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Studies of the Incretins, Glucagon-like Peptide-1 and Glucose-dependent Insulinotropic Polypeptide, and their Receptors

by

Malathy Satkunarajah

A thesis submitted in conformity with the requirements for the degree of Master of Science Graduate Department of Physiology University of Toronto

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ABSTRACT

Studies of the Incretins, Glucagon-like Peptide-1 and Glucose-dependent Insulinotropic Polypeptide, and their Receptors

Master of Science, 1998
Malathy Satkunarajah
Department of Physiology
University of Toronto

GLP-1 (Glucagon-like peptide-1) and GIP (Glucose-dependent insulinotropic polypeptide) are intestinal hormones that potentiate glucose-stimulated insulin secretion from pancreatic β cells. GLP-1, in particular, is at the forefront of diabetes research due to its antidiabetogenic actions in Type 2 diabetics. Previous studies demonstrated that GLP-1R/− mice exhibit mild diabetes despite upregulation of GIP secretion and insulinotropic action. GLP-1R/− mice were found to have diminished pancreatic insulin protein and mRNA levels, which may explain in part, the inability of GIP to fully compensate for the absence of GLP-1 activity in these mice. An evolutionary approach was used to study the functional determinants of GLP-1. The biological activities of three novel Xenopus laevis GLP-1-like peptides with ~ 70 % amino acid identity to human GLP-1 were found to be similar to that of human GLP-1. Furthermore residues at positions 12 and 23 of the Xenopus peptides, corresponding to positions 19 and 29 of mammalian GLP-1 were identified as critical to peptide activity. Study of GIP/GLP-1 receptor chimeras localized an activation domain of a GIP/GLP-1 receptor to three residues at the amino-terminus of the first transmembrane domain.
The work described here was undertaken as a part of the candidacy requirements for the M.Sc. degree in the Department of Physiology at the University of Toronto, under the supervision of Dr. Michael B. Wheeler. The experimental studies are presented in chapters 2, 3, and 4.


The experiments appearing in following figures which are included in this thesis were performed by:

- Figure 7 Dr. R.A. Pederson (University of British Columbia, Vancouver, BC)
- Figure 8 Dr. R.A. Pederson
- Figure 9 Dr. R.A. Pederson
- Figure 15 Dr. D.M. Irwin (University of Toronto, Toronto, ON) and Ms. Yi Wen
- Figure 16 Dr. D.M. Irwin and Ms. Yi Wen
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<th>Description</th>
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<tbody>
<tr>
<td>°C</td>
<td>degree celcius</td>
</tr>
<tr>
<td>μCi</td>
<td>microcurie</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μL</td>
<td>microlitre</td>
</tr>
<tr>
<td>μM</td>
<td>micromolar</td>
</tr>
<tr>
<td>A</td>
<td>adenine</td>
</tr>
<tr>
<td>Ala or A</td>
<td>alanine</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>Arg or R</td>
<td>arginine</td>
</tr>
<tr>
<td>Asn or N</td>
<td>asparagin</td>
</tr>
<tr>
<td>Asp or D</td>
<td>aspartic acid/aspartate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>B&lt;sub&gt;max&lt;/sub&gt;</td>
<td>maximum binding</td>
</tr>
<tr>
<td>B&lt;sub&gt;0&lt;/sub&gt;</td>
<td>zero binding</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>carboxyl</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine-5',3'-monophosphate</td>
</tr>
<tr>
<td>CCK</td>
<td>cholecystokinin</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CHL</td>
<td>chinese hamster lung fibroblasts</td>
</tr>
<tr>
<td>CHO</td>
<td>chinese hamster ovary cells</td>
</tr>
<tr>
<td>CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>COS-7</td>
<td>monkey kidney cells</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP responsive element</td>
</tr>
<tr>
<td>Cys or C</td>
<td>cysteine</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxyadenine triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxyctosine triphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>dGTP</td>
<td>deoxyguanine triphosphate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DP IV</td>
<td>dipeptidyl peptidase IV</td>
</tr>
<tr>
<td>dTTP</td>
<td>deoxythymidine triphosphate</td>
</tr>
<tr>
<td>EC</td>
<td>extracellular</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>half maximal excitatory capacity/dose</td>
</tr>
<tr>
<td>EI</td>
<td>enteroinsular</td>
</tr>
<tr>
<td>ES</td>
<td>embryonic stem cell</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle-stimulating hormone</td>
</tr>
<tr>
<td>G</td>
<td>guanine</td>
</tr>
<tr>
<td>GIP</td>
<td>glucose-dependent insulinotropic polypeptide/Gastric inhibitory peptide</td>
</tr>
<tr>
<td>Gin or Q</td>
<td>glutamine</td>
</tr>
<tr>
<td>GLP-1</td>
<td>glucagon-like peptide-1</td>
</tr>
<tr>
<td>GLP-1R/-/-</td>
<td>mice with a null mutation of the GLP-1 receptor gene</td>
</tr>
<tr>
<td>GLP-2</td>
<td>glucagon-like peptide-2</td>
</tr>
<tr>
<td>Glu or E</td>
<td>glutamic acid/glutamate</td>
</tr>
<tr>
<td>Gly or G</td>
<td>glycine</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>G-Protein</td>
<td>guanine nucleotide-binding regulatory protein</td>
</tr>
<tr>
<td>GRF</td>
<td>growth-hormone releasing factor</td>
</tr>
<tr>
<td>GRPP</td>
<td>glicentin-related pancreatic peptide</td>
</tr>
<tr>
<td>HG DME</td>
<td>high glucose Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>His or H</td>
<td>histidine</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>IC</td>
<td>intracellular</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>half maximal inhibitory capacity/dose</td>
</tr>
<tr>
<td>IDDM</td>
<td>insulin-dependent diabetes mellitus/Type 1 diabetes</td>
</tr>
<tr>
<td>Ile or l</td>
<td>isoleucine</td>
</tr>
</tbody>
</table>
InR1-G9  pancreatic glucagon-expressing hamster cell line
IP-1    intervening peptide-1
IP-2    intervening peptide-2
IR      immunoreactive
kb      kilobase
kDa     kilo Daltons
Leu or L leucine
LH      luteinizing hormone
Lys or K lysine
M       molar
Met or M methionine
mg      milligram
min     minute
mL      millilitre
mM      millimolar
MOPS    3-[N-Morpholino]propanesulfonic acid
MPGF    major proglucagon fragment
mRNA    messenger RNA
N       amino
NIDDM   non-insulin-dependent diabetes mellitus/Type 2 diabetes
nm      nanometre
NMR     nuclear magnetic resonance
O₂      oxygen
PACAP   pituitary adenylate cyclase activating polypeptide
PBS     phosphate buffered saline
PC      prohormone convertase
PCR     polymerase chain reaction
pg      picograms
PGK     phosphoglycerokinase
Phe or F phenylalanine
PKA     protein kinase A
PKC     protein kinase C
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tr>
<td>pM</td>
<td>picomolar</td>
</tr>
<tr>
<td>Pro or P</td>
<td>proline</td>
</tr>
<tr>
<td>PTH</td>
<td>parathyroid hormone</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>RIN 1046-38</td>
<td>rat clonal insulinoma cell line</td>
</tr>
<tr>
<td>RINm5F</td>
<td>rat insulin-secreting tumor cell line</td>
</tr>
<tr>
<td>rpm</td>
<td>rotations per minute</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>Ser or S</td>
<td>serine</td>
</tr>
<tr>
<td>SSC</td>
<td>sodium chloride—sodium citrate</td>
</tr>
<tr>
<td>SST</td>
<td>somatostatin</td>
</tr>
<tr>
<td>SST-14</td>
<td>somatostatin-14</td>
</tr>
<tr>
<td>SST-28</td>
<td>somatostatin-28</td>
</tr>
<tr>
<td>SV40Tag</td>
<td>simian virus 40 large T antigen</td>
</tr>
<tr>
<td>T</td>
<td>thymine</td>
</tr>
<tr>
<td>Thr or T</td>
<td>threonine</td>
</tr>
<tr>
<td>Tm</td>
<td>melting temperature</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane</td>
</tr>
<tr>
<td>Trp or W</td>
<td>tryptophan</td>
</tr>
<tr>
<td>TSH</td>
<td>thyroid-stimulating hormone</td>
</tr>
<tr>
<td>Tyr or Y</td>
<td>tyrosine</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>Val or V</td>
<td>valine</td>
</tr>
<tr>
<td>VIP</td>
<td>vasoactive intestinal polypeptide</td>
</tr>
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</table>
CHAPTER 1 GENERAL INTRODUCTION AND REVIEW OF THE LITERATURE

1.1 Glucose Homeostasis and Diabetes Mellitus

Glucose homeostasis depends on the balance between dietary glucose intake, glucose production by the liver, and glucose utilization by tissues such as fat, muscle, brain and kidney (1,2). Glucagon and insulin are the two main hormones involved in the regulation of blood glucose levels. Insulin is released from the pancreatic β cells upon stimulation by secretagogues such as glucose. Insulin’s main sites of action are the liver, muscle and adipose tissue. At the liver, insulin stimulates glycogenesis (in the prandial state) and inhibits glucose production (in the fasting state). In the muscle and adipose tissue, insulin stimulates glucose uptake and utilization. Therefore insulin acts to return blood glucose levels back to normal after a meal. Glucagon is secreted from pancreatic α cells when blood glucose levels fall below a minimum threshold, and acts on the liver to promote glycogenolysis and gluconeogenesis, elevating glucose levels to normal. Thus the opposing actions of glucagon and insulin are responsible for glucose homeostasis.

