ISOLATION OF A MURINE GLUCOSE-DEPENDENT INSULINOTROPIC POLYPEPTIDE (GIP) cDNA FROM A TUMOR DERIVED CELL LINE (STC^6.14) AND QUANTIFICATION OF GLUCOSE INDUCED INCREASES IN GIP mRNA

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Abstract

This thesis reports the attempts to characterize further the murine neuroendocrine cell line STC6-14 with the hope of demonstrating this cell line's potential use for intracellular studies involving GIP. These studies include the identification of a murine GIP cDNA and investigations into the role of glucose in the increase of GIP mRNA within this cell line.

Sequence analysis revealed a 537 base pair cDNA clone for murine GIP which was found to encode an open reading frame of 432 base pairs. From this sequence a 144 amino acid precursor could be predicted which was shown to code for a 43 amino acid N-terminal extension—which includes a 19-21 amino acid signal peptide, a 42 amino acid hormone and a 59 amino acid C-terminal extension. Murine GIP is predicted to differ from the human hormone by three amino acid substitutions: arginine for histidine at position 18, arginine for lysine at position 30 and serine for lysine at position 34.

GIP mRNA levels in STC6-14 cells incubated in the presence of varying glucose concentrations were investigated using a competitive-PCR method. In the presence of a 5mM glucose stimulus, 1x10^5 GIP cells were found to contain 3.9 ± 0.59 attomoles (amol) of GIP mRNA while the same number of cells contained 11.6 ± 1.4 amol when subjected to a high (25mM) glucose stimulus (p< 0.0025).

Release studies for GIP in the STC6-14 cell line were performed in conjunction with the mRNA studies to ascertain the link between GIP mRNA levels within the GIP cell and the amount of mature GIP released into the medium. In response to a low glucose (5 mM) stimulus, 3 x 10^5 STC6-14 cells released 0.84 ± 0.04% of TCC while the same number of cells were found to release 1.11 ± 0.11% of TCC when cultured in a high glucose (25 mM) media.

These investigations demonstrated that the STC6-14 cell line responds as predicted to a high glucose stimulus with an increase in the level of GIP mRNA but fails to transfer this mRNA increase into a greater GIP peptide release, most likely due to a problem within the post-translational processes of the GIP cell.
TABLE OF CONTENTS

ABSTRACT .................................................................................................................. ii

TABLE OF CONTENTS............................................................................................... iii

LIST OF FIGURES ........................................................................................................ vi

LIST OF ABBREVIATIONS ............................................................................................. vii

ACKNOWLEDGEMENTS ............................................................................................... viii

INTRODUCTION ............................................................................................................. 1

  History ......................................................................................................................... 1

  Isolation of Gastric Inhibitory Polypeptide .................................................................. 2

  Characterization of GIP .............................................................................................. 3

  Enteroinsular Axis ...................................................................................................... 3

  The GIP Receptor ...................................................................................................... 4

  The GIP Gene ............................................................................................................. 5

  GIP Gene Regulation ................................................................................................. 6

  Quantification of GIP Gene Regulation ..................................................................... 7

  Measurement of GIP ................................................................................................. 8

  Localization of GIP .................................................................................................... 9

  GIP Release in response to a Mixed Meal ................................................................. 10

  Release in Response to Carbohydrates ...................................................................... 10

  Release in Response to Fat ....................................................................................... 12
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Number</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nucleotide sequence and deduced amino acid sequence of murine GIP.</td>
<td>29</td>
</tr>
<tr>
<td>2</td>
<td>Electrophoretic gel of wild-type versus competitor GIP cDNA.</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td>Standard curve for determination of GIP mRNA levels in STC&lt;sub&gt;6-14&lt;/sub&gt; cells incubated in low glucose.</td>
<td>33</td>
</tr>
<tr>
<td>4</td>
<td>Standard curve for determination of GIP mRNA levels in STC&lt;sub&gt;6-14&lt;/sub&gt; cells incubated in high glucose.</td>
<td>34</td>
</tr>
<tr>
<td>5</td>
<td>Comparison of GIP mRNA levels in STC&lt;sub&gt;6-14&lt;/sub&gt; cells incubated in low or high glucose.</td>
<td>35</td>
</tr>
<tr>
<td>6</td>
<td>Cleavage site specificity for the endoproteases.</td>
<td>38</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

IRGIP: immunoreactive GIP
mGIP: murine GIP
PCR: polymerase chain reaction
RT: reverse transcription
DDT: dithiothreitol
IPTG: isopropyl β-D-thiogalacto-pyranoside
dATP: deoxy adenosine triphosphate
ddATP: dideoxy adenosine triphosphate
NSB: non specific binding
RIA: radioimmunoassay
EDTA: ethylenediaminetetraacetic acid
SDS: sodium dodecyl sulphate
dNTP: deoxy nucleotide triphosphate
DEPC: diethylprocarbonate
FCS: fetal calf serum
HS: horse serum
SSC: sodium citrate sodium chloride
X-gal: 5-Bromo-4-chloro-3-indolyl-β-D-galactose
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Introduction

History

The term 'hormone', meaning "I arouse to activity" in Greek, was first suggested by W.B. Hardy in an attempt to convey the meaning of a chemical messenger. The need for such a term was thrust upon the scientific community following the work of two early endocrinologists, Bayliss and Starling, in 1902. In pioneering gastrointestinal experiments, these researchers were able to show that a weak acid perfused into the canine duodenum could elicit both water and bicarbonate secretion from the pancreas, while intravenous administration of the same acid had no such effect. Moore et al. (1906) suggested that the duodenum 'supplies a chemical excitant for the internal secretion of the pancreas'. They tested the hypothesis that 'in certain cases of diabetes, the appearance of sugar in the urine might be due to functional disturbance occasioned by the absence of such an intestinal excitant of the internal secretion' (Moore et al., 1906). Such a suggestion was a radical shift of physiological thinking as this era was dominated by the findings of another early physiologist, Ivan Pavlov, who steadfastly maintained that the neuronal system was all important for internal communication.

The idea of a chemical connection between the gut and other organs continued to advance as numerous researchers recognized that food substances entering the small intestine were able to trigger an inhibition of gastric acid secretion. Kosaka and Lim (1930) introduced the term enterogastrone to describe a putative hormone which was secreted in response to fat and its digestive products in the intestinal lumen and had the effect of inhibiting gastric acid secretion. The two immediate candidates proposed as this enterogastrone were secretin (Bayliss and Starling, 1902) and cholecystokinin (CCK; Ivy and Oldburg, 1927).
Isolation of Gastric Inhibitory Polypeptide

In early studies, mucosal preparations were found to contain both secretin and CCK, as well as a host of other biologically active peptides, and were thus compromised due to their impure nature (Gray et al., 1937; Greengard et al., 1946). In 1961, Jorpes and Mutt reported a procedure which was capable of producing a CCK-free preparation of secretin which Wormsley and Grossman (1964) found capable of inhibiting gastrin stimulated gastric acid release. The structure of secretin was reported by Mutt et al. (1970) and hopes that this hormone was the elusive enterogastrone rose following the demonstration that synthetic secretin shared the same gastric acid inhibitory properties as the recently purified secretin. However both this purified product and synthetic secretin were inactive against histamine and vagally stimulated acid release thus they could not have contained the enterogastrone in question (Way, 1971).

Investigations into the role of CCK as an inhibitor of gastric acid release had shown that impure preparations of CCK were capable of inhibiting both gastrin- and histamine- stimulated acid release in the vagally denervated (Heidenheim) canine gastric pouch. In an attempt to determine whether these actions were due to the CCK in the preparations, Brown and Pederson (1970A) tested the acid secretory effects of CCK using both a 10% and 40% pure preparation. The designated purity of these preparations resulted from their ability to stimulate gall bladder activity in dogs. The two preparations were administered so that each produced a gall bladder activity level of approximately 50% of maximum. The results showed that the 40% preparation was more effective in stimulating gastric acid secretion than that of the 10% pure preparation. This uncoupling of gallbladder and acid stimulatory effects suggested that either an acid inhibitory agent had been removed or a stimulatory agent had been concentrated in the purification process. Pederson (1971) carried out experiments to determine what inhibitory effects the two preparations had on pentagastrin-stimulated acid release. Support for the inhibitory agent hypothesis was provided when the 10% pure preparation was found to be a more potent inhibitor of pentagastrin-stimulated gastric acid release as compared with the 40% pure preparation.
Characterization of GIP

Attempts to isolate the active material from these impure preparations of CCK eventually led to Brown et al. (1970) isolating a polypeptide from porcine duodenal-jejunal mucosa to a degree of purity suitable for amino acid analysis to be performed. In keeping with its postulated role, this new gastrointestinal hormone was named gastric inhibitory polypeptide, GIP (Brown and Pederson, 1970B; Brown and Dryburgh, 1971). The amino acid sequence of porcine GIP, which was first identified by Brown and Dryburgh (1971) and later corrected by Jornvall et al., (1981), put this polypeptide in the glucagon superfamily of peptides along with secretin, vasoactive intestinal polypeptide (VIP) and a host of other peptides. The designation of GIP as a member of this superfamily is apparent as GIP shares 15 of the first 26 amino acids with porcine glucagon and 9 of the first 26 amino acids with porcine secretin. This high degree of sequence homology is suggestive of a common ancestral gene linking the members of the superfamily (Bell, 1986; Campbell and Scanes, 1992).

Enteroinsular Axis

The idea of a gastrointestinal entity involved in insulin release originated with Bayliss and Starling who, in 1902, first proposed the existence of a gastrointestinal regulatory pathway for the disposal of carbohydrates. This was first shown experimentally when an intestinal extract, free of secretin activity, was able to induce a state of hypoglycemia in dogs (Zunz and Labarre, 1929; Labarre and Still, 1930). Following these investigations, Labarre (1932), introduced the term "incretin" to describe this gastrointestinal entity. In 1969, Unger and Eisentraut coined the term 'enteroinsular axis' to describe the gut involvement in insulin release. However, as Labarre (1932) had previously shown insulin release also to be under vagal control, a strict hormonal regulation of insulin secretion was never envisioned. This led Creutzfeldt (1979) to suggest that this so called
enteroinsular axis 'encompass nutrient neural and hormonal signals from the gut to the islet cells'. Creutzfeldt (1979) further defined the criteria for the hormonal part of the enteroinsular axis as being released by nutrients, particularly carbohydrates and being able to stimulate insulin secretion at physiological levels. Evidence for the involvement of GIP in this pathway was first provided by Dupre and Beck (1966) when they demonstrated that insulinotropic properties were contained within impure preparations of CCK. Subsequent studies by Rabinovitch and Dupre (1972) found this insulinotropic activity to diminish as the purity of the CCK preparation increased. Incorporating the previous work by Brown and Pederson (1970A) on the acid inhibitory actions of CCK, Dupre et al. (1973) were able to show that GIP, when intravenously infused in humans, yielded a significant rise in insulin levels, in the presence of glucose. Furthermore, it was shown that GIP had no insulinotropic properties in the euglycemic state, which suggested that GIP was only insulinotropic in the presence of elevated circulating glucose. Subsequently, in vivo experimentation has now shown GIP to have insulin secretory properties in man (Elahi et al., 1979), dog (Pederson 1971), rat (Tseng et al., 1994), the perfused rat pancreas (Pederson and Brown, 1976) and in a tumor-derived β cell line (Kieffer et al., 1993).

