ADIPOSE TISSUE EXPRESSION, ALTERNATIVE SPLICING, DESENSITIZATION AND INTERNALIZATION OF THE GLUCOSE-DEPENDENT INSULINOTROPIC POLYPEPTIDE RECEPTOR

by

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ABSTRACT

Glucose dependent insulinotropic polypeptide (GIP) is a gut hormone which plays an important role as an incretin. Thus, the GIP receptor, a member of the G-protein coupled receptor (GPCR) family, is expressed in the β-cells of the endocrine pancreas. However, it is also expressed in other tissues, including adipose tissue, where GIP exerts effects on fat metabolism. Non-insulin dependent diabetes mellitus (NIDDM), a disease characterized by impaired insulin secretion and insulin resistance, is associated with obesity. Therefore, the possibility of altered GIP receptor expression was examined in the Zucker fatty (*fa/fa*) rat, a model of obesity.

In contrast to previous studies, 11 week old obese Zucker rats did not show higher plasma glucose concentrations in response to oral glucose than did their lean littermates, but this is probably due to differences in statistical analysis. However, the 16 week old obese animals showed fasting hyperglycemia, severe glucose intolerance and greater plasma GIP responses to oral glucose than did their lean littermates, suggesting that they had become diabetic. The 11 week old lean and obese Zucker rats displayed nearly identical levels of adipose tissue GIP receptor mRNA, therefore, the adipose cell GIP receptor expression appears not to be involved in the phenotype of the animal at this stage of development.

The GIP receptor is a member of the secretin/VIP subfamily of GPCRs, several of whose members exist in alternatively spliced forms. The presence of the GIP receptor in tissues other than the endocrine pancreas suggests the possibility of different signal transduction pathways. The third intracellular (i3) loop of many GPCRs is important for

G-protein recognition and coupling, so this area of the GIP receptor was examined for alternative splicing.

An alternatively spliced variant of the GIP receptor expressed predominantly in islet tissue was detected and predicted to encode a truncated form of the GIP receptor. A second splice variant expressed predominantly in adipose tissue was predicted to encode a 28 amino acid insert located at the C-terminal end of the i3 loop.

The second and third intracellular loops of the GIP receptor contain several serine and threonine residues. As potential phosphorylation sites, these residues were examined for their ability to mediate receptor desensitization and internalization. The mutation of Ser-248, Ser-320, Thr-324 and Ser-339 to alanines resulted in a reduced ability to stimulate cyclic-adenosine monophosphate (cAMP) production in response to GIP stimulation as compared to the wild type GIP receptor (wtGIPR). The mutation of Thr-342 to alanine resulted in an increased ability to stimulate cAMP production in response to GIP stimulation in low expressing subclones. The wild type GIP receptor did not show significant desensitization in response to preincubation with GIP, so it was not possible to determine the result of mutations on receptor desensitization. GIPR/S248A was the only mutant receptor to show a decrease in internalization rate and maximum internalization as compared to the wtGIPR. Neither GIPR/S320A nor GIPR/T324A showed changes in internalization rate or maximum internalization compared to the wtGIPR, while GIPR/S339A and GIPR/T342A showed an increase in both these parameters. Therefore, Ser-339 and Thr-342 may act as negative regulators of receptor internalization.

The above studies have added to the growing knowledge about GIP's signaling pathways, its role in adipose tissue and its potential role in obesity and NIDDM.

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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*:	
α-adrenergic receptor	α-AR
β-adrenergic receptor	β-AR
Angiotensin	AT
Cholesystokinin	CCK
G-protein-coupled receptors	GPCRs
Gastric Inhibitory Polypeptide/ Glucose-dependent insulinotropic polypeptide	GIP
Glucagon-like peptide-1	GLP-1
Growth hormone	GH
GH-releasing hormone	GHRH
Leptin receptor	Ob-R
Luteinizing hormone	LH
Muscarinic acetylcholine receptor	mAChR
Parathyroid hormone	PTH
Parathyroid related peptide	PTHrP
Pituitary adenylyl cyclase-activating polypeptide	PACAP
Somatostatin	SS
Vasoactive intestinal peptide	VIP

Miscellaneous:	
β-adrenergic receptor kinase	βARK
Amino terminal	NT
Carboxy terminal	CT
Chinese hamster ovary cells	CHO-K1
Complementary DNA	cDNA
Cyclic-adenosine monophosphate	cAMP
Deoxyribonucleic acid	DNA
Dipeptidyl peptidase IV	DP IV
Extracellular	e
GPCR kinase	GRK
GPCR phosphatase	GPCRP
Guanosine triphosphate binding proteins	G-proteins
Fatty acids	FA
Free fatty acids	FFA
High performance liquid chromatography	HPLC
Immunoreactive	IR
Intracellular	i
Intracellular free calcium concentration	$[Ca^{2+}]_i$
3-isobutyl-1-methyl xanthine	IBMX
Lipoprotein lipase	LPL
Non-insulin dependent diabetes mellitus	NIDDM
Radioimmunoassay	RIA
Ribonuclease protection assay	RPA
Protein kinase A	PKA
Protein kinase C	PKC
Reverse transcription – polymerase chain reaction	RT-PCR
Ribonucleic acid	RNA
Sympathetic nervous system	SNS
Transmembrane	TM
Triglycerides	TG
Wild type	wt

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

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Ruth Ellen Gill
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CHAPTER 1

INTRODUCTION

1.1 OVERVIEW

Glucose-dependent Insulinotropic Polypeptide (GIP) or Gastric Inhibitory Polypeptide was initially discovered on the basis of its ability to inhibit gastric acid secretion (Brown and Pederson, 1970). It was later found to have an important role as an incretin, a hormone which potentiates glucose-stimulated insulin secretion in response to nutrients in the gut, when it was shown to stimulate insulin secretion in response to an oral glucose load (Dupré et al. 1973). This makes it an important part of the entero-insular axis, a term originally used by Unger and Eisentraut (1969) to describe the signaling pathways from the gut which affect the endocrine pancreas. These include the nutrient, hormonal and neuronal signals which regulate hormone secretion from the islets of Langerhans.

1.2 DISCOVERY OF GIP

In 1930, Kosaka and Lim introduced the term enterogastrone to describe a gastric inhibitory chemical messenger secreted into the blood from the small intestinal mucosa by fat. They discovered that mucosal extracts were able to inhibit meal- and histamine-stimulated acid secretion in dogs when administered in mg/kg doses. Secretin and cholecystokinin (CCK) were both considered as possible candidates as this enterogastrone. A CCK-free preparation of secretin was shown to inhibit gastrin-stimulated acid secretion (Wormsley and Grossman, 1964) but synthetic secretin was not able to inhibit vagally or histamine-stimulated acid secretion (Vagne et al. 1968). CCK

was shown to inhibit acid secretion in the Heidenhain canine gastric pouch (Gillespie and Grossman, 1964; Brown and Magee, 1967); however, the same preparation also stimulated acid secretion when administered in the fasting state (Magee and Nakamura, 1966; Murat and White, 1966). These preparations were only 10% pure so Brown and Pederson (1970) further purified the CCK to 40%. They found that the gastric inhibitory actions were reduced while the stimulatory action in the fasting state was increased. This led them to postulate that the gastric acid inhibitory activity was removed during further purification. Brown et al. (1969; 1970) purified the active substance from hog duodenal and jejunal mucosal extracts and named it gastric inhibitory polypeptide (Brown, 1971).

1.2.1 SEQUENCE HOMOLOGY OF GIP FROM DIFFERENT SPECIES

GIP is a 42-amino acid peptide which has a high degree of homology among species, suggesting an important regulatory role (Fig. 1). The sequence of porcine GIP was originally published by Brown and Dryburgh (1971) and later corrected by Jörnvall et al. (1981). The sequence of human GIP (Moody et al. 1984) differs from those of the pig and rat at two amino acid positions and from bovine and murine sequences at three positions. The gene encoding preproGIP (Inagaki et al. 1989) shares sequence homology with the glucagon gene family, which has led to speculation that there was a common ancestral gene (Bell, 1986; Campbell and Scanes, 1992).

	1	2	3	4	5	6	7	8	9	10	11
Human	Tyr	Ala	Glu	Gly	Thr	Phe	Ile	Ser	Asp	Tyr	Ser
Porcine											
Bovine											
Rat											
Mouse											

	12	13	14	15	16	17	18	19	20	21	22
Human	Ile	Ala	Met	Asp	Lys	Ile	His	Gln	Gln	Asp	Phe
Porcine							Arg				
Bovine							Arg				
Rat							Arg				
Mouse							Arg				

	23	24	25	26	27	28	29	30	31	32	33
Human	Val	Asn	Trp	Leu	Leu	Ala	Gln	Lys	Gly	Lys	Lys
Porcine											
Bovine											
Rat											
Mouse								Arg			

	34	35	36	37	38	39	40	41	42
Human	Asn	Asp	Trp	Lys	His	Asn	Ile	Thr	Gln
Porcine	Ser								
Bovine	Ser			Ile					
Rat							Leu		
Mouse	Ser								

Fig. 1. GIP Amino Acid Sequences 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).

1.2.2 GIP GENE STRUCTURE AND POSTTRANSLATIONAL PROCESSING

In 1987 the sequence of the human GIP gene was reported by Takeda et al (1987). The complementary DNA (cDNA) sequence indicates that human preproGIP is a protein of 153 amino acids with a predicted molecular weight of 17 107. Takeda et al. (1987) predicted that the signal peptide is cleaved at Gly-21, and that an amino terminal (NT)

peptide of 30 residues and a carboxyl terminal (CT) peptide of 60 residues are released from the precursor molecule, in addition to GIP. Inagaki et al. (1989) later discovered that the human GIP gene contains six exons separated by five introns, human GIP being encoded mainly by exon three. Exons 1 and 6 encode the 5'- and 3'-untranslated regions respectively. Exon 2 encodes the signal peptide and a small part of the NT peptide. Exon 4 encodes a small part of the mature peptide while exons 4 and 5 encode the remainder of a CT peptide. The entire gene spans 10 kb and is located on chromosome 17. The 5'-flanking region of the GIP gene contains a TATA motif, a binding site for SP 1 and regions similar to the consensus sequences of AP-1 and AP-2 target elements.

Like the human gene, the rat GIP gene is encoded by six exons but spans only 8.2 kb (Higashimoto and Liddle, 1993). The organization of the human gene is conserved in the rat gene; however, preproGIP is only 144 amino acids in length (Tseng et al. 1993). This is due to an 8 amino acid deletion in the NT peptide and a single amino acid deletion in the CT peptide. The putative cleavage site for the signal peptide is Gly-21 and the cleavage sites for the mature peptide are at Arg-43 and –86.

1.2.3 TISSUE DISTRIBUTION OF GIP

Human GIP mRNA has been detected only in the small intestine (Takeda et al. 1987; Inagaki et al. 1989); however, rat GIP mRNA has been detected both in the small intestine and in striated ductal cells of the rat submandibular salivary gland (Higashimoto et al. 1992; Tseng et al. 1993, 1995). Immunoreactive GIP (IR-GIP) was localized to the K cells in the mucosa of the duodenum and jejunum in humans (Polak et al. 1973; Buchan

et al. 1978) and baboons (Buchan et al. 1982). In rats and dogs IR-GIP was also found extending down to the terminal ileum (Buchan et al. 1982).

1.2.4 GIP SECRETION

GIP is released from the K cells after nutrient ingestion. After the ingestion of a mixed meal, plasma IR-GIP levels rise 5-6 fold. Absolute values range from 12-92 pM during fasting to 35-235 pM following a meal. The high variability between values is a result of poor cross-reactivity of antisera raised against GIP from different species for human GIP. There may also be large molecular weight proteins with the binding characteristics of some GIP antisera which interfere with GIP binding, and circulating fragments that cross-react to varying degrees (Jorde et al. 1983).

Carbohydrates are a major stimulus of GIP secretion. An oral glucose load has been shown to increase IR-GIP levels in human (Cataland et al. 1974), dog (Pederson et al. 1975) and rat (Pederson et al. 1982); however, intravenous glucose has no effect on secretion of IR-GIP (Cataland et al. 1974; Pederson et al. 1975). This suggests that glucose affects K cells directly. Luminal stimulation with glucose, galactose and sucrose stimulated increases in IR-GIP while fructose, mannose, 6-deoxygalactose, 2-deoxyglucose, myoinositol and lactose had no effect (Sykes et al. 1980), suggesting that sodium-dependent transport was necessary. This conclusion is supported by studies in which phloridzin, an inhibitor of glucose transport, was used to block glucose-stimulated IR-GIP release (Sykes et al. 1980; Creutzfeldt and Ebert, 1977). Glucose stimulated GIP release has also been demonstrated in cultured isolated canine K cells (Kieffer et al. 1994) and from an intestinal cell line (Kieffer et al. 1995a).

IR-GIP is also released in response to ingestion of triglycerides (TG) (Pederson et al. 1975; Cleator and Gourlay, 1975; Brown et al. 1975; Falko et al. 1975) and long-chain fatty acids (FA) (Ohneda et al. 1983; Ross and Shaffer, 1981), but not medium- or short-chain FA. The IR-GIP response to fat in humans is greater and more prolonged than to glucose(Pederson et al. 1975), but is not insulinotropic (Dupré et al. 1973; Pederson et al. 1975; Sykes et al. 1980). Beck (1989) hypothesized that fat-released GIP may have an effect on fat metabolism. A study examining the 24 h secretory pattern of GIP in humans eating a typical Western diet (~40% energy provided by fat) found that GIP levels closely paralleled that of triacylglycerol (Elliott et al. 1993). In pigs, whose diet provides less than 10% energy as fat, carbohydrate was found to be a more potent stimulator of GIP secretion than fat (Knapper et al. 1995a). It was suggested that insulin may exert a negative feedback effect on GIP secretion as the IR-GIP response to oral fat is less when intravenous glucose is infused into normal humans (Crockett et al. 1976; Creutzfeldt et al. 1980; Verdonk et al. 1980). It was also shown that insulin could inhibit fat-induced (Creutzfeldt et al. 1980), but not glucose-induced GIP release (Sykes et al. 1980; Creutzfeldt et al. 1980). Inhibition of GIP release by hyperinsulinemia has also been demonstrated in rat (Bryer-Ash et al. 1994) and human (Takahashi et al. 1991). However, during a euglycemic hyperinsulinemic clamp, fat-induced GIP secretion was not affected (Verdonk et al. 1980). Other studies have shown that IR-GIP levels were unchanged by exogenous (Service et al. 1978) or endogenous insulin (Pederson et al. 1975). Morgan (1998) suggested that the decrease in fat-stimulated IR-GIP in the presence of intravenous glucose could be a result of delayed gastric emptying resulting from hyperglycemia. A study using Pluronic L81, a surfactant that inhibits chylomicron formation in gut cells, was

able to block fat-induced IR-GIP release, thus linking it to chylomicron formation (Creutzfeldt and Ebert, 1985). Chylomicrons transport long- but not medium-chain FA which may explain why only long-chain FA stimulate IR-GIP release (Ebert and Creutzfeldt, 1984). The size of meal and proportion of carbohydrate and fat also affects GIP secretion (Hampton et al. 1986; Murphy et al. 1995). Humans who were fed a series of meals with fat content increasing from 20-80 g showed rising plasma GIP levels but no change in insulin levels (Murphy et al. 1995).

Studies examining the effect of protein on GIP secretion have shown mixed results. Meat and meat extracts had no effect on GIP secretion (Cleator and Gourlay, 1975), while intraduodenal administration of a mixture of basic amino acids, but not a mixture of aromatic amino acids, stimulated IR-GIP release (Thomas et al. 1976). The gastric infusion of peptone in dogs was also shown to stimulate IR-GIP release (Wolfe and McGuigan, 1982); however, a protein meal administered to humans failed to elicit changes in GIP secretion (Elliott et al. 1993). A study using rats in which corticosteroid or alloxan were used to enhance sodium-dependent amino acid transport by increasing Na^+K^+ATPase activity demonstrated an increase in IR-GIP release (Schulz et al. 1982). This suggests that like carbohydrates, the active transport of amino acids is coupled to GIP release.

1.2.5 REGULATION OF GIP GENE EXPRESSION

Rat GIP gene expression was shown to increase in both the small intestine and the submandibular gland in response to both glucose and fat administration (Tseng et al. 1994; Higashimoto et al. 1995), while tissue IR-GIP levels only increased in response to glucose (Higashimoto et al. 1995). GIP mRNA expression in the intestinal tumor cell line STC_{6-14}

showed a 3-fold increase in response to increasing the glucose concentration of the culture media (Schieldrop et al. 1996). Food deprivation has been shown in one study to decrease GIP mRNA levels (Sharma et al. 1992) and in another study to increase GIP mRNA and tissue IR-GIP levels (Higashimoto et al. 1995). Both the rat and human genes contain a putative cyclic-adenosine monophosphate (cAMP) response element and AP1 and AP2 sites in their 5' flanking regions (Inagaki et al. 1989; Higashimoto and Liddle, 1994). These are recognition sites for transcription factors which are regulated by cAMP/protein kinase (PK)A and PKC pathways and thus may be involved in GIP gene regulation. The GIP gene expressed in STC-1 cells has been shown to contain a GATA binding motif that promotes expression of a luciferase reporter construct (Boylan et al. 1997).

1.3 STRUCTURE-FUNCTION RELATIONSHIPS OF GIP

Several studies have shown that the truncation of GIP at the CT has only minor effects on its insulinotropic activity. Studies in the perfused rat pancreas demonstrated that porcine (p) GIP_{1-38} (Moroder et al. 1978) and bovine (b) GIP_{1-39} (Sandberg et al. 1986) have similar insulinotropic activities to GIP_{1-42}. GIP_{1-31} was found to have a receptor binding affinity an order of magnitude lower than GIP_{1-42} (Maletti et al. 1987) while synthetic human (sh) $GIP_{1-30amide}$ had equivalent insulinotropic activity to natural (n) $pGIP_{1-42}$ in the perfused rat pancreas (Wheeler et al. 1995; Morrow et al. 1996) but only 30% of the somatostatin (SS) stimulatory activity in the perfused rat stomach. $spGIP_{1-30amide}$ was found to have similar inhibitory activity to $npGIP_{1-42}$ on bombesin-stimulated amylase secretion but was much less potent at inhibiting pentagastrin stimulated acid secretion (Rossowski et al. 1992). These results suggest that the somatostatinotropic

domain of GIP falls in the CT region, while the insulinotropic domain lies in the NT region.

The results of studies examining the truncation of the NT of GIP have not been so clear. When a contaminant corresponding to $npGIP_{3-42}$ was identified in a npGIP preparation (Jörnvall et al. 1981), it proved to be biologically inactive (Brown et al. 1981; Schmidt et al. 1986a). $bGIP_{4-42}$ produced by enzymatic cleavage was found to have comparable affinity to GIP_{1-42} for the GIP receptor on hamster insulinoma cell membranes, while showing 10% of the insulinotropic activity in the perfused rat pancreas when compared to the intact peptide (Maletti et al. 1986). However, both $npGIP_{15-42}$, produced by cyanogen bromide cleavage (Pederson and Brown, 1976), and $bGIP_{17-42}$, produced by enterokinase cleavage (Carlquist et al. 1984; Maletti et al. 1986), retained definite insulinotropic activity. Maletti et al. (1986) also failed to demonstrate effects on ^{125}I-GIP binding with the fragments $bGIP_{1-16}$ and $bGIP_{19-30}$ and predicted that the insulinotropic domain fell between residues 17-38. But in a study using RINm5F cells, GIP_{17-42} failed to stimulate cAMP production whereas GIP_{1-42} was active (Gallwitz et al. 1993). In addition, unlike GIP_{1-42}, GIP_{19-42} did not stimulate insulin release in isolated islets (Schmidt et al. 1986b).

As a result of the above studies, Morrow et al. (1996) hypothesized that the insulinotropic activity of GIP must lie in residues 15-30. Using the perfused rat pancreas, they examined several fragments within this region for insulinotropic activity. Although $spGIP_{15-30}$ was found to be inactive, further cleavage to $pGIP_{17-30}$ (with enterokinase) and to $pGIP_{19-30}$ (with trypsin) restored ~30% of the insulinotropic activity of the intact peptide. These results were supported when a synthetic preparation of $spGIP_{17-30}$ was also

shown to be insulinotropic. However, the same group failed to demonstrate displacement of ^{125}I-GIP binding to β-TC3 cells with these peptides, which suggested that the site of action of the truncated peptide may be at a receptor other than the GIP receptor (Morrow, Pederson and McIntosh, personal communication).

More recently, Gelling et al. (1997a) examined several fragments for binding and cAMP production in Chinese hamster ovary (CHO-K1) cells transfected with the GIP receptor. GIP_{10-30}, $GIP_{6-30amide}$ and GIP_{7-30} were all shown to displace binding of ^{125}I-GIP, but failed to stimulate cAMP production to any significant degree. Thus, the fragments were examined for their ability to inhibit $shGIP_{1-42}$-stimulated cAMP production and were all found to be antagonists. This suggested that the domain of GIP important for insulinotropic activity and stimulation of adenylyl cyclase is found between residues 17 and 30, but that the NT of the peptide is also important for receptor binding. As only $GIP_{6-30amide}$ demonstrated receptor binding affinity equal to that of GIP_{1-42}, it was suggested that this fragment contains the high affinity binding region of GIP.

1.3.1 GIP IS METABOLIZED TO GIP_{3-42} BY DIPEPTIDYL PEPTIDASE IV (EC 3.4.14.5)

Dipeptidyl peptidase IV (DP IV) is a ubiquitously expressed selective serine protease which cleaves peptides after a penultimate N-terminal proline (Xaa-Pro) or alanine (Xaa-Ala) residue (Heins et al. 1988). The amino acids in positions P1 and P2 must be L-isomers in the trans configuration (Fischer et al. 1983) and the N-terminus must be protonated (Demuth and Heins, 1995). DP IV has been found to hydrolyse several regulatory peptides such as substance P, human α-relaxin, human pancreatic polypeptide,

human chorionic gonadotropin (hCG), prolactin, neuropeptide Y, peptide YY and β-casomorphin (Mentlein, 1988, 1993a; Nausch et al. 1990; Wang et al. 1991). Following cleavage, these peptides become susceptible to degradation by other exopeptidases (Mentlein, 1988).

Growth hormone-releasing hormone (GHRH), a member of the VIP/glucagon/secretin family has been shown to be rapidly cleaved by DP IV to biologically inactive $GHRH_{3-44}$ both *in vivo* and *in vitro* (Frohman et al. 1986). As many members of this family share sequence similarity at their amino-terminus, beginning with either Tyr-Ala, His-Ala or His-Ser (Rosselin, 1986), it was thought possible that the contamination of early porcine GIP preparations (Jörnvall et al. 1981) with GIP_{3-42} was due to DP IV degradation. It was found that GIP was indeed hydrolysed by circulating DP IV (Mentlein et al. 1993b) and that GIP_{3-42} was non-insulinotropic (Brown et al. 1981; Schmidt et al. 1986a). Degradation of GIP_{1-42} by purified placental DP IV has rapid kinetics which suggests that it is an important pathway. Using high performance liquid chromatography (HPLC), Kieffer et al. (1995b) demonstrated the rapid degradation of physiological levels of ^{125}I-GIP to ^{125}I-GIP_{3-42} following injection in rats. Within the first two minutes, more than 50% of the injected label was degraded, supporting a possible physiological role in GIP regulation. Pauly et al. (1996) has used the extremely sensitive and accurate technique of matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) to confirm that GIP_{3-42} is a major degradation product resulting from incubation with human serum. The inclusion or administration of the DP IV specific inhibitor Ile-thiazolidide could block the degradation both *in vivo* and *in vitro*

resulting in an early peak in plasma insulin and improved glucose handling in the anesthetized rat (Pauly, 1996).

In light of the degradation of GIP by DP IV, it is important to remember that as most GIP radioimmunoassays (RIAs) recognize carboxyl-terminal epitopes, they measure both biologically active and inactive forms (Brown et al. 1981; Schmidt et al. 1986a). Thus, all previously reported GIP levels are most likely an overestimation of the biologically active hormone levels, and RIAs which use amino-terminal directed antisera need to be developed.

1.4 BIOLOGICAL ACTIONS OF GIP

1.4.1 GIP AS AN ENTEROGASTRONE

GIP was originally isolated because of its gastric inhibitory properties in the denervated stomach (Brown et al. 1969, 1970; Brown, 1971; Brown and Dryburgh, 1971), but later studies in both human (Maxwell et al. 1980) and dog (Pederson and Brown, 1972; Soon-Shiong et al. 1979) showed that GIP's inhibitory effects on the innervated stomach were weak. A comparative study by Soon-Shiong et al. (1984) confirmed that GIP strongly inhibited pentagastrin-stimulated acid secretion in vagally denervated pouches (Heidenhain) but acted only weakly on the innervated gastric remnant. The GIP secretagogue oleic acid, when administered intra-duodenally, was shown to completely inhibit gastric secretion in response to liver extract consumed by dogs, while GIP infusion could only inhibit 40% of acid secretion (Yamagishi and Debas, 1980). In a study using an isolated perfused rat stomach GIP was found to stimulate the release of immunoreactive-somatostatin (IR-SS), while vagal stimulation or acetylcholine

administration inhibited IR-SS release (McIntosh et al. 1981). This implied that GIP's effects on acid secretion may be mediated via SS release from the D-cells of the stomach. The sympathetic nervous system may also act to modulate this pathway as sympathetic nervous stimulation was shown to increase gastric SS release in the perfused stomach (McIntosh et al. 1981).

1.4.2 INCRETINS AND THE ENTEROINSULAR AXIS CONCEPT

As early as the turn of the century, the possibility of a duodenal modulator of carbohydrate disposal was investigated (Bayliss and Starling, 1902). In 1906, Moore et al. suggested that diabetes was caused by the absence of a "chemical excitant" produced by the duodenum. In 1932, following studies using an intestinal extract free of secretin activity, which caused hypoglycemia in dogs, La Barre and Still proposed the term incretin to describe humoral activity from the gut that potentiates glucose disposal (Zunz and LaBarre, 1929; Brown et al. 1989). Later experiments using various intestinal extracts failed to show any effect on blood glucose levels (Loew et al. 1940), and interest in a possible insulinotropic intestinal factor waned. It was not until the development of the RIA, which could directly measure insulin levels, that investigations in this area were once again pursued. Oral glucose was demonstrated to be a more potent stimulator of insulin secretion in humans than intravenous glucose (Elrick et al. 1964; McIntyre et al. 1964), leading to the conclusion that there existed an additional modulator of insulin release, and that it was likely produced by the small intestine (McIntyre et al. 1965). In 1969, Unger and Eisentraut coined the term "enteroinsular axis" to describe the hormonal link between the gut and the pancreatic islets. Because neural connections and nutrients were also

known to have a direct effect on insulin secretion from the islets, Creutzfeldt (1979) suggested that this term should include nutrient, neural and hormonal signals from the gut to all islet cells secreting insulin (β-cells), glucagon (α-cells), SS (δ-cells) and pancreatic polypeptide (PP cells). The term "incretin" should describe a hormone of the enteroinsular axis that at physiological levels causes insulin release in the presence of glucose (Creutzfeldt, 1979).

1.4.3 GIP AS AN INCRETIN

GIP was first suspected to be an incretin in the enteroinsular axis when Rabinovitch and Dupré (1972) discovered that purification of a CCK preparation decreased its insulinotropic action. Because this was similar to the loss of acid-inhibitory activity seen by Brown and Pederson (1970), Dupré et al. (1973) hypothesized that GIP might be capable of stimulating insulin secretion. In a human study they showed that porcine GIP could stimulate insulin secretion in the presence of elevated glucose, but not in the euglycemic state. Since this time, GIP's insulinotropic properties have been demonstrated in human (Elahi et al. 1979; Andersen et al. 1978) and dog (Pederson et al. 1975) and *in vitro* with the isolated perfused rat pancreas (Pederson and Brown, 1976, 1978), isolated islets (Siegel and Creutzfeldt, 1985; Shima et al. 1988) and β-cell tumor cell lines (Kieffer et al. 1993; Lu et al. 1993a,b).

1.4.4 GIP ACTS ON OTHER ISLET CELL TYPES

The actions of GIP on the pancreatic islets are not restricted to the β-cell. GIP has been shown to stimulate the α-cell of the perfused rat pancreas to release glucagon when

glucose levels are below 5.5 mM (Pederson and Brown, 1976). GIP had no effect on mild or moderate glucose-suppressed glucagon release in humans (Elahi et al. 1979), but was able to reverse high glucose-suppressed glucagon release in the mouse (Opara and Go, 1991), suggesting a possible species difference. GIP has been shown to stimulate SS secretion from the δ-cell of the perfused rat pancreas only very weakly (Schmid et al. 1990).

1.4.5 EXTRAPANCREATIC EFFECTS OF GIP

Following a fat meal, although glucose levels are not increased, plasma GIP levels are high and do not stimulate insulin secretion (Pederson et al. 1975). This suggests a possible role for GIP in fat metabolism. GIP is involved in chylomicron metabolism, as it was seen to increase the clearance of chylomicrons in dog (Wasada et al. 1981). The action of GIP also promotes triglyceride synthesis in adipose tissue. GIP stimulated the synthesis and release of lipoprotein lipase (LPL) in mouse 3T3-L1 preadipocytes (Eckel et al. 1979) and stimulated LPL activity in rat adipose tissue explants (Knapper et al. 1995b). GIP potentiated insulin-stimulated triglyceride synthesis in rat adipose tissue (Beck and Max, 1983; Starich et al. 1985) and increased the clearance of plasma TG in the rat (Ebert et al. 1991), while it inhibited glucagon- and isoproterenol-stimulated lipolysis (Dupre et al. 1976; Ebert and Creutzfeldt, 1987; Hauner et al. 1988). GIP has also been found to stimulate fatty acid synthesis in adipose tissue explants (Oben et al. 1991) and in adipocytes (Hauner et al. 1988) at physiological concentrations. It has been shown that incubation with GIP increases glucose uptake in adipocytes both in the presence of insulin (Starich et al. 1985), and in its absence (Hauner et al. 1988). In contrast to these

lipogenic effects of GIP, a recent study using differentiated 3T3-L1 cells demonstrated GIP stimulated glycerol release which could be inhibited by insulin in a wortmannin-sensitive fashion (McIntosh et al. 1999). It was suggested that GIP-stimulated increases in circulating free fatty acids (FFA) play a role in optimizing pancreatic β-cell responsiveness to glucose and GIP, and that GIP's effects on adipocytes may be dependent upon plasma insulin levels.

GIP may be involved in hepatic metabolism although GIP receptor mRNA has not been found in the liver using in situ hybridization (Usdin et al. 1993). However, GIP infusion has been shown to increase insulin-mediated inhibition of hepatic glucose production and decrease glycogenolysis in dogs (Andersen et al. 1984), human (Elahi et al. 1986), and perfused rat liver (Hartmann et al. 1986). The existence of a different GIP receptor in liver cannot be ruled out.

1.5 THE GIP RECEPTOR

1.5.1 GIP BINDING SITES

Binding sites for GIP were originally found in transplantable hamster insulinoma cells (Maletti et al. 1984; Couvineau et al. 1984) and the insulin secreting hamster β-cell line, In 111 (Amiranoff et al. 1984, 1985). They have subsequently been identified in human insulinomas (Maletti et al. 1987) and β-TC3 cells (Kieffer et al. 1993). Both high affinity (Kd = 0.2-7 nM; 3000 binding sites/cell) and low affinity (Kd = 800 nM;1.5 x 10^5 binding sites/cell) binding sites have been demonstrated using Scatchard analysis (Maletti et al. 1984, 1987; Amiranoff et al. 1984, 1985). Most studies have found no cross reactivity in binding with other members of the glucagon/secretin peptide superfamily

(Maletti et al. 1984; Amiranoff et al. 1985; Couvineau et al. 1984); however, Kieffer et al. (1993) found that 1 μM glucagon displaced 20% of the ^{125}I-GIP binding in β-TC3 cells. More recently, very high affinity (Kd = 16-62 pM) binding sites have been identified in several areas of the rat brain (Kaplan and Vigna, 1994).

Studies using chemical (Couvineau et al. 1984) or ultraviolet irradiation (Amiranoff et al. 1986) cross-linking experiments identified a 64 kDa ^{125}I-GIP-labelled protein in membranes from β-cell tumors. This suggested a receptor with a molecular weight of 59 kDa. The GIP receptor was demonstrated to have intrachain disulfide bond(s), and be a glycoprotein containing N-acetylglucosamine, mannose and possibly sialic acid (Amiranoff et al. 1986).