Diabetes mellitus is caused by a breakdown of this important balance. It is characterized by metabolic abnormalities leading to hyperglycemia and long-term complications involving the eyes, kidneys, nerves, and blood vessels (3). The two types of diabetes mellitus are Type 1 diabetes (or insulin-dependent-diabetes mellitus, IDDM) and Type 2 diabetes (or non-insulin-dependent diabetes mellitus, NIDDM). Both types have a growing prevalence world-wide and represent a global public health problem. Thus, intense research is aimed at developing treatments and therapies for diabetes mellitus. The etiology in Type 1 is the destruction of the β cells of the pancreatic islets by an autoimmune process resulting in insulin deficiency and finally a total loss of insulin secretion (4). The current treatment is to administer exogenous insulin following blood glucose monitoring.

In Type 2 diabetes there are at least two pathological defects. The first is insulin resistance; the reduced ability of peripheral tissues to respond to insulin, leading to diminished glucose metabolism and reduced inhibition of glucose output by the liver
The second is the inability of the endocrine pancreas to fully compensate for the insulin resistance, resulting in a relative insulin deficiency (5,8,9) in the presence of elevated glycemia, although patients exhibit hyperinsulinemia. Insulin deficiency is primarily due to a defect in glucose-stimulated insulin secretion as well as a defect in the ability of glucose to potentiate other secretagogues (glucose desensitization of the pancreatic β cell) (5). Both these factors are under genetic control and are influenced by environmental factors (e.g., diet, physical fitness). Recent data indicate that impaired insulin secretion is probably the main genetic factor, and that glucose overproduction (or lack of appropriate suppression of hepatic glucose output) rather than glucose under-utilization, plays the major role in both fasting and postprandial hyperglycemia (10,11,12).

Glycemic control in Type 2 diabetic patients is improved with diet and exercise. These therapies have been shown to enhance insulin action and improve β cell function (13). Failure of diet and exercise requires the use of pharmacological agents. Current treatments are aimed at stimulating insulin secretion in an attempt to compensate for the insulin resistance and/or improving insulin sensitivity of resistant tissues. Exogenous insulin and sulfonylureas address the insulin deficiency (14). Examples of sulfonylurea compounds, which are insulin secretagogues are glibenclamide, tolazamide, and glyburide. The major drawback to insulin and sulfonylureas are that they expose the patient to the risk of hypoglycemia (15). Normally, the pancreatic β cell only secretes insulin when the blood glucose levels are above a threshold of 4.4 mM (16). However, sulfonylureas do not act in a glucose-dependent manner (15). Also, since sulfonylureas do not stimulate insulin gene transcription and biosynthesis, β cell exhaustion may result due to depletion of insulin stores as a result of their insulinotropic action. There are cases in which Type 2 diabetic patients no longer respond to sulfonylurea compounds (17). Another class of compounds, biguanides such as metformin, reduce gluconeogenesis in the liver and improve glucose utilization in the periphery, due mainly to increased insulin action (18). A third class of compounds, the thiazolidinediones, have been shown to improve insulin sensitivity in Type 2 diabetics (19). They attenuate the hyperglycemia and hyperinsulinemia in these patients (20), but do not stimulate insulin secretion. The ideal therapeutic agent should stimulate glucose-induced insulin secretion and replenish pancreatic β cell insulin stores.
Fortunately such agents, called incretins, do exist physiologically and form part of the enteroinsular axis. The physiology and molecular biology of these hormones are the focus of this thesis.

1.2 The Enteroinsular Axis

The idea that signals arising in the gut after nutrient ingestion may affect endocrine responses and the disposal of carbohydrates was first put forth in 1902 by Bayliss and Starling, the fathers of gastrointestinal endocrinology. In 1964 Elrick et al, demonstrated that plasma insulin increased more when a glucose load was administered orally compared with intravenously (21,22), even if the same amount was given via both routes (which led to higher plasma glucose concentrations with intravenous glucose). In addition, in 1966, Dupré et al, were able to stimulate insulin release by an extract of intestinal mucosa (23), thus supporting the idea of a connection between the intestine and pancreas. Over the years evidence has also accumulated to implicate neural as well as direct substrate influences on the pancreas. The extracellular events that interact to result in the secretion of appropriate levels of insulin in response to a meal include the absorption of glucose, amino acids, and fatty acids; activation of nervous reflexes involving both the central nervous system (CNS) and peripheral nervous system (PNS) evoking both excitatory and inhibitory responses; and release of regulatory peptides from the gastrointestinal tract (24). Unger and Eisentraut coined the term “enteroinsular axis” (El axis) to describe this complex connection between the gut and pancreatic islets (25). The axis gives rise to the “incretin effect”, defined by Perley and Kipnis as the ratio between the integrated insulin response to an oral glucose load and the response to an isoglycemic intravenous glucose infusion (26).

1.2.1 Components of the El axis

The endocrine pancreas is highly innervated (27,28,29). Vagal stimulation enhances insulin secretion while activation of the sympathetic nervous system attenuates insulin secretion (27). Activation of the vagus following ingestion of nutrients may augment insulin secretion via either cephalic or gastropancreatic and enteropancreatic vagovagal reflexes. A cephalic phase is documented in dogs, rats
and humans. In humans, extrinsic pancreatic nerves do not appear to contribute much to the stimulation of insulin secretion after oral glucose, because patients with a completely denervated pancreas have a normal incretin effect after an oral glucose load (30,31,32).

Incretins are hormonal factors with insulinotropic activity released from the intestine by nutrients, especially carbohydrates (33). To qualify as physiological incretins, intestinal hormones must release insulin glucose-dependently at plasma hormone concentrations achieved by ingestion of glucose or other nutrients (34). Gastrin-17 stimulates insulin secretion in humans (35). However, dose-response studies with gastrin-17 revealed that the gastrin serum concentrations necessary for insulin secretion were greater than the physiological concentrations measured after a meal (36). Secretin has also demonstrated insulinotropic activity in humans, animals and in vitro with rat islets (37,38,39). However these effects occur only at supraphysiological plasma secretin levels (40). In addition, secretin was not released by oral glucose (41). The only two physiological incretins known to date are glucose-dependent insulinotropic polypeptide (GIP(1-42), referred to as GIP for simplicity) (42,43,44,45) and N-terminally truncated glucagon-like peptide-1 (GLP-1(7-37)/(7-36)amide, referred to as GLP-1) (46,47,48).

1.2.2 Physiological Importance of the EI Axis

As early as 1967, it was estimated that 50 % of the insulin secreted after an oral glucose load was released by gastrointestinal factors (25). However these early estimations were questioned because the relative changes in insulin levels could have been due to either changes in insulin secretion or hepatic extraction of insulin (49). The subsequent use of C-peptide levels to estimate insulin secretion (since it is not degraded by the liver) confirmed early results of the significant contributions of incretins to postprandial insulin release (50,51). GLP-1 contributes ~ 75 % (52) to the EI axis while GIP contributes the other ~ 25 % (53). These results obtained with the use of GIP antisera and a GLP-1 receptor antagonist indicate that no other additional incretins are necessary to explain the full incretin effect. However the limitations of GIP antisera and the fact that the GLP-1 receptor antagonist, exendin (9-39) was subsequently shown to antagonize the GIP receptor (54,55), indicates the need for further definitive studies.
1.3 Proglucagon and GLP-1

1.3.1 Proglucagon Gene

GLP-1 is encoded by the proglucagon gene which (Figure 1a) has six exons and five introns, and is highly conserved among all vertebrate animals studied including human, rat, hamster, and guinea pig (56,57,58). The gene structure (exon-intron organization) is very similar to other members of the glucagon family of peptides which includes GIP, vasoactive intestinal polypeptide (VIP), and growth-hormone releasing factor (GRF) (59). The similarity includes the coding of each peptide by unique exons, and sequence homologies among the precursors of glucagon, VIP, GIP, and GRF. Furthermore the genes are of similar sizes, ranging from ~9 to 10 kilobases (kb) (60,61,62). Taken together these findings suggest the intriguing possibility of a common ancestral gene for the family. The human proglucagon gene is located on the long arm of chromosome 2 and spans 10 kb (60). Mammalian proglucagon gene encodes glucagon, GLP-1, and glucagon-like peptide-2 (GLP-2) on exons 3, 4, and 5, respectively. The strong sequence homologies between the three peptides (~40-50%) has led to postulations that they arose via duplication of a single ancestral gene (56,63,64). Exons 1 and 6 encode most of the 5'- and 3'-untranslated regions, respectively while exon 2 contains the signal peptide.

The proglucagon gene is expressed in three major tissues of the body, the intestinal L cell, the pancreatic α cell, and certain neurons of the brain (65,66,67). In mammals, the gene is transcribed and spliced to form one mRNA transcript, which in turn yields a single proglucagon posttranslational product, preproglucagon (65). Unlike mammals, fish and bird proglucagons were found to undergo alternative mRNA splicing that produces different 3' ends in pancreas and intestine (68). Irwin et al, demonstrated that in the trout and chicken, intestinal mRNA is spliced to one or more exons which encode GLP-2 while pancreatic mRNA terminates within the intron between the two exons encoding GLP-1 and GLP-2. Thus trout and chicken express distinct mRNA transcripts in the pancreas (encoding glucagon and GLP-1) and intestine (encoding glucagon, GLP-1, and GLP-2) (68). Alternative mRNA splicing is a means of achieving differential tissue-specific expression of peptides, however the factors that regulate the diversity in mRNA transcript formation are not completely known. In the amphibian Xenopus laevis, two distinct mRNAs are expressed, one that encodes glucagon, three
Proglucagon Gene and Posttranslational Processing

a) Gene Structure

![Gene Structure Diagram]

b) Posttranslational Products

- Pancreas:
  - GRPP
  - Glucagon
  - MPGF

- Intestine:
  - Glicentin
  - GLP-1 (1-37/36NH₂)
  - IP-2
  - GLP-2
  - GRPP
  - Oxyntomodulin
  - GLP-1 (7-37/36NH₂)

novel GLP-1-like peptides and GLP-2, and a second that encodes glucagon and three novel GLP-1-like peptides only (69). Both transcripts are found in the pancreas whereas only the former is found in the intestine and the latter in the stomach (69).