The GIP Receptor

An important step in confirming the actions of GIP at its target tissue is the demonstration of specific binding sites or receptors. Early attempts at this endeavor were inconclusive due to the possible loss of GIP receptors in the isolation process and problems associated with the use of a heterogeneous label (Brown et al., 1989). However, Maletti et al., (1984), using an HPLC purified label, were able to demonstrate the existence of high affinity GIP binding sites in membrane preparations from hamster β cell tumors. Similar studies have now identified GIP receptors in human insulinoma plasma membranes (Malleti et al., 1987), in a mouse-derived β cell line, βTC3 (Maletti et al., 1984), the pancreatic tumor cell line IN III (Amiranoff et al., 1984) and in the rat brain (Kaplan and Vigna, 1994). A novel receptor showing GIP specific activation has now been
cloned and molecular studies have placed this receptor in both tissues known to respond to GIP (pancreas, intestine and adipocyte) and in tissues with no reported GIP activity (brain and muscle; Usdin et al., 1993).

Studies of the binding of GIP to its receptor and the intracellular signaling that follow have recently been investigated by Wheeler et al. (1995). After cloning a rat islet receptor, these investigators examined the binding capabilities of various GIP fragments and found the receptor to bind both synthetic porcine and synthetic human GIP with equally high affinity. In addition, Wheeler et al. (1995) found GIP1-30 to bind with lower affinity while GIP19-30, GIP18-28 and GIP21-26 displayed no interactions with the receptor. Following the binding of GIP to this receptor it demonstrated an ability to activate two intracellular signaling pathways- cyclic adenosine monophosphate (cAMP) and intracellular calcium \([\text{Ca}^{2+}]_i\)- potentially involved in the release of insulin.

**The GIP Gene**

The sequence of the GIP gene was first reported in 1987 by Takeda et al. Analysis of the human GIP cDNA by this group showed that GIP is derived by proteolytic processing of a 153 amino acid precursor. The sequence of preproGIP indicates that it is released from the precursor by processing at single arginine residues. Following analysis of a number of polypeptide hormone precursors it would appear that processing primarily occurred at dibasic positions such as Arg-Arg or Lys-Arg (Steiner et al., 1980). However, the recent dramatic increase in elucidating precursor sequences indicates that processing frequently occurs at single basic arginine sites, especially when these are preceded by a proline residue (Schwartz, 1986).

The structural organization of preproGIP was reported as a putative signal peptide (21 amino acids), amino terminal peptide (30 amino acids), GIP (42 amino acids) and a carboxy terminal peptide (60 amino acids). Inagaki et al. (1989) described the make up of the human GIP gene as spanning 10 kilobase pairs and consisting of six exons and five introns, with exons three
and four both contributing to the mature GIP hormone. Soon after the human GIP cDNA was reported, Higashimoto et al. (1992) and Sharma et al. (1992) independently determined the rat GIP cDNA to be 144 amino acids in length, which included GIP itself, both amino and carboxy terminal peptides and a signal peptide. Overall, the translated region of rat GIP cDNA shares 77% nucleotide sequence identity with that of the corresponding human sequence. Despite the fact that the human and rat preproGIP differ in total amino acid length (153 nucleotides vs. 144 nucleotides), the 42 amino acids of the mature GIP hormone are highly conserved among those species for which the sequence is known (Jornvall et al., 1981; Carlquist et al., 1984; Chow et al., unpublished data). It is, however, worthy of note that a region of eight amino acids, present in the human precursor and consisting of 24 consecutive nucleotides, is lacking in the rat N-terminal peptide.

GIP Gene Regulation

The regulation of the GIP gene is not completely understood. In determining the structure of the GIP gene, Inagaki et al. (1989) identified three possible regulatory elements contained within the 5' flanking region. These included a binding site for Sp-1, regions closely resembling the consensus sequences of AP-1 and AP-2, and three sequences similar to a cyclic AMP (cAMP) response element (CRE). More recent studies by Someya et al. (1993), using DNase footprinting, identified only two cAMP response elements, CRE1 and CRE2, in the GIP promoter region. CRE is an inducible enhancer of genes that can be transcribed in response to increased levels of cAMP (Comb et al., 1986). With the aid of mutation studies, Someya et al. (1993) were able to confirm a cAMP-induced activation of the human GIP promoter by CRE2 located at nucleotides -164 to -149.

Previous work investigating the mechanisms by which cAMP regulates the insulin gene promoter have suggested a role for the c-jun protooncogene product, c-Jun (Inagaki et al., 1992). To examine weather c-Jun also regulates transcription of the human GIP gene, Someya et al.
(1993) used CAT co-transfection assays and found that c-Jun represses the basal promoter activity partly through two CRE's (CRE1 and CRE2). Expanding this work to include the role of c-Jun in GIP promoter activity, Someya et al (1993) found that the activity of this promoter could be repressed by c-Jun, at least partly, through two CRE's in hamster insulinoma (HIT T15) cells. Since the c-jun mRNA level increases with glucose deprivation in HIT cells (Inagaki et al., 1992), glucose may induce the GIP gene transcription by reducing the level of c-jun mRNA (Someya et al., 1993).

Quantification of GIP Gene Regulation

Understanding the regulation of gene expression depends in part on the ability to accurately measure mRNA species in defined cell populations. Conventional methods of mRNA quantification include Northern and “dot blot” hybridization and the nuclease protection assay. These techniques have been used to investigate the regulation of the GIP gene in response to stimuli as well as to detect the presence of GIP message in various tissues (Takeda et al., 1987; Inagaki et al., 1989; Higashimoto et al., 1992; Tseng et al., 1993; Higashimoto and Liddle, 1994; Higashimoto et al., 1995). These investigations have confirmed that GIP message is present in human and rat intestine as well as rat submandibular gland. In addition, Tseng et al. (1994), using Northern analysis, have reported a two-fold increase in rat GIP message following a 10% glucose meal.

While these techniques are capable of reporting relative changes in the amount of GIP gene expression, none are sensitive enough to give a quantitative picture of GIP mRNA synthesis. Recently, however, with the advent of polymerase chain reaction (PCR) technology, truly quantitative analysis of gene expression has been possible (Wang et al., 1989; Gilliland et al., 1990A; Diviacco et al., 1992; Bruna et al., 1993). Commonly termed competitive PCR, the method involves the spiking of individual reactions with a known amount of control DNA template, which is then co-amplified with the target of interest (Foley et al., 1993). Because the control template is amplified by the same primers as the endogenous template, the difference in
amplification efficiency of two templates that differ by only a small number of nucleotides, is minimal (Wang et al., 1989; Gilliland et al., 1990A).

Measurement of GIP

Studies describing the release of GIP in response to intestinal stimuli were not possible until 1974 when Kuzio et al. developed the first radioimmunoassay for GIP. This assay, employed guinea pig antisera raised in response to subcutaneously injected porcine GIP. Using this assay, Kuzio et al. (1974) reported postprandial GIP serum concentrations to be >1200pg/ml while fasting state levels were 237 ± 14pg/ml (Mean ± SEM). However, confusion concerning the physiological levels of immunoreactive GIP (IRGIP) arose when an increasing number of laboratories began reporting GIP values (Moody and Lauritsen, 1977; Morgan et al., 1978; Ebert et al., 1979; McLoughlin et al., 1979; Burhol et al., 1980; Sarson et al., 1980; Wolfe and McGuigan, 1982; Jorde et al., 1983; Sheu et al., 1987; Moody et al., 1992; Wishart et al., 1992). In an attempt to standardize the assays available at the time, Jorde et al. (1983) used identical assay conditions to measure fasting and postprandial IRGIP levels in man with seven different antisera collected from various assays. The results showed mean fasting serum concentrations ranged from 12 to 92 pmol/l while postprandial concentrations ranged between 35 and 235 pmol/l. These differences, it was concluded, resulted from the varying cross reactivities that each antiserum had for human GIP. The reasons for such discrepancies became obvious when Amaland et al. (1984) used four different antisera along with porcine standards to measure serum GIP concentrations from pigs, rats, dogs and humans. Only the tests performed on pig sera yielded similar results, leading the authors to suggest that the use of standards derived from species other than the test animal could lead to unreliable results. Following the amino acid sequencing of human GIP (Jornval et al., 1981), two further assays were produced with antisera directed against synthetic human GIP (Kreyman et al., 1987; Nauk et al., 1992). The use of this synthetic hormone in the raising of human GIP antisera has alleviated much of the discrepancies found in the early assays.
Localization of GIP

In mammals, GIP-immunoreactivity has been localized to a discrete population of mucosal endocrine cells (K cells) in the duodenum and jejunum of man (Polak et al., 1973; Buffa et al., 1975; Buchan et al., 1978; 1982), pig, dog (Buffa et al., 1975), and rats (Buchan et al., 1987). In addition, small amounts of IRGIP have been localized to the ileum of man (Ferri et al., 1983) and rat (Buchan et al., 1982). IRGIP cells occur as single entities among the surrounding epithelial cells of the intestinal villi and upper crypts (Polak et al., 1973; Buchan et al., 1978). This distribution of the IRGIP cells overlaps with that of several other peptide containing endocrine cells (Polak et al., 1973; Aiken et al., 1994). However, GIP was not co-expressed with any other enteroendocrine cell, at least in the mouse intestine (Aiken et al., 1994).

