke and Conlon, 1988) and later, on a number of insulinoma cell lines (reviewed in Fehmann *et al.*, 1995). In agreement with the somatostatinotropic activity of GLP-1, receptors have also been identified on somatostatin secreting cells (Fehmann and Habener, 1991). However, it is controversial as to whether they exist universally on α-cells, or α-cell lines (Matsumara *et al*, 1992; Fehmann and Habener, 1991; Moens *et al.*, 1996; Heller *et al.*, 1997). Studies with intact cells or cell membranes from insulinoma cell lines displayed a single class of high affinity binding sites, with Kd values ranging from 0.2-3.5 nM (reviewed in Fehmann *et al*, 1995). In similar cross-linking studies to those carried out with GIP, GLP-1 was associated with a single protein of an apparent molecular wt of 63 kDa. Unlike the GIP receptor, binding of ^{125}I-GLP-1 was partially displaced by glucagon and the related PG peptide oxyntomodulin. Complete displacement of ^{125}I-GLP-1 was obtained with GLP-1 (1-37), but with a significant shift to the right in receptor affinity (Göke and Conlon, 1988). These findings suggest that early observations of insulinotropic effects with high concentrations of GLP-1 (1-37) and oxyntomodulin were due to interaction with the GLP-1 receptor (Thorens and Widmann, 1996). No other related peptides (GIP, secretin, VIP) were shown to compete for GLP-1 binding (Fehmann *et al*, 1995).

1.6.1 ISOLATION OF THE GLP-1 RECEPTOR cDNA AND GENE

The GLP-1 receptor cDNA was isolated from a rat islet cDNA library by Thorens (1992) using an expression cloning strategy. Other groups soon followed with the

isolation of cDNAs from human islets (Dillon *et al.*, 1993; Thorens *et al.*, 1993), a human gastric tumor cell line (HGT) (Graziano *et al.*, 1993), rat lung (Lankat-Buttgereit *et al.*, 1994), and rat brain (Alvarez *et al.*, 1996). The human GLP-1 receptor gene has been mapped to the long arm of chromosome 6 (Stoffel *et al.*, 1993). Both the rat and human cDNA sequences encode for 463 amino acid receptor proteins that are highly conserved at both the amino acid (~91%) and nucleotide level (87%) (Dillon *et al.*, 1993; Thorens *et al.*, 1993; Graziano *et al.*, 1993).

1.6.2 GLP-1 RECEPTOR GENE EXPRESSION AND TISSUE DISTRIBUTION

The presence of GLP-1 receptor mRNA has been demonstrated in lung, kidney, pancreas, and brain tissues in mouse (Campos *et al.*, 1994), rat (Thorens, 1992; Bullock *et al.*, 1996; Alvarez *et al.*, 1995), and human (Wei and Mojsov, 1995). Furthermore, Wei and Mojsov (1996) have cloned and sequenced GLP-1 receptor cDNA's from brain and heart, and determined that they encode receptors with amino acid sequences identical to the pancreatic receptor. Two groups, using Northern blot analysis and RT-PCR, reported the presence of GLP-1 mRNA in liver from mouse and rat (Wheeler *et al.*, 1993; Campos *et al.*, 1994), and rat skeletal muscle (Wheeler *et al.*, 1993), while Egan and coworkers (1994) identified GLP-1 mRNA via RT-PCR in 3T3-L1 adipocytes. In contrast, two extensive studies using RNA protection assays (Wei and Mojsov, 1995; Bullock *et al.*, 1996), RT-PCR, and in situ hybridization (Bullock *et al.*, 1996) failed to demonstrate GLP-1 mRNA in adipose tissue, liver or skeletal muscle, suggesting that the extrapancreatic actions of GLP-1 in these tissues may be mediated via indirect actions, or another GLP-1 receptor. The latter of these two possibilities seems more likely given that

specific ^{125}I-GLP-1 binding has been demonstrated in membrane preparations from adipose tissue (Valverde *et al.*, 1993) and skeletal muscle (Delgado *et al.*, 1994). Interestingly, functional GLP-1 receptors and GLP-1 receptor mRNA have been demonstrated in several thyroid tumor derived cell lines (Vertongen *et al.*, 1994; Lamari *et al.*, 1996; Beak *et al.*, 1996), suggesting that GLP-1 may play a role either in the regulation of calcitonin secretion from thyroid C-cells, or as a feedback regulator of thyrotropin release from the anterior pituitary.

There is also evidence that GLP-1 receptors are involved in the regulation of exocrine secretion. ^{125}I-GLP-1 binding and Northern blot studies on highly purified parietal cells (Schmidtler *et al.*, 1994), and *in situ* hybridization studies (Bullock *et al.*, 1996), have demonstrated that parietal cells express the GLP-1 receptor. *In situ* hybridization studies also identified GLP-1 receptor mRNA within the crypts of the duodenum, and in large nucleated cells within the aveoli of the lung (Bullock *et al.*, 1996), the latter being consistent with stimulation of mucus secretion from submucosal glands in the trachea (Richter *et al.*, 1993).

While it is widely agreed that GLP-1 receptors exist on β-cells (see above references) and δ-cells (Heller and Aponte, 1995 and references therein), it still remains to be determined conclusively whether GLP-1 receptors exist on all islet α-cells or just a subpopulation. Several attempts have been made to study the intra-islet distribution of GLP-1 receptor mRNA. Bullock *et al.* (1996) observed colocalization of insulin and GLP-1 receptor mRNA, but could not determine cellular specificity. As mentioned in section 1.4.4 studies with FACS isolated islet cells suggested that the GLP-1 receptor was present on β-cells, but not α-cells (Moens *et al.*, 1996). In more recent studies Heller and

coworkers (1997), using double staining of dispersed islet cells with a GLP-1 receptor and islet hormone specific antibodies, identified the GLP-1 receptor on 90% of insulin positive cells, 76% of somatostatin positive cells and 20% of the glucagon positive cells. Using single cell RT-PCR they further demonstrated the presence of GLP-1 receptor mRNA and the PG mRNA within 2 out of 10 PG mRNA positive cells. In contrast, Ding *et al.* (1997) found that both GIP and GLP-1 stimulated glucagon secretion in FACS sorted α-cells and the majority of cells responded to GLP-1, suggesting that most, if not all, α-cells have GLP-1 receptors.

1.7 RECEPTOR SIGNAL-TRANSDUCTION PATHWAYS

1.7.1 GIP RECEPTOR SIGNAL-TRANSDUCTION MECHANISMS

GIP has been shown to stimulate adenylyl cyclase in pancreatic tumor cell lines (Amiranoff *et al.* 1984, Lu *et al.*, 1993a, Malleti *et al.*, 1987), a gastric cancer cell line (HGT-1) (Gespach *et al.*, 1984), isolated islets (Siegel and Creutzfeldt, 1985), as well as FACS sorted α- and β-cells (Moens *et al.*, 1996), with half-maximal (EC_{50}) values ranging from ~200 pM (Moens *et al.*, 1996) to 30 nM (Amiranoff *et al.*, 1984).

Although stimulation of adenylyl cyclase has been considered to be the primary mode of action for GIP, it has also been shown to increase influx of extracellular Ca^{2+} into mouse islets (Wahl *et al.* 1992), and to increase $[Ca^{2+}]i$ levels in HIT-T15 cells (Lu *et al.*, 1993a). Influx of extracellular Ca^{2+} via voltage-dependent channels (VDCC) appears to be the source of the increased $[Ca^{2+}]i$ in HIT T15 cells, as it was blocked by addition of EGTA or the voltage dependent L-type channel antagonist, nimodipine (Lu *et al.*, 1993a). However, there was no evidence that GIP increased inositol-1,4,5-

trisphosphate (IP_3) levels in HIT T15 cells, suggesting that phospholipase (PLC) mobilization of intracellular Ca^{2+} stores was not involved. In contrast Straub and Sharp (1996) reported that the phosphatidylinositol (IP) 3-kinase inhibitor, wortmannin severely decreased GIP mediated insulin release from HIT T15 cells; an observation they also made with the related peptides VIP and PACAP (Straub and Sharp, 1996). More recently Ding and Gromada (1997), examined the effect of GIP on voltage clamped individual mouse β-cells, using membrane capacitance as a measure of insulin secretion, whole-cell calcium currents to measure Ca^{2+} influx, and $[Ca^{2+}]i$ via fura-2 florescence. GIP appeared to stimulate insulin secretion with only a small increase in $[Ca^{2+}]i$ (30%), without affecting whole cell calcium currents. The increase in capacitance could be blocked by the protein kinase A (PKA) inhibitor Rp-8-bromo-cAMP, however the authors failed to determine if the $[Ca^{2+}]i$ increase from internal stores was blocked in these experiments. It was concluded that GIP increased insulin secretion by the cAMP/PKA pathway at a point distal to the increase in intracellular Ca^{2+}. The same authors have also shown that GIP stimulation of α-cells resulted in increased whole cell calcium currents that were at least in part mediated by the cAMP pathway (Ding *et al.*, 1997), suggesting that GIP receptor signaling may differ in different cellular environments.

A recent study showed GIP stimulated MAP kinase activity in both a cAMP-dependent and independent manner in CHO-K1 cells stably expressing the human GIP receptor (Kubota *et al.*, 1997). The possible contributions of this and other possible pathways, such as phospolipase A_2 (Lardinois *et al.*, 1990), and differences between pathways activated in different target tissues or cell types needs to be further explored.

1.7.2 GLP-1 RECEPTOR SIGNAL TRANSDUCTION

While some studies on the intracellular signaling of the GIP receptor have been carried-out, there is a much larger body of work examining GLP-1 receptor signal transduction. Given that the receptors share a great deal of identity, and at the physiological level appear to share a similar function, it is likely that they act though similar signaling pathways. However, the receptors differ in their peripheral effects, which may suggest that either different receptor species or isoforms exist, or that the receptors couple differentially in different cell types. It is therefore important not only to study and identify the similarities between GIP and GLP-1 receptor signaling, but also to identify differences that may exist in different cell types.

In common with GIP, GLP-1 activation of its endogenous receptor stimulates cAMP production. This has been demonstrated in β-cell lines (Drucker *et al.*, 1987; Lu *et al.*, 1993a; Widmann *et al.*, 1994), isolated β-cells (Moens *et al.*, 1996), isolated islets (Ahrén *et al.*, 1996) and cell lines transiently (COS-7) or stably expressing (CHL and CHO-K1 cells) expressing GLP-1 receptor cDNA s (Thorens, 1993; Dillon *et al.*, 1993; Wheeler *et al.*, 1993). There was good agreement between the EC_{50} values obtained in the different cellular systems (~0.5-3 nM) (Fehmann et al., 1995). Interestingly, Lu *et al.* (1993a) noted that both GIP and GLP-1 increased cAMP levels in the presence of 4 mM but not 0.4 mM glucose in HIT T15 cells, suggesting there was some glucose-dependence in incretin mediated cAMP production. However, studies in a number of cell lines (INS-1, RIN 1027-B2 and 1056A) did not demonstrate the same concentration dependence, and a glucose-dependence for incretin-stimulated cAMP generation in islet cells has not been demonstrated (Widmann et al., 1994).

Discrepancies exist in the literature concerning the effect of GLP-1 on $[Ca^{2+}]i$ in β-cell lines. There have been reports that GLP-1 induces large (Wheeler *et al.*, 1993; Holz *et al.*, 1995) or small (Ahrén *et al.*, 1996) increases in $[Ca^{2+}]i$ in HIT-T15 cells, small (Ahrén *et al.*, 1996) or no increases (Göke *et al.*; 1989) in RINmF5 cells, and large increases in both βTC-3 (Gromada and Rorsman, 1996) and β-TC6 cell lines (Holz *et al.*, 1995). Widmann and coworkers (1994) observed no increases in $[Ca^{2+}]i$ levels in any of the three cell lines (INS-1, RIN 1027-B2 and 1056A) they examined. It is hard to account for all these different observations, often using the same cell lines. However, many of these cell lines are pluripotent, and undergo differentiation or dedifferentiation depending upon the tissue culture conditions (Polak *et al.*, 1993). Additionally, it has been reported that GLP-1 mediated increases in $[Ca^{2+}]i$ may be small and relatively insignificant in dispersed islets and FACS sorted β-cells (Fridolf and Ahrén, 1993; Ahrén *et al.*, 1996; Ding and Gromada, 1997), suggesting that cell-cell interaction in the islet may be important.

One consistent observation in the above studies was that β-cell lines responding to GLP-1 with increased $[Ca^{2+}]i$, displayed a glucose-dependence to the response (Wheeler *et al.*, 1993; Holz *et al.*, 1995; Gromada *et al.*, 1996), although the glucose threshold was lower than that seen in β-cells (Cullinan *et al.*, 1994). Most groups have reported significant changes in $[Ca^{2+}]i$ in response to GLP-1 in the presence of elevated glucose levels (Yada *et al.*, 1993; Cullinan *et al.*, 1994; Holz *et al.*, 1993, 1995). In contrast, there is little agreement as to the source of the iCa^{2+}. Several studies have demonstrated that the effects of GLP-1 on Ca^{2+} can be mimicked with adenylyl cyclase activators such as forskolin, or membrane permeable cAMP analogs 8-bromo (Br)-cAMP and Sp-

adenosine-3'5'-monophospothionate triethylamine (sp-cAMP) (Yada *et al.*, 1993; Cullinan *et al.*, 1994; Holz *et al.*, 1995), or they can be blocked by protein kinase A inhibitors such as rp-cAMP or H-89, suggesting that the cAMP/PKA pathway is involved (Gromada *et al.*, 1995; Kato *et al.*, 1996). The removal of extracellular Ca^{2+} from the medium or addition of VDCC blockers often results in partial attenuation (Gromada *et al.* 1995; Holz *et al.*, 1995), complete blocking (Yada *et al.*, 1993; Cullinan *et al.*, 1994; Fridolf and Ahréns, 1993) or no effect (Wheeler *et al.*, 1993) on GLP-1-dependent Ca^{2+} responses in the different cellular systems, strongly supporting the involvement of influx from extracellular sources that may or may not be via VDCC. Recent studies have shown that GLP-1 slows calcium channel inactivation in a cAMP-dependent manner manner (Britsch *et al.*, 1995; Kato *et al.*, 1996), while other studies have identified a Na^{+}-dependence of the calcium influx, and a role for voltage-independent non specific cation channels (VINCCs) (Holz *et al.*, 1993; Fridolf and Ahréns, 1993; Kato *et al.*, 1996). It is not clear if this is a cAMP/PKA or a G-protein mediated effect on the VINCC itself (Fridolf and Ahréns, 1993; Holz *et al.*, 1993). Ding and Gromada (1997) have recently shown that neither GIP nor GLP-1 increase whole cell calcium currents in FACS sorted cells, suggesting that the small increases observed came from intracellular stores. The same authors have suggested that GLP-1 activation of the cAMP/PKA pathway may result in phosphorylation and sensitization of the ryanodine and/or IP_3 sensitive receptors to extracellular calcium mobilized by GLP-1 (Ding and Gromada, 1997; Gromada *et al.*, 1996). Many of the conflicting results are probably due to the different systems employed, and a description of the mechanisms by which GLP-1 increases iCa^{2+} remains

to be finalized. It is also likely that both GLP-1 and GIP activate alternative signal transduction pathways in different cell types and in different physiological environments.

1.8 GLUCOSE-DEPENDENCE OF GIP AND GLP-1 STIMULATED INSULIN RELEASE

GIP and GLP-1 potentiate insulin release only in the presence of elevated glucose levels. Glucose-induced insulin secretion requires glucose uptake, phosphorylation by glucokinase, and cellular metabolism. This is thought to increase the ATP/ADP ratio within the cell, resulting in the closure of ATP-dependent K^+ channels. Closure of the channels results in depolarization of the β-cell and the opening of VDCCs allowing the influx of Ca^{2+}, resulting in the exocytosis of insulin (Reviewed in Holz and Habener, 1992; Fehmann et al., 1995). Exactly how GIP and GLP-1 signal transduction pathways interact with this glucose-signaling pathway has not been determined, and there have been several hypotheses.

Increasing cAMP levels, in cells in which the Ca^{2+} levels are maintained constant, still stimulates insulin secretion suggesting that, as of yet unidentified, proteins of the exocytosis machinery may be activated by PKA phosphorylation (Ämmälä *et al.*, 1993). As mentioned in section 1.7.2 there is some evidence that GLP-1, and by extrapolation GIP, may slow VDCC inactivation and in this way potentiate insulin secretion (Britsch *et al.*, 1995; Kato *et al.*, 1996) although the extent to which this augments the influx of Ca^{2+} appears to be minimal (Ämmälä *et al.*, 1993; Thorens and Widmann, 1996). However, if calcium induced calcium release (CICR) is actually a characteristic of incretin signal transduction this may be an important priming effect that allows the eventual release of

intracellular stores or influx of Ca^{2+} via membrane VDCC (Gromada and Rorsman, 1996; Gromada *et al.*, 1996). Gromada *et al.*, (1996) suggested that the activation of PKA may lead to the phosphorylation of the IP_3 receptor and/or the ryanodine receptor resulting in their sensitization to increases in IP_3 or Ca^{2+}, respectively. It is also possible that the GLP-1-dependent increases in cAMP levels lead to activation of VINCC responsible for the Na^+-dependent influx of Ca^{2+}, augmenting membrane depolarization and activation of VDCC (Holz *et al.*, 1995; Kato *et al.*, 1996; Fridolf and Ahréns, 1993). GLP-1 may act synergistically with glucose metabolism to inactivate the ATP-dependent K^+ -channels (Holz *et al.*, 1993), however more recent studies have questioned if GLP-1 has effects on K^+ currents (Britsch *et al.*, 1995; Kato *et al.*, 1996). GLUT2 has recently been shown to be phosphorylated in β-cells in response to GLP-1 (Thorens *et al.*, 1996), resulting in a 40% decrease in the transporter's activity. If and how this phosphorylation acts to potentiate insulin secretion remains to be determined.

Both GIP and GLP-1 have also been shown to increase both proinsulin gene expression and biosynthesis in β-cell lines (Fehmann and Göke, 1995; Wang *et al.*, 1995a, 1996; Drucker *et al.*, 1987; Fehmann and Habener, 1992). Interestingly, extended (6-24h) incubations of RIN 1046-38 cells with GIP and GLP-1 both increased the expression of hexokinase I and GLUT1, but not GLUT2 or glucokinase mRNA levels (Wang *et al.*, 1995a, 1996), suggesting that the incretins are able to regulate the glucose sensing elements. It still remains to be determined if GIP and GLP-1 have similar effects in primary β-cells.

1.9 GIP AND THE GIP RECEPTOR IN NON-INSULIN DEPENDENT DIABETES MELLITUS (NIDDM)

Fasting GIP levels have been reported to be normal or elevated in individuals with NIDDM when compared to healthy individuals. GIP secretion in response to a meal has also been reported to be increased, normal or blunted in patients with NIDDM, but appears to be normal in those with IDDM (for specific references see reviews by Pederson, 1993; Crueutzfeldt and Ebert, 1993). Crueutzfeldt *et al.* (1983) determined the integrated IR-GIP response in 141 individuals with NIDDM and found a bimodal distribution with a large group of hypersecretors and a smaller group of hyposecretors when compared to normal controls. Jones *et al.* (1989a,b) demonstrated that GIP infusion under basal glycemic conditions resulted in stimulation of insulin release in individuals with NIDDM. This suggests that the β-cells in GIP hypersecretors may be constantly stimulated throughout the day.

Studies on obese patients found similar discrepancies in both fasting and food stimulated GIP release, however the test meal used and the rate of gastric emptying may have played a role in the elevated responses observed (Creutzfeldt and Ebert, 1993). When obese subjects were further subdivided on the basis of having normal or impaired oral glucose tolerance (OGT), obese individuals with impaired OGT always displayed exaggerated GIP responses, while obese or lean individuals with normal OGT displayed normal (Creutzfeldt *et al.*, 1978), or elevated GIP levels (Salera *et al.*, 1982). In contrast, in a recent study of post-menopausal women with impaired OGT, GIP levels were found to be decreased while GLP-1 levels were unchanged (Ahrén *et al.*, 1997), suggesting that a decrease in GIP secretion contributes to the impaired OGT in this group.

It is clear that individuals with NIDDM have blunted or ablated incretin responses (Nauck *et al.*, 1986, 1996; Holst *et al.*, 1997), but obese individuals are still GIP responsive (Amland *et al.*, 1985; Elahi *et al.*, 1984), and therefore may have inappropriate GIP-mediated insulin release if they are glucose-intolerant. Furthermore, it has been proposed that the hyperinsulinaemia that accompanies impaired OGT in obesity may lead to desensitization of the K-cell to normal feedback inhibition by insulin and result in hypersecretion of GIP (Creutzfeldt *et al.*, 1978). Interestingly, the hyperGIPemic response could be reversed by starvation or dietary restriction in the obese individuals with impaired OGT (Willms *et al.*, 1978; Ebert *et al.*, 1979a; Deschamps *et al.*, 1980). It has not been determined if mutations within the genes for GIP or GLP-1 are linked to NIDDM.

The glucose-dependence of both GIP and GLP-1 stimulated insulin secretion makes them prime candidates for the treatment of NIDDM. Unlike the sulfonylureas currently used to treat NIDDM, the incretins only stimulate insulin secretion in the presence of elevated glucose, and therefore never induce hypoglycemia. As mentioned above, responses to GIP infusion in individuals with NIDDM have been shown to be severely blunted or absent (Nauck *et al.*, 1993a; Jones *et al.*, 1989; Krarup *et al.*, 1987; Elahi *et al.*, 1994). However, some studies have shown that, while GIP may have little effect on insulin release in individuals with NIDDM, the incretin response is maintained for GLP-1 at supraphysiological levels (Nauck *et al.*, 1993a,b, 1996; Elahi *et al.*, 1994; Holst *et al.*, 1997). Furthermore, GLP-1 has been demonstrated to normalize fasting (Nauck *et al.*, 1993b, 1996; Nathan *et al.*, 1992), and reduce postprandial (Gutniak *et al.*, 1992, 1994) glycaemia. However, the latter finding may have been in part due to

inhibition of gastric emptying by GLP-1 resulting in slower nutrient uptake (Wettergren *et al.*, 1993; Willms *et al.*, 1996). Inhibition of gastric emptying probably also accounted for the observed decrease in postprandial glycaemic excursions in GLP-1 treated insulin-dependent diabetics (Dupre *et al.*, 1995) and may compromise its use in the treatment of NIDDM (Nauck *et al.*, 1997b).

The β-cell insensitivity to physiological levels of both GIP and GLP-1 suggests that there may be a defect at the receptor level responsible for loss of the incretin effect. The fact that GIP responsiveness is more severely affected than GLP-1 suggests that the GIP receptor signaling pathway may be impaired to a greater extent than that of the GLP-1 receptor, and that GIP plays a more important role in glucose homeostasis than GLP-1. While initial genetic studies have failed to link the GLP-1 receptor to NIDDM (Tanizawa *et al.*, 1994; Zhang *et al.*, 1994), more recent studies have identified two missense mutations in the human GIP receptor gene, Gly198→Cys (Gly198Cys) and Glu354→Gln (Glu354Gln), within the predicted second extracellular loop and sixth transmembrane domains of the receptor, respectively (Kubota *et al.*, 1996). When expressed in CHO cells the Glu354Gln mutant displayed similar function to the wild type (wt) receptor, whereas Gly198Cys displayed a shift to the right in its half-maximal stimulation value (EC_{50}). However linkage studies failed to show any linkage between NIDDM in Japanese subjects and either of the two mutations. Further studies are needed to confirm or exclude a role for the GIP receptor in NIDDM. Given that NIDDM is a polygenic disease, even if mutations exist in either incretin receptor, they may only be a minor risk factor or a major risk factor in a small number of individuals (Tanizawa *et al.*, 1994). This is exemplified by studies on transgenic mice homozygous for a targeted null

mutation in the GLP-1 receptor gene, which displayed mild fasting hyperglycaemia, and reduced glucose tolerance (Scrocchi *et al.*, 1996). Pederson (personal communication) found the same mice to have normal fasting glucose, and mild hyperglycaemia in OGTT, suggesting unidentified environmental factors, such as food source, are involved in the development of impaired glucose tolerance in this model. It also suggests that GIP is in part able to compensate for the loss of the GLP-1 stimulatory activity at the β-cell. Further examination of the contribution of GIP/GLP-1 to NIDDM and their receptors is required to determine their exact role, and possible exploitation as therapeutics, in NIDDM.

1.10 STRUCTURE-FUNCTION STUDIES OF GIP

1.10.1 IDENTIFICATION OF A BIOLOGICAL CORE IN GIP

Studies from a number of laboratories have demonstrated that limited truncation of GIP at the carboxy-terminus has relatively minor effects on its insulinotropic activity. Thus porcine GIP1-38 (Moroder *et al.*, 1978) and bovine GIP1-39 (Sandberg *et al.*, 1986) exhibited similar insulinotropic activities to GIP1-42 in the perfused rat pancreas. Further truncation produced two fragments, GIP1-31 and GIP1-30, with very interesting characteristics. The former peptide was shown by Malleti *et al.* (1987) to have a receptor binding affinity an order of magnitude lower than GIP1-42. shGIP 1-30amide was shown to be equally insulinotropic to npGIP1-42 in the perfused rat pancreas (Wheeler et al., 1995; Morrow *et al.*, 1996), but to have only 30% of the somatostatin stimulatory activity in the perfused rat stomach. Rossowski *et al.* (1992) showed that spGIP1-30amide and npGIP1-42 had similar inhibitory activity on bombesin-stimulated amylase secretion, but

spGIP1-30amide was far less potent at inhibiting pentagastrin stimulated acid release than npGIP1-42. These studies suggest that different regions of the GIP molecule are involved in different biological actions; i.e. the somatostatinotropic domain lies in the carboxy-terminal region, while the insulinotropic region exists in the amino-terminus.

Studies involving the truncation of the amino terminus of GIP produced a confusing picture. Jörnvall *et al.* (1981) identified a contaminant in a npGIP preparation that corresponded to npGIP3-42, and which was found to be biologically inactive (Brown *et al.*, 1981; Schmidt *et al.*, 1986a). The enzymatically produced bovine (b) GIP4-42 was shown to have similar affinity for the GIP receptor on hamster insulinoma cell membranes, and still retained 10% of the insulinotropic activity in the perfused rat pancreas when compared to the intact peptide (Maletti *et al.* 1986). These results were in apparent conflict with studies that showed that npGIP15-42, produced by cyanogen bromide cleavage (Pederson and Brown, 1976), and bGIP17-42, produced by enterokinase cleavage (Carlquist *et al.*, 1984; Maletti *et al.*, 1986), displayed significant insulinotropic activity. Maletti *et al.* (1986) also found that the fragments bGIP1-16 and bGIP19-30 failed to compete for ^{125}I-GIP binding, and concluded that the insulinotropic domain lay between residues 17-38. However, this was in contrast to the observations that GIP17-42 did not stimulate cAMP production in RINm5F cells (Gallwitz *et al.*, 1993), and GIP19-42 failed to stimulate insulin release from isolated islets (Schmidt *et al.*, 1986b), whereas GIP1-42 was active in both systems.

From the above observations, Morrow *et al.* (1996) predicted that the insulinotropic activity of the peptide must reside in residues 15-30, and then used the perfused rat pancreas to examine a number of fragments within this region for

insulinotropic activity. It was found that while spGIP15-30 was devoid of any significant activity, cleavage to pGIP17-30 (with enterokinase) and to pGIP19-30 (with trypsin) restored ~30% of the insulinotropic activity seen with the intact peptide. A synthetic preparation of spGIP17-30 was also found to be insulinotropic, supporting the results with the enzymatically-produced fragment. A confusing observation, made later by the same group was that these peptides failed to displace ^{125}I-GIP binding to β-TC3 cells, suggesting the peptide's site of action may be at a receptor other than the GIP receptor (Morrow, Pederson and McIntosh, personal communication).

The related hormone glucagon has recently been shown to be metabolized at target tissues to glucagon9-29 or "mini-glucagon", which has antagonistic effects on glucagon's positive ionotropic effects, and insulinotropic activity (Blache *et al.*, 1994). Ohneda and Ohneda (1994) reported that further truncation of glucagon to fragments 23-29, 21-29, 17-29 restored some insulinotropic activity to the peptides. Given that this region is well conserved between glucagon, GIP and GLP-1 it may be that it is a structurally similar region containing sequences required for receptor interaction and activation. Identification of such a common sequence should assist in the design of analogs with antagonist or improved agonist activity.

1.10.2 GIP IS METABOLIZED TO GIP 3-42 BY DIPEPTIDYL PEPTIDASE IV (EC3.4.14.5)

Dipeptidyl peptidase IV (DP IV) is a selective serine protease which preferentially hydrolyses peptides after a penultimate N-terminal proline (Xaa-Pro) or alanine residue (Xaa-ala) (Heins *et al.*, 1988), requires that amino acids in positions P1 and P2 be L-

isomers in the trans conformation (Fisher *et al.*, 1983), and the N-terminus be protonated (Demuth and Heins, 1995). DP IV has a wide tissue distribution and has been shown to degrade a number of regulatory peptides such as substance P, human α-relaxin, human pancreatic polypeptide, human chorionic gonadotropin, prolactin, neuropeptide Y, peptide YY, and β-casomorphin (Mentlein, 1988; Nausch *et al.*, 1990; Wang *et al.*, 1991; Mentlein *et al.*, 1993a). Once cleaved these peptides become susceptible to cleavage by other exopeptidases (Mentlein *et al.*, 1988). It was of particular interest that the 44 amino acid growth hormone-releasing hormone (GRH), a member of the VIP/glucagon/secretin family, was rapidly degraded by DP IV to biologically inactive GRH3-44 both *in vitro* and *in vivo* (Frohman *et al.*, 1986). Amino-terminal substituted analogs of GRH: des-amino (NH_2)-Tyr^1-, D-Tyr^1-, and D-Ala^2-GRH were all found to be resistant to DP IV cleavage (Frohman *et al.*, 1989). Earlier studies with D-Ala^2-GRH1-29amide and D-Tyr^1-GRH1-29amide, and to a lesser extent des-amino-Tyr^1-GRH1-29 truncated GRH analogs, showed they demonstrated an increased potency compared to GRH1-29amide in stimulating growth hormone (GH) release both in vivo (Lance *et al.*, 1984) and in vitro (Heiman *et al.*, 1985). It is possible that this was due to an increased biological half-life due to their resistance to DP IV degradation, however the authors thought it was more likely that the analogs had higher affinity for the GRH receptor (Lance *et al.*, 1984).

Many of the peptides of the glucagon/VIP/secretin family share a considerable sequence similarity at their amino-terminus beginning with either a Tyr-Ala, His-Ala, or His-Ser (Rosselin, 1986). It therefore seemed likely that a possible source of the contaminating GIP 3-42 in early porcine GIP preparations (Jörnvall *et al.*, 1981) was due to DP IV degradation. Mentlein *et al.* (1993b) demonstrated that GIP and GLP-1 were

substrates of circulating DP IV and cleavage renders these non-insulinotropic (Brown *et al.*, 1981; Schmidt *et al.*, 1986a; Suzuki *et al.*, 1989; Gefel *et al.*, 1990). The kinetics of DP IV degradation of GIP1-42 and GLP-1 by purified placental DP IV was rapid and suggested that this may be an important pathway for the degradation of these two peptides. This proposal was supported by Kieffer *et al.* (1995b) who demonstrated, by high performance liquid chromatography (HPLC), degradation of physiological levels of ^{125}I-GIP and ^{125}I-GLP-1 to ^{125}I-GIP3-42 and ^{125}I-GLP-1(9-36), following injection into rats. The degradation was very rapid, with over 50% of the injected label being degraded within the first 2 minutes, supporting a possible physiological role in the regulation of incretin actions. Recent studies by Pauly *et al.* (1997) using the highly sensitive and accurate technique of matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) have further confirmed that GIP 3-42 and GLP-1 (9-36) are the major degradation products resulting from incubation with human serum. This degradation was blocked both *in vitro* and *in vivo* by the inclusion or administration of the DP IV specific inhibitor Ile-thiazolidide, resulting in an early peak in plasma insulin and better glucose handling in the anesthetized rat (Pauly, 1996). Experiments in patients with NIDDM have indicated that GLP-1 is rapidly degraded to the appropriate DP IV hydrolysis product (Deacon *et al.*, 1995), suggesting that a defect in DP IV, resulting in prolonged exposure to biologically active GIP and GLP-1, does not underlie the reduced responsiveness of these individuals to incretins. It is therefore of interest to determine if DP IV inhibitors, or alternatively DP IV resistant analogs of GIP or GLP-1 can be used in the treatment of NIDDM.

1.11 STRUCTURE-FUNCTION STUDIES OF G-PROTEIN COUPLED RECEPTORS (GPCR)

Since the isolation and cloning of the β-adrenergic receptor (Dixon *et al.*, 1986), the development of molecular biological techniques has allowed the cloning of receptors without protein purification, and resulted in an explosion in the number of GPCRs isolated and identified (reviewed in Houslay, 1992; Simonsen and Lodish, 1994). Additionally, the use of site-directed mutagenesis has facilitated studies into the structure and function of GPCRs by allowing the manipulation of different regions of the receptor to determine their specific functions. In general these studies have looked at regions important to receptor binding, receptor activation, G-protein coupling, receptor desensitization, sequestration and/or downregulation. The β2-adrenergic receptor (AR) has been the most thoroughly characterized of these receptors (for recent reviews see, Hein and Kobilka, 1995; Lefkowitz et al., 1993). While many of the findings with the β2-AR can be extrapolated to other G-protein coupled receptors, many studies with individual receptors continue to identify different or additional regions involved in receptor structure and function. Given that GIP or its receptor may play a role in NIDDM, and that GIP or GIP-analogs may be useful in the treatment of NIDDM, an understanding of GIP receptor physiology and structure-function will greatly aid in identifying possible defects in diseased states, and in the design of possible useful peptidomimics.

1.11.1 RECEPTOR BINDING DOMAINS

The first step in receptor activation requires that the ligand associate with high affinity and in a specific manner with its appropriate receptor. Among the GPCRs there is diversity in the structural determinants of ligand binding (See Strader *et al.*, 1994, and Ulrich *et al.*, 1998 for reviews). The membrane-spanning regions constitute the binding domain for the cationic amine receptors (Strader *et al.*, 1994), whereas both the TM spanning segments and extracellular loops contribute to receptor binding of opioid peptides (Fukuda *et al.*, 1995; Varga *et al.*, 1996). In contrast, the binding of glycoprotein hormones is thought to be primarily to a long glycosylated extracellular domain (reviewed in Combarnous, 1992). The N-terminal (NT) extracellular domain and extracellular (EC) loops are also considered to determine binding of the neurokinins (Strader *et al.*, 1994).

Less is known regarding the binding domains of the secretin-VIP sub-class of receptors. The use of molecular biology techniques to synthesize cDNA's encoding chimeric proteins is a common technique used to examine the function of a small specific region or domains of a protein in the absence of the rest of the endogenous sequence, but allowing its expression in an appropriate manner or environment. This methodology has been used successfully in the examination of the secretin-VIP and other subclasses of receptors. Chimeric Secretin/VIP receptor studies demonstrated that the NT-domains of these proteins were involved in specific ligand binding (Holtmann *et al.*, 1995; Gourlet *et al.*, 1996). However, the NT region of the VIP receptor appeared to play a greater role in VIP binding than the secretin receptors, and EC loops one and two, but not three have been shown to also contribute to secretin binding (Holtmann *et al.*, 1995a,b, 1996). This

conclusion was supported by findings using chimeric proteins consisting of the secretin and, the more distantly related, PTH receptors (Turner *et al.*, 1996a). Receptors consisting of the PTH receptor NT and secretin receptor body bound ^{125}I-secretin and were responsive to secretin, while the inverse protein (secretin receptor NT and PTH receptor body) bound and responded to PTH. Similarly, studies on calcitonin/glucagon receptor chimeras indicated the NT-domain of the calcitonin receptor is involved in binding, but that the body of the receptor is required for signaling (Stroop *et al.*, 1995; Bergwitz *et al.*, 1996). Interestingly, neither chimera examined in that study (glucagon receptor NT/calcitonin body or calcitonin NT /glucagon body) bound or responded to glucagon, suggesting that its binding required regions present in both NT and body regions of the receptor.

Other evidence has been presented suggesting that the NT domain of the glucagon receptor is involved in ligand binding (Buggy *et al.*, 1995; Carruthers *et al.*, 1994; Unson *et al.*, 1995). While some GLP-1/glucagon receptor chimera (Buggy *et al.*, 1995) and antibody (Unson *et al.*, 1996; Buggy *et al.*, 1996) studies suggested that the membrane proximal region of the glucagon receptor was important for ligand binding, other site directed mutagenesis and receptor chimera studies suggested a more distal region (residues 29-32) was involved in ligand selectivity and binding (Graziano *et al.*, 1996). Findings by both Buggy and colleagues (1995) and Unson *et al.* (1996) suggested that the first EC domain may also be important for glucagon receptor binding. Interestingly, the extracellular domain of the closely related GLP-1 receptor, when expressed alone, has intrinsic GLP-1 binding activity (Wilmen *et al.*, 1996). By analogy it seems likely that the N-terminal region of the GIP receptor is involved in its ligand binding, however this

remains to be confirmed. It is also of interest to determine exactly how the closely related GIP, GLP-1, and glucagon receptors discriminate between their highly conserved ligands.

1.11.2 RECEPTOR-G PROTEIN COUPLING

Upon binding their ligand, GPCRs transduce the signal from the extracellular surface to the intracellular side by coupling to a hetertrimeric GTP binding (G)-protein(s), which can then initiate a variety of intracellular biochemical signals. Extensive mutagenesis and chimeric receptor studies on rhodopsin, and the β-adrenergic and cholinergic receptors, have indicated that all of the intracellular loops of the seven transmembrane class of receptors are involved in G-protein binding, although the N- and C-termini of the third intracellular loop are considered to be of primary importance for both G-protein binding and conferring specificity of action (O'Dowd *et al.*, 1988; Wong *et al.*, 1990; Liggett *et al.*, 1991; Hedin *et al.*, 1993; Burstein *et al.*, 1998). While no studies of these regions have been carried out with the GIP receptor, residues within the proximal region of the third and first intracellular loops of the closely related GLP-1 receptor (Takarh *et al.*, 1996; Mathi *et al.*, 1997; Heller *et al.* 1996), and the second and third intracellular loops of the glucagon receptor (Chicchi *et al.*, 1997) were shown to be involved in G-protein coupling.

In contrast, less is known regarding the function of the C-terminal (CT)-tail of G protein-coupled receptors. This region has been implicated in receptor desensitization and endocytosis (Reneke *et al.*, 1988; Hausdorf *et al.*, 1990b; Huang *et al.*, 1995a), and the membrane proximal region of the β_2-adrenergic receptor CT-tail has been shown to

be important for G protein coupling (O'Dowd *et al.*, 1988). Additionally, the CT-tail has also been suggested to play a role in directing transport of the receptor to the plasma membrane (Parker and Ross, 1991) and restricting lateral membrane movement (O'Dowd *et al.*, 1988; Parker and Ross, 1991). Studies on CT-tail truncated members of the secretin-VIP-glucagon family of receptors, suggested that reduction in the length of the C-terminal tail increased agonist binding of the PTH/PTH-RP (Iida-Klein *et al.*, 1995), calcitonin (Findlay *et al.*, 1994), and glucagon (Unson *et al.*, 1995) receptors, whereas effects of truncation on adenylyl cyclase responses varied from inhibition (Findlay *et al.*, 1994) to stimulation (Iida-Klein *et al.*, 1995). In more recent studies it was shown that approximately half the GLP-1 receptor's CT (Widmann *et al.*, 1997), and the majority of the glucagon receptor's CT-region (Buggy *et al.*, 1997) could be truncated with no effect on receptor affinity or expression.

1.11.3 RECEPTOR ACTIVATION AND CONSTITUTIVELY ACTIVE RECEPTORS

The binding of a receptor and its ligand, results in a conformational change that allows the receptor to associate with G-proteins, causing the G-protein to exchange bound GDP for GTP resulting in the G-protein activation. As high affinity binding only occurs when the receptor-ligand complex is also associated with a G-protein, a model accounting for the formation of such a "ternary complex" was initially proposed by DeLean and co workers (1980) and is shown in Fig. 5A. However, this model failed to explain results from studies of the α_1- (Cotecchia *et al.*, 1990; Kjelsberg *et al.*, 1992), α_2- (Ren *et al.*, 1993), and β_2- (Samama *et al.*, 1993) adrenergic receptors (AR), that identified mutations resulting in receptors that were active even in the absence of bound ligand. Nor could it

account for the observations that increasing β_2-adrenergic receptor density appeared in some cases to increase basal activity (Samama *et al.*, 1993; Adie and Milligan, 1994), and that ectopic expression of a receptor could result in high basal activity (Costa and Herz, 1989; Costa *et al.*, 1990; Tiberi and Caron, 1994; Jakubik *et al*, 1995). These data suggested that GPCR's could exist in an equilibrium between both an inactive (R) and active (R*) state (Samama *et al.*, 1993), the latter of which can associate in the absence of ligand with G-protein and account for "basal" receptor activity. This "allosteric" ternary complex model (Samama *et al.*, 1993) (Fig. 5B) illustrates the possible conformations and associations of receptor, ligand, and G-protein. In this model receptors do not have to bind their ligand to be activated. Agonists act to stabilize receptors in the R* state shifting the equilibrium to the active state resulting in an increase in receptor signaling, while inverse, or reverse, agonists act to stabilize the receptor in the inactive state (Fig. 5C) (Samama *et al.*, 1993; see Milligan *et al.*, 1995, and Scheer and Cotecchia, 1997 for recent reviews). While *in vitro* evidence for inverse agonism has existed for a long time (Milligan *et al.*, 1995; Scheer and Cotecchia, 1997), more direct evidence has been presented using transgenic mice overexpressing the β_2-adenergic receptor in a heart specific manner (Bond *et al.*, 1995). These mice displayed both increased basal cAMP levels and increased cardiac contractility, and the β_2-adenergic ligand ICI-188551 acted as an inverse agonist, decreasing ventricular systolic pressure both *in vivo* and *in vitro*.

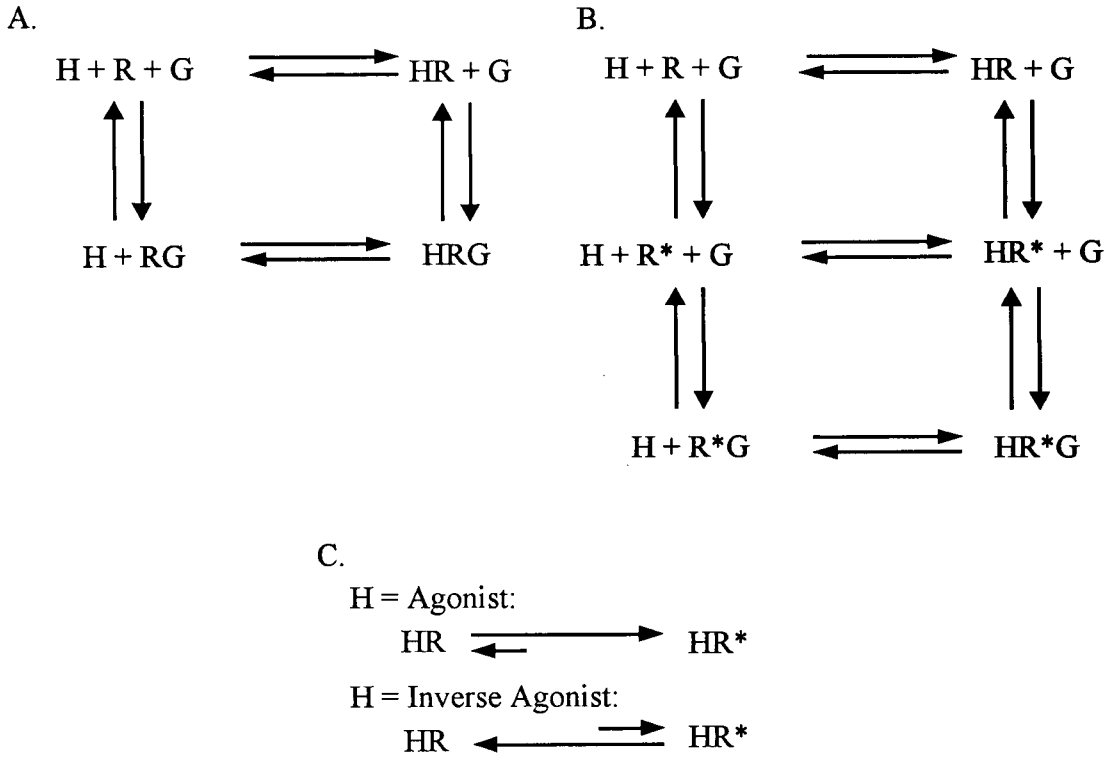


Fig. 5. The Ternary Complex Model (TCM) and Inverse Agonism. Representative diagrams of possible receptor (R)/hormone (H) and G-protein interactions. A. The TCM as originally proposed by Delean et al. (1980). B. The TCM as later modified by Samama and coworkers (1993) where it is possible for R to undergo an allosteric transition to the R* intermediate which can then interact with H and G. C. While agonists act to stabilize the R* form of the receptor, shifting the equilibrium towards the "active" form (R*), inverse agonists stabilize the majority of receptor in the inactive (R) state. Adapted from Samama et al. (1993).

Studies have also identified naturally occurring mutations in GPCRs resulting in a number of pathological states such as autosomal dominant retinitis pigmentosa and night blindness (rhodopsin receptor), familial male precocious puberty (luteinizing hormone receptor), hyperfunctioning thyroid adenomas (thyroid stimulating hormone receptor) and inherited hyperpigmentation of a mouse strain (melanocyte-stimulating hormone receptor) (reviewed in Milligan *et al.*, 1995 and Scheer and Cotecchia, 1997). Recently, the first example of such constitutively activating mutations in the glucagon/secretin/VIP receptor family were identified (Schipani *et al.*, 1995, 1996). Substitution of a histidine residue at the junction of the first intracellular loop (IC) with arginine (H170R), or a threonine in the sixth transmembrane (TM) domain with proline (T410P), results in constitutively active forms of the PTH/PTHrP receptor associated with a rare form of dwarfism, Jansen-type metaphyseal chondysplasia (Schipani *et al.*, 1995, 1996). The histidine residue at the base of the second TM is conserved in all members of the glucagon/secretin/VIP receptor family suggesting that it may play a universal role in regulating spontaneous receptor activation (Schipani *et al.*, 1995).

The availability of constitutively active receptors has assisted in the identification of inverse agonists (Samama *et al.*, 1993; Jakubik *et al.*, 1995; Gardella *et al.*, 1996). While it remains to be shown that inverse agonists have any benefit over neutral antagonists in clinical applications, it can be imagined that they may be of great use in the treatment of pathologies due to constitutively active receptors (Bond *et al.*, 1995; Milligan *et al.*, 1995).

1.11.4 RECEPTOR PHOSPHORYLATION AND DESENSITIZATION

The exposure of a receptor to high and/or continual exposure of an agonist can lead to a reduced cellular response. The attenuation of a ligand's signal can occur at many levels, including ligand removal, uptake or degradation, receptor phosphorylation, receptor sequestration or internalization and, finally, downregulation of both receptor protein and gene expression (for recent reviews see Hausdorff *et al.*, 1990a; Chuang *et al.*, 1996; Ferguson *et al.*, 1996a; Böhm *et al.*, 1997).

The term desensitization, in the case of GPCRs, usually refers to the rapid plateau or return to near basal levels seen within a few minutes of agonist stimulation. This attenuation of the cellular signaling occurs in seconds or minutes and involves receptor phosphorylation, resulting in the uncoupling of receptor from G-proteins (Hausdorff *et al.*, 1990a; Chuang *et al.*, 1996; Ferguson *et al.*, 1996a; Böhm *et al.*, 1997). The rapid phosphorylation associated with receptor desensitization is mediated by two classes of serine/threonine protein kinases: the second messenger activated protein kinases, PKA and PKC, and the G-protein coupled receptor kinases (GRKs) that preferentially phosphorylate agonist-activated GPCRs. Receptor desensitization can be functionally differentiated into agonist-dependent, or homologous desensitization, and agonist-independent or heterologous desensitization. Homologous desensitization of a receptor refers to the blunting or reduced sensitivity of a response to specific ligands of that receptor. In contrast, heterologous desensitization of a receptor is a result of the activation of a separate, different type of receptor and subsequent second messenger-dependent protein kinase phosphorylation (Hausdorff *et al.*, 1990; Ferguson *et al.*, 1996a; Chuang *et al.*, 1996; Böhm *et al.*, 1997).

While in general terms it can be said that heterologous desensitization is mediated mostly by second messenger-dependent protein kinases, and homologous desensitization is mediated mostly by GRKs, the relative contribution of the two kinase types to homologous desensitization depends on the cell type, receptor type, or even the concentration of agonist being examined (Ferguson *et al.*, 1996a; Chuang *et al.*, 1996). For example, PKA phosphorylation appears to be important for homologous desensitization of the β_2-adenergic receptor at lower agonist concentrations (Hausdorff *et al.*, 1989; Lohse *et al.*, 1990a), while at higher concentrations GRK mediated phosphorylation plays a larger role in this receptor desensitization (Hausdorff *et al.*, 1989; Lohse *et al.*, 1990a; Roth *et al.*, 1991).

As mentioned in section 1.11.2 the CT-tail of many G-protein coupled receptors have been implicated in receptor desensitization and sequestration. For the prototypic β_2-adenergic, and many other receptors, residues within the CT-tail region have been implicated in receptor phosphorylation, desensitization, and sequestration (Hausdorff *et al.*, 1990; Chuang *et al.*, 1996; Ferguson *et al.*, 1996a; Böhm *et al.*, 1997). With other receptors that either have short CT-tails, and/or few Ser/Thr residues in the CT-tail region such as the α_2-adenergic and m_2 muscarinic (m_2-AchR) receptors, the large third intracellular loops appear to be the site of GRK phosphorylation (Liggett *et al.*, 1992; Eason *et al.*, 1995; Ferguson *et al.*, 1996a). As mentioned above, GRK phosphorylation of receptors is most efficient when the receptor is activated. In addition, the residues phosphorylated differ from those modified in heterologous desensitization in both position and the fact that no identifiable consensus sequences, such as those for PKA and PKC, have been identified for the six GRK's identified to date (Ferguson *et al.*, 1996a;

Chuang *et al.*, 1996; Böhm *et al.*, 1997; Palczewski, 1997). It therefore appears that the tertiary structure of the receptor induced by receptor activation rather than exposure of a linear recognition sequence is the critical determinant for phosphorylation (Hausdorff *et al.*, 1990; Ferguson *et al.*, 1996a; Chuang *et al.*, 1996; Böhm *et al.*, 1997; Palczewski, 1997).

In studies with the rhodopsin and β_2-adenergic receptors GRK-mediated phosphorylation was not found to be sufficient for full inactivation, suggesting that some other "arresting agent" was required for full receptor desensitization. Wilden *et al.*, (1986) identified a 48-kDa protein, now called arrestin, that bound to phosphorylated rhodopsin. Benovic and co-workers (1987) noticed that a crude preparation of βARK1 (GRK-2) could fully desensitize the β_2-AR while purified βARK1 could not, suggesting that a similar arrestin like protein may exist in this system. This led to the isolation of β-arrestin 1, that was able to reestablish the ability of purified βARK-1 to desensitize β-AR in reconstitution assays (Lohse *et al.*, 1990b, 1992). To date six distinct arrestins have been identified. While it is widely accepted that phosphorylation of GPCRs by GRK-1, 2 and 3 results in the binding of arrestins, thus further uncoupling the receptor from G-proteins (Lohse *et al.*, 1990b, 1992; Pippig *et al.*, 1993), it is unclear if GRKs 4-6 target arrestins to receptors (Ferguson *et al.*, 1996; Chuang *et al.*, 1996; Böhm *et al.*, 1997; Palczewski, 1997).

Less is known about the heterologous and homologous desensitization of the secretin/glucagon/VIP receptor family. Similar to the β2-adenergic and other GPCRs, the CT-region appears to be the major site of phosphorylation in response to agonist stimulation of the receptors for secretin (Ozcelebi *et al.*, 1995; Holtmann *et al.*, 1996b),

PTH/PTHrP (Blind *et al.*, 1995, 1996), glucagon (Heurich *et al.*, 1990; Savage *et al.*, 1995; Buggy *et al.*, 1997) and GLP-1 (Widmann *et al.*, 1996a,b, 1997; Thorens and Widmann, 1996). While PKC and/or PKA phosphorylation have been implicated in the phosphorylation and desensitization of the PTH/PTHrP, glucagon and GLP-1 receptors (Blind *et al.*, 1995, 1996; Savage *et al.*, 1995; Thorens and Widmann *et al.*, 1996; Widmann *et al.*, 1996a,b), in all cases antagonism of PKC and PKA attenuated heterologous desensitization but failed to fully block the receptor desensitization due to homologous desensitization. This suggests that GRKs play a role in the desensitization of GPCRs of the secretin/glucagon/VIP receptor family (Blind *et al.*, 1995, 1996; Savage *et al.*, 1995; Thorens and Widmann, 1996; Widmann *et al.*, 1996a,b). However, to date the only direct evidence of GRK mediated phosphorylation within this subfamily of receptors is the observation that βARK 1 phosphorylates the recombinantly expressed PTH/PTHrP receptor CT-tail *in vitro* (Blind *et al.*, 1996). It remains to be demonstrated whether GRKs are involved in the *in vivo* regulation of secretin/glucagon/VIP receptor phosphorylation and desensitization. In addition, it has not been determined if arrestins play a role in the attenuation of the signals mediated by these receptors.