Studies of transgenic mice in which regions of the rat proglucagon 5'-flanking region were fused to the coding region for SV40 large T antigen (SV40Tag), indicate that the 5' sequences extending to nucleotide -1300 are sufficient to direct expression to the pancreas and brain, while expression in the gastrointestinal tract requires the sequence from -2300 to -1300 (70). This sequence contains both positive and negative regulatory elements (71). The sequence for pancreatic islet specific elements resides in approximately 300 bp of the 5'-flanking DNA (72,73). This region contains a cAMP/Ca^{2+} responsive element (CRE), an insulin responsive repressor element, a PKC responsive element, and a pancreatic $\alpha$ cell specific promoter element. Pancreatic proglucagon gene expression is stimulated by fasting and hypoglycemia and inhibited by insulin in vivo (74,75). Insulin receptors have been identified on pancreatic $\alpha$ cells (76). During feeding, insulin (and glucose) suppress glucagon secretion and production, while during fasting the inhibition on glucagon secretion is released. Studies on the regulation of intestinal proglucagon gene expression have been limited until recently due to the lack of suitable L cell lines. Nutrients stimulate intestinal proglucagon-derived peptide (PGDP) biosynthesis in both normal and resected small bowel (77). Experiments also suggest that neuroendocrine peptides such as gastrin-releasing peptide (GRP) and GIP may stimulate proglucagon gene transcription through a CRE-dependent mechanism (78).

1.3.2 Posttranslational Processing of Preproglucagon

An identical 180 amino acid (160 amino acids in human) preproglucagon precursor is found in the pancreas, intestine and brain (64,65). Differential tissue-specific posttranslational processing of preproglucagon gives rise to different peptide products in the mammalian pancreas and intestine (65,79,80) (Figure 1b). Preproglucagon is processed through the actions of a family of prohormone convertases (PCs). These endoproteolytic enzymes cleave prohormones at the C-terminal side of pairs of basic amino acid sequences or sometimes at single basic residues (81). Interestingly, within the preproglucagon molecule, PC cleavage sites
flank glicentin, oxyntomodulin, glucagon, GLP-1 and GLP-2, making these peptides potential products of PC action.

Differential tissue-specific processing occurs as a result of differential tissue-specific expression of PCs (82). In the pancreatic α cell, the major products are glucagon, glicentin related pancreatic peptide (GRPP), and the major proglucagon fragment (MPGF) (Figure 1b). The MPGF which contains the GLP-1 and GLP-2 sequences is not further processed (83). The prohormone responsible for generation of glucagon is somewhat controversial. Some studies implicate PC2 as an enzyme responsible for processing of preproglucagon to glucagon (84,85), while others have shown that PC2 only processes preproglucagon to glicentin (82,86,87). However, the localization of PC2 in pancreatic α cells suggest a role for it in the production of glucagon (88). Furthermore, studies involving mice that lack PC2 demonstrate the absence of processing of preproglucagon to glucagon (89). Thus the role of PC2 in preproglucagon processing awaits further conformation.

The major intestinal products of preproglucagon processing are glicentin, oxyntomodulin, GLP-1 and GLP-2 (Figure 1b). PC1 (also called PC3) has been shown to be responsible for intestinal preproglucagon processing (82,84,90). Localization of PC1 using immunohistochemistry has found it to be present in L cells (91), but not in pancreatic α cells (88). GLP-1(1-37) is further processed to the truncated form, GLP-1(7-37), by PC1/PC3 cleavage at arginine 6 of full length GLP-1 (82). Cleavage of the six amino acids from the N-terminus of full-length GLP-1 renders it biologically active. GLP-1(7-37) is C-terminally truncated at arginine 36 and amidated to yield GLP-1(7-36)amide (92). Peptidylglycine α-amidating monooxygenase (PAM) is most likely responsible for the amidation (92). Both truncated forms of GLP-1 have identical insulinotropic and glucagonostatic properties (93).

The proglucagon-derived peptides (PGDPs) possess diverse biological activities. A study by Thim et al, found no effects of GRPP on gastroentero-pancreatic secretion or motility (94). Both glucagon and GLP-1 are involved in glucose homeostasis while GLP-2 has recently been shown to be an intestinal growth factor (95). In rats, glicentin did not have an effect on the pancreas (96) but it did inhibit gastric acid secretion (97). It did not affect insulin secretion in mice (98). Oxyntomodulin is an efficient inhibitor of gastric and pancreatic functions in humans and also inhibits gastric motility (99). These
results were achieved with supraphysiological plasma oxyntomodulin levels and the
effects of physiological levels are as yet unknown.

1.3.3 GLP-1 Secretion

GLP-1 is secreted by L cells which are found in the distal small intestine. The L
cell is an open-type enteroendocrine cell (100,101), and is thus exposed to luminal
contents at its apical membrane and to circulating and local factors at its basolateral
membrane. *In vitro* studies using intestinal L cells in culture have demonstrated direct
stimulation of PGDP secretion by glucose and fatty acids (102,103,104,105). Plasma
GLP-1 concentrations increase after a meal (106) and in response to oral
administration of glucose (107), while intravenous glucose infusions do not stimulate
GLP-1 release (108). *In vitro* studies have shown a direct effect of mono-unsaturated
long chain fatty acids on PGDP secretion (104). Plasma GLP-1 levels increase within
10 to 15 minutes after ingestion of mixed meals. Since GLP-1 secreting L cells are
found mostly in the distal ileum and proximal colon (100,101), it is unlikely that GLP-1 is
released in direct response to luminal nutrients. Since nutrients rarely reach the ileum
before postprandial insulin secretion occurs, the role of GLP-1 as a physiological
incretin was questioned. However, in humans, GLP-1 is promptly released into the
circulation after oral ingestion of nutrients (108,109,110,111). Thus, GLP-1 secretion
appears to be induced by neural or hormonal signals arising from the proximal small
intestine (112). GIP is one of the signals, from the proximal intestine, leading to release
of GLP-1 from the L cell (113) in rats. In contrast to animal studies, human studies have
shown that infusion of exogenous GIP does not lead to increments in plasma GLP-1
(114), thus indicating species-specific differences in L cell regulation. GLP-1 secretion
also appears to be under vagal control (115).

Inhibitors of PGDP release include somatostatin (SST-14), released from the
pancreas and intestine, and SST-28, released from the intestine (102,103). As GLP-1 is
a potent stimulator of intestinal somatostatin secretion, these peptides appear to be
involved in a feedback loop. In addition to SST, insulin also appears to inhibit intestinal
PGDP secretion. Loss of insulin in an animal model of Type 1 diabetes is associated
with elevations in circulating levels of intestinal PGDPs (116), although direct effects of
insulin on the L cell have not been demonstrated. Thus insulin deficiency may indirectly
affect PGDP secretion through other endocrine and/or metabolic changes that occur in uncontrolled diabetes. The effect of insulin likely constitutes a negative feedback loop from the pancreatic β cell to the intestinal L cell, since GLP-1 is a potent stimulator of insulin secretion. Thus, the L cell is regulated both by stimulatory and inhibitory factors.

The intracellular signaling pathways involved in stimulating PGDP secretion have been investigated in several cell culture systems. Calcium appears to regulate both basal and stimulated secretion in vitro, as a calcium channel blocker inhibited PGDP release, while a calcium ionophore increased PGDP release (117). The protein kinase A (PKA) pathway has also been demonstrated to stimulate PGDP secretion (118), indicating that the direct effects of GIP on PGDP secretion in vitro are most likely mediated via this pathway. Additionally, activation of the PKA pathway also causes increased synthesis of PGDPs by stimulating proglucagon gene transcription (119). These data are consistent with the identification of a CRE element in the proglucagon gene promoter (119,120). The protein kinase C (PKC) pathway is also involved in stimulating PGDP secretion. Activators of the PKC pathway, such as phorbol esters, stimulate PGDP secretion. PKC-ξ, an isoform of PKC, has been suggested to mediate the intracellular effects of fatty acids on the L cell (104). Thus the synthesis and secretion of intestinal PGDPs is influenced by luminal, endocrine and neural factors that act through diverse intracellular signaling pathways.

1.3.4 GLP-1 Metabolism
As with all peptides or proteins, GLP-1 is subject to proteolytic cleavage. It is primarily cleaved by dipeptidyl peptidase IV (DP IV; EC 3.4.14.5) (121), a serine peptidase found in serum which cleaves peptides at penultimate N-terminal proline or alanine residues. DP IV cleaves the penultimate alanine residue of GLP-1(7-37)/(7-36)amide to generate the inactive forms, GLP-1(9-37)/(9-36)amide (122). This process has been observed both in human serum in vitro (121,123) and in the rat in vivo (122). The kidney appears to be the major site for metabolic clearance of GLP-1 (124).