Although the vast majority of GIP cells have been localized to the mammalian duodenum and jejunum, it appears that release by all K cells is not required, even in a maximal stimulation (Malfertheiner et al., 1994). In experiments involving the removal of the duodenum in dogs, Malfertheiner et al. (1994), found no significant decrease in portal vein IRGIP levels in the duodenectomized animals. These authors suggested, on the basis of their data, that the remaining K cells of the jejunum could compensate for the loss of GIP cells from the duodenum (Malfertheiner et al., 1994)

IRGIP has, in some studies, been localized to the pancreatic A cells (glucagon) in mammals (Alumets et al., 1978; Larsson and Moody, 1980) and reptiles (Buchan et al., 1982). However, attempts to block this immunoreactive signal from A cells has produced conflicting results. Leduque et al. (1982) failed to block the cross reactivity of the rabbit antiserum with human A cells using both glucagon and glicentin, yet glucagon (Buchan et al., 1978) and glicentin (Larrson and Moody, 1980) were capable of blocking this reaction in other studies. These results led Buchan et al. (1982) to compare three antisera for GIP- a novel murine derived monoclonal, and antisera both derived from guinea pig as well as rabbit. No evidence of A-cell cross reactivity was found with
either the monoclonal or the guinea pig antiserum. However, rabbit antiserum did show some cross reactivity which suggested that the previously reported pancreatic localization of GIP was most likely due to a cross reactivity of this antiserum and glucagon or the glucagon like peptides and their precursors (Buchan et al., 1982)

GIP Release in Response to a Mixed Meal

Despite the variability in reported IRGIP values, there is a general agreement that GIP levels increase 5-6 times over basal following a meal (Brown, 1974; Kuzio et al., 1974; Morgan et al., 1978; Jorde et al., 1980; Jorde et al, 1983; Amaland et al., 1984). Plasma level of GIP are elevated for approximately six hours following a meal, suggesting the existence of a raised plasma level continuing throughout the day.

GIP Release in Response to Carbohydrates

The increase in circulating IRGIP in response to oral glucose demonstrated that this simple sugar was a potent stimulatory agent (Cataland et al., 1974; Pederson et al., 1975; Elahi et al., 1979; Morgan, 1979). An increase in portal venous IRGIP occurs approximately two minutes after glucose ingestion and this is followed by an elevation of insulin at five minutes (Cataland et al., 1974). As to the molecular forms of sugars capable of stimulating the release of IRGIP glucose, galactose and sucrose were found to be stimulatory agents (Morgan et al., 1978; Sykes et al., 1980). However, Sykes et al. (1980), also determined that fructose, mannose, lactose, 6-deoxygalactose, 2-deoxyglucose and myoinositol were unable to stimulate IRGIP release. This specificity for IRGIP release relates to the mode of sugar transport across the apical cell surface of the gut epithelium. Glucose and galactose are transported by the Na\(^+\) -dependent glucose transporter, SGLT1 (Wright, 1993). In the digestion of carbohydrates, sucrose must first be broken down to its constituent simple sugars, glucose and fructose, by the intestinal brush border
enzymes before its glucose sub-unit can be transported via the Na⁺-dependent transporter (Wright, 1993). Interestingly, Morgan (1979) identified a delayed secretion of IRGIP in response to sucrose, which could be due to the time required for its hydrolysis by the brush border enzymes (Sirinek et al., 1983). In contrast, fructose is absorbed down its concentration gradient entering the epithelial cells via the facilitative transporter Glut5 (Burant et al., 1992). The failure of these other sugars to stimulate IRGIP release is best described by Sirinek et al. (1979) who postulate, on the basis of their data, that the structural integrity of the glucose molecule from the C-1 to C-4 carbon atom, a free aldehyde group on the C-1 atom and a cyclic structure are all necessary for the active transport of glucose and release of IRGIP. The transport of these sugars out of the enterocyte, across the baso-lateral membrane, is accomplished by the facilitative transporter Glut2 (Thorens, 1993).

In an attempt to begin isolating the particular mechanism responsible for IRGIP release, Morgan et al. (1985) added a non-absorbable carbohydrate, guar gum, to test meals and found a decrease in IRGIP release. As guar gum increases the viscosity of solutions, it has been suggested that addition of guar gum to meals reduces the rate of diffusion of nutrients toward the absorptive surface (Lembcke et al., 1984). These results added support to the hypothesis that the release of GIP in response to food is related to the rate of active absorption of carbohydrates (Creutzfeldt and Ebert, 1977; Sykes et al., 1980). Further evidence for the necessity of active transport of monosaccharides for IRGIP release came from Sykes et al. (1980) when they blocked the Na⁺-dependent transporter using phloridzin, a B glucoside which binds but is not transported (Hopfer, 1987), and found a cessation of IRGIP release. Additionally, Sykes et al. (1980) investigated whether monosaccharide metabolism was required for IRGIP release by using 3-O-methylglucose, a non-metabolizable glucose analogue. These investigations demonstrated a significant increase of IRGIP release, albeit somewhat less than the release observed with a glucose infusion, and thus suggested that the metabolism of sugar is not required for GIP release to occur. Finally, Fushiki et al. (1992) infused cytochalasin B into the duodenum, along with glucose, to determine the role of facilitative glucose transport in IRGIP release. Although cytochalasin is a potent competitive
inhibitor of the facilitative transporters, no significant decrease of IRGIP release was recorded and therefore the release of GIP is most likely not dependent upon the transport of glucose out of the enterocyte.

The existence of a vagal glucose sensor in the duodenum has been proposed as a final route by which GIP cells sense glucose (Fushiki et al., 1992). Mei (1978), identified a glucoreceptor which was localized to the duodenum and the proximal jejunum. Interestingly, it was found that both glucose and galactose, but not fructose, were able to stimulate this receptor (Mei, 1978). However, vagal blockade failed to affect glucose induced IRGIP release (Fushiki et al., 1992; Greenburg and Pokol-Daniel, 1994) but did affect plasma insulin responses (Greenburg and Pokol-Daniel, 1994). These authors concluded that vagal muscarinic and non vagal muscarinic pathways participate in the control of the intestinal phase of insulin secretion, but the regulation of GIP secretion is independent of vagal muscarinic influences (Greenburg and Pokol-Daniel, 1994).

GIP Release in Response to Fat

In keeping with the role of GIP as an enterogastrone, circulating levels of GIP were found to increase after the ingestion of triglyceride (Brown, 1974; Brown et al., 1975; Falko et al., 1975; Pederson et al., 1975; Ross and Dupre, 1978; Ohneda et al., 1983; Ohneda et al., 1984). The ability of fat to stimulate IRGIP release as compared to that of glucose was investigated by Krarup et al. (1985), who found that on a weight and molar basis, fat is more potent than glucose while equal potency was found with equicaloric comparisons. In order to determine whether the IRGIP secreted was in response to triglyceride itself or one of its hydrolytic products, Ross and Shaffer (1981) studied IRGIP release in healthy subjects and in subjects with defective fat lipolysis (cystic fibrosis). A ten fold increase in plasma IRGIP levels was observed in the healthy subjects as compared to those with cystic fibrosis. However, after resumption of pancreatic enzyme therapy for the cystic fibrosis patients, no significant differences in IRGIP release were identified. This clearly suggested the requirement for triglyceride hydrolysis in order to obtain normal IRGIP
responses (Ross and Shaffer, 1981). Of the products of triglyceride hydrolysis, long chain fatty acids had a greater stimulatory effect than that of glycerol, short chain fatty acids or medium chain fatty acids (Brown, 1974; Ross and Shaffer, 1981).

The potent response to luminal fatty acids by the GIP cell is not surprising considering its role in the regulation of fat metabolism. In adipose tissue, GIP has been shown to increase the activity of lipoprotein lipase (Eckel et al., 1979) while in liver (Hartmann et al., 1986) and adipose tissue (Dupre et al., 1976) it demonstrates the ability to inhibit glucagon induced lipolysis.

GIP Release in Response to Amino Acids

The original work aimed at elucidating the role of protein or amino acids in the release of IRGIP found no such stimulatory activity following ingestion of a protein (Bovril) meal (Brown, 1974). However, when mixtures of amino acids, especially those containing arginine, histidine, isoleucine, lysine and threonine were infused into the small intestine, there was a significant increase in plasma IRGIP (Thomas et al., 1976) - an increase that was not seen with the infusion of a mixture containing methionine, phenylalanine, tryptophan and valine. Thomas et al. (1978) proposed that this increase in IRGIP in response to the amino acids was due to their concentration and quantity in the small bowel. Total free amino acid concentrations after a protein rich meal are considerably less than the 158 mM solution reportedly used by Thomas et al. (1978).

In Vitro Studies of GIP Secretion

Studies into the release of GIP have, until recently, been based upon studies of circulating levels of the hormone in both man and experimental animals. Direct cellular investigations concerning the regulation of GIP stimulation, synthesis and release have traditionally been hampered by the diffuse distribution of the GIP enteroendocrine cell along the longitudinal axis of the gut. In an attempt to alleviate this problem associated with most gastrointestinal endocrine
cells, Soll et al. (1984) established a technique for the isolation and enrichment of gastric gastrin cells. This technique involves the dissection of the mucosa and subsequent collagenase digestion followed by the separation of similar cells by means of sedimentation velocities. After enriching for a specific cell type, cells can be cultured and identified by immunocytochemistry. These techniques have been modified for various cell types including somatostatin (Campos et al., 1990), enteroglucagon (Buchan et al., 1987), gastrin (Campos et al., 1989) and GIP (Kieffer et al., 1995). In the latter study, canine mucosa was isolated and subjected to collagenase digestion prior to being loaded into an elutriation chamber. This procedure of counter-flow centrifugal elutriation separates cells on the basis of their sedimentation coefficients. As the cells are pumped into a rotating chamber, their relative final position is determined when their sedimentation rate is balanced by the countercurrent flow rate. The majority of canine and porcine IRGIP cells were found to elute at a rotor speed of 2100rpm and a flow rate of 55ml/min (Kieffer et al., 1995). This technique provided a primary cell line that was enriched with GIP cells. Release experiments using this cell line showed an increased IRGIP release with increasing levels of glucose in the culture media. However, while this technique was useful for the purpose of showing GIP release, the method has proven difficult and costly and still results in low numbers of IRGIP cells.