1.5.2 THE GIP RECEPTOR cDNA

A GIP receptor cDNA was originally isolated from rat cerebral cortex (partial cDNA) and from a rat islet (RINm5F) tumor cell line (full length cDNA) (Usdin et al. 1993), and later from hamster (HIT-T15) cells (Yasuda et al. 1994) and rat pancreatic islets (Wheeler et al. 1995). It encodes a putative seven transmembrane (TM) spanning G-protein coupled receptor protein of 455 amino acids and has a predicted molecular weight of 50, 063, assuming cleavage of a putative 18 amino acid signal peptide. The amino acid sequence also contains a high degree of similarity (40-47% identity) to the GLP-1 and glucagon receptors (Yasuda et al. 1994; Usdin et al. 1993). This receptor sub-family, characterized by large NT extracellular domains, appears to have a common ancestral gene (Fehmann et al. 1995; Ulrich et al. 1998) and shares little sequence identity with other G-protein coupled receptors. The rat and hamster GIP receptor sequences

share ~86% identity while the rat islet receptor sequence differs in only one position (Glu21→Gln21) from that isolated from RINm5F cells (Wheeler et al. 1995). There are three potential glycosylation sites in the NT which supports the proposal that the receptor is a glycoprotein (Amiranoff et al. 1986) and may also explain the discrepancy in size found (~59kDa) in previous experiments (Amiranoff et al. 1986; Couvineau et al. 1984). There are many threonine and serine residues in the third intracellular (i3) loop and CT tail, which are potential phosphorylation sites (Usdin et al. 1993). Using GIP-GLP-1 receptor chimeras, Gelling et al. (1997b) demonstrated that the NT is the ligand-specific binding domain while the first TM domain (TM1) is involved in receptor activation. In a later study, Wheeler et al. (1999) used truncations of the CT tail to examine the effect on binding, cAMP production and receptor uptake and desensitization. It was observed that the majority of the CT tail was not essential for coupling to adenylyl cyclase although two truncated receptors had significantly lower (4-6-fold) EC_{50} values. It was found that truncation did result in a decrease in maximal binding levels and this was proposed to be due to a decreased efficiency of receptor insertion in the plasma membrane. Truncation decreased the rate of receptor internalization; however, studies on desensitization were inconclusive.

1.5.3 THE GIP RECEPTOR GENE

The gene of the rat GIP receptor has recently been cloned and its 5'-flanking region characterized (Boylan et al. 1999). The coding region of the gene spans ~10.2 kB and contains 13 exons, while two additional exons encode 5' and 3' untranslated sequences. Exons 3-5 and parts of exons 2 and 6 code for the NT region while the

remainder of exon 6 encodes TM1. Exon 7 encodes the first intracellular (i1) loop, the second TM domain (TM2) and most of the first extracellular (e1) loop. The remainder of the e1 loop is encoded by exon 8b, along with the third TM domain (TM3), the second intracellular (i2) loop and part of the fourth TM domain (TM4). Exon 9 codes for most of TM4 and approximately half of the second extracellular (e2) loop, while exon 10 encodes the remainder of the e2 loop and part of the fifth TM domain (TM5). Most of TM5 and part of the i3 loop are encoded by exon 11. Exon 12 codes for part of the i3 loop, the sixth TM domain (TM6), the third extracellular loop and part of TM7. The remainder of TM7 and a small portion of the CT tail are encoded by exon 13, while exon 14 codes for most of the CT tail. The 5'-flanking region was found to contain several regulatory sequences, including a cAMP response element, an octamer binding site, three SP1 sites and an initiator element. One of the SP1 sites was demonstrated to be necessary for efficient transcription in RIN38 cells.

1.5.4 TISSUE DISTRIBUTION OF THE GIP RECEPTOR

Through the use of Northern blot, RT-PCR and in situ hybridization studies, GIP receptor mRNA has been found in many tissues including pancreas, stomach, intestine, adipose tissue, adrenal cortex, heart, lung, and endothelium of major blood vessels, but not in spleen or liver (Yasuda et al. 1994; Usdin et al. 1993). Some of these tissues have not previously been considered targets for GIP and roles for the GIP receptor are as yet unknown. In situ hybridization has also detected GIP receptor mRNA in several regions of the brain including the telencephalon, diencephalon, brain stem, cerebellum and pituitary (Usdin et al. 1993). The presence of mRNA in the telencephalon agreed with the

findings of Kaplan and Vigna (1994); however, they were unable to find binding sites for GIP in the pituitary or hypothalamus. In contrast, GIP influences the anterior pituitary release of follicle-stimulating hormone and growth hormone (GH) (Ottlecz et al. 1985). The physiological relevance of GIP receptors in areas of the brain inaccessible to blood-borne peptides is questionable as GIP mRNA (Higashimoto et al. 1992; Tseng et al. 1993) has not been detected in the brain. GIP mRNA levels may be too low to detect or a homologous brain peptide may exist, or the receptor may be expressed but have no function in the brain.

A study using purified α- and β-cells and non-β-cell populations examined the expression of GIP, GLP-1 and glucagon receptors in these cells (Moens et al. 1996). Northern blot analysis revealed high expression levels for all three receptors in β-cells (~90% pure), but only the GIP receptor was found to be significantly expressed in the non- β-cell fractions (~80% α-cells, ~10% δ-cells). Total RNA was also examined in a transplantable insulinoma (MSL-G2-IN) and co-derived glucagonoma (MSL-GAN) which supported the selective expression of the GIP receptor mRNA on glucagon producing cells (Moens et al. 1996). These findings support GIP's role as a direct regulator of islet cell function, and more specifically support the findings of Pederson and Brown (1978) in the perfused rat pancreas which suggested that GIP directly affects the α-cell.

Very few studies have examined GIP receptor expression in humans. Gremlich et al. (1995) demonstrated receptor expression in islet cells. The GIP receptor was also shown to be "ectopically" expressed in adrenal cells in a group of patients with Cushing's syndrome (Lacroix et al. 1992; Reznik et al. 1992) and adrenal hyperplasia. As receptor mRNA has been found in the rat adrenal (Usdin et al. 1993), the food dependent hyper-

GIP sensitivity and resulting cortisol hypersecretion may be a result of GIP receptor overexpression or a defect in GIP regulation as opposed to abnormal expression. In fact, a recent study using RT-PCR demonstrated overexpression of the GIP receptor in the adrenals of two patients with GIP-dependent Cushing's syndrome (N'Diaye et al. 1998). The same study failed to detect a signal in adrenal adenomas or macronodular hyperplasia from patients with non-food-dependent Cushing's syndrome.

1.6 GIP RECEPTOR SIGNAL TRANSDUCTION MECHANISMS

GIP has been demonstrated to stimulate adenylyl cyclase in pancreatic tumor cell lines (Maletti et al. 1987; Lu et al. 1993a; Amiranoff et al. 1984), a gastric cancer cell line (HGT-1) (Gespach et al. 1984), isolated islets (Siegel and Creutzfeldt, 1985) and 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). In adipocytes from rat epididymal fat pad, GIP was shown to inhibit glucagon-stimulated cAMP production (Dupre et al. 1976), but a further study using a similar preparation demonstrated GIP stimulated cAMP production (Ebert and Creutzfeldt, 1987). A recent study in our laboratory using differentiated 3T3-L1 cells demonstrated GIP stimulated increases in cAMP production, coupled to increased glycerol release, which could be potentiated by the phosphodiesterase inhibitor 3-isobutyl-1-methyl xanthine (IBMX), and partially blocked by the adenylyl cyclase inhibitor MDL 12330A (McIntosh et al. 1999).

The stimulation of adenylyl cyclase is believed to be GIP's primary mode of action; however, it has also been demonstrated to increase uptake of Ca^{2+} into isolated mouse islets (Wahl et al. 1992) and to increase intracellular free calcium levels ($[Ca^{2+}]_i$) in HIT-

T15 cells (Lu et al. 1993a) and a reporter cell line transfected with the RINm5F GIP receptor (Usdin et al. 1993). The $[Ca^{2+}]_i$ response to GIP in the HIT-T15 cells could be abolished using EGTA or blockade of L-type channels with nimodipine which suggested that Ca^{2+} influx occurs through voltage-dependent Ca^{2+} channels (VDCCs) (Lu et al. 1993a). However, in another investigation using GIP receptor transfected COS cells (Wheeler et al. 1995), GIP increased $[Ca^{2+}]_i$ in a biphasic manner, with an acute transient phase followed by a sustained elevation. The acute phase was thapsigargin sensitive which indicated release from intracellular stores, while the sustained release was a result of Ca^{2+} influx through a cation channel which was unaffected by nifedipine, an L-type Ca^{2+} channel blocker. In studies using HIT-T15 cells (Lu et al. 1993a) and CHO-GR1 cells transfected with the hamster receptor (Yasuda et al. 1994) GIP failed to stimulate inositol-1, 4, 5-trisphosphate (IP_3) production suggesting that phospholipase (PLC) mobilization of $[Ca^{2+}]_i$ stores was not involved. However, a study using the phosphatidylinositol (PI) 3'-kinase inhibitor, wortmannin inhibited GIP mediated insulin release from HIT-T15 cells (Straub and Sharp, 1996). This suggested a wortmannin-sensitive signaling pathway, but its nature was unknown. A later study demonstrated GIP stimulation of 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). An investigation using voltage clamped individual mouse β-cells studied the effect of GIP on insulin secretion, as measured by membrane capacitance, Ca^{2+} influx measured by whole-cell calcium currents and $[Ca^{2+}]_i$ via fura-2 florescence (Ding and Gromada, 1997). GIP stimulated insulin secretion with only a 31% increase in $[Ca^{2+}]_i$ and with no change in whole cell calcium currents. The protein kinase A (PKA) inhibitor Rp-8-bromo-cAMP was able to block the

increase in capacitance but the authors did not attempt to block the $[Ca^{2+}]_i$ increase from internal stores in these experiments. In a separate study, the same authors demonstrated an increase in whole cell calcium currents from GIP stimulated α-cells which were in part mediated by the cAMP pathway (Ding et al. 1997). This suggests that GIP receptor signaling may vary in different cellular environments. The phospholipase A_2 signaling pathway has also been implicated in GIP actions (Lardinois et al. 1990). It would appear that GIP can act via more than one pathway depending on tissue or cell type and these need to be further elucidated.

1.7 GLUCOSE-DEPENDENCE OF GIP STIMULATED INSULIN RELEASE

Elevated glucose levels are required for GIP potentiation of insulin release. Glucose-stimulated insulin secretion involves glucose uptake, phosphorylation by glucokinase and cellular metabolism, which is believed to increase the ATP/ADP ratio within the cell. This results in the closure of ATP-dependent K^+ channels and the subsequent depolarization of the β-cell and opening of VDCCs, allowing an influx of Ca^{2+}. This ultimately results in the exocytosis of insulin (Fehmann et al. 1995; Holz and Habener, 1992). There are several hypotheses as to how the GIP signal transduction pathway interacts with this glucose-signaling pathway.

Increasing cAMP levels will stimulate insulin secretion even in cells in which the Ca^{2+} levels are maintained constant. This suggests that proteins involved in exocytosis may be activated by PKA phosphorylation (Ammala et al. 1993). There is some evidence that GLP-1 may slow VDCC inactivation and thus potentiate insulin secretion (Britsch et al. 1995; Kato et al. 1996), although the influx of Ca^{2+} seems to be only slightly increased

(Ammala et al. 1993; Thorens and Widmann, 1996). This may also be a potential pathway for GIP, therefore, if calcium induced calcium release is part of the signal transduction pathway, it may have a priming effect which permits the eventual release of intracellular stores or influx of Ca^{2+} via membrane VDCCs (Gromada et al. 1996; Gromada and Rorsman, 1996).

GIP increases proinsulin gene expression and biosynthesis in β-cell lines (Fehmann and Goke, 1995; Wang et al. 1996). RIN 1046-38 cells incubated with GIP (6-24 h) demonstrated increased expression of hexokinase I and GLUT1, but not GLUT2 or glucokinase mRNA levels (Wang et al. 1996). This suggests that GIP is able to regulate glucose sensing elements, although studies using primary β-cells have yet to be performed.

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

NIDDM is characterized by inappropriate and chronic hyperglycemia, glucose intolerance, impaired insulin secretion, peripheral and hepatic insulin resistance, leading to decreased skeletal muscle and white adipose tissue glucose utilization, and increased hepatic glucose production (McIntosh and Pederson, 1999). Usually, both insulin resistance and impaired insulin secretion are prerequisites for manifestation of NIDDM. Insulin resistance alone does not normally lead to NIDDM except in the rare case of insulin receptor mutations (Groop and Tuomi, 1997). The risk factors for NIDDM include dietary and environmental triggers and genetic susceptibility, with obesity being one of the strongest in humans (McIntosh and Pederson, 1999). As GIP exerts effects at both the pancreatic and adipose-tissue level it is a potential candidate for involvement in

obesity. Several studies have implicated an altered entero-insular axis in various obesity/diabetes syndromes characterized by disordered insulin secretion and fat deposition (Morgan, 1996). Unusually high intestinal and circulating GIP levels have been found in genetically obese (ob/ob) mice which appear to be overly sensitive to the insulinotropic effect of gastrointestinal hormones and have high adipose tissue LPL levels (Flatt et al. 1983). Normal GIP levels were found in response to nutritional stimuli in Zucker fatty (*fa/fa*) rats but the insulin-releasing actions of GIP were increased (Chan et al. 1984). In humans with NIDDM, fasting GIP levels were reported to be either normal or elevated as compared to healthy subjects (Creutzfeldt and Ebert, 1993), while nutrient induced secretion is increased, normal or blunted (Creutzfeldt and Ebert, 1993). A study examining the integrated IR-GIP response in 141 subjects with NIDDM discovered a large group of hypersecretors and a smaller group of hyposecretors when compared with normal subjects (Creutzfeldt et al. 1983). Jones and co-workers (1989a,b) found that the infusion of GIP under basal glycemic conditions stimulated insulin release in subjects with NIDDM, suggesting that the β-cells of GIP hypersecretors may be continually stimulated.

Similar inconsistencies in fasting and food stimulated GIP release have been seen in studies on obese subjects, although the test meal used and the rate of gastric emptying are thought to have contributed to the elevated responses measured (Creutzfeldt and Ebert, 1993). Obese individuals who have impaired oral glucose tolerance (OGT) have been shown to have exaggerated GIP responses, while obese or lean subjects with normal OGT have normal (Creutzfeldt et al. 1978) or elevated GIP levels (Salera et al. 1982). However, in a study of post-menopausal women with impaired OGT it was found that GIP levels were diminished (Ahren et al. 1997).

Although the incretin response of patients with NIDDM is severely impaired or abolished (Holst et al. 1997; Nauck et al. 1986; Nauck et al. 1986), obese subjects are still responsive to GIP (Amland et al. 1985; Elahi et al. 1984), and if glucose intolerant may show inappropriate GIP-mediated insulin release. The hyperinsulinemia associated with impaired OGT in obesity may result in desensitization of the K cell to normal feedback inhibition from insulin and thereby lead to hypersecretion of GIP (Creutzfeldt et al. 1978). This hyperGIPemic response of obese subjects with impaired OGT can be abolished by starvation or dietary restriction (Ebert et al. 1979; Deschamps et al. 1980; Willms et al. 1978). It should be noted that all of the secretion studies need reassessment in view of the rapid degradation of GIP by DP IV in plasma.

The β-cell insensitivity to physiological levels of GIP could potentially be a result of a defect at the receptor level. Two missense mutations have recently been identified in the human GIP receptor gene, Gly198→Cys198 (Gly198Cys) and Glu354→Gln354 (Glu354Gln) within the predicted e2 loop and TM6 of the receptor (Kubota et al. 1996). When the Glu354Gln mutant was expressed in CHO cells it showed similar function to the wild type receptor, but the Gly198Cys mutant showed a right-shifted half-maximal stimulation value (EC_{50}). Linkage studies were unable to demonstrate a linkage between NIDDM in Japanese patients and either of the two mutations.

1.9 STRUCTURE AND FUNCTION OF G-PROTEIN COUPLED RECEPTORS

The superfamily of guanine nucleotide-binding protein (G-protein) coupled receptors was first identified in the mid-1980's with the modeling and characterization of rhodopsin (Findlay and Pappin, 1986) and the β-adrenergic receptor (AR) (Dixon et al.

1986). Initially, the development of techniques to radioiodinate peptides made the localization and functional characterization of these receptors possible. With the development of molecular biological techniques, such as cDNA cloning and mutagenesis much more has been learned about this family and many more receptors have been discovered. There are several distinct families which bind ligands as diverse as photons, odorants, biogenic amines, peptides and large glycoproteins (Ulrich et al. 1998). The largest and best known of these is the rhodopsin/β-AR family. Although much that has been learned about the β-AR can be used to speculate about other receptors, it is important to study individual receptors to identify unique characteristics and functions. As GIP and/or the GIP receptor may play a role in the pathogenesis of NIDDM, it is important to further characterize these peptides.

1.9.1 RECEPTOR BINDING DOMAINS

Although there is a great variety in the structural requirements among the different GPCR's for ligand binding (Reviewed in Strader et al. 1994; Beck-Sickinger, 1996), all receptors must initially have a ligand associated with high affinity in a specific manner for activation to occur. The cationic amine receptors bind their ligands at the upper part of their TM spanning regions (Strader et al. 1994; Beck-Sickinger, 1996) while the binding domains of the opioid peptides includes the TM spanning segments and extracellular loops (Fukuda et al. 1995; Varga et al. 1996). The tachykinins bind to the top of TM2, the e1 and e2 loops and part of the NT segment (Beck-Sickinger, 1996), while the glycoprotein receptors mainly bind to ligand via a long glycosylated NT segment (Beck-Sickinger, 1996; Combarnous, 1992).

The binding domains of the secretin/VIP receptor subfamily of which the GIP receptor is a member have been characterized less than other subfamilies. However, the NT extracellular domains of these receptors appear to play an important role in high affinity binding (Ulrich et al. 1998). The use of chimeric secretin-VIP receptor constructs identified both distal and proximal segments of the NT to be important in both receptors, as well as the e1 and e2 loops of the secretin receptor (Holtmann et al. 1995, 1996a;). Studies with calcitonin/glucagon receptor chimeras suggested the NT domain of the calcitonin receptor was involved in binding, while both the NT and body regions of the glucagon receptor were necessary for binding (Stroop et al. 1995). A number of other studies of the glucagon receptor have led to the suggestion that the NT and the e1 loop are involved in ligand binding (Buggy et al. 1995; Unson et al. 1995, 1996; Carruthers et al. 1994). The extracellular domain of the GLP-1 receptor has been shown to have intrinsic binding activity when expressed alone (Wilmen et al. 1996). As mentioned in Section 1.5.2 the NT of the GIP receptor contains the high affinity binding site for GIP (Gelling et al. 1997b).

1.9.2 RECEPTOR-G-PROTEIN COUPLING

The binding of its ligand by a GPCR initiates a conformational change that results in the coupling to a heterotrimeric GTP binding (G)-protein. A variety of studies, initially with hybrid cholinergic-muscarinic receptor (Hedin et al. 1993; Wess, 1996) and AR subtypes (Dohlman et al. 1991; Hedin et al. 1993) have implicated the intracellular regions of the receptors in G-protein recognition, the most important being the i2 loop and the amino and carboxyl segments of the i3 loop (Wess, 1997). Many studies agree that these

regions cooperate to initiate appropriate G-protein recognition and activation (Liggett et al. 1991; Gomeza et al. 1996; Pin et al. 1994; Blin et al. 1995; Wong and Ross, 1994; Wong et al. 1990). Other studies with short receptor fragments led to the conclusion that the i2 and i3 loops are important in receptor-G-protein interactions (Konig et al. 1989; Munch et al. 1991) and that they cooperate together to direct appropriate G-protein recognition (Konig et al. 1989; Wade et al. 1994).

Studies using deletion mutagenesis of GPCRs generally agree that most of the CT tail is not required for efficient G-protein coupling, although some truncations have been shown to improve coupling efficacy, increase basal activity or G-protein coupling promiscuity, or render the protein functionally inactive (Reviewed in Wess, 1998). Chimeric studies with α_{2A}/β_2AR (Liggett et al. 1991) and mGluR1/mGluR3 glutamate receptors (Gomeza et al. 1996; Pin et al. 1994) have suggested that in certain receptor subfamilies, the CT tail may take part in controlling the selectivity of G-protein recognition. As mentioned in Section 1.5.2 the CT tail of the GIP receptor does not appear to be important in receptor-G-protein coupling to adenylyl cyclase (Wheeler et al. 1999), but it could be involved in coupling to other signal-transduction systems.

1.9.3 THE SECOND AND THIRD INTRACELLULAR LOOPS

Most GPCRs contain a conserved leucine residue or another hydrophobic residue in the central portion of the i2 loop (Wess, 1998). Studies in which this residue has been replaced with an alanine or a polar residue have demonstrated severely impaired G-protein coupling, suggesting that it may be a hydrophobic site for G-protein interaction (Arora et al. 1995; Moro et al. 1993a; Smit et al. 1996). The GIP receptor contains two leucine

residues in its i2 loop at positions 241 and 242, and three valines at positions 243-245. A number of studies with hybrid GPCRs have demonstrated that the i2 loop is an important region for regulating the selectivity of receptor/G-protein coupling (Reviewed in Wess, 1998). For example, insertion of the i2 loop of the α_{2B}AR, which couples to G_s, into the α_{2A} receptor, which couples to G_s only poorly, resulted in a mutant receptor which had α_{2B}-like G_s-coupling properties (Nasman et al. 1997).

Hybrid receptor studies have indicated that the i3 loop of GPCRs is extremely important for appropriate G-protein recognition, but is not the only factor which determines receptor coupling (Dohlman et al. 1991; Wess, 1996; Kobilka, 1992; Liu and Wess, 1996; Cotecchia et al. 1992; Olah, 1997; Van Leeuwen et al. 1995). The i3 loop interacts with other receptor domains, probably mainly the i2 loop, resulting in optimal coupling efficiency and selectivity (Gomeza et al. 1996; Blin et al. 1995; Wong and Ross, 1994; Wong et al. 1990). Despite varying in size from 15 amino acids (N-formyl peptide receptor) to 240 amino acids (m3 muscarinic (mACh) receptor), it has been demonstrated through deletion mutagenesis studies that only the N- and C-terminal eight to fifteen amino acids of the i3 loop are necessary for G-protein coupling (Reviewed in Strader et al. 1994). Residues in the N-terminal segment of the i3 loop have been shown to be critical for G-protein coupling selectivity in many mACh (Wess et al. 1989, 1990) and adrenergic (Liggett et al. 1991; Cotecchia et al. 1992) receptors, adrenergic/mACh hybrid receptors (Wong and Ross, 1994; Wong et al. 1990) and the A_{2a} adenosine receptor (Olah, 1997). Gain-of-function mutagenesis studies demonstrated that the N-terminal region of the i3 domain of the AT_1 angiotensin II receptor ($AT_{1A}R$) is required for coupling to $G_{q/11}$ (Wang et al. 1995), while both the N-terminal and C-terminal amino acids of the i3 loop are

necessary for $AT_{1A}R$ /G_q (Wang et al. 1995) and $\alpha_{2A}AR/G_s$ coupling selectivity (Liggett et al. 1991; Eason and Liggett, 1996). However, only the N-terminal or the C-terminal regions of the i3 domain are necessary for α_{2A}/β_2-adrenergic hybrid receptor coupling to G_i (Eason and Liggett, 1996).

Mutational analysis (Blin et al. 1995; Bluml et al. 1994a,b; Hogger et al. 1995; Hill-Eubanks et al. 1996; Liu et al. 1995), and insertion (Bluml et al. 1994c) and random mutagenesis studies (Hill-Eubanks et al. 1996) with several mAChR subtypes (m1-m5) revealed that a series of mainly hydrophobic or non-charged residues in the N-terminal segment of the i3 loop are required for receptor/G-protein coupling selectivity. They likely form a surface that corresponds to the hydrophobic side of an amphiphilic α-helix (Strader et al. 1994; Cheung et al. 1992; Altenbach et al. 1996). This conclusion has been supported by loss of function mutagenesis studies with several other GPCRs, including parathyroid hormone/parathyroid hormone-related protein (PTH/PTHrP) (Huang et al. 1996) and GLP-1 receptors (Mathi et al. 1997).

Mutational analysis of the C-terminal region of the i3 loop has shown that point mutations in this region often leads to constitutively active mutant receptors (Reviewed in Lefkowitz et al. 1993; Shenker, 1995; Shenker, 1998). From loss-of-function mutagenesis studies with several GPCRs it has been concluded that one or more positively charged residues within a stretch of charged residues found in the C-terminal segment of the i3 loop of most GPCRs are necessary for efficient G-protein activation (Hogger et al. 1995; Kunkel and Peralta, 1993; Lee et al. 1996; Obosi et al. 1997; Wang, 1997). Usually, mutation of the most C-terminal residue in this motif had the greatest effect on receptor function.

The structural and functional characteristics of the C-terminal segment of the i3 loop of various mAChR subtypes have been examined extensively by mutational analysis (Reviewed in Wess, 1996). A set of four, mainly hydrophobic, amino acids conserved among all three $G_{q/11}$-coupled mAChRs (m1, m3, m5) was demonstrated by gain-of-function studies to interact with residues in the i2 loop and the N-terminal domain of the i3 loop to determine $G_{q/11}$ coupling selectivity (Blin et al. 1995). The corresponding four amino acids found in the $G_{i/o}$-coupled m2 and m4 mAChRs were also determined to be important in the selective recognition of $G_{i/o}$-proteins (Liu et al. 1995; Kostenis et al. 1997). These hydrophobic residues are located at the i3 loop/TM6 junction, which has been hypothesized to be α-helically arranged (Strader et al. 1994; Altenbach et al. 1996), and thus form a second hydrophobic surface in the i3 loop necessary for G-protein recognition. Two amino acids at corresponding positions in bovine rhodopsin were identified by site-directed spin labeling (SDSL) to undergo a major increase in mobility upon rhodopsin activation (Altenbach et al. 1996). Among a number of G_i-coupled receptors there are two or three basic residues in the C-terminal segment of the i3 loop which are strongly conserved (Reviewed in Savarese and Fraser, 1992; Reisine and Bell, 1993). This group includes m4 mACh, α_2-adrenergic, 5-HT_{1A} serotonergic and μ-, δ-, and κ-opioid receptors. Synthetic peptides corresponding to these basic-residue rich regions have been shown to interact with G-proteins *in vitro* (Okamoto and Nishimoto, 1992; Merkouris et al. 1996; Wade et al. 1996). Site-directed mutagenesis of these residues in the μ-opioid receptor suggested that a conserved arginine residue is required for G_i activation (Wang, 1999).

Baldwin et al. (1997) developed a model which suggests that the N- and C-terminal domains of the i3 loop protrude into the center of the helical bundle where they potentially interact with residues in adjoining receptor domains. As a result of SDSL experiments (Farahbakhsh et al. 1995; Farrens et al. 1996), TM3 and TM6 are predicted to move "outward" upon receptor activation, thus creating an "opening" in the intracellular receptor surface which potentially permits the G-protein heterotrimer to interact with the i3 loop/TM junctions. Another possibility is that the N- and C-terminal domains of the i3 loop may interact in the inactive state of the receptor. These interactions are then disrupted upon ligand activation, through TM movement which allows the G-protein access to previously hidden residues (Wess, 1998).

1.9.4 RECEPTOR ACTIVATION AND CONSTITUTIVELY ACTIVE RECEPTORS

When a ligand binds to its GPCR, a conformational change is induced which allows the receptor to interact with a G-protein, resulting in the exchange of GDP for GTP and G-protein activation. The "ternary complex model" was proposed by DeLean et al. (1980) and suggests that the active form of the receptor is a ternary complex of agonist, receptor and G-protein. The agonist will only bind with high affinity when the receptor is bound to a G-protein. This model does not explain why some AR mutants could spontaneously interact with G-protein in the absence of agonist and were constitutively active (Kjelsberg et al. 1992; Cotecchia et al. 1990; Ren et al. 1993; Samama et al. 1993). It also failed to explain why increasing β_2AR density could sometimes result in higher basal activity (Samama et al. 1993; Adie and Milligan, 1994) and why ectopic receptor expression led to high basal activity (Costa et al. 1990; Costa

and Herz, 1989; Tiberi and Caron, 1994; Jakubik et al. 1995). Samama et al. (1993) developed the "allosteric" ternary complex model which proposes that GPCRs are in a state of equilibrium between an inactive (R) and active state (R*). R* is able to interact with G-protein in the absence of ligand which can explain "basal" receptor activity. When a receptor is bound by its agonist, it is stabilized in the R* state, thus shifting the equilibrium to the active state and increasing receptor signaling (Samama et al. 1993). Inverse or reverse agonists stabilize the receptor in the inactive state (Samama et al. 1993; Reviewed in Scheer and Cotecchia, 1997; Milligan et al. 1995). Inverse agonism was initially demonstrated *in vitro*, but later, a study using transgenic mice overexpressing the β_2AR in the heart showed that the β_2-adrenergic ligand ICI-188551 acted as an inverse agonist by decreasing ventricular systolic pressure both *in vivo* and *in vitro* (Bond et al. 1995).

Spontaneously occurring activating mutations in some GPCRs have been implicated in several diseases, namely autosomal dominant retinitis pigmentosa and night blindness (rhodopsin receptor), familial male precocious puberty (luteinizing hormone (LH) receptor), hyperfunctioning thyroid adenomas and familial non-autoimmune hyperthyroidism (thyroid stimulating hormone receptor), familial hypoparathyroidism (calcium sensor receptor) and inherited hyperpigmentation of a mouse strain (melanocyte-stimulating hormone receptor) (Reviewed in Scheer and Cotecchia, 1997; Milligan et al. 1995; Arvanitakis et al. 1998). The secretin/VIP receptor subfamily also includes an example of constitutively activating mutations in the PTH/PTHrP receptor, which are associated with Jansen-type metaphyseal chondysplasia, a rare type of dwarfism (Schipani et al. 1996; Schipani et al. 1995). The mutations found have been a histidine residue

replaced by an arginine at the junction of the i1 loop, or a threonine replaced by a proline in the TM6 domain. Schipani et al. (1995) has speculated that the histidine may play a universal role in regulating spontaneous receptor activation, as it is conserved among all members of the secretin/VIP receptor subfamily.

1.9.5 RECEPTOR PHOSPHORYLATION AND DESENSITIZATION

The response of GPCRs to agonists are generally not prolonged but instead are quickly attenuated through several mechanisms (Reviewed in Bohm et al. 1997a; Chuang et al. 1996; Ferguson et al. 1996a). The first mechanism of attenuation is the removal of agonist from the extracellular fluid by uptake or degradation. This is followed by receptor desensitization which takes place during the short term (seconds to minutes) exposure of cells to agonists and occurs through receptor/G-protein uncoupling. High affinity receptors are removed from the plasma membrane by receptor endocytosis, which also aids in resensitization of signaling. Over the long term (hours to days) exposure of cells to agonists, receptor down-regulation removes receptors from the cell.