1.11.5 RECEPTOR ENDOCYTOSIS

Receptor internalization appears to play a role in the desensitization of receptors in certain cellular environments, for example the μ-opioid (Pak *et al.*, 1996) and secretin receptors (Holtmann *et al.*, 1996) when expressed in CHO-K1 cells. However, it is now widely accepted that, for the majority of receptors, the kinetics of internalization are too slow to account for the majority of desensitization responses. Further, results from

studies on chemical inhibition of internalization with sucrose or concanavalin A, and internalization deficient receptor mutants, indicate that desensitization can occur independently of receptor uptake (reviewed in Hausdorff *et al.*, 1990; Ferguson *et al.*, 1996; Chuang *et al.*, 1996; Böhm *et al.*, 1997). It now seems more likely that receptor uptake is important to the resensitization of a cell to a ligand, and may also contribute to receptor downregulation (Hausdorff *et al.*, 1990; Ferguson *et al.*, 1996; Chuang *et al.*, 1996; Böhm *et al.*, 1997).

Once internalized, one or more of the following may occur to the receptor-ligand complex (reviewed in Shepherd, 1989):

1. Class I. The ligand is dissociated from the receptor due to vesicle acidification and sorted to lysosomes for degradation, while the receptor is recycled to the membrane.
2. Class II. Both receptor and ligand are recycled to the membrane.
3. Class III. Both receptor and ligand are targeted to lysosomes.
4. Class IV. Both the receptor and ligand are translocated to the opposite side of a polarized cell.

Most of the GPCRs examined to date appear to belong to the Class I group, though it is possible that a small number of receptors in each cycle are targeted to the lysosome and can, over the long term, account for the decreased receptor number observed in receptor downregulation (Hausdorff *et al.*, 1990; Ferguson *et al.*, 1996; Böhm *et al.*, 1997). It now appears likely that receptor internalization is more often involved in receptor recycling, dephosphorylation, and resensitization of the target cell to the ligand post stimulation. Evidence supporting this proposal includes: the observation that sequestered pools of the

β2-AR receptor are less phosphorylated than membrane associated receptors; these vesicles are associated with high receptor phosphatase activity; receptor resensitization can be inhibited by using phosphatase inhibitors; and finally the fact that inhibition of receptor sequestration prevents both receptor dephosphorylation and resensitization (reviewed in Furguson *et al.*, 1996; Böhm *et al.*, 1997). Recently, a membrane-associated G-protein-coupled receptor phosphatase (GPCRP) that is inactive at neutral pH but active at lower pH in vitro has been implicated in receptor dephosphorylation in the acidified endosome (Garland *et al.*, 1996; Krueger *et al.*, 1997). Given that GPCRP and the phosphorylation sites of GPCRs exist in the neutral intracellular environment, the acidification of endocytotic vesicles may act to both dissociate ligands and cause receptor-mediated conformational activation of GPCRP (Garland *et al.*, 1996; Krueger *et al.*, 1997).

There is a great deal of evidence that activated receptors uncoupled from their G-proteins are internalized into early endosomes either via clathrin coated pits, or caveolae mediated pathways. The exact pathway responsible for a given receptor, like the kinases responsible for desensitization, probably depends both on receptor specific determinants and the cellular environment it exists in (Ferguson *et al.*, 1996). For example, dominant-negative mutants of β-arrestin and dynamin, a GTPase required for clathrin coated vesicle formation, both inhibited wt β_2-AR sequestration, but not that of the angiotensin II type 1A receptor ($AT_{1A}R$), suggesting that both arrestins and dynamin are essential in β_2-adenergic clathrin-coated vesicle mediated sequestration (Ferguson *et al.*, 1996b, Zhang *et al.*, 1996). However, while $AT_{1A}R$ internalization was independent of β-arrestin and dynamin function, overexpression of β-arrestin increased the number of $AT_{1A}Rs$

internalized via clathrin coated pits, suggesting that some plasticity exists by which pathway a receptor can be internalized (Zhang *et al.*, 1996).

1.11.6 DETERMINANTS OF RECEPTOR SEQUESTERATION

To date no clear universal determinants of GPCR sequestration have been identified. It appears that multiple domains or motifs, as well as receptor phosphorylation state and receptor conformation, may all play a part individually, or combined, to induce receptor internalization (reviewed in Ferguson *et al.*, 1996a; Böhm *et al.*, 1997). Early studies suggested that regions required for G-protein coupling were also required for sequestration. In support of this was the observation that receptor coupling efficiency often correlated to a receptor's ability to be sequestered. (Strader *et al.*, 1987: Ferguson *et al.*, 1996a; Böhm *et al.*, 1997). However, further studies identified receptors that were uncoupled yet sequestered normally (Mahan *et al.*, 1985; Cheung *et al.*, 1990; Hausdorff *et al.*, 1990; Petrou *et al.*, 1997). There is also a large body of evidence indicating that the two activities (second messenger coupling and sequestration) are functionally distinguishable (reviewed in Ferguson *et al.*, 1996a; Böhm *et al.*, 1997).

It was initially suggested that receptor phosphorylation acts as an internalization signal. Subsequently it was found that removing (by CT-truncation) or mutating all PKA and GRKs phosphorylation sites, or inhibition of kinase activity failed to inhibit β2-AR sequestration. This has led to the belief that phosphorylation is not essential for receptor sequestration (Lohse et al, 1990a; Hausdorff *et al.*, 1989; Ferguson *et al.*, 1995). It has been demonstrated recently that overexpression of β-arrestin 1 or 2 rescued sequestration deficient mutants of the β2-adenergic receptor (β2-AR Y326A), an effect that was

complemented by the overexpression of GRK-2 (Ferguson *et al.*, 1996b). However, dominant-negative mutants of β-arrestin inhibited sequestration of both wt receptor and β2-AR Y326A, even when receptor phosphorylation was restored by the over-expression of GRK-2 (Ferguson *et al.*, 1996b). These observations strongly suggest that, at least in the case of the β2-AR receptor, phosphorylation acts to stabilize the conformation required for sequestration and/or promotes the association with other cellular elements required for sequestration (Hausdorff *et al.*, 1989). However, it is possible that phosphorylation is important for many other receptors, as removal of the Ser/Thr rich CT-tails of the GRP, α1b-adrenergic, PTH/PTHrP, calcitonin, $AT_{1A}R$, neurotensin, and GLP-1 receptors (Benya *et al.*, 1993; Lattion *et al.*, 1994; Findlay et al, 1994; Huang *et al.*, 1995b; Thomas *et al.*, 1995; Hermans *et al.*, 1996; Widmann *et al.*, 1996), or the middle region of the third intracellular loop of mAChRs (Lameh *et al.*, 1992: Moro *et al.*, 1993) impairs their ability to be sequestrated.

Attempts have also been made to identify endocytic signals or motifs similar to the tyrosine residues containing sequences identified for a number of single transmembrane (STM) receptors. Studies of a $NP(X)_{2,3}Y$ motif found near the cytoplasmic face of many GPCRs, that resembles the NPXY sequestration signal identified for the low density lipoprotein (LDL) and insulin receptors (Ferguson *et al.*, 1996; Böhm *et al.*, 1997), have demonstrated that mutation of the conserved tyrosine residue in both the β2-AR (Barak *et al.*, 1994) and the Neurokinin 1 receptor (NK1-R) (Böhm *et al.*, 1997b) impaired receptor internalization. However similar mutations in the GRP and $AT_{1A}R$ did not impair sequestration (Slice *et al.*, 1994; Hunyady *et al.*, 1995), suggesting that this was is not an universal GPCR internalization sequence. Further

investigation indicated that the NPXY motif may be a critical domain in receptor isomerization from low to high affinity (R→R*) (Barak *et al.*, 1995). This suggests that interaction of kinases not only requires interaction with multiple domains, but that the receptor must be in an appropriate (active like) conformation to be efficiently sequestered.

A conserved "DRY" motif (DRYXXV/IXXPL), found within the second intracellular domain of many GPCRs (but not members of the secretin/glucagon/VIP receptor family), is important for internalization of the m1 mAChR and the GnRH receptors (Moro *et al.*, 1994: Arora *et al.*, 1995). Interestingly, Tyr residues in the CT-tail of the neurokinin receptor (Böhm *et al.*, 1997b), and $AT_{1A}R$ (Thomas *et al.*, 1995), and possibly the PTH/PTHrP receptor (Huang *et al.*, 1995b) may act in a similar manner as those found in STM receptors. Additionally, negative regulatory sequences have been identified in the membrane proximal region of the thyrotropin-releasing hormone receptor (TRHR) and PTH/PTHrP receptors (Nussenzveig *et al.*, 1993; Petrou *et al.*, 1997; Huang *et al.*, 1995b). However, as stated above, none of these signals appear to be universal to GPCR's and the regions important to each receptor, or more closely related receptors, may have to be determined individually.

1.11.7 SEQUESTRATION OF THE SECRETIN/GLUCAGON/ VIP RECEPTOR FAMILY

Few studies have looked at receptor internalization of members of the secretin/glucagon/VIP receptor family, and most of these have focused on the contribution of the CT-tail region to receptor internalization. CT-truncation of the

secretin receptor had little effect on receptor sequestration in CHO-K1 cells (Holtmann *et al.*, 1996b), while internalization of the glucagon (Buggy *et al.*, 1997) and GLP-1 receptors was inhibited (Widmann *et al.*, 1995, 1997). Both glucagon and GLP-1 receptor internalization was dependent on the phosphorylation of Ser residues within the CT-tail regions (Buggy *et al.*, 1997; Widmann *et al.*, 1995, 1997). For the GLP-1 receptor, the degree of receptor internalization was dependent on the number of phosphorylation sites mutated, with the degree of inhibition being correlated to the number of sites removed. However not all sites appeared to contribute equally to receptor internalization, with the most distal of three Ser doublets in the GLP-1 receptor CT-tail being less critical than the two more internal Ser doublets for receptor internalization (Widmann *et al.*, 1996a). These findings are consistent with those found for the glucagon (Buggy *et al.*, 1997), and PTH receptors (Huang *et al.*, 1995a,b) in which the more distal regions of these two receptors could be deleted with little or no effect on sequestration.

The regulation of the more distantly related calcitonin and PTH/PTHrP receptors' internalization appears to be more complicated than that seen for the GLP-1 and glucagon receptors. Both positive and negative regulatory signals have been identified in the membrane proximal region of the PTH/PTHrP receptor (Huang *et al.*, 1995b). While the majority of the calcitonin receptor CT-tail could be deleted without impairing receptor uptake, intermediate truncations resulted in receptors that were either sequestration deficient or resulted in wild-type (wt) like or even slightly improved internalization (Findlay *et al.*, 1994). PTH/PTHrP receptor sequestration may involve phosphorylation as its CT-tail is Ser/Thr rich and is phosphorylated in response to receptor activation

(Blind *et al.*, 1995, 1996). However given the few Ser/Thr residues in the CT-tail of the calcitonin receptor, and the lack of correlation with loss of internalization with loss of these residues, it is unlikely that phosphorylation of the calcitonin CT-tail is required for sequestration (Findlay *et al.*, 1994). Given the great divergence in the CT-tail regions between members of the secretin/glucagon/VIP receptor family, it is not surprising that multiple receptor specific CT-tail regions are involved in receptor internalization.

Both the GLP-1 and PTH/PTHrP receptor appear to be internalized by via clathrin coated pits, as internalization of both could be blocked by treating cells with hypertonic sucrose (to disrupt clathrin lattices) (Widmann *et al.*, 1995; Huang *et al.*, 1995b). However, both of these observations were made in fibroblast or COS 7 cell lines, and it remains to be determined if the same endocytic pathway is important *in vivo*.

No studies to date have examined the downregulation (actual depletion of receptor number) of receptors within the secretin/glucagon/VIP family. In fact little is actually known concerning the downregulation of most GPCRs, although enhanced degradation and reduced synthesis are likely candidates (Böhm *et al.*, 1996). Downregulation of many GPCR's mRNA levels has been associated with decreased stability of the transcript following long term agonist treatment (reviewed in Böhm *et al.*, 1996). A 35 kD β-adrenergic receptor mRNA binding protein (β-ARB) has been identified that preferentially binds to one or more AUUUA pentamers found within the 3'UTR of the β2-adrenergic receptor resulting in increased agonist induced destabilization (Port *et al.*, 1992; Tholanikunnel *et al.*, 1995). The protein was also found to destabilize thrombin receptor mRNA, and it may be responsible for the post-transcriptional regulation of many other receptor mRNA's containing AUUUA pentamers within their 3'UTRs

(Tholanikunnel *et al.*, 1995). The existence of such regulation of the GIP receptor or other members of the secretin/glucagon/VIP family remains to be determined.

1.12 THESIS STUDIES: HYPOTHESES AND OBJECTIVES

Studies on numerous G-protein coupled receptors have identified regions with common functions. However, it has become apparent that in most cases it is difficult to identify distinct structural motifs, and structure-function studies have to be performed for each individual receptor. As discussed previously, it is now thought that GIP is the most important incretin in healthy individuals (Holst et al., 1997; Nauck et al., 1997a), and a defect in GIP-mediated insulin secretion may play a central role in the aetiology of NIDDM (Holst *et al.*, 1997). It is therefore important to identify structural features of both GIP and its receptor that are important for normal physiological regulation of glucose homeostasis. Studies undertaken in this thesis were designed to examine a number of questions concerning the identity, function, and structure of the rat islet GIP receptor and GIP. The rationale, specific hypotheses and objectives are listed below:

As mentioned in section 1.5.1 Usdin *et al.* (1993) originally isolated a partial cDNA clone from rat cerebral cortex, and used this sequence to isolate a full length GIP receptor cDNA clone from the rat tumor β-cell line, RINm5F. Surprisingly, the RINm5F cDNA was shown to be expressed in a wide variety of tissues not normally thought to be targets of GIP action (Usdin *et al.*, 1993). As neither GIP immunoreactivity (Buchan *et al.*, 1982), nor mRNA (Higasimoto *et al.*, 1992; Tseng *et al.*, 1993) have been detected in the brain, this suggests that the receptor isolated by Usdin et al. (1993) may encode a receptor for a highly related ligand present in the brain, and it may not be the GIP

receptor present in primary islet cells. The receptor for GIP would also be expected to display high specificity and affinity for its ligand, and activate signal transduction pathways associated with GIP-mediated insulin secretion (see section 1.7.1). To determine if the GIP mRNA isolated by Usdin et al. (1993) was also expressed in primary rat islet cells, and to more thoroughly examine its signal transduction, the following studies were undertaken.

Hypothesis 1. The pancreatic islet GIP receptor is the product of an identical or alternatively spliced form of the cDNA identified in the tumor cell line, RIN5mf, and may display major or minor sequence differences.

Objective1. To prepare a pancreatic GIP receptor cDNA from isolated rat islet mRNA and compare its sequence with that of a cDNA previously isolated from a β-cell tumor cell line, RIN5mf.

Hypothesis 2. The islet GIP receptor exhibits strong specificity with respect to ligand binding and initiation of signal-transduction pathways.

Objective 2. To compare and contrast ligand binding and activation of adenylyl cyclase by members of the secretin-glucagon superfamily in COS-7 and CHO-K1 cells expressing the pancreatic islet GIP receptor.

A great deal of controversy exists in the literature concerning the potency of synthetic human GIP in both humans (Elahi et al., 1979; Nauck et al., 1989, 1993a) and rat (Jia et al., 1995). Once isolated, the rat islet GIP receptor mRNA, when expressed in COS-7 and CHO-K1 cells allowed the examination of the potencies of different species of GIP.

Hypothesis 3. Different species of GIP display different affinity and efficacy at the rat islet GIP receptor.

Objective 3. To compare the affinity and efficacy of different commercial preparations of synthetic GIP on COS-7 and CHO-K1 cells expressing the rat

pancreatic islet GIP receptor, and compare these to the *in vivo* activity in the isolated perfused rat pancreas.

Studies to date (see section 1.10) have suggested that the biological core of GIP exists within residues 1-30, while smaller fragments may possess reduced activity (Maletti et al., 1986; Carlquist et al., 1984; Morrow et al., 1996). However, many of these peptide preparations were generated by enzymatic cleavage and may not have been pure. Furthermore, all were tested with either insulinoma cell lines or the perfused rat pancreas, both systems that contain the highly related GLP-1 and glucagon receptors, with which the fragments may crossreact, further confusing interpretation of the results. The stable rat islet GIP receptor expressing CHO-K1 cell line provided an isolated system in which to examine the ability of different GIP fragments to bind and/or activate its receptor.

Hypothesis 4. The biological core of GIP exists within the region of amino acids 1-30, and further truncation will result in fragments exhibiting high affinity and biological activity.

Objective 4. To utilize stable CHO-K1 cell lines expressing the rat islet GIP receptor to examine the effect of truncation of the biologically active GIP 1-30 fragment, to identify regions and residues important for receptor binding and activation.

While many studies concerning the structure-function of the β-AR, and the more distally related α-AR and mAchR have been carried out, far fewer have examined receptors of the secretin/glucagon/VIP family. The isolation of the rat islet GIP receptor mRNA allowed us to examine regions of the GIP receptor important to its ligand specificity, binding, and signal transduction. Based on findings presented in section 1.11 the following hypothesis were proposed:

Hypothesis 5. The amino-terminal extracellular domain of the GIP and GLP-1 receptor contains the majority of the selective ligand-binding region, while regions important for activation require multiple receptor regions.

Objective 5. To construct rat GIP/human GLP-1 receptor chimeras to define regions important for high affinity ligand binding, ligand selectivity, and receptor activation.

Hypothesis 6. The carboxy-terminal intracellular tail of the GIP receptor contains regions important for effective coupling to second messenger activation, desensitization, and sequestration.

Objective 6. To use site directed mutagenesis to construct increasing truncation of the rat islet GIP receptors carboxy-terminal tail, and compare and contrast the ability of these truncated receptors to generate cAMP, undergo desensitization, and be sequestered in response to ligand stimulation.

CHAPTER 2

METHODS

2.1. ISOLATION AND CHARACTERIZATION OF A cDNA ENCODING THE RAT GIP RECEPTOR

Isolation of the rat GIP receptor cDNA was performed in collaboration with Dr. Michael B. Wheeler. Standard molecular biology techniques used in the studies were based on those described in *Molecular Cloning, A Laboratory Manual* by Sambrook et al., 1989, and further details are provided in this section when modifications of the original procedure were made. Chemicals and enzymes were all of molecular biology grade unless otherwise stated; commercial sources are listed in the text. All primers were synthesized on an Applied Biosystems 380A DNA synthesizer in the laboratory of Dr. Ross T.A. McGillivray. They were eluted from the column with 2 ml 14.8 M NH_4OH, incubated at 55°C for 12-16h, and dried in a Speed-Vac.

2.1.1 RNA ISOLATION

Normal precautions for work with RNA were taken: all solutions used were treated with 0.1% diethyl pyrocarbonate (DEPC) for 12 h, and then autoclaved to inactivate the DEPC, or made from fresh chemical stocks with DEPC treated distilled (d)H_2O; all glassware, gel boxes, tip holders, etc. were treated with Absolve® (Dupont, Wilmington), and rinsed with DEPC treated dH_2O; and all plasticware used was RNase free grade.

Rat pancreatic Islets were isolated by Robert Pauly or Charlene Fell, as described by Van der Vliet et al., 1988. Total RNA was isolated from the rat islets using the acid guanidinium thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi (1987). Islets were removed from the isolation medium by centrifugation, and lysed in approximately 0.5 ml of solution D (4 M guanidinium isothiocyanate, 25 mM sodium citrate, 0.5 % sarcosyl, and 0.1 M 2-β-mercaptoethanol). Sequentially, 0.1 volume of 2 M sodium acetate (pH. 4) and an equal volume of buffer saturated phenol, and 0.2 volumes of chloroform/iso-amyl alcohol (49:1), were added per volume of solution D used in the initial homogenization, and the mixture vortex mixed briefly. Samples were then incubated on ice for 15-20 min., and centrifuged at 10-12,000 X g for 20 min at 4°C. The RNA containing aqueous phase (top) was transferred to a fresh RNase free tube, and mixed with an equal volume of isopropanol. After at least 1h at -20°C the precipitated RNA was sedimented at 10-12,000g for 20 min at 4°C, and the resulting pellet resuspended in 300 µl of solution D. The pellet was transferred to a fresh 1.5 ml Eppendorf tube, and precipitated with isopropanol as before. The RNA was then washed with 70% ethanol, dissolved in DEPC treated dH_2O (50-100 µl), and the concentration determined using sample absorbance at A_{260} and A_{260}/A_{280} ratios. Integrity was assessed from appearance of the RNA when run on a formaldehyde-agarose (1.1%) gel. The total RNA used for reverse transcription had an A_{260}/A_{280} ratio of at least 1.7. Total RNA was stored either as an isopropanol precipitate or in DEPC treated H_2O with 1 µl (10U) of RNase inhibitor (RNAguard, Pharmacia), until use.

2.1.2 REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

Total RNA (5 μg) from rat pancreatic islets was primed with 0.5 μg oligo deoxythymidine (dT) and 0.5 μg random hexamers (Pharmacia) by incubating at 95°C for 10 min, and then chilled on ice for 5 min. Reverse transcription (RT) was then carried out with 400U SuperScript II™ reverse transcriptase (Gibco BRL, Grand Island, NJ) in 1X RT buffer (50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3mM $MgCl_2$, and 10 mM dithiothreitol (DTT)) containing 10 U of RNase inhibitor (Pharmacia), and 1 mM dNTPs (Pharmacia), at 42°C for 1h in a total volume of 20 μL. The reaction was terminated by heating the reaction tube to 85°C for 15 min. 80 μL of dH_2O were added to the mixture to bring the total volume to 100 μL.

To amplify the coding region of the rat islet GIP receptor, cDNA oligonucleotide primers were designed based on the published sequence of Usdin et al. (1993) for a GIP receptor cDNA cloned from a rat insulinoma cell line (RINm5F), corresponding to nucleotides 163-184 (5'-AGGATGCCCCTGCGGCTGTTGC-3') and 1537-1515 (5'-GTCCTAGCAGTAACTTTCCAAGA-3'). Amplification of 1-10 μL of the cDNA was carried out with 100 pmol of each primer, 200 μM dNTPs and 5U of *Taq* polymerase (Perkin Elmer Cetus, Norwalk, CT) in 1 X PCR buffer E (67 mM Tris-HCl, pH 9.0, 1.5 mM $MgSO_4$, 166 mM NH_4SO_4, and 10 mM 2-β-mercaptoethanol) in a total volume of 50 μL. The following PCR conditions were used: denaturation at 95°C for 3 min, annealing at 62°C for 1 min, and an extension time of 1 min at 72°C for one cycle, followed by 35 cycles with 1 min denaturation, annealing and extension steps. A single product of the appropriate size (~1.4 kb) was obtained, and three individual PCR reaction products (GIP-R1, GIP-R2, and GIP-R3) were isolated, gel purified, and ligated into the TA

cloning vector pCRII (Invitrogen Co, San Diego, CA) as per the manufacturer's instructions. The pCRII vector takes advantage of the one base pair 3' overhang, usually an adenosine (A), that *Taq* polymerase adds to the end of an amplified product. The linearized vector is prepared with one base pair thymidine (T) overhang at its 3' ends, at a site within the multiple cloning site (MCS), making small "sticky ends" for the A overhangs present on the PCR product. This results in higher efficiency subcloning than other methods commonly used to subclone PCR generated products. Three individual clones (GIP-R1, GIP-R2, and GIP-R3) were mapped by restriction analysis and partial dideoxy-sequencing (T^7-Sequencing Kit, Pharmacia Biotech, Sweden). The cDNAs pGIP-R1 and pGIP-R2 were subcloned into the *Hind* III/*Xba* I site of the expression vector pcDNA 3 (Invitrogen), and the complete sequence of the pGIP-R1 coding strand was determined (See Fig 21). Alignment with the published sequence identified only one nucleotide difference in pGIP-R1, resulting in a single amino acid difference (Glu21→Gln21). This single nucleotide change was confirmed in the two other individual clones by partial sequencing, suggesting that this may be due to a polymorphism in the rat gene.

The rat and human GLP-1 receptor cDNAs were the kind gift of Dr. M. B. Wheeler and their isolation and characterization are described elsewhere (Wheeler et al., 1993; Dillon et al. 1993). Both cDNAs were sub-cloned into pcDNA 3 for use in the described experiments.

2.1.3 DNA SEQUENCING AND ELECTROPHORESIS

Purified plasmid DNA (1-2 μg) sequence was determined using a T^7-Sequencing Kit (Pharmacia) as per the manufacturer's instructions, with the following modifications. Up to 7 μL of the plasmid DNA, 10 pmol of sequencing primer (2 μL), and 2 μL of dimethylsulfoxide (DMSO) were added to a 1.5 mL tube, heat denatured for 3-5 min at 95°C, then snap frozen in a dry ice-ethanol bath. The sample was then thawed and 2 μL of annealing buffer were added. This mixture was centrifuged rapidly (<10s), and incubated at room temperature (RT) for 10 min. Six microlitres of labeling mix (3 μL labeling mixture, 1 μL α^{35}S-dATP, and 2 μL of T7 DNA polymerase) were added, mixed gently, and incubated at room temperature for 5-10 min. A microtitre plate was prepared with 2.5 μL of termination mixtures for the four nucleotides G, A, T and C, and preheated on a heating block set to 42-50°C. Sequencing mixture (4.5 μL) was added to each of the four termination mixtures, and incubated for a further 10 minutes. The reaction was stopped by the addition of loading buffer, and the samples were stored at –20 °C until separated by electrophoresis on a sequencing gel.

Samples were subjected to electrophoresis on a 6% acrylamide, 8M urea, 1X tris-borate- disodium ethylenediaminetetraacetate (EDTA) (TBE) buffered gel. The top chamber of the Model S2 sequencing apparatus (GIBCO) was filled with ½ X TBE and the bottom chamber with 200 ml of 1 X TBE. The gel was pre-warmed by running at 50-60 W for 30 min. Samples were then loaded, and subjected to electrophoresis at 60 W for 30 min. at which time 200 mL of 3 M sodium acetate was added to the bottom chamber. Electrophoresis was continued for a further 3-4 h, until the cyanol bromide (second migrating dye in loading buffer) was 3/4 the way down the gel. The gel plates were then

separated, the gel absorbed onto 3MM Whatman filter paper, and dried on a gel dryer. Once dried the gel was exposed to an autoradiograph film for 24-48h, and the sequence was read manually.

2.2 CELL CULTURE AND CELL TRANSFECTION

Generation of all stable expressing cell lines was carried out by the author. Transient expression experiments were carried out in collaboration with the laboratory of Dr. M. B. Wheeler.

2.2.1 MAMALIAN CELL CULTURE

Green Monkey Kidney (COS-7) and Chinese Hamster Ovary (CHO-K1) cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS), and DMEM/Ham's F12 supplemented with 10% newborn calf serum (NBS), respectively. All culture media contained 50 units/mL penicillin G, and 50 µg/mL streptomycin (Culture media and antibiotics from Gibco). Cells were grown in 75 cm^2 flasks until 80-90% confluency, and split 1:10-1:20 for maintenance of exponentially growing cultures.

2.2.2 TRANSIENT TRANSFECTIONS

All plasmid DNA used in transient or stable transfections was purified using Qiagen Maxi Plasmid Kits (Chatsworth, CA) as per the manufacturer's instructions. The plasmid DNA isolated had A_{260}/A_{280} ratios of 1.7-1.8. For transient expression, COS-7 cells (3.0×10^6 cells/dish) were seeded in 10 cm dishes (Becton Dickinson, Lincoln Park, NJ) and cultured in DMEM supplemented with 10% fetal bovine serum (Gibco, Grand

Island, NJ). Cells were transfected 48h. later with the appropriate cDNA at concentrations of either 5 μg (for cAMP and Ca^{++} imaging) or 10 μg (for binding experiments), using the DEAE-dextran method (Sambrook et al., 1989). Briefly, 1 mL of Chloroquine in phosphate buffered saline (PBS) (13 mg/mL) was added to 9 mL of a stock solution consisting of 11 mg/mL DEAE-dextran in PBS. To prepare the transfection medium, this solution was filter sterilized (protecting from light) just prior to transfection, and 1.2 mL added to 21.5 mL DMEM plus 2.5 mL Nu-serum (Collaborative Biomedical Products, Boston, MA). The cDNA was added to 5 mL of transfection media, mixed by inversion, added directly to the cells, and incubated for 3-4 h. The transfection medium was removed by aspiration, and the cells shocked with 2-3 mL of a solution of 10% DMSO in PBS for 1.5 min, then allowed to recover overnight in 5-7 mL. of culture medium. 12-16 h following transfection, the cells were passed into 6, 12, or 24 well plates (for cAMP or Ca^{2+} imaging studies), or 10 cm dishes (for binding or Ca^{2+} imaging studies), and cultured for an additional 48 h before experiments were conducted.

2.2.3 STABLE TRANSFECTIONS

Production of permanent (stable) CHO-K1 cell lines expressing the GIP receptor was carried out using the $CaPO_4$ co-precipitation method of Sambrook et al.(1989), with minor modifications. Cells were grown on 10 cm dishes until approximately 90% confluent. Fresh culture medium (5-7 mL) was added to the cells at least 3 h before the transfection. Ten micrograms of the cDNA was added to 62.5 μL of 2.5 M $CaCl_2$ (filter sterilized) and the total volume was brought up to 500 μl with sterile dH_2O. To this

solution, 500μL of 2 X N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-Buffered Saline (HBS) (280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 12 mM Dextrose, and 50 mM HEPES, pH 6.95) were added. The mixture was vortex mixed briefly, and incubated for 30 min at room temperature to allow a precipitate to form. The DNA/HBS/Ca^{2+} mixture (1 mL/plate) was added directly to plates containing 5-7 mL of media. After a 3.5-4h incubation, cells were then shocked for 1.5 min in 15% glycerol/1 X HBS, and allowed to recover for 12-16h. The cells were then split onto two dishes and those expressing pGIP-R1, or other constructs, were isolated by G418 selection (800 μg/mL) (Gibco), changing the media every 2-3 days as needed. After 10-12 days individual clones were isolated and further selected for high level expression by screening for GIP stimulated cAMP production and ^{125}I-GIP binding as described below. One clone, designated wtGIP-R1, was selected for further characterization. It was determined later that a pooled pGIP-R1 expressing cell line, isolated and maintained under high stringency selection (800 μg/ml), resulted in high level expression and maximal cAMP similar to the levels seen in the original wtGIP-R1 clone. In later studies pooled clones were obtained to reduce the selection time.

2.3 BINDING ANALYSIS

2.3.1 ^{125}I-GIP PREPARATION

^{125}I-GIP used in receptor studies was prepared by the chloramine T method of Kuzio et al (1974), and purified by gel filtration and High Performance Liquid Chromatography (HPLC) as described by Verchere (1991), with minor modifications. Porcine GIP, 5 μg in a silconized test tube, was dissolved in 100 μL of 0.4 M phosphate

buffer (pH 7.5), and 10 μL (1mCi) of $Na^{125}I$, and 10 μL of chloramine T (4 mg/mL in 0.4 M phosphate buffer, pH 7.5) added. The reaction was stopped 15s later by the addition of 20μL sodium metabisulphite (14.8 mg/ml in 0.4 M phosphate buffer, pH 7.5). The mixture was then applied to a column (0.5 X ~10 cm) of Sephadex® G-15 (Pharmacia), prepared in a 10 ml disposable pipette and equilibrated for 4-6h in 0.2 M acetic acid containing 2% RIA grade bovine serum albumin (BSA). On the day of the iodination 2% aprotinin (Trasylol™, Sigma) was added to the column buffer, and equilibrated for at least 1h before the iodination mixture was applied.

HPLC purification of the peak ^{125}I-GIP fractions from the gel filtration chromatography was performed on μBondapak C-18 column (Walters Associates Inc., Milford, MA) using 2 Beckman 110B solvent delivery module pumps with a programmable Beckman model 421A controller, and a model 170 Radioisotope Detector (Beckman Instruments Inc., San Ramond, CA) to monitor radioactivity eluting from the column. Separation of the different ^{125}I-GIP species was accomplished with a gradient of CH_3CN in water containing 0.1% trifluoroacetic acid run over 35 min. Fractions were injected using a needle syringe (Hamilton CO., Reno, NV), washed on to the column in 31% CH_3CN. This concentration was maintained for 10 min, following which a linear gradient to 38% CH_3CN was run over 10 min. The column was then washed by increasing the CH_3CN concentration to 70% over 5 min, where it was held for 5 min, and then returned to 31% over 5 min. The second major peak eluting at approximately 18 min post-injection was collected from multiple runs in a siliconized tube with 0.5 ml of 2.5% RIA grade BSA and 50% apoprotin. The amount of radioactive label purified was determined in a γ-counter, and aliquots of 3-6 X 10^6 cpm were lyophilized and stored at -

20°C until use. The specific activity of the label was determined to be approximately 250-350 μCi/μg using a homologous displacement assay as previously described (Verchere, 1991).

2.3.2 TRANSIENT BINDING EXPERIMENTS

Seventy two hours post transfection COS-7 cells were detached from dishes using phosphate buffered saline (PBS) containing 0.1 mM disodium ethylenediaminetetra-acetate (EDTA), washed twice in binding buffer (DMEM, supplemented with 20 mM HEPES, 0.1% bovine serum albumin (BSA), 0.05 mg/ml bacitracin, pH 7.4) (BB1) and preincubated for 30 min at 37°C. Cells (5 X 10^5/well) were then incubated for 30 min at 37°C with 50,000 cpm of radiolabeled peptide (approximately 90-130 pM), in the presence or absence of unlabeled peptide, in a final volume of 200 μl. After incubation, cell suspensions were centrifuged at 12,000 x g, washed once in ice cold binding assay buffer, and the cell associated radioactivity in the pellet measured in a γ-ray counter.

2.3.3 STABLE CELL-LINE BINDING STUDIES

While some initial studies were carried out as above, most whole cell studies with CHO-K1 cells stably expressing pGIP-R1, or other receptor constructs, were carried out with the cells attached in 24 well culture plates. Cells were washed 2X with binding buffer (BB1) with the same composition as BB1, apart from DMEM/Ham's F12 replacing DMEM. In initial studies, involving comparison of synthetic GIP preparations, cells were incubated for 1 h at either room temperature (RT) or 37°C, and saturation binding experiments with both whole cells and membranes were incubated for 4-6h at

4°C, with 50,000 cpm ^{125}I-GIP in the presence or absence of cold competing peptide. In subsequent studies, cells were incubated at 4°C for 12-16h to ensure that steady state conditions for all levels of receptor expression were achieved. At the end of the incubation period cells were washed 2X with ice cold buffer, solubilized with 0.1M NaOH (0.5 mL), and transferred to test tubes for counting of cell associated radioactivity (Wheeler et al., 1995). Non- specific binding was defined in all experiments as the cell associated activity observed in the presence of 1 μM GIP. Cell numbers were determined by counting cells trypsinized from control wells, in a haemocytometer, at the end of the experiments.

Cell membranes were isolated as described by Samama et al (1993). wtGIP-R1 cells in two 1500 cm^2 tissue culture roller bottles (Becton Dickinson) were grown until confluent. Cells were then washed twice in PBS, detached in 0.1 mM EDTA/PBS, and sedimented by centrifugation at 1200 RPM at RT. Cells were then resuspended in 10 mL of buffer A (5mM Tris-HCl, pH 7.5, 5mM EDTA) and re-sedimented by centrifugation at 40,000 X g. The pellet was resuspended in 3 ml of buffer A, homogenized in a Teflon-glass homogenizer, and centrifuged a second time. The pellet was resuspended in 3mL of the same buffer and the protein concentration determined using the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL). Approximately 26 mg total protein were obtained, diluted in buffer A to a concentration of approximately 2 mg/mL, and stored in aliquots at -80°C until use. Saturation binding studies with membranes or cells used a similar protocol and differed only in the buffers used. Whole cell experiments were carried out in the binding buffer described above, while membrane experiments were done in 50 mM Tris-HCl, pH7.2, 5 mM $MgCl_2$, 1 mM EDTA, 0.1 % RIA grade BSA,

and 0.1 mg/ml bacitracin (BB2). Transfected CHO-K1 cells (3-8 X 10^5 cells/well), or 100 µg protein/tube were incubated with increasing amounts of radiolabeled peptide (~700-1.4 x10^6 cpm) added, and allowed to incubate at 4°C for 4-6h. Cells and membranes were then centrifuged at 12000 X g and the pellet washed 2X with ice cold BB1 or BB2, respectively, and the pellet-associated binding determined. Total binding and the nonspecific binding at each concentration tested were determined as described above for cellular binding.

2.3.4 BINDING ANALYSIS

In all experiments specific binding was analyzed using the nonlinear regression analysis program Prism (GraphPad, San Diego, CA). Saturation binding analysis compared data fitted to both one and two site models using the following equations:

One site model:

$Y = Bmax*X/(Kd+X)$

Two site model:

$Y = Bmax1*X/(Kd1+X) + Bmax2*X/(Kd2+X)$

Both equations describe the binding of a ligand to a receptor that follows the law of mass action where X is the concentration of free ligand, Y is the specific binding measured, Bmax is the maximal binding, and Kd is the concentration of ligand required to reach half-maximal binding (in either cpm or molar concentration) (Fig. 6). In the two-site model Kd1 and Kd2 are the concentration of ligand required to reach half-maximal binding for two different receptor populations with different affinities for the ligand. In

both models it is assumed that the reaction has reached steady-state; only a small fraction (less than 10% in practice) of the labeled ligand is bound and therefore the free concentration is essentially the same as the original concentration added to the reaction; and that there is no cooperativity in the binding, ie. the binding of ligand to one site does not effect the affinity at another site.

Data from competitive binding experiments were fitted to both single and two site models using the following equations:

Competitive one site Model:

$Y = Bottom + (Top-Bottom)/(1+10(X-LogIC_{50}))$,

Competitive two site model:

SPAN=Bottom+Top

$Part1=Span*Fraction1/(1+10(X-LogIC_{50}1))$

$Part2=Span*(1-Fraction1)/(1+10(X-LogIC_{50}2))$

Y=Bottom + Part1 +Part2

Where Y is the bound label and X is the log value of the concentration of unlabeled competitor. Fraction one is the fraction of all sites that display affinity 1, and fraction two is the population of receptors with affinity 2. Span refers to the difference between the Bmax (Top) (maximal binding in the absence of competitor), and the bottom plateau (Bottom), or NSB (see Fig. 6B.). As the specific activity of ^{125}I-GIP was not determined for every labeling, IC_{50} values (inhibitory concentration displacing half the Bmax) are presented. The same assumptions made for saturation binding analysis are made in

competitive binding analysis. Idealized binding curves for saturation and competitive binding experiments are shown in Fig. 6.

In both saturation and competitive binding experiments, the program compared the fit of the results to both the appropriate one and two site models. The program used an F-test to compare the goodness of fit of the two equations, and the simpler equation was assumed to be correct if an F ratio corresponding to a P value of 0.05 or greater was obtained.

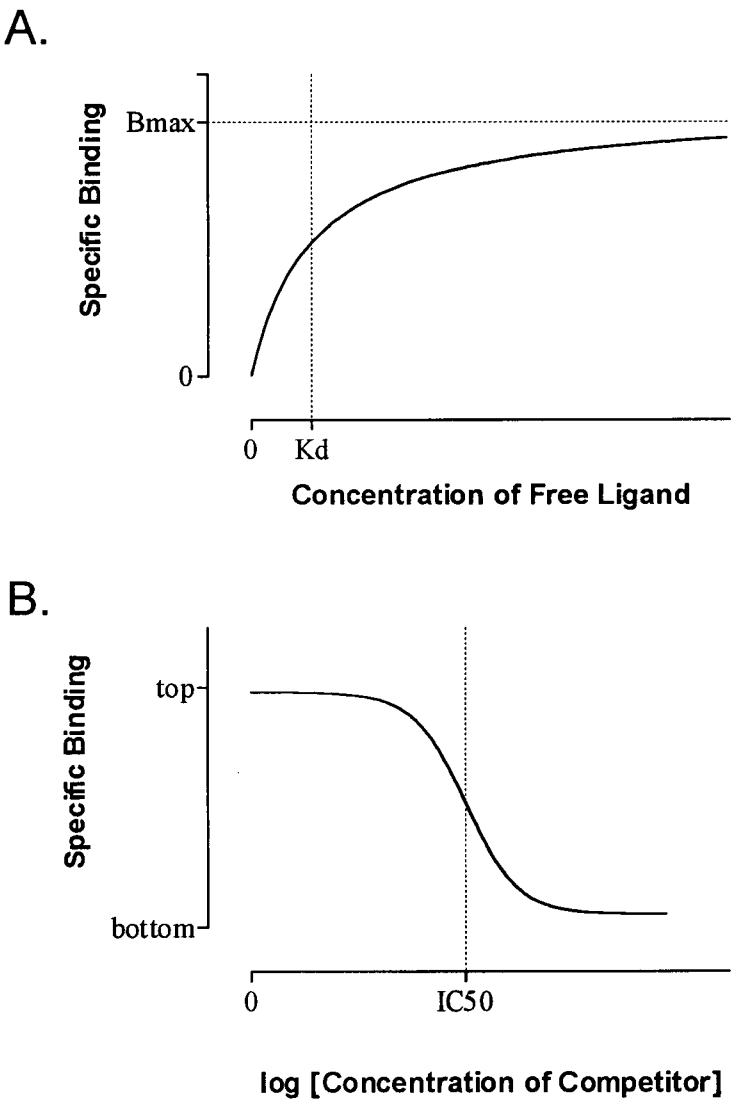


Fig. 6. Idealized Binding Curves for Saturation (A.) And Competitive (B.) Binding Studies to a Single Binding Site. The Kd is the concentration of labeled ligand required to give half-maximal binding, while the IC_{50} is the concentration of cold ligand required to inhibit half of the maximal binding of labeled ligand. See text for details.

2.4 MEASUREMENTS OF cAMP PRODUCTION

COS-7 cells (24 hours after transfection) and CHO-K1 clones expressing GIP-R1 were passed into multiwell plates and cultured for an additional 48 h. Cells were then washed in assay buffer (DMEM for COS-7 and DMEM/Ham's F12), containing 0.1% BSA and 0.05% bacitracin, and preincubated for 30-60 min, followed by a 30 min stimulation period with test agents at appropriate concentrations. In all transient experiments with COS-7 cells the stimulation media contained 1 mM isobutylmethyl-xanthine (IBMX) to prevent cAMP breakdown by phosphodiesterases and allow measurement of total cAMP production. In initial studies (as noted in the text) with CHO-K1 cells, IBMX was not included in the stimulation buffer as it was originally thought that the availability of a homogeneous population of cells expressing high levels of the GIP receptor would produce very high and consistent levels of cAMP. However, while initial studies demonstrated that GIP stimulated cAMP production in a concentration dependent manner, the variation in maximal cAMP levels obtained and estimated EC_{50} values varied greatly day to day. In later studies it was found that the addition of 1 mM IBMX in the stimulation media and normalization of cAMP levels to fmol/1000 cells gave highly consistent EC_{50} values. Maximal cAMP levels while more consistent in the presence of IBMX did vary over time, and this did not appear to correlate with passage number (both increasing and decreasing at later passages). For this reason experiments were conducted over a short a period of time as possible (30-60 days), and maximum levels were normalized to % of wtGIP-R1 maximal levels.

Following the stimulation period, cells were extracted in 0.5-1ml of 70% ethanol, transferred to Eppendorf tubes, centrifuged at 7000 rpm at 4°C to remove the cell debris, and the supernatant transferred to a second tube. Samples were then dried in a Speed-vac for a time of 6-18h and stored at -20°C until they were assayed. On the day of the assay, samples were reconstituted in 0.5 ml 0.5 M sodium acetate buffer and cAMP levels were determined using a cAMP radioimmunoassay (RIA) kit (Biomedical Technologies Inc., Stoughton, MA) as per the manufacturer's instructions.

2.5 CYTOSOLIC Ca^{2+} MEASUREMENTS

Cytosolic calcium measurements in the initial characterization of the rat islet GIP receptor signaling were carried out by Dr. M.B. Wheeler and Dr. J. Georgiou in the Departments of Medicine and Physiology, at the University of Toronto. Later studies on characterization of the GIP receptor Ca^{2+} signaling in CHO-K1 cells and the point mutant H170R (see section 3.4, Appendix A) in COS-7 cells were carried out at the University of British Columbia by Dr. P. Squires. These results were important for establishing GIP receptor linked Ca^{2+} signaling and, subsequently, in identifying the effect of cellular environment and receptor mutation on this signaling pathway. As these results were important for discussion of the results presented in this thesis, but the author did not perform the Ca^{2+} measurements himself, the methods and results are included in Appendix A.

2.6. RAT PANCREAS PERFUSIONS

The rat pancreas perfusion assays were performed with the assistance of Dr. Raymond Pederson as previously described (Pederson et al., 1982). Briefly, overnight fasted rats were anaesthetized (60mg/kg pentobarbital) and the pancreas and associated duodenum isolated. Perfusate consisted of a modified Krebs-Ringer buffer containing 3% dextran (Sigma) and 0.2 % BSA, gassed with 95% O_2/ 5% CO_2 to achieve a pH of 7.4. The peptide preparations were delivered as a linear gradient of 0-1 nM over a 45-50 minute period in the presence of 16.7 mM glucose. Immunoreactive insulin was determined using an established RIA as previously described (Pederson et al., 1982). Results are expressed as the mean integrated insulin response in mU over the 50 min. perfusion time.

2.7 SOURCES OF PEPTIDES

Synthetic peptides were obtained from the following commercial or private sources. Peptides used for comparing the biological activity of different GIP preparations and determining receptor specificity were obtained from: Peninsula Laboratories (Belmont, CA): synthetic human (sh) GIP Lot #9408164, synthetic porcine (sp)GIP Lot #033785, GLP-1 (7-37) Lot #019860, shGLP-2 Lot #008674, vasoactive intestinal peptide (VIP) Lot #015174; Novo Biolabs (Bagsvaerd, Denmark): porcine glucagon Lot #G4211963; Bachem California (Torrance, CA): shGIP Lot #ZK887, spGIP Lot #758C, exendin-4 (Ex-4) Lot #ZL765, exendin (9-39) (Ex-9-39) Lot #ZL777. Natural porcine (np) GIP was purified as described elsewhere (Brown, 1971). ShGIP 1-30 free acid (OH) and spGIP 17-30 were the kind gifts of Dr. N. Yanaihara (Shizuoka, Japan) and Dr. S. St.

Pierre (INRS Santé, Montreal, Canada) respectively. SpGIP 19-30 was prepared by tryptic digestion of spGIP 17-30, and purification of the major product by reverse phase (RP-) HPLC. The identities of the peptides were confirmed by sequence analysis. The peptides shGIP 18-21, shGIP 21-28, GLP-1 (7-36) 21-28 and glucagon 21-28 were synthesized by solid-phase techniques by the Nucleic Acid-Protein Service Unit, UBC, and purified by RP- HPLC.

As it was determined that both sh and spGIP were equipotent and had identical affinity for the rat islet GIP receptor, all further peptides for investigations were based on the human sequence, and shGIP preparations were obtained from Bachem (Torrence, CA.) or Hukabel (Montreal, QUE). Dr. T. O'Dorisio, S. Cataland and O. Succek (University of Ohio, Columbus) kindly provided GIP 10-30. GIP 7-30, D-Ala^2-GIP, D-Ala^2-GLP-1, and GIP 3-42 were obtained from Hukabel (Montreal, QUE). GIP 1-30amide, GIP 6-30amide, D-Ala^2-GIP 1-30amide, D-Tyr^1-GIP 1-30amide, Desamino-Tyr^1-GIP 1-30amide, D-Glu3-GIP 1-30amide, and D-Ala^4-GIP 1-30amide were synthesized by Dr. D.H. Coy (Peptide Research, Department of Medicine, Tulane School of Medicine, New Orleans, LA, USA). All peptides were shown to be homogeneous on HPLC and of the correct molecular weight by MALDI-TOF Mass Spectrometry.

2.8 SITE-DIRECTED MUTAGENESIS OF THE RAT ISLET PANCREATIC GIP RECEPTOR

The experimental design of mutant forms of the receptors was carried out in collaboration with Dr. Michael B. Wheeler. Several different methodologies were utilized, and specific constructs and the methodologies used for their production are described below.

2.8.1 OLIGONUCLEOTIDE PHOSPHORYLATION

Non-PCR based oligonucleotide-directed mutagenesis protocols require that the 5' end of the mutant oligonucleotide is phosphorylated to allow the ligation of the 5' termini of the synthetic oligonucleotide in the extension and ligation reaction step (see below). In all protocols utilized below the same basic protocol was used to phosphorylate the primers: 1-2.5 μg of the mutagenic oligonucleotide in 1 X kinase buffer (70 mM Tris-HCl, pH 7.6, 10mM $MgCl_2$) plus 1 mM rATP was incubated with 10-25 U of T_4 polynucleotide kinase at 37°C for 1h. Incubating the mixture at 65°C for 10 min. terminated the reaction.

2.8.2 CONSTRUCTION AND EXPRESSION OF H170R

Double stranded oligonucleotide-directed mutagenesis was utilized to generate a point mutation in the GIP receptor resulting in the substitution of an arginine at position 170 in place of the endogenous histidine residue. This mutation in the related PTH/PTHrP receptor had been shown previously to result in a receptor with constitutive signaling activity (Schipani et al., 1995, 1996). A commercial kit (Morph™ Site-Specific Plasmid DNA Mutagenesis Kit, 5-prime 3-prime, Boulder, CO) utilized two steps allowing the use of double stranded template, while obtaining an acceptable rate of mutagenesis. The mutagenic primer, corresponding to coding nucleotides 501-520 (5'-ATTACATTCGCATGAACCTG-3'), was designed with a single base change (underlined) at position 510 (A→G) resulting in the amino acid substitution of an

arginine for histidine at amino acid residue 170. The protocol is described briefly below. All materials, except primers and template, were from 5-prime 3-prime (Boulder, CO).

1. Double stranded template (~30 fmol; pBKS-/GIP-R1) and 100 ng of the 5'-phosphorylated mutagenic primer, in a total volume of 20 µl 1 X MORPH™ Annealing buffer, were denatured at 100°C for 5 min, cooled in an ice water bath for 5 min, and incubated at RT for 30 min.

2. A replacement strand was synthesized *in vitro* resulting in a mixture of hemi-methylated half-mutant plasmid and, fully methylated wild type (wt) target plasmid (Fig.7). The reaction conditions were as follows: 8 µL of 3.75 X MORPH™ Synthesis buffer (buffered 4 dNTP's and rATP solution) along with 3U of T_4 DNA polymerase and 4U of T_4 DNA ligase were added to the primer template mixture and incubated at 37°C for 2h. Heating the reaction to 85°C for 15 min. stopped the reaction.

3. The next step involved the addition of the restriction enzyme *Dpn* I, and digestion of the mixture for 30 min. The preference of *Dpn* I for the fully methylated target plasmid DNA resulted in the preferential degradation of the wild type target DNA into linear DNA of low transformation efficiency, while the hemi-methylated heteroduplex mutant DNA was left in the circular form, and "transformation competent". This eliminated much of the non-mutant dsDNA that would increase

the number of wild type progeny in the subsequent transformation into an *E. coli* host.

4. Immediately after the 30 min. digestion the entire reaction volume was transformed into the MORPH mutS$^-$ cells. The use of a strain of *E. coli* (mutS$^-$) deficient in methyl-dependent DNA repair system prevented the normal tendency to identify the methylated strand as correct and a higher loss of mutant than non-mutant plasmid in the propagation step. In these studies approximately 40-60% colonies isolated and screened carried mutant plasmids. Six clones containing the mutation were identified by dideoxy-sequencing, two of which were subcloned into the *Hind* III/*Xba* I site of pcDNA 3 for expression.