1.3.5 GLP-1 Structure/Function
GLP-1 shares sequence homology with members of the glucagon/secretin family of peptides. Members of this family include secretin, glucagon, GIP, GLP-2, VIP, GRF,
pituitary adenylate cyclase activating polypeptide (PACAP), and parathyroid hormone (PTH). The amino acid sequences of some of the family members are shown in Figure 2. Exendin-3 (125) and exendin-4 (126) isolated from venom of the Gila monster lizard *Heloderma horridum* and *Heloderma suspectum*, respectively, exhibit approximately 50% amino acid identity to mammalian GLP-1. Exendin-3 increases amylase secretion (126) while exendin-4(1-39) binds to the GLP-1 receptor and stimulates glucose-dependent insulin secretion in islet cells in vitro (127, 128) and in animal studies in vivo (52). Truncation studies of exendin-4 revealed that exendin-4(9-39)amide acts as a high potency antagonist of the GLP-1 receptor (128), and also antagonizes the GIP receptor (54,55). The presence of a penultimate glycine, instead of alanine, at position 2 in exendin-4 raises the possibility that exendin-4 will be more resistant to degradation by DP IV compared to mammalian GLP-1s with a position 2 alanine. Indeed preliminary studies have suggested that exendin-4 may be more potent than native GLP-1 in studies examining insulin secretion in vivo (129). The molecular cloning of proglucagon cDNAs from *Xenopus laevis* revealed the structure of three unique GLP-1-related molecules (69). The structures and activities of these peptides are presented in chapter 3. Very recently, two GLP-1 peptides were also isolated and purified from the pancreas of the cane toad, *Bufo marinus* (130). These peptides with 32- and 37-amino acid residues, GLP-32 and GLP-37, respectively, produced concentration dependent increases in insulin release from glucose-responsive rat insulinoma-derived BRIN-BD11 cells (130). The first 5 residues of these peptides are identical to that of human GLP-1, suggesting important functional conservation.

2D ¹H NMR was used to determine the structure of mammalian GLP-1 (7-36)amide bound to a dodecylphosphocholine micelle (131). In this membrane-like environment, GLP-1 has a structure similar to that observed for glucagon. GLP-1 was found to consist of an N-terminal random coil segment (residues 1-7), two helical segments (residues 7-14 and 18-29), and a linker region (residues 15-17) (131). In addition, the C-terminal helix was found to be highly helical, and more stable than the N-terminal helix (131). Since the N terminus is more conserved than the C terminus and shows greater sequence homology among the glucagon family of peptides, it was speculated that it is involved in high affinity receptor binding and receptor activation rather than in specific receptor recognition. In support of this hypothesis, N-terminal
### Sequences of Peptides in the Glucagon Family

<table>
<thead>
<tr>
<th>Peptide</th>
<th>GLP-1</th>
<th>GIP</th>
<th>Exendin-4</th>
<th>Glucagon</th>
<th>GLP-2</th>
<th>VIP</th>
<th>Secretin</th>
<th>PACAP-38</th>
<th>GRF</th>
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<td>1/7</td>
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<tr>
<td>GLP-1</td>
<td>HAPGGQFTSDVSYYMEGDAKEEFLIAVLKXVSSGSDamide</td>
<td>YAVEGEDAIRSDYSIAMDKIHQQDFVWNWLLAQKGKNDWKNHNTQ</td>
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<td>Secretin</td>
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<td>PACAP-38</td>
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**Figure 2:** Comparison of the GLP-1 sequence to the sequence of other peptides in the glucagon family. The sequence of exendin-4 is also shown. Identical regions are highlighted. Position 7 of GLP-1 corresponds to position 1 of the other peptides. Adapted from: Adelhorst K, Hedegaard BB, Knudsen LB, and Kirk O: Structure-activity studies of glucagon-like peptide-1. *J Biol Chem* 269: 6275-6278, 1994.
truncation of GLP-1 by 2 amino acids resulted in weak agonist activity, while an 8 amino acid truncation eliminated all activity (132). Particularly important is the N-terminal histidine, since its deletion in GLP-1 produced a decrease in binding affinity (133). Furthermore, N-terminal extension of GLP-1 by one amino acid resulted in significant loss of receptor affinity. Alanine substitutions of GLP-1 residues to identify side-chain functional groups required for interaction with the GLP-1 receptor (endogenous alanines were substituted with the corresponding amino acid in glucagon) revealed that substitutions at positions 7, 10, 12, 13, 15, 28, and 29 caused a great loss of receptor affinity (134,135). Far-UV CD spectra (Far-ultraviolet circular dichroism, allows measurement of secondary structures such as helices in peptides) of the above alanine substitutions indicated that only the GLP-1 analogs with alanines at position 28 and 29 had altered CD scans compared to wildtype GLP-1. Thus these substitutions had disrupted part of the alleged helical structure of the molecules (134), indicating that rather than being involved in receptor interaction, Phe28 and Ile29 may, consequently, be more important for the secondary structure of GLP-1, contributing to a conformation that is recognized by the receptor.

Changes of amino acids to their D-isomer forms at positions 9, 11, 12, 14, 15, 19, 20, 28, 32, and 33 led to significant reduction of receptor affinity, with 11 and 14 being extremely sensitive and thus possibly serving as a link between the N-terminal tetrapeptide and the mid-sequence of GLP-1 (136). Exchange to D isomer allows the identification of local parameters important for receptor interaction and thus side-chains important for receptor interaction (136). In contrast, the region from 15 to 27 showed no significant loss of affinity when changed to D isomer. Compared to N-terminal residues, C-terminal residues are less conserved suggesting a possible role in receptor recognition rather than high affinity binding. When residues 16-27 or 31-36 were replaced with alanine, no reduction in affinity was observed (136). The authors speculated that these amino acids may serve as spacer components between active residues, and therefore, when developing stable analogs, these residues may be replaced with a non-peptide framework. Truncation of two or three residues from the C-terminal of GLP-1(7-37) shifted the binding affinity by two orders of magnitude from that of wildtype GLP-1(7-37), indicating that the C-terminal is involved in receptor interaction (133). Therefore, studies involving amino acid substitutions and deletions of GLP-1
provide evidence for the importance of both the N- and C-termini of GLP-1 in binding and functional activity of the peptide.

1.4 ProGIP and GIP
1.4.1 The ProGIP Gene

GIP is encoded by the proGIP gene (Figure 3a) which is similar in structure to the mammalian proglucagon gene with 6 exons and 5 introns (137,138). To date the rat and human sequences have been cloned and show a high degree of amino acid sequence identity (137,138). The human gene is located on the long arm of chromosome 17 and spans 10 kilobases (138). Exons 2, 3, and 4 encode the preproGIP precursor protein which is 153 amino acids in length. The structural organization of human preproGIP was reported by Moody et al, to be putative signal peptide, 21 amino acids; NH₂-terminal peptide, 30 amino acids; GIP, 42 residues; and COOH-terminal peptide, 60 residues (139). Most of the sequence for the mature GIP is contained in exon 3 with the remaining in exon 4. Exons 1 and 6 encode the 5' and 3' untranslated regions, respectively.

Transcripts for the gene have been found predominantly in the intestine, specifically in the duodenum, upper jejunum and rat ileum (140,141). Examination of the tissue distribution of GIP mRNA by northern blotting revealed that GIP was present only in the duodenum; no signals were detected in the pancreas or liver (138). In contrast to GLP-1 expression, Takeda et al, showed that GIP was not expressed in the brain (137). It is also expressed in submandibular gland of the rat (142,143). The 5' flanking region of the gene codes for regulatory elements including a TATA motif, binding site for SP 1 and regions resembling consensus sequences of AP-1 and AP-2 target elements (138). These factors are involved in the regulation of gene expression by protein kinases A and C (144,145,146). Additional sequences were identified that were similar to a CRE. A hybrid gene containing the GIP promoter/enhancer region, linked to a reporter gene, was expressed in hamster insulinoma HIT-T15 cells and was stimulated by forskolin (147). In rats, the intraduodenal infusion of fat resulted in elevated duodenal GIP mRNA levels (143).
ProGIP Gene and Posttranslational Processing

a) Gene Structure

![Gene Structure Diagram]

b) Posttranslational Product

![Posttranslational Product Diagram]

1.4.2 Posttranslational Processing of PreproGIP

ProGIP protein is processed (Figure 3b) to one main end product with biological significance, GIP(1-42) (148,149). The sequence indicates that proteolytic processing at single arginine residues at either end of the GIP sequence would yield GIP(1-42). GIP(7-42) was among three peptides purified from the upper part of the pig small intestine and was shown to exhibit antibacterial activity (150). Although smaller GIP fragments have been isolated, they appear to be the result of enzymatic cleavage during the purification procedure (148,151).