Another option for the study of GIP at the cellular level lies in the use of tumor-derived cell lines. Rindi et al. (1990) describe the development of a murine-derived cell line, named STC-1, which is derived from a neuroendocrine tumor found in transgenic mice. The general strategy behind the production of such a cell line is the construction of a hybrid gene consisting of the promoter region from an endocrine cell and the coding region of an oncogene. After transformation of this hybrid gene to fertilized murine eggs via microinjection, the resulting mice are monitored for the production of tumors (Hanahan, 1984). For the STC-1 cell line specifically, mice were raised harbouring either the rat insulin promoter (RIP) linked to the early region of the SV40 oncogene (RIP1/TAG2) or the rat insulin promoter linked to the polyoma small T antigen (RIP2/PyST1). Individually these mice showed no abnormalities beyond the expected pancreatic tumors, however, when the two strains were bred to produce double transgenics of the lineage
RIP1-TAG2/RIP2-PyST1 (Grant et al., 1991) additional intestinal tumors were observed (Rindi et al., 1990). Following immunocytochemistry, the resulting cell line, named STC-1 (Secretin producing Tumor Cells) was found to consist of approximately 54% secretin expressing cells, 10% glicentin, 7% GIP, 7% gastrin-cholecystokinin, 5% glucagon-like polypeptide 1, 2% pancreatic polypeptide, 2% neurotensin and 1% somatostatin (Rindi et al., 1990). At present, this cell line has been used to investigate the release and/or intracellular mechanisms of that release in a number of its constituent cell types. These include GLP-1 (Abello et al., 1994), proglucagon (Ehrlich et al., 1994; Gajic and Drucker, 1993), CCK (Mangel et al., 1993; Chang et al., 1994; Snow et al., 1994), secretin (Wheeler et al., 1992) and GIP (Boylan et al., 1994). While the latter investigation was successful in the use of this cell line, data from immunocytochemical (ICC) studies indicated that only a small number of these cells were in fact immunoreactive for GIP.

In an attempt to increase the proportion of GIP cells within the STC-1 population, this cell line was sub-cloned to produce the stable cell line STC6-14 (Kieffer et al., 1995). Briefly, an average of 1 cell per well was plated in multi well plates and cultured until confluency. Cells from RIA positive wells were passaged into progressively larger wells to increase cell number and at the same time immunostained to determine the number of GIP cells within the population. The most stable clone, STC6-14, was found to contain approximately 30% immunoreactive GIP cells as well as approximately 30% immunoreactive somatostatin cells (Keiffer et al., 1995). In release experiments carried out on this cell line, a dose dependent relationship was found to exist between the level of glucose in culture and amount of IRGIP release, suggesting that this cell line acted in a manner closely resembling a normal endocrine cell (Kieffer et al., 1995). Interestingly, the increase observed over the 5-20 mM range was lower than the increase seen in the enriched cultures of canine and porcine cells, which consisted of only 10% and 8% IRGIP, respectively. It was hypothesized that this result could be attributed to the inhibitory actions of somatostatin on GIP release. To test this hypothesis, Kieffer et al. (1995) added the somatostatin antibody SOMA-10 to the graded glucose concentrations and found a significant rise in IRGIP in all but 20 mM glucose. This result is in agreement with the known actions of somatostatin as an inhibitor of intestinal
endocrine release. It was therefore concluded that this new cell line, STC6-14, by virtue of endogenous GIP release and glucose responsiveness, would serve as a suitable model for the study of GIP release and the intracellular mechanisms involved in that release (Kieffer et al., 1995).

Thesis rationale

The present knowledge of GIP depicts this hormone as having major roles in the insulinotropic actions on the β cell as well as in the regulation of fat metabolism. There is also much known about the specific secretagogues involved in the release of GIP and the level of release associated with each type of stimulus.

However, the vast majority of this information is taken from data on the circulating levels of GIP and very little is known regarding the cellular events which precipitate this release. It is imperative now that we begin to understand the intracellular mechanisms that are involved in the recognition of these secretagogues and the subsequent pathways leading ultimately to the release of GIP into the systemic circulation.

Until recently, studies into the intracellular events of GIP release have been difficult to undertake due to the lack of any permanent cell line which expresses this peptide. Now, with the use of the STC6-14 cell line, consisting of 30% IRGIP cells, it should be possible for investigations to center on the internal events of release while at the same time manipulating the cellular environment to control any paracrine, neurocrine or luminal influences.

The first aim of these investigations was to characterize the STC6-14 cell line by confirming the existence of GIP with the use of DNA sequence analysis. Following this positive identification for murine GIP, a quantitative analysis of GIP mRNA, in response to glucose, was employed using both Northern analysis as well as a more accurate quantitative approach, the reverse transcription-polymerase chain reaction (RT-PCR).
Materials and Methods

1. Culture of STC<sub>6,14</sub> cell line

Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco Laboratories, Burlington, Ont.) supplemented with 25 mM glucose, 12.5% horse serum (HS, Gibco), 2.5% fetal calf serum (FCS, Sigma Chemicals, St. Louis, MO.), penicillin (Gibco, 50 units/ml) and streptomycin (Gibco, 50 units/ml) at 37°C in 5% CO<sub>2</sub> in air using 250 ml Falcon tissue culture flasks (Becton and Dickinson Canada Inc., Toronto, Ont.). The cells were subcultured every three days or when their confluency reached approximately 80% by harvesting with trypsin-EDTA (Sigma).

2. Extraction of Murine GIP RNA

Total RNA from approximately 1x10<sup>7</sup> STC<sub>6,14</sub> cells was extracted using the acid-phenol method of Chomczynski and Sacchi (1987). Briefly, the cells were lysed directly in the culture flask with 4M guanidine thiocyanate (Fisher Scientific, Nepean, Ont.), 0.5% N-lauroylsarcosine (Sigma), 25mM tri-sodium citrate (BDH Inc., Toronto Ont.) and 100mM 2-mercaptoethanol (BDH Inc.)(Solution D). This suspension was then mixed sequentially with 100µl 2M sodium acetate (BDH Inc.), 1ml buffer saturated phenol (Sigma) and 200µl of a 49:1 chloroform:isoamyl alcohol mixture (Fisher), for each 1ml of lysis solution used. The suspension was then vortexed, left on ice for 15 minutes and then centrifuged at 10,000g for 20 minutes at 4°C (Savant, Hicksville, N.Y.). The aqueous layer was removed and mixed with 1ml of isopropanol (BDH) and the RNA precipitated at -20°C. This solution was again centrifuged (Baxter Biofuge, West Germany) as before, and the RNA pellet resuspended in 0.3ml Solution D and an equal volume of isopropanol and placed at -20°C for 1 hour. Following a final sedimentation at 10,000g (Baxter Biofuge), the RNA pellet was resuspended in a small volume of distilled water (dH<sub>2</sub>O) treated with
diethylpyrocarbonate (DEPC, BDH). RNA yields were quantified by spectrophotometric absorption at 260nM.

3. Reverse-Transcription - Polymerase Chain Reaction amplification of murine GIP mRNA.

Using 100ng of isolated murine total RNA, single stranded complementary DNA (cDNA) was synthesized. The reverse transcription (RT) reaction was performed at 37°C for 1 hour in RT buffer containing 10U RNA Guard, 1mM dithiothreitol (DTT), 200U Superscript RT (all from Gibco), 1mM dNTP's (Pharmacia, Uppsala, Sweden) and 100pmol random hexamers as primers. After the reaction was complete the enzyme was inactivated by heating the reaction to 68°C for 10 minutes. To amplify the murine GIP cDNA the polymerase chain reaction (PCR) was employed using primers derived from conserved sequences found in both rat (Tseng et al., 1992) and hamster (Chow et al., unpublished data) preproGIP (Table 1). All primers were synthesized on the PCR mate oligo synthesizer (Applied Biosystems, Foster City, CA.). The PCR protocol was carried out in a PCR thermocycler (Perkin-Elmer Cetus, Norwalk, CT.) for a total of 30 cycles under the following conditions: 30 sec. denaturation at 94°C, 30 sec. annealing at 52°C and 30 sec. extension at 72°C.

Table 1: Primer sequences and their corresponding positions on the murine GIP precursor

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIP-1</td>
<td>5’&gt;TGGCTCGAGGACACAACCTA&gt;3’</td>
<td>-26 to -6</td>
</tr>
<tr>
<td>GIP-2</td>
<td>5’&gt;TATGCAGAGKGGACTTTCAT&gt;3’</td>
<td>130 to 150</td>
</tr>
<tr>
<td>GIP-3</td>
<td>5’&gt;GTTGTGTTTCAGGCAGTATTCT&gt;3’</td>
<td>226 to 246</td>
</tr>
<tr>
<td>GIP-4</td>
<td>5’&gt;TTTTTCCTCGAGGCTCAAGAC&gt;3’</td>
<td>533 to 553</td>
</tr>
</tbody>
</table>
4. Cloning Strategy

The amplified GIP cDNA product was ligated into the pGEM-T plasmid vector (Stratagene, La Jolla, CA) as follows: 2.5 picomoles (pmol) of insert, 75 femtomoles (fmol) of pGEM-T vector and 2U of T4 DNA ligase were combined in T4 DNA ligase buffer (10X ligation buffer: 300mM Tris-HCl, 100mM MgCl₂, 100mM DTT and 10mM adenosine triphosphate (ATP)) in a final reaction volume of 20 μl and incubated overnight at 16°C. Ten microliters of the ligation mix were then used to transform 100μl of competent E. coli strain DH5α. Briefly, this involved the addition of 10μl of the ligation mix to 100μl of ice-cold competent E. coli. This mixture was then incubated for 30 min. on ice followed by 2 min. at 42°C. One milliliter of sterile Luria-Bertani (LB) broth (10g bacto tryptone, 5g bacto-yeast extract and 10g NaCl per liter of dH₂O) was added and the suspension was incubated on an incubator shaker (New Brunswick Scientific, Edison, N.J.) for 60 min at 37°C. The cells were then recovered by centrifugation (Sorval MC 12V, Delaware) at 10,000g for 2 min and resuspended in 100 μl of LB media. The transformed cells were then spread on LB agar plates containing 100 μg/ml ampicillin (Sigma), 50 μg/ml X-gal (Sigma) and 50 μg/ml IPTG (Sigma) and incubated overnight at 37°C. Successful transformants, appearing as colourless colonies, were inoculated into 5 ml LB media containing 50 μg/ml ampicillin (Sigma) and grown overnight at 37°C with vigorous shaking. The plasmid DNA was isolated from these cells using the Magic Miniprep DNA Purification System (Promega, Madison, WI).