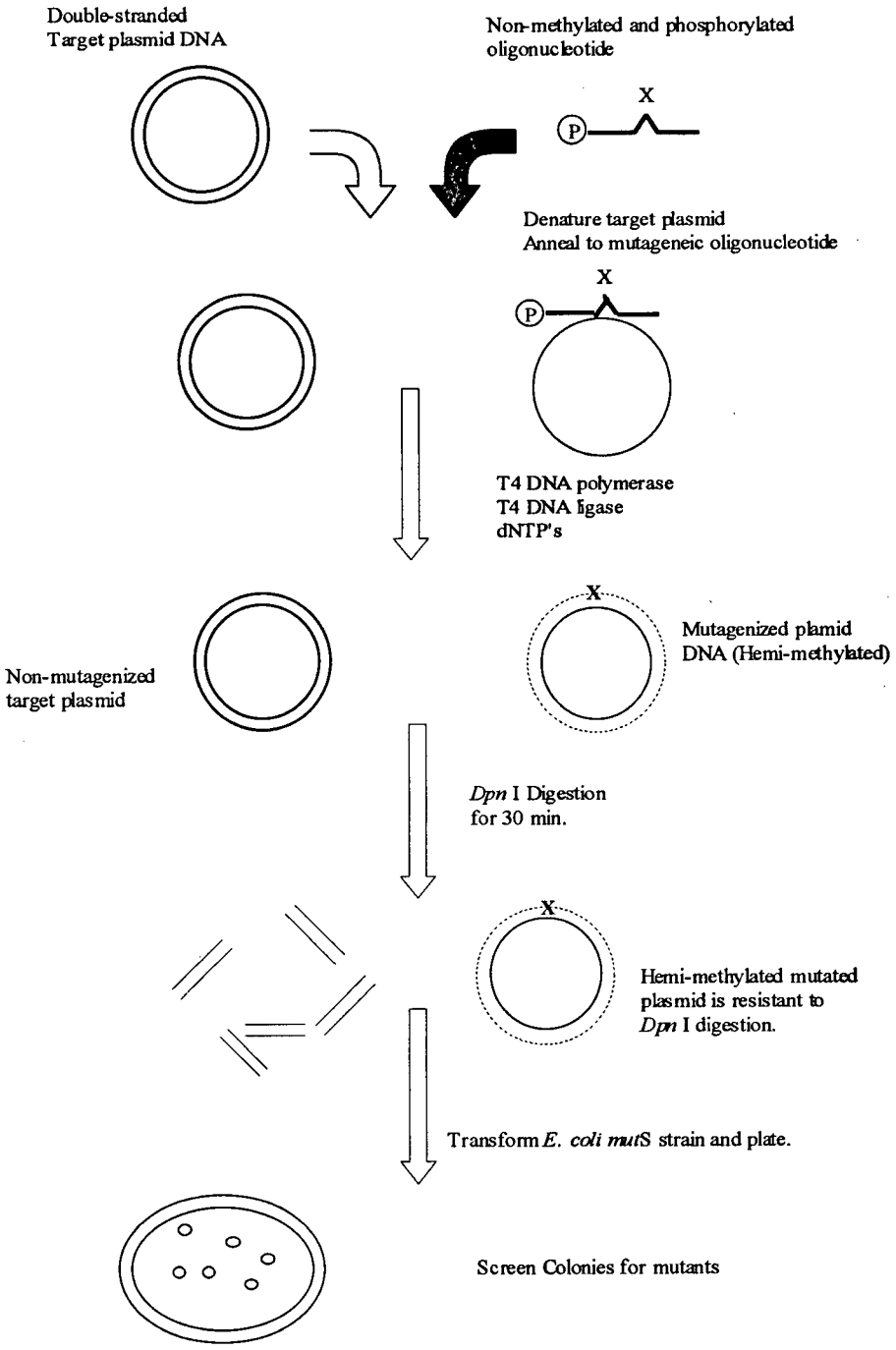


Fig. 7. Double-stranded DNA Mutagenesis. Generation of the GIP H170R receptor mutant was carried out using the commercially available mutagenesis kit (Morph™ Site-Specific Plasmid DNA Mutagenesis Kit, 5-prime 3-prime). See text for detail.

2.8.3 CONSTRUCTION AND EXPRESSION OF CHIMERIC RECEPTORS

The rGIP-R and hGLP-1R wild type (wt) receptor cDNAs were cloned into the expression vector pBKS$^-$ (Stratagene, San Diego, CA) for modification. Receptor cDNAs were endonuclease-digested utilizing common endogenous sites or sites introduced into homologous regions (see below for specific constructions) to facilitate chimeric construction. Recombinant receptors were generated by ligating the N-terminal fragment of one receptor to the C-terminal fragment of the other into the *Hind* III/*Xba* I site of pcDNA 3 for expression (Invitrogen, San Diego, CA). Introduction of mutations and the generation of common restriction sites were performed using the strand selection technique developed by Kunkel (1985), and two different PCR based methodologies. The chimeric forms generated and the methodologies used are described below:

2.8.4 USE OF ENDOGENOUS RESTRICTION SITES

Several chimeric receptors did not require the introduction of non-endogenous occurring restriction sites. CH-1: generated using the *Kpn* I site at coding nucleotide 264 in both receptors, resulting in a receptor encoding the first 88 amino acids of the GIP receptor and residues 99-463 of the human GLP-1 receptor. CH-4 and CH-6: N-terminal regions of the receptors, consisting of the extracellular NT domain, first EC loop, TM domains 1 and 2, and part of 3, and the first intracellular loop, were exchanged using the common *Sca* I restriction sites at nucleotide 664 in rGIP-R and nucleotide 703 in hGLP-1R (Fig. 8). The general methodology is outlined in Fig.8, and the cartoons of the individual receptors are presented in Fig. 9.

2.8.5 SINGLE STRANDED OLIGONUCLEOTIDE-DIRECTED MUTAGENESIS WITH STRAND SELECTION

This methodology of ssDNA oligonucleotide mutagenesis originally described by Kunkel (1985) utilizes *E. coli* strains that are dUTPase (*dut*-) and uracil N-glycosylase (*ung*-) deficient, and is outlined in Fig. 10. These strains have increased levels dUTP due to the lack of functional dUTPase, resulting in the incorporation of dUTP rather than dTTP into the replicating DNA. Due to the lack of uracil N-glycosylase, the spuriously incorporated dUTP molecules are not removed by normal DNA repair mechanisms. M13 derived plasmids with target sequences can be transformed into a *dut/ung*$^-$ strain (such as CJ236) to generate dUTP containing ssDNA template. This template can then be used to synthesize a mutant heteroduplex molecule in the presence of dTTP, which is then transformed into an *ung*+ strain. The uracil containing parental strand is selected against, while the mutant strand is preferentially replicated, increasing the number of mutant progeny to as high as 80%. The following chimeric receptor cDNAs were produced utilizing this methodology.

1. CH-2 and CH-5: The putative NT domain of hGLP-1R was replaced by all but the two distal amino acids of the rGIP-R NT, and vice versa, by introducing a *Xho* I restriction site at nucleotide 392 in GIP-R and nucleotide 424 in GLP-1R (Fig. 11). This modification resulted in no amino acid changes in the

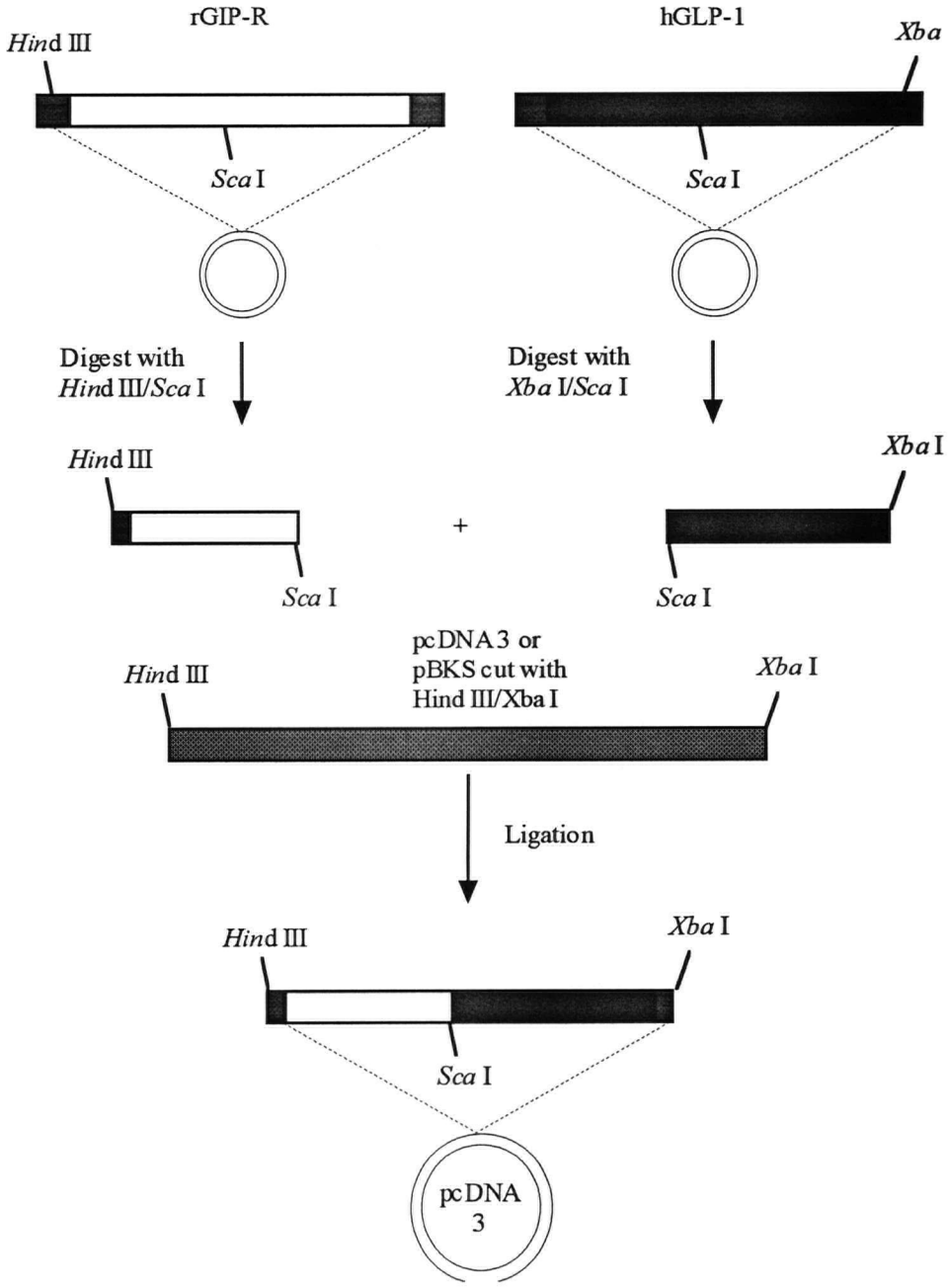


Fig. 8. Construction of Chimeric GIP/GLP-1 Receptors Using Endogenous Restriction Sites. When possible restriction sites common to the GIP and GLP-1 receptors were utilized to construct cDNA's encoding chimeric receptors. Generation of the construct CH-4 was accomplished by utilizing common *Sca* I sites in the two receptor cDNAs.

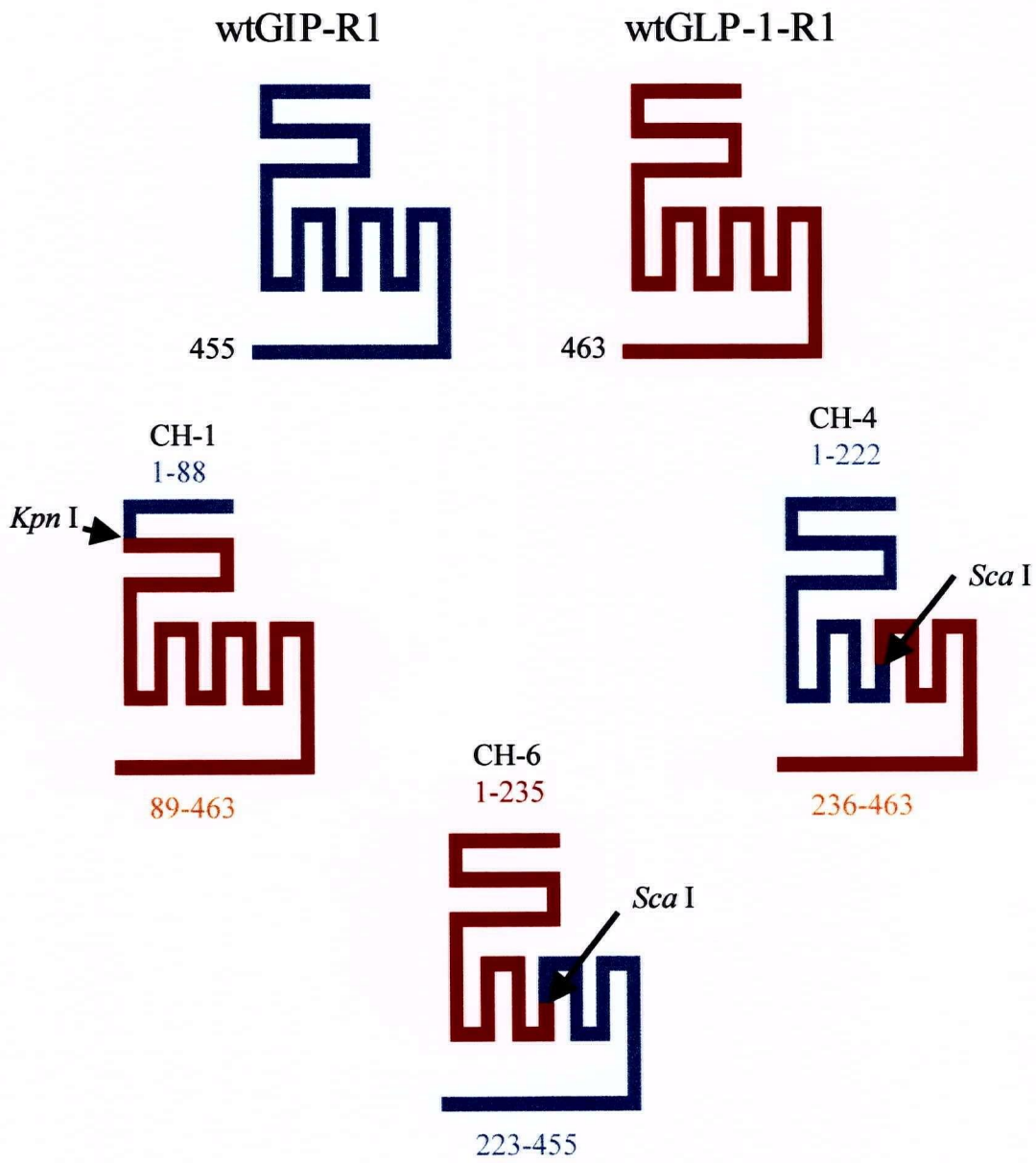


Fig. 9. Predicted Topography of Chimeric Receptors Constructed Using Endogenous Restriction Sites. Regions corresponding to GIP-R are in blue, those of the GLP-1-R are in red. See text for details

wtGIP-R1, and a Phe to Glu substitution at amino acid 143 in the wtGLP-1 receptor. The latter modification to the GLP-1 receptor was shown to generate a receptor that was functionally similar to the wtGLP-1R.

2. CH-3: constructed by introducing a *Nhe* I site at nucleotide 480 of the hGLP-1 cDNA (Leu to Ile substitution at amino acid 161), allowing ligation of the N-terminal *Hind* III/*Nhe* I fragment of rGIP-R1 to the carboxy-terminus of hGLP-R1 (Fig. 11).

2.8.6 POLYMERASE CHAIN REACTION MUTAGENESIS

The method of Vallette *et al.* (1988) was used to introduce silent *Bss* HII sites at coding nucleotides 539 and 567 by introducing a single nucleotide change of A→C at codons 180 and 190, of GIP-R1 and GLP-R1 respectively. Full-length clones, with the introduced *Bss* HII restriction site, were obtained as the result of three rounds of the polymerase chain reaction (see Fig. 12). The Expand High Fidelity PCR system (Boehringer Mannheim, Laval, Quebec), a commercial mixture of two thermal DNA polymerases, *Taq* and *Pwo*, was used to reduce the occurrence of PCR mutations

1. Round 1: 100 pmol of an appropriate mutagenic primer (A) and the vector specific flanking primer (B; 5'-GGAGTACTAGTAACCCTGGCCCCAGTCACGACGTTGTAA-3') (Fig.12), ~1 fmol of pBKS-/GIP-R1 or pBKS⁻/hGLP-1 R template, 200 μM dNTPs, and 2.6 U of Expand High Fidelity PCR system were added together in a total volume of 50 μl, in 1X Expand Buffer, supplemented with 2 mM $MgCl_2$ (Boehringer

Mannheim) and cycled 25 cycles: 30s denaturation at 95°C, 30s anneal at 58°C, and a 1.5 min extension at 72°C. Buffer conditions did not change in any of the following steps.

2. Round 2: ~600 fmol (200 ng) of gel purified Round 1 PCR product used to linear amplify 1 fmol of the same template from which it was derived, under similar conditions: 2 min denaturation at 95°C, 30s anneal at 55°C, and 2 min extensions at 72°C.

3. Round 3: 100 pmol of primers C (5'-CGAGAAACAGCTATGACCAT-3') and D (5'-GAAGTACTAGTAACCCTGGC-3') (Fig.12) were added to the Round 2 reaction mixture and cycled 25 times as follows: 30s denaturation at 95°C, 30s anneal at 55°C, and 2 min extensions at 72°C. The PCR products of three individual reactions were gel purified, and restriction digested with *Hind* III/*Xba* I and subcloned into pcDNA 3 for expression, and further manipulation. Clones were transiently expressed, and binding studies were performed as a quick screen for extraneous PCR errors prior to use in generating chimeric receptors. cDNAs used to construct both CH-7 and CH-8 were sequenced on one strand to confirm no other PCR errors occurred.

The N-terminal *Hind* III/*Bss*H II fragment of the GIP-R1 cDNA was ligated to the GLP-R1 *Bss*H II/*Xba* I fragment (CH-7) in the *Hind* III/*Xba* I sites of pcDNA 3 for expression and sequencing (Fig. 13). The inverse of this construct was also generated in a similar way and designated CH-8 (Fig. 13).

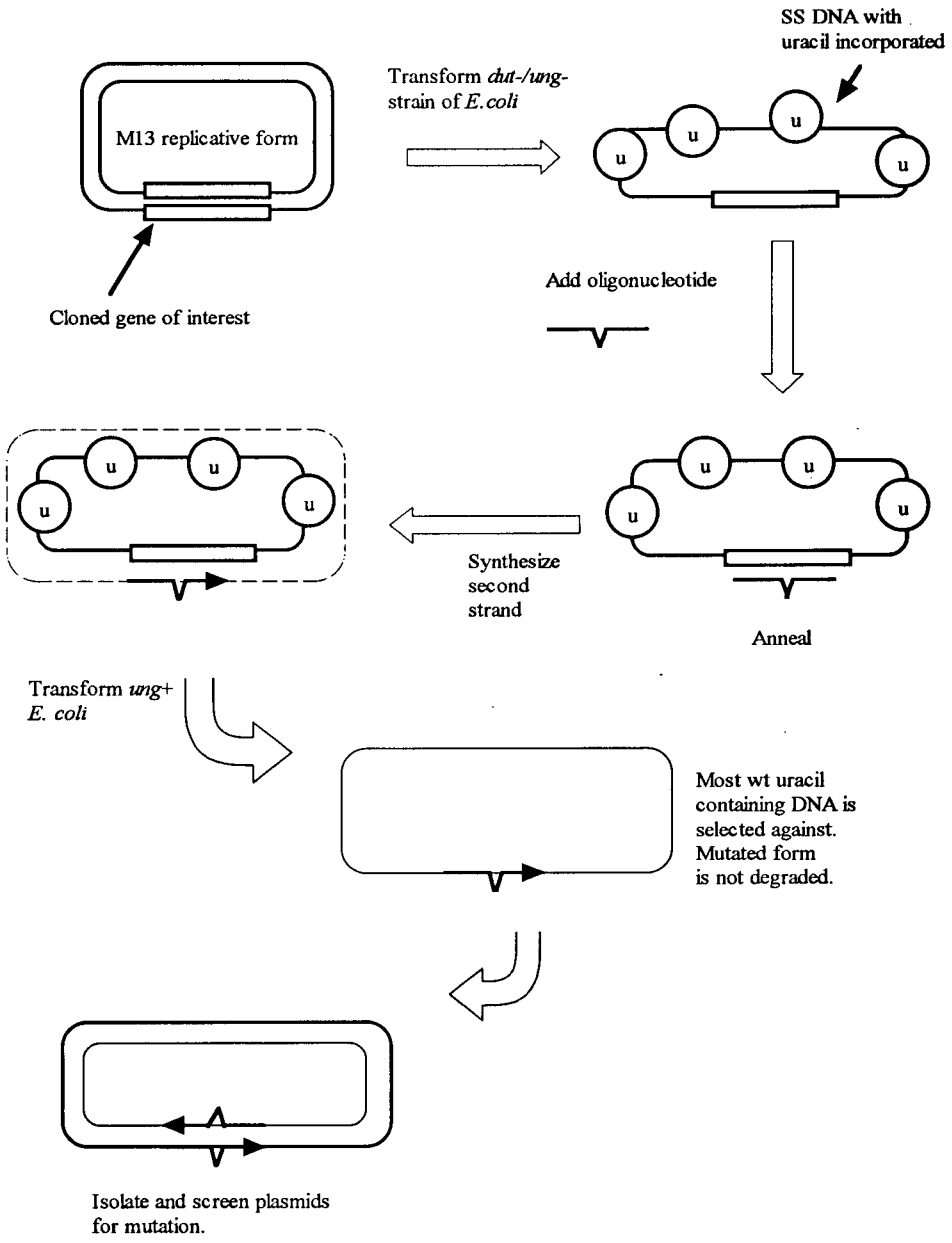


Fig. 10. Single Stranded Oligonucleotide-directed Mutagenesis with Strand Selection. The method originally described by Kunkel (1985) was used to generate chimeric receptors CH-2, CH-5 and CH-3. See text for details.

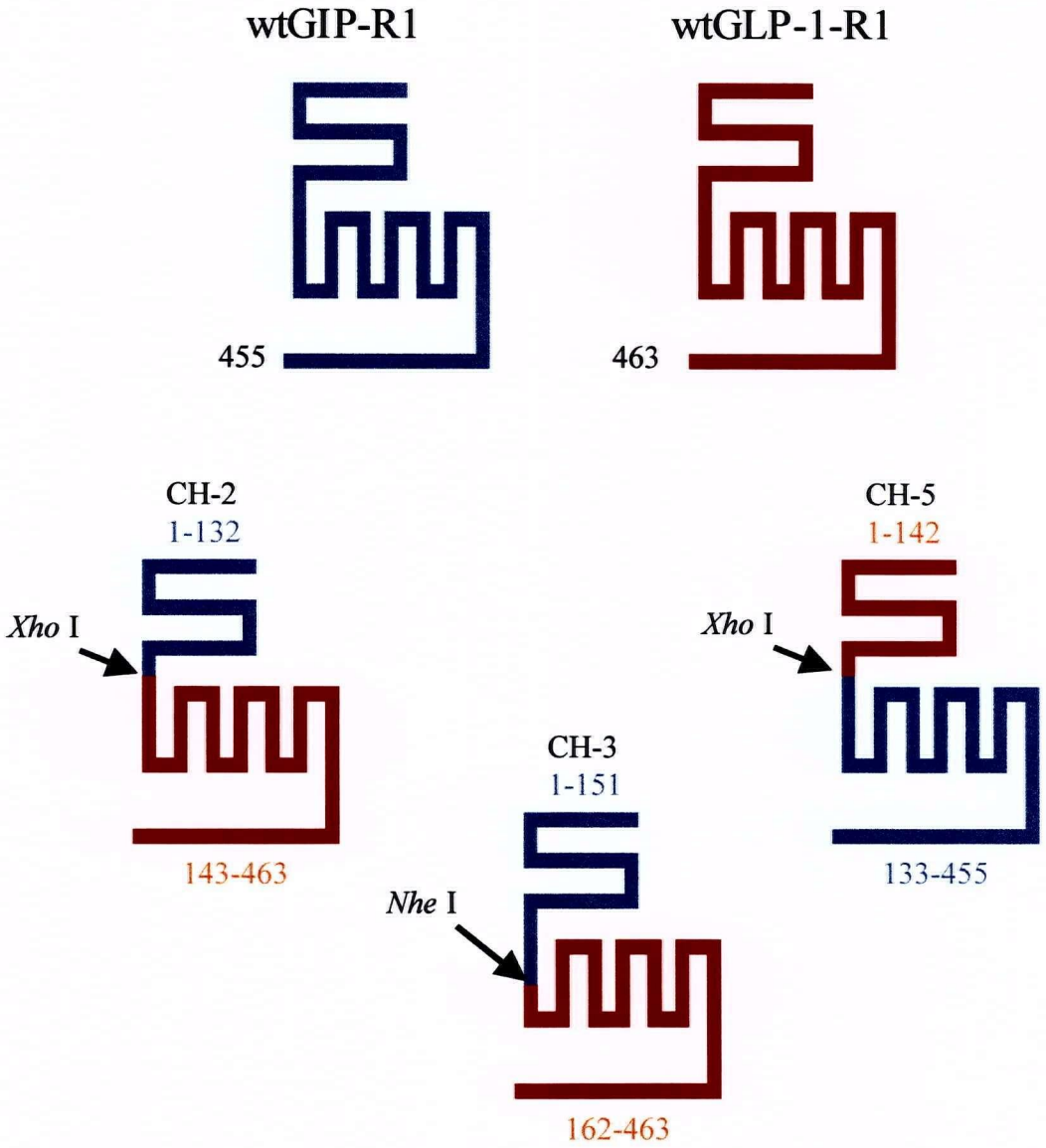


Fig. 11. Predicted Topography of Chimeric Receptors Constructed Using Single Stranded Oligonucleotide-directed Mutagenesis. Regions corresponding to GIP-R are in blue, those of the GLP-1-R are in red. See text for details

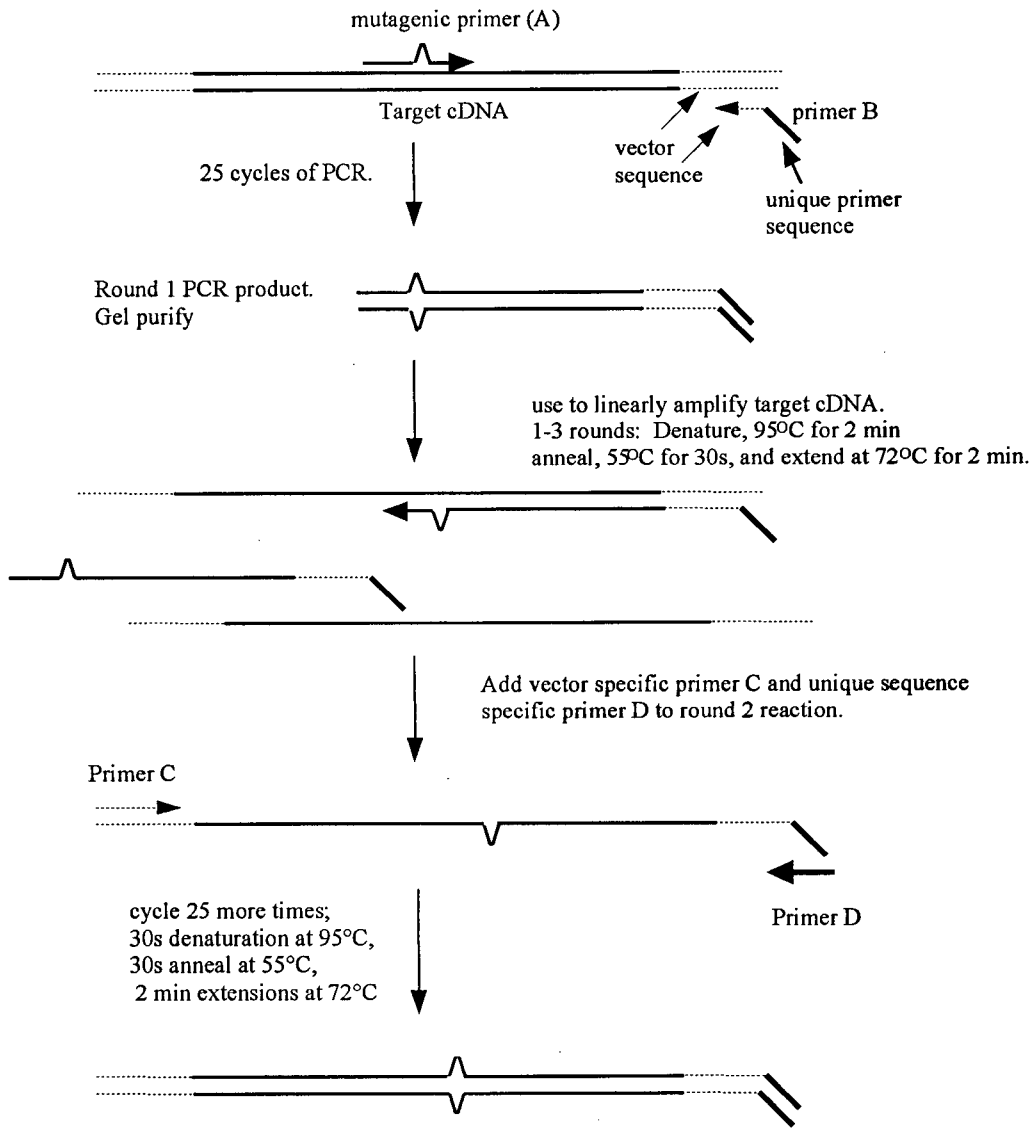


Fig. 12. PCR Based Oligonucleotide-directed Mutagenesis. A PCR based strategy was used to introduce *Bss*H II restriction sites into both the GIP and GLP-1 receptor cDNAs. This modification was used to construct the chimeric receptors CH- 7 and CH-8 and are shown in Fig. 13.

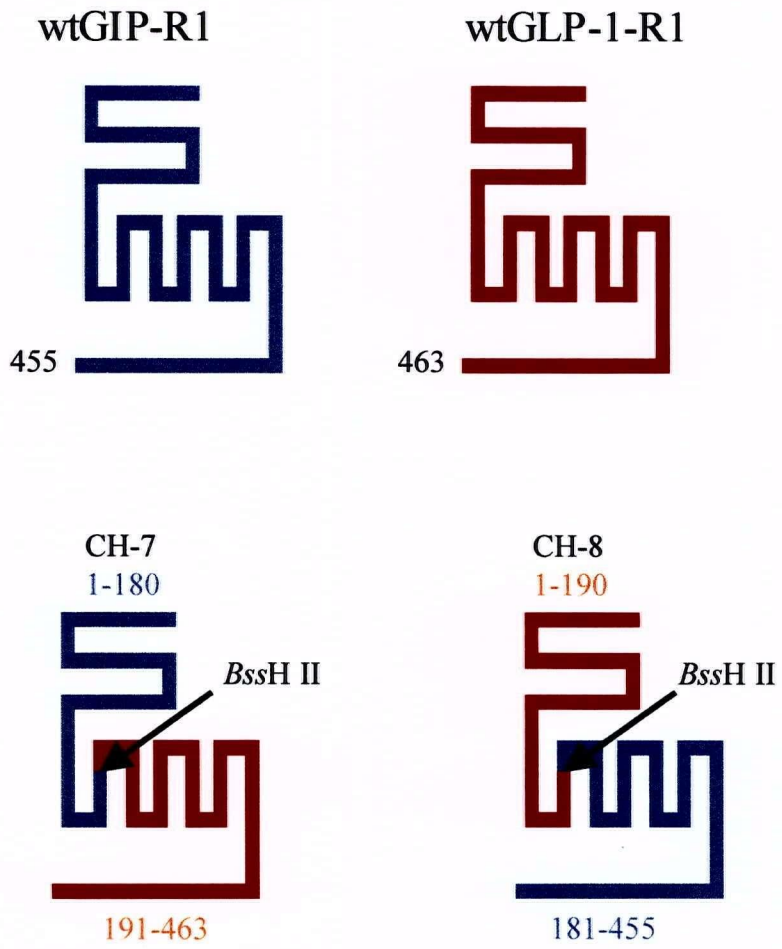


Fig. 13. Predicted Topography of Chimeric Receptors Constructed Using Polymerase Chain-reaction Oligonucleotide-directed Mutagenesis. Regions corresponding to GIP-R are in blue, those of the GLP-1-R are in red. See text for details

The second methodology used PCR to introduce a type II restriction enzyme recognition sequence (*Eam* 1104 I) at the 5' and 3' end of PCR fragments. The fact that this Type II restriction enzyme cleaves at regions outside its consensus sequence allows the production of sticky DNA fragments with sticky ends unrelated to the recognition sequence. This allowed the two cDNAs of interest to be ligated together at structurally homologous regions (i.e. transmembrane domains), requiring only one conserved amino acid at the point of ligation (see Fig.14). A mutagenesis kit (Seamless™ Cloning Kit, Stratagene) was used as described below.

5'β-Gal	5'-ATACTCTTCACCATGATTACGCAAGCGC-3'
3'β-Gal	5'-ATACTCTTCATGGTCATAGCTGTTTCCTG-3'
GIP Phe159	5'-ATACTCTTCTGAACAAACTTAAAATGAGT-3'
GLP Phe169	5'-ATACTCTTCCTTCAGACACCTGCACCTGC-3'

Fig. 14. Primers Used In the Construction of CH-9.

Primers were designed with three random nucleotides, *Eam* 1104 I sequences (underlined), followed by receptor specific sequences one nucleotide prior to the codon (bold) used for ligation (Fig. 14). Two other primers, designed with vector specific sequences (5' and 3'β-Gal), were also used in conjunction with one of the receptor specific primers as shown in Fig. 15.

A recipient (GLP-R1 carboxy terminal amino acids 169-463 plus vector) and insert (GIP-R1 NH_3^+-terminal to amino acids 1-159) PCR products were obtained as follows: 10-15 fmol of target vector (pBKS/GIP-R1 or GLP-R1) in 1 X *Pfu* Buffer, 200 μM of each dNTP, 20 μM of each of the appropriate primers (see Fig. 14 and 15), and 2.5

U *Pfu* polymerase were cycled as follows: one cycle 95°C for 3 min, 58°C for 1min, and 72° for 6 min (GLP-R1 reaction) or 1 min (GIP-R1 reaction), followed by 12 cycles of 95°C for 45s, 58°C for 45s, and 72° for 6 min (GLP-R1 reaction) or 1 min (GIP-R1 reaction). *Pfu* polymerase was used as it is a "proof reading" polymerase (having 3"-5' exonuclease activity) with a lower error rate than *Taq*.

This was followed by the addition of 50 µl of one times *Pfu* buffer containing 200 µM dATP, dGTP, dTTP, and 1 mM 5-methyl (^{m5})dCTP, and the reactions were cycled 5 more times as above. PCR products where then extracted once with phenol-chloroform (1:1), and precipitated with 0.1 vol of 3M sodium acetate and 2.5 vol of ice cold ethanol (98%). The pellet was resuspended in 50 µl of TE (10 mM tris-HCl, pH 7.5, 1mM EDTA) and run on agarose gels to estimate PCR product concentration.

10 µl of each PCR product in 1X Universal buffer (Stratagene), were then digested with 24U of *Eam* 1104 I in a total volume of 50 µl, at 37°C for 1 hour. The presence of the ^{m5}dCTP in the PCR products renders endogenous *Eam* 1104 I sites that exist within the vector or target sequences resistant to digestion, while the non-methylated sites within the primers can be digested.

Five-fifteen µl of this digestion mixture were then ligated in 1 X ligase buffer, with 1mM rATP, 0.25 U T_4 Ligase (Stratagene), and 4U of *Eam* 1104 I in a total volume of 20 µl, at 37°C for 30 min. 2-5 µl of the ligation reation were transformed into Epicurian Coli XL1-Blue MRF' (Statagene) supercompetent cells. Only one clone corresponding to the expected ligation product was obtained. It was cloned into pcDNA 3 (Invitrogen) for expression and sequencing, and was designated CH-9 (Fig. 16).

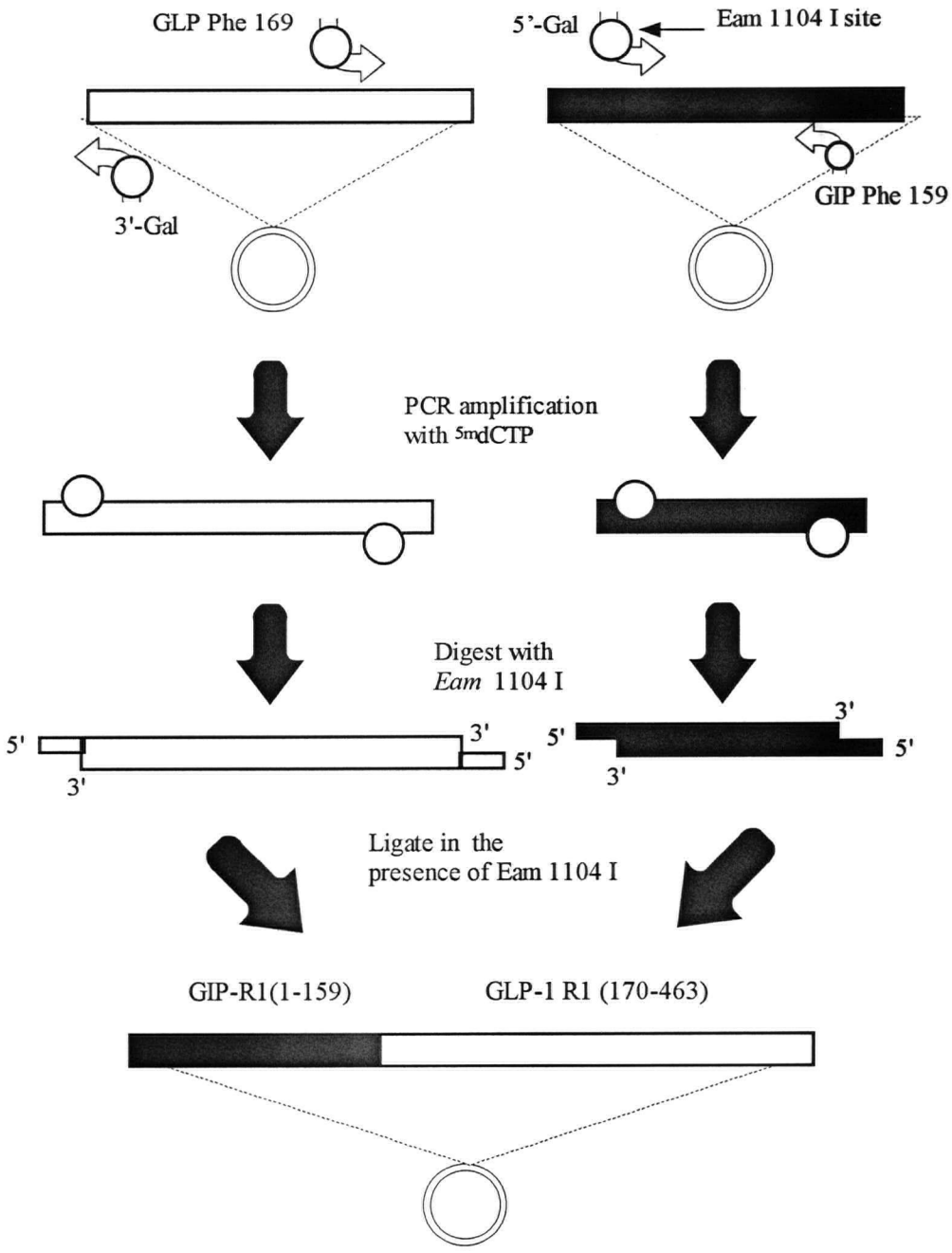


Fig. 15. PCR Based Strategy for the Construction of CH-9. Primers containing *Eam* 1104 I sites allowed the generation of 3 base pair overhangs 4 nucleotides from the recognition sequence. This allowed the two cDNAs to be joined at a single conserved amino acid.

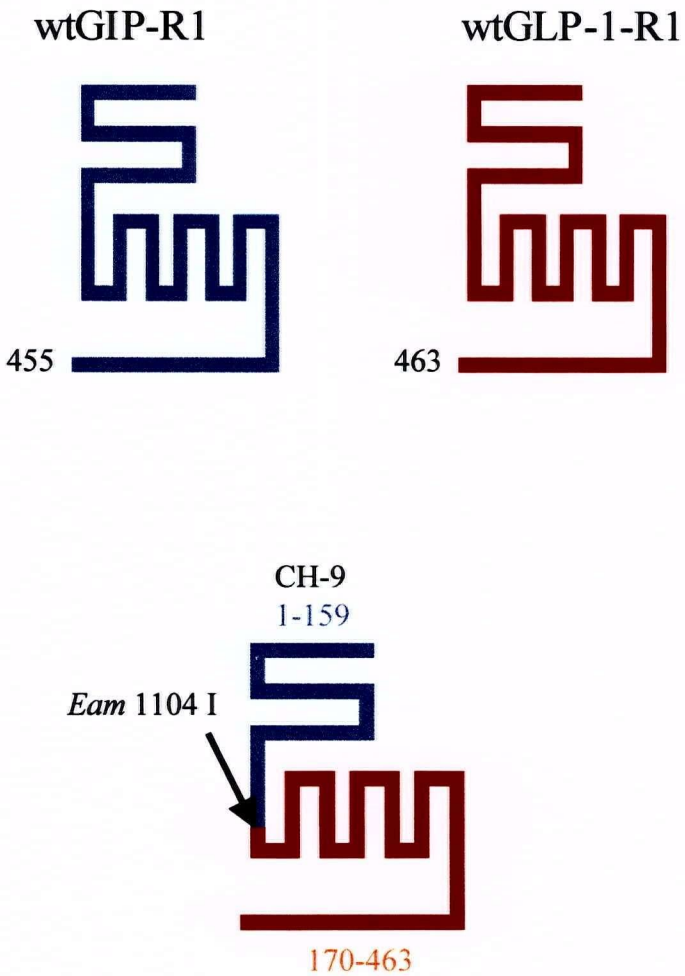


Fig. 16. Predicted Topography of the Chimeric Receptor CH-9. CH-9 was constructed using a polymerase chain-reaction oligonucleotide-directed mutagenesis methodology. Regions corresponding to GIP-R are in blue, those of the GLP-1-R are in red. See text for details

2.8.7 GENERATION OF CARBOXY-TERMINAL TRUNCATED FORMS OF THE RAT GIP RECEPTOR

Preparation of cDNAs encoding truncated forms of the GIP-R1 utilized primers directed to specific regions of the CT tail of the receptor designed to introduce a stop codon at the site of interest (Fig. 17). These primers were used with a common 5' primer specific for the beginning of the coding region (5'-AGGATGCCCCTGCGGCTGTTGC-3') to generate cDNA encoding truncated forms of the receptor.

1255-1236, 5'-CTACTGCCCCAGGTGCGGACGTG-3' (418)
1216-1196, 5'-CTAGAGACGCAGACGGCGGATCTC-3' (405)
1198-1175, 5'-CTAGATCTCCGACTGTACCTCTTTGTTGAT-3' (400)
400ala$_5$, 5'-CTATGCTGCTGCTGCTGCGATCTCCGACTGTACCTCTTTGTT-3'
386ala$_9$,
5'CTATGCTGCTGCTGCTGCTGCTGCTGCTACCTCTTTGTTGATGAAGCAG-3'

Fig. 17. Primers Used In the Construction of Carboxy-Terminal Truncated GIP Receptors.

All PCR products were initially cloned into pCR II (Invitrogen), before subcloning into the *Hind* III/ *Xba* I, or *Not* I/ *Nsi* I sites of pcDNA 3 (Invitrogen) for sequencing and expression.

One other truncated receptor was generated by digesting the wt rGIP-R in the vector Bluescript-KS with *Sac*I at nucleotide number 1278 and religating with the vector's *Sac* I site prior to insertion into the expression vector. The resulting construct

encoded the first 427 amino acids of the GIP receptor plus 6 unrelated amino acids (QRVGCI) encoded for by the vector sequence and was designated GIP-R-427+. A construct with the membrane proximal residues 397-400 deleted (GIP-R-ΔQSEI) was generated using the ssDNA oligonucleotide mutagenesis strategy described in section 2.7.5. Figures 18 and 19 illustrate the CT-tail truncation and modifications resulting from the mutagenesis.

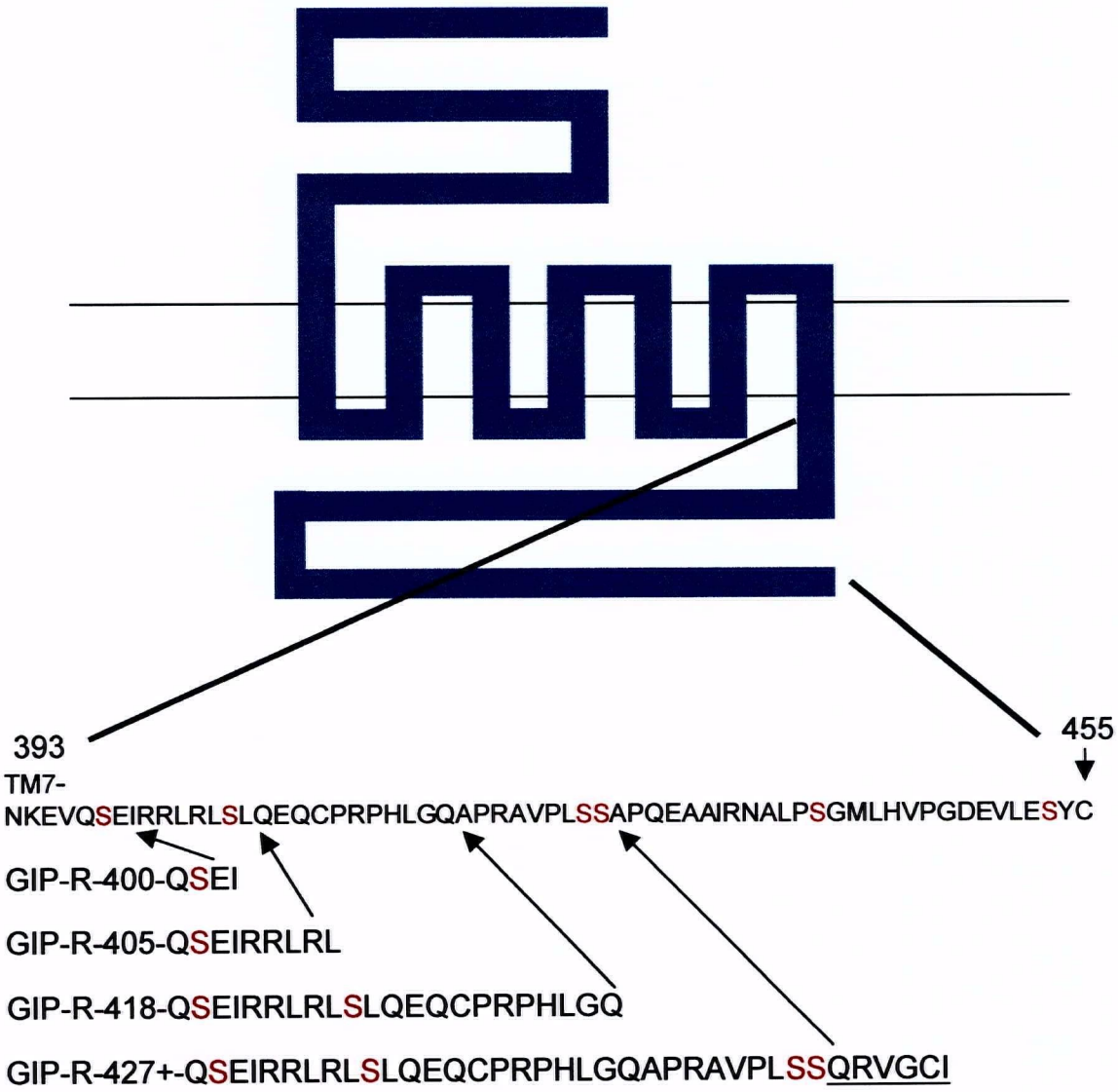
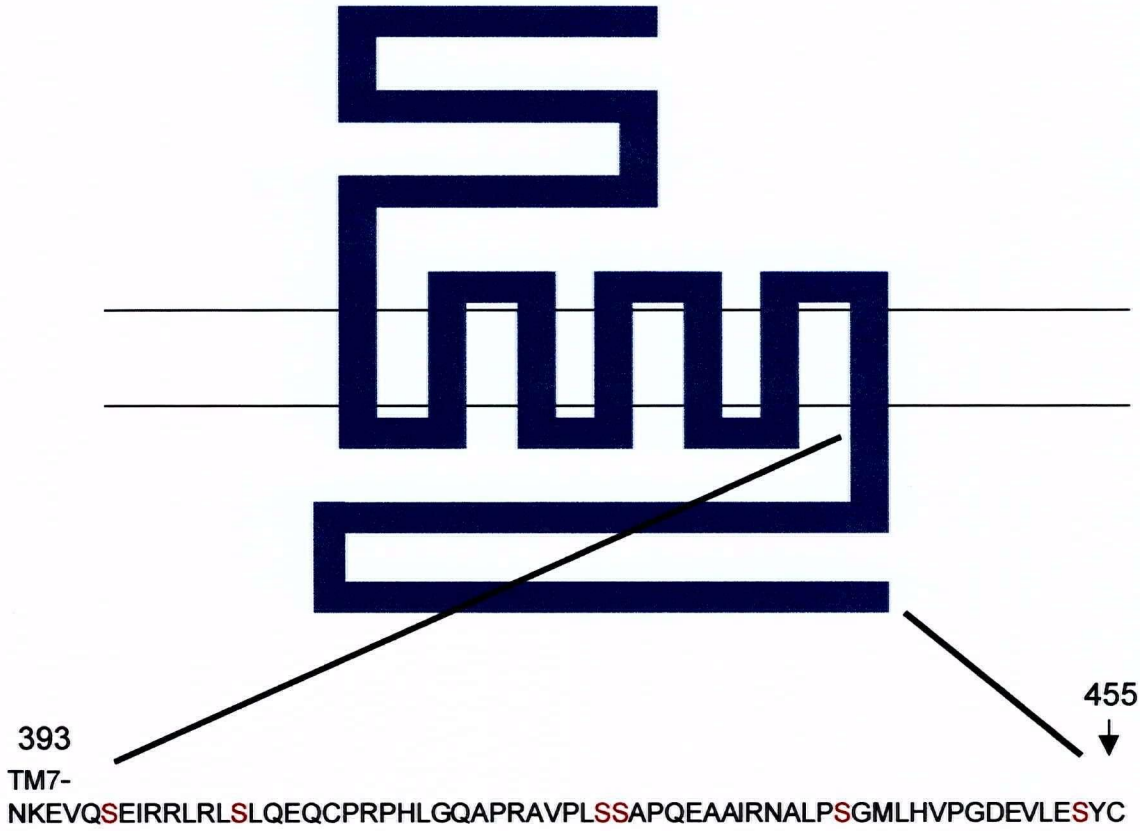


Fig. 18. Carboxy-terminal Truncation of the Rat Islet GIP Receptor. The single letter code for amino acids is used. Four cDNAs were constructed encoding forms of the rat GIP receptor carboxy-terminal truncated at the indicated residues. Serine residues, which are potential phosphorylation sites, are in red text. The six residues resulting from vector sequence in construct GIP-R-427+ are underlined. See Text for details.



GIP-R-396A_9 = TM7-NKEVAAAAAAAAA

GIP-R-400A_5 = TM7-NKEVQSEIAAAAA

GIP-R-ΔQSEI = TM7-NKEVRRLRLSLQEQCPRPHLGQAPRAVPLSSAPQEA
AIRNALPSGMLHVPGDEVLESYC

Fig. 19. Modified Carboxy-Terminal Tail forms of the Rat Islet GIP Receptor. The single letter code for amino acids is used. Serine residues, which are potential phosphorylation sites, are in red text. TM7 = transmembrane region 7. See Text for details.

2.9 RECEPTOR DESENSITIZATION STUDIES.

In desensitization studies, the CHO-K1 cell line wtGIP-R1 was preincubated in the presence or absence of various concentrations of GIP for 0-120 min. in cAMP assay buffer as described in section 2.4. After this initial preincubation, cells were washed three times with assay buffer, and then stimulated for 15-20 min. with various concentrations of GIP in the presence or absence of IBMX. Cells were then extracted in 70% ethanol, and assayed for cAMP production as described in section 2.

CHAPTER 3

RESULTS

3.1 ISOLATION AND CHARACTERIZATION OF A cDNA ENCODING THE RAT GIP RECEPTOR

Total RNA was isolated from purified rat pancreatic islets and first strand cDNA prepared using the polymerase chain reaction with oligonucleotide primers designed to amplify the coding region of the rat islet GIP receptor cDNA, based on the published sequence of the RIN5mF cell receptor cDNA (Usdin *et al.*, 1993). A single product of appropriate size (~1.4Kb) was amplified, as determined by agarose gel electrophoresis (Fig. 20). Three individual PCR reaction products (GIP-R1, GIP-R2, and GIP-R3) were subcloned into the TA cloning vector pCRII (Invitrogen). Subsequently, a *Hind* III/*Xba* I fragment of the cDNA pGIP-R1 was subcloned into the *Hind* III/*Xba* I site of the expression vector pcDNA 3, and the complete sequence of the coding strand was determined (Fig. 21). Alignment with the published sequence identified only one nucleotide difference in pGIP-R1, resulting in a single amino acid difference (Glu21→Gln21). This single nucleotide change was confirmed in the two other independently generated clones by partial sequencing, suggesting that it may be due to a single nucleotide polymorphism (SNP) in the rat gene. The predicted secondary structure of the seven-transmembrane receptor is presented in Fig. 22.