1.4.3 GIP Secretion

Indirect immunofluorescence using conventional antisera demonstrated that immunoreactive GIP cells were confined to the mucosa of the upper small intestine (duodenum and jejunum) (152). These cells are endocrine K cells which are diffusely distributed throughout the small intestine (140). GIP secretion is linked to nutrient absorption. In humans plasma immunoreactive GIP levels increase 5 to 6 times after a mixed meal and remain elevated for over 6 hours in humans (153). Glucose administered orally serves as a stimulus for GIP secretion. Intravenous administration of glucose does not cause changes in plasma GIP levels indicating a requirement for luminal stimulation by glucose (44,154,155,156). GIP release occurs in a dose-dependent manner in response to glucose in isolated and cultured GIP cells (157,158). Thus glucose is a secretagogue acting directly on endocrine K cells. Ingestion of triglycerides also releases IR-GIP in humans and dogs (44,155,159,160,161). The response is greater and more prolonged than to glucose. This may be due to the slowing of gastric emptying by fat which presents a more prolonged stimulus for GIP release than does glucose. Long-chain fatty acids are stimulatory whereas medium-chain fatty acids have little effect (162,163). Fat induced IR-GIP was found not to be insulinotropic in the fasting state, as is the case with exogenously infused GIP (42,44,164). This led Beck to suggest that fat-released GIP may be exerting an effect on fat metabolism (165).
1.4.4 GIP Metabolism

The degradation of GIP(1-42) to its inactive form GIP(3-42) is similar to that of GLP-1, and occurs through the action of DP IV both in vitro (121,123) and in vivo (122).

1.4.5 GIP Structure

GIP was first isolated for its ability to inhibit gastric acid secretion by Brown et al, and was thus named gastric inhibitory polypeptide (148,166). Subsequently it was shown to be a potent stimulator of insulin release, hence its other name glucose-dependent insulinotropic polypeptide (42,43,44). The sequence for GIP is shown in Figure 2 (148,167). GIP has a high degree of conservation among species suggesting an important regulatory role in metabolism. There are only 2 amino acid differences between human and porcine GIP, at positions 18 and 34 (168) and only one amino acid difference between human and bovine GIP, at position 37 (169). Some studies have been aimed at elucidating the regions of GIP that confer its activity. GIP (3-42) has no ability to release insulin and does not antagonize GIP(1-42) (170). In contrast, Maletti et al, reported both receptor binding and insulinotropic activity in the 17-42 GIP fragment (171). Early work by Pederson et al, using the perfused rat pancreas with GIP(1-14) versus GIP(15-43) indicated the insulinotropic activity of the molecule resided in the C-terminal region (172). Interestingly, a study reported on the dissociation of the actions of porcine GIP(1-30) on insulin release from the pancreas versus somatostatin release from the stomach (173). Although GIP(1-30) was equipotent with GIP(1-42) in stimulating insulin release, it had 75 % less activity in stimulating somatostatin release compared to the full-length molecule. This suggested that different regions of the molecule may be necessary for binding to receptors in the stomach and the pancreas. A study of the effects of truncated forms of GIP, GIP(10-30), GIP(6-30)amide, and GIP(7-30), on the binding of [125]GIP(1-42) to GIP receptors in transfected Chinese hamster ovary cells (CHO-K1 cells), and on cAMP responses to GIP(1-42), reveal all three truncated peptides to be receptor antagonists, with GIP(6-30)amide exhibiting receptor binding affinity equivalent to that of GIP(1-42) (174). Thus GIP(6-30)amide appears to be a potent antagonist of the GIP receptor in vitro and contains the high affinity binding region of the GIP molecule.
1.5 Receptors

1.5.1 GPCR Superfamily

The receptors for GLP-1 and GIP belong to the superfamily of G protein-coupled receptors (GPCRs). Nearly 2000 GPCRs have been reported since the first receptor, bovine rhodopsin was cloned in 1983 (175). Most members of this family of cell surface receptors mediate their intracellular actions by a pathway that involves activation of one or more guanine nucleotide-binding regulatory proteins (G proteins). These receptors form a large and functionally diverse superfamily. The receptors are separated into A, B, and C classes. The A class of rhodopsin-like receptors have a relatively small extracellular N-terminal domain. The secretin/glucagon/VIP family of receptors which includes the receptors for GIP and GLP-1 are in the B class. These receptors have a relatively larger extracellular N-terminal domain. Other members of the B class include receptors for PACAP, calcitonin, PTH/PTH-related peptide, and GRF (176). These B class receptors are all structurally related with the most conserved residues residing in the putative membrane spanning regions. There is about 27 to 49 % amino acid identity among these receptor proteins while the sequence identity to receptors of other classes of GPCRs is less than 10 %. The metabotropic glutamate receptors form the C class subfamily of GPCRs. These receptors are present in the central and peripheral nervous system, where they regulate a wide variety of functions in response to the agonist glutamate (177).

Although the superfamily has a common unifying motif of seven transmembrane domains, the receptors are quite diverse and possess unique properties. The most obvious of which are the diversity of ligands, which range from small amines such as catecholamines, to small peptides such as GLP-1 and GIP to large glycoproteins such as thyroid-stimulating hormone (TSH) and luteinizing hormone (LH). GPCR domains involved in ligand binding and activation are just as diverse as the chemical structures of the endogenous ligands. For example, the biogenic amines, epinephrine, norepinephrine, dopamine, and histamine enter and bind the TM core (178). The family of receptors for thrombin and other proteases is activated by proteolysis of the N-terminal segment. The new N-terminal segment acts as a tethered ligand and interacts with extracellular loops of the remaining receptor (179,180). Glycoprotein hormones, LH, FSH, and TSH, are the largest (30-40 kDa) and most complex GPCR agonists. These
hormones initially bind exclusively to the N-terminal segment with high affinity. The hormone-N-terminal segment complex undergoes conformational changes (181) and makes secondary interactions with the membrane-associated domain, thus generating a signal (182).

Members of the B class of GPCRs have seven hydrophobic transmembrane (TM) domains, with an extracellular (EC) N terminus and a cytosolic C terminus. The adoption of a seven TM structure allows for placement of the N- and C-terminal segments at opposite membrane surfaces. It allows for glycosylation and ligand binding at the N-terminal segment, and phosphorylation and palmitoylation at the C-terminal segment for desensitization and internalization (183). Each putative transmembrane domain is made up of approximately 20-30 amino acids. The membrane spanning regions are α helical in structure. This secondary structure allows for the most favorable interaction with the hydrophobic lipid bilayer. There are three extracellular (EC) loops and three intracellular (IC) loops that link the TM regions.

All class B receptors contain an amino-terminal hydrophobic domain, which is presumed to serve as a signal sequence followed by a relatively long (115-160 residues) hydrophilic domain, preceding the seven TM domains. The EC N terminal domain has a series of conserved cysteine residues which are thought to form disulfide bonds (184). These bonds contribute to the conformation of the receptors and confer structural stability. There are two conserved cysteines on the EC loop 1 and 2. The receptors also contain glycosylation sites. A sequon with the consensus sequence Asn-X-Ser/Thr, where X is any amino acid except proline or glutamate, is the site of N-linked glycosylation, although only one-third of such sequences in glycoproteins are actually glycosylated (185). Asparagine is the acceptor for N-linked oligosaccharide chains. The glycosylated asparagine is generally located in or near regions predicted to contain a reverse turn (186,187). Sites of glycosylation mark extracellular positions within membrane proteins. Protein glycosylation has been shown to be important for a variety of functions, including normal protein folding, stability, intracellular trafficking, and cell surface expression, although the role of N-linked glycosylation may differ in various membrane proteins (188). The secretin/VIP/glucacon subfamily of receptors generally have three N-linked glycosylation sites. The C terminal cytoplasmic domains of the receptors have PKA/PKC phosphorylation consensus sites that are thought to be
involved in receptor desensitization and internalization. All members of the secretin/glucagon/VIP subfamily cloned to date are coupled to the $G_s$ G-protein. Many receptors have also been reported to couple to other G-proteins, leading to increases in intracellular calcium levels and inositol triphosphate hydrolysis (189). The second and third intracellular loops have been implicated in interactions with G proteins.

1.5.2 GLP-1 Receptor

The actions of GLP-1 are mediated through high-affinity membrane receptors (Figure 4). GLP-1 receptors were first identified on rat insulinoma-derived RINm5F cells (190). Analysis of binding experiments performed with RINm5F cells revealed that GLP-1 binds to a single class of binding sites (191). In cross-linking studies with $[^{125}]$GLP-1 a single band with an apparent molecular mass of 63 kDa was labeled (192). The rat and human β cell GLP-1 receptor cDNAs have been cloned (127,193). At the amino acid level the rat and human GLP-1 receptors share 90 % sequence identity. The human GLP-1 receptor has been localized to 6p21, the long arm of chromosome 6 (194). The receptor consists of 463 amino acids. The GLP-1 receptor is expressed in stomach, intestine, liver, kidney, islets, lung, heart, skeletal muscle, and adipose tissue (193,195,196,197). The highest levels of GLP-1 receptor mRNA transcripts are found in lung and islets (193), while the presence of GLP-1 receptors in peripheral tissues such as liver, muscle and fat is controversial (193,197,198,199). The GLP-1 receptor is regulated via mRNA expression, receptor internalization and receptor desensitization. In insulin-secreting cells, the expression of the GLP-1 receptor is negatively regulated upon activation of various second messenger systems. The steady state levels of rat GLP-1 receptor mRNA in RINm5F cells are downregulated by dexamethasone and agents that increase intracellular cAMP levels (200). This includes regulation by GLP-1 itself (200). Treatment of these cells with forskolin resulted in a 40 % reduction in GLP-1 receptor mRNA levels, while phorbol 12-myristate-13-acetate, a protein kinase C activator, caused a 20 % decrease in receptor mRNA levels. Down-regulation of the GLP-1 receptor number in RINm5F cells resulted after treatment of the cells with dexamethasone, although the affinity of the receptor for GLP-1 was not affected (201).
Human GLP-1 Receptor

Legend
- Hydrophobic residues
- Hydrophilic residues
- Positively charged residues
- Negatively charged residues