The desensitization of GPCRs involves the rapid attenuation of cell signaling as seen by the generation of second messengers and can occur through two different mechanisms. Homologous desensitization is the deactivation of one receptor as a result of agonist activation, while heterologous desensitization is the deactivation of one receptor by the activation of another (Bohm et al. 1997a; Chuang et al. 1996; Ferguson et al. 1996a). Desensitization is mediated through receptor phosphorylation by serine/threonine kinases, and results in the uncoupling of the activated receptor from its G-protein. Homologous desensitization is mediated through the G-protein-coupled receptor kinases

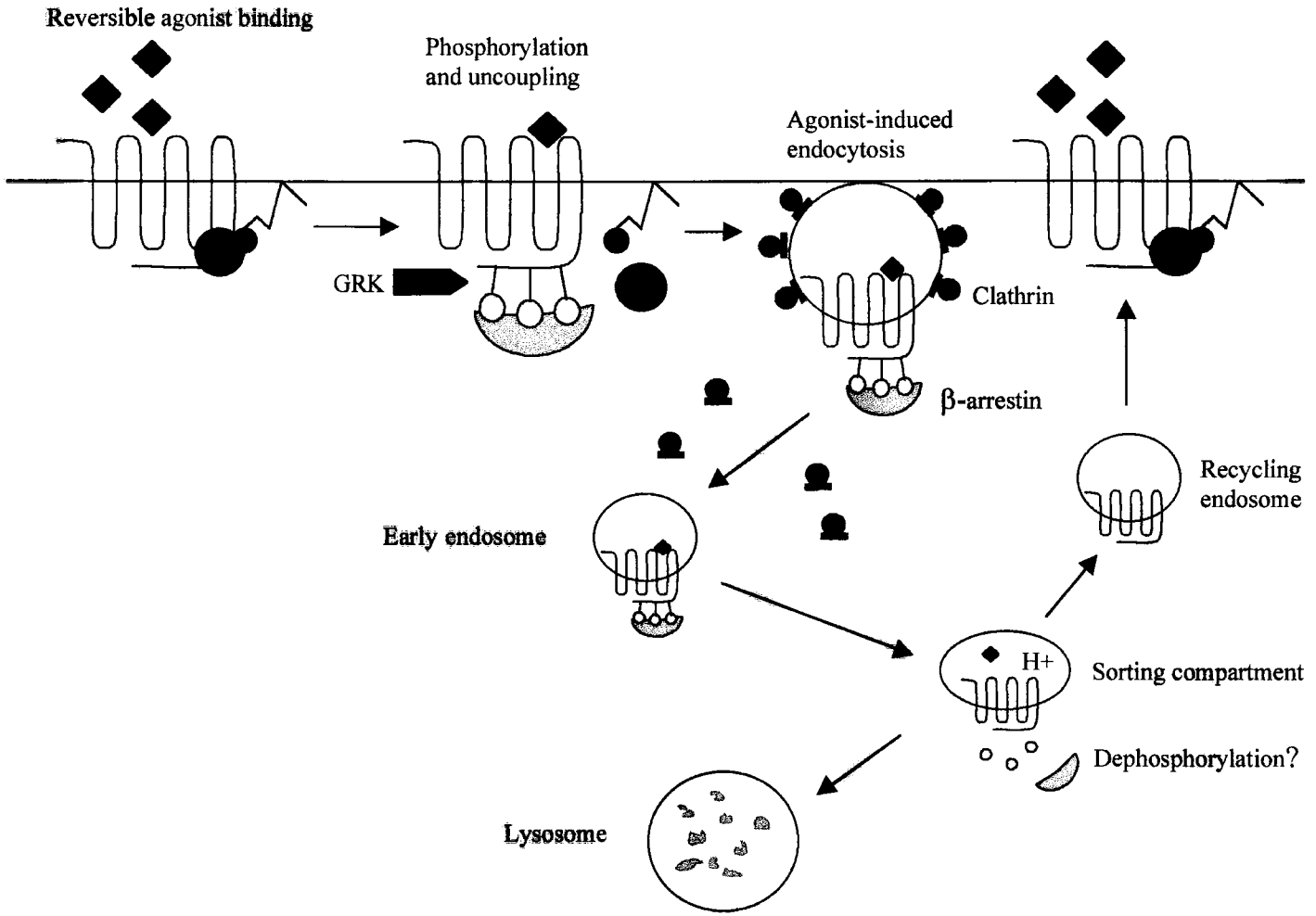

Fig. 2. Proposed Pathway for Desensitization and Sequestration of G-Protein Coupled Receptors. See text for details. Adapted from Bohm et al. (1997).

(GRKs) and their cofactors the arrestins (Fig. 2). GRKs will only phosphorylate agonist-bound receptors and therefore can only be involved in homologous desensitization. Heterologous desensitization is mediated by the second messenger dependent kinases PKA and protein kinase C (PKC), which phosphorylate different sites of the receptor than GRKs, and are not dependent on receptor occupancy by agonist (Bohm et al. 1997a; Chuang et al. 1996; Ferguson et al. 1996a).

At one time it was thought that homologous and heterologous desensitization were totally different processes, but it is now known that agonist-dependent desensitization can also be modulated by PKA and PKC (Chuang et al. 1996; Ferguson et al. 1996a). The second messenger dependent protein kinases contribute to different extents depending on agonist concentration, cell type and the particular GPCR. For example, the β_2AR is most efficiently desensitized at low agonist concentrations via PKA phosphorylation (Hausdorff et al. 1989; Lohse et al. 1990a); however, at higher agonist concentrations, the contribution of GRK phosphorylation becomes increasingly significant (Hausdorff et al. 1989; Roth et al. 1991; Lohse et al. 1990a).

GRK-mediated desensitization usually involves the phosphorylation of several serine and threonine residues of the GPCR, but it is thought that the initial phosphorylation events are the most physiologically relevant (Ohguro et al. 1993). Many receptors have several potential phosphorylation sites in their CT tail which are necessary for desensitization and sequestration, such as the β_2AR (Bohm et al. 1997a; Chuang et al. 1996; Ferguson et al. 1996a). Other receptors which have shorter CT tails or tails that contain few serine or threonine residues, have longer i3 loops which usually contain several potential phosphorylation sites. For example, both the α_2AR (Eason et al. 1995;

Liggett et al. 1992) and the m2 mAChR (Nakata et al. 1994) have large i3 loops containing phosphorylation sites necessary for desensitization. There does not appear to be a consensus sequence for any of the six known GRKs as there is for PKA and PKC. Instead, it has been proposed that the tertiary structure of the receptor resulting from ligand activation directs phosphorylation rather than a linear recognition sequence (Bohm et al. 1997a; Chuang et al. 1996; Ferguson et al. 1996a; Palczewski, 1997).

Arrestins were originally discovered when early studies with rhodopsin and β_2AR showed that GRK-mediated phosphorylation was not sufficient for full receptor desensitization. This "arresting agent" was identified by Wilden et al. (1986) to be a 48 kDa protein that bound to rhodopsin. When it was observed that a crude preparation of βARK 1 could completely desensitize β_2AR while purified βARK could not, the existence of an arrestin in this system was thought likely (Benovic et al. 1989). It was soon discovered that β-arrestin 1 along with βARK could fully desensitize β_2AR activity (Lohse et al. 1990b, 1992). Six distinct arrestins have been identified at this point. It is known that GRK-1, -2 and –3 require the binding of arrestins for receptor phosphorylation (Lohse et al. 1990b, 1992; Pippig et al. 1993); however, it is as yet unknown whether GRK-4, -5 and –6 also require arrestin binding (Bohm et al. 1997a; Chuang et al. 1996; Ferguson et al. 1996a; Palczewski, 1997).

Not a lot is known about the homologous and heterologous desensitization of the secretin/VIP receptor subfamily. The major site of phosphorylation has been shown to be the CT tail in the receptors for secretin (Ozcelebi et al. 1995; Holtmann et al. 1996b), PTH/PTHrP (Blind et al. 1995, 1996), glucagon (Buggy et al. 1997; Savage et al. 1995; Heurich et al. 1996) and GLP-1 (Thorens and Widmann, 1996; Widmann et al. 1996a,b,

1997). The phosphorylation and desensitization of the PTH/PTHrP, glucagon and GLP-1 receptors is known to involve PKA and/or PKC phosphorylation (Thorens and Widmann, 1996; Blind et al. 1995, 1996; Savage et al. 1995; Widmann et al. 1996a,b). However, while antagonism of PKA and PKC was able to inhibit the heterologous desensitization of each receptor, it could only partially inhibit homologous desensitization. It seems likely therefore, that GRKs are involved in the desensitization of GPCRs of the secretin/VIP subfamily (Thorens and Widmann, 1996; Blind et al. 1995, 1996; Savage et al. 1995; Widmann et al. 1996a,b). The phosphorylation of the recombinantly expressed PTH/PTHrP receptor CT tail *in vitro* by βARK 1 has been demonstrated (Blind et al. 1996), but no other evidence of GRK involvement in the desensitization of this receptor subfamily has been seen. It is also not yet known whether arrestins are involved in this process.

1.9.6 RECEPTOR ENDOCYTOSIS

Once a GPCR has been desensitized it is sequestered in a membrane-associated intracellular compartment (Fig. 2). This process is mainly associated with receptor resensitization, and although it is involved somewhat with the desensitization of a few receptors (Holtmann et al. 1996b; Pak et al. 1996), the internalization process is too slow for most desensitization events (Roth et al. 1991). Studies using inhibitors of internalization such as concanavalin A and sucrose have also shown that receptor desensitization is independent of receptor sequestration (Yu et al. 1993; Pippig et al. 1995). When stimulation of the cell is chronic, the receptor is down-regulated, a process in which receptor endocytosis may also be involved (Reviewed in Bohm et al. 1997a;

Chuang et al. 1996; Ferguson et al. 1996a). However, it is believed that the main function of GPCR internalization is to mediate receptor dephosphorylation and resensitization (Garland et al. 1996; Yu et al. 1993; Pippig et al. 1995; Sibley et al. 1986). Receptors isolated from a sequestered pool of membranes were shown to be phosphorylated less than receptors isolated from plasma membranes (Sibley et al. 1986). Sequestered vesicular membranes are associated with high levels of receptor phosphatase activity (Sibley et al. 1986), while phosphatase inhibitors are able to block GPCR resensitization (Garland et al. 1996; Pippig et al. 1995). As well, both dephosphorylation and resensitization of the β_2AR were blocked by pharmacological inhibition of sequestration (Pippig et al. 1995). A recent study demonstrated that a membrane-associated G-protein-coupled receptor phosphatase (GPCRP), which is activated *in vitro* by lowering the pH from neutral, is responsible for receptor dephosphorylation in the acidified endosome (Garland et al. 1996; Krueger et al. 1997). The phosphorylation sites and the GPCRP both exist in the neutral intracellular environment, therefore, it is possible that the acidification of endocytic vesicles is responsible for dissociation of ligands and the receptor-mediated conformational activation of GPCRP (Garland et al. 1996; Krueger et al. 1997).

Endocytosis is an agonist-induced process which has been demonstrated for many GPCRs. Most GPCRs, such as the NK1-R for SP (Grady et al. 1995a, 1996; Garland et al. 1996; Mantyh et al. 1995), the β_2AR (von Zastrow and Kobilka, 1992; Yu et al. 1993), and receptors for gastrin-releasing peptide (GRP) (Grady et al. 1995b), endothelin A (Chun et al. 1995), CCK (Roettger et al. 1995) and thyrotropin-releasing hormone (Ashworth et al. 1995), are internalized with their agonist into endosomes where they are

dissociated by acidification. The agonist is then degraded in lysosomes while the receptor is recycled (Bohm et al. 1997a; Ferguson et al. 1996a), although a small proportion of receptors may be sorted and targeted to lysosomes for degradation (von Zastrow and Kobilka, 1992). Some receptors such as the thrombin receptor and the proteinase-activated receptor-2 follow a different pathway (Hoxie et al. 1993; Bohm et al. 1996; Hein et al. 1994). These receptors are activated by cleavage and then internalized and targeted to lysosomes for degradation.

The internalization of GPCRs is probably accomplished via clathrin-coated pits and caveolae as there is evidence supporting both mechanisms (Ferguson et al. 1996a). The mechanism of choice is probably dependent on the individual receptor and its cellular environment. Both mechanisms have been implicated in the internalization of the β_2AR (von Zastrow and Kobilka, 1992). Dominant-negative mutants of β-arrestin and dynamin, a GTPase necessary for clathrin coated vesicle formation, both blocked the internalization of the β_2AR, but not of the $AT_{1A}R$ (Ferguson et al. 1996b; Zhang et al. 1996). Despite the $AT_{1A}R$ independence from β-arrestin and dynamin mediation, over-expression of β-arrestin was able to increase the number of receptors sequestered via clathrin-coated vesicles (Zhang et al. 1996), which suggests some flexibility in the internalization pathways.

1.9.7 DETERMINANTS OF RECEPTOR SEQUESTRATION

Sequestration via clathrin-coated pits requires the interaction of the endocytic machinery with particular domains of a given GPCR. It does not appear that there is an endocytic motif that is common to all GPCRs. Instead there seems to be several motifs

which differ among the various receptors. It was initially believed that the domains necessary for G-protein coupling were also needed for sequestration, and in fact it was found that there was an approximate correlation between GPCR coupling efficiency and the ability to be internalized (Bohm et al. 1997a; Ferguson et al. 1996a; Strader et al. 1987). But later evidence indicated that some uncoupled receptors retained the ability to internalize (Hausdorff et al. 1990; Cheung et al. 1990; Mahan et al. 1985; Petrou et al. 1997) and other studies have confirmed that these two processes are functionally distinct (Reviewed in Bohm et al. 1997a; Ferguson et al. 1996a).

The CT tail has proven to be critical for the internalization of several GPCRs, including the receptors for SP, GRP, thyrotropin stimulating hormone, AT II, gonadotropin releasing hormone (GnRH), bombesin, neurotensin, and PTH/PTHrP and the α_{1B}AR, AT_{1A}R and SSTR3 (Kreienkamp et al. 1998; Bohm et al. 1997b; Benya et al. 1993; Huang et al. 1995a; Nussenzveig et al. 1993; Thomas et al. 1995; Lattion et al. 1994; Tseng et al. 1995; Hermans et al. 1996; Roth et al. 1997), while it is not important for the internalization of the m1 mAChR (Lameh et al. 1992). More interestingly, truncation studies with the LH/CG receptor and the avian β1 or β2ARs have shown that removal of the CT tail actually increases the sequestration rate, suggesting that they contain motifs that impair endocytosis (Rodriguez et al. 1992; Parker et al. 1995; Bouvier et al. 1988). Furthermore, the SSTR4 has a single Thr residue which blocks internalization of this receptor subtype (Kreienkamp et al. 1998; Roth et al. 1997).

Tyrosine-containing endocytic motifs may be of importance in the internalization of some GPCRs. Such motifs have been well characterized in single TM proteins and typically consist of six residues forming an exposed β-turn which interacts directly with

clathrin-associated proteins (Reviewed in Bohm et al. 1997a). Studies have shown that Tyr residues in the CT tail of the neurokinin (NK)1-R and the AT II receptor are critical for internalization, although the surrounding residues do not always fit the typical consensus sequence for tyrosine endocytic motifs (Bohm et al. 1997b; Thomas et al. 1995). However, tyrosines in the CT tail of the β_2AR and m2 mAChR are not necessary for receptor sequestration (Valiquette et al. 1990; Goldman and Nathanson, 1994). There is also a highly conserved tyrosine containing motif ($NP(X)_{2,3}Y$) in TM7 of many GPCRs, which is very similar to the endocytic motifs of the low-density lipoprotein and insulin receptors (Bohm et al. 1997a; Ferguson et al. 1996a). Mutation experiments have demonstrated the importance of this domain for the internalization of the β_2AR (Barak et al. 1994, 1995; Ferguson et al. 1995) and the NK1-R (Bohm et al. 1997b), but not for the $AT_{1A}R$ (Hunyady et al. 1995) or the GRP receptor (Slice et al. 1994). It is believed that the $NP(X)_{2,3}Y$ motif may actually act to regulate the receptor conformation necessary for normal interactions with agonist and G-protein (Barak et al. 1995), probably by regulating the isomerization of GPCRs from their low- to high affinity conformation ($R \rightarrow R^*$) in response to agonist. This has been supported by studies with the m4 mAChR (Van Koppen et al. 1994) and the GnRH receptor (Arora et al. 1995).

In 1986, Sibley et al. proposed that phosphorylation might be a prerequisite not only for GPCR uncoupling, but also for internalization. This was shown to be the case for the mAChR (Lameh et al. 1992; Moro et al. 1993b), but it did not hold true for the β_2AR (Hausdorff et al. 1989; Lohse et al. 1990a). Later studies demonstrated increased m2 mAChR internalization as a result of βark 1 overexpression and overexpression of a dominant-negative βark 1 slowed the sequestration of the m2 mAChR when expressed in

COS 7 cells (Tsuga et al. 1994). Studies with a sequestration-defective mutant β_2AR-Y326A demonstrated a role for GRK-mediated phosphorylation; however, this does not appear to be positively required for internalization (Reviewed in Ferguson et al. 1996a). Overexpression of β-arrestin 1 or 2 was able to rescue β_2AR-Y326A sequestration and this was potentiated by overexpression of GRK-2 (Ferguson et al. 1996b). These findings support the suggestion that phosphorylation both promotes internalization by stabilizing the necessary β_2AR conformation and the association with other cellular elements needed for internalization (Hausdorff et al. 1989). Phosphorylation has been implicated in the sequestration of other GPCRs as demonstrated by the detrimental effects of deleting the Ser/Thr rich CT tails of the GRP, α_{1b}AR, AT_{1A}R and neurotensin receptors (Benya et al. 1993; Thomas et al. 1995; Lattion et al. 1994; Hermans et al. 1996).

The i2 loop of many GPCRs (but not members of the secretin/VIP receptor family) contains a highly conserved DRYXXV/IXXPL motif which influences sequestration (Ferguson et al. 1996a). In both the m1 mAChR and the GnRH receptor, mutation of the leucine residue was shown to hinder internalization (Arora et al. 1995; Moro et al. 1993a). The replacement of serine 140 by a tyrosine residue in the i2 loop of the GnRH receptor also increased receptor internalization by 60% and was linked to an increase in agonist binding affinity (Arora et al. 1995).

1.9.8 SEQUESTRATION OF THE SECRETIN/VIP RECEPTOR SUBFAMILY

As yet little is known about sequestration of the secretin/VIP receptor subfamily. The effects of CT truncation of members of this family have been mixed. While truncation

of the last 27 residues of the secretin receptor CT tail had only minor effects on internalization in CHO-K1 cells (Holtmann et al. 1996b), the same treatment of the glucagon (Buggy et al. 1997) and GLP-1 receptors abolished internalization (Widmann et al. 1995, 1997). Mutation studies have shown that sequestration of both the glucagon and GLP-1 receptors is contingent on phosphorylation of Ser residues within the CT tail (Buggy et al. 1997; Widmann et al. 1995, 1997) and the degree of internalization of the GLP-1 receptor depends on the number of phosphorylation sites mutated. Of the three Ser doublets in the GLP-1 receptor CT tail, the most distal Ser doublet appears to be less important to receptor sequestration than the two more proximal Ser doublets (Widmann et al. 1996a). This is similar to the glucagon (Buggy et al. 1997) and PTH receptors (Huang et al. 1995a,b) in which the more distal domains of the CT tails could be removed with little or no effect on internalization.

Unlike the GLP-1 and glucagon receptors, the calcitonin and PTH/PTHrP receptors appear to contain both positive and negative regulatory domains in their CT tails (Huang et al. 1995a; Findlay et al. 1994). Deletion of most of the calcitonin receptor CT tail had no effect on receptor internalization; however, intermediate truncations appeared to cause either internalization deficient or wild type like receptors or even improved internalization (Findlay et al. 1994). The CT tail of the PTH/PTHrP receptor is rich in Ser/Thr residues and is phosphorylated as a result of receptor activation, therefore its internalization may involve phosphorylation (Blind et al. 1995, 1996). The same is probably not true for the calcitonin receptor as its CT tail contains few Ser/Thr residues and deletion of these residues does not result in loss of sequestration (Findlay et al. 1994).

Sequestration via clathrin coated pits has been implicated for both the GLP-1 and PTH/PTHrP receptors, as treating cells with hypertonic sucrose (to disrupt clathrin lattices) inhibited their sequestration (Huang et al. 1995a; Widmann et al. 1995). These experiments were performed with fibroblast or COS 7 cell lines, so these observations must be confirmed with primary cells.

A recent study by Wheeler et al. (1999) examined the role of the six serine residues in the CT-tail of the GIP receptor in receptor sequestration. Mutation of serines 426, 427 and 440 to alanine resulted in a decrease in maximum internalization for all three mutant receptors, and a decrease in initial internalization rate for S426A and S427A. A complete CT serine knockout mutant showed an initial internalization rate of only 46% that of the wild type receptor, and a decrease in maximum internalization.

1.9.9 RECEPTOR DOWN-REGULATION

Receptor down-regulation is the reduction of total receptor number in a cell as the result of long-term exposure to agonist. Although little is known about this process in GPCRs, it is believed that increased degradation and decreased synthesis play a role (Reviewed in Bohm et al. 1997a). Destabilization of receptor mRNA has been shown to contribute to down-regulation in several GPCRs (Bohm et al. 1997a). A 35 kDa βAR mRNA binding protein (βARB) has been found to increase agonist induced destabilization by binding preferentially to one or more AUUUA pentamers in the 3' untranslated region of the β_2AR (Port et al. 1992; Tholanikunnel et al. 1995). The thrombin receptor has also been shown to undergo agonist induced down-regulation via mRNA destabilization and may very well interact with βARB (Tholanikunnel et al. 1995), as may other members of

the GPCR family. As yet, nothing is known about receptor down-regulation of members of the secretin/VIP receptor subfamily.

1.9.10 ALTERNATIVE SPLICING OF G-PROTEIN COUPLED RECEPTORS

The genomic DNA of most eukaryotes contains introns which must be removed from the primary RNA transcript (pre-mRNA) to produce an mRNA molecule which codes directly for a protein (Reviewed in Alberts et al. 1994; Chew, 1997). This procedure, known as RNA splicing, is achieved in the cell nucleus by a complex of small nuclear RNAs (snRNAs) and proteins known as the spliceosome. Introns contain splice sites at their 5' and 3' ends which are recognized by the splicing apparatus and used to excise the extraneous sequence. In some pre-mRNA molecules there are alternative splice sites which if used, result in mRNAs that contain different combinations of exons. For example the insulin receptor gene contains 22 exons, with alternative splicing of exon 11, while the calcitonin/calcitonin gene-related peptide (CGRP) gene has 6 exons with alternative splicing of exon 4 (Chew, 1997). When exon 4 is included it ends in a polyadenylation signal and the resulting product is calcitonin. When exon 4 is spliced out, exons 5 and 6 are included and the resulting product is CGRP. Alternative splicing is an important means of governing gene expression in higher eukaryotes (Smith et al. 1989) and developmental and tissue-specific factors, including hormonal and metabolic signals, contribute to the regulation of alternative splicing events (Chew, 1997). For instance, both glucose and insulin have been shown to affect the splicing of exon 11 of the human insulin receptor gene, while tissue specific factors dictate the production of calcitonin in the thyroid gland and CGRP in neural tissues (Chew, 1997).

Many alternatively spliced variants are expressed at varying levels in different tissues. While exon 11 is included in the insulin receptor mRNA at only low levels in most tissues, it is found in over 50% of the transcripts produced in the liver (Chew, 1997). The additional amino acid residues encoded by exon 11 affect the function and signaling of the insulin receptor (Kosaki et al. 1995). Sometimes alternative splicing can be used to produce receptor isoforms with opposite and competing functions, as is the case with the thyroid hormone receptor (THR) (Katz and Lazar, 1993). The THR gene erbAα exists in two mRNA isoforms, α1 and α2. The protein encoded by α1 is the functional THR, while that encoded by α2 is deficient in the TH binding domain. The erbAα gene contains an alternative 5' splice site, which if excluded results in the inclusion of the α1 exon, ending in a polyadenylation signal. When the alternative 5' splice site is chosen the transcript splices to the α2 exon, thus excluding the α1 exon.

1.9.11 ALTERNATIVE SPLICING OF MEMBERS OF THE SECRETIN/VIP G-PROTEIN COUPLED RECEPTOR SUBFAMILY

The secretin/VIP subfamily of GPCRs contains several examples of alternative splicing among its members. In 1992, while cloning the receptor for GHRH, Mayo (Mayo, 1992) discovered a rat cDNA clone with an additional 123 bases which was predicted to encode a 41 amino acid insert in the i3 loop. This study was the first demonstration of alternative splicing among members of the secretin/VIP subfamily of GPCRs. Later studies using normal human pituitary and GH-secreting pituitary tumors found two alternatively spliced variants of the GHRH receptor (Motomura et al. 1998; Hashimoto et al. 1995), which were hypothesized to be products of alternative splicing

and predicted to truncate their encoded proteins. One of the spliced variants, predicted to be truncated at the C-terminal end of the i3 loop, was unable to transduce a signal stimulated by GHRH when expressed in COS 7 cells (Hashimoto et al. 1995). It has recently been shown using co-expression of this splice variant and the wild type GHRH receptor that the truncated receptor acts as a dominant-negative inhibitor of the wild type receptor (Tang et al. 1995).

The rat pituitary adenylyl cyclase-activating polypeptide (PACAP) type I receptor (-I-R) was discovered by several groups simultaneously to exist in at least five alternatively spliced forms due to two insertions in the CT end of the i3 loop (Spengler et al. 1993; Svoboda et al. 1993; Hosoya et al. 1993; Morrow et al. 1993; Hashimoto et al. 1993; Pisegna and Wank, 1993). A sixth splicing variant was discovered soon after (Journot et al. 1994). The mouse PACAP-I-R gene was later found to express splicing variants due to the same two insertions (Aino et al. 1995). The rat isoforms of PACAP-I-R were discovered to be differentially expressed in several anterior pituitary cell types, thus suggesting differing physiological roles (Vertongen et al. 1995). More recently, an alternative splice site has been discovered in the NT domain of the PACAP-I-R gene which results in deletion of a 21 amino acid sequence (Pantaloni et al. 1996). This splice variant was found to be localized to only a few of the tissues in which the gene is normally expressed, and it appears to modulate selectivity for agonist binding.

The rat calcitonin receptor has two isoforms, CT1a and CT1b, which are alternatively spliced in the e2 domain (Sexton et al. 1993; Albrandt et al. 1993), resulting in the presence (CT1b) or absence (CT1a) of a 37-amino acid insert. The two isoforms show altered ligand recognition and binding kinetics when stably expressed in human

embryonic kidney (HEK)-293 cells (Houssami et al. 1994). An examination of mRNA levels in rat kidney showed that it expresses the CT1a isoform exclusively (Firsov et al. 1995). Interestingly, the human calcitonin receptor is alternatively spliced in its i1 loop resulting in two isoforms differing by a 16 amino acid insert (Gorn et al. 1992, 1995; Kuestner et al. 1994; Nakamura et al. 1995; Nussenzveig et al. 1995; Egerton et al. 1995), while a third isoform results from deletion of both the 16 amino acid insert and the first 47 amino acids of the NT extracellular domain (Albrandt et al. 1995). Characterization of the two human calcitonin receptor isoforms differing at only the i1 loop showed the insert-containing receptor to have altered signaling and internalization as compared to the insert-negative isoform in baby hamster kidney (BHK) cells (Moore et al. 1995). The presence of the insert negative isoform was recently demonstrated in TT cells, a cell line derived from medullary thyroid carcinoma (MTC), and in two cases of MTC (Frendo et al. 1998). In fact, it was discovered that only the insert-negative form was expressed in MTC and normal thyroids. The rabbit calcitonin receptor also exists in two isoforms, one homologous to the CT1a isoform, while the other is a spliced variant with a deletion of exon 13, which encodes 14 amino acids in TM7 (Shyu et al. 1996). The two splice variants show differing expression among various tissues and very different ligand recognition and signal transduction properties.

Using RT-PCR, Maget et al. (1994) found evidence suggesting the rat glucagon receptor may exist in several isoforms due to alternative splicing at the 5' end of the gene. They believe that two of these variants are expressed in the liver. Iwanji (1995) also observed two differently sized receptors in canine liver and kidney which he speculates may be due to alternative splicing. Dunphy et al. (1998) failed to detect multiple receptor

isoforms using RT-PCR Southern analysis; however, only the sequence between TM3 and TM6 was analyzed. Further analysis of the glucagon receptor is needed to confirm the existence of alternative spliced variants.

Alternatively spliced variants of the GIP receptor have been discovered while the current studies were in progress. Two isoforms of the human receptor which differ by a 27 amino acid insert at the juxtamembrane domain of the CT tail were cloned from a pancreatic islet cDNA library (Gremlich et al. 1995). Both variants bind GIP_{1-42} and stimulate cAMP production. Another variant was discovered encoding a truncated receptor with the deletion of exons 9 and 10, but was found to be non-functional (Gremlich et al. 1995). More recently alternative splicing has been observed in the GIP receptor expressed in the adrenals of normal adults and those with GIP-dependent Cushing's syndrome (N'Diaye et al. 1998). One isoform lacks exon 4, which encodes a section of the amino terminal domain, while the other lacks exon 9 which encodes approximately 2/3 of TM4 and half of the e2 loop. A possible splice variant of the rat GIP receptor has also been discovered which contains a newly identified 49 base pair exon termed 8a and is predicted to encode a truncated receptor with an insert in the e1 loop (Boylan et al. 1999).

1.10 HYPOTHESES AND OBJECTIVES

NIDDM is characterized by impaired insulin secretion and insulin resistance. The risk factors for this disease include dietary and environmental triggers and genetic susceptibility, with obesity being one of the strongest predictors in humans (McIntosh and Pederson, 1999). Several studies have implicated an altered entero-insular axis in various

obesity/diabetes syndromes characterized by disordered insulin secretion and fat deposition (Morgan, 1996), including studies with animal models of obesity (Flatt et al. 1983; Chan et al. 1984). GIP exerts its effects not only at the level of the pancreas, but also in adipose tissue, and therefore is a potential candidate for involvement in obesity. The obese Zucker rat is characterized by hyperlipidemia, mild hyperglycemia and hyperinsulinemia. In the strain under study in our laboratory the obese animals have also been shown to have normal circulating GIP levels, but abnormal pancreatic responses to exogenous GIP, characterized by a loss of the normal glucose threshold requirement for GIP action. Consequently it is possible that insulin secretion is stimulated by GIP under basal conditions *in vivo*. We postulated that GIP action on adipose tissue may also be altered in the obese rat, and this may be associated with changes in GIP receptor expression.

> Hypothesis 1. GIP receptor expression in adipose tissue is altered in animal models of obesity and /or NIDDM.
>
> Objective 1. To examine GIP receptor expression in adipose tissue of the fatty Zucker rat as a model of obesity and compare this to the lean Zucker rat.

GIP receptor mRNA has been detected in many tissues outside of the pancreas, including the stomach and adipose tissue, suggesting that GIP may have multiple functions in addition to its major role as an incretin (Usdin et al. 1993). Studies with $GIP_{1\text{-}30}NH_2$ showed a tissue specific reduction in activity when compared to $GIP_{1\text{-}42}$ (Pederson et al. 1990; Rossowski et al. 1992), which suggests that there may be different receptor isoforms among different tissues. As discussed in Section 1.9.11, the GIP receptor belongs to the secretin/VIP subfamily of GPCRs, several of whose members exist in differentially spliced forms. In fact, in recent years, several isoforms of the GIP receptor

have been discovered with alternative splicing occurring in the CT tail (Gremlich et al. 1995), the NT domain (N'Diaye et al. 1998), TM4 and the e2 loop (N'Diaye et al. 1998), and the i1 loop (Boylan et al. 1999). Receptor isoforms with alternative splicing of the i3 loop have also been found for the GHRH receptor (Mayo, 1992) and the PACAP-I-R (Spengler et al. 1993; Svoboda et al. 1993; Hosoya et al. 1993; Morrow et al. 1993; Hashimoto et al. 1993; Pisegna and Wank, 1993), and these have been associated with different signal transduction pathways. As mention in Section 1.9.3, the i3 loop of many GPCRs is important for G-protein recognition and coupling. Therefore, it is possible that the GIP receptor may exist in isoforms with alternative splicing of the i3 loop, which might be involved in actions of GIP not yet discovered or fully characterized.

> Hypothesis 2. The third intracellular loop of the GIP receptor exists in alternatively spliced forms.
>
> Objective 2. To use PCR to examine the third intracellular loop for the presence of cDNAs coding for alternatively spiced forms of the GIP receptor.

As mentioned previously in Section 1.9.5, homologous desensitization of GPCRs is mediated through receptor phosphorylation by serine/threonine kinases (Bohm et al. 1997a; Chuang et al. 1996; Ferguson et al. 1996a). These phosphorylation sites are present in the intracellular domains, often in the i3 loop. Phosphorylation may also be important for the internalization of some GPCRs as reviewed in Section 1.9.7, including some members of the secretin/VIP subfamily (Widmann et al. 1996a, 1997; Huang et al. 1995a; Findlay et al. 1994). There are several serines and threonines in, or adjacent to, the second and third intracellular loops of the GIP receptor, therefore they may be involved in receptor desensitization and internalization.

Hypothesis 3. The serine and threonine residues of the second and third intracellular loops of the GIP receptor are involved in receptor desensitization and internalization.

Objective 3. To use site-directed mutagenesis to generate mutant forms of the GIP receptor with alanine substituting for serine and threonine in the second and third intracellular loops, and to examine their ability to generate cAMP, undergo desensitization and be internalized in response to ligand stimulation.

CHAPTER 2

METHODS

2.1 ANIMAL EXPERIMENTS

A colony of Zucker rats was bred in the Physiology Department at the University of British Columbia. Six obese (*fa/fa*) and six lean (*Fa/?*) male Zucker rats of approximately 16 weeks of age and weighing 320-420 g were used initially. The obese rats are characterized by hyperphagia, hyperlipidemia and mild glucose intolerance and develop severe hyperinsulinemia as early as 21 days of age (McIntosh and Pederson, 1999). Oral glucose tolerance tests were performed on overnight fasted, conscious unrestrained rats and epididymal adipose tissue was collected the following day to be used for RNase protection analysis. A further six obese and six lean male Zucker rats of approximately 11 weeks of age and weighing 260-375 g underwent the same treatment and their epididymal adipose tissue was collected for RT-PCR analysis. In addition, a lean male was used for the isolation of pancreatic islets and removal of various tissues for PCR analysis in the search for splice variants.