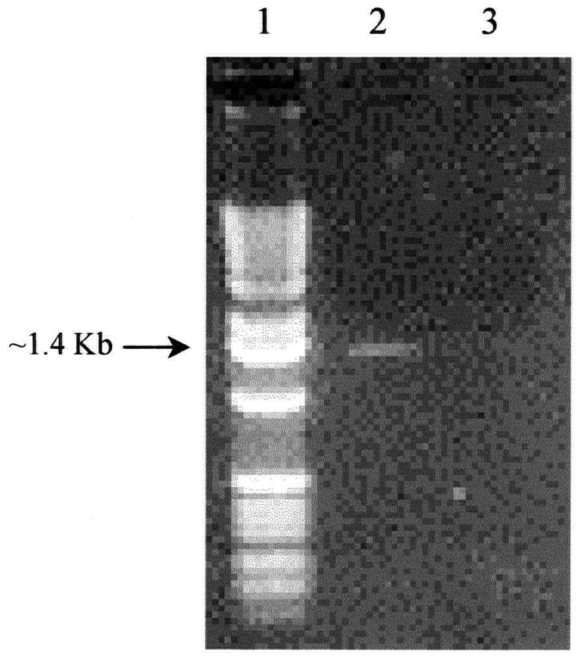


Fig. 20. Amplification of a cDNA Encoding the Rat Islet GIP Receptor Using the Polymerase Chain Reaction. Lane 1, 1 Kb ladder; Lane 2, Rat Islet 1st strand cDNA as template; Lane 3, dH_2O negative control. A single product of approximately 1400 bp was obtained following amplification of rat islet cDNA. Clones from three individual PCR reaction were subcloned for further sequence analysis.

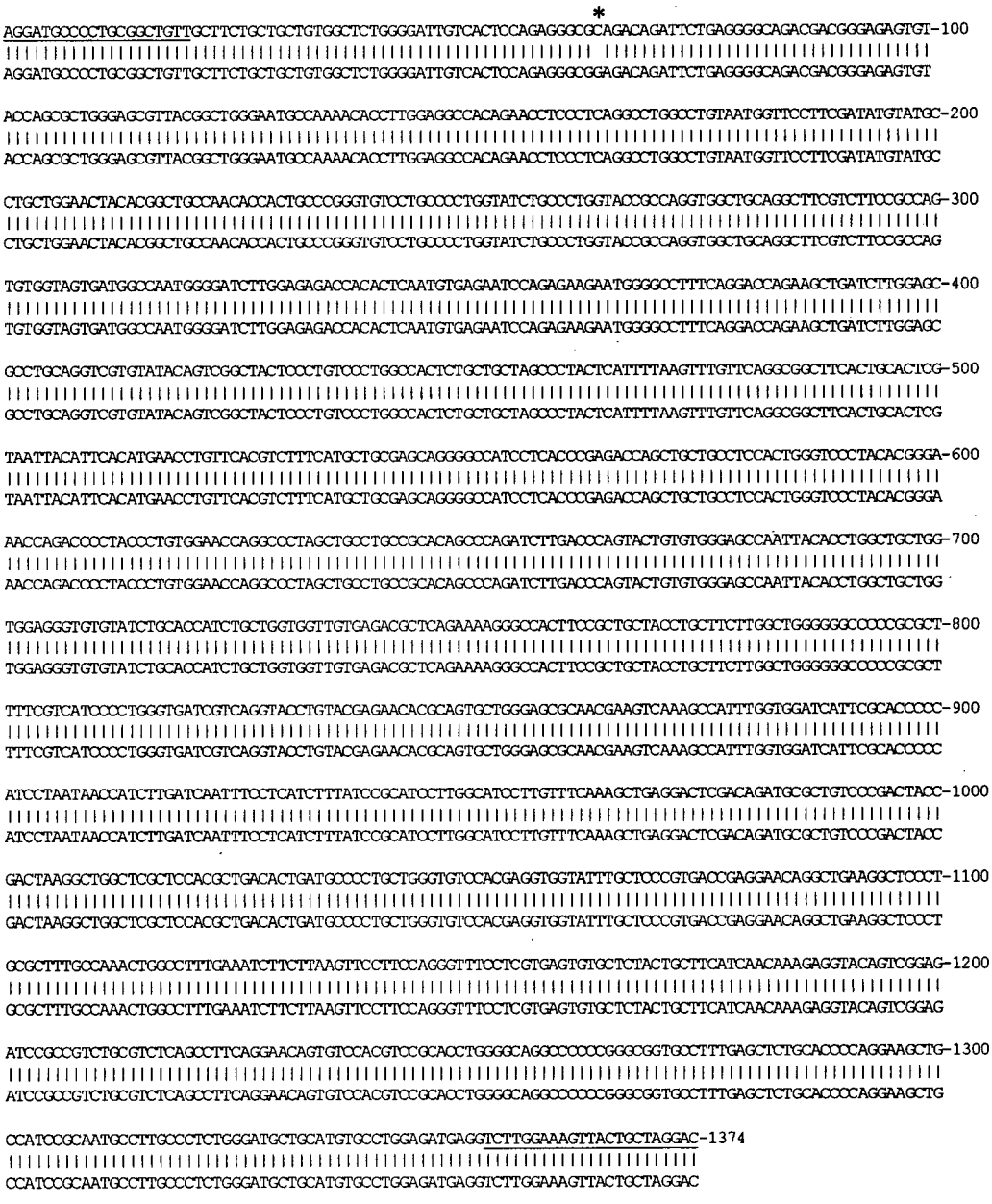


Fig. 21. Sequence Alignment Of The Rat Islet Pancreatic GIP Receptor cDNA Isolated Via RT-PCR (Top) to that of the Previously Published Sequence of Usdin *et al.* (1993) (Bottom). Sequence of the primers used in PCR reactions are underlined and the one nucleotide difference found in three individual clones isolated is noted by *.

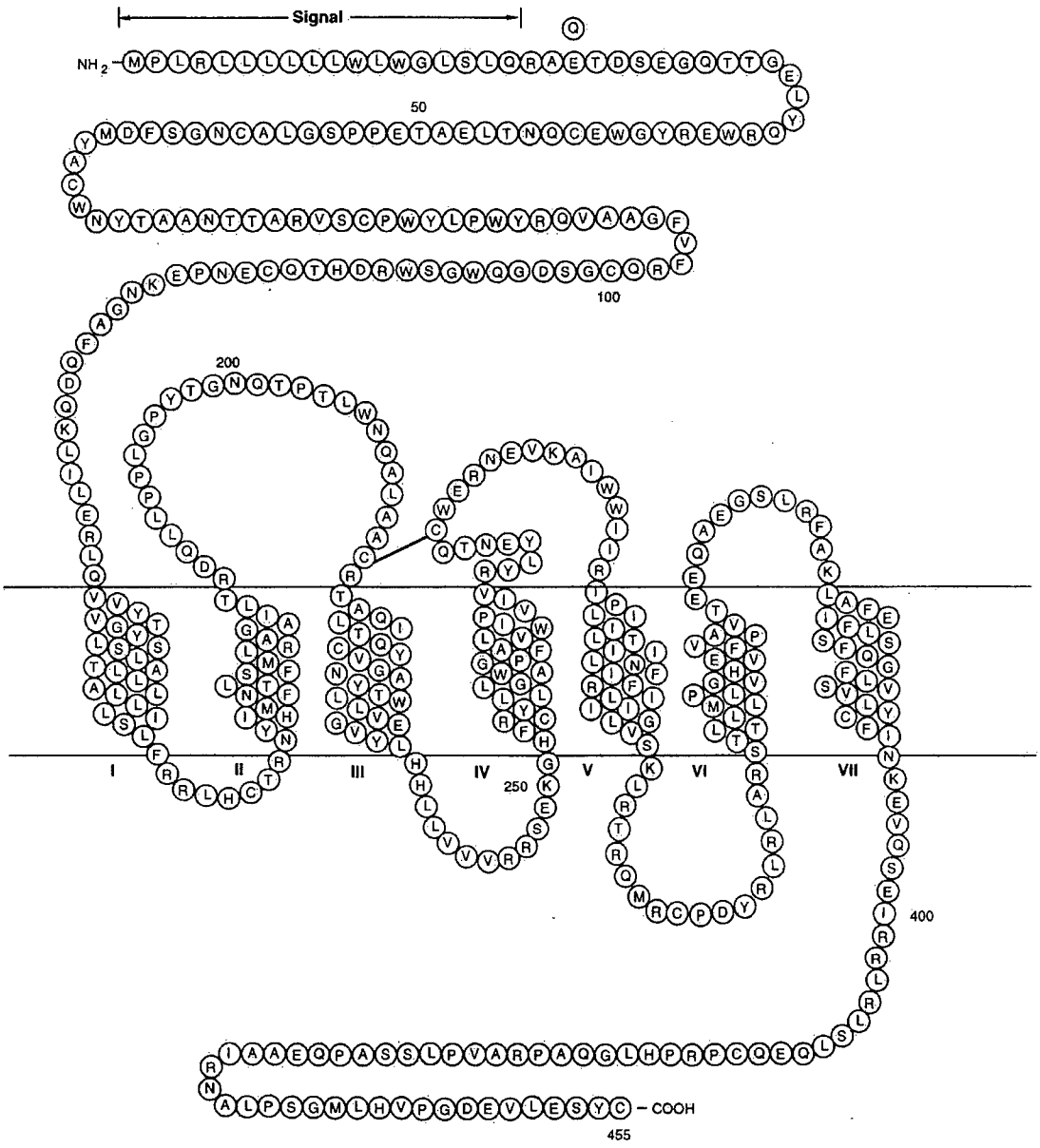


Fig. 22. The Predicted Secondary Structure of the Rat Islet GIP Receptor.

3.2 CHARACTERIZATION OF GIP RECEPTOR BINDING

Initial studies on GIP action used peptide purified from pig intestines (pGIP), and it was shown to be strongly insulinotropic in rats (Pederson and Brown, 1976), humans (Andersen *et al.*, 1978), and other species (reviewed in Brown *et al.*, 1989; Pederson, 1994). However, it was subsequently reported that synthetic preparations of human GIP exhibited greatly reduced insulinotropic activity compared to both the native porcine hormone and synthetic preparations of pGIP (Nauck *et al.*, 1989, 1993a). In comparative studies, using the isolated perfused rat pancreas, Jia and co-workers (1995) showed that commercially available preparations of shGIP exhibited much lower insulinotropic activity than synthetic pGIP preparation. It was suggested that the sequence differences between the human and porcine peptides could account for the difference in potency, but that problems in chemical synthesis were the most likely source. To establish that the PCR-amplified cDNA isolated from rat islet tissue encoded a GIP-specific receptor, and to compare receptor binding affinity of spGIP and shGIP preparations, binding analyses were initially performed on COS-7 cells transiently expressing GIP-R1. Both peptide preparations inhibited the specific binding of ^{125}I-spGIP to COS-7 cells in a concentration-dependent manner and with similar potencies (Fig. 23). The IC_{50} values for displacement were 7.6 ± 1.2 nM and 8.9 ± 1.8 nM (n = 3) for spGIP and shGIP, respectively (Fig. 23). Similar displacement results were obtained with COS-7 cells transfected with a second GIP receptor cDNA, GIP-R2 (spGIP IC_{50} 7.2 nM; shGIP IC_{50} 8.0nM, n =2). There was a complete absence of specific binding to control non-transfected COS-7 cells, to cells transfected with the expression vector pcDNA 3, or to the rat GLP-1 receptor.

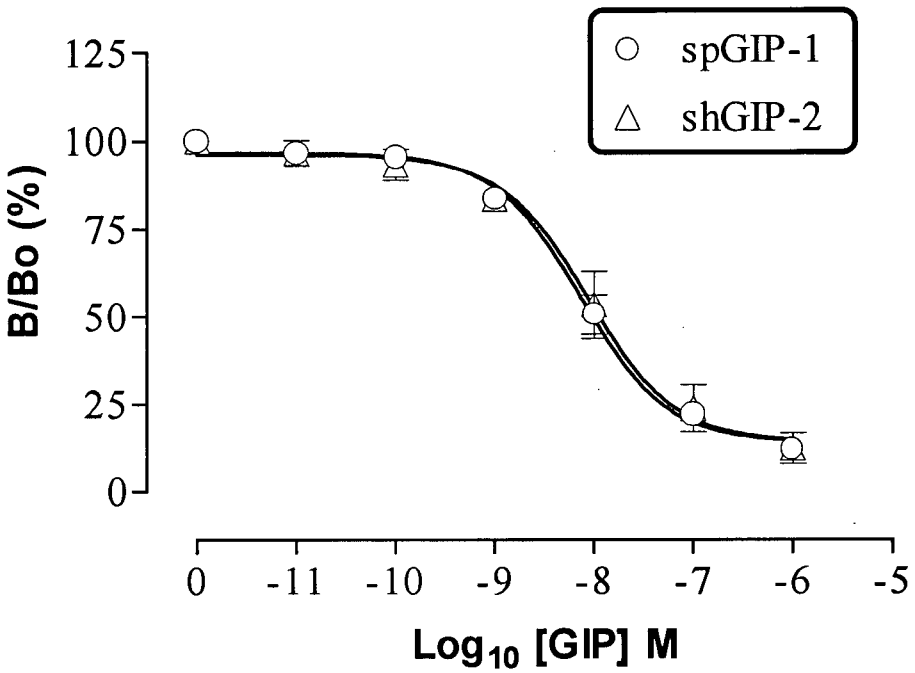


Fig. 23. Displacement of ^{125}I-spGIP Binding From COS-7 Cells Transiently Expressing GIP-R1. Both spGIP and shGIP displayed similar affinity for the rat islet GIP receptor when expressed in COS-7 cells (see Text for details). Curves are representative of 3 individual experiments.

Since stable CHO-K1 cell lines expressing GIP-R1 provided a more practical approach to assess ligand binding, the high level expressing stable CHO-K1 clone, referred to as wtGIP-R1, was used to characterize GIP receptor binding further. Saturation isotherms obtained with both intact cells and membranes gave monophasic binding curves with Kd values of 204 ± 17 pM and 334 ± 94 pM (n = 3-6), respectively (Fig 24). wtGIP-R1 was determined to express approximately 12-15 X 10^4 receptors/cell or 11.8 ± 0.10 fmol of receptors/mg of cell membrane.

In competitive binding studies, data were found to fit most consistently to a one site model at all temperatures examined (4°C, RT, and 37°C). IC_{50} values were lower at 4°C (1.2 –1.7 nM) than at room temperature (3.1-3.7 nM) or 37°C (1.2-8.9 nM) for spGIP (Table 1). Interestingly, Bmax values obtained at 37°C were consistently decreased in comparison to those obtained at either 4°C, after a 4 hour incubation, or RT after a 1 hour incubation (Table 1). As experiments performed at room temperature gave more reproducible IC_{50} values then those at 37°C, with a convenient investigation time, all initial experiments were performed at RT. Given that Jia *et al.* (1995) had seen a marked difference in the insulinotropic potencies of spGIP and shGIP, it was surprising that no differences between the affinities of these peptides was observed in COS-7 cells. To determine if differences in the quality of different synthetic preparations could account for the lack of biological activity, the wtGIP-R1 cell line was used to examine the affinity of two different spGIP and shGIP preparations in competitive binding experiments. Again, there were no differences between mean IC_{50} values for different preparations of either porcine or human GIP [shGIP-1 (Bachem) 2.6 ± 0.8 nM, spGIP-1 (Bachem) 3.7 ± 1.5 nM, shGIP-2 (Peninsula) 3.1 ± 0.9nM, spGIP-2 (Peninsula), 3.6 ± 0.4 nM], or

between the preparations from the two different commercial suppliers ($P > 0.05$, $n = 3$; Fig. 25, Table 2). Natural porcine GIP (enterogastrone III; EGIII) also had a similar IC_{50} value (~3nM, Table 2), further validating the use of more readily available synthetic preparations for GIP studies. Non-transfected CHO-K1 cells or cells transfected with vector alone did not display specific ^{125}I-GIP binding.

Competitive binding		
Conditions	IC_{50} (nM) n = 2	Bmax (% of 4°C Bmax) n = 2
4°C, 4h	1.2-1.7	100%
RT, 1h	3.1-3.7	89%
37°C, 1h	1.2-8.9	54%

Table 1. Summary of Preliminary Binding Experiments. Experiments were carried out with wtGIP-R1 cells to optimize conditions for later experiments.

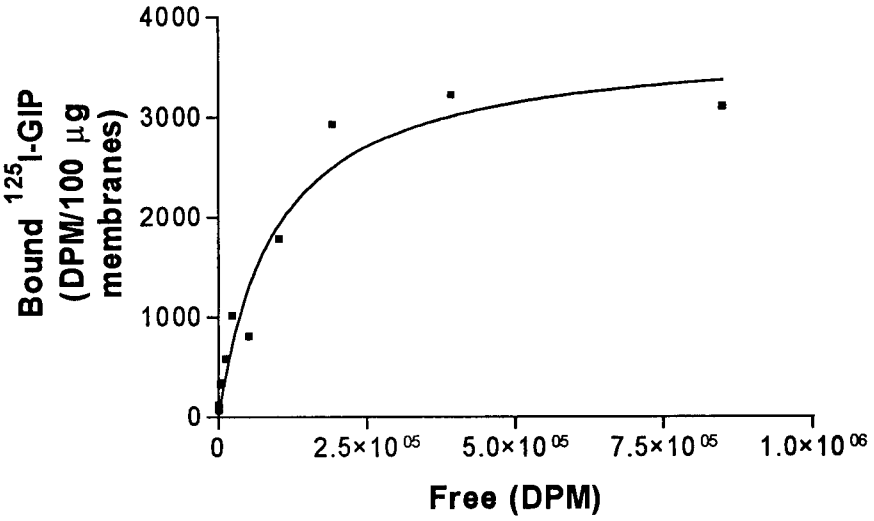
	GIP preparation				
	spGIP-1	spGIP-2	shGIP-1	shGIP-2	EG III
IC_{50} (nM)	3.7 ±1.5	3.6 ± 0.4	2.6 ± 0.8	3.1 ± 0.9	3.0*

Table 2. Summary of Competition Binding Studies Comparing Different GIP Preparations. EG III (Enterogastrone III) = porcine GIP purified from intestine. n=3 for all data except * where n = 2.

The binding specificity of the receptor was examined further in wtGIP-R1 cells, using several structurally related mammalian peptides, including GLP-1(7-36) (tGLP-1), GLP-2, glucagon, and VIP (Fig. 26). In addition, given that the GLP-1 agonist, exendin (Ex)-4, and antagonist, Ex (9-39), venom peptides isolated from *Heloderma suspectum*,

have been demonstrated to stimulate and antagonize, respectively, the incretin response in the rat (Kolligs *et al.*, 1995; Wang *et al.*, 1995), it was of interest to determine whether these peptides interact with the rat islet GIP receptor. Interestingly, while none of the structurally related mammalian hormones tested inhibited ^{125}I-spGIP binding, both Ex (9-39) and Ex-4 demonstrated significant low affinity binding to the GIP receptor; with ~39% and 21% displacement of ^{125}I-GIP binding, respectively, at a concentration of 1μM (Fig. 27). Although these results demonstrated the high specificity of the GIP receptor for its native ligand, they also suggest that Ex (9-39), an antagonist of the GLP-1 receptor, may have a similar action at the GIP receptor when used in the micromolar or higher concentration range.

A.



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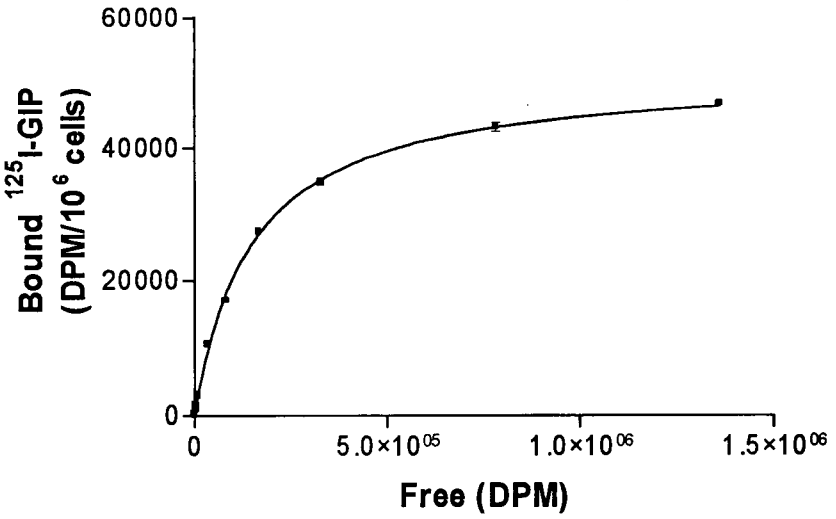


Fig. 24. Saturation binding curve for isolated wtGIP-R1 cell membranes (A) and intact cells (B). Data were analyzed using the curve-fitting program PRISM (GraphPad). The curve was monophasic for both membranes and cells expressing the rat islet GIP receptor, and three individual experiments yielded mean Kd values of 334 ± 94 pM (membranes) and 204 ± 17 pM and a binding capacity of 59 ± 0.5 pM/mg of membrane protein (124,560 ± 14,800 sites/cell).

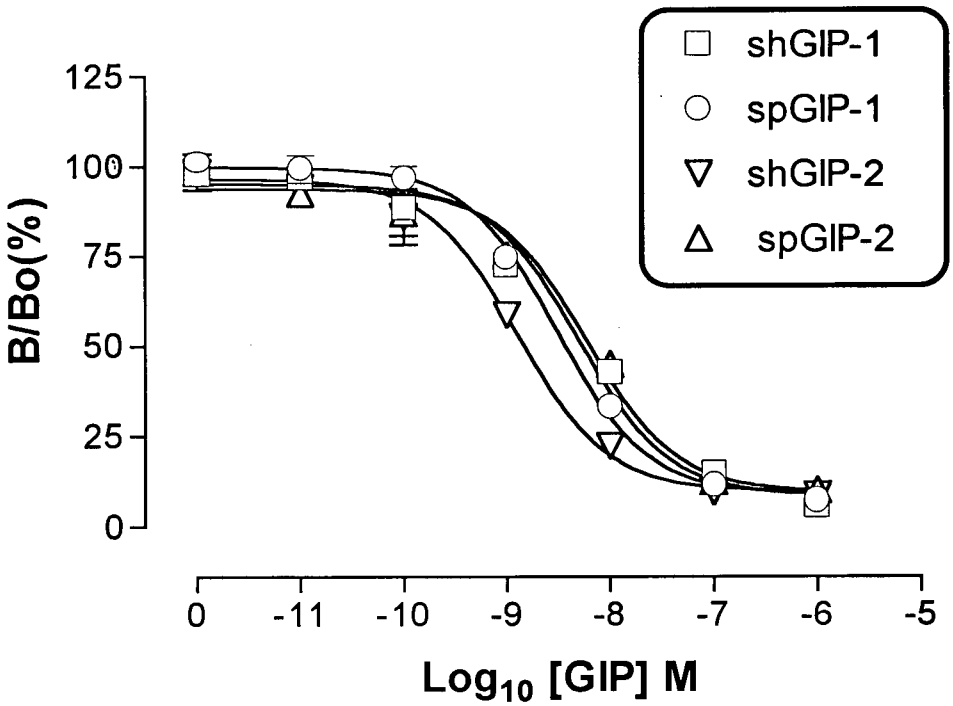


Fig. 25. Displacement of ^{125}I-spGIP Binding from wtGIP-R1 Cells. A CHO-K1 cell-line stably expressing pGIP-R was used to compare the affinity of two shGIP and spGIP preparations. No significant differences between the IC_{50} values obtained were observed (see Text and Table 2). Curves are representative of 3 individual experiments.

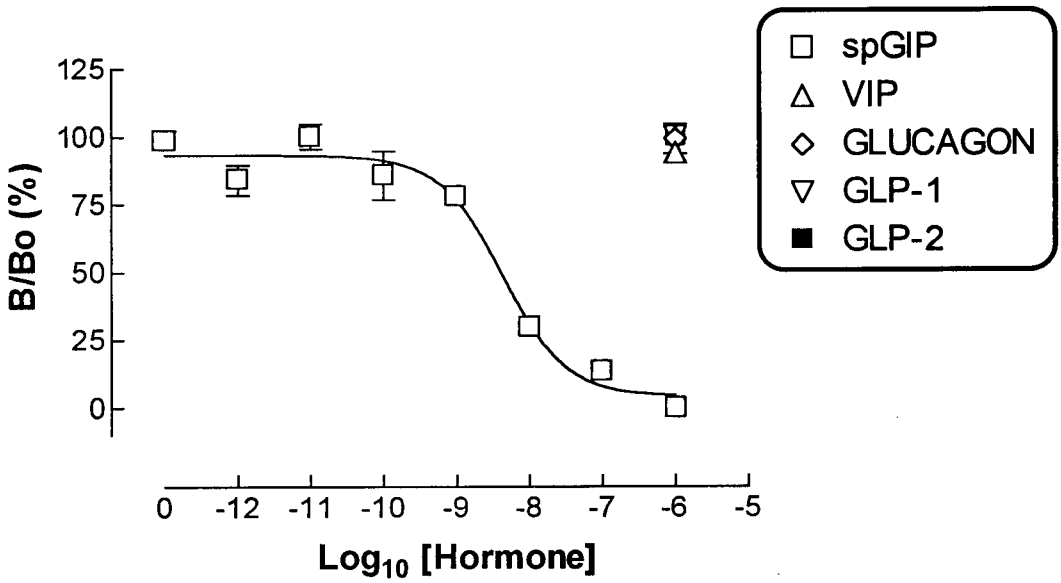


Fig. 26. Displacement of ^{125}I-spGIP Binding from wtGIP-R1 Cells by Peptide Hormones of the Glucagon/VIP/Secretin Family. None of the related hormones tested displaced specifically bound ^{125}I-spGIP. Curves are representative of 3 individual experiments.

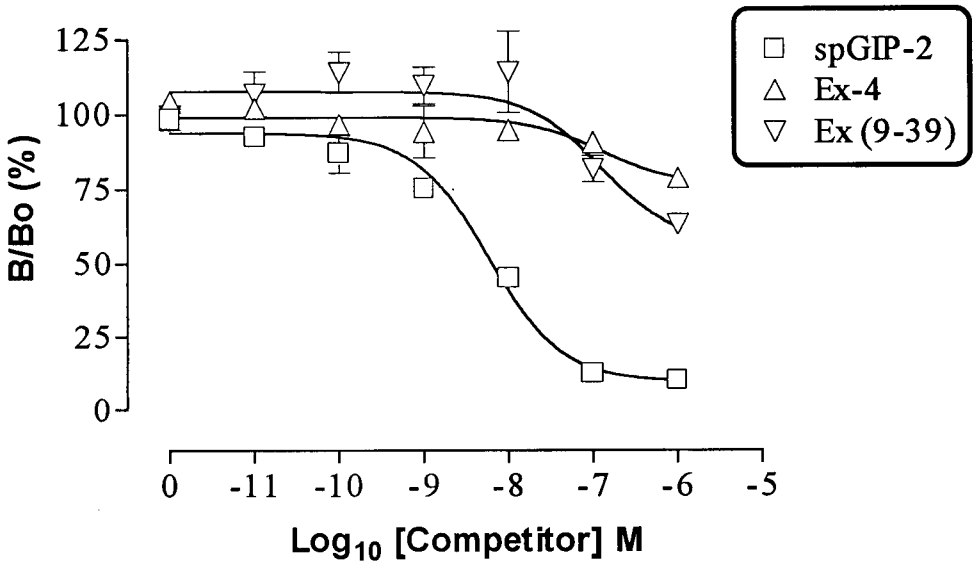


Fig. 27. Displacement of ^{125}I-spGIP Binding from wtGIP-R1 Cells by the GLP-1 Receptor Agonist, Exendin (Ex)-4 and the Truncated Antagonist Form Ex (9-39). Ex-4 and Ex (9-39) inhibited binding by 21% and 39%, respectively, at 1 μM. Curves are representative of 3 individual experiments.

3.3 EFFECTS OF RAT ISLET GIP RECEPTOR EXPRESSION ON cAMP FORMATION

To correlate GIP receptor binding to activation of the adenylyl cyclase (AC) system, cAMP accumulation was determined by radioimmunoassay in COS-7 cells expressing pGIP-R1. In the presence of the phosphodiesterase inhibitor, IBMX (1 mM), synthetic porcine GIP evoked a concentration-dependent increase in cAMP accumulation (EC_{50} = 870 ± 150 pM). This effect was not significantly different from that observed with shGIP (810 ± 160 pM) (Figure 28), indicating that the human and porcine species of GIP also share similar biological activities. No significant increases in cAMP accumulation were observed with any of the structurally related peptides tested previously in binding experiments, with the exception of Ex-4, which produced a small response (2.6 + 0.3 fold over basal) at the highest concentration tested (1μM). In control experiments, with cells expressing the vector, or the GLP-1 receptor (GLP-1R), GIP (100 nM) was unable to evoke a cAMP response, further demonstrating the specificity of responses in wtGIP-R transfected COS-7 cells.

The wtGIP-R1 cell line was subsequently used to examine the effects of different GIP preparations (Summarized in Table 3), related hormones, and fragments on GIP-stimulated activation of cAMP production. Due to the large numbers of cells expressing GIP-R1 in each well, it was anticipated that the addition of IBMX would not be required for cAMP assays in these studies. Comparisons of cAMP responses to different synthetic GIP preparations did not reveal any significant differences at any GIP concentration examined (Table 3). The preparation of synthetic human GIP used in the present study also produced an identical insulin response to spGIP in the isolated perfused rat pancreas

(integrated 50 min insulin responses: shGIP-1 172.7 ±17.5 mU, spGIP 176.2 ± 32.7 mU, shGIP-2 175.0 ± 26.8 mU, spGIP-2, 180.8 ± 17.5 mU, Table 3) in contrast to the commercial preparations of shGIP assayed by an identical procedure in earlier studies (Jia *et al.*, 1995).

Treatment	shGIP-1	spGIP-1	shGIP-2	spGIP-2
GIP (nM)	Cyclic AMP			
0.1	5.8 ± 0.3	6.0 ± 0.1	5.7 ± 0.6	6.5 ± 0.3
1.0	7.6 ± 1.0	7.8 ± 0.3	6.6 ± 0.5	8.2 ± 0.4
10	9.6 ± 1.2	9.4 ± 1.2	8.8 ± 0.3	10.0 ± 2.5
	Integrated insulin			
1.0	172.7 ±17.5	176.2 ± 32.7	175.0 ± 26.8	180.8 ± 17.5

Table 3. The Effect of Different GIP Preparations on cAMP Accumulation in wtGIP-R1 Cells and Insulin Release from the Isolated Perfused Rat Pancreas. cAMP levels are expressed as fold-increase over basal, and insulin levels as integrated insulin responses in mU of insulin over 50 min. Data = mean ± S.E.M. of ≥ 3 individual experiments.

Since the GLP-1 receptor antagonist Ex (9-39) and agonist Ex-4, displayed binding to the rat pancreatic islet GIP receptor, the ability of these peptides to alter cAMP accumulation in the absence and presence of GIP was examined. In wtGIP-R1 cells, and in contrast to experiments with transiently transfected cells, Ex-4 did not increase cAMP measurably at concentrations as high as 1μM, nor did it antagonize GIP-stimulated cAMP increases (Fig. 29A). Importantly, Ex (9-39) did not have any effect on basal cAMP accumulation, or on responses to 10 nM spGIP (Fig. 29A). In agreement with studies reported by Fehmann *et al.* (1994), both Ex-4 and Ex (9-39) exerted their expected agonist and antagonist effects, respectively, in cells expressing the GLP-1 receptor (Figure 29B). As binding data would predict, none of the structurally related mammalian hormones (GLP-1, GLP-2, glucagon or VIP) had any effect on cAMP accumulation in wtGIP-R1 cells at concentrations as high as 1 μM (Fig. 30).

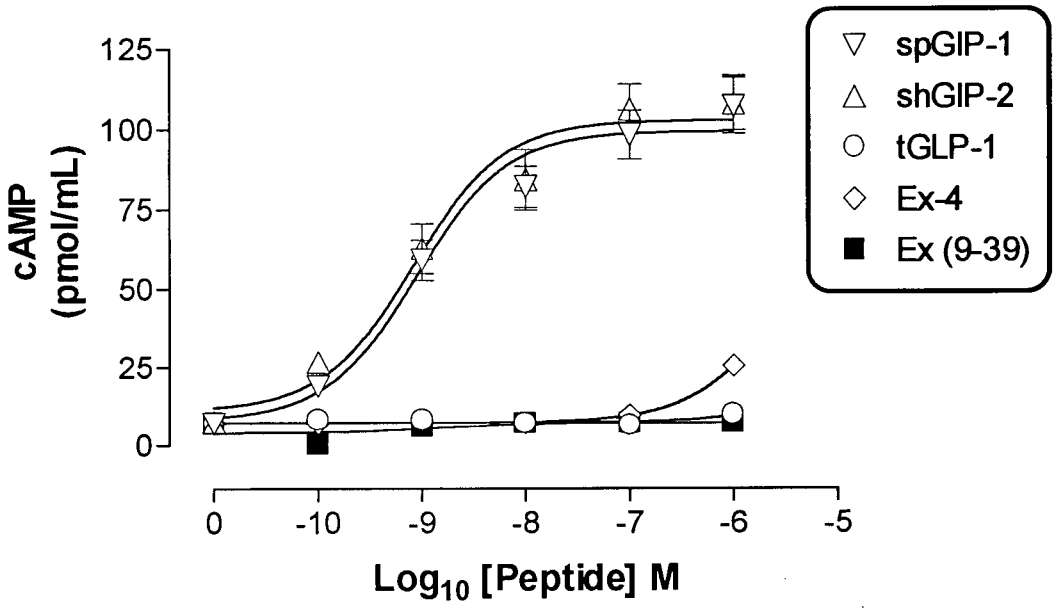


Fig. 28. Stimulation of cAMP Formation in COS-7 Cells Transiently Expressing pGIP-R1 in Response to Secretin/Glucagon Related Peptides. EC_{50} values for shGIP-1 and shGIP-2 were not significantly different ($P > 0.05$). Ex-4 caused a 2.6 fold increase over basal at a concentration of 1 μM.

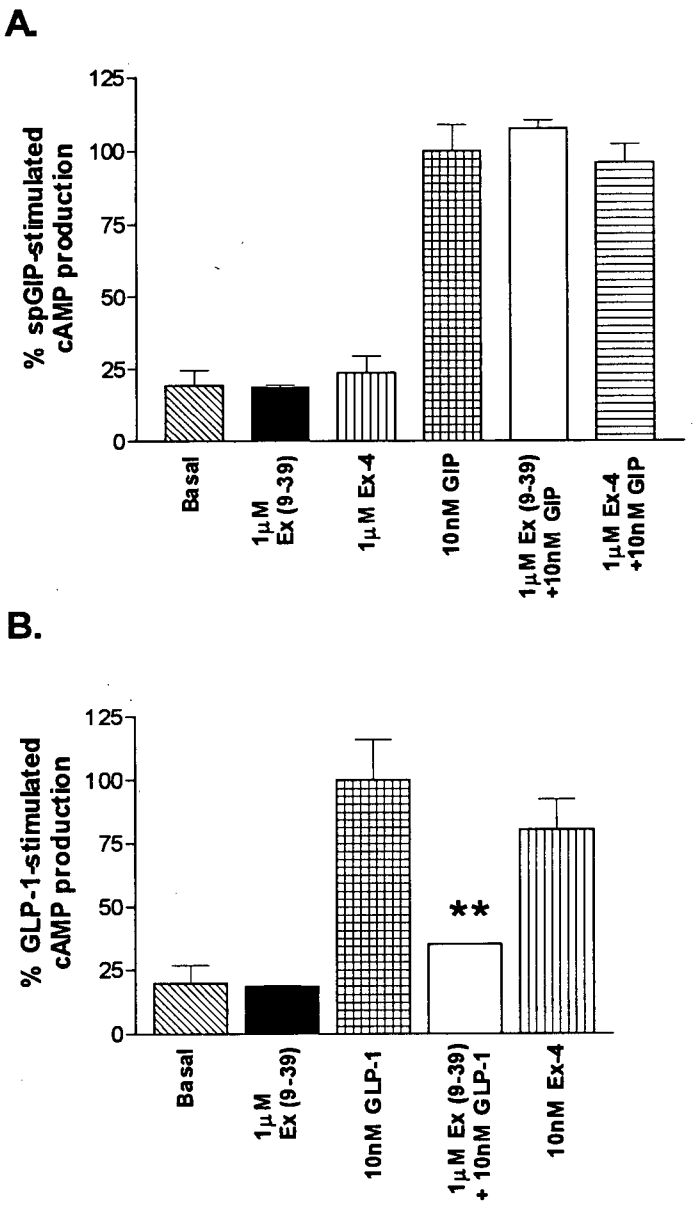


Figure 29. The Effect of Ex-4 and Ex (9-39) on spGIP-stimulated cAMP Formation in the Stable CHO-K1 Clone wtGIP-R1 (A), and on GLP-1-stimulated cAMP Formation in Cells Stably Expressing the Rat GLP-1 Receptor (wtGLP-1-R1) (B). Neither form of Exendins influenced GIP-stimulated cAMP production in wtGIP-R1 cells (A), whereas Ex-4 increased cAMP levels and Ex(9-39) inhibited 10 nM GLP-1-stimulated cAMP production in wtGLP-1-R1cells (B).

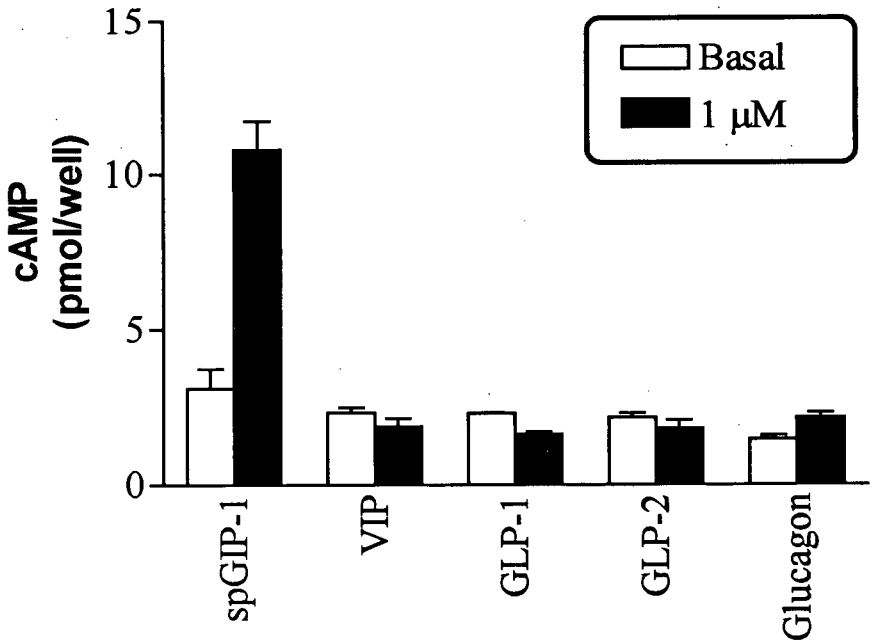


Fig. 30. Cyclic-AMP Formation in wtGIP-R1 Cells in Response to 1 μM Secretin/Glucagon Related Peptides. spGIP was the only peptide tested that elicited a significant increase in cAMP accumulation in a CHO-K1 cell line expressing the rat islet GIP receptor. The data are representative of two individual experiments carried out in triplicate.

3.4 LOCALIZATION OF THE CORE GIP BINDING REGION

In studies aimed at localizing the region of GIP that is required for receptor binding, the ability of several GIP fragments (shGIP 1-30amide, spGIP 17-30 and spGIP 19-30) to displace ^{125}I-spGIP binding from wtGIP-R1 cells was first examined. In addition, based on the recent identification of the region 21-29 as a biologically active fragment of glucagon (Ohneda and Ohneda, 1994), several fragments with sequences based on the homologous region 21-26 in GIP, GLP-1 and glucagon were synthesized and tested. These included: GIP 21-26, GIP 18-28, GLP-1 21-26, and Glucagon 21-26 (Glu 21-26). To increase the sensitivity of the system, and allow true steady state conditions to be obtained all of these, and later, competition binding experiments were carried out at 4°C for 12-16 h. A synthetic preparation of GIP 1-30-free acid (OH), prepared by Dr. N. Yanaihara (Shizuoka, Japan) was found to exhibit significantly reduced affinity (IC_{50} = 39 ± 17 nM) when compared to shGIP (Table 4). In contrast, GIP 1-30amide from an alternative source (Dr. D.H. Coy, Tulane School of Medicine, New Orleans, LA) displayed nearly identical affinity for the rat islet GIP receptor to shGIP (IC_{50}: shGIP 1.2 ± 0.5 nM; n = 7 vs. shGIP 1-30amide 3.1 ± 0.9 nM, n = 6) (Fig. 31). MALDI-TOF analysis of the two GIP 1-30 preparations indicated that GIP 1-30-OH contained contaminating additional peptides of lower molecular mass, while the GIP 1-30amide preparation was of high purity (Dr. H.-U. Demuth, personal communication). An under-estimation of the GIP-1-30-OH peptide mass in the stock solutions therefore probably accounted for the decreased apparent affinity observed. In contrast to the high affinity binding of GIP1-30amide, neither the fragments sharing homology within the

region of residues GIP 21-26 nor the peptides spGIP 17-30 or spGIP 19-30, displaced specific ^{125}I-GIP binding (Fig. 31, Table 4).

	cAMP Studies	Binding Studies	
Peptide	IC_{50} (nM)	EC_{50} (pM)	Inhibition of 1 nM GIP stimulated cAMP production
shGIP #1	1.21 ± 0.46	112 ± 25	
shGIP 1-30-OH	39 ± 17*	ND	ND
shGIP 1-30amide	3.01 ± 0.69*	120 ± 45	ND
GIP 17-30	-	-	-
GIP 19-30	-	-	-
GIP 21-26	-	-	-
GIP18-28	-	-	-
GLP-1 21-26	-	-	-
Glu 21-26	-	-	-
shGIP #2	2.39 ± 1.15	310 ± 26	
GIP15-42	1980 ± 420*	-	+ (40.9 ± 3.6%)
GIP 10-30	562 ± 37*	-	+ (50.8 ± 2.3%)
GIP 7-30	177 ± 25*	+/-	+ (73.0 ± 0.7%)
GIP 6-30amide	3.08 ± 0.57	+/-	+ (94.0 ± 2.2%)

Table 4. Summary of GIP Fragment Binding and cAMP Studies. Control shGIP values (#1 and #2) precede the data for the peptides tested. Maximal cAMP inhibition observed is in parenthesis. (-) no displacement, cAMP accumulation, or inhibition of cAMP accumulation observed; (+) strong or (+/-) weak stimulation of cAMP accumulation or inhibition. Data are representative of $n \geq 3$ experiments carried out in triplicate. * = significantly different from responses to shGIP 1-42 ($p < 0.05$ or less).

Results of initial cAMP studies with wtGIP-R1 cells proved to be highly variable from day to day, making estimates of EC_{50} values difficult to calculate. The addition of 1 mM IBMX to inhibit phosphodiesterase activity and stabilize cAMP for the duration of the stimulation period alleviated this problem, without affecting basal cAMP levels (~1-3 fmol/1000 cells). Interestingly while EC_{50} values were very consistent, maximal cAMP levels shifted over time (compare maximal levels observed in Fig. 32 with Fig. 39B). This necessitated that a group of experiments be completed within a limited time range.

The addition of 1 mM IBMX to the incubation buffer was performed in all further experiments.

GIP 1-30amide was the only truncated form of GIP tested that stimulated cAMP production. Despite the small shift in affinity observed in binding experiments, cAMP responses to GIP 1-30amide did not differ from GIP[2] in either maximal levels achieved (GIP 351 ± 63 fmol/1000 cells vs. GIP 1-30amide 347 ± 24 fmol/1000 cells), nor in efficacy (EC_{50} values: GIP 112 ± 25 pM vs. GIP 1-30amide 120 ± 45 pM, n =4-5) (Fig. 32). None of the fragments tested potentiated or antagonized 10nM shGIP-stimulated cAMP accumulation (Table 4).

Examination of the predicted secondary structure of GIP using PCGENE indicated that GIP should share a "coil-helix-coil " structure exhibited by members of the glucagon superfamily of peptides (Bodanszky, 1974; Sasaki *et al.*, 1975) (Fig. 33). It was predicated that the high affinity binding region of the peptide may be localized to, and require, the secondary structure found within residues 10-30 (comprising the putative large central helical stretch). To test this theory, a number of synthetic fragments (based on the human sequence) were synthesized corresponding to residues GIP 10-30 and amino-terminally extended forms, GIP 7-30 and GIP 6-30amide. In addition a carboxy-terminal fragment, GIP 15-42 corresponding to the predicted "turn-helix" region (Bodanszky, 1974) of GIP, allowed examination of the contribution of this region to binding and activation of the receptor.

[2] For brevity, GIP will be used to refer to the full-length molecule GIP 1-42.

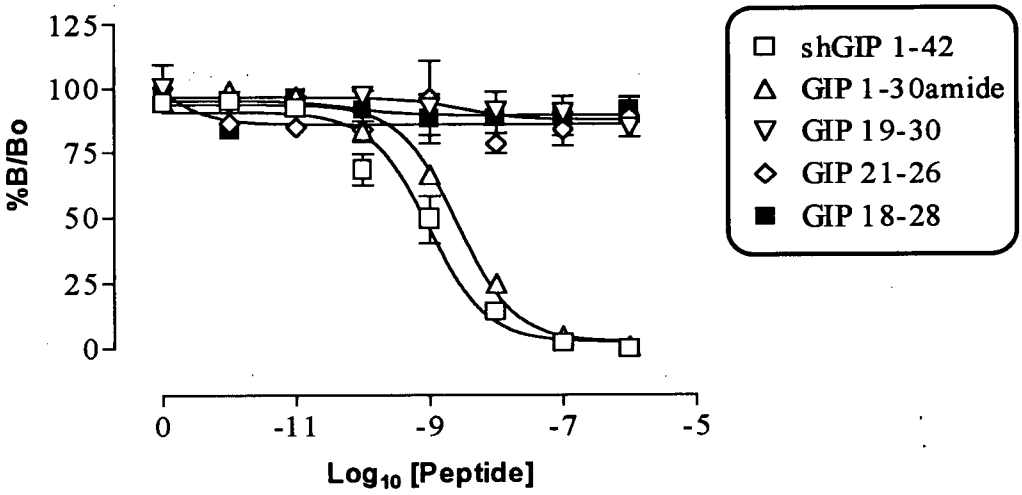


Fig. 31. Displacement of ^{125}I-GIP by different truncated forms of shGIP. Of the fragments tested only GIP1-30amide displaced ^{125}I-GIP binding from wtGIP-R1 cells. There was a small but significant shift to right in the IC_{50} value obtained for GIP 1-30amide in comparison to the full length peptide (GIP1-30amide: 3.01± 0.69 nM, n = 6, vs. shGIP 1-42: 1.21 ± 0.46 nM, n = 7: $P < 0.05$).

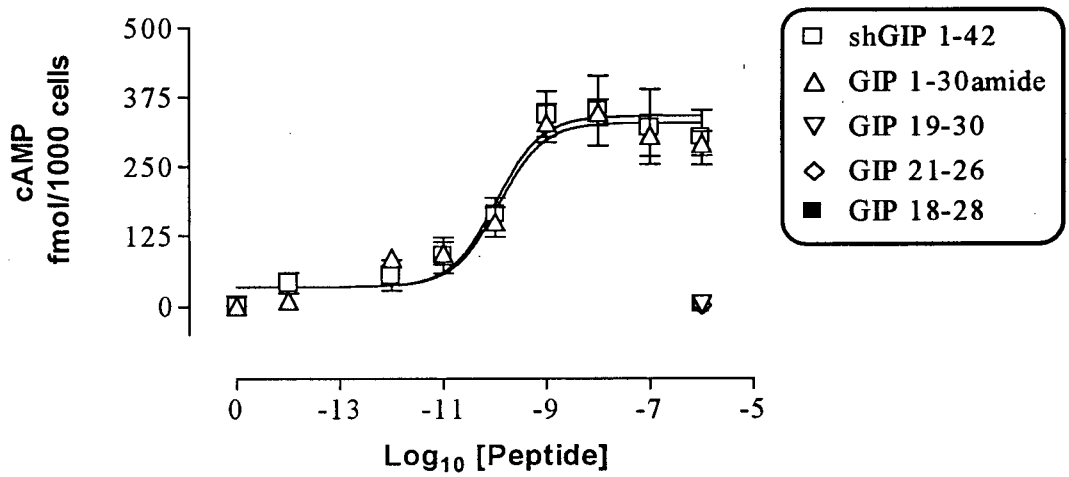


Fig. 32. Stimulation of cAMP Accumulation in wtGIP-R1 Cells by shGIP and Fragments of shGIP. GIP 1-30amide was the only peptide tested that increased cAMP levels in a concentration dependent manner. There were no differences in efficacy or maximal cAMP levels between GIP 1-30amide and shGIP 1-42 (See Table 4).

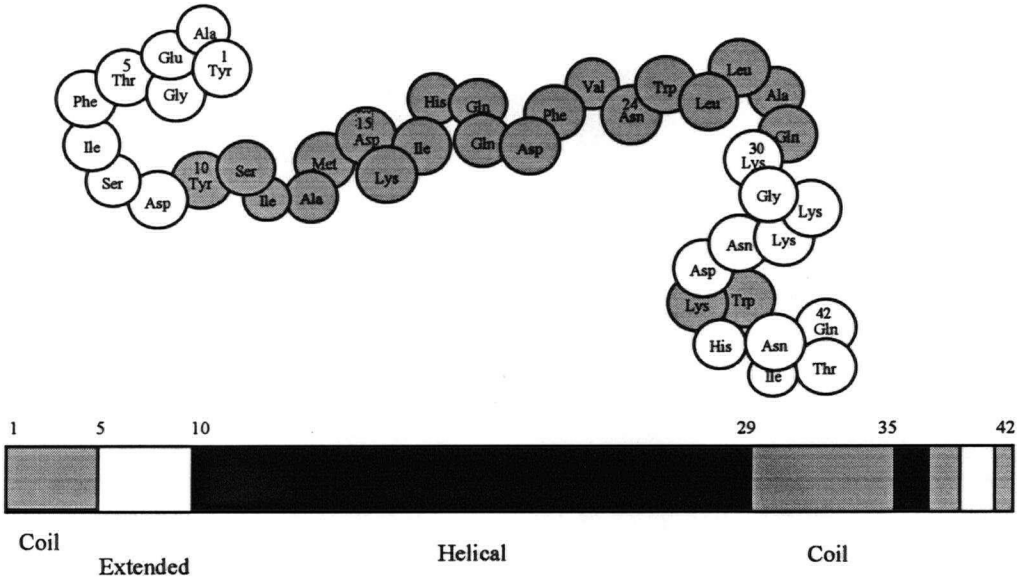


Fig. 33. Predicted Secondary Structure of shGIP. The method of GGBSM was used to predict the secondary structure using the sequence analysis package PCGENE (IntelliGenetics, 1995).

Competitive binding experiments on wtGIP-R1 cells with ^{125}I-GIP revealed no significant difference between the receptor binding affinities for GIP and GIP 6-30amide, with mean IC_{50} values of 2.39 ± 1.15 nM and 3.08 ± 0.57 nM (n = 3), respectively (Fig. 34A, Table 4). Both GIP 7-30 and GIP 10-30 also displaced ^{125}I-GIP in a concentration-dependent manner, but the mean IC_{50} values, 177.15 ± 25.09 nM and 562.36 ± 37.47 nM (n = 3) respectively (Fig. 34A), were approximately 74 (GIP 7-30) and 235 (GIP 10-30) - fold greater than that for GIP. GIP 15-42 displayed the lowest affinity of the fragments (an approximate 641-fold decrease in affinity) with a mean IC_{50} value of 1.98 ± 0.42 μM (n = 3). Neither GIP 10-30 nor GIP 15-42 fully displaced ^{125}I-GIP to non-specific binding levels obtained with 1 μM GIP. The maximal displacements obtained with GIP 10-30 and GIP 15-42 were 93.9 ± 1.8% and 74.4 ± 2.0% (n = 3), respectively, at 10 μM peptide (Fig. 34A).

Synthetic human GIP, stimulated cAMP production in wtGIP-R1 cells in a concentration-dependent manner to a maximum of 138 ± 31 fmol/1000 cells (n = 3) at a concentration of 1 μM, with an EC_{50} of 310 ± 26 pM (Fig. 34B). While GIP 10-30 and GIP 15-42 produced no change in cAMP levels at concentrations as high as 10 μM, both GIP 6-30amide and GIP 7-30 consistently caused small increases in mean cAMP levels, of 1.58 ± 0.54 and 3.49 ± 2.96 fmol/1000 cells (n = 3) respectively, at a concentration of 10 μM (Fig. 34B, Table 4). However, these increases were less than 1.14 and 2.53% respectively of those obtained with GIP at a concentration of 1 μM.