5. Sequence Analysis of Murine GIP cDNA

The sequence of the cloned GIP insert was confirmed for both strands using the Sequenase version 2.0 DNA sequencing kit (United States Biochemical (USB), Cleveland, OH.) which is modeled after the dideoxy chain termination method of Sanger et al. (1977). Briefly, 2 pmol of pGEM-T mGIP was added to 10 pmol of either T7 or SP6 primer and 1μl of dimethyl sulfoxide (DMSO) in a final volume of 10 μl. The samples were then heated to 95°C for 3 min and snap
frozen in dry ice/ethanol for 5 min. To the annealed template-primer the following was added: 2 µl
0.1M DTT, 2 µl of a 1 in 12 diluted labeling mix (7.5 µM each of dGTP, dCTP and dTTP; USB),
0.8 µl [α-35S] dATP (Du Pont, Wilmington, DE) and 2 µl of a 1 in 8 dilution of Sequenase enzyme
in enzyme dilution buffer (USB). After 6-7 min at room temperature, 3.5 µl of this solution was
added to each of 4 wells on a 96 well microtitre plate (Nunc, Kamstrup, Denmark). To each well
2.5 µl of the appropriate termination mix (ddTTP, ddCTP, ddATP or ddGTP) was added and the
plate was incubated for 10 min at 37°C at which point 4 µl of Stop Solution was added to each well
to terminate the reaction. After a brief heating to 80°C, 4 µl from each well (designated G, A, T or
C) was added to the appropriate lanes of a previously prepared 6% urea gel. The gel was allowed
to run for 90 min at 150V on the Model 2 sequencing gel electrophoresis apparatus (Gibco).

6. Radioimmunoassay for GIP

The radioimmunoassay (RIA) for GIP was first described by Kuzio et al. (1974) and
modified by Morgan et al. (1978). GIP was iodinated with 125I (Dupont, Willmington, DE.) in a
modified protocol of the chloramine-T method by Greenwood et al. (1963). The standards for this
RIA were prepared from a stock of porcine GIP and diluted to a final concentration range down to
78pg/ml. The rabbit anti-porcine GIP serum used for the RIA (LMR34) was generously supplied
by Dr. L.M. Morgan (University of Surrey, UK) and was used at a final dilution of 1:3 x 10^5.

On the first day of the assay, 200 µl of assay buffer (40 mM phosphate buffer (pH 6.5) with
5% charcoal extracted plasma and 2% aprotinin) were added to each tube (except total counts)
followed by either 100 µl of porcine GIP standard or 100 µl of sample and 100 µl of GIP
antibodies (LMR34). For the non-specific binding (NSB) and zero-binding (Bo) tubes, 100 µl of
assay buffer were added instead of antibody or standard, respectively. All tubes were mixed and
placed at 4°C for 24 hours. On the following day, 100 µl of porcine GIP label (5000 cpm/100 µl)
were added to each tube, mixed and placed at 4°C for a further 24 hours. On the last day of the
assay, 500 µl of polyethylene glycol (250 g/l PEG 8000; Fisher) was added to all tubes except total
counts, mixed well and centrifuged at 1000 x g for 45 min. The supernatant was then decanted and the pellets allowed to dry for 12 hours before radioactive counting for 2 min on a gamma counter (LKB Wallace 1277 Gammamaster).

7. Release Experiments

Approximately 3.5x10^5 cells in 1 ml of culture medium were plated in each well of collagen-coated Costar 12-well plates and cultured for 2 days. On the third day, the medium was removed by pipette and replaced with 1 ml of culture medium containing 5 mM glucose and incubated for 5 h. This low glucose culture medium was then removed by pipette and the cells were washed with 1 ml of release medium consisting of DMEM supplemented with 5 mM glucose, 1% FCS and 2% aprotinin (Trasylol, 200 kallikrein-inhibiting units/ml, Miles Pharmaceuticals, Rexdale, Ont.). After removal of the wash solution, 1 ml of the appropriate release medium was added to each well and the cells were incubated an additional 2 h. At the end of 2 h, the release medium was removed and placed in 1.5 ml microcentrifuge tubes on ice. The remaining cells were lysed with 1 ml of solution D (as previously described) and stored for subsequent RNA analysis using a competitive PCR technique. The tubes containing the release medium were then centrifuged at 7000g (Baxter Biofuge) and 4°C for 5 min., the supernatant removed and then stored at -20°C for subsequent immunoreactive GIP (IRGIP) assay. IRGIP secretory values were recorded as a percentage of total cell content (%TCC) of IRGIP as measured from extracts. Total cell count per well was determined following the release protocol by harvesting cells from control wells using Trypsin-EDTA.

8. Production of GIP DNA Probe

A 410 bp DNA fragment of murine GIP was created using primer 1 and primer 4 (Table 1) in a PCR protocol for use as a probe in Northern analysis. This DNA was radiolabelled with [α-
\[ \text{\( ^{32}\text{P}\) deoxycytidine triphosphate (\([\alpha-^{32}\text{P}]dCTP\)}, \text{using the Klenow fragment of DNA polymerase I} \]

and random hexamers as primers (Pharmacia) in a reaction mix that was incubated for two hours at 37°C followed by 10 min at 68°C to inactivate the enzyme. The efficiency of the labeling reaction was assessed by TCA precipitation and only those probes that showed a 60% or greater incorporation were used in the hybridization reaction.

9. Northern-blot analysis of GIP mRNA

Approximately 1x10^7 STC-14 cells were cultured, as earlier described, for a total of three days. On the fourth day the cells were subjected to the same low and high glucose media as described in the release experiments protocol. After the two hour incubation in either high or low glucose, the cells were lysed with 4 ml of solution D and stored at -70°C until needed for RNA extraction. Total RNA was isolated from this mix by the acid-phenol method (Chomczynski and Sacchi, 1987) as described above. Following total RNA quantification by a UV spectrophotometer at both 260 and 280 nm, a total of 30 \( \mu \)g of RNA from either low or high glucose stimulated cells was denatured in gel running buffer (0.04 M 3-(N-morpholino) propanesulphonic acid, 10 mM sodium acetate (BDH), 0.5 mM EDTA (BDH), 50% formamide and 6% formaldehyde). The RNA was then electrophoresed on a 1.5% agarose 6% formaldehyde gel. The integrity of the RNA was assessed by the visualization of 28S and 18S ribosomal RNA bands with ethidium bromide staining. After electrophoresis at 13 V/cm, the RNA was transferred from the gel to a nylon membrane (Hybond N\(^+\), Amersham) by capillary action using the Posi-blot transfer system (Stratagene, CA). Hybridization was then performed using the 410 bp fragment of murine GIP cDNA, produced using primers GIP-2 and GIP-4 (Table 1), as the radiolabelled probe. The blots were prehybridized for 1 hour at 42°C in 5X SSC (1X SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 10X Denhardt’s solution, 50% formamide (vol/vol), 50 mM NaPO\(_4\), 1% sodium dodecyl sulphate (SDS; Gibco) and 10 \( \mu \)g/ml denatured herring sperm DNA. The membranes were then hybridized at 42°C for 18 hours in 5X SSC, 10X Denhardt’s solution, 50% formamide, 20 mM NaPO\(_4\), 0.5% SDS and
20 μg/ml of herring sperm DNA and approximately 1X10⁷ counts per minute (cpm) of the probe per 100 cm² of membrane. After hybridization, blots were washed once at room temperature for 15 min in 1X SSC and 1% SDS, once at room temperature in 0.5X SSC and 0.5% SDS for 15 min, twice at room temperature in 0.5X SSC and 0.5% SDS for 15 min each and once at 50°C in 0.1X SSC and 0.1% SDS for 30 min. The membranes were then placed, while still wet, into a developing cartridge for the Molecular Dynamics phosphoimager SI for a period of 1 hour. The probe signal strength for each preparation was then determined by the phosphoimager using the IP lab gel software connected to a MacIntosh computer.

10. Production of a cDNA Competitive Template

Following removal of the release medium, the cells from all wells, except control, were lysed with 1ml of solution D (prepared as above) and the total RNA extracted as previously described. 2.5 ug of this total RNA was used in a RT reaction (as above) which contained 10 pmol of the mutagenic primer GIP-A (5’- AATTTAATACGATCCACTATAGGGATACGC AGAGGGGACTTTTCAT- 3’) which produced a 330 nt cDNA fragment identical to wildtype murine GIP except for an 80 nt internal deletion. The competitor template was then amplified by adding 10 μl of the reaction to a standard PCR (as above) with 10 pmol of primer GIP-B (5’- TTTCTCGAGGCTCAAGACTTTATTGGTTAGGTCAGTCACTGAGACCTG- 3’), containing 20 nt corresponding to the 5’ region of murine GIP. The reaction underwent 30 cycles of the following conditions: 30s at 94°C, 30s at 52°C and 30s at 72°C. The PCR product was then isolated on a 2% agarose gel and purified via electroelution followed by a single phenol:chloroform extraction and ethanol precipitation. The amount of cDNA present was determined using a UV spectrophotometer at an optical density of 260 nm. To verify the integrity of the competitor, 1:2 serial dilutions were prepared of the cDNA and added to six tubes of a PCR reaction along with 10 pmol each of primer GIP-2 and GIP-4 (Table 1). To ensure the competitor was free of any cDNA contaminants, a control PCR reaction was set up in which one tube received all ingredients for the
PCR except for the cDNA template The PCR products were then run on a 2% agarose gel containing 0.5 mg/ml ethidium bromide and then visualized using a UV transilluminator and photographed. The competitor was deemed suitable for use in mRNA analysis if the photograph revealed a decreasing concentration of cDNA product in the first six tubes and no product was visible in the control tube.

11. mRNA Quantification using a Competitive Reverse Transcription-Polymerase Chain Reaction Protocol

To set up a competitive RT-PCR, 100 ng of total RNA from approximately 3.5x10⁵ STC₆₋₁₄ cells were added to each of 7 microcentrifuge tubes containing the standard RT ingredients (as previously described). To help control the inter-tube variation, a master mix of all ingredients used in both the RT and PCR reactions (except for reverse transcriptase and Taq DNA polymerase) was prepared for each separate run. Following the RT, 10 µl of each of the reactions were added to a separate tube containing a standard PCR mix. A 1:2 serial dilution was then made of the cDNA competitor in a concentration range of 70 attomoles to 2 attomoles (amol) with each tube then receiving one of the concentrations of competitor. The PCR reaction followed a protocol of 94°C for 30s, 52°C for 30s and 72°C for 30s with a total of 50 cycles. The products were then separated on a 2% agarose gel containing ethidium bromide, visualized under UV light and photographed.