Figure 4: Schematic of the amino acid sequence of the human GLP-1 receptor showing the TM, EC, and IC domains. The 6 cysteines in the N terminal domain and the 2 cysteines in EC 1 and EC 2 are highly conserved among members of the secretin/glucagon/VIP receptor family (←). The 3 potential glycosylation sites are also indicated (↗). CT is the cytoplasmic domain. The amino acids are indicated in single letter codes.
The GLP-1 receptor is sensitive to glycosidase treatment and has three potential N-linked glycosylation sites (at residues 63, 83, 150 on the rat receptor). It is still under investigation as to which sites are actually glycosylated. Glycosylation is important for the biosynthetic process of the GLP-1 receptor and may be involved in regular receptor function (202). Glycosylation of the β₂-adrenergic receptor was found to be important for receptor expression by not for binding or functional activity (189). Such studies for the GLP-1 receptor are forthcoming. The ligand binding domain of the receptor is thought to include the N-terminal extracellular domain. Göke et al, isolated and purified the N-terminal domain and demonstrated that it had intrinsic binding activity (203). In addition, it was covalently attached to GLP-1 by cross-linking experiments, demonstrating that there was a close physical interaction between the ligand and the N-terminal domain. The N-terminal EC domain was also shown to contain molecular determinants for both agonist binding affinity and selectivity. A series of chimeric receptors in which 4-6 amino acids in the N-terminal EC domain of the human GLP-1 receptor were replaced with the analogous region of the human glucagon receptor were constructed and expressed in Monkey kidney cells (COS-7 cells) (204). One particular chimera in which amino acids 29-32 were swapped for residues in the glucagon receptor, displayed a 7 to 10-fold decrease in affinity for GLP-1. This decrease in affinity also results in a 50-fold decrease in selectivity of the receptor for GLP-1. Site-directed mutagenesis studies of tryptophan residues (to alanine) in the N-terminal EC domain of the rat GLP-1 receptor revealed five of these resides to be essential for its ligand binding ability (205). Of the 6 tryptophans, at positions 39, 72, 87, 91, 110, and 120, number 72 and 110 are highly conserved within the receptor subfamily. Tryptophan 87 to alanine exhibited wild-type binding characteristics. The third IC loop and in particular, lysine at position 334 was found to be important for efficient coupling of the rat receptor to adenylyl cyclase (206). Furthermore, three substitution mutations, V327A, I328A, and V331A resulted in significantly lower GLP-1-stimulated cAMP production without reductions in receptor expression. These residues are localized to the predicted junction of the fifth TM helix and the third IC loop.
1.5.3 GIP Receptor

The rat GIP receptor (Figure 5) is 455 amino acids long and has a predicted molecular weight of ~52 kDa, including the signal peptide. It has three potential N-linked glycosylation sites at positions 59, 69, and 74 of the EC N terminal domain (207). The third cytoplasmic loop and C-terminus are rich in threonine and serine residues, which are potential phosphorylation sites and may be involved in receptor desensitization and internalization (207). The ligand binding domain of the receptor has been localized primarily to the EC N terminal domain using chimeric GIP/GLP-1 receptors (208). Detailed structure/function data on the GIP receptor is still forthcoming as the receptor was only cloned in 1993 (207). The isolated hamster sequence shares ~86% identity with the rat homologue (209). The amino acid sequences of both the rat and hamster receptors share 40-47% identity with the GLP-1 and glucagon sequences (207,209). Secretin, VIP, glucagon, GLP-1 and GLP-2 have no effect on $[^{125}\text{I}]$GIP binding. Surprisingly, exendin(9-39), a GLP-1 receptor antagonist, and exendin-4(1-39), a GLP-1 receptor agonist, demonstrated low affinity for the GIP receptor, with 39% and 21% displacement of 1 µM labeled GIP respectively (54). Additionally GIP(6-30)amide exhibited high affinity antagonism of the GIP receptor (174).

The presence of GIP receptors was first demonstrated in a transplantable hamster insulinoma (210) and an insulin-secreting hamster cell line In 111 (211,212). Both studies identified high and low affinity receptors. The mRNA for the GIP receptor was detected in the pancreas, stomach, duodenum, proximal small intestine, adipose tissue, adrenal gland, brain, and pituitary (207). Studies of the biological actions of GIP have identified several responsive tissues including stomach, pancreas, fat, and liver.

1.5.4 Signal Transduction of Incretin Receptors

Both the GLP-1 and GIP receptors are coupled to stimulatory G proteins and hence to the adenylate cyclase cascade. In addition, stimulation of the GLP-1 receptor leads to an increase in free cytosolic calcium levels which initiates the insulin secretory process (213,214). A schematic of the signaling pathways in the pancreatic β cell leading to insulin secretion, including signaling through the GLP-1 and GIP receptors is shown in Figure 6. Glucose-stimulated insulin secretion requires the uptake of glucose (through GLUT 2 transporters) and its subsequent metabolism to produce a rise in
Figure 5: Schematic of the amino acid sequence of the rat GIP receptor showing the TM, EC, and IC domains. The 6 cysteine residues in the N terminal domain and the 2 cysteines in EC 1 and EC 2 are indicated (→). The 3 potential glycosylation sites are also indicated (↗). CT is the cytoplasmic domain. The amino acids are indicated in single letter codes.
Figure 6: Signal transduction pathways leading to insulin secretion in the pancreatic B cell. Upon glucose entry into the cell, it is metabolized to produce ATP. The change in ATP:ADP ratio closes the $K_{ATP}$ channel, thus depolarizing the plasma membrane and opening voltage dependent $Ca^{2+}$ channels. Increase in intracellular $Ca^{2+}$ initiates insulin secretory granule exocytosis. GLP-1 and GIP bind to their respective high affinity receptors on the cell surface and cause an increase in cAMP. cAMP phosphorylates PKA, activating it. PKA then has potential sites of action including the $K_{ATP}$ channel, the voltage dependent $Ca^{2+}$ channel and SNARE proteins, all of which have potential PKA phosphorylation sites. Adapted from: Fehmann HC, Göke R, Göke R: Cell and molecular biology of the incretin hormones glucagon-like peptide-1 and glucose-dependent insulin releasing polypeptide. Endocr Rev 16: 390-410, 1995.
cytoplasmic ATP:ADP ratio. The change in the ratio induces ATP-dependent K⁺ channels to close. This blocks K⁺ efflux from the cell, causing the plasma membrane to depolarize. Voltage-gated calcium channels are opened upon membrane depolarization and Ca²⁺ enters the cells causing the free cytosolic calcium concentrations ([Ca²⁺]ᵢ) to increase. Ca²⁺ is a trigger for exocytosis of insulin-containing secretory granules (215,216,217). This results in docking of the vesicles to the membrane, fusion of the vesicle and expulsion of the insulin granules into the circulation. Besides glucose, GLP-1, and GIP, cholecystokinin (CCK) and arginine are other insulin secretagogues.

Drucker et al, demonstrated that GLP-1 increased intracellular cAMP levels in a rat islet cell line (218). An increase in cAMP could result either by the stimulation of its production by adenylyl cyclase or by the inhibition of its degradation through inhibition of phosphodiesterase. Studies by Göke et al, revealed that stable guanidine nucleotides significantly reduced the binding of GLP-1(7-36)amide to membranes of RINm5F cells (which express GLP-1 receptors), as well as the affinity of GLP-1 for its receptor (219). These results indicate that the receptor is coupled to stimulatory G proteins, Gₛ. Gₛ proteins are known to activate adenylate cyclase, thus GLP-1 receptor activation leads to increases in cAMP levels through the adenylate cyclase system. This finding has been confirmed by several groups (193,220,221,222). A direct stimulation of adenylate cyclase and PKA were shown in response to GLP-1 (223). Recently, loss of GLP-1 mediated insulin secretion was observed in RIIα knockout mice islets (224). RIIα is a regulatory subunit of PKA. GIP also produced an increase in cAMP in various experimental systems (210,211,212,225,226). So upon ligand binding, the receptors become activated which in turn activate the G-protein signaling system. The Gₛ protein activates adenylate cyclase which increases the intracellular levels of cAMP. However, studies have also shown that at supraphysiological concentrations, GLP-1 decreases cAMP production (180). This response is thought to occur due to a rapid and reversible desensitization of the GLP-1 receptor. cAMP activates protein kinase A and it is thought that PKA may phosphorylate various molecules in the β cell to trigger insulin secretion. These molecules include the voltage-gated Ca²⁺ channels, K<sub>ATP</sub> channels, both of which have PKA phosphorylation consensus sites, and the SNARE proteins.
GLP-1 has also been shown to increase the concentration of free cytosolic calcium $[\text{Ca}^{2+}]_i$ which is followed by a potentiation of glucose-induced insulin release in rat pancreatic $\beta$ cells (227,228,229,230). The increase in $[\text{Ca}^{2+}]_i$ is inhibited by blockers of L-type voltage-dependent channels. Therefore the rise of $[\text{Ca}^{2+}]_i$ is most likely due to increased influx through voltage-dependent channels activated by GLP-1. This effect is only seen in the presence of elevated glucose (231). In COS cells transfected with the GLP-1 receptor, both adenylate cyclase and phospholipase C were activated (228). This resulted in the release of $\text{Ca}^{2+}$ from intracellular stores (228). However, phospholipase C pathway is not activated in Chinese hamster lung cells (CHL cells) stably expressing the human GLP-1 receptor (232). Thus GLP-1 is able to couple to different intracellular signaling pathways depending on the cell type. Although the rise in $[\text{Ca}^{2+}]_i$ is necessary for GLP-1 to potentiate glucose-induced insulin release, GLP-1’s effects on insulin gene transcription are mediated mainly through the cAMP-CRE pathway (233).