As the fragments exhibited extremely weak, or absent, agonist activity, their ability to antagonize 1 nM GIP-stimulated cAMP production was examined. In

agreement with the competitive binding studies, the order of potency of the fragments as inhibitors of GIP-stimulated cAMP production was GIP 6-30amide > GIP 7-30> GIP 10-30> GIP 15-42 (Fig. 35, Table 4). GIP 10-30 and GIP 15-42 caused significant inhibition only at a concentration of 10 μM (50.8 ± 2.3% and 40.9 ± 3.6%, respectively. n = 3). In contrast, GIP 7-30 inhibited GIP-induced responses by 34.2 ± 3.3% and 73.0 ± 0.7% at 1.0 and 10 μM respectively (n = 3). GIP 6-30amide was the most potent antagonist, significantly inhibiting cAMP production by 58.0 ± 2.5%, 87.0 ± 0.4%, and 94.0 ± 2.2% (n = 3), at concentrations of 0.1, 1.0 and 10 μM respectively (Fig. 35, Table 4). In some experiments it was observed that the peptide antagonists actually decreased basal cAMP, levels suggesting they may be acting as inverse agonists (Milligan et al., 1995; Scheer and Cotecchia, 1997), stabilizing receptors in the low affinity, G-protein uncoupled state as discussed in section 1.11.3. To examine the nature of the antagonism of these peptides in more detail, site directed mutagenesis was used in an attempt to generate a constitutively active form of the GIP receptor. Based on several reports of constitutively active receptors within the Secretin/VIP family, both naturally occurring (Schipani *et al.*, 1995, 1996) and generated by mutagenesis (Schipani *et al.*, 1995), GIP receptor mutants were prepared containing arginine substituted for histidine at position 170 at the intracellular face of the predicted second transmembrane domain. The resulting receptor construct was referred to as H170R.

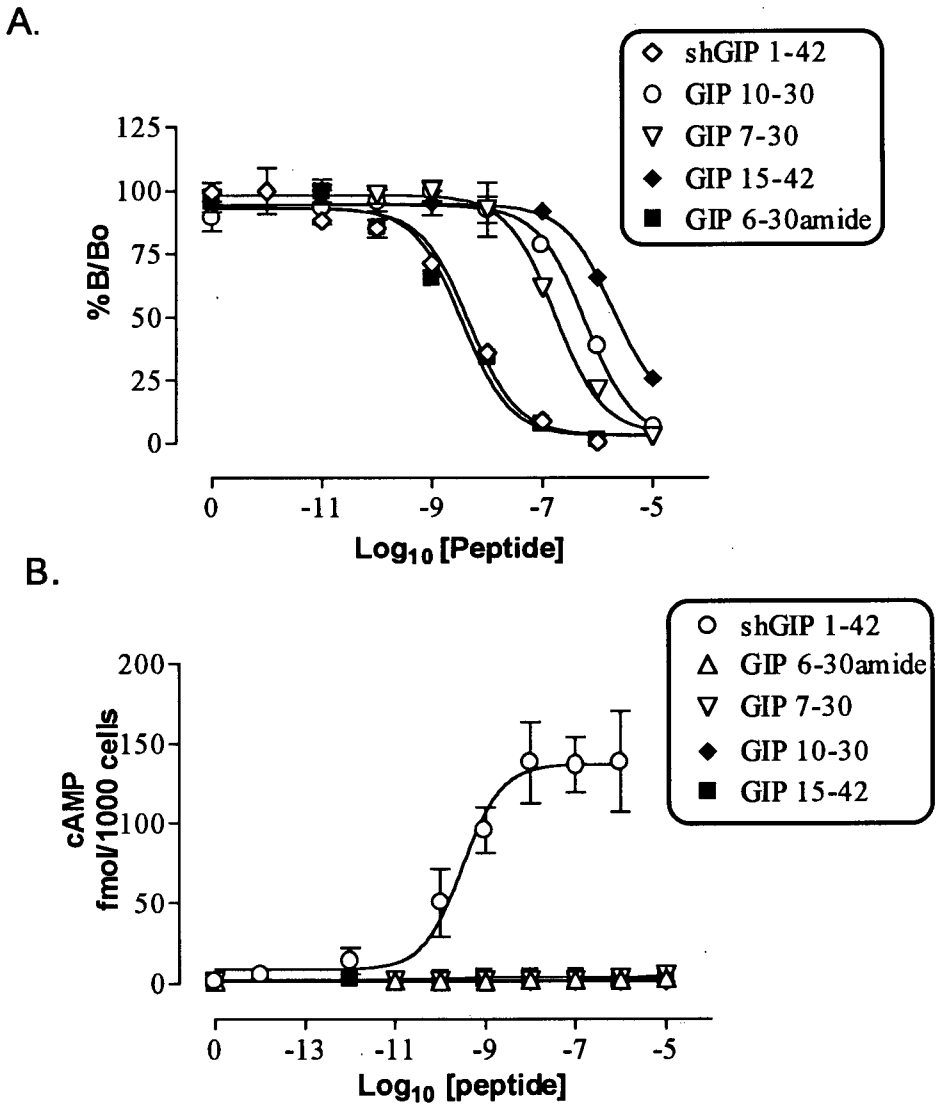


Fig. 34. Displacement of ^{125}I-GIP Binding to CHO-K1 Cells Expressing the Rat Islet GIP Receptor (wtGIP-R1 Cells) by Truncated Forms of GIP (A), and Stimulation of cAMP Production in wtGIP-R1 Cells by the Same Peptides (B). Data are representative of three individual experiments, each carried out in triplicate. See text and Table 6 for details.

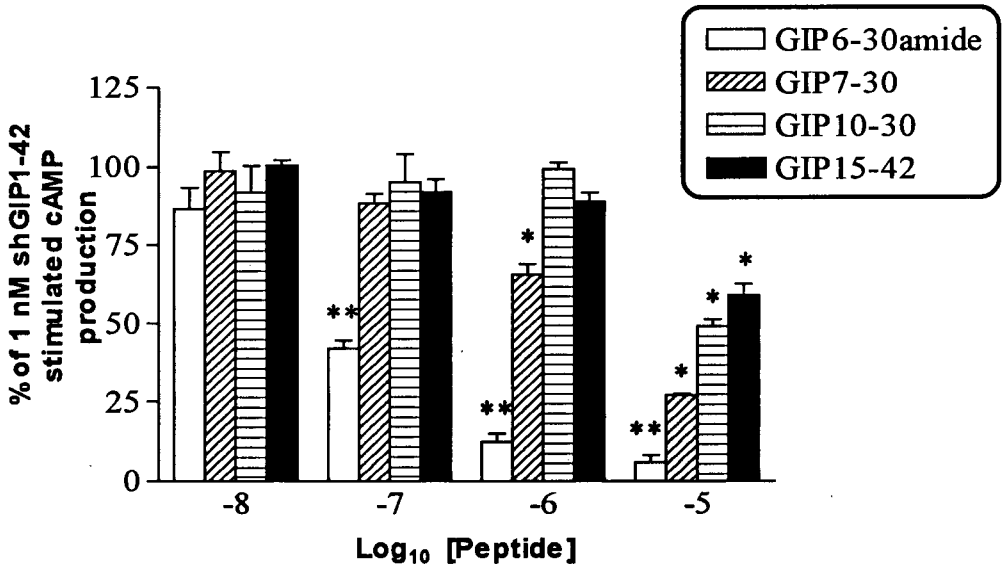
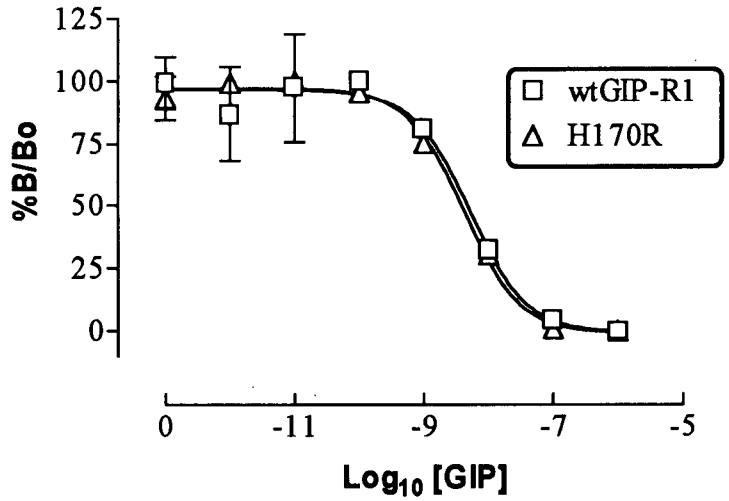


Fig. 35. Inhibition of 1 nM GIP 1-42 Stimulated cAMP Production in wtGIP-R1 Cells. Cells were preincubated with GIP fragments for 15 min. prior to stimulation with 1 nM GIP 1-42 for 30 min. Significant inhibition of cAMP production occurred at concentrations of 100 nM GIP 6-30amide, 1 μM GIP 7-30, 10 μM GIP 10-30 and GIP 15-42. * = significance at $p<0.05$ and ** = significance at $p<0.01$.

In transient experiments, COS-7 cells expressing H170R, displayed similar affinity to shGIP (IC_{50}: H170R 4.18 ± 0.4 nM vs., GIP-R1 5.65 ± 3.96 nM. n =3) in competitive binding studies (Fig. 36A), and similar expression levels (Bmax, 78.3 ± 2.9% of wt, n=3) when compared to cells expressing the wild type receptor (Fig. 36A). In cyclic AMP studies with cells transiently expressing the H170R or wtGIP-R1 cDNAs, no significant differences in basal or 10 nM-stimulated cAMP production (Fig. 36B) were evident. In addition, Ca^{2+} imaging experiments revealed no differences between the $[Ca^{2+}]i$ responses of COS-7 cells expressing wt and H170R cDNAs (See Appendix A, Fig. A3). It was therefore not possible to examine whether the GIP fragments are capable of demonstrating reverse agonism.

A.



B.

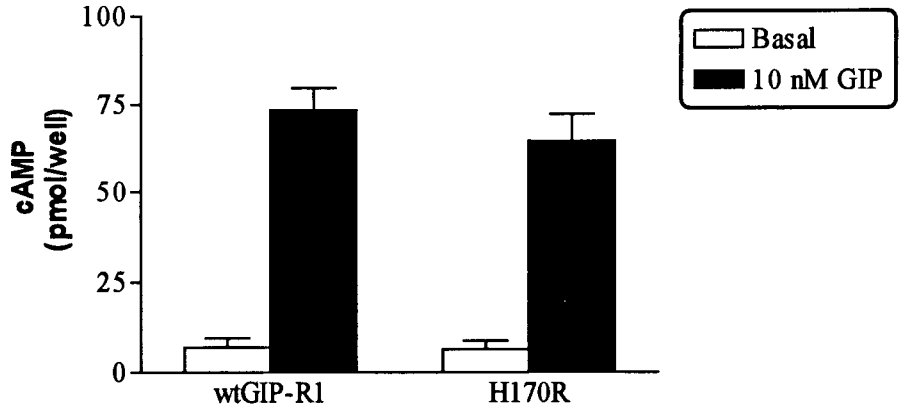


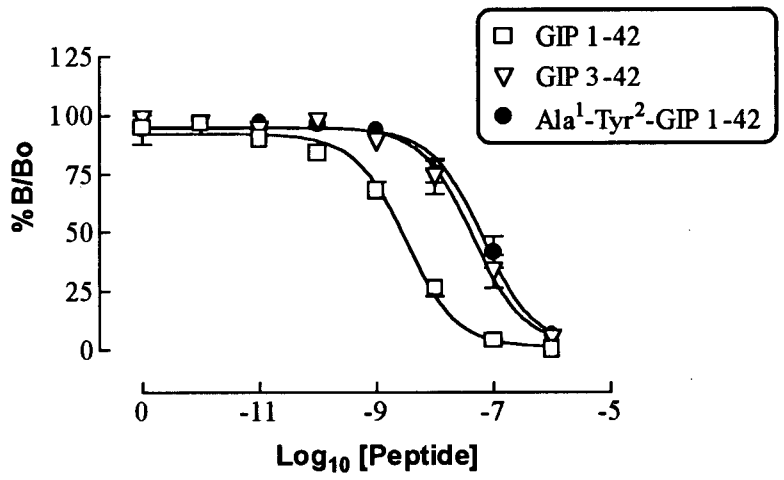
Fig. 36. A. Displacement of ^{125}IGIP From COS-7 Cells Expressing wtGIP-R1 and H170R cDNAs by shGIP. While H170R expression levels (Bmax) were 78.3 ± 2.9% of wtGIP-R1, there was no significant difference between the point mutant and the wtGIP-R1 receptors in their affinity for ligand.
B. 10 nM shGIP-stimulated cAMP Accumulation in COS-7 Cells Transiently Expressing wtGIP-R1and H170R Forms of the GIP Receptor. No changes in basal or 10 nM GIP-stimulated cAMP levels were observed.

3.5 EXAMINATION OF RESIDUES IN GIP IMPORTANT FOR RECEPTOR ACTIVATION

It has been shown previously that the first two residues at the amino-terminus of GIP (Tyr^1-Ala^2) and GLP-1 (His^1-Ala^2) are required for biological activity (Brown *et al.*, 1981; Schmidt *et al.*, 1986a; Suzuki *et al.*, 1989; Gefel *et al.*, 1990), and that GIP and GLP-1 are metabolized by DP IV to GIP 3-42 and GLP-1 9-36amide (Mentlein *et al.*, 1993b; Kieffer *et al.*, 1995b; Pauly *et al.*, 1997), respectively. The effect of removing residues 1 and 2 from the N-terminus of GIP and reversing the order of the first two amino acids on receptor binding and receptor activation was examined. Both GIP 3-42 and Ala^1-Tyr^2-GIP displayed reduced affinity for the receptor in competition binding studies (GIP 3-42 IC_{50} = 58.42 ± 18.76 nM, n = 5; Ala^1-Tyr^2-GIP IC_{50} = 67.04 ± 20.26 nM, n = 3 vs. GIP 3.56 ± 0.81 nM, n = 6.) (Fig. 37A, Table 5). Both peptides were found to be devoid of the ability to stimulate cAMP production in wtGIP-R1 cells at concentrations as high as 1 μM (Fig. 37B). However, both peptides antagonized GIP-stimulated cAMP accumulation by 45.59 ± 5.23 % and 91.04 ± 0.95 % (GIP 3-42), and 40.54 ± 4.62 % and 90.91 ± 0.42 % (Ala^1-Tyr^2-GIP), at 1 μM and 10 μM (n = 3-7) respectively (Fig. 38).

The observation that removing or modifying the first two residues of GIP reduced the affinity of the peptides below that for GIP 6-30amide, suggested that the sequence and/or conformation of the N-terminal putative "coiled region" was important for both high affinity binding and receptor activation.

A.



B.

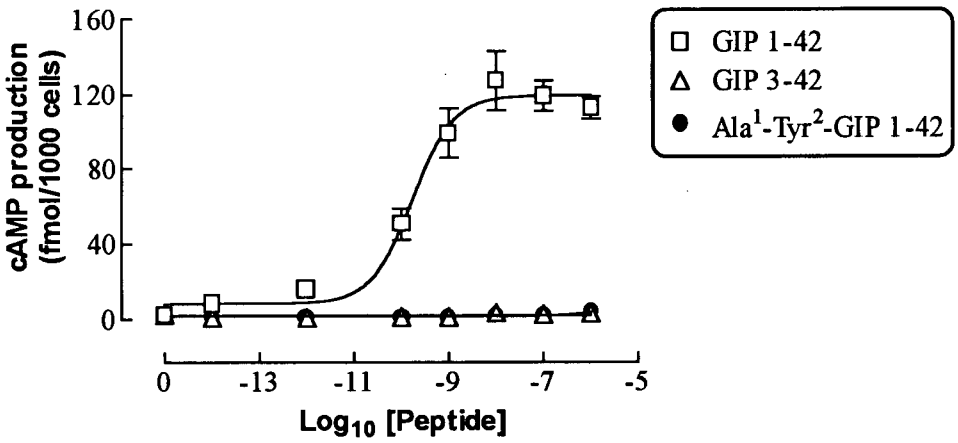


Fig. 37. Displacement of ^{125}I-GIP Binding (A), and Stimulation of cAMP Production (B) by GIP 1-42, N-terminal Truncated (GIP 3-42), and Sequence Modified (Ala^1-Tyr^2-GIP 1-42) Analogs. Mean IC_{50} and EC_{50} values are summarized in Table 5.

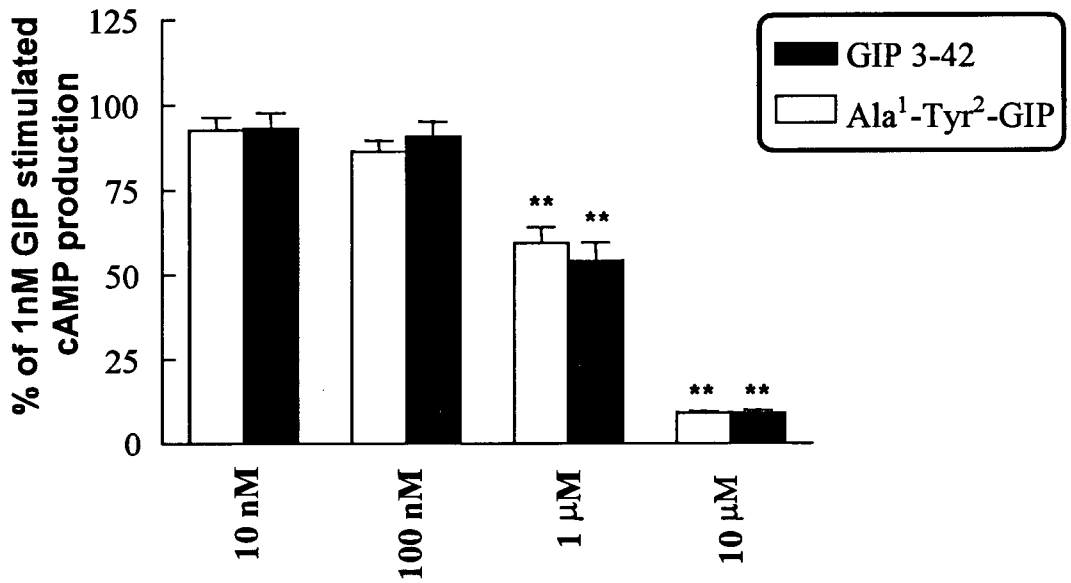
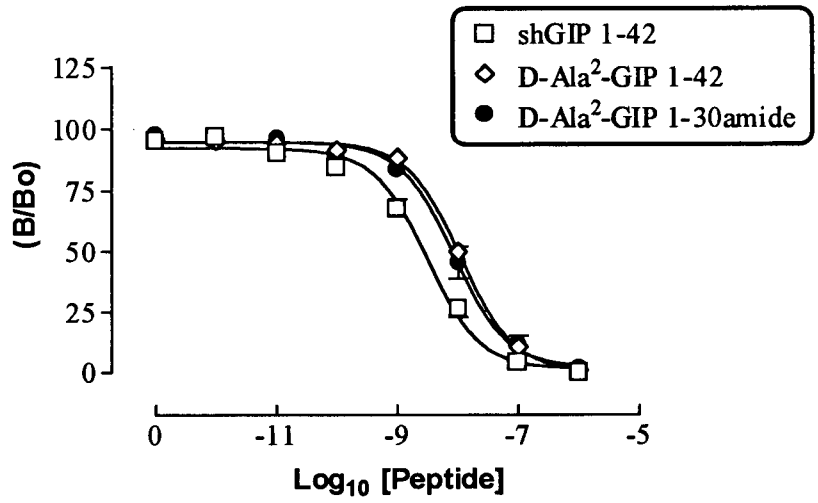


Fig. 38. GIP 3-42 and Ala[1]-Tyr[2]-GIP Inhibit 1nM GIP-stimulated cAMP Production. wtGIP-R1 cells were preincubated with the peptides at the given concentrations, then stimulated with 1nM GIP in the presence of the GIP fragment or modified form at the appropriate concentration. Data shown are the mean ± S.E.M. of 3-7 individual measurements. ** = significantly different from responses to shGIP 1-42 ($p < 0.01$ or less).

Additional amino terminally modified forms of GIP, GLP-1 and GIP1-30amide were synthesized to examine the contribution of specific N-terminal residues for receptor affinity and activation. As mentioned earlier, dipeptidyl peptidase IV attenuates the biological activity of both GIP and GLP-1 (Mentlein *et al.*, 1993b; Kieffer *et al.*, 1995b; Pauly *et al.*, 1997). Requirements for enzymatic activity of this enzyme are that amino acids in the P1 and P2 positions are L-isomers in the trans conformation (Fisher *et al.*, 1983) and that the N-terminus is protonated. An Desamino-Tyrosine amino-terminal deprotonated form (3-phenyl propionic acid (Ppa^1)-GIP 1-30amide) and a number of D-isomer substituted forms (see Table 5) of truncated GIP were synthesized for us, by Drs. H. U. Demuth and D. Coy to allow examinination of the contribution of the N-terminal residues to receptor affinity and activation. Additionally, these studies incorporated attempts at synthesizing DP IV resistant forms of GIP.

D-Ala^2-GIP and D-Ala^2-GIP 1-30amide were found to exhibit a slight shift to the right in their IC_{50} (11.52 ± 1.08 nM and 10.26 ±2.76 nM, n = 5, respectively) and EC_{50} (1.78 ± 0.86 nM, n = 4 and 681 ± 210 pM, respectively, n = 5) values, when compared to GIP binding (IC_{50} = 3.56 ± 0.8, n=6) and cAMP production (EC_{50} = 248 ± 68 nM, n=7) (Fig.39, Table 5). D-Ala^2-GLP-1 displayed a similar small decrease in potency in cAMP production (EC_{50} = 692 ± 177 pM , n = 3) and a small shift to the right in displacement of ^{125}I-GLP-1 (IC_{50} = 5.85 ± 3.52 nM), in comparison to GLP-1 (EC_{50} = 255 ± 36 pM, and IC_{50} = 1.17 ± 0.31, n=3) when tested on the wtGLP-R1 cell line (Fig. 40). These small differences, while statistically different for the GIP analogs, were not different for the single GLP-1 analog tested.

A.



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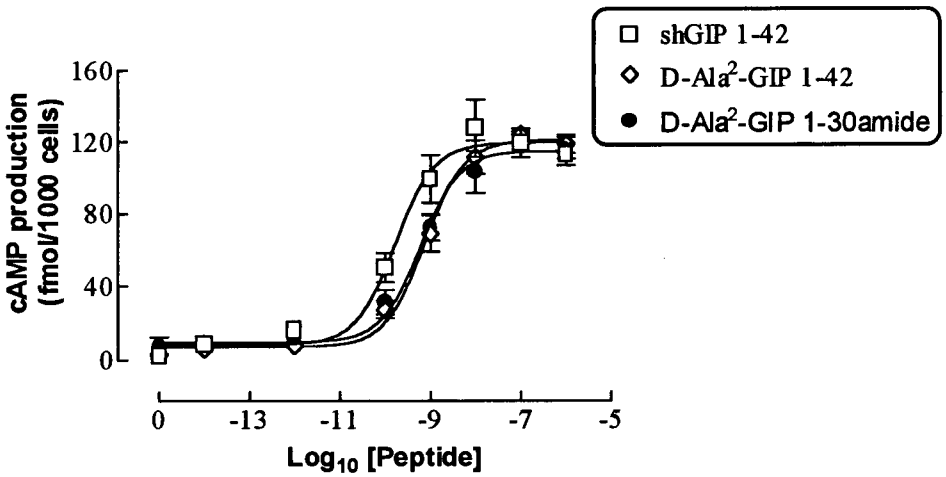
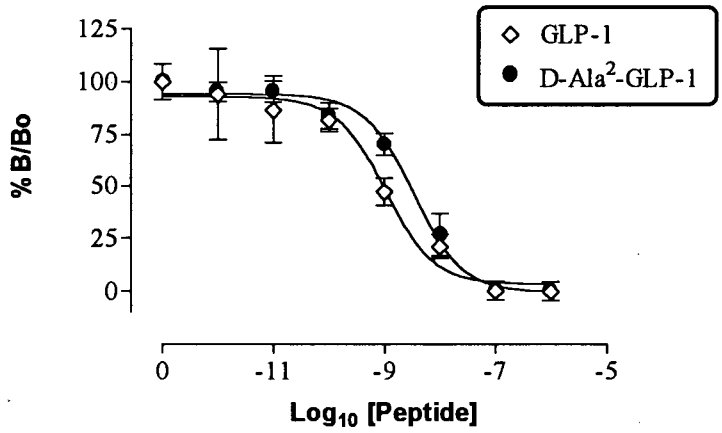


Fig. 39. Displacement of ^{125}I-GIP Binding (A), and Stimulation of cAMP Production (B) by shGIP and D-Ala^2-analogs. Mean IC_{50} and EC_{50} values are summarized in Table 5.

A.



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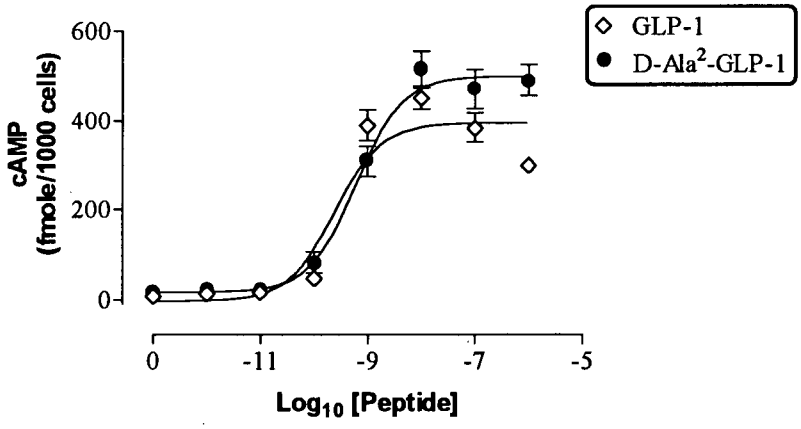
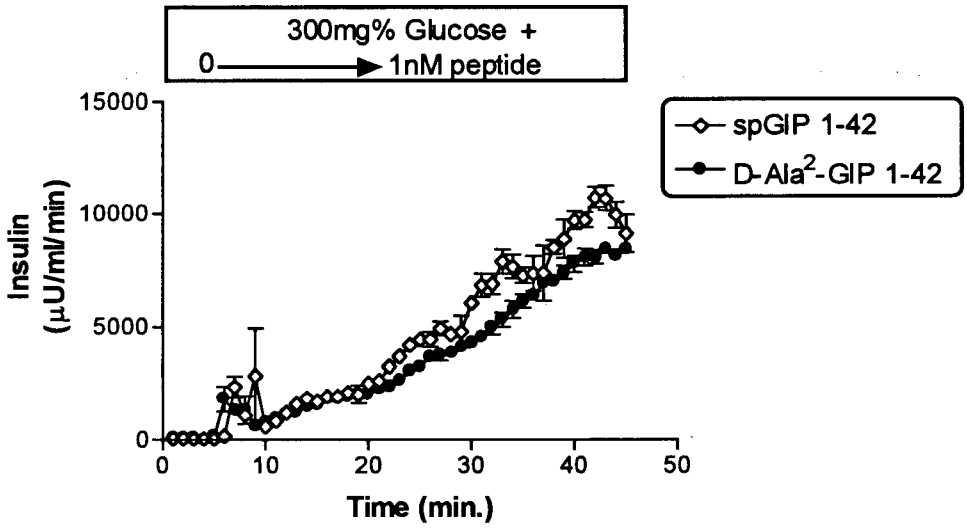


Fig. 40. Displacement of ^{125}I-GLP-1 Binding (A), and Stimulation of cAMP Production (B) by GLP-1, and the D-Stereoisomer Substituted Analog, D-Ala^2-GLP-1. Mean IC_{50} values were GLP-1: 1.17 ± 0.31 nM and D-Ala^2-GLP-1: 5.39 ± 3.52 nM (A). Mean EC_{50} values were GLP-1: 255 ± 36 pM, and D-Ala^2-GLP-1: 692 ± 177 pM (B).

Results consistent with the similar cAMP responses were obtained in the perfused rat pancreas, with D-Ala^2-GIP having a marked but significantly smaller integrated insulin response in comparison to the endogenous hormone (GIP =188.3 ± 6.1 mU, n = 5, vs. D-Ala^2-GIP = 154.5 ± 4.9 mU) (Fig. 41). D-Ala^2-GLP-1 did not differ significantly in its stimulation of insulin secretion when compared to GLP-1 (160.1 ± 3.240 mU, n = 4 vs. 174.7 ± 15.4 mU n = 6, respectively) (Fig. 42).

Other analogs were tested only in binding and cAMP studies. In binding analysis of the analogs (Fig. 43A, Table 5), displacement curves for both D-Ala^4-GIP 1-30amide and D-Tyr^1-GIP 1-30amide were shifted to the right in comparison to GIP [IC_{50}s = 30.67 ± 6.62 nM and 29.28 ± 6.83 nM, respectively, vs. GIP IC_{50} = 3.56 ± 0.81. $P < 0.05$, (n = 4-6)]. Ppa^1-GIP 1-30amide and D-glu^3-GIP 1-30amide did not differ significantly from GIP in their affinity for the rat islet GIP receptor (Fig. 43A, Table. 5). As would be predicted from the binding studies, in cAMP experiments D-Ala^4-GIP 1-30amide and D-Tyr^1-GIP 1-30amide displayed displacement curves significantly shifted to the right, with EC_{50} values of 158.4 ± 85.3 nM and 13.6 ± 0.7 nM, respectively (n=4), compared to 248 ± 68 pM (n = 7) for GIP. While D-Glu^3-GIP 1-30amide displayed a small shift in efficacy (EC_{50} = 469 ± 126 pM) compared to GIP, Ppa^1-GIP 1-30amide, despite its high affinity, had a greater reduction in receptor activation (EC_{50} = 930 ± 143 pM).

A.



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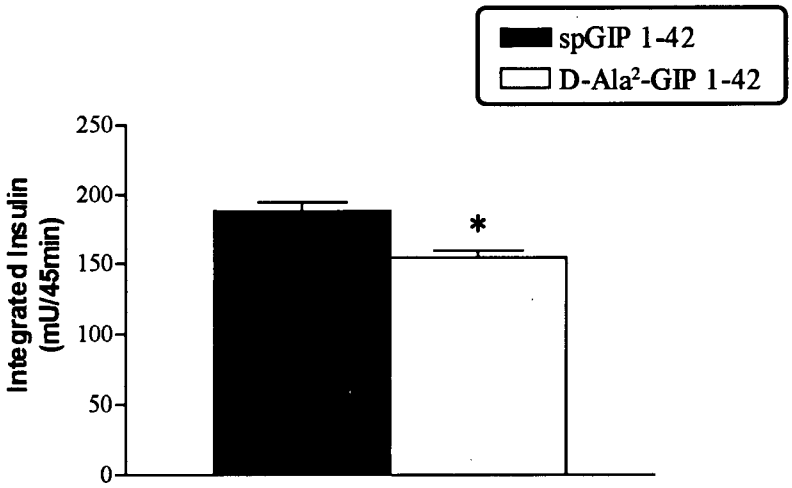


Fig. 41. Comparison of spGIP 1-42 and D-Ala^2-GIP 1-42 in the Isolated Perfused Rat Pancreas. A. A linear gradient of 0 to 1 nM of the indicated peptide was perfused in the presence of 300 mg% glucose.
B. Integrated insulin release (area under the curve) was slightly decreased for D-Ala^2-GIP 1-42 (154.5 ± 4.9 mU) in comparison to spGIP 1-42 (188.3 ± 6.1).($p < 0.05$, n = 4-5).

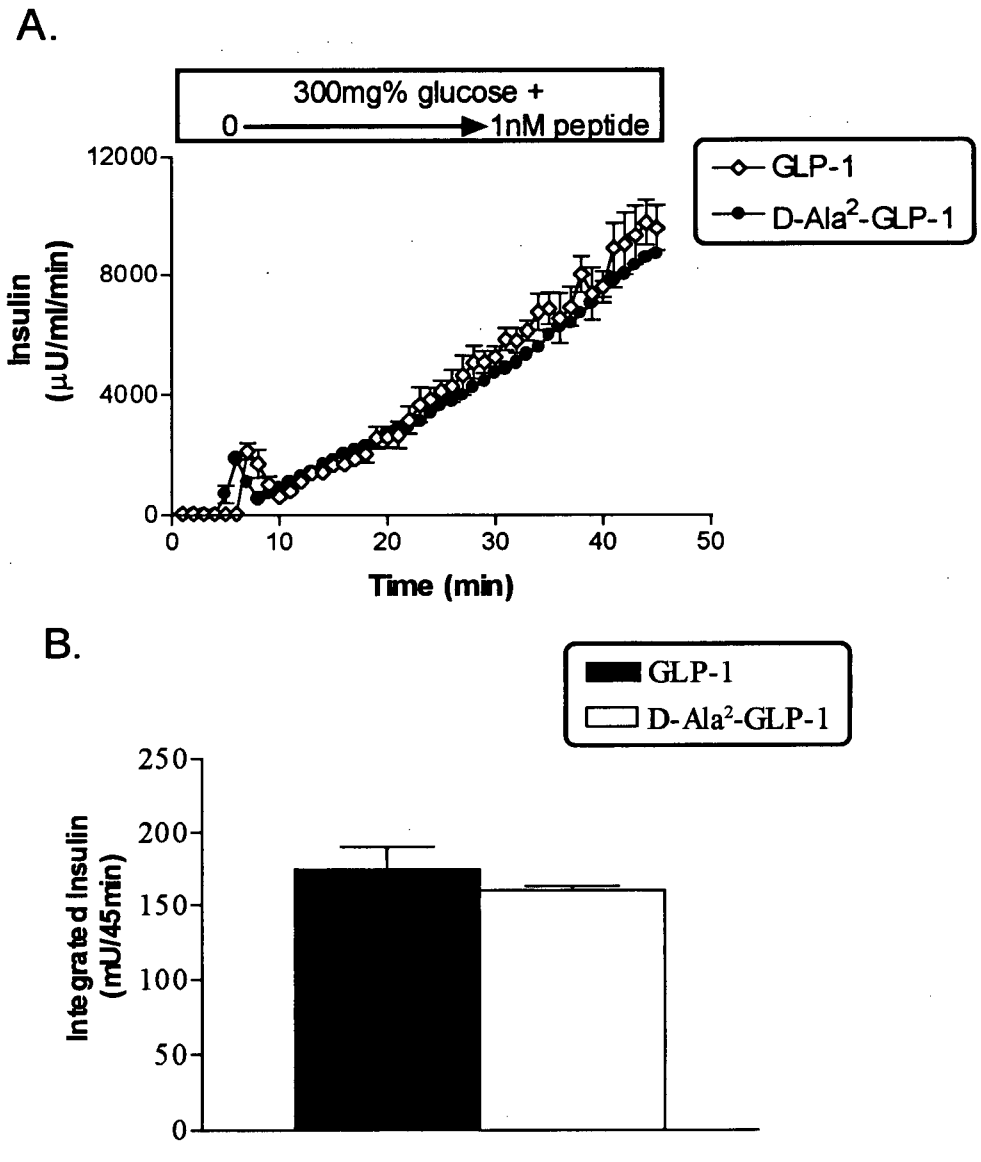


Fig. 42. Comparison of GLP-1 and D-Ala^2-GLP-1 in the Isolated Perfused Rat Pancreas. A. A linear gradient of 0 to 1 nM of the indicated peptide was perfused in the presence of 300 mg% glucose.
B. Integrated insulin release (area under the curve) was similar for both D-Ala^2-GLP-1 and GLP-1 (n = 4-6).

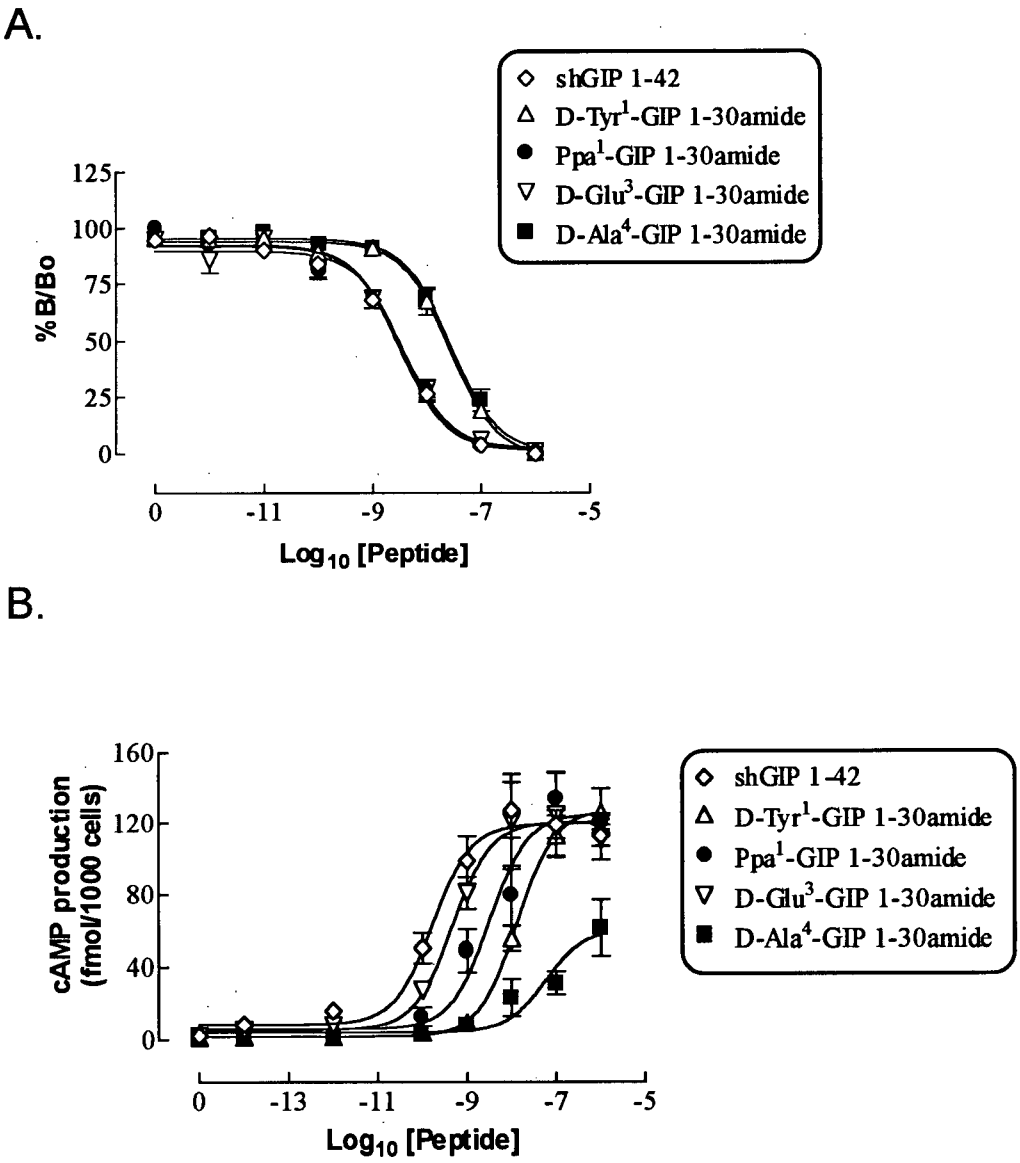


Fig. 43. Displacement of ^{125}I-GIP Binding (A) and Stimulation of cAMP Production (B) by shGIP 1-42, and GIP 1-30amide Analogs. Mean IC_{50} and EC_{50} values are summarized in Table 5.

Analog	IC_{50} (nM)	EC_{50} (nM)	% of maximal GIP cAMP production (%)
hGIP	3.56 ± 0.81	0.248 ± 0.068	100%
D-Ala^2-GIP	11.52 ± 1.08*	1.780 ± 0.864	102.2 ± 3.03
D-Ala^2-GIP 1-30amide	10.26 ± 2.76*	0.681 ± 0.210	94.5 ± 3.7
D-Tyr^1-GIP 1-30amide	29.28 ± 6.83*	13.6 ± 0.7*	113.6 ± 4.79
Ppa^1- GIP 1-30amide	4.85 ± 1.33	0.930 ± 0.143	107.4 ± 7.71
D-Glu^3-GIP 1-30amide	3.84 ± 0.55	0.469 ± 0.126	103.5 ± 7.08
D-Ala^4-GIP 1-30amide	30.67 ± 6.62*	158.4 ± 85.3*	50.9 ± 7.6*
GIP 3-42	58.42 ± 18.76*	-	-
Ala^1-Tyr^2-GIP	67.04 ± 20.26*	-	-

Table 5. Summary of Competitive Binding (IC_{50}) and cAMP Responses (EC_{50} and % of maximal GIP cAMP production) with GIP analogs. IC_{50} and EC_{50} values were determined by nonlinear regression analysis (n=3-7). (-) = non-detectable. (*) = differ from GIP by at least $P < 0.05$ as determined by one way ANOVA.

3.6 GIP AND GLP-1 RECEPTOR CHIMERAS

Studies with structurally related receptors of the glucagon superfamily, have suggested that the amino-terminal (NT) region of the secretin, VIP (Holtmann et al., 1995a,b; Gourlet et al., 1996), and glucagon (Buggy et al., 1995; Carruthers et al., 1994; Unson et al., 1995, 1996; Garziano et al., 1996) receptors play a role in ligand binding. Therefore, in an attempt at identifying regions of the GIP and GLP-1 receptors required for ligand binding and receptor activation, GIP/GLP-1 receptor chimeric cDNAs encoding differing portions of one receptor's NT-domain ligated to the C-terminal coding regions of the other were constructed as described in Sections 2.7.3-2.7.6, and depicted in Fig. 44.

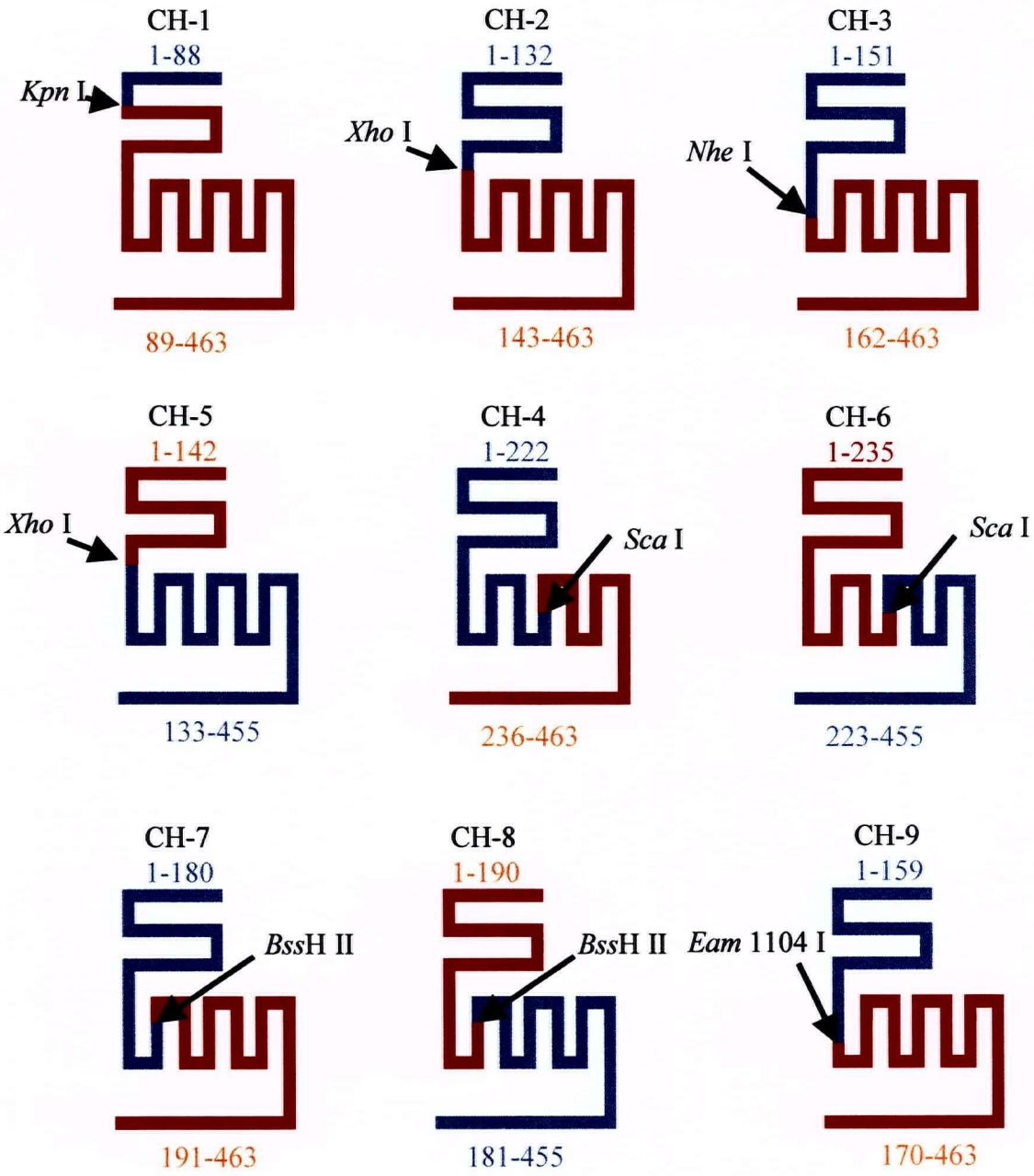


Fig. 44. Predicted Topography of the Chimeric Receptors. Regions corresponding to GIP-R are in blue, those of the GLP-1-R are in red.

3.6.1 LIGAND BINDING OF RECEPTOR CHIMERAS

Receptor binding and displacement studies were performed to determine the effect of domain exchange on ligand-receptor binding. Most receptor constructs were analyzed in both transient and stable expression systems. For initial localization of binding specificity, constructs were generated encompassing the putative NT domain, intracellular domain (IC)-1, extracellular domain (EC)-1, and part of the TM-3 of each receptor, displayed on the corresponding C-termini (CH-4, CH-6, Fig. 44). Interestingly, CH-4 displayed near normal affinity for GIP when expressed transiently or stably when compared to the wtGIP-R: IC_{50} values (nM) in COS-7 cells CH-4 2.74 ± 0.87 vs. wt 6.42 ± 1.22; CHO-K1 cells CH-4 8.33 ± 0.14 vs. wt 1.33 ± 0.19, respectively (Fig. 45; Table 6). The similar affinity was observed despite the fact that this and subsequent constructs were not expressed with the same efficiency as the wt receptors (Table 6). CH-4 did not bind ^{125}I-GLP-1, and therefore lacked the necessary regions for high affinity GLP-1 binding (Fig. 46, Table 6). Neither CH-5 nor CH-6 displayed detectable binding of ^{125}I-spGIP or ^{125}I-GLP-1 (Table 6). To date receptor antibodies for the GIP or GLP-1 receptors are not available, and it was therefore not possible to establish whether the lack of binding was due to absent cell surface expression of the chimeric receptors, or loss of the ability of the modified receptors to bind ligand.

To localize GIP binding specificity further, smaller portions of the GIP-R NT were used: CH-3, encompassing the first NT domain and a portion of TM-1 of the GIP receptor, and CH-2, in which only the first 132 amino acids of the GIP NT domain was displayed on the GLP-1 R (Fig. 44), both bound GIP, however there was a 5 to 10 fold shift in affinity compared to wtGIP-R (Fig. 45, Table 6). CH-1, consisting of the first 88

amino acids of the GIP receptor and C-terminal residues 89 to 463 of the GLP-1 receptor (Fig. 44), bound neither ^{125}I-GIP (Fig. 45; Table 6) nor ^{125}I-GLP-1 in either COS-7 or CHO-K1 cells. Thus, despite an apparently minor shift in binding affinity, it is quite clear that the majority of GIP-R specificity for its ligand is localized to the first 132 amino acids of the GIP NT domain.

In an attempt at restoration of binding affinity to that seen with CH-4, constructs encoding chimeric receptors with further NT extensions of the GIP receptor into the first transmembrane domain (CH-9) and into the second transmembrane (CH-7) were constructed. However, all these receptors failed to bind ^{125}I-GIP (Fig. 45; Table 6). While both COS-7 and CHO-K1 cells expressing the hGLP-1 receptor bound ^{125}I-GLP-1 with high affinity (IC_{50} 7.5 ± 1.2 nM and 1.47 ± 0.42 nM, respectively) none of the chimeric receptors tested were capable of binding ^{125}I-GLP-1 (Fig. 46, Table 6). Therefore it appears that GLP-1 receptor requires multiple receptor regions in order to bind GLP-1.

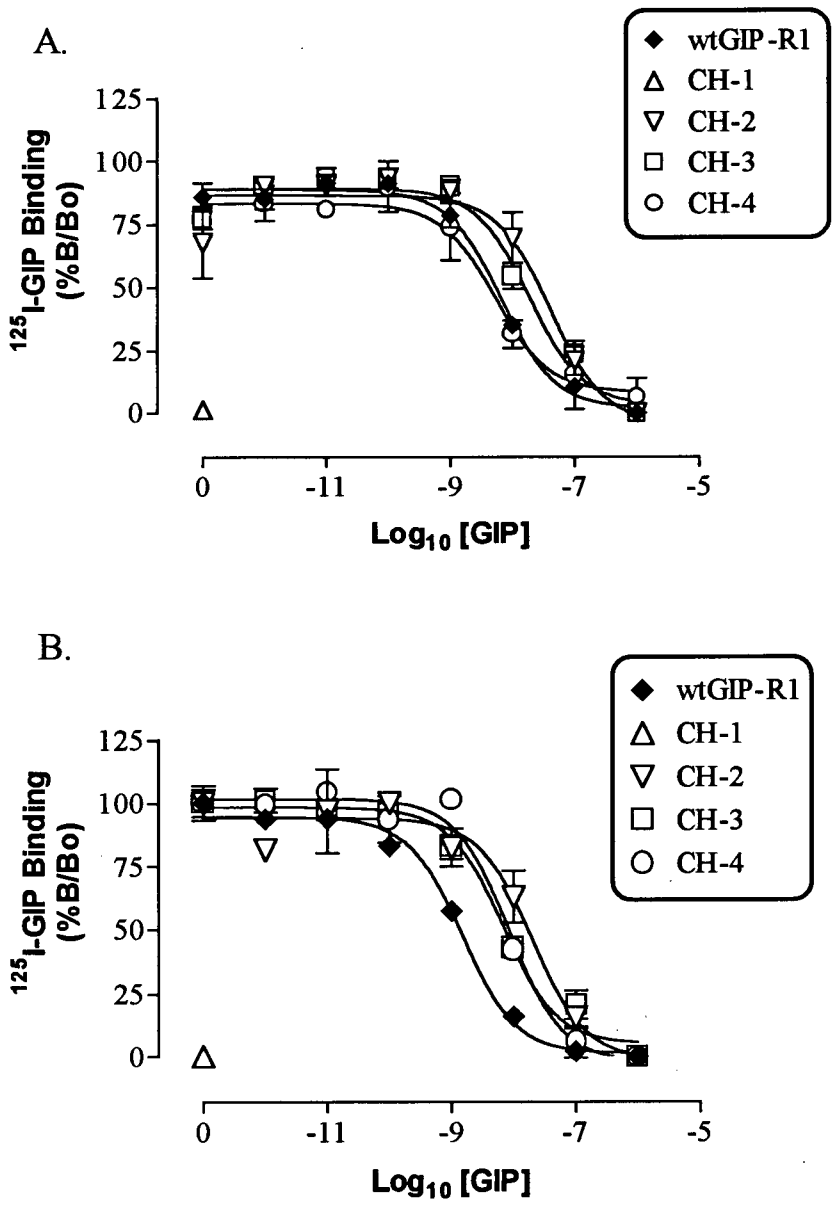


Fig. 45. Displacement of ^{125}I-GIP Binding by GIP in COS-7 (A) and CHO-K1 (B) Cells Expressing Wild Type or Chimeric Receptors. Data are the means ± S.E.M. of 4-7 individual experiments. Expression level and IC_{50} values are summarized in Table 6.