The resulting photographic negative was placed on a scanning flatbed densitometer (Bio Rad 660, Hercules, CA) and the band densities of both the competitor and wild type GIP cDNA were determined. The density of the competitor bands were corrected to compensate for the 80 bp deletion before being recorded.
12. Perifusion protocol

The perifusion of the STC₆-14 cells was investigated using an Endotronics Acusyst-s culture and perifusion apparatus (Endotronics Inc., Coon Rapids, MN.) linked to an Endotronics automated perifusion system pump (APS-10, Endotronics Inc.). This system consists of an incubation compartment linked to a media pumping system that allows for the long term perifusion of cell cultures. In addition the system allows for the maintenance of a constant temperature and gaseous environment. Approximately 5x10⁶ STC₆-14 cells were grown for three days with 0.33 ml of a cytodex-3 slurry (Pharmacia) in 10 ml of DMEM culture media (as previously described) supplemented with 25 mM of glucose. On the fourth day cell covered beads were transferred into an Endotronics microchamber (200 μl volume; Endotronics Inc.) which was bordered on each side by a single sheet of Nytex mesh supported with a large pore metal screen. The flow rate across the cells was set for 6 ml/hr by setting the APS-10 pump at 7.5 ml/hr per microchamber used and the acusyst-s perifusion system at 6% of its maximum outflow.

The perifusion protocol consisted of a 12 h perifusion with 25 mM glucose culture medium (as previously described) followed by 5 h of a 5 mM glucose release medium (as previously described) and finally 5 h of a 25 mM glucose release media. The outflow from each microchamber was channeled to a fraction collector set to advance every hour from the 18th to 20th hour of the experiment and then every 20 min for the last 5 h. After each tube was filled the perifusate was placed at -20°C for future radioimmunoassay for GIP.

Following the 26 h protocol the microchamber was disassembled and the coated beads were removed. The cells were separated from the beads using Trypsin/EDTA (Sigma) and then assessed for viability by Trypan Blue staining (Sigma).
Expression of Results

The GIP release data were calculated as mean ± standard error of the mean (SEM) for a %TCC to reduce any variation between cell culture flasks. The GIP mRNA quantification data was also calculated as mean ± SEM. Statistical significance was determined for both studies by Student’s t test and was set at the 5% level.
Results

1. Sequence Analysis

Using PCR primers derived from both rat and hamster preproGIP, a murine partial cDNA for GIP was identified (Figure 1). This sequence consists of 537 bp and encodes an open reading frame of 432 bp—indicating the GIP precursor contains 144 amino acids. The structural organization of the murine preproGIP was found to consist of a 43 amino acid N-terminal extension, containing a 19 to 21 amino acid signal peptide, a 42 amino acid hormone and a 59 amino acid C-terminal extension. Although the 3' primers position did not allow for a positive identification of the poly (A) tail, it was possible to identify a putative poly (A) adenylation signal (AATAAA) corresponding to positions 532 to 537 inclusive (Figure 1).

By analysis of the GIP sequence, murine GIP is predicted to differ from the human hormone by 3 amino acid substitutions; arginine for histidine at position 18, arginine for lysine at position 30 and serine for lysine at position 34. Overall the translated region of murine GIP cDNA shares 77% sequence identity with that of the human sequence. At the carboxy terminus of the peptide, the arginine residue at position 84 of the precursor apparently serves as a single basic cleavage site for the removal of the 59 amino acids making up the carboxy-terminal extension peptide. In a similar fashion the arginine residue bordering the amino terminus of the mature peptide (position 43 of the precursor) acts as a monobasic cleavage site for further proteolytic processes which release the amino terminal peptide. The two new arginine residues found within the mature murine hormone are not predicted to direct any new proteolytic processes due to the lack of appropriate surrounding residues.
Figure 1: The nucleotide sequence and deduced amino acid sequence of murine GIP. The numbering of the nucleotides starts from the first adenine of the initiating methionine and ends at the nucleotide just prior to the poly (A) tract. The mature GIP peptide is underlined and the polyadenylation signal is in bold type.
Northern analysis of GIP mRNA

The Northern-blot analysis protocol for quantification of GIP mRNA levels in the STC_{6-14} cell line resulted in no detectable levels being picked up by the phosphoimager. Numerous modifications of this protocol were attempted but no GIP mRNA bands were detected.

GIP mRNA Synthesis in Response to a Glucose Stimulus

Following a two hour incubation in either 5 mM or 25 mM glucose, the cells were lysed for analysis of GIP mRNA content. After reverse transcription and PCR amplification of both the control and target template, these 2 cDNA species were subjected to electrophoretic analysis. The resulting gel showed a gradual increase in target cDNA as the amount of starting control cDNA was decreased (Figure 2). In response to the 5 mM glucose stimulus the 3.5 x 10^{5} STC_{6-14} cells contained 3.9 amol of GIP mRNA (Figure 3). In 25 mM glucose media the same number of STC_{6-14} cells produced 11.7 amol of GIP mRNA (Figure 4). The increase in GIP mRNA associated with a high glucose culture medium corresponds to a 3-fold increase over basal levels (p<0.0025)(Figure 5). In terms of mRNA copy number, this PCR technique predicted that the 5 mM glucose condition gave rise to an average of 23 molecules of GIP mRNA per GIP cell while the 25 mM conditions showed a 3 fold increase with 70 molecules of GIP mRNA per GIP cell.

IRGIP Release in Response to Glucose

The effect of varying glucose concentrations on the release of IRGIP was investigated for the STC_{6-14} cell line. With the cell line containing approximately 30% GIP cells, the 3.5 x 10^{5} cells plated represents 1 x 10^{5} GIP cells. When these cells were subjected to a basal (5 mM) level of glucose, they were found to release 0.84 ± 0.04% of total cell content (TCC ± SEM). Under the
same culture conditions, and GIP cell number, a 25 mM glucose medium produced a release of 1.11± 0.11% of TCC.

**Perifusion Studies**

Perfusion of $3.5 \times 10^5$ STC6-14 cells for a period of 26 h resulted in the cell population within the microchambers being 89% viable as determined by Trypan Blue staining.
Figure 2. Electrophoretic gel of wild-type versus competitor GIP cDNA. Lanes 1-6 contain both a fixed concentration of wild-type GIP cDNA (top bands; A) and a 1:2 dilution series of competitor cDNA (bottom bands; B) (5.8-0.18 amol). As the amount of starting competitor cDNA is decreased in subsequent tubes, the end amount of wild-type cDNA increases.
Figure 3. Standard curve for determination of GIP mRNA levels in STC<sub>6-14</sub> cells incubated in low glucose. Each point is plotted as the mean ± the standard error of the mean (Mean ± SEM) for 4 separate competitive PCR runs. The linear regression line was found to have an $r^2$ value (goodness of fit) of 0.98.
Figure 4. Standard curve for determination of GIP mRNA levels in STC6-14 cells incubated in high glucose. Each point is plotted as the mean ± SEM for 4 separate competitive PCR runs. The $r^2$ value for the regression line is 0.98.
Figure 5. Comparison of GIP mRNA levels in STC6-14 cells incubated in either low (5 mM) or high (25 mM) glucose (* represents significance to a level of p<0.05).
Discussion

A major problem associated with the molecular analysis of the intracellular events involved in GIP release is the diffuse distribution of the endocrine cells within the small intestine. Ideally, analysis of these mechanisms would be undertaken in permanent cell lines which express GIP, thus providing virtually unlimited numbers of cells with which to work. In addition, the use of cell culture allows for a tightly controlled environment and therefore a knowledge of the possible influences contained within the defined media. However, a problem associated with the use of these cell lines is the fact that they are by definition, abnormal. For this reason, any potential cell line must first be carefully scrutinized before it can be deemed suitable for the use in cellular studies.

Kieffer et al. (1995) have already partially characterized the STC6-14 cell line. A comparison was made of the elution time of synthetic porcine GIP and the immunoreactive GIP (IR-GIP) expressed in the STC6-14 cell line, using reverse phase high performance liquid chromatography (HPLC), and it was found that STC6-14 IR-GIP eluted as a mono-component at the same retention time as that of synthetic GIP. In addition, Kieffer et al. (1995) examined the IR-GIP release in response to glucose in this cell line and found that, like the primary cultures of canine GIP cells, concentrations of glucose from 10-20 mM significantly increased IRGIP release. A further increase of IR-GIP release was demonstrated with the addition of anti-somatostatin antibodies into the culture media, thus suggesting that the STC6-14 cells are inhibited by somatostatin. This inhibition has been shown previously to exist in humans (Salera et al, 1982), rats (Ho et al., 1987) and dogs (Pederson et al., 1975), with it most likely occurring in vivo via a paracrine pathway. Finally, these researchers were able to show, using immunocytochemistry (ICC), that the two major peptides in the STC6-14 cell line, GIP and somatostatin (SS), were released by separate cells. The objective of the first part of these investigations was to characterize the STC6-14 cell line further by identifying the murine cDNA sequence for GIP, thus allowing further molecular studies into the regulation of GIP release.
From the sequence analysis, a murine preproGIP was identified which consisted of 537 base pairs and was predicted to encode for a signal peptide, an amino terminal peptide, the GIP hormone and a carboxy terminal peptide. The proteolytic processing of the larger precursor polypeptide is predicted to be similar to the processing described for other hormones of the glucagon superfamily. However, there is nothing in the literature to date describing any proteolytic processing of the GIP precursor.

The signal peptide most likely consists of 19 or 21 amino acids, with the cleavage point being after one of the glycine residues at these positions. This area of the precursor molecule consists of numerous hydrophobic residues which most likely act to direct the newly synthesized polypeptide into the lumen of the endoplasmic reticulum (Blobel and Dobberstein, 1975). The identified signal peptide shows a similarity with those of other secretory peptides, namely a region of hydrophobic residues preceded by a positively charged residue near the amino terminus (Funckes et al., 1983).