2.1.1 ORAL GLUCOSE TOLERANCE TEST

Obese or lean rats were administered oral glucose through a gavage tube (1g/kg, 40% wt/vol dextrose at time 0). At 5, 10, 20, 30 and 60 min following the glucose challenge, blood samples were collected from the tail vein using 250 µl heparinised blood collecting tubes (Fisher Scientific, Nepean, ON). The samples were stored on ice until centrifugation (10,000 x g, 4 °C, 30 min) and then plasma was collected and frozen at –20 °C until glucose and GIP analysis

Glucose analysis was performed using the glucose oxidase method (Beckman Glucose Analyzer 2, Fullerton, CA).

2.1.2 MEASUREMENT OF GIP

Serum GIP levels were measured using a GIP RIA, but due to a lack of plasma, it was not possible to measure insulin levels. Plasma samples (100 µl each) were aliquoted into borosilicate tubes containing 200 µl Assay buffer (5% charcoal extracted plasma (CEP), 2% Trasylol, 0.4 M sodium phosphate Buffer, pH 6.5). The total counts (TC) tube received no buffer, the non-specific binding tube (NSB) 400 µl buffer and the blank tube 300 µl buffer. The control tube had buffer containing no CEP. Nine GIP standard tubes were also set up ranging from 7.8 to 2000 pg/100 µl. One hundred µl GIP antibody was added to all tubes except TC and NSB and they were vortex mixed, covered and incubated for 24 h at 4 °C. One hundred µl of ^{125}I-GIP (5000 cpm) was added to each tube and they were mixed by vortexing, covered and incubated for 24 h at 4 °C. Five hundred µl of 25% Polyethylene Glycol 8000 was added to all tubes except TC, they were vortex mixed well and centrifuged at 3000 rpm for 45 min. The tubes were decanted gently, inverted on tissue for 2-24 h and counted in a gamma counter.

2.1.3 STATISTICAL ANALYSIS OF PLASMA VALUES

Differences between lean and obese values were compared using a t-test ($P < 0.05$). This test assumes that two populations have the same variance. Where the variances were different, Welch's correction, which assumes different variances, was applied to the test.

2.1.4 ISOLATION OF RAT ISLETS

Pancreatic Islets from Zucker rats were isolated by collagenase digestion of the pancreas and purification in a discontinuous dextran gradient using a modification of the method of Van der Vliet et al. (1988). A midline incision was made from the pubis to the xiphisternum and the bowel retracted to expose the common bile duct and the pancreas. The common bile duct was clamped at the sphincter of Oddi and cannulated. The pancreas was inflated slowly using 15 ml of ice cold collagenase solution (Type XI, 0.25 mg/ml, Sigma, St. Louis, MO), excised, and trimmed of excess fat. The pancreas was placed in a 50 ml polypropylene tube and incubated in a water bath at 37 °C for ~15 min for enzymatic digestion with vigorous shaking at 0, 5 and 10 min. Digestion was stopped with the addition of ice cold Hank's Balanced Salt Solution (HBSS) (Sigma, St. Louis, MO) and the digested tissue dispersed with repeated pipetting into a 10 ml serological pipette. The tissue was centrifuged for 4 min at 100 X *g* and then washed 2X with 40 ml HBSS and further centrifugation. The tissue was dispersed in another 40 ml HBSS and the suspension was then filtered through plastic mesh (pore size of 1mm^2) to remove large particles of undigested tissue. The tissue was recentrifuged and the pellet resuspended in 10 ml of 29% dextran T-70 (in 1 X HBSS) (Amersham Pharmacia Biotech, Baie d'Orfe, PQ). Using a serological pipette, a 4 ml layer of 29% dextran was layered underneath the suspension, and 5.5 ml layers of 23% and 11% dextran were layered on top. The gradient was centrifuged for 5 min at 50 X *g* and 10 min at 450 X *g*. This caused the islets to migrate to the interface between the 23 and 11% dextran layers while the exocrine tissue remained in the 29% dextran. The islets and the 11% dextran were removed to a petri dish using a siliconized glass pipette. Using a dissecting

microscope the islets were removed to a second petri dish and washed in 1 X HBSS until no connecting tissue remained.

2.2 CULTURE AND DIFFERENTIATION OF 3T3-L1 CELLS

3T3-L1 cells (American Type Culture Collection; ATCC) were cultured in DMEM containing high glucose supplemented with 5% newborn calf serum (NCS) and containing 50 Units (U)/ml penicillin G and 50 μg/ml streptomycin (culture media and antibiotics from Gibco BRL, Grand Island, NJ) in 12 or 24 well culture plates. Cells were induced to differentiate into the adipocyte phenotype by a modification of the method described by Rentsch and Chiesi (1996). Two days after cells were confluent, the medium was supplemented with dexamethasone (0.6 μM) for 72 h. Cells were then cultured in DMEM high glucose medium containing 10% fetal calf serum (FCS). Differentiation was complete in 7 days.

2.3 ISOLATION OF RNA

2.3.1 ISOLATION OF RNA FOR RIBONUCLEASE PROTECTION ANALYSIS

RNA was isolated from cultured 3T3-L1 cells or the epididymal adipose tissue of Zucker rats for RNase protection analysis using the Qiagen Rneasy Mini Kit. Cells from 7 X 10 cm culture dishes were washed in PBS, trypsinized, and pelleted by centrifugation at 1200 X *g* for 5 min. The cells were resuspended in 600 μl lysis buffer (Buffer RLT*), vortex mixed well and homogenized by passing 5X through an 18 syringe needle. Tissue was homogenized in 350 μl Buffer RLT/20 mg tissue and snap frozen. On the day of

isolation the lysate was thawed, centrifuged for 3 min at 12 500 rpm in a microfuge tube and the supernatant transferred to a new tube. One volume of 70% ethanol was added to the lysate and mixed by pipetting. The sample was applied to an Rneasy mini spin column placed in a 2 ml collection tube, 700 µl at a time, and centrifuged for 15 sec at 12 500 rpm. The flow-through was discarded, the column incubated with 700 µl wash buffer (Buffer RW1) for 5 min, and centrifuged for 15 sec at 12 500 rpm. The column was transferred to a new 2 ml collection tube and washed 2X with 500 µl wash buffer (Buffer RPE). After the second wash the column was centrifuged for 2 min at maximum speed to dry the membrane. The column was transferred to a 1.5 ml collection tube and the RNA eluted 2X by pipetting 30 µl of DEPC-treated dH_2O onto the membrane, incubating for 10 min and centrifuging at 12 500 rpm for 1 min. RNA was treated with DNase (1 U/µg RNA; Gibco BRL, Life Technologies, Burlington, ON) at 37 °C for 30 min, then heated at 75 °C for 5 min. RNA to be used immediately was stored at –20 °C while long term storage was at -70 °C. RNA was quantitated by measuring the A_{260}.

2.3.2 ISOLATION OF RNA FOR REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION ANALYSIS

Total RNA was isolated from the epididymal adipose tissue of Zucker rats for RT-PCR analysis using a modification of the method of Chomczynski and Sacchi (1987) (Trizol; Gibco BRL, Life Technologies, Burlington, ON). Fresh tissue was homogenized in 1 ml Trizol/50-100 mg tissue in 50 ml conical Falcon tubes. Samples were centrifuged (3000 rpm, 4 °C, 10 min) and the supernatant transferred to fresh tubes where they were

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* Buffer details are not supplied by the manufacturer.

incubated at room temperature for 5 min. Two hundred µl chloroform per 1 ml Trizol were added, samples were shaken vigorously for 15 sec and incubated at room temperature for 10 min during which time samples were aliquoted to 1.5 ml microcentrifuge tubes. Samples were then centrifuged (12 000 X *g*, 4 °C, 15 min) and the aqueous phase (top) transferred to fresh microcentrifuge tubes. Five hundred µl isopropanol per 1 ml Trizol were added, and samples mixed and incubated for 10 min. Samples were then centrifuged (12 000 X *g*, 4 °C, 10 min), the supernatant removed and the RNA pellet washed with 75% ethanol. RNA was centrifuged (7 500 X *g*, 4 °C, 5 min), the supernatant removed and the pellets air-dried for 10 min. The pellets were dissolved in 15 µl water and incubated at 60 °C for 10 min. RNA to be used immediately was treated with DNase (1 U/µg RNA; Gibco BRL, Life Technologies, Burlington, ON) at 37 °C for 30 min, then heated at 75 °C for 5 min and stored at –20 °C. RNA was stored long term at –70 °C. RNA was quantified by measuring the A_{260}.

2.3.3 ISOLATION OF RNA FOR ANALYSIS OF THE GIP RECEPTOR THIRD INTRACELLULAR LOOP

Total RNA was isolated from adipose tissue and pancreatic islets of a lean male Zucker rat using the acid guanidinium thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi (1987). Tissue was homogenized in 1 ml solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7; 0.5% sarcosyl, 0.1 M 2-mercaptoethanol)/100 mg tissue in 50 ml conical Falcon tubes, and islets (200-400) were lysed in 1 ml solution D in 15 ml conical tubes. The homogenates were passed 5X through a 22 gauge syringe needle, snap frozen and stored at -70 °C until use.

Sequentially, 0.1 volume 2 M sodium acetate, pH 4, 1 volume phenol (water saturated), 0.2 volume chloroform-isoamyl alcohol (49:1) were added to the homogenate and mixed by inversion. The final reaction was shaken vigorously for 10 sec, incubated on ice for 15 min, and centrifuged at 10 000 *g* for 20 min at 4 °C. The RNA containing aqueous phase was transferred to a fresh tube, 1 ml isopropanol was added, and the mixture incubated at -20 °C for a minimum of 1 h. The sample was centrifuged at 10 000 *g* for 20 min and the RNA pellet dissolved in 0.3 ml solution D. The solution was transferred to a fresh 1.5 ml microcentrifuge tube and precipitated with 1 volume isopropanol at -20 °C for 1 h. The sample was centrifuged for 10 min at 4 °C, washed with 75% ethanol and the RNA resuspended in DEPC-treated dH_2O containing RNase inhibitor (RNAguard, Amersham Pharmacia Biotech, Baie d'Orfe, PQ). RNA to be used immediately was stored at –20 °C while long term storage was at -70 °C. RNA was quantitated by measuring the A_{260}.

2.4 RIBONUCLEASE PROTECTION ASSAY

A ribonuclease (RNase) protection assay (RPA) was used to detect the presence of the GIP receptor mRNA in extracts from 3T3-L1 cells and epididymal adipose tissue from Zucker rats. An RPA is a very sensitive procedure which allows the detection and quantitation of an RNA molecule from among total cellular RNA. An RNA probe complementary to part of the target RNA is allowed to hybridize to the target RNA. Single stranded RNA is digested while the double stranded RNA is protected from digestion. When present in molar excess over the target fragment, the probe can be used to measure the amount of target RNA in the sample (Fig. 3).

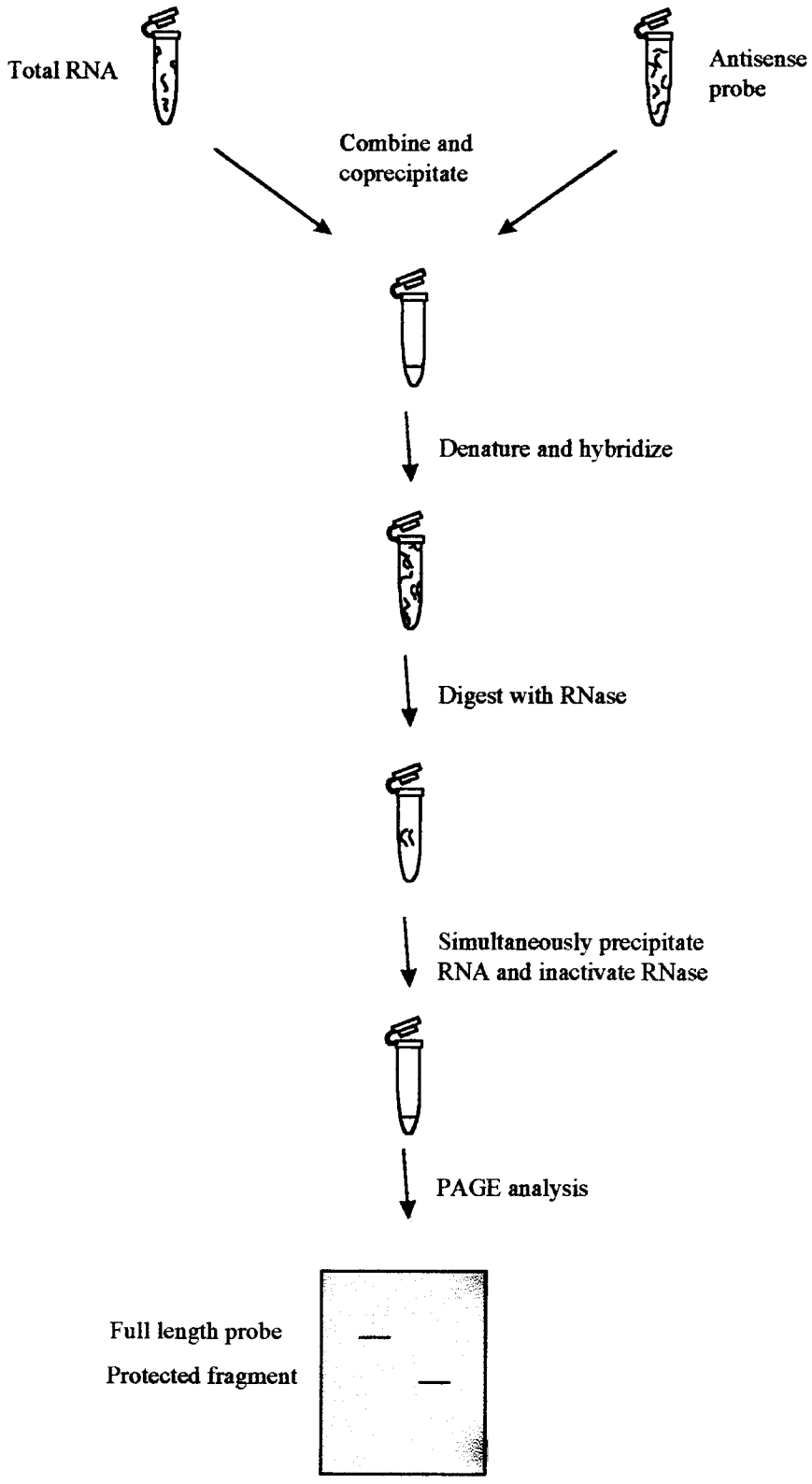


Fig. 3. Ribonuclease Protection Assay. See text for details. Adapted from Ambion RPA II™.

An antisense probe, complementary to nucleotides 1295-1534 of the C-terminal tail of the GIP receptor, was transcribed using the Megascript™ SP6 kit (Ambion, Inc., Austin, TX). The DNA template was prepared by linearizing the GIP receptor with the restriction enzyme Afl II, which cuts after base 1294, and the linearized receptor was gel purified. The RNA probe was transcribed for 2 h at RT in a total volume of 20 µl containing 2 µg template DNA, 1 X Transcription Buffer, 0.5 mM each ATP, CTP, GTP and UTP Solutions, 20 U SP6 and 10 U RNase Inhibitor. The enzyme was denatured for 2 min at 95 °C, transferred to an ice-water bath, 2 U Dnase I added and the mixture incubated at 37 °C for 15 min. The transcript was electrophoresed on a 1.5 mm thick 5% polyacrylamide 8 M urea gel and visualized by UV shadowing. The RNA band was excised with a sterile scalpel blade and eluted in 150-350 µl Probe Elution Buffer overnight at RT. The next day the probe was precipitated with 3 volumes EtOH, centrifuged at 12 000 *g* at 4 °C for 15 min., washed with 70% EtOH and resuspended in TE to a concentration of 50 ng/µl. A β-actin control probe was prepared using the same procedure with a pTRI-β-actin-mouse plasmid DNA template from the Megascript™ SP6 kit. An 18S ribosomal control probe was also prepared using the same procedure with a pTRI RNA 18S antisense control template (Ambion, Inc., Austin, TX). The probes were labeled with psoralen-biotin using the BrightStar™ Psoralen-Biotin Nonisotopic Labeling Kit (Ambion, Inc., Austin, TX). The RNA probe (0.5 µg) was mixed with 1 µl Psoralen-Biotin reagent in a microtiter plate in the dark, and while on ice placed directly under a 365 nm UV lamp for 45 min. The sample was then diluted with 89 µl 1 X TE and

extracted twice with 200 µl dH_2O-saturated n-Butanol. RNA probes, labeled and unlabeled were stored at –20 °C for short term and –70 °C for long term.

An RPA was performed using the RPA II™ Kit (Ambion, Inc., Austin, TX). Five µg total RNA were coprecipitated in a 1.5 ml microcentrifuge tube with 200 ng GIP receptor probe. In later experiments 420 ng of the 18S ribosomal probe were also added. Control tubes with 2.5-10 µg/µl mouse liver total RNA and β-actin probe (1 µl of the total reaction) and two control tubes with 5 µg Yeast RNA and 200 ng GIP receptor probe were also prepared. The concentration of ammonium acetate (NH_4OAc) was adjusted to 0.5 M with 5 M NH_4OAc and 2.5 volumes of EtOH were added, mixed, and placed at –20 °C for 15 min. The RNA was pelleted at 12 000 *g* and 4 °C for 15 min and then dissolved in 20 µl Hybridization Buffer. The RNA was denatured at 90 °C for 4 min and incubated in a hybridization oven at 44 °C for 18 h. An RNase A/RNnase T1 Mix was diluted 1:50 in RNase Digestion Buffer and 200 µl added to each sample tube and one yeast control tube. The single stranded RNA was digested at 37 °C for 30 min and 300 µl RNase Inactivation/Precipitation Solution, 100 µl EtOH and 1.5 µl Yeast RNA were added. The samples were precipitated at –20 °C for 15 min, centrifuged at 12 000 *g* and 4 °C for 15 min and resuspended in 18 µl Gel Loading Buffer II. The double stranded RNA was separated on a 0.5 mm thick 5% polyacrylamide 8 M urea gel until the bromophenol blue dye band had just run off the gel. The RNA was transferred to a positively charged nylon membrane (BrightStar™-Plus Membrane, Ambion, Inc., Austin, TX) by electroblotting at 200 mA for 1 h and then crosslinked in a microwave oven for 2.5 min at full power. Biotinylated RNA was detected using a BrightStar™ Biodetect™

Nonisotopic Detection Kit (Ambion, Inc., Austin, TX). The membrane was washed 2 X 5 min in 1 X Wash Buffer (1ml/cm^2), 2 X 5 min in Blocking Buffer (0.5 ml/cm^2) and 30 min in Blocking Buffer (1ml/cm^2). The membrane was then washed for 30 min in Conjugate Solution (10 ml Blocking Buffer plus 1µl Streptavidin•Alkaline Phosphatase Conjugate/100 cm^2), a covalent conjugate which binds the biotinylated probe. The membrane was washed for 15 min in Blocking Buffer (0.5 ml/cm^2), 3 X 15 min in 1 X Wash Buffer (1ml/cm^2) and 2 X 2 min in 1 X Assay Buffer (0.5 ml/cm^2). The membrane was then washed for 5 min in CDP-Star™ (5 ml/100 cm^2), an alkaline phosphatase-reactive 1,2-dioxetane chemiluminescent substrate. The membrane was blotted quickly on filter paper, wrapped in plastic wrap, and exposed to an autoradiograph film for 5 min. Due to problems with the procedure it was decided to quantify mRNA levels using real-time PCR.

2.5 REVERSE TRANSCRIPTION AND POLYMERASE CHAIN REACTION

2.5.1 IDENTIFICATION OF ADIPOSE TISSUE GIP RECEPTOR USING RT-PCR

Total RNA (3 µg) from adipose tissue of 11 week old Zucker rats, isolated as described in Section 2.3.2, was primed with 30 pmol of a reverse primer (5'-ATGTGCCTGGAGATGAGGTCTTG-3') complementary to nucleotides 1497-1519 of the CT tail. The mixture was denatured at 85 °C for 10 min and then put on ice for 2 min. The tube was centrifuged briefly and placed at 50 °C. A mix containing 50mM Tris-HCl (pH 8.3), 75mM KCl, 3mM $MgCl_2$, 2mM dNTPs, 1mM dithiothreitol (DTT), and 1 U RNase inhibitor (Amersham Pharmacia Biotech, Baie d'Orfe, PQ) was added, and 2 min later 340 U Superscript II Rnase H$^-$ Reverse Transcriptase (Gibco BRL, Grand

Island, NJ) was added to make a total volume of 20µl. The reaction was incubated at 50 °C for 60 min and then the enzyme was inactivated at 75 °C for 10 min.

The C-terminal tail region of the rat GIP receptor was amplified using cDNA oligonucleotide primers corresponding to nucleotides 1271-1293 (5'-GCCAAACTGGCCTTTGAAATCTT-3') and 1497-1519 (ATGTGCCTGGAGATGAGGTCTTG-3'). Four µl of the reverse transcription product were amplified in a 25 µl polymerase chain reaction (PCR) containing: 67 mM Tris-HCl, 3 mM $MgSO_4$, 166 mM $(NH_4)_2SO_4$, 10mM β-mercaptoethanol (pH 8.3), 2.5 mM $MgCl_2$, 400 µM dNTPs, 5 pmol of each primer and 1 U of *Taq* DNA Polymerase. PCR conditions were as follows: 3 min denaturation at 95 °C, annealing at 59 °C for 45 sec, extension for 45 sec at 72 °C for one cycle followed by 35 cycles with 30 sec denaturation, 45 sec annealing and extension segments and a final extension of 5 min. Contamination from an unknown source became a problem and although steps were taken to resolve this, they were unsuccessful. Therefore, it was decided to measure GIP receptor mRNA levels using real-time PCR.

2.5.2 REAL-TIME PCR ANALYSIS OF GIP RECEPTOR LEVELS

Real-time PCR utilizes a fluorogenic probe which hybridizes within the target sequence (Orlando et al. 1998). The probe contains a fluorescent reporter dye (6-carboxy-fluorescein (FAM)) covalently linked to the 5' end and a quencher dye (6-carboxy-tetramethylrhodamine (TAMRA)) covalently linked to the 3' end (Fig. 4A). When the probe is intact, the reporter fluorescence is suppressed because of its closeness to the quencher emitter. During PCR cycling, the probe is cleaved by the 5'→3'

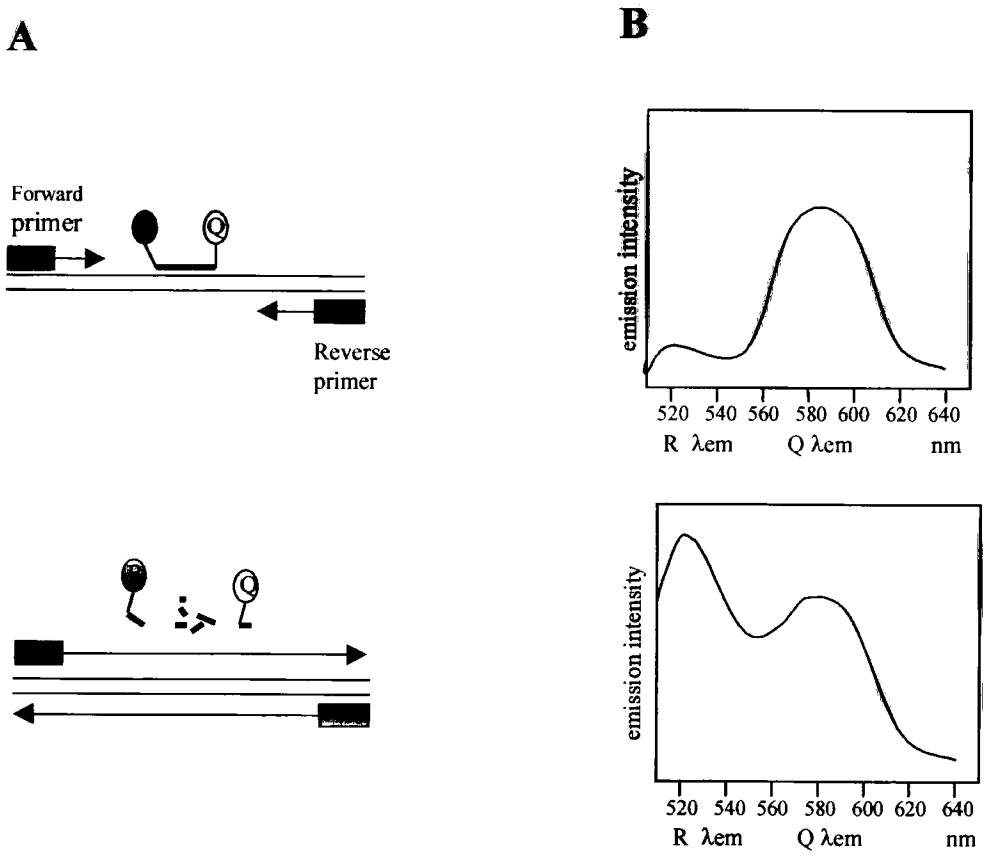


Fig. 4. Fluorescence Emission by TaqMan™ Probe Reaction in Real-Time PCR. A. The fluorogenic probe anneals to the target sequence and is cleaved by the 5' to 3' exonuclease activity of Taq DNA polymerase. B. While the fluorogenic probe is intact the quencher dye suppresses reporter fluorescence. When the probe is cleaved, reporter fluorescence emissions are released. See text for details. Adapted from Orlando et al. (1998).

exonucleolytic activity of Taq polymerase and the fluorescence emission of the reporter dye increases without affecting the quencher dye emission (Fig. 4B). The exonuclease activity of Taq polymerase will act only when the fluorogenic probe is annealed to the target sequence and the enzyme is unable to hydrolyze the probe when it is free in solution. Thus, the increase of fluorescence is directly proportional to the amount of specific PCR product.

Total RNA (2 μg) from the 11 week old Zucker rats was primed with 30 pmol of a reverse primer (5'-ATGTGCCTGGAGATGAGGTCTTG-3') complementary to nucleotides 1497-1519 of the CT tail. The reaction then proceeded as described in Section 2.5.2.

Two μl (200 ng) of rat adipose tissue cDNA were used in the real-time PCR reaction to measure GIP receptor expression, while 2 μl (20 ng) cDNA were used in the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control PCR reaction. The PCR reaction mix consisted of 2.5 μl of 10x TaqMan™ Buffer A (PE Applied Biosystems, Foster City, CA), 10mM $MgCl_2$, 200 μM dATP, dCTP, dGTP and 400 μM dUTP, 200 nM GIP receptor 5' forward primer (5'-CCGCGCTTTTCGTCATCC-3'), 200 nM GIP receptor 3' reverse primer (5'-CCACCAAATGGCTTTGACTT-3'), 200 nM GIP receptor probe (5'-CCCAGCACTGCGTGTTCTCGTACAGG-3'), 0.01 U/μl AmpErase UNG* (PE Applied Biosystems), and 0.025 U/μl of AmpliTaq Gold (PE Applied

*AmpEraseUNG or Uracil DNA Glycosylase catalyzes the removal of uracil from double stranded DNA which has been synthesized in the presence of dUTP. dUTP is substituted for dTTP during PCR amplification. Subsequent PCR reaction mixtures are treated with UNG before amplification and the abasic polynucleotides are cleaved during the initial denaturation step. This prevents contamination by products of previous PCR reactions.

Biosystems). The GAPDH control reactions were performed as above with the exception that the primers and probe were purchased from PE Applied Biosystems and were directed towards rodent GAPDH. PCR reactions were done in triplicate in the PE Applied Biosystems 7700 sequence detection system. The reaction profile included a 10 min preincubation at 50 °C to allow the UNG to degrade any previous PCR contamination and a further 10 min incubation at 94 °C to activate the AmpliTaq Gold. Following these preincubations, a two-step PCR protocol was carried out: a denaturation step at 94 °C for 15 sec followed by a 1 min annealing/extension step at 60 °C. Fluorescence was measured during the annealing/extension steps over 40 cycles and used to calculate a cycle threshold (Ct), i.e. the point at which the reaction is in the exponential phase and is detectable by the hardware. All reactions followed the typical sigmoidal reaction profile, and Ct was used as a measure of GIP receptor presence. The GIP receptor Ct data was corrected for GAPDH Ct data and then expressed as a ratio of GIP receptor mRNA to GAPDH mRNA in the PCR sample.

2.5.3 REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION ANALYSIS OF THE GIP RECEPTOR THIRD INTRACELLULAR LOOP

Total RNA (1-5 μg) from adipose tissue and pancreatic islets of a lean male Zucker rat was primed with 0.5 μg oligo deoxythymidine (dT) and 0.5 μg random hexamers (Amersham Pharmacia Biotech, Baie d'Orfe, PQ) in a total volume of 11 μl in a 500 μl microcentrifuge tube. The mixture was denatured at 70 °C for 10 min, centrifuged briefly, allowed to anneal at RT for 10 min, then chilled on ice for 2 min. Reverse transcription took place at 42 °C for 1 h with 200 U Superscript II Rnase H$^-$ Reverse

Transcriptase (Gibco BRL, Grand Island, NJ) in a solution containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 0.5 mM dNTPs, 10 mM DTT, and 10 U RNase inhibitor in a final volume of 20 µl. The reaction was terminated by adding 80 µl dH_20 and heating at 95 °C for 5 min.

The third intracellular loop of the GIP receptor was amplified using cDNA oligonucleotide primers complimentary to nucleotides 1049-1070 (5'-ATCATTCGCACCCCCATCCTAA-3') and 1209-1231 (5'-GTGTCCACGAGGTGGTATTTGCT-3') of TM5 and TM6. One µl of the reverse transcription product was amplified in a total PCR volume of 50 µl containing 67 mM Tris-HCl, 3 mM $MgSO_4$, 166 mM $(NH_4)_2SO_4$, 10mM β-mercaptoethanol (pH 8.3), 2.5 mM $MgCl_2$, 200 µM dNTPs, 5 pmol of each primer and 0.5 U of *Taq* DNA Polymerase. PCR was run for 35 cycles with segments of 1 min each. The denaturation temperature was 94 °C and the elongation was 72 °C. The first 5 annealing segments took place at 68 °C, 66 °C, 64 °C, 62 °C, and 60 °C and the remaining 30 cycles at 58 °C. The PCR products were purified on a 2% agarose gel, the bands of interest excised, and electroeluted. Purified PCR products were ligated into pGEM™-T (Promega, Madison, WI) using the pGEM™-T Vector System I. The ligation products were used to transform competent DH5α cells as described previously. Three colonies from each transformation plate were used to inoculate LB cultures and plasmid DNA was isolated and sequenced.

Some of the plasmid DNA originating from rat islets was sequenced by the Nucleic Acid Protein Sequencing Unit (University of British Columbia, Vancouver, BC). The remaining plasmid DNA originating from rat islets and rat adipose tissue was sequenced as described in Section 2.6.

2.6 PLASMID DNA SEQUENCE ANALYSIS

Purified plasmid DNA sequence was determined using a ^{T7}Sequencing(Kit (Amersham Pharmacia Biotech, Baie d'Orfe, PQ) with some modifications. Template DNA (1.5-2 μg) was combined with 10 pmol primer and 1 μl dimethylsulfoxide (DMSO) in a total volume of 13 μl. The mixture was denatured at 95 °C for 5 min, snap frozen in a dry ice/ethanol bath, thawed, centrifuged briefly and 2 μl Annealing Buffer were added. The reaction was incubated at RT for 5-10 min and then 3 μl Labeling Mix-dATP, 0.6-1.0 μl [α-^{35}S]dATPαS and 2 μl diluted (1:7) T7 DNA Polymerase were added. The mixture was incubated at RT for 10 min and 4.5 μl removed and added to 2.5 μl each of the four termination mixtures (G, A, T and C) previously aliquoted in a microtitre plate and preheated to 37 °C. The termination reaction was incubated at 37 °C for 5 min and then stopped with 5 μl Stop Solution. The sequence reactions were stored at -20 °C until separation by acrylamide gel electrophoresis.