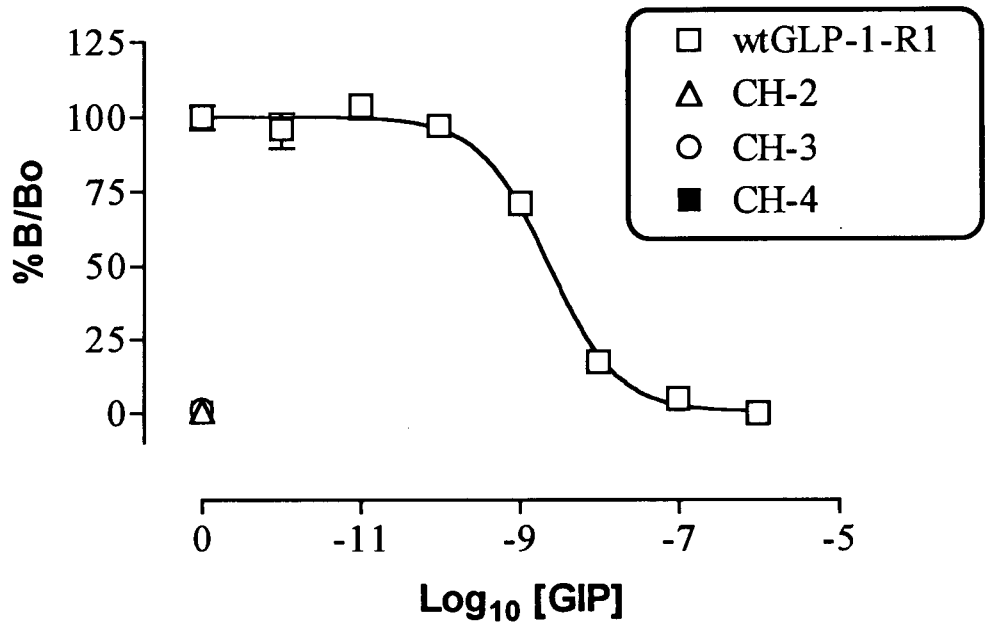


Fig. 46. Displacement of ^{125}I-GLP-1 by GLP-1 in CHO-K1 Cells Expressing the wtGLP-1 Receptor. Curves are representative of at least three individual experiments. Expression levels (Bmax) and IC_{50} values are summarized in Table 6.

A.

Receptor	COS-7 Bmax (% of wt)	CHO-K1 Bmax (% of wt)	COS-7 IC_{50} (nM)	CHO-K1 IC_{50} (nM)
wtGIP-R1	100	100	6.42 ± 1.22	1.33 ± 0.19
wtGLP-1-R1	-	-	-	-
CH-1	-	-	-	-
CH-2	15.5 ± 1.5*	6.4 ± 1.9*	31.7 ± 8.18*	27.8 ± 11.9*
CH-3	27.9 ± 8.7*	4.8 ±1.7*	23.6 ± 10.6	9.04 ± 1.07*
CH-4	15.2 ± 1.5*	13.3 ± 3.1*	2.74 ± 0.87	8.33 ± 0.14*
CH-5	-	-	-	-
CH-6	-	-	-	-
CH-7	-	ND	-	ND
CH-8	-	ND	-	ND
CH-9	-	-	-	-

B.

Receptor	COS-7 Bmax (% of wt)	CHO-K1 Bmax (% of wt)	COS-7 IC_{50} (nM)	CHO-K1 IC_{50} (nM)
wtGIP-R1	-	-	-	-
wtGLP-1-R1	100	100	7.5 ±1.2	1.47 ± 0.42
CH-1	-	-	-	-
CH-2	-	-	-	-
CH-3	-	-	-	-
CH-4	-	-	-	-
CH-5	-	-	-	-
CH-6	-	-	-	-
CH-7	-	ND	-	ND
CH-8	-	ND	-	ND
CH-9	-	-	-	-

Table 6. Binding of ^{125}I-GIP (Bmax and IC_{50}) (A) and ^{125}I-GLP-1 (B) to GIP/GLP-1 Receptor Chimeras in COS-7 and CHO-K1 Cells. IC_{50} and EC_{50} values were calculated by nonlinear regression analysis (n = 3-7). ND = Not determined; - = Non-detectable.

3.6.2 CYCLIC AMP RESPONSES OF RECEPTOR CHIMERAS

In COS-7 cells expressing the rat wild type GIP receptor, treatment with 10 nM GIP resulted in stimulation of cAMP production to a maximum value of 1323 ± 141 pmol/well (n = 9), compared with 25.5 ± 17.2 pmol/well (n = 12) in controls wells (Fig

47A). In COS-7 cells expressing CH-3 and CH-4 increases in cAMP production in response to 10 nM GIP were 37.6 ± 3.3% and 20.5 ± 3.8% (n=3) of that seen with the wtGIP receptor (Fig. 47A, Table 7). In agreement with the binding data, none of the chimeras consisting of the NT-region of the hGLP-R1 responded to 100 nM GLP-1 stimulation (Table 7). Unexpectedly, however, COS-7 cells expressing chimeras CH-2 and CH-3 responded to stimulation with 100 nM GLP-1 with 84.8 ± 9.6% and 102.5 ± 23.0%, respectively (n=3), of the cyclic AMP response of cells expressing hGLP-R1 (Fig. 48A). The failure to detect significant binding of ^{125}I-GLP-1 in these cells is probably a result of the presence of sufficient receptors to stimulate measurable cyclic AMP responses, but of either insufficient number or too low an affinity to allow the detection of ^{125}I-GLP-1 binding.

Stably transfected CHO-K1 cells were used to examine the cAMP responsiveness of the different receptor forms more fully. The maximal increases in cAMP levels, at concentrations up to 1 μM GIP (Fig. 47B), were: CH-3 29.3 ± 10.2% and CH-4 42.4 ± 11.2% of wt GIP-R1 cell line responses (180.1 ± 21.2 fmol/1000 cells, n=7). In agreement with COS-7 experiments, cells expressing CH-2 showed no significant cAMP responses to GIP up to 1 μM. The mean EC_{50} value for CH-4 (Fig. 47B, Table 7) was not significantly different from that of the wtGIP-R1, however there was a significant shift to the right in the concentration-response curve for CH-3 ($p<0.05$) (Fig. 47B, Table 7). It is unlikely that this and other shifts in EC_{50} values are due to reduced receptor expression, since a low level expressing clone, wtGIP-R8, exhibited an EC_{50} value similar to that seen with the high level expressing wtGIP-R1 cell line (summarized in Table 8).

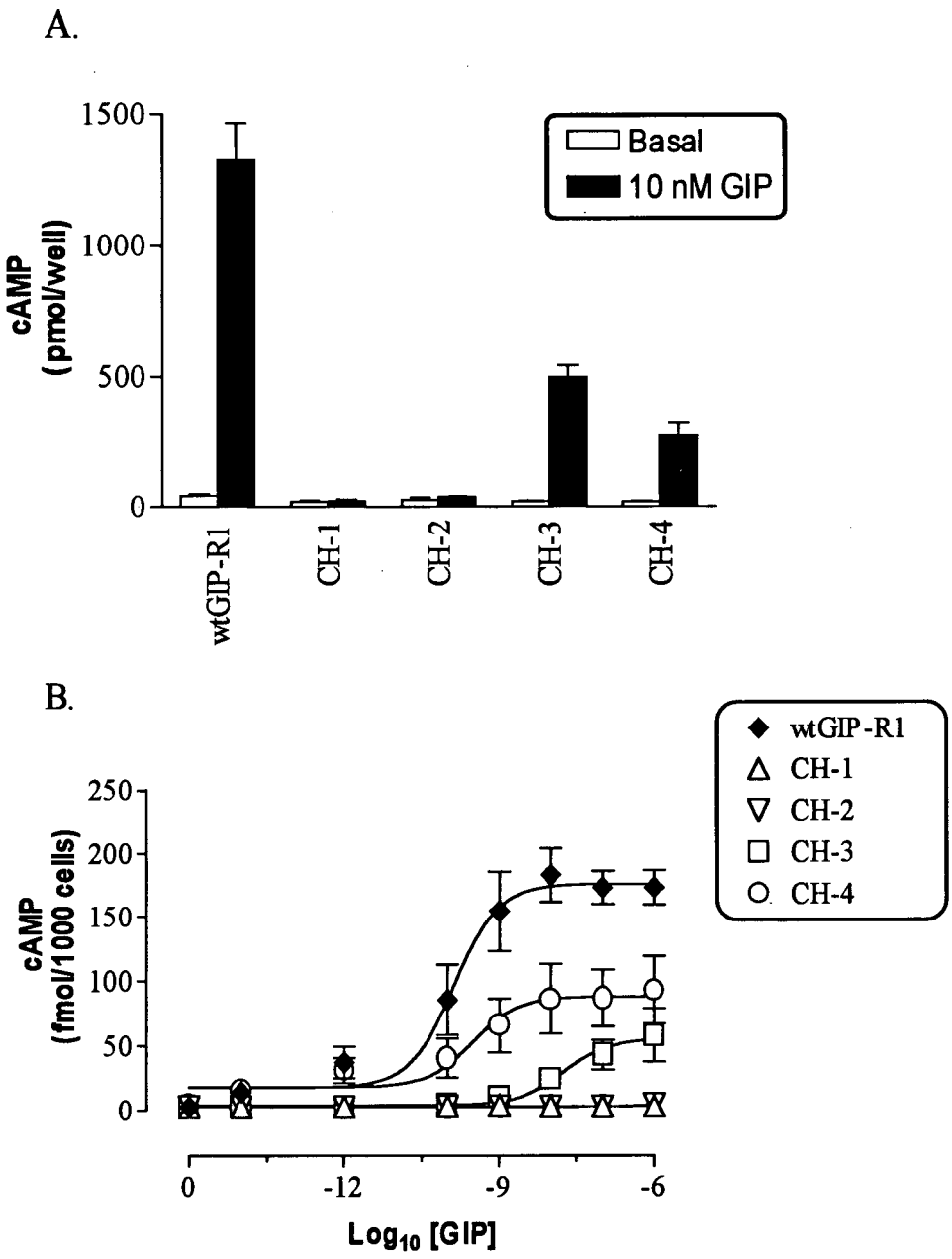


Fig. 47. Stimulation by GIP of cAMP Production in COS-7 (A), and CHO-K1 (B) Cells Expressing the Wild Type GIP Receptor or GIP/GLP-1 Chimeric Receptors. Data are the mean ± S.E.M. of 4-7 individual experiments. Maximal cAMP increases and EC_{50} values are summarized in Table 7.

As seen in the transient expression experiments, GLP-1 stimulated cAMP production in a concentration-dependent manner in CH-2 and CH-3 expressing cell lines (Figure 48B). The levels of cAMP production in CH-2 and CH-3 cells were 30.5 ± 2.0% and 13.2 ± 1.9%, respectively, of the maximal levels seen in CHO-K1 cells stably expressing the GLP-1 receptor (wtGLP-R1). The EC_{50} values for CH-2 (81.4 ± 19.6 nM, n=3) and CH-3 (5.99 ± 0.68 nM, n=3) were shifted to the right compared to wt GLP-R1 (103 ± 11 pM, n=3). In agreement with COS-7 cell line studies none of the other chimeras responded with increased cAMP production in the presence of GLP-1 (Fig. 48, Table 7).

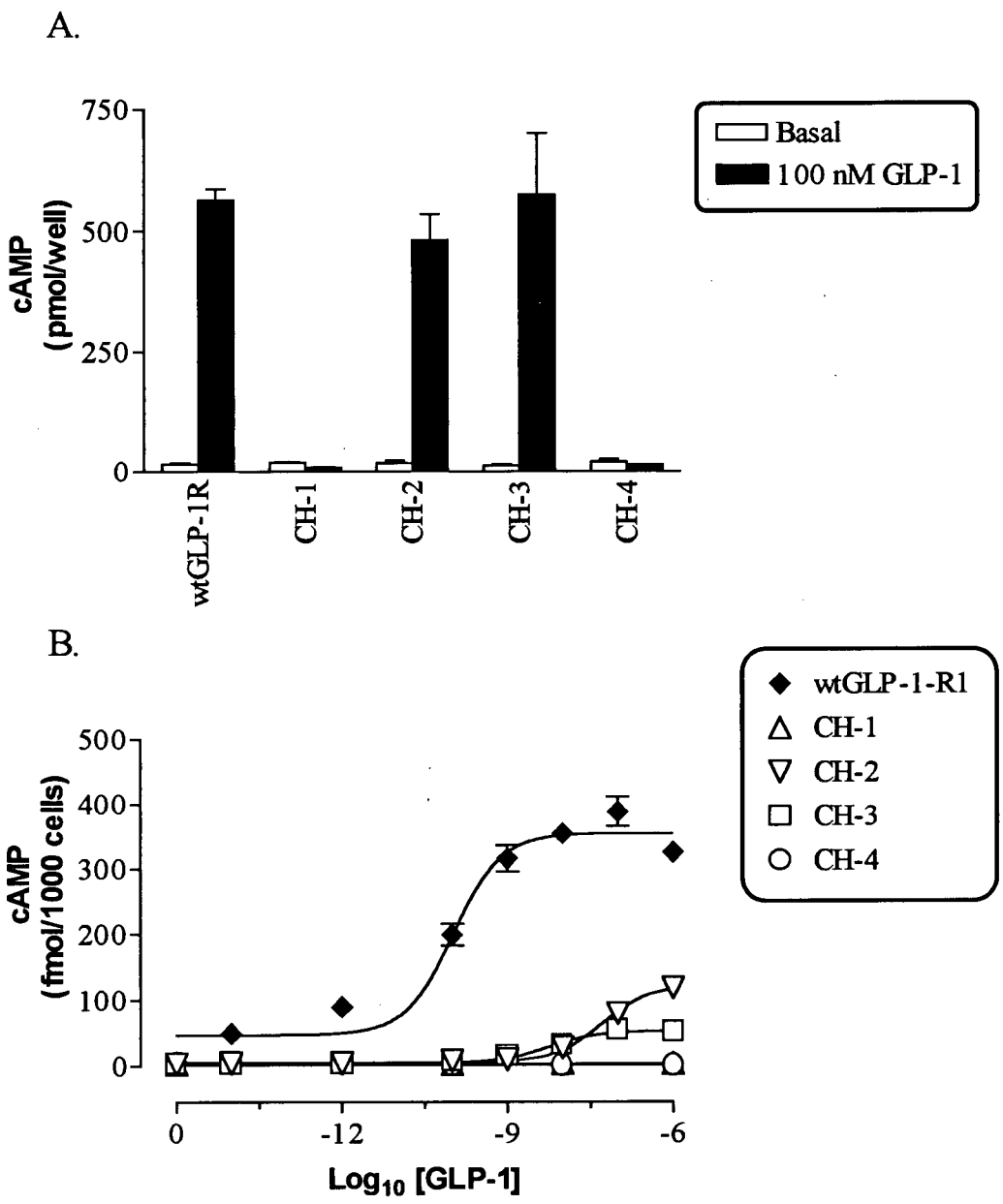


Fig. 48. Stimulation by GLP-1 of cAMP Production in COS-7 (A), and CHO-K1 (B) Cells Expressing the Wild Type GLP-1 or GIP/GLP-1 Chimeric Receptors. Data are the mean ± S.E.M. of 3-7 individual experiments. Maximal cAMP increases and EC_{50} values are summarized in Table 7.

Receptor	GIP-Stimulated cAMP accumulation			GLP-1-Stimulated cAMP accumulation		
	COS-7 % of wt max	CHO % of wt max	CHO EC_{50} (nM)	COS-7 % of wt max	CHO % of wt max	CHO EC_{50} (nM)
wtGIP-R1	100	100	0.49 ± 0.21	-	-	-
wtGLP-R1	-	-	-	100	100	0.103 ± 0.011
CH-1	-	-	-	-	-	-
CH-2	-	-	ND	84.8 ± 9.6	30.5 ± 2.0*	81.4 ± 19.6 *
CH-3	37.6 ± 3.3*	29.3 ± 10.2*	17.1±3.5*	102.5 ± 23.0	13.2 ± 1.9*	5.99 ± 0.68 *
CH-4	20.5 ± 3.8*	42.4 ± 11.2*	0.47 ± 0.17	-	-	-
CH-5	-	-	-	-	-	-
CH-6	-	-	-	-	-	-
CH-7	-	ND	ND	-	ND	ND
CH-8	-	ND	ND	-	ND	ND
CH-9	-	-	-	-	-	-

Table 7. Cyclic AMP Responses to GIP and GLP-in COS-7 and CHO-K1 Cells Expressing Chimeric Receptors. Data are expressed as % of maximal wild type levels and EC_{50} values, calculated by nonlinear regression analysis (n=3-7). ND = Not determined; - = Non-detectable.* = significantly different from wt ($p \leq 0.05$, n = 3-7).

Cell line	Binding		cAMP production.	
	Bmax (% of wt)	IC_{50} (nM)	% of wt Max.	EC_{50} (pM)
wtGIP-R1	100%	4.87	100%	490 ± 210
wtGIP-R8	4.9%	1.37	9.59 ± 0.29	70.3 ± 43

Table 8. Summary of ^{125}I-GIP binding (Bmax and IC_{50}, n =2) and cAMP Responses to GIP, with wtGIP-R1 and wtGIP-R8 CHO-K1 Cell Lines. Data expressed as % of maximal wild type levels (n = 3) for the low-level, stable GIP receptor expressing CHO-K1 cell line, wtGIP-R8.

3.7 TRUNCATION OF THE CARBOXY-TERMINAL TAIL OF THE GIP RECEPTOR

As discussed in sections 1.11.2, 1.11.4, and 1.11.5 the carboxy-terminal (CT) tail of G-protein linked receptors has been identified as a region that is important for G-protein interaction, receptor desensitization, and sequestration. A number of carboxy-terminal truncated forms of the receptor were constructed to examine the effect on receptor binding and activation of adenylyl cyclase, desensitization, and receptor internalization. Receptor truncations were designed to examine the effects of loss of a given CT-tail region on receptor binding and signaling, and the effect of loss of possible serine (Ser) phosphorylation sites on receptor desensitization and internalization.

One receptor construct was derived using a convenient CT restriction site (*Sac* I), which resulted in removal of the most distal 30 amino acids of the receptor (GIP-R-427+), including two Ser residues (440 and 453). Since an additional six amino acids encoding vector sequence (QRVGCI) were included, the receptor's final length was 433 residues (Fig. 49). The other constructs prepared were:

GIP-R-418-further truncation to 418 residues, resulting in the deletion of an additional two Ser residues (426 and 427).

GIP-R-405-truncation to 405 residues resulted in the loss of a putative casein kinase II phosphorylation site (Ser406).

GIP-R-400-truncated by a further 5 residues resulted in the deletion of a highly basic sequence (RRLRL) within a region shown to be important to G-protein coupling in the β2-adenergic receptor (O'Dowd et al., 1988; Liggett et al., 1991).

Fig. 49 depicts the different constructs generated.

3.7.1 EFFECT OF CARBOXY-TERMINAL TAIL TRUNCATION ON LIGAND BINDING

Receptors truncated at residues 405, 418 and 427+ exhibited high affinity binding in competition binding studies similar to that seen in cells expressing the 455 amino acid wild type receptor (wtGIP-R1), when expressed both transiently in COS-7 cells (Table 11), and stable CHO-K1 cell lines (Fig. 50A, Table 9). Receptor expression level as determined from Bmax values indicated that GIP-R-427+ was expressed at least as efficiently (125 ± 33%, CHO-K1) as the wt receptor in both cell systems (Table 9). However, in cells expressing the 418 and 405 amino acid forms of the receptor, maximal binding was 74 ± 16% and 29 ± 3% of that seen with the wtGIP-R1 cell line, respectively, suggesting these receptors were not as efficiently expressed at the membrane level as the longer forms of the protein. In contrast, when the receptor was truncated at amino acid 400 no detectable ^{125}I-GIP binding was observed (Fig. 50A). The

other three constructs bound GIP with affinity similar to that of the full length GIP receptor in both COS-7 (Table 9) and CHO-K1 cells (Fig 50A, Table 11).

In order to determine whether GIP-R-400 is expressed it would be preferable to determine its localization (membrane or intracellular) by immunocytochemistry. However, this was not possible due to the lack of a GIP receptor antibody. As the CT-tail length appeared to be important for efficient expression at the cell surface, an alternative approach to examine the importance of residues 401-405 was taken. A sixth construct with five alanine ($400A_5$) residues added on to GIP-R-400 was constructed to give a similar length to that of 405 (Fig. 51). Both COS-7 (Table 9) and CHO-K1 cells, stably expressing the $400A_5$ receptor, specifically bound ^{125}I-GIP with a small but significant shift to the right in the displacement curve compared to the wtGIP-R1 cell-line (Fig. 50B, Table 9). Two other constructs were then generated to examine the region just distal to the seventh transmembrane domain, one with residues 397-405 replaced with 9 Ala residues (GIP-R-$396A_9$), and the other with the residues 397-400 deleted (GIP-R-ΔQSEI). Neither of the constructs displayed specific ^{125}I-GIP binding in competition binding experiments (Fig. 50B, Table 11).

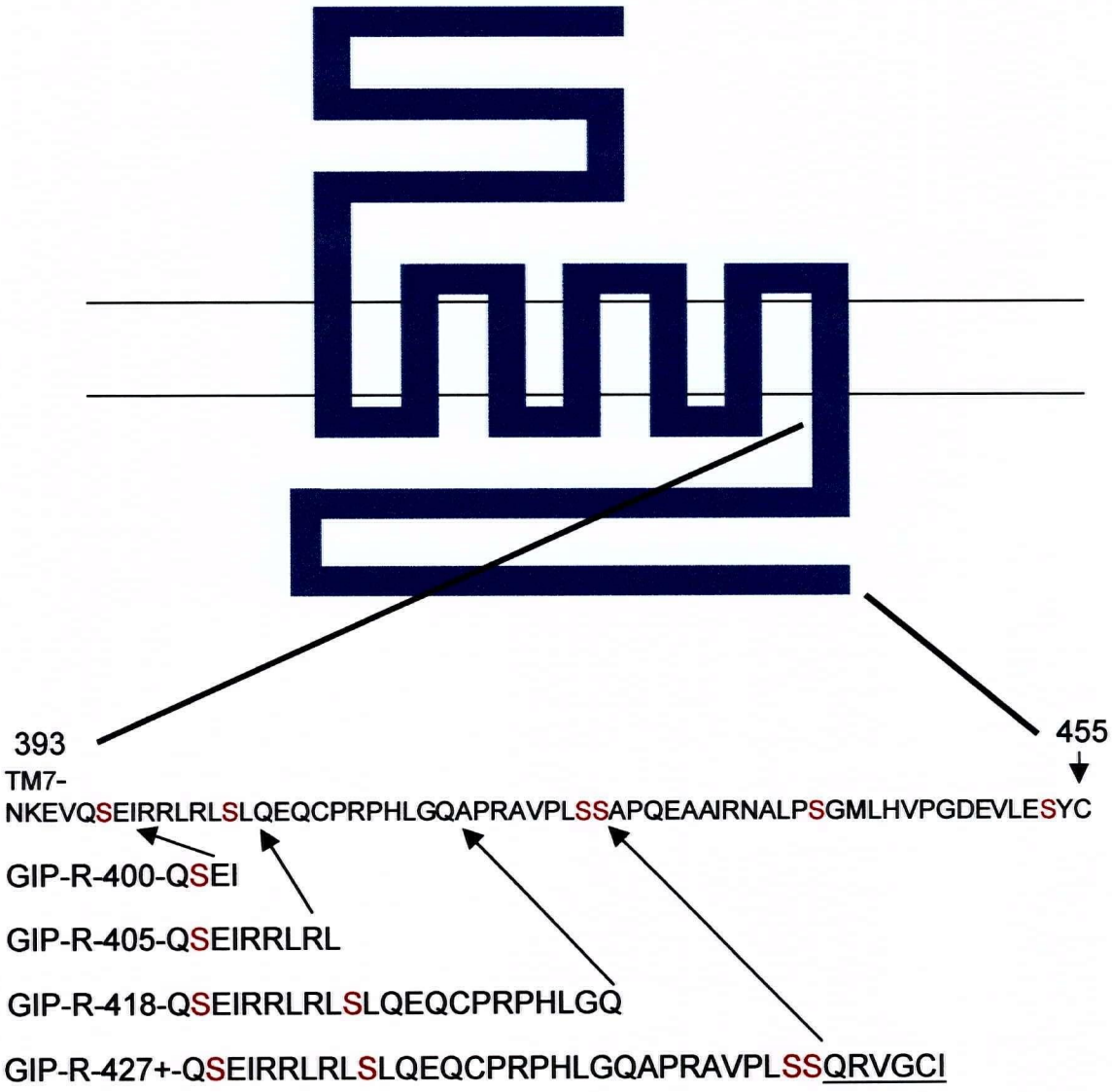
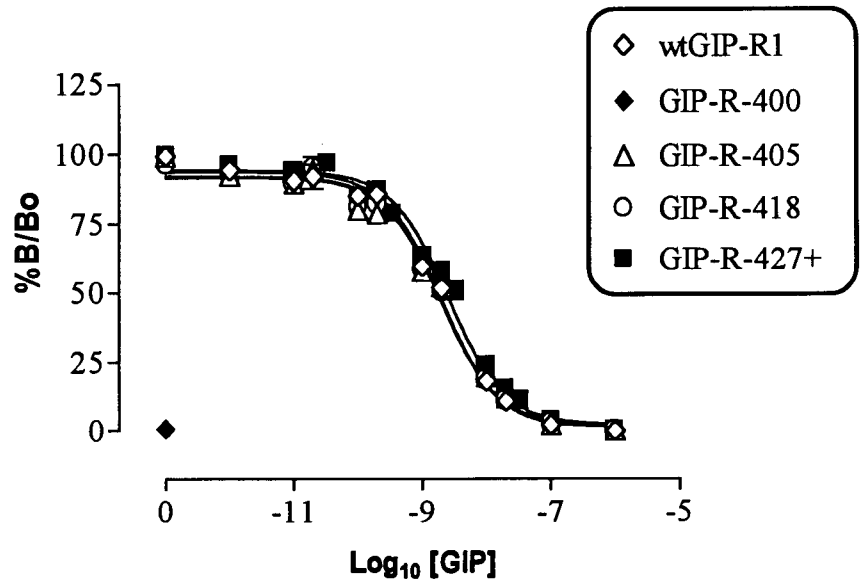


Fig. 49. Carboxy-terminal Truncation of the Rat Islet GIP Receptor.

A.



B.

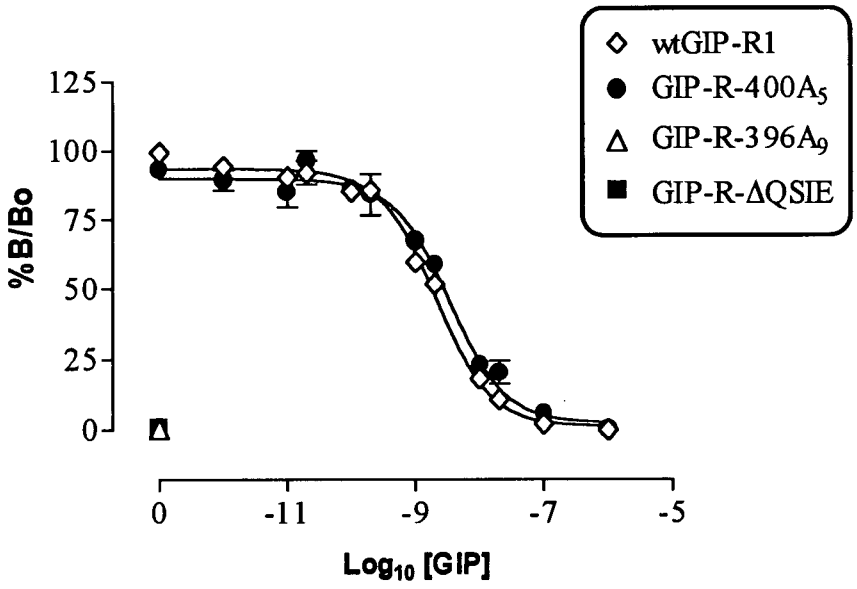
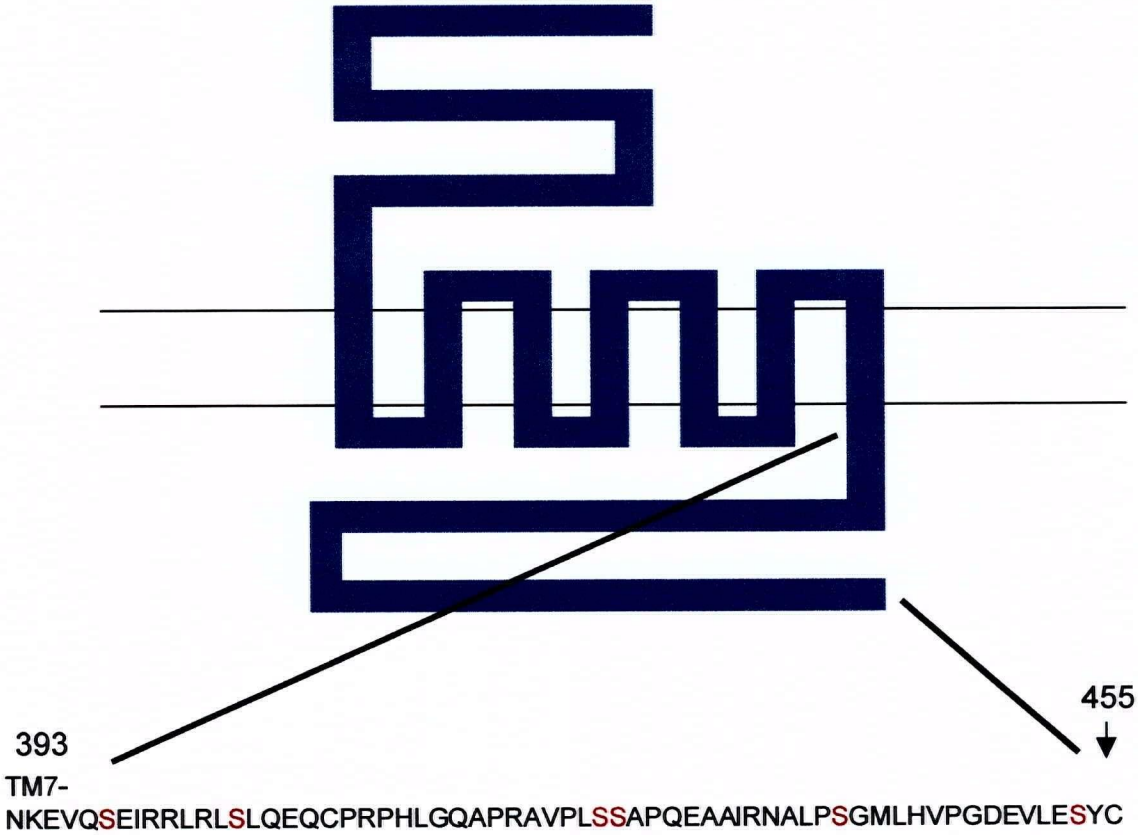


Fig. 50. Displacement of ^{125}I-GIP by GIP in CHO-K1 Cell-lines Stably Expressing Carboxy-Terminal truncated and wtGIP Receptors. Curves are representative of at least 4 individual experiments. Expression levels (Bmax) and IC_{50} values are summarized in Table 9.

	COS-7		CHO-K1	
Construct	IC_{50} (nM)	Bmax % of wt	IC_{50} (nM)	Bmax % of wt
wtGIP-R1	1.79	100.0	2.24 ± 0.35	100
GIP427+	1.73	100.8	2.73 ± 0.23	125.5 ± 33.8
GIP418	0.42	70.4	2.28 ± 0.27	74.0 ± 16.1
GIP405	1.47	29.8	2.16 ± 0.12	29.3 ± 2.94*
GIP400	-	-	-	-
GIP400ala_5	0.42	ND	3.79 ± 0.21 *	12.0 ± 1.1*
GIP396ala	-	-	-	-
GIP-QSIE	-	-	-	-

Table 9. Summary of Binding Experiments with Carboxy-Terminal Tail Truncated Forms of the Rat GIP Receptor. (-) = not detectable, ND = not done. (*) = significance difference from wt, $p < 0.05$, n =6.



GIP-R-396A_9 = TM7-NKEVAAAAAAAAA

GIP-R-400A_5 = TM7-NKEVQSEIAAAAA

GIP-R-ΔQSEI = TM7-NKEVRRLRLSLQEQCPRPHLGQAPRAVPLSSAPQEA
AIRNALPSGMLHVPGDEVLESYC

Fig. 51. Modified Carboxy-Terminal Tail forms of the Rat Islet GIP Receptor.

3.7.2 EFFECT OF CARBOXY-TERMINAL TAIL TRUNCATION ON cAMP PRODUCTION

To assess the effect of truncation on signal transduction, cAMP responses to GIP were determined with cells expressing the truncated receptors both transiently in COS-7 cells and stably in CHO-K1 cells. In COS-7 cells, maximal GIP (10 nM)-stimulated cAMP levels observed for receptors GIP-R-427+ (100.3 ± 6.5 pmol/well) and GIP-R-418 (93.4 ± 14.6 pmol/well) were not significantly different ($p \leq 0.05$, $n = 3$) from those with the wtGIP-R (104 ± 17 pmol/well) ($p > 0.05$; n=3). However, GIP-R-405 (60.3 ± 12.7 pmol/well) and GIP-R-400 (5.3 ± 0.7 pmol/well) displayed significant decreases in maximal cAMP production (57.7 ± 12.1% and 5.7 ± 0.7% of wtGIP-R maximum, respectively) (n=3, $p<0.05$)) (Fig. 52A, Table 10). Of particular interest was the fact that extension of the receptor tail length to 405 amino acids, in the GIP-R-400A_5 construct, restored the levels of cAMP production to 67.7 ± 6.9% of GIP-R-405 (Fig. 52B). However, neither GIP-R-386A_9 nor GIP-R-ΔQSIE responded to 10 nM GIP stimulation (Fig. 52B, Table 10), suggesting that either these receptors were not expressed, or that regions important to G-protein coupling were changed or deleted.

When expressed in CHO-K1 cells, maximal cAMP production with all of the truncated receptors was decreased when compared to that obtained in the wtGIP-R1 cell line (Fig. 53, Table 10). Surprisingly, even though there was only a small decrease in the affinity of GIP-R-405A_5 for GIP, there was a large increase in the EC_{50} value (1.16 ± 0.32 μM) when compared to the wt receptor (69 ± 27 pM) (Fig. 53, Table 10). None of the other truncated forms of the receptor differed significantly from the wt receptor in their EC_{50} values (Fig. 53, Table 10). In agreement with results in COS-7 experiments,

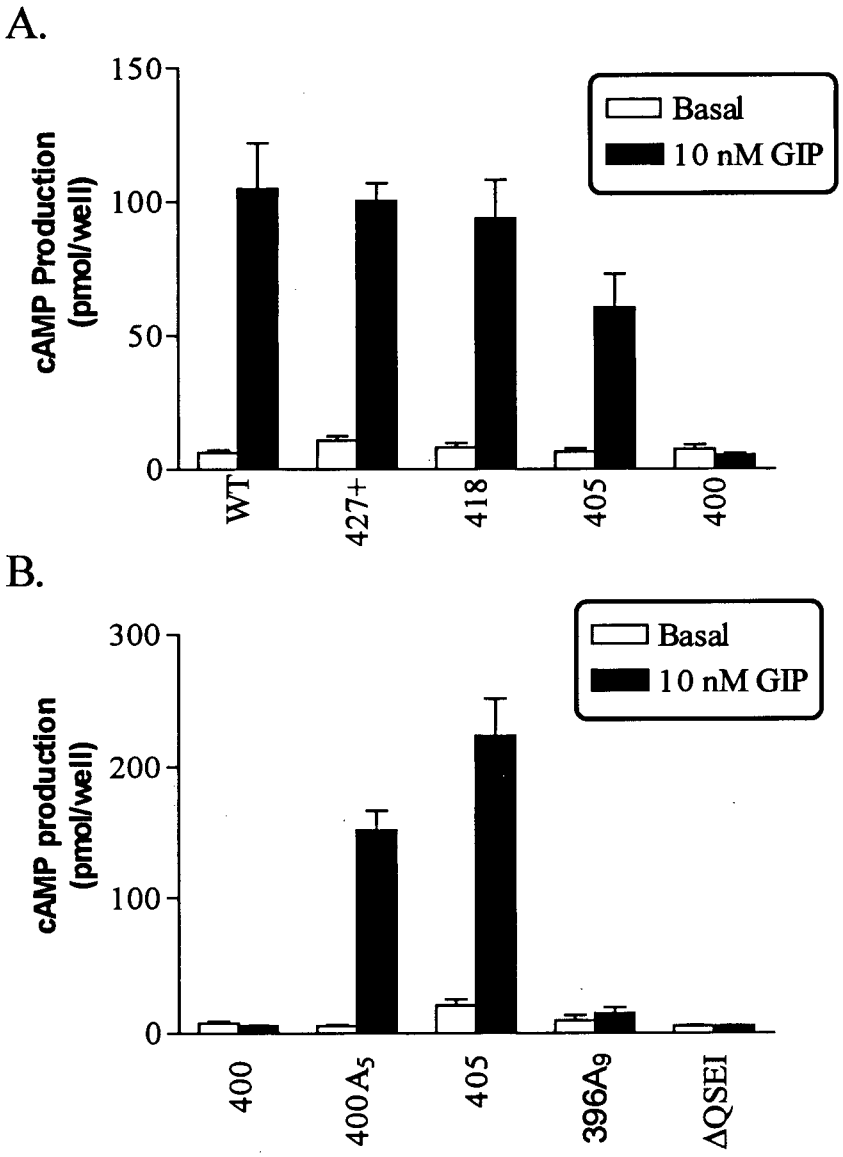
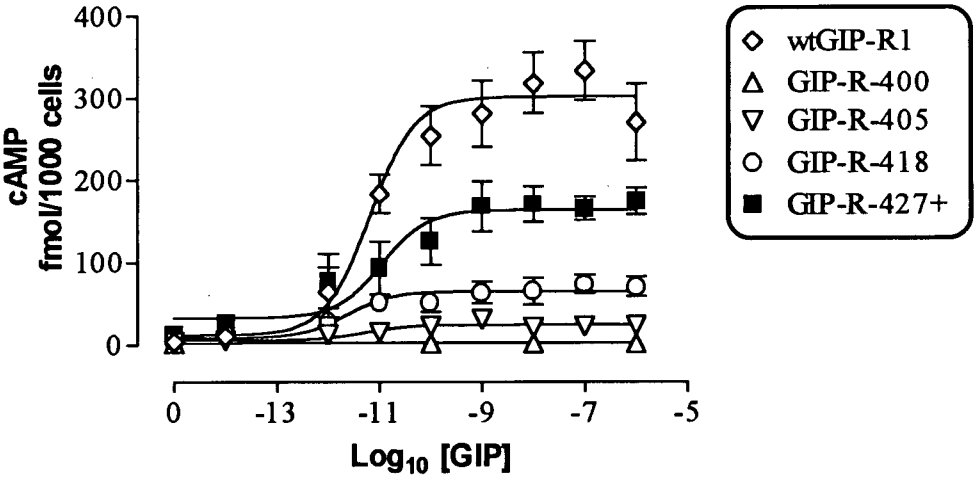


Fig. 52. 10 nM GIP-stimulated cAMP Production in Carboxy-Terminal Tail Truncated Forms of the GIP Receptor. A: Of all the receptor constructs initially examined, only 400 did not respond to 10 nM GIP stimulation, while receptors with truncations up to amino acid 405 responded. B: When the 400 construct was extended to a length of 405 amino acids by the addition of 5 alanine residues $400A_5$, cAMP responsiveness was restored to 67.7 ± 6.9% of that seen with the GIP-R-405. A third construct with residues 397-400 deleted (ΔQSEI) was also found to not respond to 10 nM GIP stimulation.

A.



B.

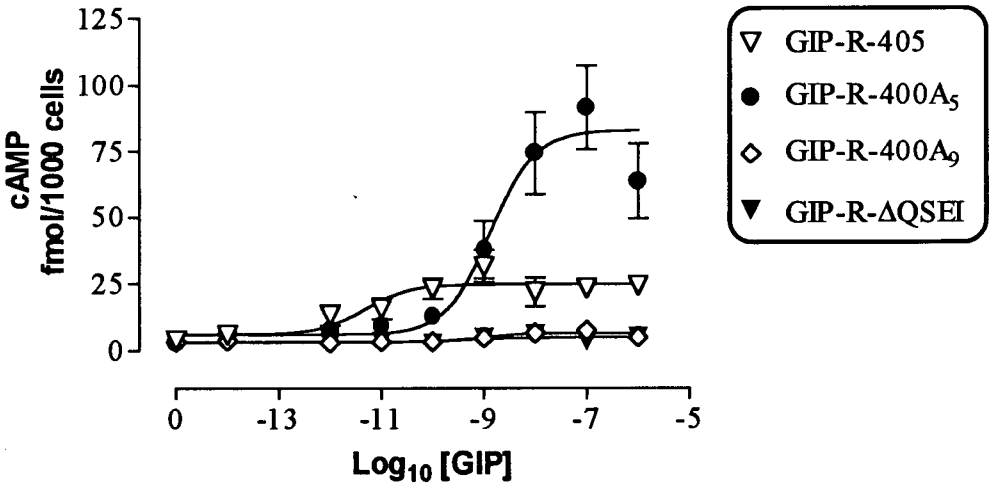


Fig. 53. Stimulation by GIP of cAMP Production with Carboxy-Terminal Truncated (A), Deletion, and Alanine Substituted GIP Receptor Mutants (B) Expressed in CHO-K1 Cells. Data are the mean ± S.E.M. of 3-6 individual experiments. Maximal cAMP increases and EC_{50} values are summarized in Table 10.

neither GIP-R-396A_9 nor GIP-R-ΔQSEI were responsive to GIP stimulation at concentrations as high as 1μM (Fig. 53, Table 10).

Receptor	COS-7	CHO-K1	
	% of wt max	% of wt max	EC_{50} (pM)
wtGIP-R1 (455)	100	100	69 ± 27
427+	95.9 ± 6.2	55.1 ± 12.5 *	47 ±20
418	89.3 ± 14.0	23.3 ± 4.9*	15 ± 0.9
405	57.7 ± 12.1	8.1 ± 1.9*	11 ± 0.7
400	5.0 ± 0.7*	-	-
400ala_5	-	31.3 ± 6.8*	1163 ± 320 *
396ala_9	-	-	-
ΔQSEI	-	-	-

Table 10. Summary of cAMP Experiments with Carboxy-Terminal Tail Truncated Forms of the Rat GIP Receptor. (-) = not detectable. (*) =significance difference from wt, $p < 0.05$, n =3-6.

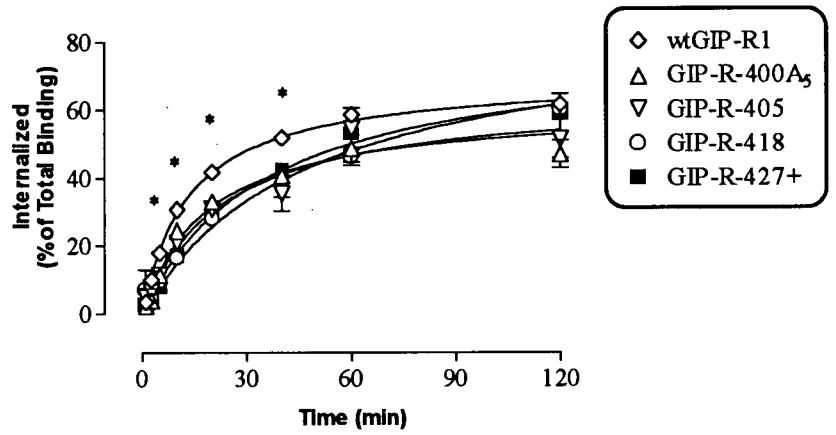
3.7.3 EFFECT OF CARBOXY-TERMINAL TAIL TRUNCATION ON RECEPTOR UPTAKE AND DESENSITIZATION

As serine and threonine residues are often phosphorylated in response to receptor activation, and can lead to both homologous receptor desensitization and receptor sequestration, the effect of truncation of the CT-tail on receptor uptake and homologous desensitization was examined.

All the constructs were internalized over time as assessed by the increase in acid resistant pool in receptor uptake studies (Fig. 54A). The wtGIP-R1 receptor clone

displayed a rapid increase in acid resistant binding over time, reaching maximal levels (64.9 ± 2.7% of the total bound) within 120 minutes (Fig. 54A, Table 11). Maximal internalization of the truncated receptors did not differ significantly from that seen for the full length GIP-R-455 cell line at time points from 60-120 min (Fig. 54A, Table 11). Further incubation times of up to 4 hours failed to reveal a difference in maximal uptake of the different receptor constructs in CHO-K1 cells. Closer examination of the initial uptake suggested that it was linear in nature, and the rate of initial uptake was examined for the different constructs (Fig. 54B). Surprisingly, truncation of the tail by 28 residues (GIP-R-427+) and 37 residues (GIP-R-418) caused significant decreases in the rate of internalization over the first 10 min compared to that seen for the wt cell line (Fig. 54B, Table 11). However, further truncation of the C-terminal tail by 50 amino acids (GIP-R-405) partially restored the rate of receptor uptake and, with the GIP-R-400A_5 construct, the rate of uptake was not significantly different from that of the wt receptor (Fig. 54B, Table 11). These data suggest that truncation of the more distal region of the receptor interfered with receptor sequestration, possibly by stabilizing a conformation unable to interact as efficiently with other proteins involved in the receptor uptake machinery. Further truncation of the receptor to 405 amino acids (GIP-R-405 and GIP-R-400A_5) appeared to restore efficient receptor sequestration activity to the GIP receptor, possibly by relieving conformational inhibition. More detailed mutational analysis is required to determine if this is due to removal of a negative sequestration motif or exposure of a positive sequestration signal, as has been suggested for other receptors (Huang et al., 1995b; Findlay et al., 1994).

A.



B.

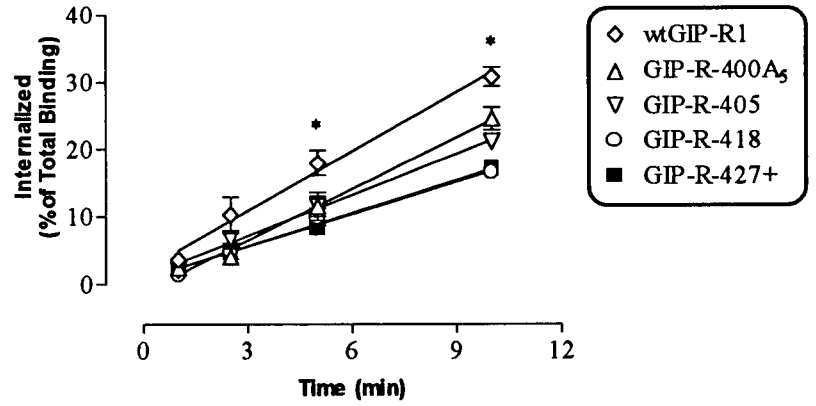


Fig. 54. The Effect of Carboxy-Terminal Tail Truncation of the Rat Islet GIP Receptor on Receptor Sequestration in CHO-K1 Cell Lines. A: No significant difference in maximal receptor sequestration was observed between the wtGIP receptor and four truncated receptors examined. However, uptake of wtGIR-R1was more rapid and was significantly greater than that seen for some CT truncated receptors at time points 5-40 min. B: Initial rate of receptor uptake was greater for the wtGIP-R1 receptor than that seen for GIP-R-418, GIP-R-427+, and GIP-R-405, while no significant difference between GIP-400A_5 and the wt GIP-R1 uptake rates was observed. Maximal sequestration (% of total binding), and rate of internalization (Slope) values are summarized in Table 11.

Construct	Maximal Internalization (% of Total Bound)	Slope (%/min)
GIP-R-455	64.9 ± 2.7	2.94 ± 0.26
GIP-R-400A_5	61.8 ± 2.9	2.53 ± 0.24
GIP-R-405	60.3 ± 3.3	2.02 ± 0.16 *
GIP-R-418	73.8 ± 2.4	1.64 ± 0.16 *
GIP-R-427	69.8 ± 1.0	1.60 ± 0.08 *

Table 11. Maximal Receptor Internalization and Initial Slope Values for Receptor Uptake Over the Initial 10 Minutes. Values are the mean ± S.E.M. of at least 3-4 individual determinations. * = Significant difference from wtGIP-R1, $p<0.05$.

Attempts at examining receptor desensitization with the wtGIP-R1 cell line were undertaken, but were without success (Fig. 55). wtGIP-R1 cells pre-incubated with GIP (1 nM-1μM), for 15 min-2 hours did not differ in their subsequent responses to 1 or 10 nM GIP, in comparison to untreated wtGIP-R1 cells. Nor did their EC_{50} values differ significantly if dose-response curves were generated after pre-treatment. This suggests that the GIP receptor does not undergo homologous desensitization, or at least not in CHO-K1 cells.

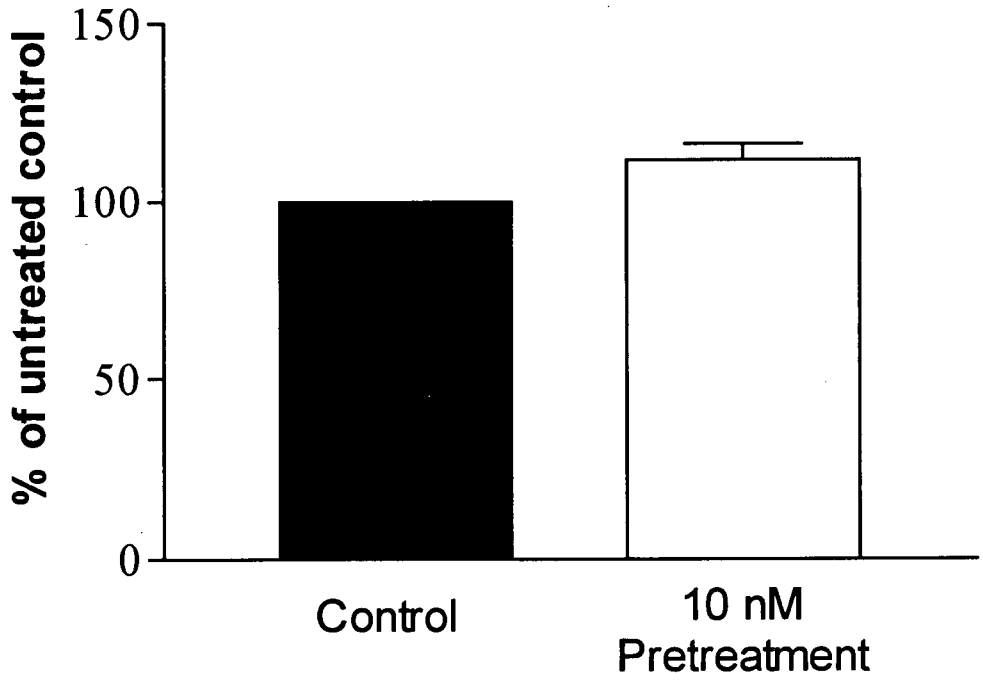


Fig. 55. Lack of Desensitization of GIP-mediated cAMP Production in wtGIP-R1 Cells. Cells were pre-incubated with 10 nM GIP for 20 min., washed three times with assay buffer, then challenged again with 10 nM GIP. cAMP levels were normalized to % of untreated cells .

CHAPTER 4

DISCUSSION

4.1 ISOLATION AND CHARACTERIZATION OF RAT PANCREATIC ISLET GIP RECEPTOR cDNAs

The pancreatic islet GIP receptor has proven difficult to study due to its apparent low level of expression, and difficulties in obtaining islets in sufficient quantities for detailed investigations. In light of these limitations much of the information regarding GIP receptor expression has been obtained from membranes of multipotential islet-derived tumor cells that express a variety of neuroendocrine peptide receptors (reviewed in Pederson, 1993; McIntosh *et al.*, 1996; Fehmann *et al.*, 1995). The first objective of the studies undertaken in the current thesis was to isolate cDNAs encoding the rat islet GIP receptor to enable study of its ligand binding and intracellular signaling properties.

4.1.1 THE RAT ISLET GIP RECEPTOR cDNA

Islet receptor cDNAs (GIP-R1, GIP-R2 and GIP-R3) were isolated, and sequencing demonstrated that they were homologous to the cDNA obtained by PCR from a rat tumor cell line (RINm5F) (Usdin *et al.*, 1993). However all three receptor cDNAs differed from the sequence reported by Usdin *et al.* (1993) at one nucleotide (Fig. 21) resulting in a single amino acid difference (Glu21→Gln21) (Fig. 22), suggesting that this is a single nucleotide polymorphism (SNP) in the rat gene, rather than a PCR based sequencing error. Nevertheless, it is evident that the GIP receptor expressed endogenously in rat islets

(Wheeler *et al.*, 1995) is the same as that expressed in RINm5F cells and the brain (Usdin *et al.*, 1993).

Similar GIP receptor cDNAs to that described in this Thesis have also been isolated from the hamster cell line HIT-T15 (Yasuda *et al.* 1994), human islets (Gremlich *et al.*, 1995), and a human insulinoma cDNA library (Volz *et al.*, 1995). The human 466 and hamster 462 amino acid GIP receptors displayed a high degree of identity with the rat, 79%, and 86% respectively (Volz *et al.*, 1995; Yasuda *et al.*, 1994), with the greatest divergence residing in the signal peptide and carboxy-terminal tail regions (Gremlich *et al.*, 1995). Both groups that isolated human receptor cDNAs reported partial cDNAs. One of these displayed a small deletion in the second extracellular loop (Gremlich *et al.*, 1995), and the second was an alternatively spliced, truncated form of the receptor due to the deletion of a 62 base pair exon, which resulted in a frame shift within the fourth transmembrane domain (Volz *et al.*, 1995). The receptors were non-functional when expressed in CHL cells. Gremlich *et al.* (1995) also isolated both a short and long form of the human receptor; the latter most likely arising from a partially spliced pre-mRNA resulting in the insertion of an additional 27 amino acids at the juxtamembrane region of the cytoplasmic tail. Surprisingly, this long form was shown to be expressed and to function in a similar fashion to the fully spliced cDNA, encoding a receptor of 466 amino acids (Gremlich *et al.*, 1995).