The GLP-1 receptor has most recently been shown to stimulate two important mitogen-activated protein (MAP) kinase pathways due to coupling to $G_i$ proteins (234). GLP-1 activated extracellular signal-regulated protein kinase (ERK 1 & 2) and p38 MAP kinase in Chinese hamster ovary (CHO) cells. GLP-1 induced p38 MAP kinase activation was not found to depend on elevated intracellular cAMP levels. GLP-1’s ability to enhance p38 MAP kinase was mimicked by lysophosphatidic acid, whose receptor interacts with pertussis toxin-sensitive $G_i$ proteins (234). Therefore GLP-1 receptors can couple to different intracellular pathways depending on the cell type.

1.6 **Incretin Hormone Actions**

1.6.1 **Actions of GLP-1 and GIP on the Pancreas**

GLP-1 receptors are found on pancreatic $\alpha$, $\beta$, and $\delta$ cells (235,236), while GIP receptors are found on pancreatic $\beta$ cells (171,210). Both GLP-1 and GIP have no direct effect on exocrine pancreatic secretion in vitro (237,238). GLP-1 and GIP stimulate insulin release from pancreatic $\beta$ cells in a glucose-dependent manner (239,240,241). In the rat pancreas, the glucose threshold for potentiation by GIP and GLP-1 is 4.5 mM, and at higher glucose concentrations, the effect is amplified (242).
GLP-1 and GIP also potentiate amino-acid induced insulin secretion (243). Their effects on insulin secretion are additive (244,245). In addition, both these peptides have been shown to stimulate insulin gene transcription and biosynthesis (218,246). The effects of GLP-1 are present even in patients with severe Type 2 diabetes (114,247). In addition, GLP-1 can overcome decreased responsiveness of the β cell in Type 2 diabetic patients thereby enhancing glucose-stimulated insulin secretion (114).

GLP-1 has the ability to inhibit glucagon secretion (248,249). These effects are mediated both directly through α cells and indirectly through stimulation of somatostatin and insulin secretion. GLP-1 infusion in C-peptide negative diabetic dogs lowered circulating plasma glucagon, suggesting that the glucagonostatic effects of GLP-1 are partly independent of circulating insulin (250). However, in the isolated rat α cells, GLP-1 stimulated glucagon secretion. This effect was inhibited by somatostatin, indicating that the glucagonostatic effects of GLP-1 may be indirect through a paracrine effect on somatostatin secretion (251). In glucagon-producing InR1-G9 cells, GLP-1 directly inhibited glucagon secretion in the absence of somatostatin (252). Thus the inhibitory effects of GLP-1 on the α cell still remain unclear. In the perfused rat pancreas, GIP stimulated glucagon secretion in the presence of glucose levels of 5 mM or lower only (239). In contrast, GIP infusion in humans during normoglycemic and hyperglycemic conditions did not affect plasma glucagon levels (253). GLP-1 stimulates somatostatin secretion. Stimulation of somatostatin secretion from isolated human islets is not dependent upon elevated glucose concentrations (254). GIP is a very weak stimulator of somatostatin secretion in the perfused rat pancreas (255). Pancreatic δ cells are much less sensitive to GIP than to GLP-1 (255). The effects of GIP and GLP-1 on glucagon release might be explained by their effects on somatostatin secretion.

In addition to their effects on islet hormone secretion, the incretin hormones regulate the expression of several islet genes, including the genes encoding insulin, GLP-1 receptors and the neuroendocrine chaperone 7B2. Both GLP-1 and GIP stimulate proinsulin gene expression in the presence of elevated glucose levels. Treatment of different insulinoma cell lines with GLP-1 increases the cellular levels of proinsulin mRNA (128,223,233). Most studies have shown these effects to be due to a direct stimulatory action on proinsulin gene transcription rather than mRNA stabilization. However one study revealed that GLP-1 stabilized proinsulin mRNA levels (256).
addition to increasing insulin gene transcription, GLP-1 has also been shown to inhibit insulin mRNA degradation in a insulin-secreting cell line, RIN 1046-38 (256). RIN1046-38 cells, a rat clonal insulinoma cell line were investigated for the effects of exposure to GLP-1 (256). Incubation of cells with glucose plus 1 or 10 nM GLP-1 for 12 or 24 hours significantly increased insulin release by about 3-fold, intracellular insulin content by 1.5-fold, and insulin mRNA by almost 2.5-fold. Treatment of cells with a transcription inhibitor, actinomycin D, demonstrated that elevated insulin mRNA levels after a GLP-1 exposure are mainly due to stabilization of the mRNA. Actinomycin D blunted the GLP-1 effect on insulin release but did not affect GLP-1-mediated elevation of insulin mRNA. This was explained by the fact that actinomycin D inhibited the transcription of the proteins necessary for insulin biosynthesis and insulin release, such as GLUT-1 and hexokinase I. Studies using incorporation of [3H]leucine into proinsulin showed that GLP-1 stimulates proinsulin biosynthesis as well (233). Thus GLP-1 helps to restore the intracellular insulin pool. The effects on insulin gene transcription appear to be mediated through a cAMP-responsive element. Ca²⁺ is not able to regulate insulin gene transcription (257). Thus the release of insulin and the stimulation of insulin gene transcription seem to be regulated independently by different second messengers. GIP has also been shown to stimulate the transcription of the insulin gene (246).

Incubation of an insulin-secreting cell line, RINm5F with GLP-1 caused downregulation of GLP-1 receptor mRNA levels. The molecular mechanism of this effect was characterized with actinomycin D as an inhibitor of RNA polymerase. Incubation of cells with the inhibitor caused a decrease in GLP-1 receptor mRNA levels, however, GLP-1 did not influence receptor biosynthesis (200). 7B2 is a protein that is expressed in neuroendocrine cells and functions as an endogenous inhibitor and chaperone of the prohormone convertase PC2. PC2 is involved in the generation of mature insulin from its precursor in endocrine pancreatic cells. A hybrid gene containing the promoter of the 7B2 gene linked to the transcriptional reporter gene CAT was transfected into βTC-1 cells (258). Treatment of cells with forskolin or GLP-1 increased CAT activity, indicating a stimulatory effect on 7B2 gene transcription by these agents.
1.6.2 Extra-pancreatic Actions of GLP-1

Cells bodies immunoreactive for GLP-1 have been found in the nucleus tractus of solitarius and the ventral and dorsal part of the medullary reticular nucleus, while nerve fibers immunoreactive for GLP-1 were found throughout the brain with highest concentrations in midline hypothalamic structures (259,260,261). Interestingly, these nuclei are known to receive afferent inputs from gastrointestinal organs including the stomach and intestine (262). RIA quantitation of GLP-1 revealed large concentrations in the hypothalamus and brain stem (263,264). Perfusion of GLP-1 induced release of glutamine and glutamic acid in the rat basal ganglia (265). These observations suggest that GLP-1 may act as a neurotransmitter in the brain (266). In support of this, GLP-1 receptors and mRNA transcripts have been found in different regions of the central nervous system and pituitary (267,268). GLP-1 receptors are mainly localized to hypothalamic nuclei involved in regulation of digestive behavior. Intraventricular administration of GLP-1 profoundly inhibits food intake in rats (267,268,269). This effect seems to be highly specific since appropriate doses of exendin(9-39), a GLP-1 antagonist, abolish the effects of GLP-1 (267). Thus Turton et al, concluded that GLP-1 was a satiety factor (267). However, their study is controversial due to the supraphysiological doses of GLP-1 used. Furthermore, mice with a targeted disruption of the GLP-1 receptor gene were found to gain weight normally (270).

As discussed in section 1.5.2, GLP-1 receptors are localized in a number of extra-pancreatic tissues, including the gastrointestinal tract. GLP-1 has been found to be a potent inhibitor of gastric motility and postprandial acid secretion (271). In the stomach, GLP-1 inhibits gastric emptying (gastric motility) and thus attenuates meal-associated glucose excursion (272,273). Although this action may important in postprandial glucose control, the dependence on glucose levels for insulin secretion by GLP-1 could potentially diminish the effects of GLP-1. In the rat lung, GLP-1 has been demonstrated to stimulate mucus production and relax pulmonary arteries in the in vitro (274). Since GLP-1 receptor knock-out mice do not exhibit any obvious abnormalities in pulmonary function (270), the physiological relevance of the above findings is not clear. GLP-1 receptors have been reported on 3T3 L adipocyte cells (277) as well as on solubilized membranes of rat epididymal adipose tissue (275), and GLP-1 was reported to stimulate the lipolysis in isolated rat adipocytes (276). Based on studies in rodents,
where GLP-1 may have direct peripheral actions on adipocytes (277), skeletal muscle (278), and hepatocytes (279), it was speculated that part of GLP-1’s glucose-lowering effect in humans could also be attributed to its direct effects on peripheral tissues (280,281). However recent studies involving hyperinsulinemic, euglycemic clamp techniques and endocrine clamps, using high doses of somatostatin to eliminate changes in secretion of pancreatic glucoregulatory hormones (282,283), indicate that the effects of GLP-1 on glucose disposal are due to its effects on pancreatic hormone secretion.