Samples were separated by electrophoresis on a 6% acrylamide, 8 M urea, 1 X tris-borate-EDTA (TBE) buffered gel in a Model S2 sequencing apparatus (Gibco BRL, Grand Island, NJ). The top chamber was filled with 1 X TBE and the bottom with 250 ml 0.5 X TBE. The gel was pre-warmed by running at 60 W for 30-60 min. Samples were then loaded and run at 60 W for 3 hours. An equal volume of 3 M sodium acetate was then added to the bottom chamber and samples were re-loaded in adjacent lanes and run for another 3 hours until the cyanol bromide (second migrating dye in loading buffer) was ¾ the way down the gel. The plates were then separated, the gel was transferred to 3MM Whatman filter paper and dried on a gel dryer. The gel was exposed to an autoradiograph film (Kodak, Rochester, NY) for 60-72 h and the sequence was read manually.

2.7 SITE-DIRECTED MUTAGENESIS OF THE RAT PANCREATIC GIP RECEPTOR

Several point mutations were made in the rat GIP receptor using the Morph™ Site-Specific Plasmid DNA Mutagenesis Kit (5-prime(3-prime, Boulder, CO) (Fig. 5). A single base change was made at nucleotide 908 (T→G) resulting in the amino acid substitution of an alanine for a serine at amino acid residue 248 in the i2 loop. Single base changes were made at nucleotides 1124 (T→G) and 1181 (T→G) also resulting in the substitution of an alanine for a serine at amino acid residues 320 in TM5 and 339 in TM6. Single base changes were made at nucleotides 1136 (A→G), 1184 (A→G) and 1190 (A→G) resulting in the substitution of an alanine for a threonine at amino acid residues 324 in the i3 loop and 340 and 342 in TM6. Mutagenic primers corresponding to coding nucleotides were designed as follows (single base change is underlined): nucleotides

899-916 (5'-GTGAGACGCGCAGAAAAG-3')

1114-1134 (5'-CATCCTTGTTGCAAAGCTGAG-3')

1125-1147 (5'-CAAAGCTGAGGGCTCGACAGATG-3')

1175-1192 (5'-GCTCGCGCCACGCTGACA-3')

1172-1198 (5'-CTGGCTCGCTCCGCGCTGACACTGATG-3')

1180-1198 (5'-CTCCACGCTGGCACTGATGC-3')

Each mutagenic oligonucleotide (2.5 µl) was phosphorylated at the 5-prime end with 25 U T4 Polynucleotide Kinase (Amersham Pharmacia Biotech, Baie d'Orfe, PQ) in a 50 µl total reaction volume containing 70 mM Tris-Cl, pH 7.6, 10 mM $MgCl_2$, 5 mM Dithiothreitol and 1 mM ATP. The reaction was incubated at 37 °C for 1 h and then terminated by heating at 65 °C for 10 min. One hundred ng of each phosphorylated

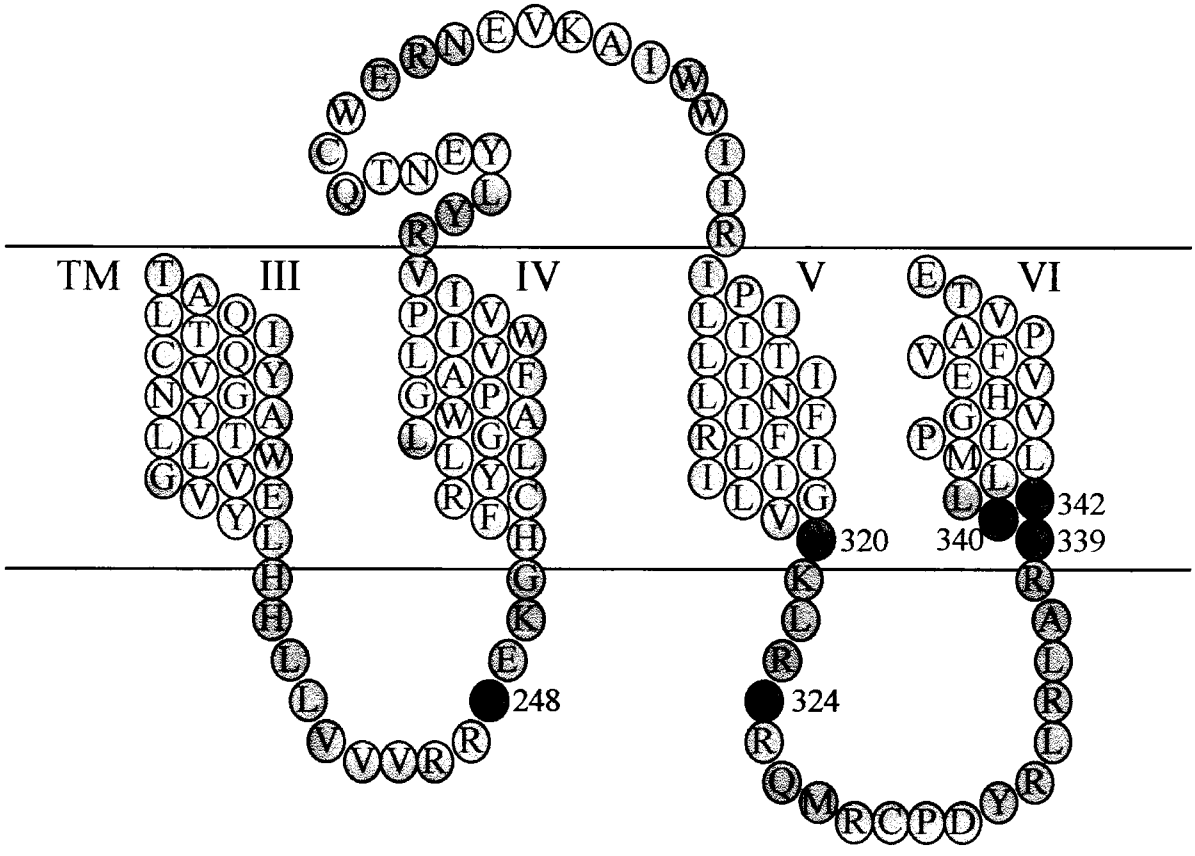


Fig. 5. Amino Acid Sequence of the GIP Receptor from TM III to VI. Sites of mutation are highlighted in purple.

mutagenic oligonucleotide was added to 30 fmol double stranded template (pBKS/GIPR1) in a total reaction volume of 20 µl 1 x Morph™ Annealing Buffer. The mixture was denatured at 100 °C for 5 min, placed in an ice-water bath for 5 min and allowed to anneal at RT for 30 min. A replacement strand was synthesized by adding 8 µl Morph™ Synthesis Buffer, 3 U T4 DNA Polymerase and 4 U T4 DNA Ligase to the primer-template mixture and incubating at 37 °C for 2 h. The reaction was terminated by heating at 85 °C for 15 min. The reaction resulted in a mixture of hemi-methylated half-mutant plasmid and fully methylated wild type target plasmid This mixture was incubated with the restriction endonuclease *Dpn* I at 37 °C for 30 min which digested the methylated non-mutated target plasmid DNA into linear molecules of low transformation efficiency. The circular half-mutant plasmid DNA was left transformation competent. Each mutagenesis reaction was transformed into 200 µl of competent *E. Coli* MORPH™ *mutS* cells. This strain of *E. Coli* has had the methylation-specific repair system inactivated so that when it finds base mismatches it will not recognize the methylated strand as correct but will randomly pick one of the strands as correct and repair the other one. This means that approximately 50% of the colonies will contain the mutation. The transformed cells were plated on Luria Broth (LB) (1% Bactotryptone, 0.5% yeast extract, 1% NaCl) plates containing 0.01% Ampicillin (Sigma, St. Louis, MO) and incubated overnight at 37 °C.

Ten to 15 colonies were picked for screening and grown up overnight in 3.5 ml LB (0.01% Ampicillin) at 37 °C in a shaking platform at 300 rpm. Plasmid DNA was isolated using standard procedures. Cells were centrifuged at 7000 rpm in a microcentrifuge resuspended in 150 µl lysis buffer (50mM glucose, 25mM Tris-HCl, pH 8, 10mM disodium ethylenediaminetetraacetate (EDTA)) by vortex mixing and incubated at RT for

5 min. Three hundred µl freshly prepared alkaline solution (0.2 M NaOH, 1% SDS) were added, and samples were mixed by inversion and placed on ice for 5 min. Two hundred µl of ice cold 7.5 M ammonium acetate (pH 7.6) was added, and samples were mixed gently by inversion and placed on ice for 5 min. Samples were then centrifuged at 12 500 rpm for 5 min and the supernatant was transferred to fresh microcentrifuge tubes. 0.6 volumes of isopropanol was added, samples vortex mixed and incubated at RT for 10 min. The supernatant was discarded, 100 µl of 2 M ammonium acetate (pH 7.4) were added, pellets were vortex mixed violently, incubated on ice for 5 min and centrifuged at 12 500 rpm for 5 min. The supernatant was transferred to a set of fresh tubes, 100 µl of isopropanol added and samples were mixed and incubated at RT for 10 min. Samples were centrifuged at 12 500 rpm for 10 min, the supernatant was discarded, the pellets were resuspended in 20 µl dH_2O (0.005% Rnase, Amersham Pharmacia Biotech, Baie d'Orfe, PQ) and incubated at RT for 15 min. The presence of plasmid DNA containing an insert was verified by a restriction digest of 1 µl DNA with *Hind* III and *Xho* I at 37 °C for 1 h. Digested plasmid DNA was visualized on a 1% agarose gel stained with ethidium bromide (Sigma, St. Louis, MO) and concentration of DNA was estimated for sequencing. The DNA was sequenced as described previously in Section 2.6.

Plasmid DNA which contained the appropriate mutation was subjected to digestion with the restriction enzymes *Hind* III and *Xba* I thus removing the insert from pBKS. The DNA was gel purified and electroeluted, and ligated into pcDNA3. Transformation competent DH5α cells were transformed with 5 µl of the ligation product, plated on LB (0.01% Ampicillin) agar plates and incubated overnight at 37 °C.

Two or three colonies were picked for further screening and of those containing the appropriate mutation, one was chosen to use for transfection.

Plasmid DNA was isolated using a Qiagen Plasmid Midi kit (Chatsworth, CA). A single colony was used to inoculate 2 ml LB (0.01% Ampicillin) which was incubated for 6-8 h at 37 °C and 300 rpm. This culture was then diluted 1/500 into 25 ml LB (0.01% Amp) and incubated overnight at 37 °C and 300 rpm. Cells were centrifuged at 6000 X *g* for 15 min at 4 °C and the supernatant removed. Cells were resuspended in 4 ml Resuspension Buffer (50mM Tris-HCl, pH 8, 10mM EDTA, 100 μg/ml Rnase A), 4 ml Lysis Buffer (200mM NaOH, 1% SDS) was added, the mixture was mixed by inversion six times and incubated at RT for 5 min. Four ml Neutralization Buffer (3.0 M potassium acetate, pH 5.5) was added, the mixture was inverted six times, incubated on ice for 15 min and centrifuged at 20 000 X *g* for 30 min at 4 °C . The supernatant was removed and re-centrifuged at 20 000 X *g* for 15 min at 4 °C . The supernatant was removed and applied to a Qiagen-tip 100 previously equilibrated by application of 4 ml Equilibration Buffer (750 mM NaCl, 50mM MOPS, pH 7, 15% isopropanol, 0.15% Triton (X-100). The Qiagen-tip was washed twice with 10 ml Wash Buffer (1.0 M NaCl, 50 mM MOPS, pH 7, 15% isopropanol) and the DNA eluted with 5 ml Elution Buffer (1.25 M NaCl, 50mM Tris, Tris(Cl, pH 8.5, 15% isopropanol). The DNA was precipitated with 3.5 ml isopropanol and centrifuged at 15 000 X *g* for 30 min at 4 °C . The supernatant was decanted and the DNA pellet washed twice with 2 ml 70% ethanol and centrifuged at 15 000 X *g* for 10 min. The supernatant was decanted, the pellet air dried for 10 min and resuspended in 500 μl Tris-EDTA (TE) (10 mM TrisCl, pH 8.0, 1mM EDTA). The plasmid DNA isolated had an A_{260}/A_{280} ratio of 1.6-1.8.

2.8 CELL CULTURE AND TRANSFECTION

Chinese Hamster Ovary (CHO-K1) cells were cultured in DMEM/Ham's F12 supplemented with 10% NCS and containing 50 U/ml penicillin G and 50 μg/ml streptomycin (culture media and antibiotics from Gibco BRL, Grand Island, NJ). Cells were grown at 37 °C in 75 cm^2 flasks until 80-100 % confluent, and split 1:10-1:20 for maintenance of exponentially growing cultures.

Stable CHO-K1 cell lines expressing the mutated forms of the rat GIP receptor were produced by using the $CaPO_4$ co-precipitation method of Sambrook et al. (1989) with minor modifications. Cells were grown on 10 cm dishes until 80-100% confluent. The cells were fed with 5-7 ml fresh culture media 2-4 h before the transfection. Ten μg of DNA were added to 62.5 μl of 2.5 M $CaCl_2$ and the total volume brought up to 500 μl with sterile water. An equal volume 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) was added and the mixture vortex mixed. The precipitate was then allowed to form at RT for 30-60 min. The entire mixture was then added to one plate and incubated at 37 °C for 4 h. The cells were shocked with 15% glycerol/1 X HBS for 1.5 min and allowed to recover in 5-7 ml fresh growth medium overnight. The cells were then split onto 2 plates and those expressing the rat GIP receptor were isolated by G418 selection (800 μg/ml) (Gibco BRL, Grand Island, NJ), changing the media every 2-3 days as needed. After 8-9 days individual clones were isolated and further selected for high or low level expression by screening for ^{125}I-GIP binding. When confluent, pooled clones were tested for GIP

receptor expression by binding analysis. The GIPR/T340A mutant did not display competitive binding of GIP so no further experiments were performed with this mutant.

2.9 BINDING ANALYSIS

2.9.1 ^{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 a few modifications. Five μg of porcine GIP in a siliconized test tube was dissolved in 100 μl of 0.4 M phosphate buffer (pH 7.5) and 10 μl (1mCi) of Na^{125}I was added. Ten μl of chloramine T (4 mg/ml in 0.4 M phosphate buffer, pH 7.5) was added and 15 sec later the reaction was stopped with the addition of 20 μl sodium metabisulphite (14.8 mg/ml in 0.4 M phosphate buffer, pH 7.5). The mixture was applied to a column (0.5 X 10 cm) of Sephadex® G-15 (Amersham Pharmacia Biotech, Baie d'Orfe, PQ) which had been equilibrated for 4-6 h in 0.2 M acetic acid containing 2% RIA grade bovine serum albumin (BSA). On the day of iodination 2% aprotinin (Trasylol, Sigma, St. Louis, MO) was added to the column buffer and was allowed to equilibrated for at least 1 h before the iodination mixture was applied.

The peak ^{125}I-GIP fractions from the gel filtration chromatography were purified by HPLC on a μBondapak C-18 column (Walters Associates Inc., Milford, MA) using two 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. The different ^{125}I-GIP species were separated using a gradient of CH_3CN in water containing 0.1%

trifluoroacetic acid run over 35 min. Fractions of 100-300 µl were injected using a needle syringe (Hamilton CO., Reno, NV) and washed on to the column in 31% CH_3CN over a 10 min period. A linear gradient to 38% was run over the next 10 min. The column was then washed by increasing the CH_3CN concentration to 70% over 5 min, holding it at 70% for 5 min and returning to 31% over the next 5 min. The second major peak which eluted at approximately 18 min post-injection was pooled from multiple runs in a siliconized tube with 0.5 ml of 2.5% RIA grade BSA and 50% aprotinin. A γ-counter was used to determine the amount of purified radioactive label and aliquots of 3×10^6 cpm were lyophilized and stored at -20 °C.

2.9.2 BINDING EXPERIMENTS

Cells were prepared for binding assays by seeding onto 24 well plates at a density of 50 000 cells/well and incubating at 37 °C for 48 h. Four wells were seeded at the same density to be counted for data normalization. On the day of the assay cells were washed twice with ice cold assay buffer (DMEM/Ham's F12 supplemented with 25mM HEPES and 0.1% BSA, pH 7.2). At this point cells were put at 4 °C until ready to begin the assay. Plates were emptied of existing assay buffer and 160 µl assay buffer supplemented with 0.01 ml Trasylol/ml buffer were added to each well. Twenty µl serially diluted $GIP_{1\text{-}30}NH_2$ was then added to each well in sets of three to give final concentrations of 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M. Twenty µl (~50 000 cpm) of ^{125}I-GIP were added to each well and the plates were incubated at 4 °C overnight. The next day cells were washed 2X in 0.5 ml ice cold assay buffer and 1 ml 0.1 M NaOH was added to each

well. The contents of the wells were transferred to borosilicate tubes and counted in a γ-counter.

When screening for subclones and when performing binding analysis on low expressing subclones, only NSB (2 μM $GIP_{1-30}NH_2$) and zero binding (no cold GIP) were tested.

2.9.3 BINDING ANALYSIS

The analysis program Prism (GraphPad, San Diego, CA) was used to analyze specific binding. Competitive binding analysis compared data fitted to a one site model using the following equation:

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

This equation describes the binding of a ligand to a receptor where Y is the bound label and X is the log value of the concentration of unlabeled competitor. Top is the top of the curve or B_{max} while Bottom is the bottom plateau or NSB (see Fig. 6). The specific activity of ^{125}I-GIP was not determined for every labeling, therefore IC_{50} values (inhibitory concentration displacing half the B_{max}) are presented. An idealized binding curve for competitive binding experiments is shown in Fig. 6. This model assumes that the reaction is at a steady-state; the fraction of labeled ligand bound is small (less than 10%) and thus the free concentration is in essence identical to the original concentration added to the reaction; and there is no cooperativity in binding, i.e. the binding of ligand to one site has no effect on the affinity at another site.

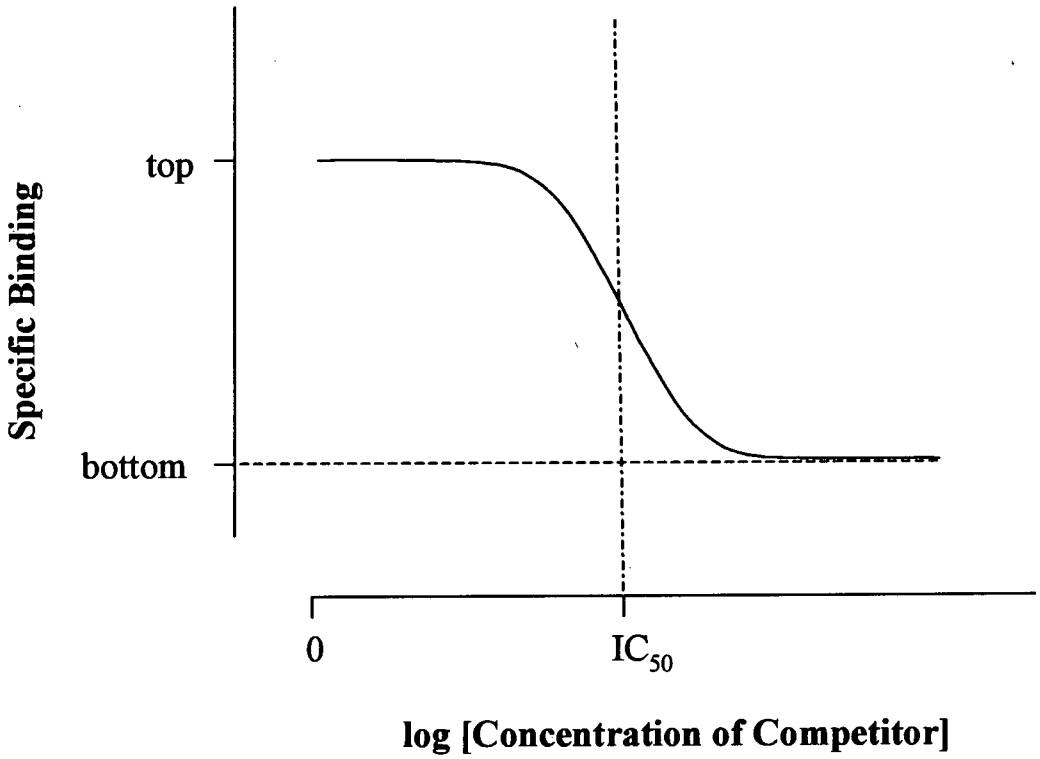


Fig. 6 Idealized Binding Curve for Competitive Binding Studies to a Single Binding Site. The IC_{50} is the concentration of cold ligand required to inhibit half of the maximal binding of labeled ligand. See text for details.

2.10 MEASUREMENT OF cAMP PRODUCTION

Cells were prepared for cAMP assays by seeding onto 24 well plates at a density of 50 000 cells/well and incubating at 37 °C for 48 h. Four wells were seeded at the same density to be counted for data normalization. On the day of the assay, cells were washed once with assay buffer and incubated in 1 ml assay buffer at 37 °C for 1 h. Plates were emptied of existing buffer and 160 µl assay buffer supplemented with 0.5 mM IBMX were added to each well. Twenty µl $GIP_{1-30}NH_2$ (2 µM) were then added to three wells while three wells received no GIP. Cells were incubated at 37 °C for 30 min at which time media was removed and 1 ml 70% ethanol was added to each well. Cells were scraped off the plate using a 1 ml syringe plunger and contents of each well transferred to a 1.5 ml microcentrifuge tube. Samples were centrifuged for 10 min at 12 000 rpm and 4 °C and the supernatant transferred to a new set of tubes. The samples were dried in a Speed-vac and stored at -20 °C until they were assayed. Samples were resuspended in 400 µl or 1.5 ml 0.5 M sodium acetate buffer and cAMP levels were determined using a cAMP RIA kit (Biomedical Technologies Inc., Stoughton, MA) as per the manufacturer's instructions.

Student's t test ($P < 0.05$) was used to compare GIP stimulated cAMP production by wild type GIP receptor with that of mutant GIP receptors.

2.11 RECEPTOR DESENSITIZATION STUDIES

Cells were prepared for desensitization studies by seeding into 12 wells of 24 well plates at a density of 60 000 cells/well and incubating at 37 °C for 48 h. Four wells were seeded at the same density to be counted for data normalization and six wells were seeded for binding. On the day of the assay, cells were washed once in 37 °C assay buffer. Assay

buffer (180 µl) was added to all the wells, 20 µl $GIP_{1-30}NH_2$ (1 µM) was added to half the wells and cells were incubated in a 37 °C water bath for 60 min. At the end of this pre-incubation cells were washed 2X in 1 ml assay buffer over 10 min. One hundred and eighty µl assay buffer supplemented with 0.5 mM IBMX were added to all wells. Half of the control wells and half of the pre-treated cells were stimulated with 20 µl $GIP_{1-30}NH_2$ (100 nM) and incubated in a 37 °C water bath for 30 min. One ml 70% ethanol was added to each well and the protocol as described for the cAMP assay in Section 2.10 was followed from that point.

2.12 RECEPTOR INTERNALIZATION STUDIES

Cells were prepared for internalization studies by seeding into 24 well plates at a density of 60 000 cells/well and incubating at 37 °C for 48 h. On the day of the assay cells were washed 2X in assay buffer, 180 µl assay buffer were added to all wells and the plate placed in a 37 °C water bath. In quadruplicate 20 µl of 1µM $GIP_{1-30}NH_2$ was added at 60, 25, 15 and 7.5 min before time zero. The NSB and zero binding wells received no GIP. At time zero cells were washed once in 1 ml ice cold stripping buffer (150 mM NaCl, 50 mM Glycine, pH 3) and incubated on ice for 5 min in 1 ml stripping buffer. Cells were then washed twice in assay buffer, 180 µl assay buffer supplemented with 0.01 ml Trasylol/ml buffer and 20 µl (~50 000 cpm) ^{125}I-GIP were added to all wells and 20 µl $GIP_{1-30}NH_2$ (5 µM) were added to the NSB wells. The cells were incubated at 4 °C for 4 h and then washed 2X in 0.5 ml ice cold assay buffer. One ml 0.1 M NaOH was added to each well and the contents of the wells were transferred to borosilicate tubes and counted in a γ-counter.

2.13 DESENSITIZATION AND INTERNALIZATION ANALYSIS

Student's t test ($P < 0.05$) was used in the desensitization experiments, to compare GIP stimulated cAMP production with basal cAMP levels, and to compare control with desensitized GIP stimulated cAMP production.

Internalization rate over the first 15 min was determined by performing a linear regression and taking the slope of the line. A one-way analysis of variance (ANOVA) was used in the internalization studies to compare wtGIP receptor with mutant receptor values for IC_{50}, B_{max}, internalization rate and maximum internalization. The post hoc Dunnett's test was then used to compare individual mutant receptor values to that of wild type receptor.

CHAPTER 3

RESULTS

3.1 CHARACTERIZATION OF GIP RECEPTORS IN ADIPOSE TISSUE OF ZUCKER RATS

NIDDM is a disease associated with obesity. As GIP exerts effects on adipose tissue, the expression of the GIP receptor in this tissue is of interest in relation to obesity. The Zucker fatty (*fa/fa*) rat is a model of obesity and therefore was used to study GIP receptor expression in adipose tissue.

3.1.1 ORAL GLUCOSE TOLERANCE IN ZUCKER RATS

An oral glucose tolerance test was performed on fasted Zucker rats and plasma glucose levels were measured. At 11 weeks of age, the obese and lean animals did not have significantly different plasma glucose concentrations by overall analysis of variance (Fig. 7). At 16 weeks of age, the obese animals had significantly higher plasma glucose concentrations by overall analysis of variance (Fig. 8).

3.1.2 GIP RESPONSES TO ORAL GLUCOSE

GIP plasma levels of the 16 week old rats were measured by RIA as described in Section 2.1.2 (Fig. 9). The obese animals had significantly higher plasma GIP levels than did their lean littermates by overall analysis of variance.

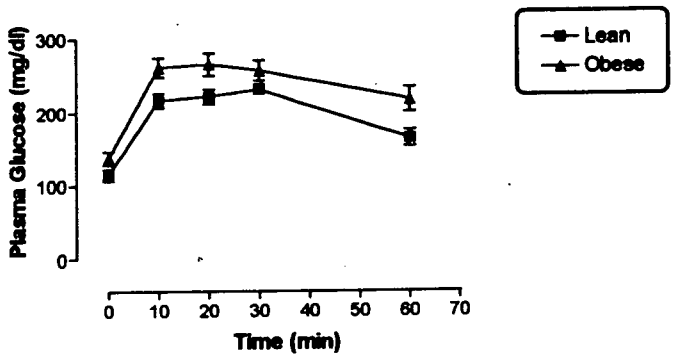


Fig. 7. Oral Glucose Tolerance Test Performed on 11 Week Old Lean and Obese Zucker Rats. Data represent mean ± S.E. (n = 6). Lean and obese values were not significantly different by overall analysis of variance (P < 0.02)

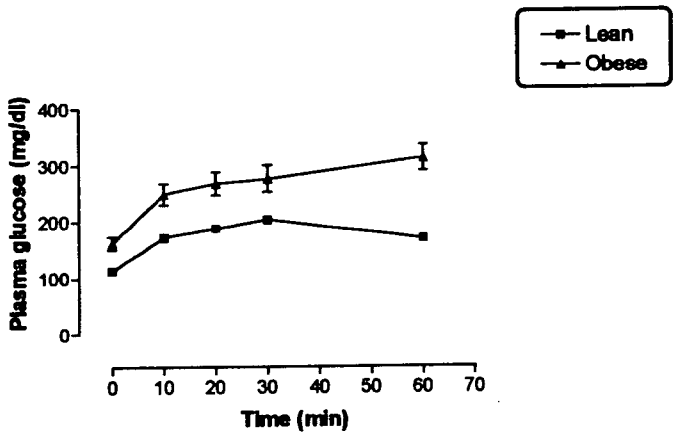


Fig. 8. Oral Glucose Tolerance Test Performed on 16 Week Old Lean and Obese Zucker Rats. Data represent mean ± S.E. (n = 6). Lean and obese values were significantly different by overall analysis of variance (P < 0.05).

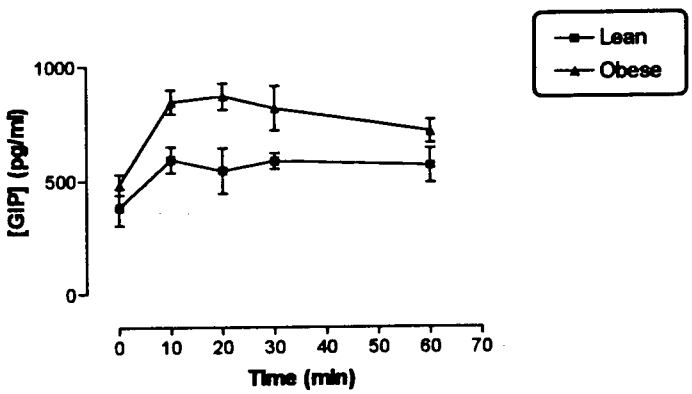


Fig. 9. Plasma GIP Concentration During Oral Glucose Tolerance Test in 16 week old Lean and Obese Zucker Rats. Data represent mean ± S.E. (n = 6). Lean and obese values were significantly different by overall analysis of variance (P < 0.03).

3.1.3 CHARACTERIZATION OF GIP RECEPTOR MESSENGER RNA IN ADIPOSE TISSUE

3.1.3.1 RIBONUCLEASE PROTECTION ASSAY

Total RNA was isolated from rat epididymal adipose tissue and subjected to a ribonuclease protection assay as described in Section 2.4. A protected fragment of the expected size (~300 bases) for the GIP receptor probe was detected and, as expected, the undigested yeast control had a band ~330 bases, while there was absence of a band with the digested yeast control (Fig. 10). A protected fragment of the expected size (250 bases) was also observed with each of the β-actin control lanes. A ribonuclease protection assay on RNA isolated from 3T3-L1 cells also showed a band of ~300 bases using the GIP receptor probe (Fig. 11). While experiments to optimize the conditions were still being performed, a problem with the GIP receptor probe developed. The GIP receptor band was no longer visible although an 18S control band of 80 bases was visible. It was decided to use PCR to examine GIP receptor expression.

3.1.3.2 REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION DETECTION OF GIP RECEPTOR

Total RNA was isolated from epididymal adipose tissue of 11 week old rats as described in Section 2.3.2 and first strand cDNA was prepared using an oligonucleotide primer complimentary to the CT tail of the GIP receptor, as described in Section 2.5.1. The cDNA was subjected to PCR, and a band of 500 bp was observed, using primers complimentary to the CT tail of the GIP receptor (Fig. 12). While these conditions were being optimized so that competitive PCR could be attempted, a problem with contamination from an unknown source developed. Although many steps were taken to

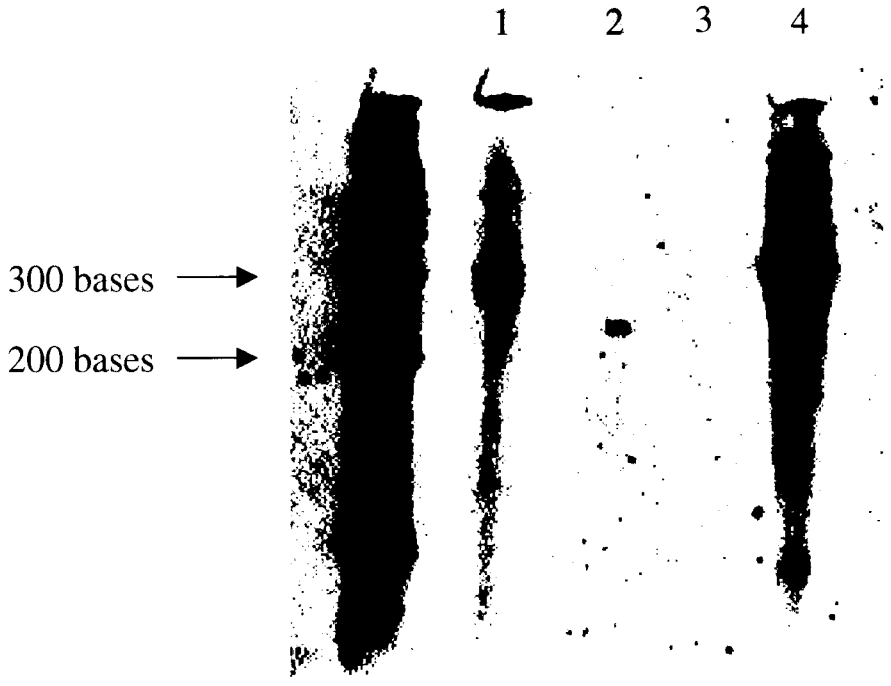


Fig. 10. Autoradiograph of a Ribonuclease Protection Assay of Rat Adipose RNA Using a Probe Complimentary to the GIP Receptor. Lane 1. GIP receptor. Lane 2. β-actin control. Lane 3. Digested yeast control. Lane 4. Undigested yeast control.