The GIP receptor gene has been mapped to human chromosome 19 (Gremlich *et al.*, 1995; Stoffel *et al.*, 1995). Cloning and sequencing (Yamada *et al.*, 1995) revealed that the ~13.8 kb gene contains 14 exons. The exon-intron organization is conserved among members of the VIP/glucagon/secretin receptor family (Yamada *et al.*, 1995),

being similar to that seen with the genes for both the mouse parathyroid hormone receptor (McCuaig *et al.*, 1994), and the human and mouse glucagon receptors (Lok *et al.*, 1994; Burcélin *et al.*, 1995).

All GPCRs share the same basic secondary structure consisting of an extracellular amino-terminal "head", seven transmembrane domains, with three extracellular (EC) and three intracellular (IC) loops, and an intracellular carboxy-terminus (CT) tail (see Fig. 22). The rat pancreatic GIP receptor is a member of the Secretin/Glucagon/VIP receptor sub-family (Usdin *et al.*, 1993; Donnelly, 1997; Ulrich *et al.*, 1998) of the GPCR superfamily (Probst *et al.*, 1992). Members of the Secretin/Glucagon/VIP receptor family lack any significant sequence identity to other GPCRs, such as the β-adrenergic and rhodopsin receptors, but share substantial identity with each other (Segre and Goldring, 1993; Donnelly, 1997; Ulrich *et al.*, 1998). Members of this family bind moderately large peptides, and include the receptors for pituitary adenylate cyclase-activating polypeptide (PACAP) (Spengler *et al.*, 1993), calcitonin (Lin *et al.*, 1991), calcitonin gene-related peptide (Aiyar *et al.*, 1996), parathyroid hormone/parathyroid hormone-related peptide (PTH/PTHrP) (Jüppner *et al.*, 1991), growth hormone-releasing factor (GHRH) (Godfrey *et al.*, 1993), corticotropin-releasing factor (CRF) (Chang *et al.*, 1993), glucagon (Jelinek *et al.*, 1993), GLP-1 (Thorens, 1992) and insect diuretic hormone (Reagan, 1994). As mentioned in section 1.5.1 the rat islet GIP receptor shares the greatest identity with the glucagon and GLP-1 receptors (~40%) (Fig. 56).

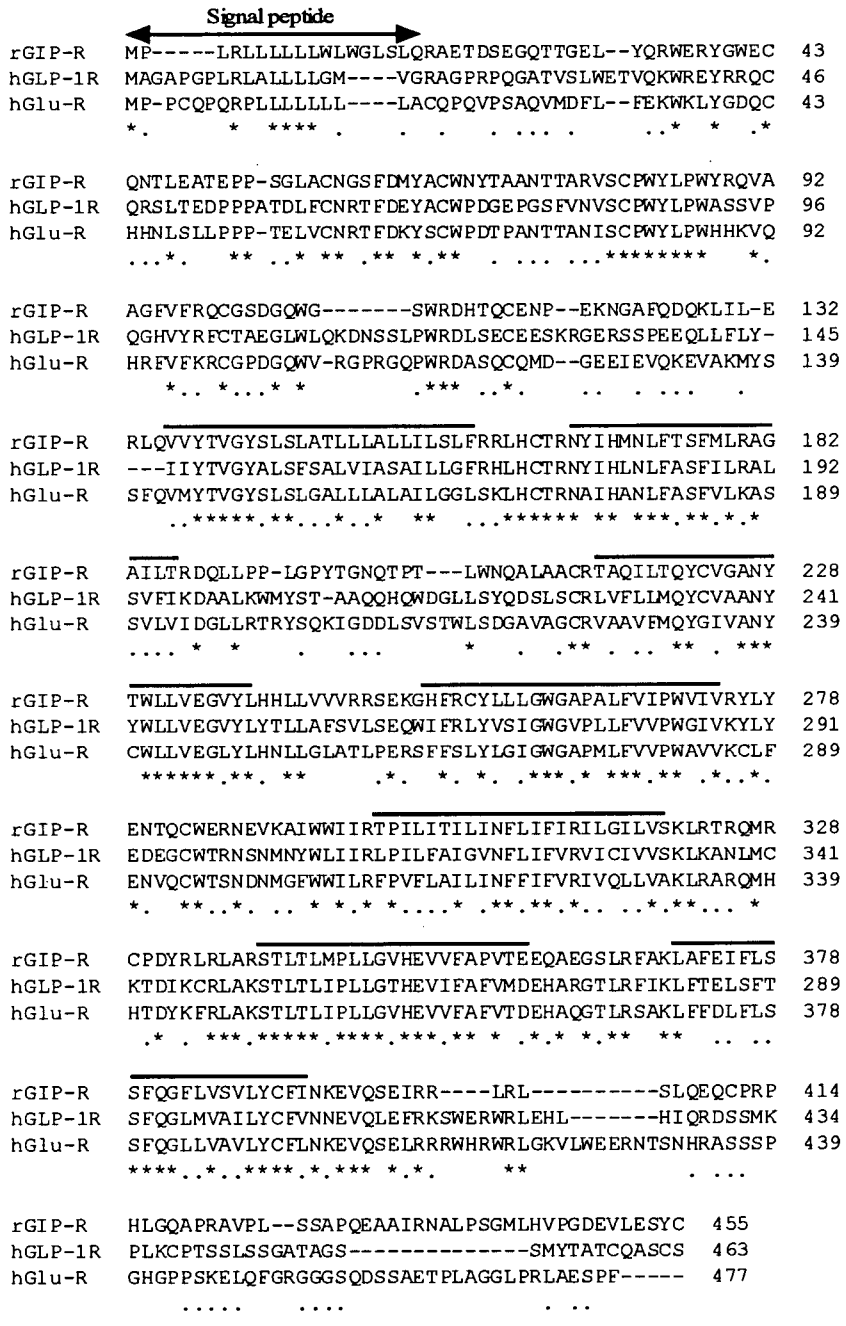


Fig. 56. Alignment of the Amino Acid Sequences of the Rat GIP, Human GLP-1, and Human Glucagon Receptors. Amino acids are presented as their single letter abbreviations. * = residues completely conserved, . = residues well conserved. Putative transmembrane domains are overlined. Alignment was carried out using the PC Gene software package (IntelliGenetics, 1995).

The receptor has a large amino-terminal head (~135 amino acids) with three putative N-linked glycosylation sites (asparagines 59, 69, and 74) and 6 conserved cysteine residues, as well as two other highly conserved cysteine residues in the first and second EC loops that are thought to form disulfide bonds that may be important in the maintenance of receptor structure.

4.1.2 CHARACTERIZATION OF GIP RECEPTOR BINDING AND SIGNALING VIA cAMP

In initial binding studies, cells expressing GIP-R1 transiently, or stably, demonstrated a single class of high affinity binding sites (IC_{50} = 1.2-7.9 nM) in competitive binding studies (Fig. 23, Table 1), values within the range of those reported in tumor cell lines (reviewed in Pederson, 1993;McIntosh *et al.*, 1996), and with expressed receptor cDNAs from the hamster β-cell line, HIT-T15 (Yasuda *et al.* 1994), human islets (Gremlich *et al.*, 1995), and a human insulinoma cDNA library (Volz *et al.*, 1995). Intact wtGIP-R1 cells, and membranes purified from these cells, both bound ^{125}I-spGIP in a concentration dependent manner in saturation binding studies (Fig. 24). Non-linear regression analysis indicated that the isotherms were monophasic, with Kd values of 200-300 pM, and an expression level of 12-15 X 10^3 receptors per cell, or 59.2 ± 5.2 pmol of receptors/mg of protein. In addition, synthetic porcine GIP yielded concentration-dependent increases in cAMP in wtGIP-R1 cells with an EC_{50} of 8.7 x10^{-10} M (Fig. 28), a value which is in agreement with those previously reported in HIT cells (Lu *et al.*, 1993a) and the originally isolated RIN cell cDNA (Usdin *et al.*, 1993). More recently, others have demonstrated that the cells expressing hamster (Yasuda *et al.*, 1994)

and human (Gremlich *et al.*, 1995; Volz *et al.*, 1995) GIP receptors responded to GIP with increases in cAMP levels, with EC_{50} values ranging from 300 pM (Usdin *et al.*, 1995) to 15 nM (Yasuda *et al.*, 1994). Volz *et al.* (1995) reported a technically inaccurate value of 0.12 fM, since these authors extrapolated to below tested peptide concentrations. The considerable range of reported EC_{50} values probably results from technical differences in the determination of cAMP levels, and in the cell lines used in the experiments. Importantly, none of the related peptide hormones tested in the current study (GLP-1, GLP-2, glucagon, or VIP) displaced ^{125}I-spGIP (Fig. 26) or stimulated cAMP production (Fig. 30) in the nM range, demonstrating that GIP-R1 encoded a rat islet GIP-specific receptor. This lack of cross reactivity has been confirmed by others for the rat (Usdin *et al.*, 1993), hamster (Yasuda *et al.*, 1994), and human receptor (Gremlich *et al.*, 1995; Volz *et al.*, 1995) indicating the absolute specificity of the GIP receptor from different animal species for its ligand.

Recently, the use of the GLP-1 receptor antagonist Ex (9-39) has provided important information supporting a role for GLP-1 in the enteroinsular axis (Kolligs et al., 1995; Wang et al., 1995; D'Alessio et al., 1996; Schirra et al., 1998). In the current studies, exendin-4 and exendin (9-39) were the only non-GIP peptides that displaced ^{125}I-spGIP binding (Fig. 27), a finding that was later confirmed with the human GIP receptor (Gremlich *et al.*, 1995), suggesting that at high concentrations these peptides may antagonize or potentiate GIP action. While COS-7 cells expressing GIP-R1 displayed a small increase in cAMP levels at the highest concentration of Ex-4 tested (1 μM), neither Ex-4 nor Ex (9-39) increased cAMP levels in the equivalent CHO-K1 cell line (wtGIP-R1) (Fig. 28 and 29B). In addition, neither Ex (9-39) nor Ex-4 were able to inhibit or

augment GIP stimulated cAMP production in wtGIP-R1 cells (Fig. 29A), while in parallel studies with cells expressing the rat GLP-1 receptor, Ex (9-39) inhibited GLP-1-stimulated cAMP production by approximately 65% (Fig. 29B). These findings suggest that interaction of the venom peptides with the GIP receptor is very weak, and support recent studies in the rat (Kölligs *et al.*, 1995; Wang *et al.*, 1995) and humans (Schirra et al., 1998) indicating that Ex (9-39) does not alter the contribution of GIP to the enteroinsular axis *in vivo*, or decrease its insulinotropic action in RIN5AH cells (Wang *et al.*, 1995) at the concentrations used.

Although GIP has potent insulinotropic properties and facilitates glucose disposal, controversy exists in the literature as to the relative effectiveness of GIP and GLP-1 in stimulating insulin release. Some investigators have found GIP to be equipotent to GLP-1 (Schmid *et al.*, 1990; Suzuki *et al.*, 1990) while others have found that GLP-1 has markedly greater insulinotropic activity (Siegel *et al.*, 1992; Shima *et al.*, 1988). In addition, GIP was shown to be strongly insulinotropic in humans in one study (Nauck *et al.*, 1989) while a subsequent report by the same authors demonstrated that GIP was a poor stimulant of insulin secretion (Nauck *et al.*, 1993a). Some of these differences may be explained by variability in the relative potencies among several commercially available GIP preparations. Having a single species of the rat GIP receptor expressed in COS-7 and CHO-K1 cells provides an ideal model with which to address this issue. The present studies revealed no significant differences between porcine and human GIP formulations tested with respect to binding properties or ability to stimulate cAMP accumulation. This suggests that the rat GIP receptor does not have preferential affinity for either peptide, and that the His18→Arg18 and Ser34→Asn34 amino acid differences

between porcine and human GIPs do not influence ligand-receptor interaction. These studies also revealed no significant difference in the affinities or potencies of synthetic GIP preparations obtained from two different suppliers (Fig. 26, Table 2, and Table 3). Unlike earlier peptide preparations (Jia *et al.*, 1995), the current batches of spGIP and shGIP were also equipotent as insulinotropic agents in the isolated perfused rat pancreas (Table 3). Since porcine and human GIP bind and activate the rat islet GIP receptor equally, such a heterologous system can be used for structure-activity studies on these peptides. Also, while the present studies cannot conclusively confirm that the discrepancies seen in the literature concerning the insulinotropic potency of shGIP are due to variations in the synthetic preparations, they do indicate that GIP-R1 expressing cell lines represent an ideal model to assess such preparations in the future.

4.1.3 EFFECTS OF GIP ON INTRACELLULAR CALCIUM

Usdin *et al.* (1993) examined the RINm5F GIP receptor signaling pathway utilizing a cell line consisting of HEK293 cells expressing apo-aequorin which, when reconstituted with the chromophore coelenterazine, emits light on the binding of calcium. Cells transfected with the RINm5F cell GIP receptor displayed increases in $[Ca^{2+}]i$ in response to GIP, however the mechanism underlying the increase was not determined. As discussed in section 1.7.1 and 1.7.2 there has been considerable discrepancy in the literature concerning the effects of both GIP and GLP-1 on changes in intracellular calcium. However, characteristics of Ca^{2+}-signaling in response to GLP-1 have been more extensively examined, and provide clues as to how GIP and its receptor may signal through this system. Real time spectrofluorimetry studies on GIP-dependent Ca^{2+}

responses were performed in Toronto using cells transfected with the wild type cDNA described in the current Thesis (see Appendix A). The data are discussed here since both the design and interpretation of experiments were performed collaboratively.

COS-7 cells expressing the GIP receptor displayed a biphasic $[Ca^{2+}]i$ response in the presence of GIP (50 nM) with an acute transient phase, termed P1, which then decreased to a sustained level over basal, termed P2 (Fig. A1). Phase P2 was abolished under conditions in which extracellular calcium was removed from the medium, suggesting that this response was dependent on Ca^{2+} influx across the plasma membrane, while P1 persisted under Ca^{2+} free conditions, strongly suggesting that it represents Ca^{2+} release from intracellular stores (Fig A1, Table A1).

To examine the nature of P1 further, cells were treated with the endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin. As thapsigargin is known to deplete the intracellular inositol triphosphate-sensitive Ca^{2+} stores (Lytton *et al.*, 1991), loss of the peak transient phase after this treatment supports the possibility that P1 represents a GIP-induced mobilization of Ca^{2+} from intracellular sources. Although the ability of the GIP receptor to couple to intracellular mobilization of Ca^{2+} has not previously been described in pancreatic islets, the related incretin GLP-1 in part increases $[Ca^{2+}]i$ through the release of intracellular Ca^{2+} stores in mouse (Cullinan *et al.*, 1994) and human (Gromada *et al.*, 1998a,b) β-cells. Furthermore, L-type Ca^{2+}-channel blockers do not block the $[Ca^{2+}]i$ response in mouse and human β-cells once the initial GLP-1 mediated increase has been initiated (Cullinan *et al.*, 1994; Gromada *et al.*, 1998a), suggesting that entrance of Ca^{2+} from extracellular sources acts as a feed forward stimulus of Ca^{2+} release (calcium-induced calcium release; CICR). A similar response to GIP does not appear to

be present in COS-7 cells, as the transient (P1) response was unchanged in the absence of extracellular Ca^{2+}. It is possible that the overexpression of the GIP receptor results in inappropriate coupling to G-proteins and mobilization of Ca^{2+} from intracellular sources, alternatively it may be that GIP receptor is acting via an alternative Ca^{2+}-signaling pathway in non-beta cells such as COS-7 cells.

Given the contradictions in the literature concerning GIP and GLP-1 mediated increases of $[Ca^{2+}]i$, it is likely that the cellular environment in which the response is observed is very important. For example both ourselves, with CHO-K1 cells expressing the rat islet GIP receptor (Appendix A), and others (Gremlich *et al.*, 1995; Volz *et al.*, 1995) with the GIP receptor expressed in Chinese hamster lymphoblast (CHL) cells, have observed that GIP stimulation of the receptor in these cellular environments resulted in no increase in $[Ca^{2+}]i$ levels. In addition, studies on several tumor cell lines (Lu *et al.*, 1993a; Widmann *et al.*, 1994) and on CHO-GR1 cells stably transfected with the hamster GIP receptor revealed no GIP-dependent increases in IP_3 levels (Yasuda *et al.*, 1994). An IP_3-mediated increase in $[Ca^{2+}]i$ is therefore unlikely. Similar contradictions exist concerning the GLP-1 receptor. Interaction of GLP-1 with its receptor results in the activation of a number of other signal transduction pathways that, as with GIP receptor signal transduction, appear to depend on the cell type used for expression studies. For example, expression of the receptor transiently in COS-7 cells was reported by one group to increase levels of IP_3 and $[Ca^{2+}]i$ (Wheeler *et al.*, 1993), while a second group did not see consistent increases in IP_3 levels, and no effect on $[Ca^{2+}]i$ when expressed in the same cell line (Widmann *et al.*, 1994). Dillon and coworkers (1993) reported that GLP-1 increased $[Ca^{2+}]i$ levels in COS-7 cells expressing the human GLP-1 receptor, as with the

GIP receptor, but no GLP-1-dependent increases in $[Ca^{2+}]i$ were observed in CHL cells expressing the human GLP-1 receptor (Thorens *et al.*, 1993). This was unlikely to be due to species differences, as the rat GLP-1 receptor also failed to increase $[Ca^{2+}]i$ in CHL cells (Widmann *et al.*, 1994). In contrast, GLP-1 was found to stimulate large increases in $[Ca^{2+}]i$ in the human embryonic kidney cell line (HEK293) stably expressing the human GLP-1 receptor (Gromada *et al.*, 1995).

Receptors for several other members of the Secretin/VIP receptor family, including those for PTH/PTHrP (Abou-Samra *et al.*, 1992), glucagon (Jelinek *et al.*, 1993), calcitonin (Cal) (Force *et al.*, 1992; Houssami *et al.*, 1994), and the PACAP receptor family (Spengler *et al.*, 1993), are capable of mobilizing intracellular Ca^{2+} stores. Given the apparently wide distribution of the GIP and the GLP-1 receptor in extrapancreatic tissues, it is quite possible that, as with glucagon and PTH/PTHrP receptors, the GIP receptor couples to different species of G-protein and signal transduction pathways in different tissues (Spengler *et al.*, 1993; Wakelam *et al.*, 1986).

How are the effects of GIP and GLP-1 on cAMP production and intracellular Ca^{2+} levels linked to insulin secretion? Lu *et al.* (1993a) using the glucose responsive HIT-T15 cell line as a model, demonstrated that insulin secretion was associated with both GIP-induced increases in cAMP levels and Ca^{2+} influx through VDCCs. This correlates with findings that GLP-1 stimulated increases in $[Ca^{2+}]i$ can be blocked with L-type channel blockers, both in isolated β-cells (Fridolf and Ahrén, 1993; Holz *et al.*, 1995; Yada *et al.*, 1993) and cell lines (Gromada *et al.*, 1995; Lu *et al.*, 1993a). The overall conclusion from such studies is that GLP-1, and probably GIP, target either the ATP-sensitive K^+ channel and/or VDCC to increase $[Ca^{2+}]i$ through cAMP-dependent protein kinases. The

current findings, that GIP evoked a concentration-dependent increase in cAMP accumulation, support this model. However, since COS-7 cells do not bind dihydropyridines, and do not exhibit $[Ca^{2+}]i$ responses to KCl or forskolin (M.B. Wheeler, personal communication), it is unlikely that the GIP-induced increases in Ca^{2+} influx were mediated by cAMP-dependent effects on VDCCs. This possibility is supported by the observation that increases in $[Ca^{2+}]i$ during the sustained phase (P2) were not sensitive to the L-type calcium channel antagonist nifedipine. Since P2 was dependent on extracellular Ca^{2+}, GIP may activate a voltage-independent Ca^{2+}/cation channel. Recent studies using the mouse insulin-secreting tumor cell line ßTC6 (Holtz *et al.*, 1995) and rat islets demonstrated that a component of the tGLP-1 induced increase in $[Ca^{2+}]i$ is not sensitive to nifedipine or membrane depolarization (Holtz *et al.*, 1995, Kato *et al.*, 1996). Holz and coworkers (1985) suggested that, in addition to its actions on ATP-sensitive K^+ channels, GLP-1 modulates β-cell Ca^{2+} influx through voltage-independent Ca^{2+} channels or non-specific cation channels. A similar conductance, that is activated by maitotoxin, has been identified in mouse β-cells (Worley *et al.*, 1994), and a β-cell line (Leech and Habener, 1997). In addition Soergel and co-workers (1990) have demonstrated that this maitotoxin-activated conductance is associated with stimulation of insulin secretion from the HIT β-cell line. Whether the voltage-independent Ca^{2+} influx evoked by GIP in COS-7 cells and GLP-1 in ßTC6 cells represent the same receptor-mediated Ca^{2+} entry pathway remains to be determined. Also undetermined is the significance of the heterogeneity in the Ca^{2+} flux patterns observed in individual cells (Fig. A2). It is possible that the magnitude of the Ca^{2+} response is dependent on the level of receptor expression, representing another potential mode of agonist regulation. Such a

relationship has been documented for the PTH/PTHrP (Guo *et al.*, 1994) and the calcitonin receptor isoforms C1a and C1b, where an increase in the Ca^{2+} sensitivity to CT was positively correlated with receptor number (Houssami *et al.*, 1994).

In summary, it is likely that GIP, like GLP-1, stimulates insulin release via cAMP-dependent pathways at sites both proximal and distal to increases in $[Ca^{2+}]i$. Such pathways probably involve phosphorylation of ATP-sensitive K^+ channels, VDCCs, and/or VINCCs, resulting in potentiation of glucose-dependent cellular depolarization, increased intracellular calcium levels, and increased recruitment of secretory granules from the reserve pool to the readily releasable pool (Ding *et al.*, 1997; Gromada *et al.*, 1998a, b). However in other cell types the modes of action may differ, and it will important to examine the effects of GIP and GLP-1 in such cells to determine whether there are cell-specific signal-transduction pathways.

4.2 IDENTIFICATION OF THE CORE GIP BINDING REGION

In humans, GIP has been shown to be a potent incretin (Brown *et al.*, 1989; Pederson *et al.*, 1993; Fehmann *et al.*, 1995) and, since incretins and their analogs have promising therapeutic potential for the treatment of NIDDM (Nauck *et al.*, 1989; Gutniak *et al.*, 1992; Byrne and Göke, 1996; Todd *et al.*, 1997; Nauck et al.,1997a), it is important to develop an understanding of the regional sequence requirements for receptor interaction. As discussed in section 1.10, structure-activity studies on GIP showed that limited truncation at the C-terminus had relatively minor effects on its insulinotropic activity. Thus, GIP 1-38 (Moroder *et al.*, 1978) and GIP 1-39 (Sandberg *et al.*, 1986) were found to exhibit similar activity to GIP 1-42 in the perfused rat pancreas. GIP 1-31

was shown subsequently by Maletti *et al.* (1987) to exhibit 10-fold weaker receptor binding affinity than GIP 1-42, but to have similar adenylyl cyclase stimulating activity. A further analog, GIP 1-30amide, was also shown to stimulate insulin secretion (Pederson *et al.*, 1990) and inhibit bombesin-stimulated amylase secretion (Rossowski *et al.*, 1992). Further studies on N-terminally truncated forms of GIP showed that GIP 17-42 possessed significant insulinotropic activity (Maletti *et al.*, 1986; Carlquist *et al.*, 1984). Morrow and co-workers (1996) examined the effect of shorter GIP fragments in the perfused rat pancreas, based on the premise that amino acids 15-30 appeared to be crucial for activity, and showed that GIP 17-30 and GIP 19-30 exhibited low, but significant, insulinotropic activity. Overall these studies suggested that the region of GIP important for insulinotropic activity lies between residues 17 and 30, but that the N-terminus of the molecule is also important for receptor binding. However, many of the fragments used in these studies were produced enzymatically, and tested in bioassays complicated by the presence of GLP-1 and glucagon receptors, with which the fragments could conceivably cross-react. The cloning of the rat islet GIP receptor and its expression in CHO-K1 cells provided an ideal system to reexamine, in isolation, the interaction of some of these fragments with the GIP receptor, as well as better define which residues were involved in both receptor binding and receptor activation.

None of the fragments generated based on the conserved region of amino acids 21-28 of GIP, GLP-1 and glucagon (GIP 18-28, GIP 21-26, GLP-1 21-26, and Glucagon 21-26), affected ^{125}I-GIP binding or cAMP production. Interestingly, GIP 17-30 and GIP 19-30 neither displaced ^{125}I-GIP binding, nor stimulated cAMP accumulation in wtGIP-R1 cells (Fig 31,32, and Table 4), despite having been demonstrated to exert some

insulinotropic activity in the perfused pancreas (Morrow *et al.*, 1996). This suggests that they may be acting either via interaction with another related receptor or an unidentified isoform of the GIP receptor. However, preliminary studies indicate that neither of these fragments interact with the rat GLP-1 receptor (P. Dan and C.H.S. McIntosh, personal communication). In contrast, GIP 1-30amide displayed high affinity GIP receptor binding (3.01 ± 0.69 nM) and, despite a small shift to the left in its displacement curve compared to GIP (1.21 ± 0.46 nM) (Fig. 31, Table 4), exhibited equivalent activity in cAMP experiments both in its EC_{50} and maximal cAMP levels obtained (Fig.32, Table 4).

4.3 GIP 6-30AMIDE CONTAINS THE HIGH AFFINITY BINDING REGION OF GIP AND IS A POTENT INHIBITOR OF GIP ACTION IN VITRO

While these observations confirmed that GIP 1-30amide interacted with the GIP receptor, and that the fragment contained the majority of the determinants for high affinity binding, the exact N-terminal requirements of receptor binding were still unclear. It was reasoned that limited truncation of GIP 1-30 at the N-terminus should retain the region which is important for receptor binding and could result in peptides with either agonist or antagonist activity. Examination of the predicted secondary structure (PC Gene) (see Fig. 33), and comparison to the crystal (Sasaki *et al.*, 1976) and lipid/aqueous interface NMR (Braun *et al.*, 1983) structures of glucagon, suggested that residues 10-30 contain a putative extended alpha helical region when GIP is associated with its receptor (Sasaki *et al.*, 1976). This is a conformation that has been suggested to play an important role in receptor binding for members of the glucagon superfamily (Bodanszky, 1974).

Interaction with the rat GIP receptor with this predicted α-helical stretch (GIP 10-30), and two other fragments with amino-terminal extensions from the α-helix boundary predicted to begin at Tyr^{10} (GIP 7-30 and GIP 6-30amide), were therefore studied. Importantly, the results showed that the core region responsible for high affinity binding lies within residues 6-30, since GIP 6-30amide displays a receptor affinity equivalent to that of the intact peptide (IC_{50}s = 2.39 ± 1.15 nM and 3.08 ± 0.57 nM, respectively). Further truncation of the N-terminus, by one and four amino acids, resulted in peptides with 74-fold (GIP 7-30) and 235-fold (GIP 10-30) lower affinity, as determined from competitive binding assays. Truncation of the full length GIP polypeptide by 15 amino acids (GIP 15-42) resulted in a 641-fold decrease in the peptide affinity when compared to that observed for the endogenous peptide (Fig. 34A). Despite the ability of all four peptides to bind to the GIP receptor, they demonstrated either extremely weak or absent stimulant activity on adenylyl cyclase (Fig.34B), suggesting that they could act as antagonists. This possibility was confirmed, with GIP 15-42, GIP 10-30, and GIP 7-30, inhibiting GIP (1 nM)-stimulated cAMP production in the μM range, and GIP 6-30amide inhibiting by 58 ± 2.5% at a concentration of 100 nM (Fig 35).

The current results regarding the antagonist activity of GIP 7-30 are in agreement with a recent report that C-terminally amidated rat GIP 7-30 inhibited GIP stimulated cAMP production in cells transfected with GIP receptor cDNA, and in β-TC3 cells, and displaced ^{125}I-GIP binding to β-TC3 cells (Tseng *et al.*, 1996a). However, although *in vitro* GIP 6-30amide exhibits much higher receptor binding affinity and is a more potent inhibitor of GIP stimulated adenylyl cyclase activity than GIP 7-30, it has not so far proven possible to show inhibition of insulin release with either GIP 6-30amide or 7-30

during oral glucose tolerance tests in the rat (Pederson and McIntosh, personal communication). One possible explanation for this discrepancy is that Tseng *et al.* (1996a) assessed the inhibition by GIP 7-30amide of responses to a GIP infusion in the absence of stimulating glucose levels in the anesthetized rat, and of insulin release following a 30 min feeding period, after an overnight fast. Both protocols resulted in low level insulin secretion (Tseng *et al.*, 1996a). It therefore appears that while GIP 7-30 and GIP 6-30amide may be useful for antagonizing GIP action in cell culture and other *in vitro* applications, they are of limited use in examining the *in vivo* actions of GIP, probably due to increased degradation and short half-life in the whole animal.

The observation that N-terminally truncated fragments of GIP could still bind to the receptor, but did not result in activation, suggests that while the binding core is located between residues 6-30, other residues in the N-terminus are critical for receptor activation. This appears to be a common theme for ligands of the secretin/glucagon/VIP receptor family, as the carboxy-terminal region of glucagon (Hruby, 1982), secretin, VIP (Gourlet *et al.*, 1996; Robberecht *et al.*, 1986; Turner et al., 1986), calcitonin, and PTH (Bergwitz *et al.*, 1996; Stroop *et al.*, 1996) have been shown to contain the core sequences critical for binding, while the amino-termini of the ligands are required for receptor activation. Additionally, several N-terminally truncated VIP (Turner *et al.*, 1986), secretin (Konig *et al.*, 1984; Kofod *et al.*, 1991), and PTH/PTHrP (Gardella *et al.*, 1996) fragments have been demonstrated to antagonize the action of these hormones *in vitro*. However, in a similar fashion to GIP 7-30 and GIP 6-30amide, these fragments were of limited use due to their lack of potency (Ulrich *et al.*, 1998).

Recently, the PTH/PTHrP antagonists [Leu^{11}, D-Trp^{12}]hPTHrP-(7-34)amide and [D-Trp^{12}, Tyr^{34}]bPTH-(7-34)amide were demonstrated, using constitutively active mutants of the human PTH/PTHrP receptor (Schipani *et al.*, 1995, 1996), to be the first examples of inverse agonism within the secretin/glucagon/VIP receptor family (Gardella *et al.*, 1996). As discussed in section 1.11.3, GPCRs can exist in either the inactive R or active R* conformation, with receptors in the basal state existing at an equilibrium that favours the inactive R form (Samama *et al.*, 1992; Milligan *et al.*, 1995; Scheer and Cotecchia, 1997). As inverse agonists stabilize the receptor in the inactive R form, they may be of use in the treatment of some disease states resulting from constitutively active receptors, or increased basal receptor sensitivity due to ectopic production and/or overexpression of a receptor.

Given that some forms of food-dependent Cushing's syndrome are characterized by the presence of either ectopic production or overexpression of the GIP receptor in the adrenal (Lacroix *et al.*, 1992; Reznik *et al.*, 1992), a GIP receptor inverse agonist may be useful in treatment of this disease. However, the GIP receptor point mutant generated in these studies, H170R, did not differ from the wt receptor in its affinity for GIP, in basal or stimulated cAMP production (Fig. 36), or in its ability to invoke increases in $[Ca^{2+}]i$ (Appendix B, Fig. A3). It was therefore not possible to determine if any of the peptides found to antagonize GIP-stimulated cAMP production could act as inverse agonists.

Tseng and Lin (1997) recently confirmed that rat receptors containing the H170R mutation, when expressed in human embryonic kidney cells, did not differ from the wild type in affinity or in stimulation of cAMP production. However, mutations of the homologous His residue in the glucagon receptor did result in increased basal cAMP

levels, with a blunted glucagon-stimulated response (Hjorth *et al.*, 1998), while point mutants of both the glucagon and GIP receptors at conserved Thr352 and Thr340 residues, at the base of the 6th transmembrane domain, resulted in receptors with increased basal cAMP production but unaltered ligand-induced cAMP production. This mutation is similar to a second naturally occurring mutation identified originally in the PTH/PTHrP receptor (Schipani *et al.*, 1995, 1996), suggesting that it is located in a region involved in tethering the receptor in an inactive R state, and/or transducing the ligand binding signal within this family of GPCRs. However, it is important to note that while a T340P mutation resulted in constitutively active GIP and PTH/PTHrP receptors, a T352A but not a T352P mutation resulted in constitutive activation of the glucagon receptor, indicating that differences exist in the sequence requirements for this activity in different receptors.

It has been demonstrated that the N-terminal residues His^{1}, Gly^{4}, Phe^{6}, Ile^{7}, Asp^{9}, and the C-terminal residues Phe^{22} and Ile^{23} of GLP-1 are critical for binding (Gallwitz *et al.*, 1994; Adelhorst *et al.*, 1994), while His^{1} (Unson *et al.*, 1991), Phe^{6} (Unson *et al.*, 1993; Azizeh *et al.*, 1997), Asp^{15}, and Tyr^{10} (Azizeh *et al.*, 1996) of glucagon have been demonstrated to be important for receptor binding and activation. Similarly, in the current study, it was found that addition of the hydrophobic amino acids Ile^{7} and Phe^{6}, dramatically increased the binding affinity of GIP fragments 7-30 and 6-30amide for the GIP receptor, when compared to the GIP fragment 10-30 (Fig. 34, Table 4). This region is thought to form a small hydrophobic patch in the three related peptide hormones, and it appears to be required for receptor binding by glucagon (Azizeh *et al.*, 1997), GLP-1 (Gallwitz *et al.*, 1994; Adelhorst *et al.*, 1994) and GIP. Studies directed at examining

GLP-1 and glucagon structure/activity using chimeric peptides indicated that the divergent carboxy-terminal residues of GLP-1 are important for the ligand's ability to discriminate between the GLP-1 and glucagon receptors, while the N-terminal residues of glucagon are important for binding to its receptor (Hjorth *et al.*, 1994). In addition, a chimeric peptide consisting of the N-terminal 14 amino acids of glucagon and the carboxy-terminal 15 residues of GLP-1 bound to both receptors with high affinity (Hjorth *et al.*, 1994). In contrast, substitution of GIP residues at non-conserved positions in the N-terminal 22 amino acids of GLP-1 disrupted binding to the GLP-1 receptor, especially $Tyr^{13} \rightarrow Ala^{13}$ and $Glu^{15} \rightarrow Asp^{15}$, while none of the chimeric peptides bound effectively to the GIP receptor, suggesting that GIP required both N- and C-terminal residues for efficient binding (Gallwitz *et al.*, 1996).

Comparison of the sequences of the three peptides (see Fig. 2) indicates that the central region of GIP (~residues 12-20) has the least identity, further supporting a role for this region in GIP receptor specific interactions. These observations, taken together with data presented in the current Thesis, support the conclusion that N-terminal residues of the peptides in the glucagon/secretin/VIP superfamily are important for receptor recognition and activation. However, for the closely related peptides GIP, glucagon, and GLP-1 divergence of amino acid sequence in different regions of the peptides allows nearly absolute discrimination by the ligands between the three known receptors, but differences in ligand binding domains must also play a part in ligand selectivity.

4.4 EXAMINATION OF AMINO-TERMINAL RESIDUES OF GIP IMPORTANT FOR RECEPTOR ACTIVATION.

The importance of the N-terminal residues His^1 or Tyr^1 has been demonstrated for a number of members of the secretin/glucagon/VIP superfamily. Substitution, truncation, or modification of L-His^1 in analogs of glucagon (Lin *et al.*, 1975; Sueiras-Diaz *et al.*, 1984; McKee *et al.*, 1986; Unson *et al.*, 1991) and GLP-1 (Gefel *et al.*, 1990; Gallwitz *et al.*, 1996) resulted in peptides with some reduction in affinity, but with a much greater reduction in their ability to activate their appropriate receptors (Adelhorst *et al.*, 1994; Unson *et al.*, 1987). Interestingly, substitution of D-Tyr^1 and D-Ala^2 for the endogenous L-isomers in truncated GRH (GRH 1-29) resulted in peptides with increased *in vivo* potency (Lance *et al.*, 1984; Heiman *et al.*, 1984). Important studies by Frohmann *et al.* (1986) indicated that GRH was metabolized to biologically inactive GRH 3-44 both *in vivo* and *in vitro* by the enzyme DP IV (Frohman *et al.*, 1989), and that des-amino-Tyr^1-, D-Tyr^1-, and D-Ala^2-GRH were resistant to cleavage by this enzyme, suggesting that increased biological half-life may play a role in the increased potency previously observed.

It has now been well established that DP IV is involved in the degradation of GIP and GLP-1 to GIP 3-42 and GLP-1 9-36amide (Mentlein *et al.*, 1993b; Kieffer *et al.*, 1995b; Pauly *et al.*, 1997), rendering these fragments non-insulinotropic (Brown *et al.*, 1981; Schmidt *et al.*, 1986a; Suzuki *et al.*, 1989; Gefel *et al.*, 1990). The current studies have shown that more extensive degradation results in peptides, such as GIP 6-30, GIP 7-30 and GIP 15-42, that interact with the GIP receptor but do not activate GIP-receptor mediated cAMP production. However, since these fragments also inhibit GIP-mediated

stimulation of cAMP production, it suggested that the major product of GIP metabolism *in vivo*, GIP 3-42, might also inhibit the action of the intact peptide *in vivo*. Both GIP 3-42 and an analog with the first two amino acids inverted (Ala^1-Tyr^2-GIP) were found to display reduced affinity in competition binding studies and to be devoid of the ability to generate cAMP (Fig. 37) at concentrations as high as 1 μM. As predicted, given the previous N-terminal fragment data, both analogs were capable of inhibiting GIP (1nM)-stimulated cAMP production in the μM range (Fig. 38). The reduced affinity (~16-18 fold) of this fragment compared to that of GIP 6-30amide, suggests that extension of the GIP fragment from Phe^6 to Glu^3 reduced the ability of the fragment to bind to the receptor. Given that Tyr^1 appears to be a critical residue for both binding and receptor activation, the presence of an aromatic hydrophobic residue such as Phe^6 rather than the more hydrophilic Glu^3 may account for the difference in binding especially if, as has been suggested for a number of other member of the glucagon superfamily, the N-terminus associates with regions within the transmembrane regions of the receptor (Turner *et al.*, 1996a, b).

It has recently been suggested that GLP-1 9-36amide may act as an antagonist of the GLP-1 receptor (Grandt *et al.*, 1996; Knudsen and Pridal, 1996). However it is clear from the current studies and that of Knudsen and Pridal (1996) that the reduced affinity of the metabolites, and their relatively low circulating concentrations, would preclude significant inhibition of hormone-receptor interaction *in vivo*.

In order to define the contribution of the N-terminal amino acids for receptor activation further, additional N-terminally modified analogs of GIP were examined. In parallel with these studies, attempts were initiated at developing DPIV-resistant forms of

GIP, that could form the basis for future development of long-acting analogs with equal or greater insulinotropic activity. This involved studying the effect on receptor binding and ability to stimulate cAMP production of changing individually the structure (Ppa^1-GIP 1-30amide), chirality (D-Tyr^1-GIP 1-30amide, D-Ala^2-GIP, D-Ala^2-GIP 1-30, D-Glu^3-GIP 1-30amide), or identity (D-Ala^4-GIP 1-30amide) of the first four amino acids of the GIP molecule. As mentioned in the Results section, all of these peptides, including Ala^1-Tyr^2-GIP and GIP 3-42, but excepting D-Glu^3- and D-Ala^4-GIP 1-30amide, were resistant to DP IV degradation *in vitro* (D.-H. Demuth, personal communication).

D-Ala^2-GIP and the C-terminally truncated D-Ala^2-GIP 1-30amide displayed similar shifts in their affinity (11.52 ± 1.08 nM and 10.26 ± 2.76 nM, respectively) and efficacy (EC_{50}s; 1.78 ± 0.86 nM and 0.68 ± 0.21 nM, respectively) compared to those for GIP (IC_{50}: 3.56 ± 0.81 nM; EC_{50}: 0.25 ± 0.07 nM) in studies with wtGIP-R1 cells (Fig.39, Table 5). A similar small, but significant, decrease was seen in the integrated insulin response to D-Ala^2-GIP compared to the endogenous hormone (154.5 ± 4.9 mU vs. 188.3 ± 6.1 mU, respectively) in the isolated perfused rat pancreas (Fig. 41), suggesting that modifications to the shorter fragment resulting in changes in affinity or potency should accurately reflect changes made to the full length hormone. In contrast, D-Ala^2-GLP-1 had nearly identical affinity and efficacy to GLP-1, when tested on CHO-K1 cells stably expressing the GLP-1 receptor (Fig. 40) and in the isolated perfused rat pancreas (Fig. 42), suggesting that the chirality of Ala^2 is less important for receptor recognition and activation than with GIP.

Similar to studies on the importance of His^1 in glucagon (Sueiras-Diaz *et al.*, 1984; McKee *et al.*, 1986), or Tyr^1 in GRH (Lance *et al.*, 1984; Heiman *et al.*, 1984),

modification of Tyr^1 in GIP resulted in variable changes in binding and signalling, depending on the modification made. The substitution of des-aminoTyr (Ppa^1) in GIP 1-30amide led to no change in receptor affinity but resulted in a 3- to 4-fold shift in efficacy (Fig. 43, Table 5), while D-Tyr^1-GIP 1-30amide displayed an approximate 8-fold decrease in receptor affinity which was associated with a surprising 55-fold reduction in its EC_{50} value in comparison to GIP (Fig. 43; Table 5). The smaller shift in efficacy seen for Ppa^1-GIP 1-30amide, compared to that seen for D-Tyr^1-GIP 1-30amide, suggests that the chirality of the Tyr^1 is more important than the protonated amide group for receptor activation.

The substitution of L-Ala^3 with D-Glu^3 or L-Gly^4 with D-Ala^4 in GIP 1-30amide did not result in analogs that were resistant to DP IV hydrolysis (D-H Demuth, personal communication). D-Glu^3-GIP 1-30amide did not differ significantly from GIP in affinity (IC_{50}s: 3.84 ± 0.55 nM vs. 3.56 ± 0.81 nM, respectively) or efficacy (EC_{50}s: 0.248 ± 0.068 nM vs. 0.469 ± 0.126 nM, respectively) (Fig. 43, Table 5). The glycine at position 4 is completely conserved in glucagon, GLP-1, and most other members of the glucagon superfamily, and introduction of D-Ala^4 into GIP 1-30amide resulted in an 8-fold decrease in affinity, similar to that seen for D-Tyr^1-GIP 1-30amide, and a 639- fold decrease in EC_{50} value compared to those of GIP (Fig. 43: Table 5). D-Ala^4-GIP 1-30amide was also the only peptide tested that differed from GIP in maximal cAMP levels achieved (50.9 ± 7.6% of GIP max.) in concentration-response studies (Fig. 43, Table 5), suggesting that addition of even the small space filling methyl group/and or altering the chirality of alanine resulted in some steric hindrance to binding, and greatly disrupted structures required for receptor activation. Given that substitution of L-Ala at the

conserved position in GLP-1 resulted in a similar decrease in affinity and potency it seems likely that a conformational change rather than the chirality is responsible (Gallwitz *et al.*, 1994; Adelhorst *et al.*, 1994).

These studies indicate that DP IV resistant forms of GIP can be synthesized that are of high (Ppa^1-GIP 1-30amide) or slightly decreased (D-Ala^2-GIP, D-Ala^2-GIP 1-30amide) affinity and efficacy. Recent studies have indicated that similar modifications of Ala^2 of GLP-1 with Ser, Gly, Thr or alpha-aminoisobutyric acid, resulted in DP IV-resistant GLP-1 analogs that displayed similar shifts in receptor affinity and stimulatory activity in the perfused porcine pancreas when compared to GLP-1 (Deacon *et al.*, 1998a). Initial studies by Pederson (personal communication) have failed to demonstrate that D-Ala^2-GIP, D-Ala^2-GIP 1-30amide, and D-Ala^2-GLP-1 display any increased insulinotropic ability *in vivo* when compared to the endogenous peptides. This may be due to the fact that DP IV resistant analogs of GIP, like those of GLP-1 (Deacon *et al.*, 1998a), have increased N-terminal stability but are still cleared by other mechanisms such as renal metabolism (Deacon *et al.*, 1996) or degradation by other proteases to fragments non-detectable by RIA (Deacon *et al.*, 1998a). Interestingly, the first pharmaceutical reports of combined DP IV-resistant and fatty-acylated forms of GLP-1 have just appeared (Clodfelter *et al.*, 1998). Acylation was reported to decrease the plasma clearance of the peptide, and the DP IV stability extends its activity, resulting in increased biological activity *in vivo* (Clodfelter *et al.*, 1998). Whether similar modifications of GIP can result in increased biological activity remains to be determined.

4.5 LOCALIZATION OF GIP RECEPTOR REGIONS IMPORTANT FOR LIGAND BINDING

A chimeric receptor approach has been shown to be useful for delineating regions important for ligand binding and signal-transduction (Strader *et al.*, 1994; Ulrich *et al.*, 1998). As discussed in section 1.11.1, from previous studies on GPCRs it has been concluded that ligand binding involves the extracellular NT region of glycoprotein receptors (reviewed in Combarnous, 1992) and TM domains for cationic amines and small neuropeptides (Strader *et al.*, 1994). Peptides of the secretin-glucagon family are probably too large to be completely accommodated by a transmembrane pocket since their structures are predicted to fold into a relatively large one or two-helical conformation. In recent years significant evidence has appeared implicating the large cysteine rich, and therefore presumably highly folded, extracellular NT domain as a likely ligand binding target for members of the secretin-glucagon family of receptors (Holtmann *et al.*, 1995; Gourlet *et al.*, 1996; Stroop *et al.*, 1995; Bergwitz *et al.*, 1996; Buggy *et al.* 1995, 1996; Carruthers *et al.*, 1994; Unson *et al.*, 1995, 1996; Wilman *et al.*, 1996). Most of this information has been concerned with the receptors for secretin, VIP, CT, and PTH/PTHrP. Much less is known concerning the binding of GIP, GLP-1, and glucagon to their receptors, and the current studies were designed to define more closely regions important for binding and signaling of GIP and GLP-1.

Chimeras, consisting of portions of the GIP and GLP-1 receptors, which share approximately 40% overall sequence identity (~35% in the NT) (Usdin *et al.*, 1993), and appear to signal via identical intracellular mechanisms (Usdin *et al.*, 1993; Wheeler *et al.*, 1995; Thorens *et al.*, 1992; Lu *et al.*, 1993a; Gromada *et al.*, 1998b), were utilized to

define regions involved in ligand binding. One important finding of the present study is that expression of the first 132 N-terminal residues of the GIP receptor on the body of the GLP-1 receptor (CH-2) bound ^{125}I-GIP with slightly reduced affinity in COS-7 cells (IC_{50}s: 31.7 ± 8.18 nM (CH-2) vs. 6.42 ± 1.22 nM (wtGIP-R1)) and CHO-K1 cells (IC_{50}s: 27.8 ± 11.9 nM (CH-2) vs. 1.33 ± 0.19 nM (wtGIP-R1)). Extension of the GIP receptor to include the first 222 amino acids, consisting of the NT, EC-1, TM-1 and -2, and part of TM-3 (CH-4) was sufficient for full ligand binding when expressed in COS-7 cells, or with a slight decrease in affinity seen in the more sensitive CHO-K1 cell line (Fig. 47, Table 6). In contrast, in cAMP studies, GIP-stimulated cAMP production was only observed in cells expressing CH-4, suggesting that while the N-terminus of the GIP receptor was sufficient, when expressed on the body of the GLP-1 receptor, to bind GIP, additional receptor regions, critical to receptor activation, existed within the region of TM-1 to TM-3.

To address this question, a number of other chimeric receptors, consisting of further C-terminal extensions, were constructed (CH-3, CH-9, and CH-7; See Fig. 44), however only CH-3, which extended the N-terminus an additional 19 amino acids into the first TM-domain, displayed ^{125}I-GIP specific binding with an affinity equal to that seen for CH-4 in CHO-K1 cells (IC_{50}s: 9.04 ± 1.07 nM (CH-3) vs. 8.33 ± 0.14 nM (CH-4)) (Fig. 45; Table 6). Addition of these 17 amino acids, putatively of TM-1, also restored cAMP responsiveness to the chimeric receptor, indicating that the first transmembrane region of the GIP receptor is important both for GIP binding and receptor activation.

All the chimeras that displayed GIP-specific binding and cAMP production, exhibited decreased expression levels (Table 6) and, predictably, lower maximal cAMP

production (Fig. 47, Table 7). Indeed the extension of the CH-3 by an additional 8 amino acids (CH-9) or into TM-2 (CH-7) resulted in receptors that did not appear to be expressed. However without GIP or GLP-1 receptor specific antibodies it was not possible to determine if the receptors were inactive, or just not expressed at the cell membrane. It is difficult to ascertain why these hybrids were expressed less efficiently than the wt receptor, however it is suggested that the most likely explanation is less efficient processing, since receptor binding affinities and, in the case of CH-4, its EC_{50} value were not dramatically altered.

While all chimeric receptors did not display detectable ^{125}I-GLP-1 (Fig. 46, Table 6), CH-2 and CH-3 both responded with increases in cAMP levels when stimulated with GLP-1 (Fig. 47, Table 7). Surprisingly, maximal cAMP production in COS-7 cells was the same as that seen for cells expressing the wtGLP-1 receptor. In contrast, GLP-1 stimulation in CHO-K1 cell lines expressing CH-2 and CH-3 resulted in maximal cAMP levels that were 30.5 ± 2.0 % and 13.2 ± 1.9 %, respectively, of those seen in the wtGLP-1 cell line. In addition, there is no obvious explanation for the apparent higher efficacy of GLP-1 with CH-2 (81.4 ± 19.6 nM) than CH-3 receptors, which are expressed at a higher level and consist of less of the GLP-1 receptor body. However, despite these discrepancies it is apparent that the body of the receptor is capable of interacting with GLP-1 and stimulating cAMP accumulation.

The observation that none of the chimeras consisting of GLP-1 N-terminal regions expressed on the C-terminal GIP receptor (CH-5, CH-6, and CH-8) bound or signaled in response to GIP or GLP-1 may also have been due to inefficient processing. Alternatively, binding of GLP-1 and glucagon may be more complex than that for GIP

and have absolute requirements for multiple binding regions. Observations that glucagon/Calcitonin (Cal) (Stroop *et al.*, 1995, 1996), and GLP-1(NT)/glucagon (Buggy *et al.*, 1995) receptor chimeras have been generated that are expressed at the cell surface, but have disrupted ligand binding, suggest that it is possible that GLP-1 binding was disrupted in all constructs examined in the current studies. Again, without receptor antibodies this remains to be determined.

Importantly, the studies described here show that the GIP receptor NT demonstrates high affinity binding of GIP in the presence of a heterologous first extracellular loop, and that the distal part of NT and TM-1 appear to be important for cAMP activation of both the GIP and GLP-1 receptors. The reduced binding affinity seen for GIP with CH-2 compared to CH-4 suggests that the first EC loop is involved in ligand binding and that TM-domains 1-3 may also be involved. Additionally, the reduction in affinity observed in CHO-K1 cells expressing CH-4, compared to the wt receptor, suggest that regions in the CT of the GIP receptor, most likely EC-loop 3, and/or TM-domains 4, 5, 6 and 7 may contribute to ligand binding. The importance of the NT and first extracellular loop for binding is similar to the glucagon receptor, as deduced from studies on deletion/truncation mutants (Carruthers *et al.*, 1994; Unson *et al.*, 1995), and GLP-1/glucagon receptor chimeras (Buggy *et al.*, 1995). However, in addition to the NT-domain and first extracellular loop, the third EC-loop has also been implicated in ligand binding of the PTH/PTHrP (Lee *et al.*, 1994) and PTH2 (Clark *et al.*, 1998), and glucagon (Unson *et al.*, 1995; Buggy *et al.*, 1995) receptors.

There are both similarities and major differences between the NT requirements for the GIP/GLP-1 receptor sub-group and other members of the secretin-glucagon family.

Holtmann *et al.* (1995) showed that secretin-VIP receptor chimeras, consisting of the complete NT of the secretin receptor and the C-terminus of the VIP receptor, exhibited low affinity responses to secretin, whereas this region of the GIP receptor expressed on the GLP-1R C-terminus was capable of ligand binding with an affinity similar to that of the wt receptor, albeit with a significantly reduced level of binding.