The presence of a single arginine residue at both position 43 and 86 of the GIP precursor most likely serve as cleavage sites for the endoproteases which are capable of releasing the mature peptide from both the amino and carboxy terminal peptides. However, because there are two additional arginine residues in the mature form of murine GIP which are absent from the human hormone, cleavage must require more than the presence of an arginine residue. It would therefore seem beneficial to begin with an overview of the current knowledge regarding the substrate specificity of the specific endoproteases.

Devi (1991) proposed four rules governing monobasic cleavages by surveying approximately 110 monobasic sequences. These rules are 1) a basic residue (primarily arginine) is present at position -4,-6 or -8 from the site of cleavage; 2) a hydrophobic aliphatic residue is never present at position 1; 3) a cysteine residue is never present around the cleavage site and 4) an aromatic residue is never present at position -2 (Figure 4). Nakayama et al. (1992), examined other cleavage sites through the expression of various prorenin mutants around the dibasic
arginine-lysine site and concluded that for cleavage to occur at either dibasic or mono-arginyl sites similar rules as those found by Devi (1991) must apply.

Figure 6. Cleavage site specificity for the endoproteases. The position 0 indicates the site of cleavage with the basic residue at position -1 in most cases. In some peptides the cleavage site is at 0 with the basic residue at position 1

Transferring this information to the murine GIP sequence, one finds that the arginine residues bordering the mature hormone within either the amino or carboxy terminals adhere to these rules while the two arginine residues within the murine hormone (positions 18 and 30) do not. Therefore the rules proposed by Devi (1991) and Nakayama et al. (1992) can account for why most single arginine residues which are ubiquitous within precursor sequences are never cleaved (Nakayama et al., 1992).

It should be emphasized that the particular endoproteases responsible for these terminal cleavages have not yet been defined for GIP. However, by knowing the substrate specificity for the different endoproteases found to function within endocrine cells, it should be possible to predict those which are most likely to exist within the GIP cell. These prohormone processing proteases, PC1(also called PC3), PC2, PC4, PC5 (also called PC6) and furin, belong to the family of mammalian subtilisin related proprotein convertases (PC) possessing homology to the yeast Kex2 protease (Smeekins et al., 1991; Azaryan et al., 1995). PC1 and PC2 are soluble proteases which are expressed solely in endocrine tissue, suggesting a key role in prohormone processing (Patel and Galanopoulou, 1995). Both of these endoproteases have been shown to process
hormone precursors at lysine-arginine and arginine-arginine type dibasic sites (Benajannet et al., 1991; Thomas et al., 1991; Nakayama et al., 1992). In addition, PCI has been shown to cleave at single basic sites when the cleavage site is not flanked by a proline residue (Nakayama et al., 1992). By contrast, furin is a membrane bound protease capable of monobasic cleavage and exhibits an ubiquitous distribution in all tissues and cells (Galanopoulou et al., 1993).

The monobasic cleavage site at the carboxy terminal of the murine GIP hormone follows all the rules for cleavage previously laid out and is therefore most likely cleaved by the PCI endopeptidase within the secretory vesicles of the regulatory pathway that endocrine cells utilize. However, the amino terminal monobasic site lies adjacent to a proline residue and is therefore unlikely to be acted upon by PCI, even though there is an arginine residue at the -4 position. It has been shown in cells containing prorenin mutants transfected with individual endoproteases (Nakayama et al., 1992) that an entity separate from the PCI class of endoprotease is responsible for the cleavage of proline associated monobasic sites. The most likely candidate for this action is the furin endoprotease, firstly because furin has been shown by the experiments on pro-renin mutants not to be hampered by the presence of a proline residue. Furthermore, furin cleaves pro-peptides typically at R-X-X-R sequence motifs with the additional requirements of there being an arginine at position -1 and at least two of the -6,-4 or -2 sites containing a basic residue (Watanabe et al., 1992). Considering these requirements, furin or at least a furin like enzyme, would be the best protease candidate because the murine GIP amino terminal sequence possesses the substrate specificity for cleavage while other identified endoproteases have demonstrated little or no effectiveness at similar sites.

An understanding of the precise localization of the proteases predicted to be involved in GIP post-translational processing is important because it may shed light on the particular pathway- regulatory or constitutive- used for peptide release. All peptides targeted for release into the extracellular compartment originate in the endoplasmic reticulum and are then transported and distributed via transport vesicles to their correct destination. For the regulated pathway, peptides are shuttled through the Golgi apparatus which ends at the trans Golgi network, a special
term for the trans most (exit) cisterna of the Golgi stack. From here, the peptides are collected into secretory granules which only fuse with the plasma membrane in response to a physiological stimulus (Rothman and Wieland, 1996). The constitutive pathway shares this same route up to the point of the trans Golgi network, where the secretory proteins are then collected within vesicles that are immediately targeted for the plasma membrane. Hence, the precise localization of the endoproteases should now provide a hint as to the pathways in which they function. Furin has been localized to the Golgi apparatus which therefore indicates its involvement in the processing of proteins that are normally secreted via the constitutive pathway or the early part of the regulatory pathway (Hasaka et al., 1991; Molloy et al., 1994). In contrast, PC1 and PC2 have been found within the secretory granules of endocrine and neuroendocrine cells (Halban and Irmiger, 1994; Seidah et al., 1994), while PC1 has also been demonstrated to effectively cleave prosomatostatin in the constitutively secreting cell line COS-7. This suggests the presence of PC1 in cellular structures occurring before the separation of the regulated and constitutive pathways at the trans Golgi apparatus (Patel and Galanopoulou, 1995).

The localization of these endopeptidases sets up the interesting possibility that within certain abnormal cells, preproGLP could be completely processed, via furin or PC1, within the constitutive pathway. The other alternative is that this processing occurs within normal cells but the endoprotease activity begins with furin removing the amino terminal peptide before the trans golgi apparatus and the carboxy terminal is removed by PC1 in the secretory vesicle of the regulatory pathway.

In order to manipulate the release of any peptide hormone properly, it is essential that a full understanding of the mechanisms involved in this release are realized. For the GIP cell, it is known that an increase in glucose or fat within the intestinal lumen will lead to increased GIP release into the systemic circulation (Brown, 1974; Cataland et al., 1974; Brown et al., 1975; Falko et al., 1975; Pederson et al., 1975b; Ross and Dupre, 1978; Morgan et al., 1979; Krarup et al., 1985). An understanding of the regulation of gene expression depends in part on the ability to accurately measure mRNA species in defined cell populations (Gilliland et al., 1990A). Present
understanding of the regulation of GIP gene expression is minimal. It is well known that a glucose concentration increase will lead to a greater release of GIP. However, to date there are no data on the intracellular mechanisms involved in this release for a defined GIP cell population—where intracellular studies are best performed. To investigate the intracellular link between the release of GIP and the apparent glucose stimulus, the STC6-14 cell line was used in a Northern blot technique to assess mRNA levels following their culture in either a basal (5 mM) or elevated (25 mM) glucose media concentration.

A prerequisite for Northern blotting is the availability of cloned DNA or synthetic DNA sequences that can be used as probes for the gene transcripts of interest. The previous work on the cloning of the murine preproGIP from this cell line therefore provides the ideal material from which to produce a murine GIP probe. Unfortunately, even with the availability of this completely homologous probe, no GIP message was detected for this cell line. There are undoubtedly many problems that can be associated with the lack of any radioisotope signal—such as procedural errors, RNA degradation or simply that such low levels of GIP mRNA were expressed that this technique was unable to detect it. However, the fact that over 20 individual Northern-blot analyses were carried out, using a variety of subtle changes to the standard protocol, suggests a procedural error would be unlikely to explain the complete lack of results. In addition, many possible deviations from the “ideal” GIP Northern protocol would most likely only decrease the signal strength but not eradicate it entirely.

The possibility of RNA degradation is always a popular explanation because of the abundant RNases present throughout a laboratory setting. However, in this case, the cells were lysed directly in the tissue culture flask by a 4 M guanidine thiocyanate mixture—an extremely potent RNnase inhibitor. Final and complete removal of this inhibitor did not occur until the purified total RNA was about to be loaded onto a formaldehyde gel for electrophoresis. In addition, the integrity of the RNA product was verified by the visualization of both 18S and 28S ribosomal RNA bands, using ethidium bromide staining, before the RNA was transferred to the nylon membrane. Finally, the possibility that this technique was not sensitive enough for mRNA
detection is brought into question by the positive Northern analyses obtained for GIP mRNA by other researchers using similar quantities of total RNA (Inagaki et al., 1989; Higashimoto et al., 1993; Tseng et al., 1994). However, these past results were shown on intestinal tissue extracts from various animals while the present work detailed here was the first attempt to obtain similar results in a cell line that was suitable for intracellular studies involving GIP. In an attempt to ascertain whether the Northern analysis failed due to inadequate levels of GIP mRNA contained in these cell cultures, the more sensitive and truly quantitative technique of competitive PCR was employed.

It has been shown that the combined use of reverse transcription followed by the polymerase chain reaction (RT-PCR) is between 1,000-10,000 fold more sensitive than the traditional RNA blot techniques (Byrne et al., 1988; Wang et al., 1989; Mocharla et al., 1990). However, early attempts at quantifying mRNA via this technique proved difficult because the initial exponential nature of PCR amplification meant that small tube-to-tube variations in amplification efficiency would result in dramatic changes in product yields, making reproducible results very hard to show. Another problem with this technique was that any quantification attempt had to remain in the exponential phase (usually 10-15 cycles, depending on starting amounts; Gilliland et al., 1990B). As the components of the PCR mix (primers, nucleotides or enzyme) begin to be consumed in the reaction or lose activity, the efficiency of amplification starts to decrease and the reaction enters the plateau phase. Because the exact efficiency changes cannot be recorded during this transition, any quantification at this stage is inaccurate.

In the present investigations, a competitive-PCR approach was used in order to alleviate many of the problems encountered in PCR quantification. This technique involves the spiking of individual reactions with a known amount of a control cDNA. The control template consisted of the 410 bp target GIP cDNA with an 80 bp internal deletion. This similarity of control cDNA to the target cDNA means that amplification efficiency, between the two templates, should be almost completely identical throughout the PCR reaction. In this situation both the control and target
templates are being amplified by the same primers which means that there is no longer any necessity for the reaction to remain within the exponential phase.