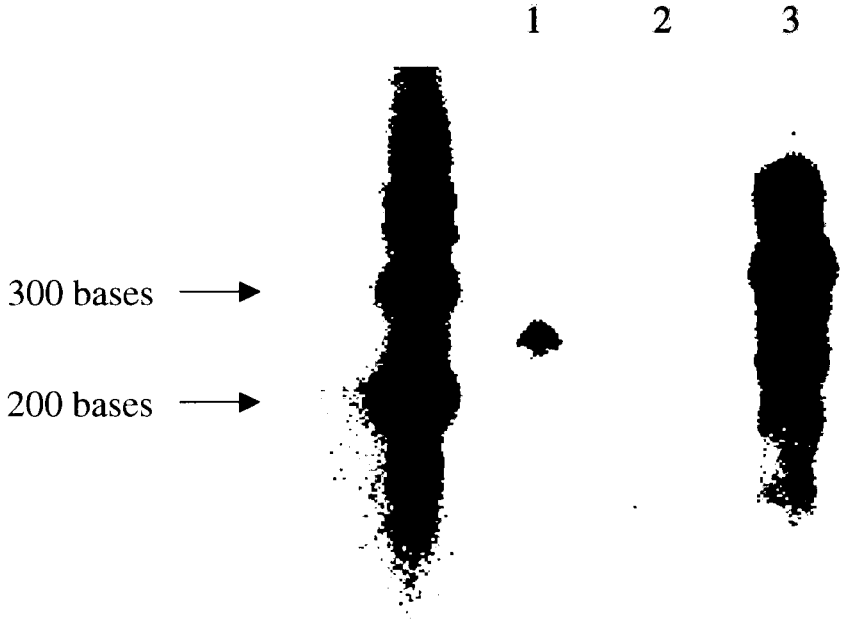


Fig. 11. Autoradiograph of a Ribonuclease Protection Assay of 3T3-L1 RNA Using a Probe Complimentary to the GIP Receptor. Lane 1. GIP receptor. Lane 2. Digested yeast control. Lane 3. Undigested yeast control.

resolve this, they were unsuccessful. As the Real-Time PCR system was being set up, it was decided to analyze the RNA samples using this method.

3.1.3.3 REAL-TIME PCR DETERMINATION OF GIP RECEPTOR LEVELS

Total RNA was isolated from epididymal adipose tissue of 11 week old rats as described in Section 2.3.2 and first strand cDNA was prepared using both an oligonucleotide primer complimentary to the CT tail of the GIP receptor and random hexamers, as described in Section 2.5.2. The GIP receptor target sequence was amplified and its amount expressed as a ratio of GIP receptor mRNA to GAPDH mRNA present in the PCR reaction. There was no difference in mRNA expression levels between lean and obese rats (Fig. 13).

3.2 THE SEARCH FOR ALTERNATIVE SPLICE SITES IN THE THIRD INTRACELLULAR LOOP OF THE RAT GIP RECEPTOR

Total RNA was isolated from the pancreatic islets and epididymal adipose tissue of a lean male Zucker rat, as described in Section 2.3.3. First strand cDNA was synthesized as described in Section 2.5.3 and the i3 loop of the GIP receptor was amplified using PCR. The primers used were expected to generate a product of 183 bp, and such a band was seen in both islet and adipose tissue lanes when separated by agarose gel electrophoresis and visualized (Fig. 14). In both samples there were additional bands of approximately 350 and 450 bp. The 450 bp band was very strong in the adipose sample but weak in the islet sample, while the 350 bp band was strong in the islet sample but barely visible in the adipose tissue sample. It was decided to examine both the 350 bp islet

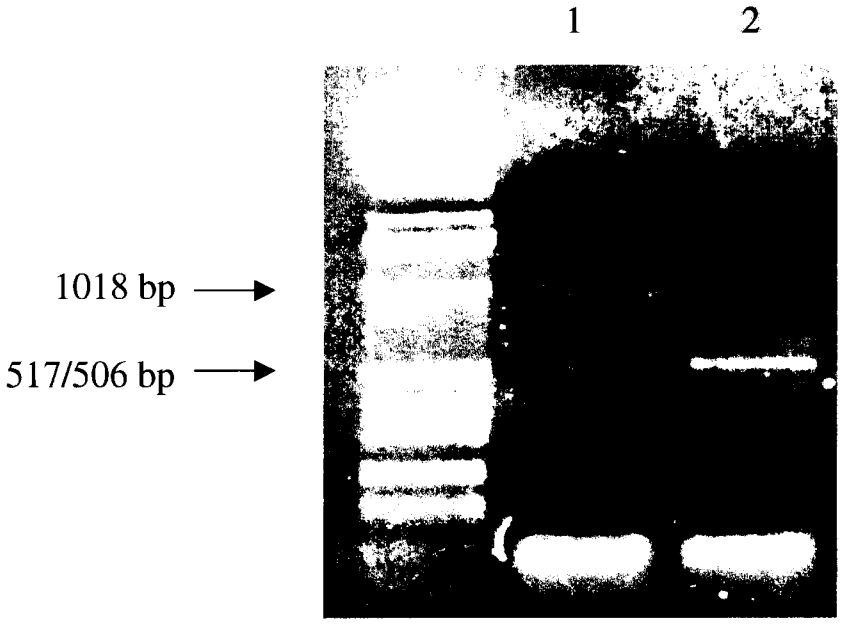


Fig. 12. Amplification of a cDNA Encoding the C-Terminal Tail of the Rat GIP Receptor From Adipose RNA Using RT-PCR. Lane 1. Negative control. Lane 2. A band of ~500 bp was amplified. See Section 2.5.1 for details.

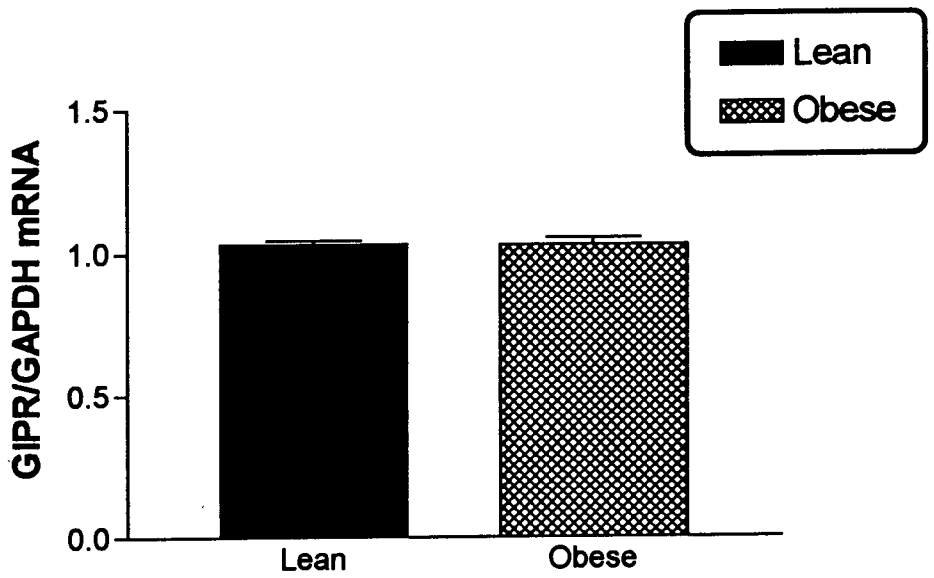


Fig. 13. Ratio of GIP Receptor (GIPR)/GAPDH mRNA in11 Week Old Lean and Obese Zucker Adipose Tissue. Data represent mean ± S.E. (n = 6).

product and the 450 bp adipose product further. The PCR products were ligated into the cloning vector pGEM™-T (Section 2.5.3) and the sequences determined (Sections 2.5.3 and 2.6). The islet product was found to contain a 129 base insert located 34 nucleotides from the first nucleotide of the 5' primer. When the sequence was translated, a stop codon (TGA) was found 32 amino acid residues into the sequence. This indicates that if expressed, this sequence would encode a receptor truncated in the i3 loop. The adipose tissue product was found to contain the 129 base insert found in the islet product; however, it contained an additional 84 base insert. This insert encodes 28 amino acid residues, contains no stop codons and is located between Arg-335 and Leu-336 (Fig. 15).

3.3 SITE-DIRECTED MUTAGENESIS OF THE RAT PANCREATIC GIP RECEPTOR

Single point mutations were made in the i2 and i3 loops of the GIP receptor, in which serines 248, 320 and 339, and threonines 324, 340 and 342 were each replaced with an alanine. Each mutant receptor was stably transfected into CHO-K1 cells, and in preliminary binding studies, all mutants except GIPR/T340A demonstrated binding of ^{125}I-GIP and competitive displacement by GIP_{1-42} (Fig. 16a). As GIPR/T340A did not bind ^{125}I-GIP, no further experiments were performed with this mutant. The wild type and mutant receptors were expressed at varying levels in the CHO-K1 cells, as can be seen by the variation in maximal binding in Fig. 16a. However, when expressed as a percentage of zero binding, the mutant receptors showed similar displacement of ^{125}I-GIP by GIP_{1-42} to that of the wild type GIP receptor (wtGIPR) (Fig. 16b), with the expected sigmoidal curve and IC_{50} values ranging from 1.77 - 15.2 nM (Table 1).

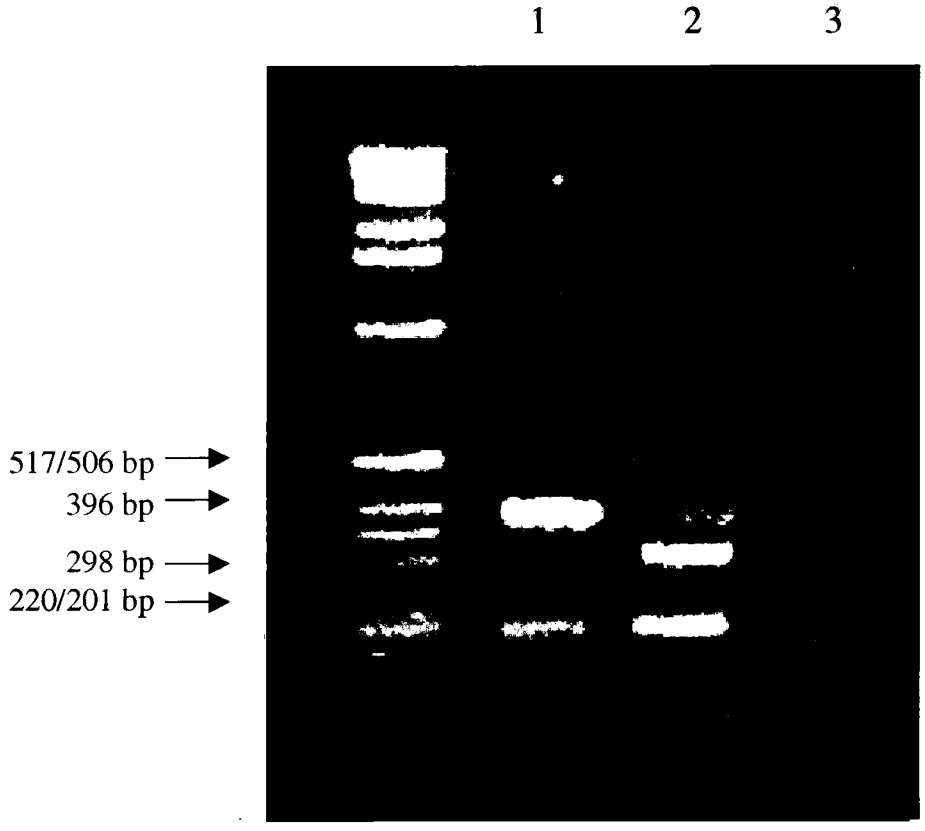
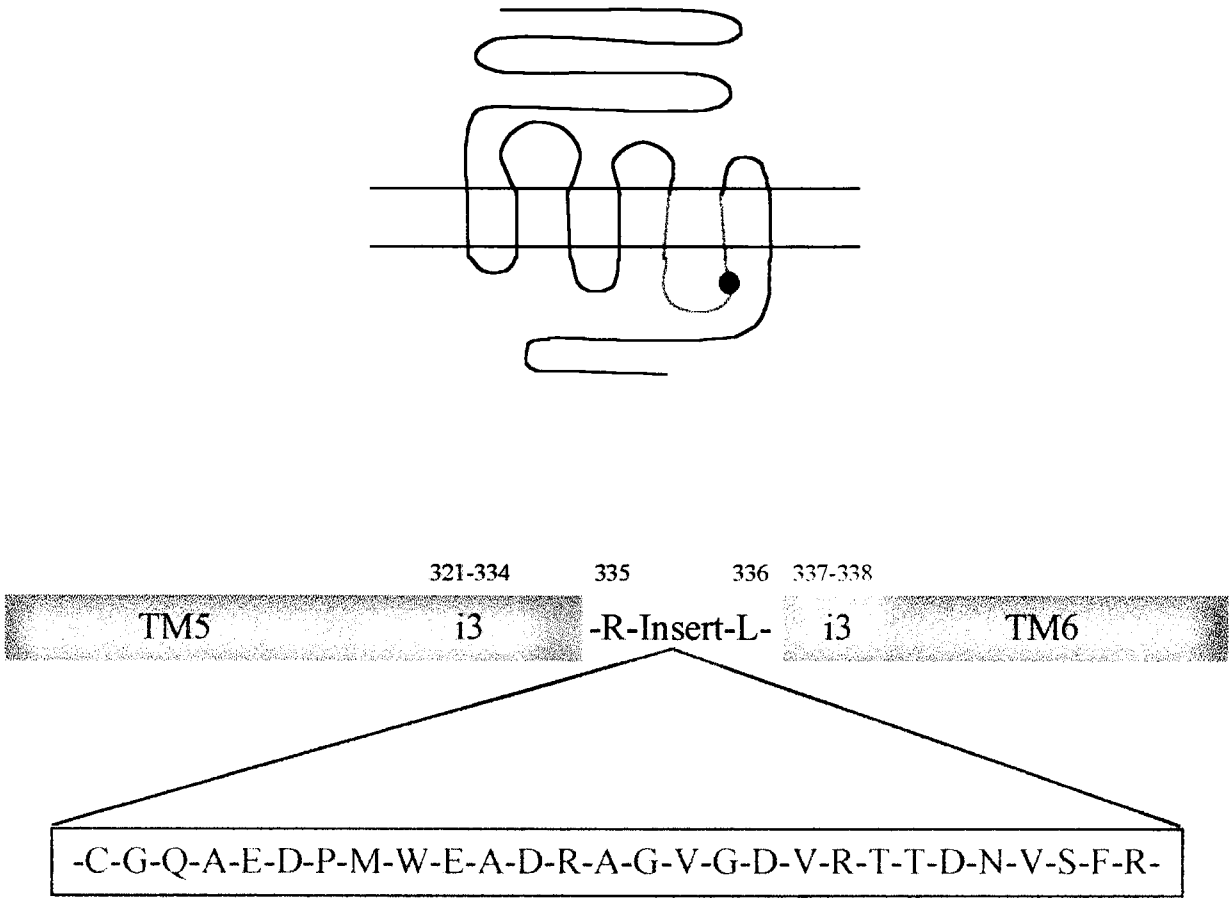


Fig. 14. Amplification of cDNA Encoding the Third Intracellular Loop of the Rat GIP Receptor Using RT-PCR. Bands of 200, 350 and 450 bp were amplified. Lane 1. Adipose tissue Lane 2. Pancreatic islets Lane 3. Negative control.

a.

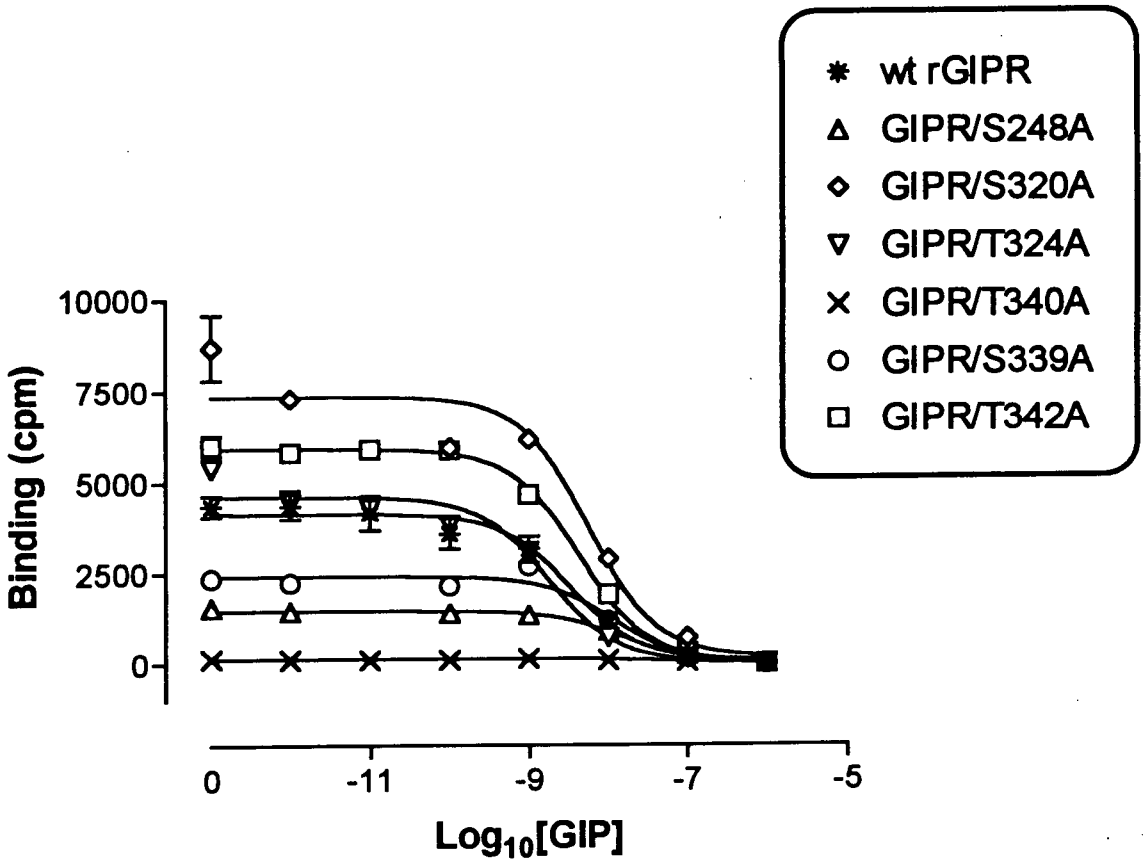


b.

5' - TGT GGG CAG GCT GAG GAC CCA ATG TGG
GAA GCA GAT AGG GCC GGA GTA GGG GAC
GTG CGG ACC ACT GAT AAC TGT CTT TCC - 3'

Fig. 15. Alternatively Spliced Variant of the GIP Receptor. a. A 28 amino acid insert was found in the i3 loop between Arg-335 and Leu-336. • denotes insert. b. Nucleotide sequence of the insert.

a.



b.

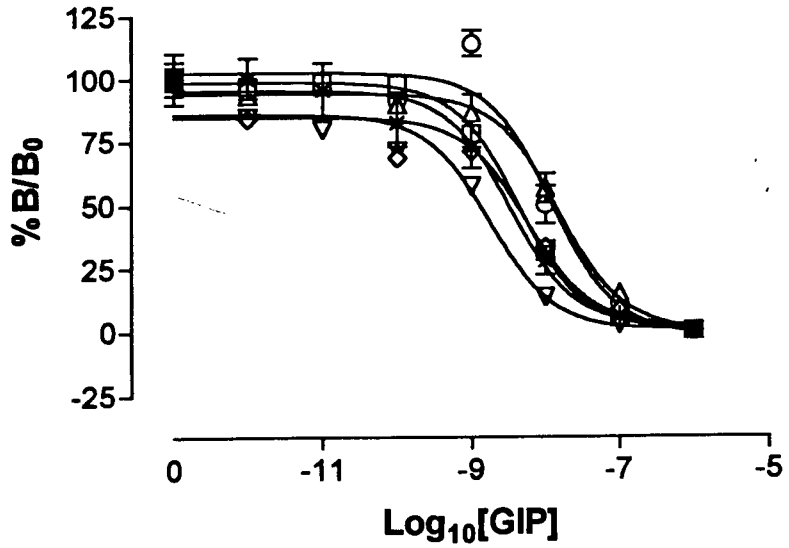


Fig. 16. Displacement of ^{125}I-GIP Binding by GIP_{1-42} in CHO-K1 Cells Expressing Wild Type GIP Receptor (wtGIPR) or Mutant GIP Receptors. Cells were incubated for 24 h. n = 1 a. Specific binding measured in cpm. b.Binding expressed as a percentage of zero binding. See Table 1 for IC_{50} values.

Receptor	wtGIPR	GIPR/ S248A	GIPR/ S320A	GIPR/ T324A	GIPR/ S339A	GIPR/ T342A
IC_{50} (nM)	3.33	15.2	5.68	1.77	12.2	4.35

Table 1. IC_{50} Values of the Competitive Displacement of ^{125}I-GIP by GIP_{1-42} in CHO-K1 Cells Stably Transfected with wtGIPR and Mutant GIPR. (n = 1)

Because the transfected cells represent a heterogeneous population with varying receptor expression levels, individual colonies were screened for expression levels similar to wtGIPR, by measuring zero binding and non-specific binding. Subclones with a specific binding of ~5000 cpm and ~500 cpm were chosen for the high and low expressing subclones, respectively. High expressing subclones were used for internalization experiments as these cells contain high enough levels of receptors to show competitive binding. However, subclones expressing high levels of the GIP receptor do not show desensitization, because there are too many receptors which are not desensitized and continue to stimulate cAMP production. Low expressing subclones contain few enough receptors that once desensitized, very few are still able to stimulate adenylyl cyclase. Therefore, low expressing subclones were used for the desensitization experiments.

3.3.1 RECEPTOR DESENSITIZATION EXPERIMENTS

On the day of the experiment a binding assay was performed and expression levels ranged from 46-286% of wild type expression (Table 2). Fig. 17 shows the effect of stimulation with 10 nM GIP_{1-30} on cAMP production, while basal values and the actual increase in cAMP over basal is shown in Table 2. The concentration of 10 nM GIP_{1-30} was chosen as it has been shown in previous studies in our laboratory to give the

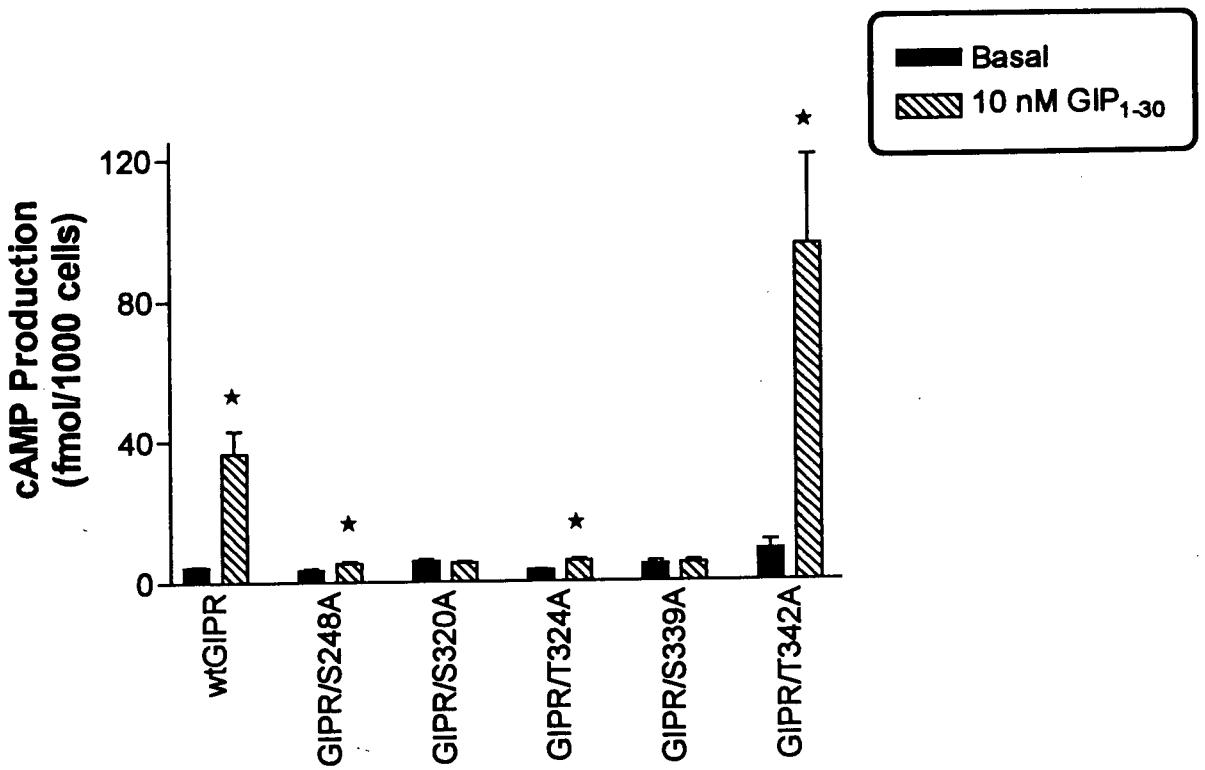


Fig. 17. Stimulation by 10 nM GIP_{1-30} of cAMP Accumulation in CHO-K1 Cells Expressing Low Levels of Wild Type GIP Receptor (wtGIPR) or Mutant GIP Receptors. Data are represented as mean ± S.E. (n = 5). ★ indicates a significant increase over basal ($p < 0.05$).

Receptor	Expression Levels	Basal cAMP Production	Increase in cAMP Over Basal	Decrease in cAMP Response After Preincubation
	(%)	(fmol/1000 cells)	(fmol/1000 cells)	(%)
wtGIPR	100	4.3 ± 0.30	32.3 ± 6.4*	54 ± 6
GIPR/S248A	185 ± 23	3.4 ± 0.42	1.9 ± 0.47*	11 ± 0.47
GIPR/S320A	46 ± 13	5.8 ± 0.60	-0.53 ± 0.43	8.9 ± 0.43
GIPR/T324A	182 ± 50	3.1 ± 0.32	2.8 ± 0.53*	11 ± 0.53
GIPR/S339A	102 ± 34	4.7 ± 0.81	0.47 ± 0.73	-1.1 ± 0.73
GIPR/T342A	286 ± 40	8.8 ± 2.7	87 ± 25*	32 ± 25

Table 2. GIP_{1-30} Stimulated cAMP Production in CHO-K1 Cells Stably Transfected with wtGIPR and Mutant GIPR, Expressed at Low Levels. cAMP production was stimulated with 10 nM GIP_{1-30}. Cells were pre-incubated with 100 nM GIP_{1-30}. Data represent mean ± S.E. (n = 3-5). Basal cAMP values were significantly different from wtGIPR by analysis of variance ($p < 0.05$), but only GIPR/T342A was significantly different from wtGIPR by the post hoc Dunnett's test ($p < 0.05$). * indicates significant increase in cAMP production over basal, $p < 0.05$.

optimal stimulation of cAMP production. Wild type GIPR cells showed an 8.5-fold increase in cAMP production over basal, while GIPR/S320A and GIPR/S339A cells showed no significant change in cAMP production from basal. While GIPR/S248A and GIPR/T324A cells showed only 1.5-fold and 1.9-fold increases, respectively, GIPR/T342A cells showed an 11-fold increase in cAMP over basal. GIPR/T342A cells were the only ones that showed any significant difference in basal cAMP production compared to wtGIPR, and they showed the maximal cAMP response at 95 ± 25 fmol/1000 cells, compared to wtGIPR at 37 ± 6.4 fmol/1000 cells.

Fig. 18 and Table 2 shows the effect of pre-incubation with 100 nM GIP_{1-30} on subsequent cAMP production in response to 10 nM GIP_{1-30}. Although wtGIPR cells

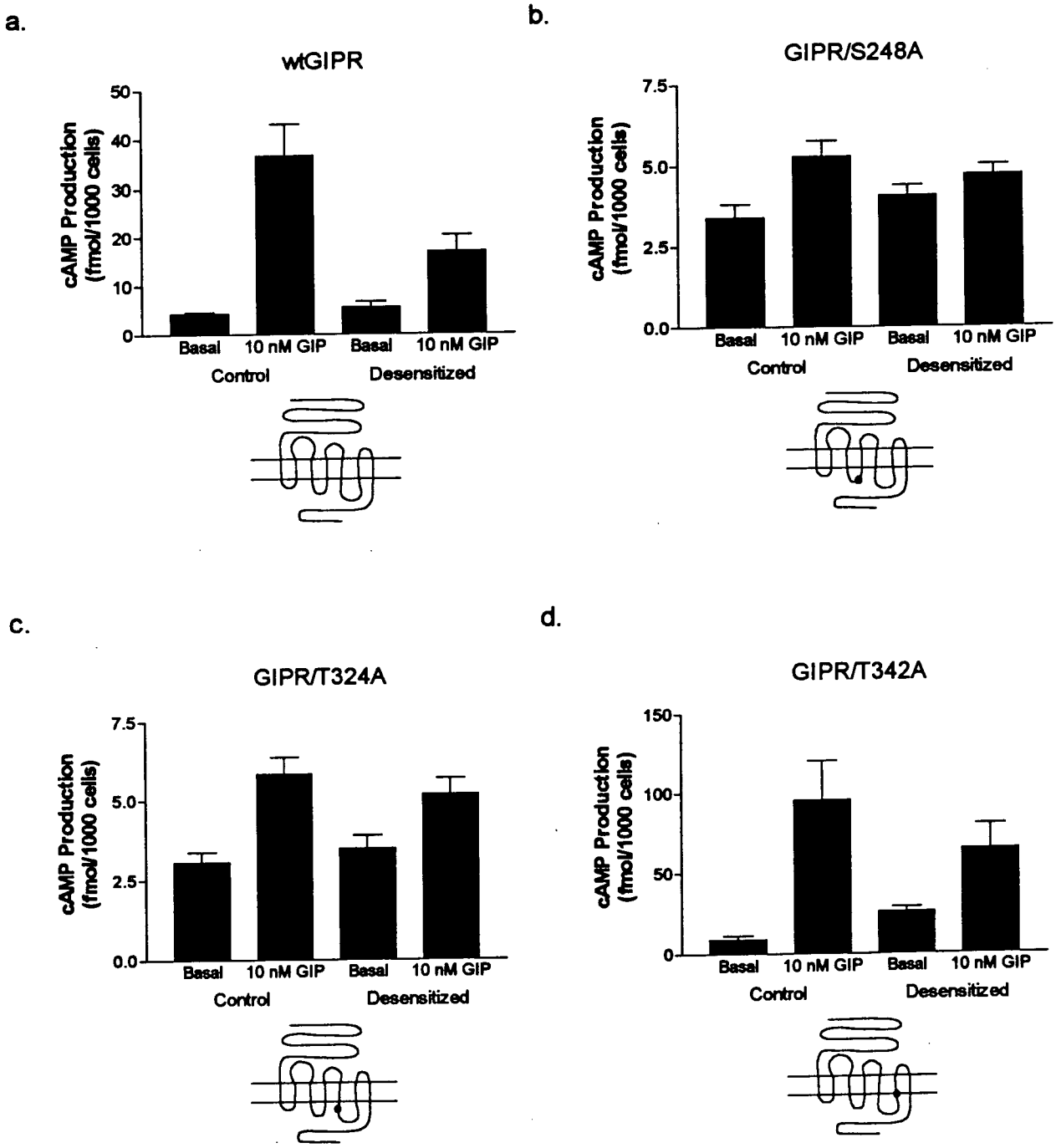


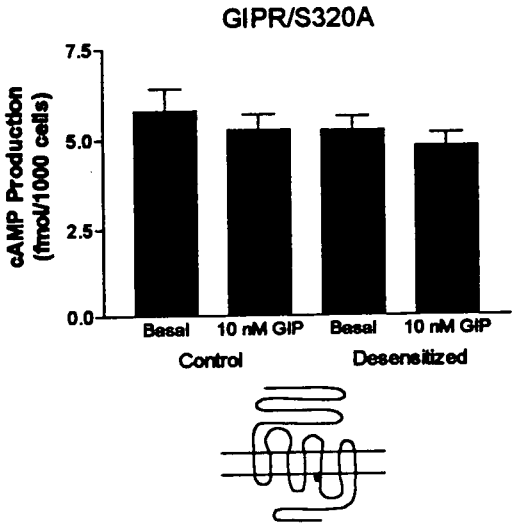
Fig. 18. Stimulation by 10 nM GIP_{1-30} of cAMP Accumulation After Incubation With or Without 100 nM GIP_{1-30} in CHO-K1 Cells Expressing Low Levels of Wild Type GIP Receptor (wtGIPR) or Mutant GIP Receptors. Data are represented as mean ± S.E. (n = 3-5). Control data is that which appears in Fig. 17. • indicates site of mutation. a.wtGIPR b.GIPR/S248A c.GIPR/T324A d.GIPR/T342A

appeared to show a decrease in mean values for GIP_{1-30} stimulated cAMP production after pre-incubation with 100 nM GIP_{1-30}, it did not reach significance. However, this clone has shown desensitization under similar conditions in our lab (Hinke et al. 2000) and an increase in the n value would likely result in significance. GIPR/S248A, GIPR/T324A and GIPR/T342A cells did not show changes in GIP_{1-30} stimulated cAMP production after preincubation with 100 nM GIP_{1-30}. Point mutations of other GPCRs, for example, the β_2AR (Hausdorff et al. 1990), have been shown to diminish the ability of these receptors to desensitize, so this may be the case here. As there was no control stimulation of cAMP over basal with GIPR/S320A and GIPR/S339A (Fig. 19), it is not possible to determine whether mutation of these residues results in a change in cAMP production after preincubation with 100 nM GIP_{1-30}.