The requirement of the GIP receptor for the NT, EC-1, and associated TM domains, for maximal signaling is similar to the secretin receptor, whereas only the extracellular NT was required for high affinity binding and signal-transduction in the VIP receptor. Similar studies with Cal/Glucagon (GR) receptors revealed that expression of the N-terminal region of the Cal receptor on the GR receptor body bound Cal with a slight reduction in affinity (12 nM vs 0.3 nM) compared to the wt Cal receptor, but did not signal in response to glucagon or Cal (Stroop *et al.*, 1995), similar to the chimera CH-2 described in this Thesis. In contrast, chimeras consisting of the GR N-terminus and Cal receptor body did not bind detectable amounts of ^{125}I-glucagon or ^{125}I-Cal, but did respond with increased cAMP levels when stimulated with Cal, in a manner similar to CH-2 when stimulated with GLP-1 (Stroop *et al.*, 1995).

The observation that the GR N-terminus lacked binding ability when expressed on the Cal receptor body, and the fact that others have demonstrated that the glucagon receptor can be expressed with its N-terminus deleted, suggests that glucagon (Unson *et al.*, 1995; Hjorth *et al.*, 1998) requires multiple regions, both N-terminal and more distal, to efficiently bind and signal. However the GLP-1 receptor body, like that of the Cal receptor, appears to act as a low affinity binding site that can be activated by its ligand. The inability to determine if CH-5 (GLP-1 NT/GIP receptor body) was expressed or not

prevents conclusions regarding the similarity of the involvement of the GIP receptor body in binding and signaling to the Cal and GLP-1 receptors or to that of the glucagon receptor.

The complexity of the phenotypes seen with the different chimeric receptors argues against a single model of receptor binding and activation. However the most interesting observation here was that extension of the GIP extracellular N-terminus by 19 amino acids into the TM-1 (CH-3) restored GIP-dependent cAMP responsiveness, but did not disrupt GLP-1 signaling. In addition, extension of the GIP receptor sequence to include TM-2, EC-1, and part of TM-3, restored the absolute GIP specificity of the chimera CH-4. This suggests that the first 2/3 of TM-1 of the GLP-1 receptor, or the two amino acids predicted to exist at the membrane border, in some way act as a selective filter, discriminating between GIP and GLP, while the GIP specific filter exists somewhere in the bottom of TM-1, TM-2, EC-1, and possibly TM-3.

Recently, the second transmembrane domain of the PTH receptor has been shown to contain a single Ile234 residue that, when changed to the corresponding Asn192 residue in the secretin receptor results, in a receptor that responds to both ligands. The reciprocal substitution in the secretin receptor (Asn192Ile) results in a secretin receptor that can signal in response to both PTH and secretin. In addition, point mutations of charged residues in the TM-domains of the PTH and secretin receptor suggest they are important for receptor activation and ligand binding (Turner *et al.*, 1996a), supporting an argument for TM-domains playing a role in ligand binding and receptor activation by peptide hormones, somewhat like that seen for cationic amines and small neuropeptides

(Strader *et al.*, 1994). Further studies are required to determine if, and exactly what, residue(s) are involved in the proposed GIP and GLP-1 filters.

While no studies reported in the current Thesis examined which specific regions of GIP associate with the different regions of the receptor, studies with hybrid PTH(1-34)/Cal ligands and N-terminal PTH/Cal receptor chimeras indicated that the C-terminal regions of the peptides bind to the N-terminus of their respective receptors, and that the N-terminus of the ligand associates with the body of the receptor (Bergwitz *et al.*, 1996). In addition, Stroop and co-workers (1995) demonstrated, using GR/Cal receptor chimeras, high affinity binding associated with the N-terminus of the receptor and a lower affinity association with the body of the Cal receptor, which helped decrease the off rate and allow activation by Cal. Additional studies using a number of analogs of Cal, showed that the ability of an analog to form an α-helical stretch was positively correlated with the analog's affinity for the NT-Cal/GR chimera (Stroop *et al.*, 1996). While the ability to activate the NT-GR/Cal receptor chimeras was also dependent on the analogs helical nature, conservation of the N-terminal sequence residues 1-6 was also essential for receptor activation (Stroop *et al.*, 1996).

Both Stroop and Co-workers (1996) and Hjorth and Schwartz (1996) have proposed similar models for the calcitonin, GLP-1, glucagon, and other members of the secretin/glucagon/VIP receptor family. In these models the disulfide bonds formed by six conserved Cys residues in the N-terminus form a globular domain that initially binds or captures the ligand with lower affinity. Once tethered, the peptide is brought into close proximity to the external face of the receptor, resulting in high affinity binding and receptor activation. In light of the results from the GIP/GLP-1 receptor chimera and

structure-activity studies examining residues important for GIP receptor activation, it is tempting to speculate that the C-terminal 6-30 amino acids of the GIP molecule interact with the N-terminal region and specific external loops of the receptor, resulting in high affinity binding similar to that of GIP 1-30amide or GIP. The N-terminal 6 residues essential for activation of the receptor could do so by interaction with regions within a binding pocket including TM-1 and, possibly, TM-2 and EC loop 1. A similar model for the binding of GLP-1 to its receptor can also be visualized.

While more detailed receptor mutagenesis, perhaps coupled with hybrid ligand studies, are required to explore this proposed model, the GIP chimeras described in the current Thesis should provide a basis on which to plan future experiments. NT-GR/GIP receptor chimeras may be expressed more efficiently then the NT-GLP-1/GIP receptor chimeras, and thus aid in defining regions of the GIP receptor body important for receptor activation and ligand selectivity.

4.6 EXAMINATION OF THE CARBOXY-TERMINAL TAIL DOMAIN OF THE GIP RECEPTOR

As discussed in section 1.11.2 the intracellular loops of the heptahelical receptors have been implicated in G-protein recognition, coupling and activation (O'Dowd *et al.*, 1988; Wong *et al.*, 1990; Liggett *et al.*, 1991; Hedin *et al.*, 1993; Burstein *et al.*, 1998; Takarh *et al.*, 1996; Huang *et al.*, 1996; Mathi *et al.*, 1997; Heller *et al.*, 1997; Chicchi *et al.*, 1997), but there is no consensus as to the importance of the C-terminal tail with regard to these functions. Indeed, there appears to be considerable variability in the importance of this region among the different G-protein coupled receptor types. For

example, O'Dowd *et al.* (1988) showed that the N-terminus of the C-terminal tail of the human β2-adrenergic receptor was critical for coupling to G protein activation of adenylyl cyclase, whereas shortening of the C-terminus of the avian β-adrenergic receptor resulted in increased basal and agonist-stimulated cyclic AMP production, and reductions in agonist EC_{50} values (Parker and Ross, 1991). There have been few studies on the importance of the C-terminal tail of the secretin-VIP receptor family, and the only relatively consistent finding has been an increase in affinity for agonists with CT-truncated mutants, as reported for the PTH/PTH-RP (Iida-Klein *et al.*, 1995), calcitonin (Findlay *et al.*, 1994), and glucagon (Unson *et al.*, 1995) receptors. In the case of the GIP receptor, removal of up to 50 amino acids from the C-terminal tail had no significant effect on receptor binding affinity (IC_{50}s: 2.73 ± 0.35 nM (wtGIP-R1) vs. 2.16 ± 0.12 nM (GIP-R-405)). This is similar to the human glucagon receptor, for which 62 of the amino acids in the CT were shown not to be required for binding (Buggy *et al.*, 1997).

It is evident from the GIP-induced cyclic AMP responses of the truncation mutants that the majority of the C-terminus of the receptor is not essential for coupling to adenylyl cyclase, since a mutant consisting of as few as thirteen of the sixty-three amino acids was capable of stimulating adenylyl cyclase. The ability to remove a substantial portion of the C-terminal tail while retaining G protein coupling is in agreement with studies on other heptahelical receptors. In similar mutational analysis experiments to those described here, progressive truncations of the C-terminal tail of the opossum (Huang *et al.*, 1995a) and human (Schneider *et al.*, 1994) PTH/PTH-RP receptors resulted in no significant alterations in cyclic AMP production. Similarly, removal of the distal two-thirds of the TSH (Chazenbalk *et al.*, 1990) and luteinizing hormone/chorionic gonadotropin

receptors (Rodriguez *et al.*, 1992) also resulted in no change in ligand activation of adenylyl cyclase. In contrast, C-terminally truncated forms of both the rat PTH/PTHrP (Iida-Klein *et al.*, 1995) and avian β-adrenergic (Parker and Ross, 1991) receptors were found to signal adenylyl cyclase with much higher efficacy than the wt receptor, and evidence was presented suggesting that the C-terminal tail decreases PTH/PTHrP receptor affinity for Gs (Iida-Klein *et al.*, 1995).

The situation with the PTH/PTHrP is probably complicated, however, since pertussis toxin-sensitive inhibitory effects of PTH on adenylyl cyclase were observed only in wt receptors, and it was proposed that the CT plays a crucial role in interactions between receptors and inhibitory G-proteins. In contrast to the PHT/PTHrP receptor, a reduction, rather than an increase, in maximal cAMP production was observed in the current studies with truncated GIP receptors. This can probably be partially explained by reduced plasma membrane expression levels, as discussed further below. Nevertheless, significantly lower EC_{50} values (4-6-fold) were obtained for GIP-R-418 and GIP-R-405 (Table 10).

One possible interpretation of this result is that CT shortening removes specific amino acids that induce less efficient receptor-induced Gs coupling to adenylyl cyclase. The truncated region contains both serine and threonine residues, and phosphorylation could result in such reduced coupling. Although it is not known whether the C-terminus of the GIP receptor is phosphorylated following agonist binding, it seems probable since phosphorylation of the closely related GLP-1 receptor by both protein kinase C and other receptor kinases has been established (Widmann *et al.*, 1997). Another possible explanation, which has been proposed for the GLP-1 receptor, is that over-expression

leads to desensitization of the receptor, a decrease in EC_{50} (Fehmann *et al.*, 1997), and reduced maximal rates of cAMP production. If expression level plays a role with the GIP receptor, then the increase in efficacy observed with GIP-R-418 and GIP-R-405 may have been due to the lower expression levels (Table 9, 10). Indeed in earlier studies using the GIP-R8 clone that expressed the GIP receptor at ~5 % of that seen for wtGIP-R1, EC_{50} values were decreased 7-fold, while maximal cAMP levels were ~10% of that seen for the wt receptor (Table 8).

All receptor truncations, except GIP-R-427+, resulted in some decrease in maximal binding levels. Truncation probably decreases the efficiency of receptor insertion in the plasma membrane, and the decrease in maximal cyclase stimulation with GIP-R-405 reflects this reduced membrane expression. Cells transfected with the truncated mutant GIP-R-400 exhibited neither binding nor an ability to stimulate adenylyl cyclase. Although this could be due to either lack of receptor expression in the plasma membrane, or dramatically reduced agonist binding to an expressed receptor, the former is more likely. There is probably a minimum length for efficient folding of a heptahelical receptor and its translocation from the endoplasmic reticulum and insertion into the plasma membrane. Such a lack of expression explains the inability to detect biological responses with these severely truncated mutants.

Similar suggestions were made to explain the lack of detectable binding with extensively truncated PTH-PTH-RP receptors (Huang *et al.*,1995a) and, more recently, with the human glucagon receptor (Buggy *et al.*, 1997). In the latter study, using similar CT mutation and alanine substitution techniques to those used here, it was shown conclusively that, as with the GIP receptor, the majority of the CT of the glucagon

receptor could be deleted without compromising membrane insertion. However a glucagon receptor mutant (RT410) equivalent, apart from one less amino acid, to GIP-R-400 was shown by immunostaining not to be expressed in the plasma membrane, whereas a further mutant, RT415, almost equivalent to GIP-R-405 was expressed. Unfortunately, the absence of a GIP receptor antibody precluded us from performing similar immunostaining studies to those of Buggy *et al.* (1997).

More recently it has been shown that extension of the rat gonadotropin-releasing hormone receptor (GnRHR) CT, which normally consists of only 2 amino-acids, with an additional 51 amino acids from the catfish (ct) GnRHR CT tail had no effect on binding affinity, but increased the number of the receptor binding sites ~5-fold, and augmented the IP_3 response (Lin *et al.*, 1998). In addition, truncation of the catfish receptor tail-region of these chimeras resulted in decreased expression compared to the full-length chimera.

Taken together, the three studies provide convincing evidence that a C-terminal peptide length of between 10 and 15 amino acids, in the case of the GIP and glucagon receptors, is necessary for membrane expression of this receptor sub-family. In contrast, for some receptors, such as the mammalian GnRHR, as few as ~2 CT amino acids are sufficient for expression. However this may have required changes in mammalian GnRHR expression to correct for the apparent decreases in efficient membrane expression and decreased coupling to some second messenger pathways (Lin *et al.*, 1998).

Interestingly, COS-7 cells expressing the truncated mutants had similar expression levels, but higher maximal cAMP levels when compared to maximal levels seen with the

truncated CHO-K1 cell lines (Table 9, 10). The reason for this is unclear, however it may be that the receptor expression in the COS-7 system is so high that enough spare receptors exist to activate near wt-maximal Gs-mediated adenylyl cyclase activity, even in the case of disrupted coupling. In the case of the CHO-K1 system receptor expression is lower, and therefore more sensitive to both alterations in receptor coupling and changes in receptor levels. For example, GIP-R-427+ was expressed at similar levels to those seen as the wt receptor in both systems. However, while its EC_{50} value did not differ from that seen for the wtGIP-R1 cell line (wtGIP-R1: 69 ± 27 pM vs.GIP-R-427+: 47 ± 20 pM), maximal cAMP levels were significantly decreased in the CHO-K1 cell system.

If the above suggestion is correct, given the relative expression levels (Table 9) of the truncated receptor in the CHO-K1 system, there was a definite decrease in the ability of the receptor to stimulate maximal cAMP levels (Table 10). This suggests that some of the distal 50 amino acids act to couple the GIP receptor in a positive manner to Gs-mediated adenylyl cyclase activity. In the case of GIP-R-427+, the additional six vector-encoding amino acids do not appear to be responsible for the loss of coupling as recent studies with the GIP receptor truncated at 427 using site directed mutagenesis produced a receptor with very similar characteristics to those seen with GIP-R-427+ (M.B. Wheeler and C.H.S. McIntosh, personal communication.). Further, GIP-R-405 was expressed at ~ 30%, and maximal cAMP values were only 8.1 ± 1.9%, of those obtained with the wtGIP-R1 cell line. In contrast, as discussed above, the wtGIP-R8 clone expression levels, and maximal cAMP accumulation, were 4.9% and 9.59 ± 0.29% respectively of wtGIP-R1 levels. These results threfore suggest that GIP-R-427+, GIP-R-418, and GIP-R-405 coupled ~2-, 3-, and 3.5-fold less efficiently to cAMP production, respectively,

assuming that a receptor with a membrane expression level of 10% would be expected to display maximal cAMP levels of at least 10% that of the wt receptor clone.

Two possibilities were considered regarding the structure of the CT necessary for membrane expression. Either the specific sequence R-R-L-R-L, at positions 401-405, was required (O'Dowd *et al.*, 1988; Liggett *et al.*, 1991), or the chain length itself was the determining factor, and the specific amino acids were immaterial. A mutant receptor was therefore prepared which extended the C-terminal chain with five alanines to produce a 405 amino acid protein (GIP-R-400A_5). The level of receptor binding and the maximal level of cAMP production with this receptor mutant were similar to those produced with GIP-R-405. However, there was a marked increase in the EC_{50} value for cAMP production (1163 ± 320 pM) compared to the full-length receptor (69 ± 27 pM) indicating that specific amino acids within the 400-405 region influence the efficacy of G protein coupling.

Attempts were made to define the importance of the membrane proximal region further, but were unsuccessful due to the fact that neither GIP-R-396A_9 nor the deletion mutant GIP-R-ΔQSEI bound ^{125}I-GIP, and therefore their expression or lack thereof could not be determined. However, despite the decrease in EC_{50} observed with GIP-R-400A_5, what is important is that there must be considerable redundancy in the specific amino acids in this region of the CT which can allow G-protein coupling, and the length of the tail appears to be the critical factor.

Since the proximal CT of the glucagon receptor (403-415 NKEVQSELRRRW) is almost identical to that of the GIP receptor (393-405 NKEVQSEIRRLRL) it is likely that a similar level of redundancy exists for this receptor. Specificity of G-protein binding in

the secretin-VIP receptor family probably resides elsewhere within the intracellular domains, and evidence has been presented recently indicating a critical role for a single amino acid (K334) in the N-terminal portion of the IC3 loop of the GLP-1 receptor for efficient coupling to adenylyl cyclase (Takhar *et al.*, 1996). This region is highly conserved among the secretin-VIP family, and will probably prove equally important for GIP receptor activation.

The C-terminal tails of a number of G-protein coupled receptors, including those for yeast α-mating factor (Reneke *et al.*, 1988), calcitonin (Nygard *et al.*, 1997) and GLP-1 (Widmann *et al.*, 1997) have been shown to be phosphorylated on serine and threonine residues, and to be involved in both receptor desensitization and internalization, although the majority of the CT of the β-adrenergic receptor was not required for receptor sequestration (Strader *et al.*, 1987).

Studies on desensitization and internalization of the GIP receptor failed to produce unambiguous evidence for a major role for the CT in these events. In fact we were unable to demonstrate receptor desensitization in either the high level expressing wtGIP-R1 clone (Fig. 55) or the low-level expressing clone wtGIP-R8. We failed to find any major effect of receptor truncation on short-term desensitization of cAMP signaling in response to GIP. A similar observation was also made by Buggy *et al.* (1997) with the homologous glucagon receptor, although homologous and heterologous glucagon receptor desensitization has been demonstrated in hepatocytes (Savage *et al.*, 1995). Additionally, both the human and rat GLP-1 receptor have been shown previously to undergo homologous desensitization in response to receptor phosphorylation (Widmann *et al.*, 1995, 1997). The protocol used by us was similar to that described by Widmann *et al*

(1997) for the GLP-1 receptor, and the CT of the GIP receptor contains serines that could potentially be involved in phosphorylation and subsequent desensitization. However, there is only one putative casein kinase II site, and no consensus sequences for PKA or PKC phosphorylation in the C-terminus (PCGENE).

Other workers have shown that reduced responsiveness to chronic infusion of 10 nM GIP occurs in rats after approximately 4 hours (Tseng *et al.*, 1996b). In expression studies using a β-galactosidase reporter cell line, desensitization of the cAMP response was observed after 16 hours (Tseng *et al.*, 1996b). Incubation periods greater than 2-4 hours were not examined in our studies as the rapid desensitization of the receptor was of initial interest. It is possible that the GIP receptor does not undergo rapid desensitization, either *in vivo* or *in vitro*, and long-term down regulation resulting in loss of membrane localized receptor is the mechanism by which GIP responsiveness is attenuated, in a manner analogous to that seen for the μ-opioid receptor (Pak *et al.*, 1996). Alternatively, it is possible that expression of the GIP receptor in cells in which it is not normally found, such as CHO-K1 cells, may ablate its ability to desensitize homologously, possibly due to lack of the appropriate GRK. Expression of the wt GIP receptor in islet cell lines, as well as determining the GRKs expressed in the specific islet cell types, should help clarify if the GIP receptor, and truncated mutants described here, are susceptible to desensitization, and the cellular components required.

It has been suggested that the maintenance of an insulin response to pharmacological GLP-1 treatment and the loss of GIP responses in NIDDM patients (Holst *et al.*, 1997), may be due to a defect in GIP receptor function or expression (Holst *et al.*, 1997). The delineation of potential pathways of desensitization and down-

regulation of the GIP receptor is therefore important since chronic elevation of GIP levels has been reported in some NIDDM patients (Ebert and Creutzfeldt, 1980; Elahi *et al.*, 1994). Such a condition may lead to long term desensitization (see below), resulting in a decreased GIP responsiveness. One possible scenario is that the GIP receptor does not undergo rapid desensitization but that it is internalized (see below) and cycled in response to ligand activation. Over time this could lead to GIP receptor down-regulation at the protein level due to increased receptor uptake, degradation, and/or decreased receptor expression. In contrast, GLP-1 receptors, which are rapidly desensitized, may have reduced overall receptor cycling and degradation. Since both receptors signal via identical second messenger pathways it is conceivable that elevated GIP levels may also lead to heterologous down-regulation of GLP-1 receptors, and be partially responsible for the apparent defect in GLP-1 responsiveness in NIDDM patients.

All CT truncated GIP receptor forms were internalized to a similar maximal level (~60-70%) over time, in response to ligand exposure (~80-100 pM GIP) (Fig. 54, Table 11). However the wt receptor internalization was more rapid at early time points suggesting that truncation may have decreased the rate at which the receptor was internalized. Examination of initial uptake rates from time points 1-10 min revealed small changes in the initial rate of uptake (% Total binding /min), although it is unclear as to whether these would have a major impact on overall responsiveness to GIP (Fig. 54). However it does suggest that either a positive internalization signal exists in the distal 27 amino acids, and/or truncation allows exposure of a negative sequestration signal even when the receptor is unoccupied.

Interestingly, GIP-R-400A_5 in which residues 401-405 were replaced with 5 Ala residues restored the internalization rate to that seen for the wt receptor (Table 11), suggesting that the membrane proximal basic L-R-R-L-R sequence may be acting as a negative internalization signal similar to those described for the PTH/PTHrP and thyrotropin-releasing hormone receptors (Huang *et al.*, 1995b; Petrou *et al.*, 1997). However more detailed analysis of this region is required to confirm if such a sequence exists.

These findings are in contrast to the closely related GLP-1 and glucagon receptors, in which internalization was shown to be very sensitive to removal of CT serine residues (Widmann *et al.*, 1997; Buggy *et al.*, 1997). It is possible that experimental differences explain these divergent results, but it is alternatively possible that other regions of the internal domains of the GIP receptor are involved in the processes leading to internalization and desensitization. Further studies are required to determine the importance of the more membrane proximal residues to desensitization and internalization.

4.7 CONCLUSIONS AND FUTURE STUDIES

The cloning of the cDNA and gene for the GIP receptor has expanded the horizons of incretin research. The involvement of GIP/and or its receptor in NIDDM has long been speculated upon (Pederson *et al.*, 1993; Crueutzfeldt and Ebert, 1993; Holst *et al.*, 1997), and the studies described here are the first to examine the structure-function relationships of both GIP and its cloned receptor in isolation from other complicating factors present in cell lines and whole animal bioassays. These findings are important if

the intelligent design of therapeutic analogs of GIP for the treatment of NIDDM and other pathophysiological states, such as food-dependent Cushings disease (Lacroix *et al.*, 1992; Reznik *et al.*, 1992) are to be undertaken. Importantly these studies have shown:

- A cDNA isolated from pancreatic islets was shown to encode a cDNA identical to that initially isolated by Usdin and co-workers (1993) from the CNS and a pluripotent β-cell line cDNA library, establishing that GIP receptors encoded by these different cell types are products of the same gene. A fact that has been further confirmed by the cloning of the human homologue from a human islet cDNA library (Gremlich et al., 1995).

- Heterologous expression of the GIP-R1 cDNA in COS-7 and CHO-K1 cells resulted in both ^{125}I-GIP specific binding and GIP-stimulated cAMP production with IC_{50} and EC_{50} values comparable to those observed by others in cell lines and *in vivo*. Surprisingly, GIP stimulated an increase in $[Ca^{2+}]i$ in COS-7 cells and not in CHO-K1 cells, suggesting the cellular environment in which the receptor is expressed may effect the signal transduction pathways activated. GIP-stimulated changes in $[Ca^{2+}]i$ in COS-7 cells were biphasic with a rapid thapsigargin sensitive peak, resulting from release from intracellular stores, followed by a lower level extended phase that was due to influx of extracellular Ca^{2+} that was not via VDCCs. The exact nature of this influx remains to be determined, however it may be that a nonspecific cation channel similar to that described for rat β-cells and some β-cell lines (Holz *et al.*, 1995; Kato *et al.*, 1996) is involved.

- Synthetic preparations of porcine and human GIP displayed similar affinity and efficacy with cloned rat islet GIP receptors. In addition, all peptide preparations displayed similar potency in the isolated perfused rat pancreas, unlike a previous shGIP preparation (Jia *et al.*, 1995). While these data cannot confirm that much of the controversy concerning the potency of GIP in different systems is due to the heterogeneity of GIP preparations, the wtGIP-R1 cell line provides a quick and easy bioassay to examine preparations of GIP. Indeed it was this assay that allowed us to determine that one batch of shGIP was devoid of biological activity. It was later determined via N-terminal sequencing and MALDI-TOF spectrometry that this preparation had the first two amino acids reversed (Ala^1-Tyr^2-GIP).

- It was demonstrated using the high level GIP receptor expressing CHO-K1 cell line, wtGIP-R1, that the majority of the residues of GIP required for high affinity binding and receptor activation reside within the region 1-30. In addition it was demonstrated that N-terminal truncation of this fragment resulted in peptides with reduced binding affinity, and antagonist activity. One fragment, GIP 6-30amide, displayed equal affinity to GIP1-42 for the GIP receptor, but did not activate the GIP receptor at concentrations as high as 10 μM. While GIP 6-30amide proved to be potent antagonist of GIP *in vitro*, it did not display any antagonist activity *in vivo* (R.A. Pederson, personal communication).

- Modification of the N-terminal residues of GIP, to render the peptide DP IV resistant, had varying effects on both receptor affinity and ability to activate adenylyl cyclase. Importantly, the modification of Tyr^1 and Ala^2 to Ppa^1 and D-Ala^2, respectively, demonstrate that it is possible to design GIP analogs resistant to DP IV degradation with only small effects on affinity and efficacy. Furthermore, modifying the stereochemistry of Tyr^1 (D-Tyr^1-GIP 1-30amide) and the sequence of Gly^4 (D-Ala^4-GIP 1-30amide) had the greatest effect on affinity and efficacy at the GIP receptor. This suggests that Tyr^1 of GIP is involved in a stereospecific interaction with the receptor that is important to receptor activation, while substitution of the highly conserved Gly^4 must also disrupt the ability of the N-terminus of the GIP molecule to assume an active conformation. These observations, along with the N-terminal truncation data indicate that the central, putatively α-helical region of GIP, is important for receptor binding while the N-terminal 4-6 amino acids are important for both binding and receptor activation. This appears to be part of a conserved structural architecture of members of the glucagon superfamily (Bergwitz *et al.*, 1996; Stroop *et al.*, 1996; Hjorth and Schwartz, 1996; Ulrich *et al.*, 1998).

- Similar to other members of the secretin/glucagon/VIP receptor family (Holtmann *et al.*, 1995; Gourlet *et al.*, 1996; Turner *et al.*, 1996a; Stroop *et al.*, 1995; Bergwitz *et al.*, 1996; Wilman *et al.*, 1996), the N-terminus of the GIP receptor appears to contain the majority of the structural requirements for high affinity GIP binding. Interestingly while GIP did not stimulate cAMP production of one chimeric receptor consisting of the N-terminus of the GIP receptor when expressed on the body of the GLP-1

receptor, GLP-1, which did not appear to bind, was able to stimulate cAMP production. Extension of the EC N-terminus of the GIP receptor by 19 amino acids into the first TM domain partially restored GIP responsiveness and did not affect GLP-1 activation. Further extension of the GIP receptor protein to TM-3 further restored cAMP production in response to GIP and ablated any responsiveness to GLP-1. This suggests that regions important to GIP receptor activation exist in the first 2/3 of the TM-domain. Additionally this region of the GLP-1 receptor appears to contain a ligand-specific "filter" sequence, as has been describe for the PTH/PTHrP and secretin receptors (Turner *et al.*, 1996b). Additional regions of the GIP receptor, which may include the remainder of TM-1, TM-2, EC loop 1, and possibly TM-3, appear to be required to maintain the GIP-specific selectivity of the receptor.

- It was found that the majority of the CT-tail of the GIP receptor can be truncated with no effect on receptor affinity, and only minor effects on receptor coupling to cAMP generation and receptor internalization. However, truncation of the CT tail by greater than 27 specific amino acids resulted in reduced expression levels at the plasma membrane as determined from Bmax levels obtained in competition binding studies. This observation is similar to that seen for the glucagon (Buggy *et al.*, 1997), PTH (Huang *et al.*, 1995a) and CT (Findlay *et al.*, 1994) receptors, although different effects were observed on receptor affinity and G-protein coupling. Interestingly it was not possible to demonstrate homologous desensitization of the wt GIP receptor in the CHO-K1 cell system, suggesting that the GIP receptor does not undergo rapid homologous desensitization or at least not in the CHO-K1 cell system employed.

The described studies provide a basis on which to develop further research on important aspects of the physiology of GIP and its receptor. Areas in which they are likely to have an important impact and further developments that are likely to arise are outlined below.

The transfected cell lines can clarify the confusion in the literature over human responsiveness to synthetic human GIP preparations. It had been noted previously that some shGIP preparations exhibited peptide heterogeneity and low biological activity (Jia *et al.*, 1995), and this may explain some of the variability in responsiveness of NIDDM patients reported. It is therefore essential that clinical studies using synthetic preparations of GIP are repeated, both in healthy individuals and individuals with NIDDM, with well standardized, biologically active peptide, to establish whether GIP or GIP analogs are potential therapeutic agents in the treatment of some patients with NIDDM. Since both shGIP and spGIP were equipotent in binding and stimulating the heterologously expressed rat islet GIP receptor this system is appropriate for screening different GIP preparations.

Knowledge of the minimal structural requirements of high affinity GIP binding of the GIP antagonist, GIP 6-30amide and N-terminal sequence requirements provides some clues as to structural motifs that theraputic analogs will have to maintain to conserve binding affinity and/or receptor activation. Interestingly, although GIP 6-30amide was found to be a potent antagonist, and the analogs D-Ala^2-GIP and D-Ala^2-GIP 1-30amide were DP IV-resistant, *in vitro*, neither antagonism of GIP action nor prolonged biological activity, respectively, were observed *in vivo*. This suggests that other considerations such

as systemic half-life due to renal clearance (Deacon *et al.*, 1996) will have to be considered.

The recent report that a DP IV-resistant analog of GLP-1, with the addition of a fatty acyl-chain, had improved biological activity (Clodfelter *et al.*, 1998), suggests that similar modification of the DP IV resistant GIP analogs and the high affinity antagonist GIP 6-30amide may improve activities of these peptides *in vivo* by increasing their half life. Another exciting possible therapeutic strategy is the use of DP IV antagonists to prolong the biological half-life of endogenously released GIP and GLP-1, a strategy that has been shown to inhibit GIP and GLP-1 degradation (Kieffer *et al.*, 1995b; Pauly *et al.*, 1996; Deacon *et al.*, 1998b) and improve glucose tolerance in the rats (Pauly *et al.*, 1998).

The major drawback of using peptide based analogs to treat any pathology is that they require some form of invasive application, although there are reports of using a GLP-1 buccal tablet (Gutnaik *et al.*, 1996, 1997), and other investigators have suggested that microencapsulation may prove to be suitable for administration of GLP-1 analogs (Nauck *et al.*, 1997a). In the case of NIDDM, an analog, most likely non-peptide in nature, with a long biological half life that could be taken orally and that acts via the GIP or GLP-1 receptors would be preferred over the more complicated and expensive peptide formulation. It is therefore important to understand the structural requirements of the peptide critical for receptor activation since this region, or even specific residues within this region, could be targeted. The studies presented here suggest that such analogs should interact with the N-terminus for binding and at least the first TM domain in order to activate the GIP receptor. However, it appears that regions of TM-2, similar to the

PTH and secretin receptors (Turner *et al.*, 1996a, b), may also be involved. Additional screening of individual residues in this region may allow the design of small inorganic GIP-mimics that specifically interact with the activation region of the GIP receptor, and may or may not require interactions with other such regions of the receptor.

Interestingly, the majority of the GIP receptor CT tail could be removed with only small effects on coupling with adenylyl cyclase and receptor internalization, and no effect on receptor affinity. However the membrane proximal region of the tail appears to be important for receptor expression, and may contain residues or sequences that are involved in the inhibition of receptor sequestration. Alanine scanning of this region should help to identify which, if any, of the residues are involved. However it appears that other regions of the GIP receptor are involved in G-protein coupling, similar to those described for the GLP-1 receptor (Takhar *et al.*, 1996; Mathi *et al.*, 1997).

Desensitization of the GIP response was not observed in CHO-K1 cells expressing the wt GIP receptor cDNA. It would be surprising if the GIP receptor was dramatically different from the glucagon and GLP-1 receptors (Widmann *et al.*, 1995, 1997; Savage *et al.*, 1995). Indeed the fact that Buggy and co-workers (1997) did not observe homologous desensitization in response to agonist stimulation of the glucagon receptor expressed in CHO-K1 cells, while its rapid desensitization has been demonstrated in isolated hepatocytes, suggests that both the GIP and glucagon receptors require cellular elements to undergo homologous desensitization that are not present in CHO-K1 cells. However 4 hours of GIP infusion were required to elicit desensitization with *in vivo* studies in rats (Tseng *et al.*, 1996b). This slow time frame of desensitization suggests that *in vivo* desensitization is at least in part due to downregulation of the

receptor at the protein level. Such a response may have important implications in some NIDDM individuals that display elevated GIP levels, and could account for the lack of a GIP response and reduced responsiveness to GLP-1. However such a possibility is just speculative at this point and examination of desensitization in islets and β-cell lines should help to clarify the role of desensitization in GIP receptor physiology.

While the exact role of GIP in NIDDM remains to be determined, available evidence indicates that GIP is the most important incretin, at least in healthy individuals (Holst *et al.*, 1997; Nauck *et al.*, 1997a). It is therefore essential to develop a greater understanding of GIP receptor function, both in healthy and diseased states, and to elucidate further the structure-function requirements of GIP and its receptor, in order to develop GIP analogs that may prove useful in the treatment of NIDDM. It is hoped that the work described in this Thesis will help direct future research into these areas.

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Appendix A.

CYTOSOLIC Ca^{2+} MEASUREMENTS

CUVETTE FLUORIMETRY:

Free cytosolic calcium concentrations $[Ca^{2+}]i$ were determined using a Hitachi F2000 spectrophotometer as described by Nasmith and Grinstein (1997). Briefly, COS-7 cells were loaded in DMEM with 2 μM fura-2 AM (Molecular Probes, Eugene, OR) for 20 min at 37°C. Aliquots of these cell suspensions were washed by sedimentation, and approximately 2.5 x 10^5 cells resuspended in KRPD buffer (140 mM NaCl, 4 mM KCl, 10 mM glucose, 10 mM HEPES, 1 mM MgCl2, with or without 1 mM $CaCl_2$, pH 7.4) and placed into a cuvette (#. 67.775, Sarstedt, Germany) with magnetic stirring in a thermostatically controlled (37°C) chamber. Fluorescence was measured at 37°C with excitation at 335 nm and emission at 510 nm. $[Ca^{2+}]i$ calibration was performed using ionomycin and Mn^{2+} with a Kd of 224 nM and a ratio Fmax/Fmin of 3 as previously determined (Nasmith and Grinstein, 1997).

SINGLE-CELL MICROFLUORIMETRY

A confocal laser scanning microscope (CLSM, Biorad-600) was used to analyze GIP-evoked Ca^{2+} fluxes in individual COS-7 cells transfected with pGIP-R1. Briefly, COS-7 cells were loaded with 10μM INDO-3AM (Molecular probes), for 1hr in KPRD /DMEM V/V, 0.5% DMSO, and 0.01% pluronic acid. Subsequently, cells were washed twice in DMEM and changes in $[Ca^{2+}]i$ analyzed in the presence of 50nM GIP. Fluo-3 AM was excited using the 488 nm line of the argon laser, and emitted fluorescence

detected through a low pass filter with cutoff at 515 nm. Images were collected digitally and a false color scale generated for quantitative purposes, where blue corresponds to lower and red to higher [Ca^{2+}] levels. The changes in fluorescence were measured using CONRAD, a program for PC analysis and the preparation of confocal images, written by T.A. Goldthorpe, Department of Physiology, University of Toronto.

Studies carried out by Dr. Paul E. Squires were essentially carried out as described above for the cuvette studies. Briefly, CHO-K1 or COS-7 cells were loaded with 5 μM fura-2-AM in DMEM at 37°C for 20 min. Individual cells were imaged with an Attofluor™ digital fluorescence microscopy system (Atto Instruments, Rockville, MD). All records were corrected for background fluorescence. In contrast to the cuvette studies, only the relative changes in [Ca^{2+}]i are presented.

RESULTS

EFFECTS OF GIP ON [Ca^{2+}]i

Usdin et al (1993) demonstrated that the RINm5F cell GIP receptor when expressed in a calcium reporter cell line (HEK293 expressing apo-aequorin), yielded an increase in [Ca^{2+}]i in the presence of 100 nM GIP. The present series of experiments were designed to examine the linkage between GIP-R1 and [Ca^{2+}]i. In COS-7 cells expressing GIP-R1 and loaded with the intracellular Ca^{2+} indicator fura 2-AM, 50 nM GIP increased [Ca^{2+}]i with an acute transient phase, followed by a sustained elevation of [Ca^{2+}]i (Fig. A1.A). The net increases in the transient (P1: Δ[Ca^{2+}]i) and sustained phases (P2: Δ[Ca^{2+}]i) were 114±18.1 and 36± 6.1 nM respectively ($n \geq 3$). Δ[Ca^{2+}]i P1 was further shown to be concentration-dependent with net increases of 49 ± 3.8 and 11 ± 4.5 at 5 nM and 0.5 nM

spGIP respectively (n = 3). To determine whether the source of the increased $[Ca^{2+}]i$ elicited by GIP was from an intracellular or extracellular source, the above experiment was repeated first in a nominally Ca^{2+} free environment (Table A1), and then in the presence of 4 mM EGTA (Fig. A2.B). Under both conditions the transient first phase response was reduced but not eliminated (70±7.6 and 40±7.6 nM respectively vs. 114±8.1 nM in controls, $p \leq 0.05$). In contrast, the second phase responses were completely eliminated, in fact spGIP appeared to induce Ca^{2+} efflux from the cell (-10 ± 2.4 and -17 ± 2.4 nM). These data are consistent with the transient increase of $[Ca^{2+}]i$ originating primarily from an intracellular Ca^{2+} pool, and the sustained phase of $[Ca^{2+}]i$ increase resulting from an extracellular source(s).

Treatment	COS-7 Δ $[Ca^{2+}]i$ P1 (nM)	COS-7 Δ $[Ca^{2+}]i$ P2 (nM)
50 nM GIP	114 ± 8.1	36 ± 6.1
50 nM GIP + 10 μM nifedipine	110 ± 5.3	42 ± 2.9
50 nM GIP (Ca2+-free media)	70 ± 7.6	-10 ± 2.4
50 nM GIP + 4 mM EGTA	40 ± 7.6	-17 ± 2.4

Table A1. Effects of spGIP on $[Ca^{2+}]i$ in COS-7 cells expressing GIP-R1. Data presented are the mean ± SEM of at least three individual experiments. P1, peak transient phase; P2, plateau phase.

To characterize further the first phase $[Ca^{2+}]i$ response, cells were exposed to the sarcoplasmic/ endoplasmic reticulum Ca^{2+} ATPase inhibitor thapsigargin in nominally Ca^{2+} free medium (Fig. A2.D). Thapsigargin (50 nM) initially caused an increase in $[Ca^{2+}]i$ followed by a plateau phase suggesting depletion of intracellular Ca^{2+} stores. Subsequent addition of GIP (50 nM) did not elicit an increase in $[Ca^{2+}]i$ strongly suggesting that the P1 response was primarily due to the mobilization of Ca^{2+} from intracellular stores. To characterize the Ca^{2+} entry pathway, COS-7 cells expressing GIP-

R1 were pretreated with the L-type VDCC blocker nifedipine (10 μM) (Fig A1.C). Nifedipine had no effect on either the sustained increase in $[Ca^{2+}]i$ (42±2.9 vs. 36±6.1 nM, $p>0.05$), or the immediate acute rise in $[Ca^{2+}]i$ (110±5.3 vs. 114± 8.1, $p>0.05$) (Table A1). This result is in contrast to previous findings in insulin-secreting HIT cells (Lu et al., 1993) and more recently in the βTC6-F7 cell line (unpublished results) where EGTA (4 mM) pretreatment, or L-type Ca^{2+} channel blockers prevented spGIP induced increases in $[Ca^{2+}]i$. The activation of voltage-sensitive Ca^{2+} channels by spGIP in COS cells was further discounted since KCl, used at a concentration that should depolarize the cell (50 mM), was unable to stimulate Ca^{2+} entry. The P2 response was also not elicited by forskolin (10 μM) or IBMX (data not shown) indicating that spGIP-induced increases in $[Ca^{2+}]i$ were unlikely to be mediated by a protein kinase A (PKA)-mediated pathway.

Confocal microscopy was used to determine the relative number of COS-7 cells responding to spGIP and to examine Ca^{2+} fluxes in individual cells. In cells loaded with fluo 3-AM the majority were observed to have similar resting Ca^{2+} fluorescence levels (Fig. A2.B). In response to the addition of 50 nM GIP, approximately 10-60% of cells in any given field showed an increase in Ca^{2+} fluorescence intensity (Fig. A2.C). This percentage is similar to that observed in control transfection experiments using pCMV b-gal (Invitrogen) as a reporter system to assess transfection efficiency. The changes in fluorescence for each cell (indicated by numbers in Fig. A2.B) were normalized to respective control values (DF/F) and plotted against time (Fig. A2.D). The pattern of fluorescence, although similar among cells, varied greatly in overall intensity. When averaged however, the calcium response pattern was remarkably similar to that observed by fluorimetry (Fig. A1.A). That is, a rapid initial phase followed by a sustained second

phase. This is in direct contrast to the observations in CHO-K1 cells expressing GIP-R1. No GIP-stimulated changes in $[Ca^{2+}]i$ were observed in the wtGIP-R1 cell line, suggesting that the cellular environment the receptor is expressed in may determine the signal transduction pathways activated by a receptor. Alternatively extremely high expression levels obtained with the transient COS-7 cell line may be required to activate the Ca^{2+}-signaling pathway.

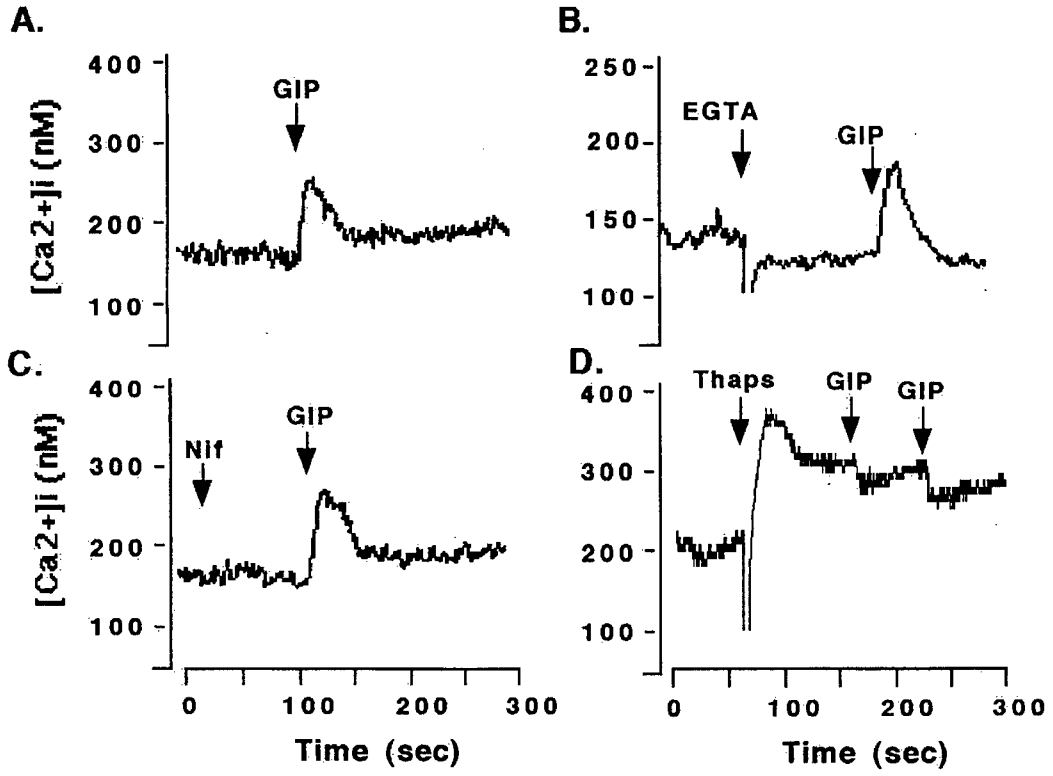

Figure A1. Effects of spGIP on $[Ca^{2+}]_i$ in suspensions of COS-7 cells. (A) The effect of spGIP on $[Ca^{2+}]_i$ was measured in COS-7 cell suspensions 72 h post-transfection with pGIP-R1. the cells were loaded with fura-2 and then spGIP (50nM) was added at time point indicated by the arrow. In control experiments GIP was unable to evoke a $[Ca^{2+}]_i$ response in cells expressing the GLP-1 receptor under identical conditions (not shown). (B) to determine the source of the spGIP-induced increase in $[Ca^{2+}]_i$, GIP-R1 transfected COS-7 cells were pre incubated in 4mM EGTA and stimulated with 50mM spGIP. (C) Alternatively, the cells were pretreated with 10μM nifedipine or (D) the Ca^{2+}-ATPase inhibitor, thapsigargin (50nM). tracings are representative of at least three independent experiments.

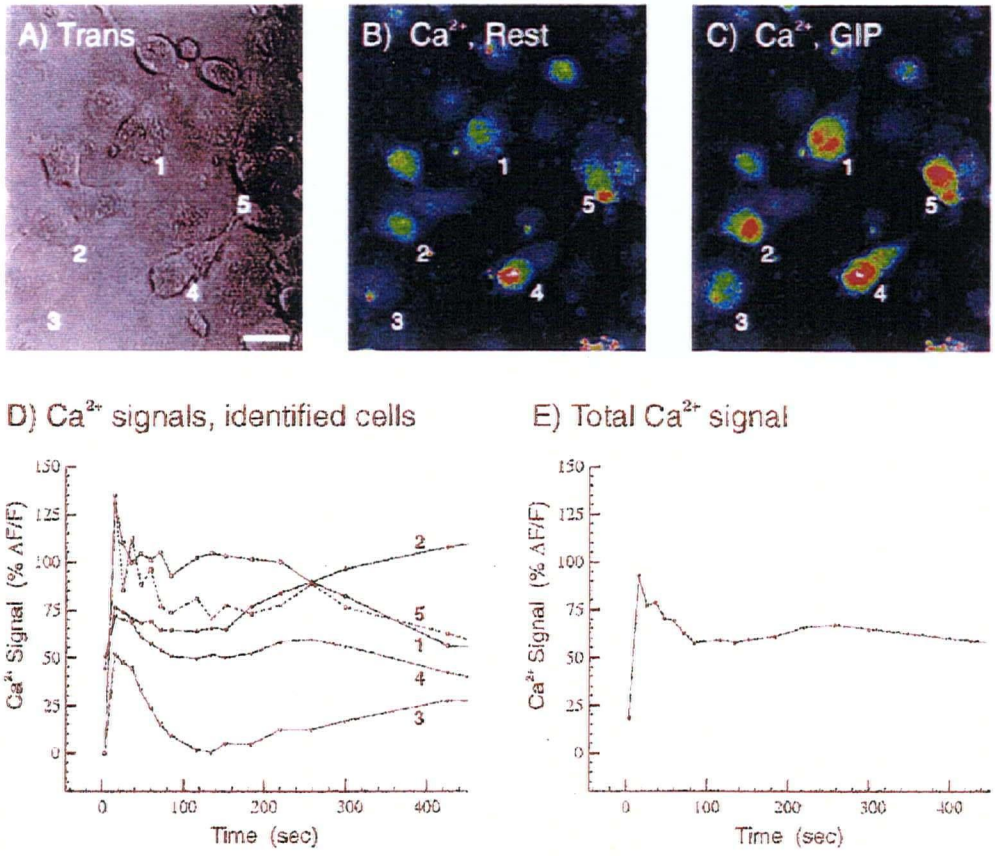

Figure A2. Effect of spGIP on $[Ca^{2+}]_i$ in individual COS-7 cells. Confocal microscopy was employed to determine the pattern of changes in $[Ca^{2+}]_i$. **(A)** Nonconfocal image acquired using the Bio-Rad transmitted light attachment showing COS-7 cells transfected with GIP-R1. Scale bar, 50 μm. **(B)** Confocal image of the same cells as in (A) loaded with fluo-3 AM, showing resting Ca^{2+} levels. Relative fluorescence appears in colour scale, with blue representing lower and white representing higher Ca^{2+} fluorescence. **(C)** In response to 50nM spGIP, fluorescence was followed in five identified responding cells (1-5). **(D)** Changes in Ca^{2+} fluorescence, normalized to resting fluorescence (%ΔF/F) for each cell, were analyzed over time from the identified cells (1-5). GIP was added at time zero. **(E)** The Ca^{2+} signals for cells 1-5 were averaged to show the Ca^{2+} signal in a population.

THE EFFECTS OF THE H170R POINT MUTATION ON GIP-MEDIATED $[Ca^{2+}]i$ SIGNALING.

Responses of COS-7 cells expressing the GIP-R1 cDNA to 10 nM GIP were similar to those originally obtained using single cell fluorimetry except the onset of the response was slightly delayed and the secondary phase appeared to have an decreased duration (Fig. A3.A). COS-7 cells expressing the point GIP receptor point mutant H170R did not appear to differ in their Ca^{2+}-responsiveness compared to cell expressing the wt GIP receptor cDNA (Fig. A3.B). It was concluded that the substitution of His 17s0 with Arg did not affect the receptor's ability to couple to changes in $[Ca^{2+}]i$.

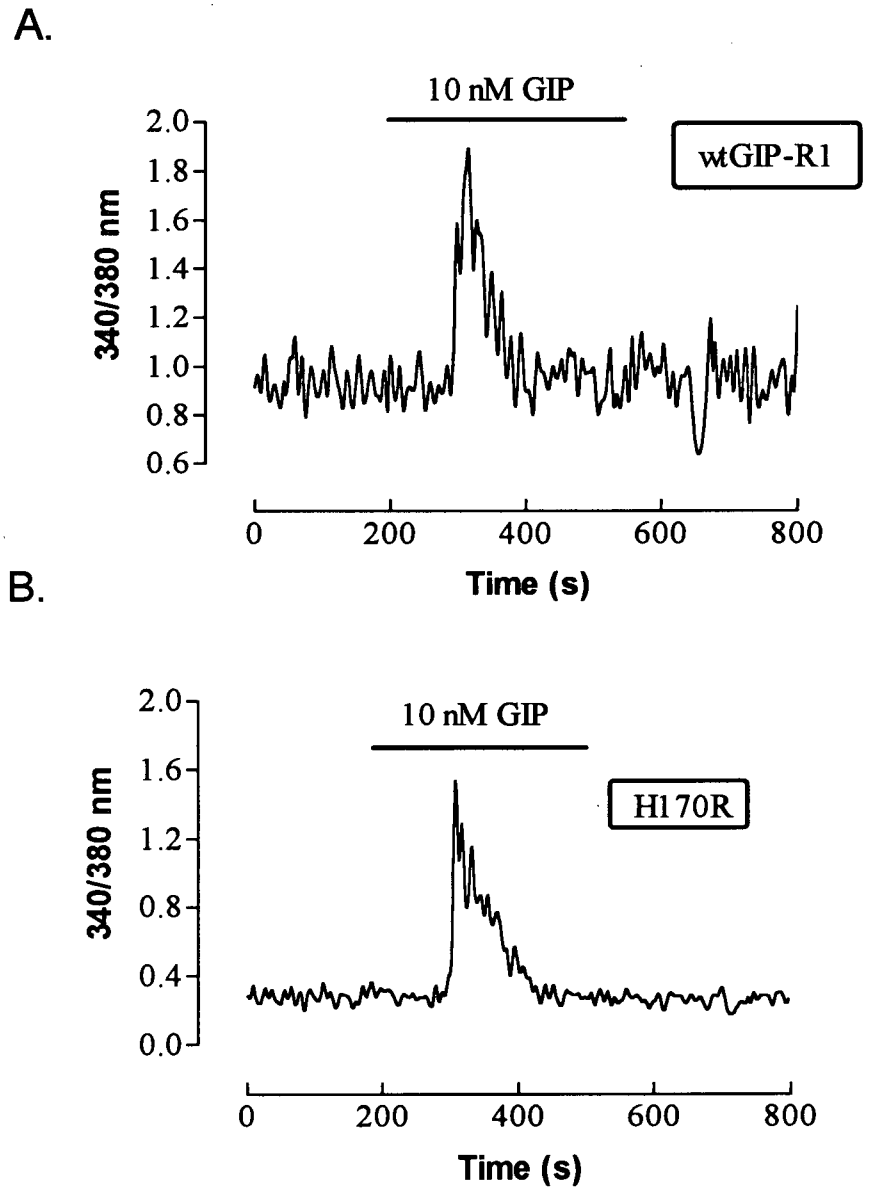

Fig. A3. Changes in $[Ca^{2+}]i$ in Response to 10 nM GIP Treatment in COS-7 Cells Expressing GIP-R1(A) or H170R (B). 10 nM GIP stimulated rapid $[Ca^{2+}]i$ transients followed by slower secondary decay in $[Ca^{2+}]i$. Experiments are representative of 3 individual transfections. The number of cells responding on a given coverslip varied from 5-60%.