The 3-fold increase in GIP mRNA levels observed upon stimulation with 25 mM glucose seems to be in line with the previous studies on GIP gene regulation. Tseng et al. (1994), using Northern-blot analysis, examined GIP mRNA levels in rats consuming 7-8 ml of either water or a 10% glucose solution. Their results showed the glucose fed rats exhibited a 2.6-fold increase in GIP message after 2 hours and a 3-fold increase after 4 hours. Similarly, Higashimoto et al. (1995) using the ribonuclease protection assay showed that rats fed 15ml of a 37.5% (w/v) glucose solution over a 24 hr period had a 4-fold increase in GIP mRNA as compared to rats fed an equal amount of saline. However, a major difference exists between the latter two experiments and the present work. Both Tseng et al. (1994) and Higashimoto et al. (1995) report GIP levels following an \textit{in vivo} experiment while the present study reports GIP levels from a well defined and controlled \textit{in vitro} study. The absolute level of GIP release would therefore be more accurately expressed in this present study because the cell culture environment provides far less opportunity for interference with the released GIP molecule. The use of cell culture for analysis of GIP mRNA is also being investigated by Tseng et al. (1994). These researchers, however, are using the STC-1 cell line, which consists of only approximately 7% GIP cells, and quantitating the mRNA via Northern blots. Although these previous studies failed to truly quantitate GIP mRNA, their results are in line with those from the STC\textsubscript{6-14} cell line and thus indicate that this competitive PCR technique is capable of accurate GIP mRNA quantification.

The present work has also reported the mRNA copy number per GIP cell for the STC\textsubscript{6-14} cell line. In the unstimulated state (5 mM glucose) it was estimated that there were 23 GIP mRNA molecules per GIP cell in culture. This increased to an estimated 70 molecules per GIP cell in the stimulated state (25 mM glucose) over a period of 2 h. These figures seem in line with previously reported observations. However, the previous data are limited and to date no other work has reported the mRNA copy number for a peptide hormone, which could be vastly different from any other cell type. Presently the reported copy numbers for a wide variety of cell types
encompasses a range from under 10 molecules per cell to over 22,000 (Gilliland et al., 1990A; Shin et al., 1992; Sklar et al., 1992; Trapnell, 1993; Riedy et al., 1995). A possible reason so few researchers report their values as an absolute number of mRNA molecules per cell could be due to the fact that this competitive PCR technique is not accurate enough for such sensitive measurements. In the investigations carried out for this thesis, such precise quantification is compromised by the fact that there was no internal control for the reverse transcription reaction. In the current work, and that of others (Becker-Andre and Hahlbrock, 1989; Gilliland et al., 1990A; Gilliland et al., 1990B; Seibert and Larrick, 1992; Apostolakos et al., 1993; Murphy et al., 1993), it was assumed that an efficiency of 100% existed for the RT reaction. However, in an attempt to determine the efficiency of different RT enzymes, Kotewicz et al. (1988) added 1μg of RNA to tubes containing different reverse transcriptases and found that only 24% to 34% of the cDNA transcripts were of full length. Yet, any exact measurement of RT efficiency must be dependent upon the specific RNA being transcribed due to the fact that the different secondary structures will affect reverse transcription to variable degrees (Brooks et al., 1995). In addition to the inaccuracies of the RT step, the problems associated with the accurate addition of the cDNA competitor to each tube—its initial quantification, pipetting errors—would limit the ability of this technique to define something as precise as the number of mRNA molecules per cell in a mixed cell population. Therefore, the competitive PCR employed here is capable of reporting quantitative mRNA level increases as well as molar concentrations of specific mRNA species, but should not be used to determine mRNA copy numbers per cell.

The final investigation of this thesis involved the measuring of GIP release from the STC6.14 cells in an attempt to correlate the mRNA levels with a corresponding level of peptide release. In order to keep the results of these two experiments as closely linked as possible, the culture medium which bathed the cells for mRNA quantification was itself used for the release studies. In response to basal glucose (5 mM) media, approximately $1 \times 10^5$ GIP cells were found to release $0.84 \pm 0.04\%$ of the total cell content (%TCC) of GIP. This value only slightly increased with a high glucose medium to $1.11 \pm 0.11\%$ TCC. These release profiles do not match those which
could be predicted from the 3-fold increase in GIP message. As well, these results do not match those found by Kieffer et al. (1995) who found an increase from 2.20 ± 0.17% TCC at low (5 mM) glucose to 4.20 ± 0.42% TCC at 20 mM glucose.

The difference between the levels of release reported here and those reported by Kieffer et al. (1995) is not easily explained. However, when comparing the release work done by Kieffer and colleagues on both the STC6-14 cell line (1995) and on primary cell cultures of canine and porcine gut epithelial cells enriched for GIP (1994), one finds many additional secretagogues (Ca**, cAMP and K+) in the latter study which were capable of significantly increasing GIP release. In addition to these observations, the one major difference between the experimental protocol used by Kieffer et al. (1995) and the one employed in this study was that the latter study used the STC6-14 cells between passages 26-39 while this study used the same cells from passages 48-56. This difference is significant since it has been shown that both the hormonal phenotype of a specific cell line (Philippe et al, 1986) and the relative proportion of a released hormone (Drucker et al., 1992) can change with cell passage or tumor growth. Related to the present study is the fact that such altered hormonal processing has recently been shown to occur for glucagon in the STC6-14 cell line’s parent cell line, STC-1 (Erhlich et al., 1994).

In order to explain the mechanisms which may lead to this change in transcription/release profiles, it is convenient to divide the intracellular events into two temporal phases: those involved in the gene transcription and the accumulation of mRNA and the events of post translational processing. Intracellular studies into the role of glucose on gene expression and accumulation have understandably centered on the workings of the β cell in the production of insulin. These studies have shown that glucose is capable of both stimulating insulin gene expression (Welsh et al., 1985; Hammonds et al., 1987) and stabilizing insulin mRNA (Hammonds et al., 1987; Melloul et al., 1993; Sharma and Stein, 1994). The pathway responsible for this glucose induced increase in insulin mRNA was postulated to occur through cAMP (Inagaki et al., 1992). Evidence for this came from the fact that glucose could be shown to increase cAMP levels in β cells (Charles et al., 1975) and that analogues of cAMP augmented insulin mRNA levels in these same cells
(Hammonds et al., 1987) and in clonal cell lines (Nielson et al., 1985). The identification of a cAMP response element within the promoter region of the GIP gene certainly adds credence to this hypothesis (Someya et al., 1993).

More recent investigations have, however, identified numerous transcription factors that respond to a glucose stimulus (see German and Wang, 1994 for a review). The isolation of these new factors in glucose-induced gene expression are certainly compelling, but at present are simply shrouding this process in more uncertainty—especially for genes such as GIP which has not yet had any of these transcription factors mapped to its upstream elements.

Moving away from the exact mechanism of glucose induced GIP expression, it is generally accepted that glucose is capable of increasing the intracellular levels of GIP mRNA. This would indicate that in terms of gene expression, the STC–14 cell line responds to a glucose stimulus in an appropriate manner and could therefore be used further to investigate the precise mechanisms involved in this glucose stimulus.

By combining the decreased GIP release with the previous conclusion, that these cells respond normally to a glucose stimulus up to the point of translation, it could be concluded that the problem lies within the realm of post-translational processing. This sort of problem seems common for tumor derived cells as it has been implicated in the abnormal processing of many propeptides including that for proglucagon in the related cell line STC-1 (Ehrlich et al., 1994). Using this cell line, Ehrlich et al. (1994) found a 17-fold increase in intracellular glucagon-like immunoreactivity (GLI) as compared to the levels detected in the RIN 1056A cell line. However, these researchers detected no difference in the plasma levels of GLI in mice containing these tumors. In addition, Berger et al. (1984) found a lack of correlation between gene expression, tumor peptide content and plasma levels of circulating peptides in patients with pancreatic endocrine tumors. This could suggest that tumor specific differences exist in the efficiency of translation, processing and secretion of a prohormone.

The specific mechanisms involved in glucose stimulation of mRNA have been investigated in the pancreatic β cell. Using a hybridization method to determine the levels and subcellular
localization of insulin mRNA, Itoh and Okamoto (1980) found that glucose stimulated cells had a 10-fold increase in proinsulin synthesis with only a small increase in the amount of insulin mRNA bound to the polysomes. This would suggest that proinsulin synthesis is mainly regulated by enhancing the translation efficiency of insulin mRNA. Now while this may indicate a possible mechanism for the impaired GIP release, there is greater evidence that the most common problems for tumor cells occur later in the pathway.

In order to change a release profile in terms of its %TCC there must be an increase in intracellular peptide with respect to the released peptide. It is therefore hard to explain the lower %TCC results obtained by this study, as compared to those reported by Kieffer et al. (1995), by a decrease in peptide synthesis. The amount of intracellular peptide, be it prohormone or completely processed GIP, is higher in relation to the released peptide in this study than it was for the studies by Kieffer et al. (1995). Similar results were obtained by Galanopoulou et al. (1995) when they found presomatostatin to be significantly processed by furin or furin-like enzymes in the constitutive cell lines COS-7 and PC12. However, this processing was also found to be inefficient based on the intracellular quantity of the processed precursor presomatostatin. In contrast, the regulated cell line AtT-20 was capable of processing almost all of the presomatostatin. This illustrates that although processing of endogenous prohormones at basic residues can occur in the constitutive pathway, efficient processing is believed to require targeting to the regulatory pathway (Thomas et al., 1988; Galanopoulou et al., 1995; Hosaka et al., 1991).

Conclusions and Future Work

The mechanism for GIP release in the STC6-14 cell line therefore appears to be compromised at the level of prohormone processing. In addition, the release profile of this peptide, along with the localization of the required enzymes, suggests that GIP is being released via the constitutive pathway. At present no investigations have centered on the pathways of GIP processing within this cell line. Results from such experiments could effectively open the door for
the use of this cell line in intracellular studies involving GIP or, conversely, eliminate it for all but those studies centered on the regulation of GIP mRNA synthesis- a process which so far seems to operate in a manner closely approximating that seen in the normal GIP cell.

Clearly one of the first questions that needs to be answered is whether or not the STC\textsubscript{6-14} cell line displays the characteristic biphasic GIP release profile, which is associated with the regulated pathway, on stimulation with higher levels of glucose. For this, there is now in place a perifusion protocol which should enable one to ascertain the level of GIP release over a longer period of time. If this cell line does show a considerable biphasic release, the question should shift to the changes in release profiles that subsequent passages display. If however, this cell line does not show a typical regulatory release profile, even at low passage numbers, its uses will obviously be limited.
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