3.3.2 RECEPTOR INTERNALIZATION EXPERIMENTS

Binding and cAMP assays were performed on the high expressing subclones of the mutant GIP receptors. Fig. 20 shows the binding and competitive displacement of ^{125}I-GIP by GIP_{1-30}. IC_{50} values were similar for each of the mutant receptors, ranging from $1.13 \pm 0.16 - 1.85 \pm 0.52$ nM, and were not significantly different from the wild type receptor value of 1.31 ± 0.23 nM (Table 3). The maximum binding (B_{max}) of each mutant receptor when calculated as a percentage of wild type, gives an indication of the expression level of each receptor. These values varied from $35 \pm 1.9 - 120 \pm 40\%$ of wild type, and although this range of values was significantly different using an ANOVA, the individual values were not significantly different from the wild type value using the post hoc Dunnett's test (Table 3). Fig. 21 shows the effect of stimulation with 10 nM GIP_{1-30}

a.



b.

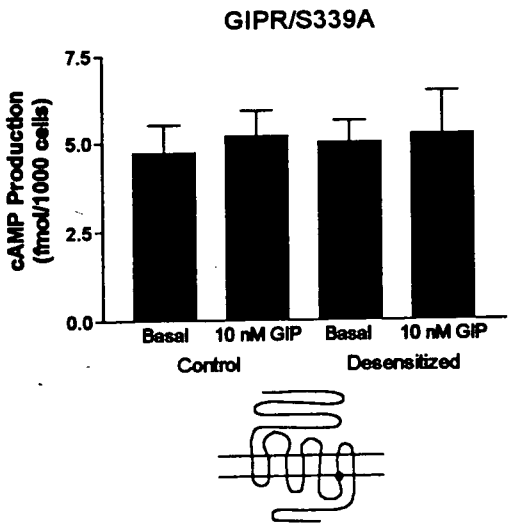


Fig. 19. Stimulation by 10 nM GIP_{1-30} of cAMP Accumulation After Incubation With or Without 100 nM GIP_{1-30} in CHO-K1 Cells Expressing Low Levels of Mutant GIP Receptors. Data are represented as mean ± S.E. (n = 3-4). Control data is that which appears in Fig. 17. • indicates site of mutation. a.GIPR/S320A b.GIPR/S339A

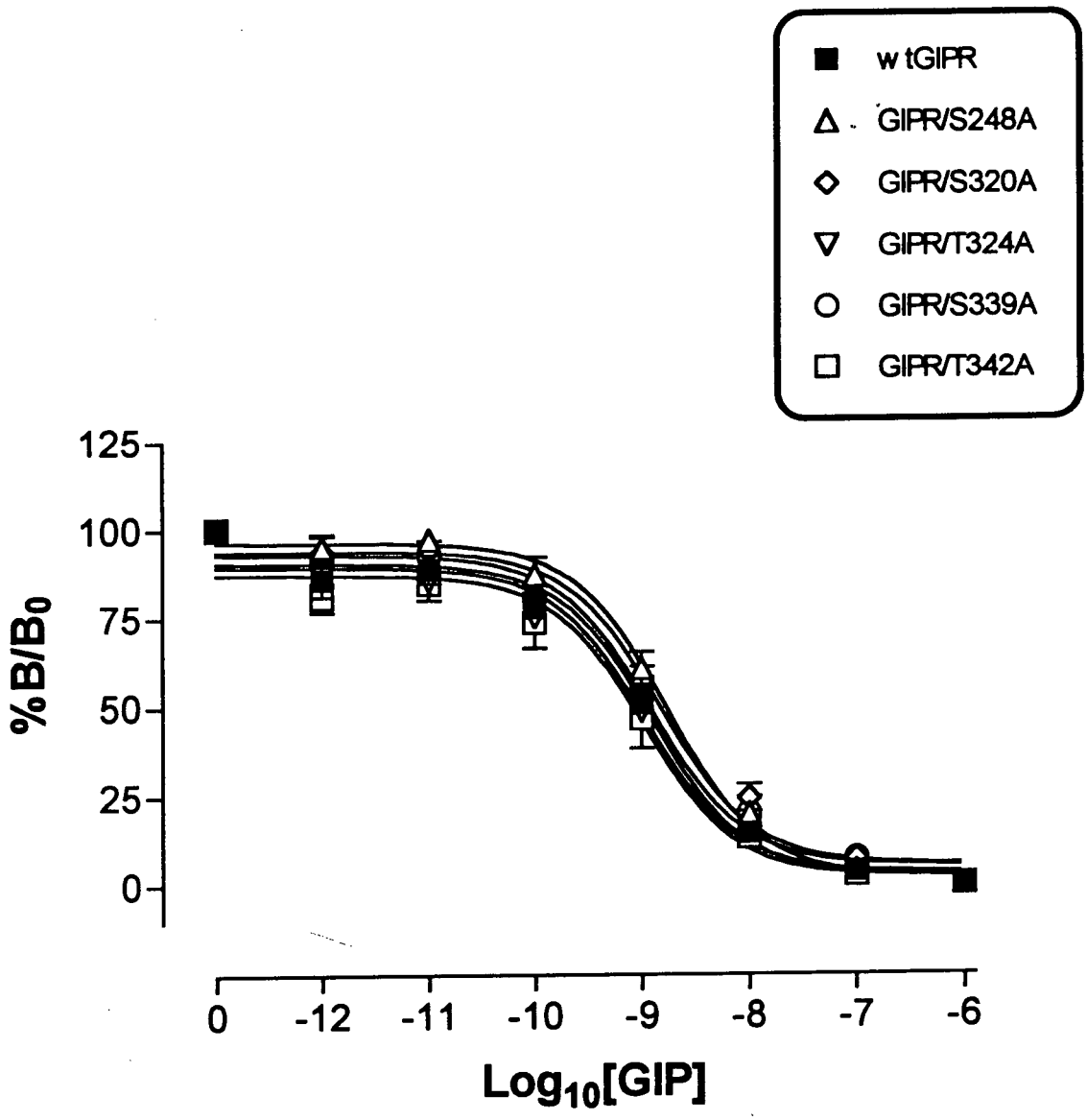


Fig. 20. Displacement of ^{125}I-GIP Binding by GIP_{1-30} in CHO-K1 Cells Expressing High Levels of Wild Type GIP Receptor (w tGIPR) or Mutant GIP Receptors. Data are expressed as mean ± S.E (n = 6). No significant differences betw een the IC_{50} values obtained w ere observed.

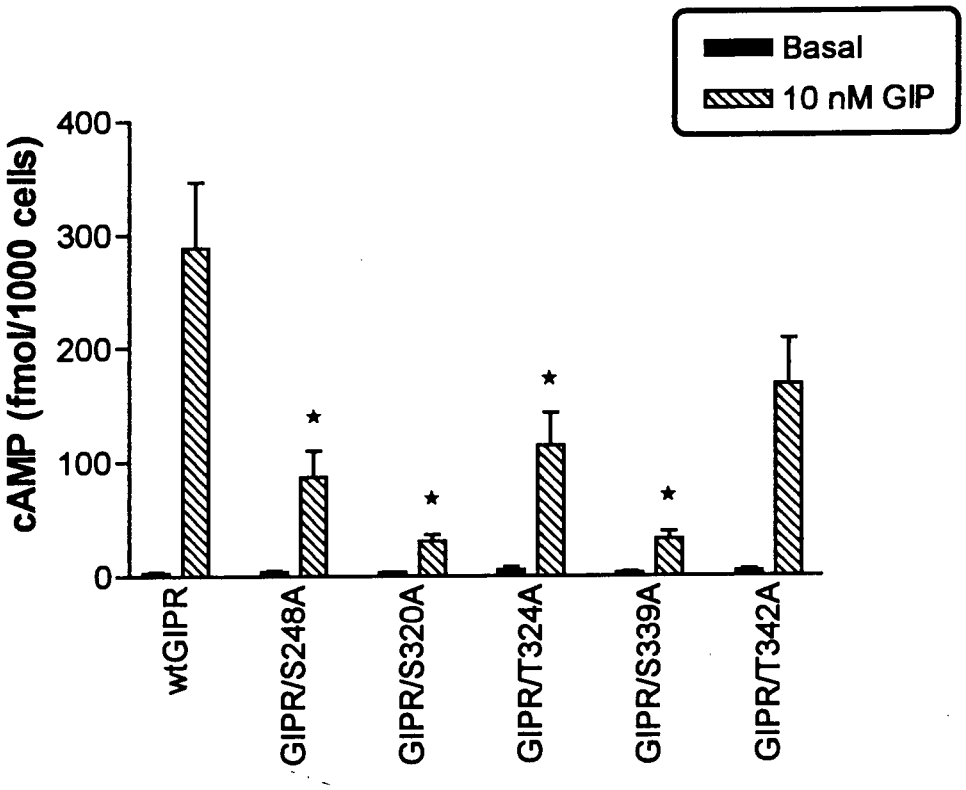


Fig. 21. Stimulation by 10 nM GIP_{1-30} of cAMP Accumulation in CHO-K1 Cells Expressing High Levels of Wild Type GIP Receptor (wtGIPR) or Mutant GIP Receptors. Data are represented as mean ± S.E. (n = 5-6). ⋆ indicates a significant change from cells expressing wild type using a t test.

Receptor	IC_{50}	B_{max}	Internalization rate over first 15 min	Maximum internalization
	nM	% wild type	%/min	%
wtGIPR	1.31 ± 0.23	100	1.33 ± 0.26	33.9 ± 2.9
GIPR/S248A	1.81 ± 0.31	120 ± 40	0.61 ± 0.11*	23.8 ± 2.0
GIPR/S320A	1.85 ± 0.52	35 ± 1.9	1.30 ± 0.25	37.6 ± 4.3
GIPR/T324A	1.13 ± 0.16	67 ± 13	1.17 ± 0.03	41.0 ± 4.3
GIPR/S339A	1.51 ± 0.47	42 ± 1.5	2.47 ± 0.88*	51.2 ± 6.9**
GIPR/T342A	1.23 ± 0.46	125 ± 22	2.04 ± 0.5*	50.6 ± 2.8**

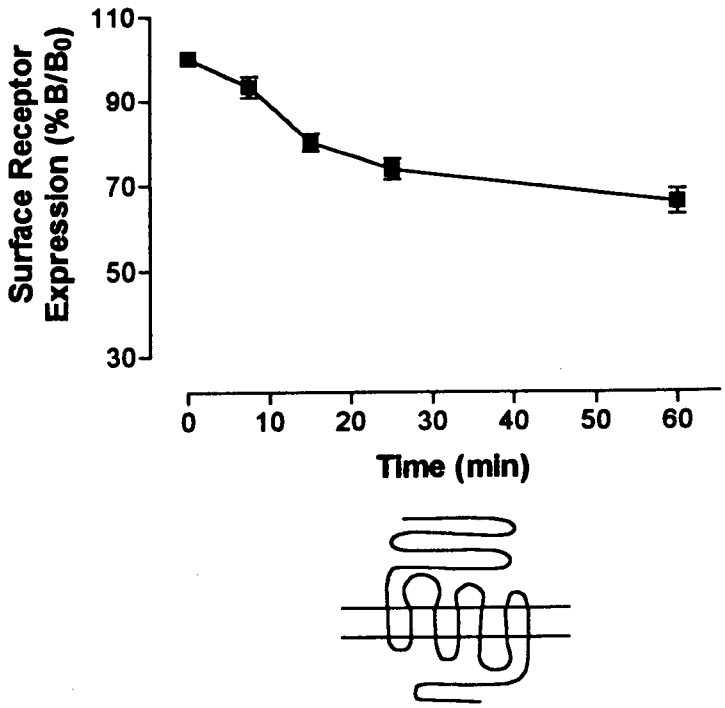
Table 3. Binding and Internalization Characteristics of CHO-K1 Cells Stably Transfected with wtGIPR and Mutant GIPR. Data represent mean ± S.E. (n = 3-10). IC_{50} values were not significantly different from wtGIPR by analysis of variance or the post hoc Dunnett's test. B_{max} values were significantly different from wtGIPR by analysis of variance ($p < 0.05$) but not by Dunnett's test. * and ** indicates significant differences from wtGIPR, $p < 0.01$ and 0.05, respectively.

on cAMP production. Cells expressing wtGIPR showed a 95-fold increase in GIP_{1-30} stimulated cAMP production over basal. GIPR/T342A cells showed only a 35–fold increase, but this value was not significantly lower than the wild type value. GIPR/S248A, GIPR/S320A, GIPR/T324A and GIPR/S339A showed significantly lower values than wtGIPR with 21–fold, 9-fold, 19-fold and 10–fold increases in cAMP production, respectively.

Internalization experiments were also performed on the high expressing subclones of the mutant GIP receptors as described in Section 2.12. Fig. 22 shows the decrease in surface receptor binding which occurs after incubation with 100 nM GIP_{1-30} over one hour. GIPR/S248A shows a smaller decrease in surface receptor expression at 15, 25 and

60 min, than wtGIPR, while GIPR/S320A and GIPR/T324A show similar decreases and GIPR/S339A and GIPR/T342A show greater decreases in surface receptor binding at all time points than wtGIPR. In Fig. 23a, the loss of receptor binding is expressed as the percent internalized. The maximal internalization of GIPR/S339A and GIPR/T342A was significantly greater than that of wtGIPR, while the remaining mutants did not differ significantly from wtGIPR (Table 3). Fig. 23b shows the internalization over the first 15 min. GIPR/S339A and GIPR/T342A showed significantly greater rates of internalization than wtGIPR while GIPR/S248A showed a significantly lower rate of internalization than wtGIPR (Table 3). The rate of internalization of GIPR/S320A and GIPR/T324A did not differ significantly from wtGIPR.

a.



b.

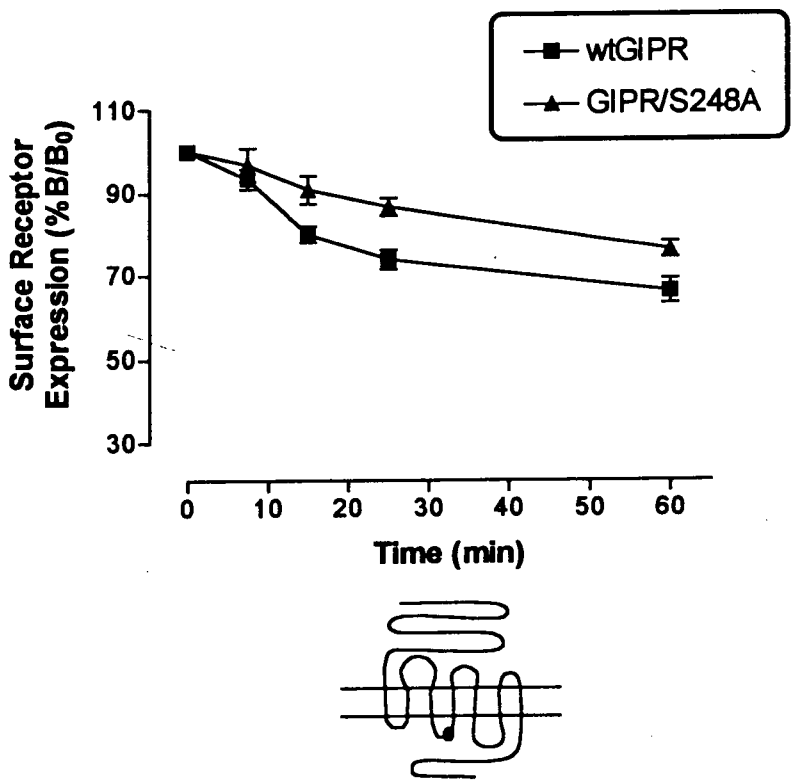
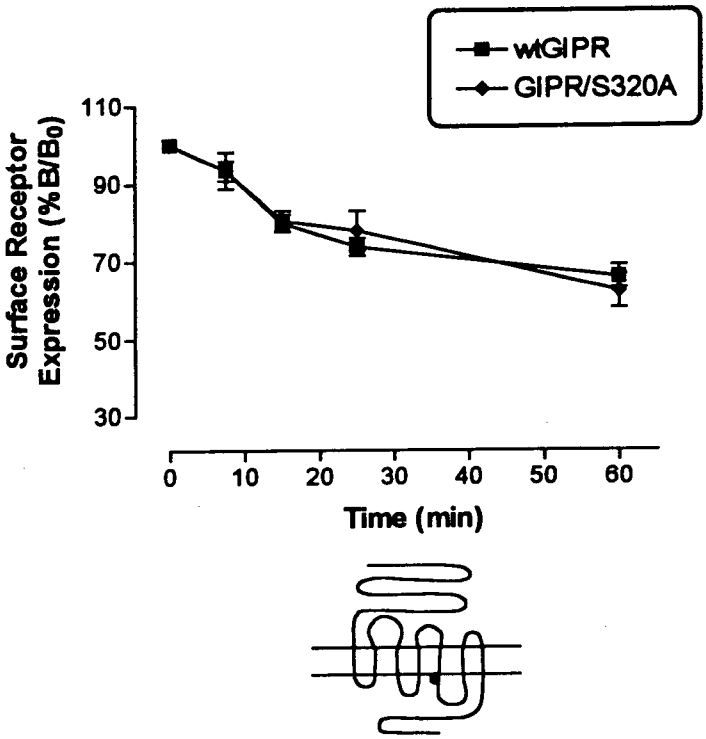


Fig. 22. Loss of Surface Receptor Expression During Incubation with 100 nM GIP_{1-30} Over 60 min. Wild Type GIPR and mutant GIP receptors were expressed at high levels in CHO-K1 cells. Data are represented as mean ± S.E. • indicates site of mutation. a. wtGIPR, n = 10. b. wtGIPR and GIPR/S248A, n = 6-10.

c.



d.

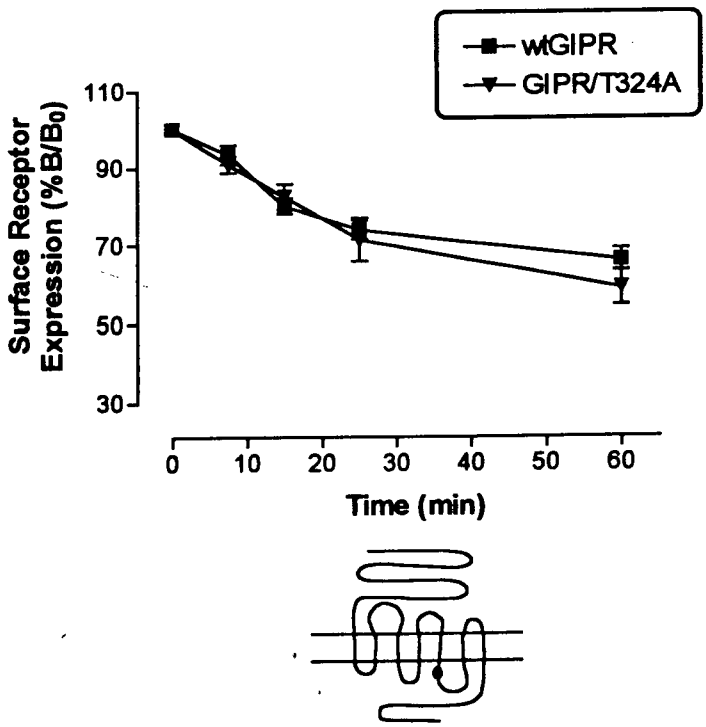
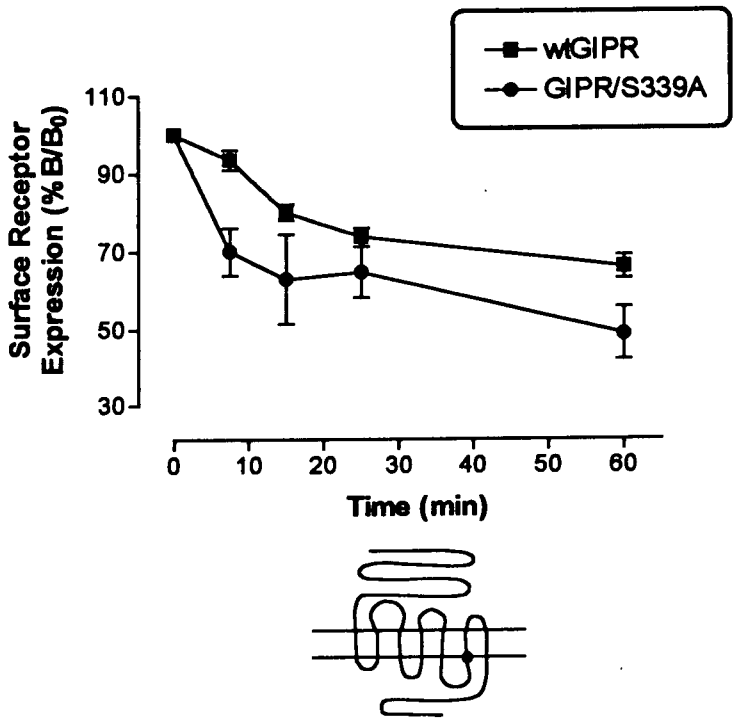


Fig. 22. Loss of Surface Receptor Expression During Incubation with 100 nM GIP_{1-30} Over 60 min. Wild type GIPR and mutant GIP receptors were expressed at high levels in CHO-K1 cells. Data are represented as mean ± S.E. • indicates site of mutation. c. wtGIPR and GIPR/S320A, n = 3-10. d. wtGIPR and GIPR/T324A, n = 4-10.

e.



f.

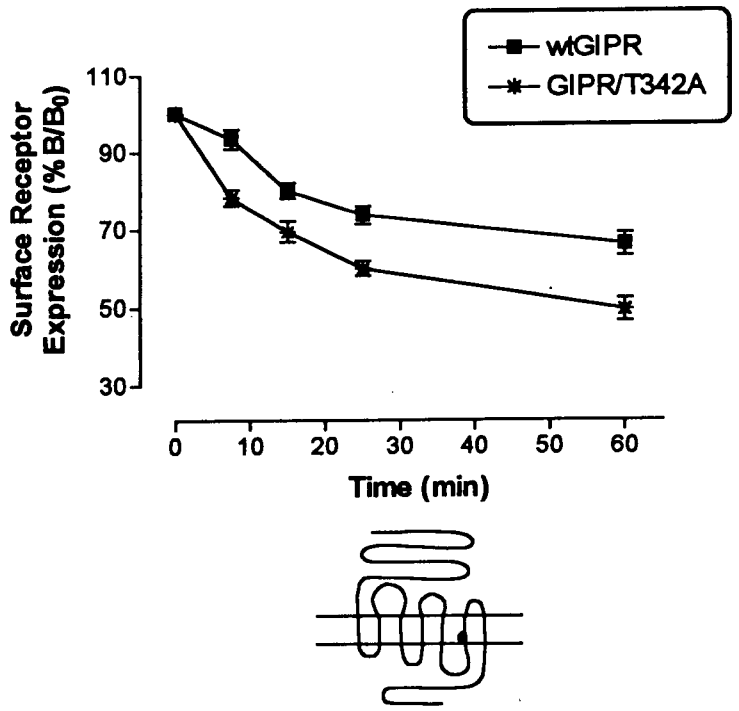
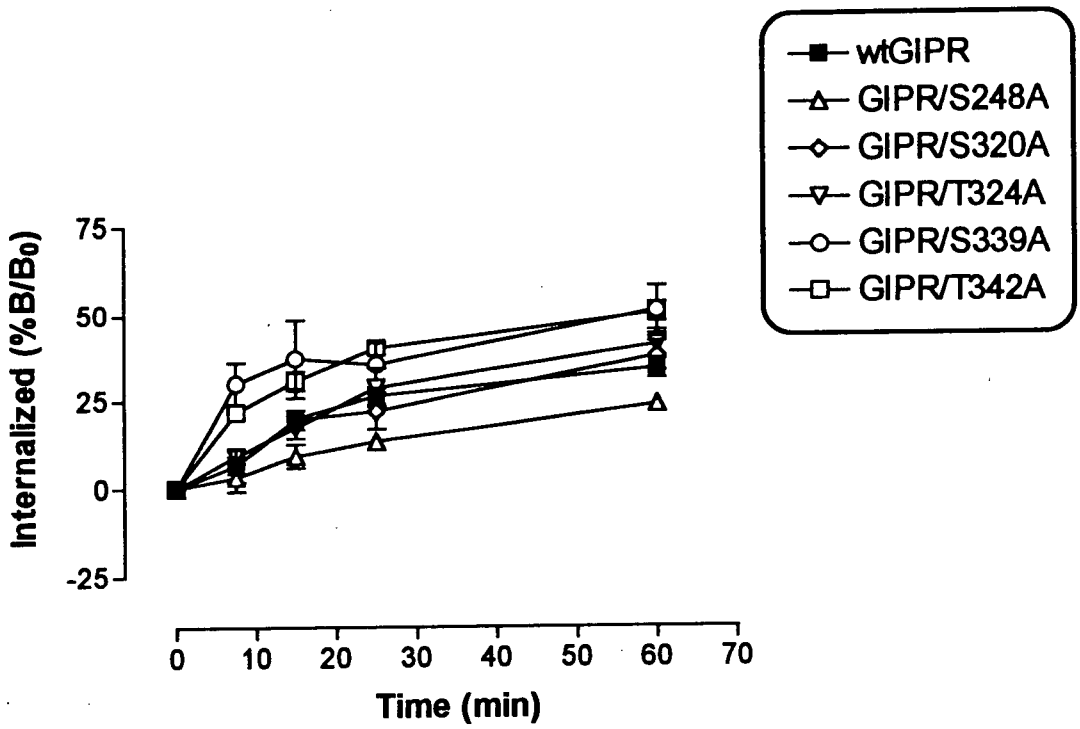


Fig. 22. Loss of Surface Receptor Expression During Incubation with 100 nM GIP_{1-30} Over 60 min. Wild type GIPR and mutant GIP receptors were expressed at high levels in CHO-K1 cells. Data are represented as mean ± S.E. • indicates site of mutation. e. wtGIPR and GIPR/S339A, n= 7-10. f. wtGIPR and GIPR/T342A, n = 6-10.

a.



b.

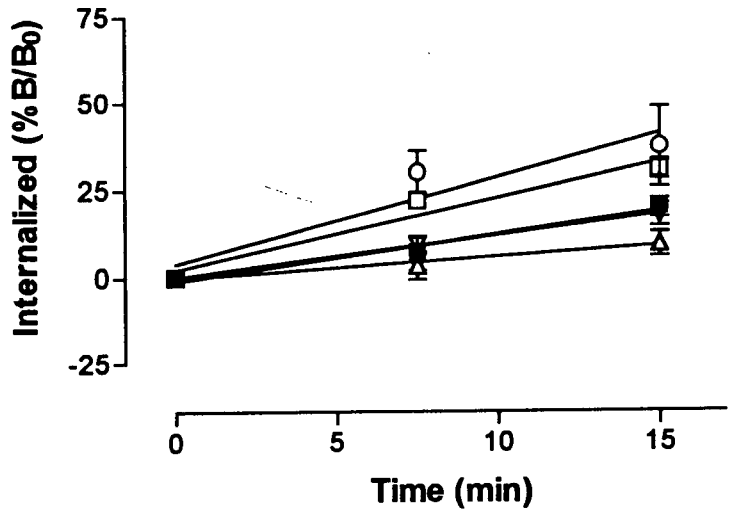


Fig. 23. Internalization Kinetics of Wild Type GIP Receptor (wtGIPR) and GIP Receptor Mutants in Transfected CHO-K1 Cells. a. Internalization over 1 h. b. Internalization over first 15 min. Data are represented as mean ± S.E.; n = 3-10. See Table 3 for statistical analysis.

CHAPTER 4

DISCUSSION

4.1 THE ROLE OF GIP IN FAT METABOLISM

Triglyceride ingestion increases plasma GIP levels through a pathway involving metabolism (Ross and Shaffer, 1981; Yoshidome et al. 1995) and absorption of FA (Ebert and Creutzfeldt, 1980; O'Dorisio et al. 1976) and GIP may be involved in TG disposal. Chylomicron-associated TG clearance from the blood in dogs is enhanced by GIP administration (Wasada et al. 1981), while peak plasma TG levels during an intraduodenal fat infusion in rats can be lowered by GIP administration (Ebert et al. 1991). GIP is also active in cultured preadipocytes where it stimulates LPL activity (Eckel et al. 1979), thus potentially increasing cellular uptake of TG. GIP may also be involved in adipocyte lipid metabolism as it potentiates the incorporation of FA (Beck, 1989) and glucose (Hauner et al. 1988) into lipids and it inhibits glucagon-stimulated lipolysis and cAMP production (Dupre et al. 1976; Ebert and Creutzfeldt, 1987). These studies appear to indicate an anabolic role for GIP in adipose tissue; however, GIP's main effects in the pancreas are mediated via cAMP (Wheeler et al. 1995; Gelling et al. 1997b), a substance which mediates catabolic effects in adipose tissue. Rising levels of cAMP in adipocytes leads to the stimulation of hormone-sensitive lipase activity, and the release of glycerol and FA (Yeaman, 1990). GIP stimulation of cAMP production has recently been demonstrated by direct measurement, and through the use of the adenylyl cyclase inhibitor MDL 12330A, in the stimulation of lipolysis in 3T3-L1 cells (McIntosh et al. 1999). In the same study, GIP stimulated lipolysis was inhibited by 10^{-10} M insulin, a concentration comparable to that seen circulating in the early postprandial period. This suggests that GIP may only

stimulate lipolysis during fasting. As circulating FA have been shown to be necessary for optimal glucose stimulation of insulin secretion following fasting (Stein et al. 1996), it could be that GIP is able to stimulate lipolysis when insulin concentrations are too low to be inhibitory, so that circulating FA levels are at an optimum for glucose and GIP stimulated insulin secretion (McIntosh et al. 1999).

4.1.1 GIP RECEPTOR LEVELS IN THE ZUCKER FATTY RAT

The Zucker fatty (*fa/fa*) rat, as a model of obesity, was used to examine GIP receptor expression in adipose tissue. The Zucker fatty rat contains a mutation (*fa*) which arose spontaneously in an outbred stock of the Zucker laboratory (Zucker and Zucker, 1961). The main characteristics of the obese (*fa/fa*) Zucker rat are hyperphagia (Bray and York, 1972), hyperlipidemia (Zucker and Zucker, 1962) and mild glucose intolerance (Ionescu et al. 1985), with obesity and profound hyperinsulinemia developing as early as 21 days of age (Zucker and Antoniades, 1972; Chan et al. 1985). The pancreatic islets are abnormally large (Shino et al. 1973) and respond to a glucose stimulus with a left-shift in the glucose-concentration response curve (Chan et al. 1984, 1985; Jia et al. 1995). The fasting hyperinsulinemia is probably due in part to this increased pancreatic sensitivity to low glucose levels. However, GIP and GLP-1, which normally only potentiate insulin secretion at a glucose concentration of 5 to 6 mM or higher, actually stimulate insulin secretion at basal glucose levels (Chan et al. 1984; Jia et al. 1995).

In view of the altered pancreatic responsiveness to GIP it was considered possible that fat cell responses are also altered, and that this may be associated with changes in adipose tissue GIP receptor expression. The current study was therefore established with

this premise in mind. The 11 week old obese Zucker rats appeared to have higher plasma glucose concentrations in response to oral glucose than did their lean littermates, however, this did not reach statistical significance. Rats from the UBC colony have previously been shown to have mild glucose intolerance, but differences in statistical analysis (analysis of variance as opposed to Student's t test) probably account for the difference in results seen here. The 16 week old obese animals showed fasting hyperglycemia and more severe glucose intolerance, which suggests that they may have become diabetic. The 16 week old obese rats also demonstrated greater plasma GIP responses to oral glucose than the lean animals. Obese Zucker rats normally display similar GIP responses to oral glucose as their lean littermates (Chan et al. 1984). The increases in glucose intolerance and circulating GIP in the obese rats may be a result of NIDDM. This suggests that the UBC colony is becoming more like the Zucker Diabetic Fatty (ZDF) rat which shows fasting hyperglycemia and is insulin resistant (McIntosh and Pederson, 1999). This rat developed from the inbreeding of a substrain of hyperglycemic *fa/fa* rats (Peterson et al. 1990). The pre-diabetic rats show normal glucose tolerance, but their islet β-cells are unable to respond normally to changes in glucose (Tokuyama et al. 1995), resulting in an unusually large insulin response to glucose. The males of this strain develop obesity, insulin resistance and overt NIDDM between 7 and 10 weeks of age, while their average plasma glucose is greater than 22 mM.

The 11 week old lean and obese Zucker rats displayed nearly identical levels of adipose GIP receptor mRNA. Although the *fa/fa* rat is hyperinsulinemic, GIP levels are normal at 11 weeks (Chan et al. 1984). This suggests that the adipose cell GIP receptor expression may not to be involved in the phenotype of the animal at this stage of

development, although a defective receptor cannot be ruled out. Additionally, mRNA levels also do not necessarily reflect actual protein levels and it is possible that there are changes at the level of translation or subsequent incorporation of receptor protein into the membrane. In addition, the rat is still relatively young at 11 weeks of age, and fasting hyperglycemia and elevated GIP levels only develop later. They may also develop altered GIP receptor expression at a later time.

While the current study was underway, the identity of the genetic defect in the Zucker rat was clarified, and shown to be a single point mutation (CAG→CCG) in the *fa* gene resulting in a Gln269Pro substitution in the extracellular domain of the receptor (Ob-R) for the newly discovered hormone, leptin (Chua et al. 1996; Iida et al. 1996). In transient transfection studies with rat OB-R cDNA containing this mutation it was demonstrated that there is a severe loss in surface leptin binding (Yamashita et al. 1997), as well as abnormal signaling in the pathways that involve early response genes (c-fos, c-jun, jun-B) (Yamashita et al. 1997) and Signal Transducers and Activators of Transcription (STAT) 5B (White et al. 1997). Although a leptin receptor deficiency evidently had no effect on GIP receptor levels it could be involved in the altered pancreatic response to GIP. A short discussion to outline the characteristics and actions of leptin is therefore necessary.

4.1.2 LEPTIN

Leptin was originally discovered by positional cloning of the mouse *ob/ob* gene (Zhang et al. 1994) that contains a point mutation. This mouse is characterized by obesity which is a result of a deficiency of the *ob* gene product, leptin, and can be reversed by

leptin replacement (Pelleymounter et al. 1995; Halaas et al. 1995; Campfield et al. 1995). Leptin is a 146 amino acid secreted protein which is considered part of the hematopoietic cytokine family on the basis of its structure (Reviewed in McIntosh and Pederson, 1999). The mouse leptin shares 84% and 95 to 96% sequence identity with the human and rat proteins, respectively (Zhang et al. 1994; Murakami and Shima, 1995). The Ob-R is predicted on the basis of cDNA sequence to be a single membrane-spanning protein belonging to the class I cytokine receptor family (Tartaglia et al. 1995; Tartaglia, 1997). The human and mouse receptors share a high degree of homology (Chen et al. 1996). The Zucker fatty rat has abnormally high circulating leptin and *ob* mRNA levels in its white adipose tissue, as do all other models of genetic and diet-induced obesity that have been examined (Weigle and Kuijper, 1996; Campfield et al. 1996; Saladin et al. 1996). This implies that the size of the adipose tissue mass is reflected in the level of *ob* gene expression and leptin synthesis (Campfield et al. 1996; Saladin et al. 1996). There is indeed a positive correlation in adult humans among body mass index (BMI), body fat, *ob* mRNA levels and circulating leptin levels (Campfield et al. 1996; Saladin et al. 1996; Caro et al. 1996; Considine and Caro, 1996; Considine et al. 1996; Lonnqvist et al. 1995), with the highest correlation found between circulating leptin and percentage body fat (Considine and Caro, 1996; Considine et al. 1996). However in children, leptin is important in maintaining energy balance, as plasma leptin levels correlate with total energy expenditure and the level of physical activity, independent of body weight (Salbe et al. 1997). It has been proposed that the elevated levels of plasma leptin found in human obesity reflect resistance to leptin's actions (Banks et al. 1996; Golden et al. 1997; Malik and Young, 1996; Schwartz et al. 1996b), while other studies suggest that an early leptin

deficiency may contribute to obesity and NIDDM in some groups (Ravussin et al. 1997; Havel et al. 1996; Rosenbaum et al. 1996).

Mature adipocytes are the main site of *ob* gene expression (Masuzaki et al. 1995), with all rat fat deposits containing *ob* mRNA (Moinat et al. 1995; Tsuruo et al. 1996). It has been proposed that major determinants of *ob* gene expression may be cell stretching and/or lipid content, as the level of *ob* gene expression does reflect the size of the adipose depot (McIntosh and Pederson, 1999). Adipocyte leptin levels have also been shown to be affected by nutrient intake (Saladin et al. 1996; Frederich et al. 1995a; Becker et al. 1995; Masuzaki et al. 1996), and a role for insulin in the regulation of leptin gene expression has been suggested by several studies (McIntosh and Pederson, 1999; Saladin et al. 1995, 1996; Zimmet et al. 1996; Kolaczynski et al. 1996).

An important role for leptin in body weight homeostasis and the regulation of energy balance has been established, in which leptin signals information to the brain about the size of energy stores and activates centers involved in regulating food intake and energy expenditure (Caro et al. 1996; Spiegelman and Flier, 1996; Flier, 1995). Studies with *ob/ob* mice have shown that leptin will decrease food intake, percentage body fat and body weight, and circulating glucose and insulin concentrations (Pelleymounter et al. 1995; Halaas et al. 1995; Campfield et al. 1995; Schwartz et al. 1996a; Stephens et al. 1995; Weigle et al. 1995). Normal mice also reduce their food intake in response to leptin, although to a lesser degree than *ob/ob* mice (Halaas et al. 1995; Campfield et al. 1995).

Leptin also acts via the sympathetic nervous system to increase energy expenditure (Haynes et al. 1997), and acts directly on adipocyte metabolism to increase glucose

utilization and lipolysis (Siegrist-Kaiser et al. 1997). Normal rats administered a recombinant adenovirus vector overexpressing leptin, showed a large reduction in whole body fat, accompanied by decreased circulating TG and insulin (Chen et al. 1996). These studies imply a possible lipoatrophic role for leptin, in which leptin deficiency contributes to insulin resistance through an increase in FFAs (Reviewed in McIntosh and Pederson, 1999). Leptin may also directly inhibit insulin actions (Cohen et al. 1996).

A hypothesis to explain the peripheral effects of leptin has recently been presented by Unger (1999). He proposes that leptin protects nonadipocytes from lipotoxicity by limiting their storage of TG while directing this storage to the adipocytes. He points out that nonadipocytes maintain their TG content within a narrow range (Koyama et al. 1997a; Lee et al. 1994), regardless of food intake, while that of adipocytes varies considerably. Normal islets cultured in FA show only a small rise in TG content because the FA are directed into oxidative rather than lipogenic pathways (Zhou et al. 1998; Shimabukuro et al. 1997), while islets from ZDF rats which have defective leptin receptors, display an increase in TG accumulation when cultured with the same FA concentrations (Lee et al. 1997), suggesting that leptin plays a role in this pathway. Unger suggests that nonadipocytes contain a small FA reserve in the form of TG for the purpose of maintaining structural and signaling components of the cell. If this reserve was used for fuel, the normal functioning of the cell would be compromised, and as the TG storage capacity of nonadipocytes is small, a specialized storage facility, the adipocyte, developed. Adipocytes are capable of storing large quantities of fuel which can then be used by nonadipocytes as needed without compromising the upkeep of the cell. He proposes that a signal was needed during times of TG deposition to prevent equal distribution to all cells

and promote storage in the adipocytes, and this signal is leptin. To support this hypothesis Unger points to the rise in adipocyte leptin mRNA which is associated with an increase in food intake (Saladin et al. 1995; Frederich et al. 1995b). The ZDF rat with its defective Ob-R receptor also fits this model. The nonadipocyte tissues have a significantly greater TG content (Lee et al. 1994, 1997), which increases concomitantly with the increase in adipocyte TG (Unger, 1995; Lee et al. 1994). As the islet TG content rises, the β cells initially become hyperplastic with increased insulin production (Hirose et al. 1996; Milburn et al. 1995), but eventually undergo lipoapoptosis (Shimabukuro et al. 1998) and diabetes is manifested. There are also morphological changes to the islets (disorganization of the islet architecture, loss of the glucose transporter GLUT-2, and mitochondrial abnormalities) which point to lipotoxicity. In islets from normal rats with functioning Ob-Rs, adenovirus-mediated ectopic overexpression of the leptin gene results in fat-depleted islets with low basal insulin secretion and an inability to respond to glucose and arginine stimulation, both of which can be restored with the addition of FA (Koyama et al. 1997b).

Therefore, Unger's hypothesis suggests that with an increase in plasma TG, and increased TG storage in adipocytes, leptin would signal nonadipocytes to direct FA to oxidative rather than lipogenic pathways. At the same time it would signal the brain to decrease food intake and increase energy expenditure, thus eventually lowering circulating glucose and TG. In light of what is now known about leptin and the *ob* gene, one can speculate as to what may be occurring in the *fa/fa* rat. GIP stimulates insulin secretion at basal glucose levels in this animal, while a glucose concentration of 5 to 6 mM is usually required (Chan et al. 1984; Jia et al. 1995), possibly contributing to hyperinsulinemia. Chronic insulin administration to humans increases *ob* gene and circulating leptin levels

(Saladin et al. 1996; Kolaczynski et al. 1996), which suggests a long term effect of insulin through a trophic effect on fat cells (McIntosh and Pederson, 1999). Plasma leptin and adipose *ob* mRNA levels are known to be elevated in the *fa/fa* rat (Weigle and Kuijper, 1996; Campfield et al. 1996; Saladin et al. 1996); however, the mutation in the Ob-R probably results in a functional loss of leptin in this animal. This would mean a lack of signaling to the brain about the need to decrease food intake and increase energy expenditure (Caro et al. 1996; Spiegelman and Flier, 1996; Flier, 1995). Leptin has been suggested to have a lipoatrophic role, so that a leptin deficiency may contribute to insulin resistance through an increase in FFAs (Reviewed in McIntosh and Pederson, 1999), thus explaining the insulin resistance seen in the *fa/fa* rat.

4.2 ALTERNATIVE SPLICE VARIANTS OF THE GIP RECEPTOR

Although GIP's primary role defined to date is the stimulation of insulin secretion from the endocrine pancreas, GIP receptor mRNA has been detected in many tissues outside of the pancreas, including adipose tissue (Usdin et al. 1993). This suggests that GIP may have additional functions in addition to acting as an incretin. Indeed, as mentioned previously in Section 4.1, GIP appears to stimulate a catabolic pathway in adipose tissue (McIntosh et al. 1999), and is known to have gastric inhibitory properties (Section 1.4.1). The GIP receptor belongs to the secretin/VIP subfamily of GPCRs, several of whose members exist in differentially spliced forms, which leads to the possibility of alternatively spliced forms of the GIP receptor also being expressed. Alternative functions of GIP may be mediated fully or in part via alternative isoforms of

the GIP receptor. For this reason the GIP receptor was examined for the existence of alternatively spliced variants.

When the i3 loop of the rat GIP receptor was amplified using PCR, additional products were found using islet and adipose cDNA. The translation of the islet sequence indicates a truncated form of the GIP receptor. The insert begins in the middle of TM5 and extends into the ic loop where a stop codon occurs. Whether this truncated receptor is actually expressed *in vivo* is unclear. An alternatively spliced variant of the GHRH receptor was discovered which was truncated at the C-terminal end of the i3 loop, but it was unable to transduce a signal stimulated by GHRH when expressed in COS 7 cells (Hashimoto et al. 1995). However, when co-expressed with the wtGHRH receptor, it acted as a dominant-negative inhibitor of GHRH-induced cellular signaling (Tang et al. 1995). A mutated form of the β_2AR which is truncated at the C-terminal end of the i3 loop was found to be incapable of ligand binding (Kobilka et al. 1987), but this was not tested with the truncated GHRH receptor (Motomura et al. 1998). Further studies would be needed to determine a role, if any, for this truncated form of the GIP receptor.

The adipose tissue sequence is more interesting, in that it appears to encode a splice variant which may actually be expressed *in vivo*. This potential splice variant contains a 28 amino acid insert located at the C-terminal end of the i3 loop, flanked on either side by Arg-335 and Leu-336 (Fig. 5). This would more than double the size of the i3 loop. The insert contains three potential phosphorylation sites, two threonines and a serine, which could be involved in receptor desensitization and internalization (Section 1.9.5-1.9.8). Serines and threonines are phosphorylation sites for GRKs, the kinases which mediate homologous desensitization, and for PKA and PKC, which mediate

heterologous desensitization (Bohm et al. 1997a; Chuang et al. 1996; Ferguson et al. 1996a). Phosphorylation has also been implicated in receptor internalization in several GPCR receptors (Ferguson et al. 1996a).

As discussed in Section 1.9.3, the i3 loop of GPCRs is extremely important for appropriate G-protein recognition (Wess, 1998). It is predicted to interact with other receptor domains, chiefly the i2 loop, resulting in optimal coupling efficiency and selectivity (Gomeza et al. 1996; Blin et al. 1995; Wong and Ross, 1994; Wong et al. 1990). The N- and C-terminal eight to fifteen amino acids of the i3 loop are particularly critical for appropriate G-protein coupling (Strader et al. 1994). A series of mainly hydrophobic or non-charged residues in the N-terminus of the i3 loop have been demonstrated to be essential for receptor/G-protein coupling selectivity in several GPCRs, including subtypes of the mAChR (Blin et al. 1995; Bluml et al. 1994a,b,c; Hogger et al. 1995; Hill-Eubanks et al. 1996; Liu et al. 1995), the PTH/PTHrP receptor (Huang et al. 1996) and the GLP-1 receptor (Mathi et al. 1997). The GIP receptor has a leucine residue at position 322 in the N-terminal half of its i3 loop, but does not have a run of hydrophobic or non-charged residues (Fig. 5). One or more positively charged residues within a stretch of charged residues in the C-terminus of most GPCRs are required for efficient G-protein activation (Hogger et al. 1995; Kunkel and Peralta, 1993; Lee et al. 1996; Obosi et al. 1997; Wang, 1997). The GIP receptor has arginines at positions 333, 335 and 338 in the C-terminus half of its i3 loop (Fig. 5). The addition of the 28 amino acids (Fig. 15) would still mean three positively charged residues in the C-terminal 15 amino acids of the i3 loop, and would not change the sequence of the N-terminal amino acids. A set of four, mainly hydrophobic, amino acids have been identified as important to

G-protein coupling selectivity in the mAChRs (Blin et al. 1995; Liu et al. 1995; Kostenis et al. 1997). These residues are located at the i3 loop/TM6 junction and are predicted to be α-helically arranged as an extension of TM6, and forming a hydrophobic surface (Strader et al. 1994; Altenbach et al. 1996). In the m1, m3 and m5 muscarinic receptors these residues correspond to Ala-488, Ala-489, Leu-492 and Ser-493 (Blin et al. 1995), and in the m2 and m4 receptors they correspond to Val-385, Thr-386, Ile-389 and Leu-390 (Liu et al. 1995; Kostenis et al. 1997). The GIP receptor has a similar motif at the i3 loop/TM6 junction, corresponding to Leu-336, Ala-337, Thr-340 and Leu-341 (Fig. 5), which is not disrupted by the presence of the 28 amino acid insert. By simply examining the sequence of the insert in relation to the i3 loop of the GIP receptor, the presence of the insert does not appear to change the presence or placement of residues important for G-protein coupling and selectivity. However, further studies need to be performed to examine the binding and signaling characteristics of this alternatively spliced variant of the GIP receptor. An altered i3 loop may still mean coupling to alternate G-proteins and the initiation of alternate signaling pathways. Given that this splice variant appears to be most strongly expressed in adipose tissue, an alternative signaling pathway is possible, as GIP's actions here are different from its actions in the pancreas. GIP may also have a catabolic role in adipose tissue as seen by its ability to stimulate glycerol release (McIntosh et al. 1999), although previous studies indicate that GIP has lipogenic effects (Beck and Max, 1983; Eckel et al. 1979; Oben et al. 1991; Starich et al. 1985; Hauner et al. 1988; Knapper et al. 1995b; Ebert et al. 1991). Clearly, more studies need to be undertaken to clarify GIP's actions and the pathways through which these actions are mediated.

4.3 THE IMPORTANCE OF THE SERINE AND THREONINE RESIDUES IN THE INRACELLULAR LOOPS OF THE RAT PANCREATIC GIP RECEPTOR

4.3.1 THE ROLE OF SERINE AND THREONINE RESIDUES IN GIP STIMULATED cAMP PRODUCTION

As already discussed in Sections 1.9.2, 1.9.3 and 4.2, the i2 and i3 loops of many GPCRs are of primary importance in G-protein recognition and receptor/G-protein coupling (Wess, 1998). The importance of the i2 loop for regulating the selectivity of receptor/G-protein coupling has been demonstrated using hybrid GPCRs (Reviewed in Wess, 1998). In addition to aiding in appropriate G-protein recognition (Wess, 1998), the i3 loop is believed to interact with other receptor domains, mainly the i2 loop, to optimize coupling efficiency and selectivity (Gomeza et al. 1996; Blin et al. 1995; Wong and Ross, 1994; Wong et al. 1990).

In the current study, mutations of serine and threonine residues in the i2 and i3 loops of the GIP receptor affected GIP stimulated cAMP production in both low expressing and high expressing subclones. In most cases the mutations resulted in a decrease in cAMP production compared to the wtGIPR. GIPR/S320A and GIPR/S339A showed the most drastic decrease with no noticeable increase in cAMP production over basal in the low expressing subclones. The GIPR/S320A and GIPR/S339A high expressing subclones showed significant increases in cAMP production over basal, but these increases were very small compared to that obtained with the wtGIPR. The cAMP response to GIP stimulation by the low expressing subclones of GIPR/S248A and GIPR/T324A are 3 and 4 fold lower, respectively, than the response of the wtGIPR, while the responses of the high expressing subclones are almost 5 times lower. Binding in these four mutant receptors did not appear to be affected, so it appears that these mutations

impair the ability of the GIP receptor to transduce its signal. As both the i2 and i3 loops are important for G-protein recognition and the i3 loop has a role in coupling efficiency and selectivity, the mutation of these serines and threonines is probably disrupting the ability of these domains to act effectively. The mutations of Ser-320 and Ser-339, which are located at the boundaries of the i3 loop and TM domains seem to disrupt signaling to a greater extent than Ser-248 and Thr-324, which are located toward the middle of the intracellular loops. Ser-320 and Ser-339 may, therefore, be critical residues in receptor/ G-protein coupling. As discussed in Section 4.2, sets of four, mainly hydrophobic, amino acids have been identified as important to G-protein coupling selectivity in the mAChRs (Blin et al. 1995; Liu et al. 1995; Kostenis et al. 1997). These residues are located at the i3 loop/TM6 junction and are predicted to be α-helically arranged as an extension of TM6, where they form a hydrophobic surface (Strader et al. 1994; Altenbach et al. 1996). The GIP receptor has a similar motif at the i3 loop/TM6 junction, corresponding to Leu-336, Ala-337, Thr-340 and Leu-341 (Fig. 5). By mutating Ser-339 to an alanine, the pattern of the α-helix may be disrupted or changed, thus changing the surface which interacts with the G-protein and impairing G-protein coupling. Interestingly, the GIPR/T342A high expressing subclone did not show a significant reduction in GIP stimulated cAMP production compared to wtGIPR, while the low expressing subclone showed a 3.4-fold increase in GIP stimulated cAMP production compared to wtGIPR. The mutation of Thr-342 may be causing a change in conformation of the receptor which allows more efficient receptor/G-protein coupling.

4.3.2 THE ROLE OF SERINE AND THREONINE RESIDUES IN GIP RECEPTOR DESENSITIZATION

Phosphorylation by serine/threonine kinases of residues in the intracellular loops and CT tail of GPCRs mediates receptor desensitization (Bohm et al. 1997a; Chuang et al. 1996; Ferguson et al. 1996a) and is often involved in receptor sequestration (Ferguson et al. 1996a). Homologous desensitization (Fig. 2) is the deactivation of one receptor type as a result of agonist activation, and results in the uncoupling of the activated receptor from its G-protein (Bohm et al. 1997a; Chuang et al. 1996; Ferguson et al. 1996a). It is usually mediated via receptor phosphorylation by the GRKs in cooperation with their cofactors the arrestins (Bohm et al. 1997a; Chuang et al. 1996; Ferguson et al. 1996a), but is also at times effected through PKA and PKC (Chuang et al. 1996; Ferguson et al. 1996a).

GRK-mediated desensitization of GPCRs (see Section 1.9.5) generally requires the phosphorylation of several serine and threonine residues, with the initial phosphorylation events probably being the most important (Ohguro et al. 1993). Many receptors, like the β_2AR, contain several potential phosphorylation sites in their CT tails, which are crucial for desensitization (Bohm et al. 1997a; Chuang et al. 1996; Ferguson et al. 1996a). Among members of the secretin/VIP receptor subfamily, the CT tail is the major site of phosphorylation in receptors for secretin (Ozcelebi et al. 1995; Holtmann et al. 1996b), PTH/PTHrP (Blind et al. 1995, 1996), glucagon (Buggy et al. 1997; Savage et al. 1995; Heurich et al. 1996), and GLP-1 (Thorens and Widmann, 1996; Widmann et al. 1996a,b, 1997). Phosphorylation by PKA and/or PKC is known to be involved in, but not totally responsible for the desensitization of PTH/PTHrP, glucagon and GLP-1 receptors

(Thorens and Widmann, 1996; Blind et al. 1995, 1996; Savage et al. 1995; Widmann et al. 1996a,b). The phosphorylation of these receptors by GRKs is suspected, but as yet only the *in vitro* phosphorylation of the recombinantly expressed PTH/PTHrP receptor CT tail by βARK has been demonstrated (Blind et al. 1996).

The GIP receptor contains six serines in its CT tail. A study by Tseng (1998), in which Ser-406 was replaced by an arginine, claimed that desensitization was partially attenuated in this mutant as compared to the wild type receptor. However, desensitization was defined as the residual ability of cells to increase cellular cAMP levels in cells preincubated with 10^{-7} M GIP for 24 h. Desensitization takes place during short term (seconds to minutes) exposure of cells to agonists and occurs through receptor/G-protein uncoupling (Bohm et al. 1997a; Chuang et al. 1996; Ferguson et al. 1996a). After a 24 h incubation period, an inability to increase cellular cAMP levels would most likely be due in large part to receptor sequestration or down-regulation, which occurs over the long term (hours to days) exposure of cells to agonists (Bohm et al. 1997a; Ferguson et al. 1996a).

The wild type GIP receptor appeared to show a decrease in GIP stimulated cAMP production after preincubation with GIP, but this was not significant. Previous experiments in our laboratory have shown a significant decrease in GIP stimulated cAMP production after preincubation with GIP under similar conditions (Hinke et al. 2000). As the sample size was small (n = 3), it is likely that increasing the sample size would result in significance between the two GIP stimulated groups. GIPR/S248A, GIPR/T324A and GIPR/T342A did not show reductions in GIP stimulated cAMP production after preincubation with GIP compared to wtGIPR. As the decrease in GIP stimulated cAMP production did not reach significance, it is not possible to say whether the mutation of

these residues has any effect on desensitization. However, as mentioned in Section 3.3.1, point mutations of other GPCRs, for example, the β_2AR (Hausdorff et al. 1990), have been shown to diminish the ability of these receptors to desensitize, so this may be the case here.

4.3.3 THE ROLE OF SERINE AND THREONINE RESIDUES IN GIP RECEPTOR INTERNALIZATION

GPCR internalization, as discussed in Section 1.9.6, is agonist-induced and usually involves the uptake of receptor and agonist into lysosomes where they are dissociated by acidification (Fig. 2) (Bohm et al. 1997a; Ferguson et al. 1996a). The agonist is degraded in the lysosomes while the receptor is recycled (Bohm et al. 1997a; Ferguson et al. 1996a), although a small percentage of receptors may be sorted and targeted to lysosomes for degradation (von Zastrow and Kobilka, 1992). Internalization is most likely accomplished via clathrin-coated pits or caveolae, the mechanism depending on the individual receptor and its cellular environment (Ferguson et al. 1996a). During resensitization, the receptors are probably dephosphorylated before being returned to the plasma membranes (Bohm et al. 1997a; Ferguson et al. 1996a).

There is no single endocytic motif common to all GPCRs, but several which differ among the various receptors (Section 1.9.7). The CT tail (Elrick et al. 1964; Ferguson et al. 1996a), tyrosine-containing motifs (Elrick et al. 1964; Ferguson et al. 1996a) and a highly conserved DRYXXV/IXXPL motif (Ferguson et al. 1996a) have all been implicated in the sequestration of GPCRs. Receptor phosphorylation has also been implicated in the internalization of several GPCRs (Reviewed in Ferguson et al. 1996a).

Experiments using a sequestration-defective mutant β_2AR-Y326A found that GRK-mediated phosphorylation was not absolutely required for internalization, but probably acted to promote internalization by stabilizing the needed β_2AR conformation and the association with other cellular elements necessary for internalization (Ferguson et al. 1996a,b; Hausdorff et al. 1989). Phosphorylation of Ser/Thr residues appears to regulate sequestration in a number of receptors of the secretin/VIP subfamily, as discussed in Section 1.9.8. Serines in the CT tail of receptors for glucagon, GLP-1, and PTH/PTHrP, have been shown to play a role in sequestration (Buggy et al. 1997; Widmann et al. 1995, 1997), with the degree of internalization dependent on the number of sites phosphorylated (Buggy et al. 1997; Widmann et al. 1996a; Huang et al. 1995a,b).

While the selective serine and threonine mutations to the GIP receptor appeared to remove sites involved in desensitization, the effects on internalization were somewhat different. When preincubated with GIP over one hour, the wtGIPR showed an initial internalization rate of 1.33 %/min with a maximum internalization of 40% of total binding. GIPR/S248A was the only mutant receptor to show a decrease in both internalization rate and maximum internalization from wtGIPR values. The initial internalization rate of this mutant was less than half that of the wtGIPR. The maximum internalization was also less than that of the wtGIPR but this difference was not significant, and more experiments are needed to establish whether there is a reduction. Phosphorylation of this serine residue may aid in the efficient sequestration of the GIP receptor. As occurs with the β_2AR (Ferguson et al. 1996a,b; Hausdorff et al. 1989), phosphorylation of this residue may act to stabilize the receptor conformation to aid in the association with the cellular elements

involved in the internalization process. Thus, it acts to increase the rate of receptor internalization, but does not effect the amount of maximum internalization.

Neither GIPR/S320A nor GIPR/T324A showed changes in internalization rate or maximum internalization compared to wtGIPR, and these residues therefore, do not appear to be involved in GIP receptor sequestration. GIPR/S339A and GIPR/T342A both showed an increase in the rate of internalization and maximum internalization compared to wtGIPR. The phosphorylation of Ser-339 and Thr-342 may negatively regulate the mechanism of receptor internalization, and act as a check to counterbalance receptor sequestration. Removal of regulatory elements by truncation of the CT tail has been shown to increase the sequestration rate in some GPCRs, namely the LH/CG receptor and the avian β_1 or β_2AR (Rodriguez et al. 1992; Parker et al. 1995; Bouvier et al. 1988). The CT tail of the PTH/PTHrP receptor is rich in Ser/Thr residues and appears to contain both positive and negative regulatory elements (Huang et al. 1995a). A recent study by Wheeler et al. (1999) examined the role of the six serine residues in the CT-tail of the GIP receptor in receptor sequestration. Mutation of serines 426, 427 and 440 to alanine resulted in a decrease in maximum internalization for all three mutants, and a decrease in initial internalization rate for S426A and S427A. A complete CT serine knockout mutant showed an initial internalization rate of only 46% that of the wild type receptor, and a decrease in maximum internalization. However, none of these residues appear to negatively regulate internalization. It appears that the CT tail and the i2 loop are the more important domains for positively regulating receptor internalization, while the i3 loop contains negative regulatory elements for this process.

4.4 CONCLUSIONS AND FUTURE DIRECTIONS

Understanding the changes or lack of changes in adipose GIP receptor expression with obesity and NIDDM is a step towards understanding these abnormal states. The discovery of an alternatively spliced variant of the GIP receptor, most strongly expressed in adipose tissue which may have an alternative signaling pathway is also important for better understanding the role of GIP in adipose tissue and its potential contribution to disease states. The discovery that the mutation of the serine and threonine residues in the i2 and i3 loops of the GIP receptor has an effect on cAMP production, desensitization and internalization will lead to a better understanding of how GIP exerts its effects at its target tissues and how these effects are regulated. Once these processes are fully understood and combined with the knowledge of the roles of GIP in both physiological and pathophysiological states, the possibility of modulating GIP's actions as a treatment for obesity and/or NIDDM can be examined. However, there is much that still needs to be studied in these areas.

The 11 week old *fa/fa* rat did not show changes in GIP receptor mRNA compared to the lean rats; however, as stated in Section 4.1.1, this does not necessarily reflect protein levels. Therefore, an examination of GIP receptor levels by Western analysis would be useful. It would also be interesting to examine both GIP receptor mRNA and protein levels in older *fa/fa* rats from the UBC colony, as the 16 week old rats showed fasting hyperglycemia and increased GIP levels. The discovery of an insert in the i3 loop of the GIP receptor which likely encodes a truncated form of the GIP receptor, suggests that this form of the receptor may not be expressed. However, a truncated form of the GHRH receptor, when co-expressed with the wtGHRH receptor, acted as a dominant-

negative inhibitor of GHRH-induced cellular signaling (Tang et al. 1995). Therefore, it would be of interest to attempt to express this truncated GIP receptor and measure its ability to bind GIP, and examine its effects, if any, on GIP-induced signaling via the wtGIP receptor.

The discovery of a 28 amino acid insert in the C-terminal end of the i3 loop of the GIP receptor points to a possibly functional alternatively spliced variant. This receptor needs to be examined for its ability to bind ligand and transduce a signal. As this receptor variant's mRNA was most strongly expressed in adipose tissue, where GIP's actions are different from its actions in the pancreas, it may signal through alternative pathways not utilized in the β-cell. Currently, a project is underway in our laboratory, in which Western analysis is being used to detect phosphorylation of enzymes which may be involved in the signaling pathways of GIP. It will be important to also carry out these experiments using this alternatively spliced variant of the GIP receptor. This insert also contains three potential phosphorylation sites which should be examined for roles in desensitization and internalization.

The mutation of Ser-248, Ser-320, Thr-324 and Ser-339 appeared to impair the signaling ability of the GIP receptor. It would be of interest to examine the effects on cAMP production of mutating all four residues together. The desensitization of the wtGIPR did not reach significance as it has with previous experiments in our laboratory, so this experiment needs to be repeated to increase the sample size. When this is done, the effects of mutating Ser-248, Thr-324 and Thr-342 on receptor desensitization can be determined.

The mutation of Thr-342 appeared to increase both GIP-stimulated cAMP production and receptor internalization, while mutation of Ser-339 also increased internalization. It would be interesting to examine the effect on internalization of mutating both of these residues. If they both act as negative regulatory elements, the two mutations together may even further increase internalization.

Ser-248 was the only residue that when mutated, decreased internalization. As the serines in the CT-tail of the GIP receptor have been shown to be important in regulating its internalization (Wheeler et al. 1999), a knockout of Ser-248 in addition to the CT serines may prove to further hinder internalization.

The discovery of an alternatively spliced variant of the GIP receptor and the experiments which have begun to examine GIP receptor expression in adipose tissue during obesity, will both contribute to a better understanding of GIP's role in this tissue and the potential changes to its actions which may occur during obesity and NIDDM. Discovering how the serine and threonine residues in the i2 and i3 loops of the GIP receptor contribute to GIP receptor signaling and the regulation of the signal through receptor desensitization and internalization have also contributed to the growing knowledge about GIP and its signaling pathways.

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