1.6.3 Extra-pancreatic Actions of GIP

GIP inhibits gastric acid secretion at supraphysiological infusion rates in man (284). GIP also has insulin like effects on other organs and tissues such as adipose tissue and the liver. Fat-induced release of GIP has no effect on insulin secretion in the absence of hyperglycemia, suggesting a role for this peptide in fat metabolism. Evidence exists of GIP, directly or in conjunction with insulin to increase fat storage in adipose tissue (285). GIP has insulin-like effects on the liver, reducing hepatic glucose production and inhibiting glucagon-stimulated glycogenolysis (286,287). These effects are more pronounced in the presence of insulin, although GIP does not affect hepatic insulin extraction. In spite of these actions, GIP receptor mRNA was not detected in liver using in situ hybridization (207).

1.7 Pancreatic Hormones

1.7.1 Insulin

In all species studied to date, the insulin gene is present as a single copy with the exception of the rat and mouse which have 2 non-allelic insulin genes (288). The insulin gene is regulated by a cAMP response element. The secretory vesicles containing proinsulin are formed in the trans Golgi network. Proinsulin is converted to insulin by prohormone convertases in the secretory vesicles. Mature Insulin is ~6 kDa and consists of a 21 amino acid A-chain and a 30 amino acid B-chain connected by two interchain disulfide bonds. C-peptide is cleaved off during processing and is secreted
along with the bioactive insulin. C-peptide has recently been shown to prevent vascular and neural dysfunction in diabetic rats (289).

The primary effects of insulin are to stimulate glucose transport into peripheral tissues such as muscle and adipose, and inhibit hepatic gluconeogenesis. In addition to its primary effects on glucose homeostasis, insulin also promotes a number of cellular events including regulation of ion and amino acid transport, lipid metabolism, glycogen synthesis, gene transcription and turnover, protein synthesis and degradation, and DNA synthesis (290). The insulin receptor is a member of the protein tyrosine kinase receptor family. It is a heterodimer composed of two α subunits and two β subunits. The β subunit has tyrosine kinase activity and a complex intracellular signaling pathway is initiated upon insulin binding and autophosphorylation of the β subunits.

### 1.7.2 Glucagon

Glucagon is encoded by the proglucagon gene and is released upon posttranslational processing of preproglucagon in pancreatic α cells (described earlier). Glucagon is a 29 amino acid peptide hormone secreted from pancreatic α cells. Glucagon plays a central role in the physiology of blood glucose regulation. It acts as a counter-regulatory hormone to insulin, to increase hepatic glucose production and decrease glucose utilization in non-hepatic tissues. In man, glucagon secretion from α cells is elevated and pulsatile under fasting conditions (291). Glucagon secretion is inhibited after a mixed meal.

The glucagon receptor has been cloned from rat and human liver and is confirmed to be a member of the superfamily of seven-transmembrane domain G protein-coupled receptors (292). The receptor is coupled to a stimulatory G protein. Binding of glucagon to its hepatic receptor is known to result in a number of effects, including the intracellular accumulation of cAMP and subsequent activation of protein kinase A, and the mobilization of intracellular Ca^{2+}. The mobilization of Ca^{2+} from intracellular stores occurs by signaling through inositol triphosphate/phospholipase C (292,293). Besides the liver, glucagon receptors have been found in some brain regions, the kidneys, heart, adipose tissue, pancreatic islets, stomach and intestine (294).
1.7.3 **Somatostatin**

Somatostatin (SST) is a neuropeptide originally isolated from the hypothalamus as a growth hormone inhibitory substance (295). It is found in vertebrates, invertebrates and plants and is widely distributed in many body tissues, acting as an inhibitory peptide. SST is typically found in neurons or endocrine-like cells throughout the central and peripheral nervous systems, in the endocrine pancreas, and in the gut (296). There are 2 naturally occurring bioactive SST products, SST-14 (14 amino acids long) and SST-28 (28 amino acids long) (297,298). In mammals these two peptides are encoded on a single gene and are derived from a common precursor which is differentially processed to generate tissue-specific products (299,300,301). SST-14 is the pancreatic form secreted from pancreatic δ cells.

The effects of SST are mediated through a family of G protein coupled receptors. Five human SST receptors (hSSTRs) have recently been cloned and are functionally coupled to inhibition of adenylyl cyclase via pertussis toxin sensitive GTP binding proteins (302). SSTRs are negatively coupled to adenylyl cyclase via a G\_i protein. Receptor activation leads to inhibition of adenylyl cyclase and a fall in intracellular cAMP levels (303). SSTRs have been shown to occur in varying densities in brain, gut, pituitary, endocrine, and exocrine pancreas, adrenals, thyroid, kidneys, and immune cells (304). Expression cloning of SSTRs has revealed the existence of separate SST-14 and SST-28 selective receptors (302). Their actions include inhibition of virtually every known endocrine and exocrine secretion, modulation of neuropeptide and neurotransmitter systems, behavioral and autonomic effects of centrally administered SST, and effects on gastrointestinal and biliary motility, vascular smooth muscle tone, and intestinal absorption of nutrients and ions (302). SST is also a physiological regulator of islet cells and gastrointestinal functions (302). SST suppresses glucagon and insulin secretion.

1.8 **The Incretins in Diabetes**

Studies of the incretin effect in normal, obese, and Type 2 diabetic subjects revealed that the incretin effect was reduced in diabetic patients compared to normal subjects (26). Although the incretin effect was significantly diminished in Type 2
diabetics, GIP secretion was normal (50). However, despite the normal GIP output, its
insulinotropic effect is reduced in Type 2 diabetics (108), leading to the speculation that
the defect lies in reduced sensitivity of the pancreatic β cell toward GIP (305). In
support of this, supraphysiological concentrations of GIP do not significantly stimulate
insulin secretion in Type 2 diabetics (114). However the possibility of a defect in
another incretin was not excluded by these studies. In contrast to GIP, supraphysiological concentrations of GLP-1(7-36) amide have been shown to overcome
decreased β cell responsiveness and stimulate insulin secretion in Type 2 diabetics
(114,306,307). In addition, it has been shown to be effective in patients with Type 2
diabetes in whom sulfonylurea treatment has failed (247). Although both GLP-1 and
GIP stimulate insulin secretion, GLP-1 but not GIP, inhibits gastric emptying and lowers
circulating glucagon in Type 2 diabetic patients (114,273). In addition to GLP-1’s
beneficial actions in Type 2 diabetic patients, GLP-1 has also proven beneficial in Type
1 diabetic patients. GLP-1 lowered postprandial blood glucose and meal-related insulin
requirements Type 1 diabetic patients in association with a reduction in circulating
glucagon and somatostatin (307). These effects are likely due in large part to a delay in
gastric emptying and inhibition of glucagon secretion (308). Inhibition of glucagon
secretion is the primary determinant of the reduction in fasting glycemia observed in
Type 1 diabetic patients infused with GLP-1 (309).

1.9 GLP-1 as a Therapeutic Agent

1.9.1 Problems with Native GLP-1

It was demonstrated that both subcutaneously and intravenously administered
GLP-1 are rapidly degraded by DP IV at the amino-terminus in patients with Type 2
diabetes and healthy subjects (310). The half-life of GLP-1 is approximately 1.5
minutes in vivo (122). GLP-1(9-36)amide has been shown to bind to the GLP-1
receptor and acts as a competitive antagonist of the GLP-1 receptor (311) in vivo,
although it binds with lower affinity. The relatively short half-life of GLP-1 limits the
achievement of good 24-hour metabolic control. In addition, to avoid degradation of
GLP-1 by digestive enzymes, it must be administered intravenously or subcutaneously,
making it a less practical drug. So clearly, more stable GLP-1 analogs are required if
GLP-1 is to be a successful treatment for Type 2 diabetes.

Subcutaneous injections of 0.15 nmol/kg body weight produced no side effects. However, once this dose was exceeded in normal volunteers, the majority of subjects had side effects including nausea, vomiting, chills, and thermal sensations (312). These side effects may be explained by the presence of GLP-1 receptors in the brain (267,268). It should be noted however that no side effects have been reported when GLP-1 is administered leading to plasma concentrations in the physiological range. GLP-1 receptors are found in the stomach and GLP-1 is known to inhibit gastric emptying. A near complete inhibition of gastric emptying was reported in patients with Type 2 diabetes who received intravenous GLP-1 (7-36) amide (plasma levels of ~70 pmoles/L) during the ingestion of a liquid meal (273).

1.9.2 Approaches to Solve the Problems

In summary, the main problems with the use of GLP-1 as a drug are, the limited duration of its action, the method of administration, and its side effects. The approaches taken to solve these problems are to use inhibitors of GLP-1-cleaving enzymes, to make structural modifications of the peptide (DP IV resistant analogs), to develop oral agonists or nonpeptidergic oral agonists, and to develop more advanced delivery systems (e.g. coating GLP-1 to decrease its intra-gut destruction). The use of inhibitors of DP IV as therapeutics does not seem feasible since DP IV is a ubiquitous enzyme that is involved in the metabolism of other proteins such as GLP-2. However a recent study by Pauly et al, with the DP IV inhibitor, led to improved glucose tolerance in rats (313). DP IV resistant analogs of GLP-1, which possess an amino acid substitution at position 8 of GLP-1, exhibit extended metabolic stability and improved biological activity in the isolated perfused porcine pancreas (314). Approaches for enhancing and simplifying GLP-1 delivery include the development of GLP-1-containing tablets for buccal adsorption. Preliminary studies have demonstrated transmucosal absorption of bioactive GLP-1 in fasting human subjects associated with increased levels of insulin and decreased glucagon and blood glucose (315), suggesting a promising alternative administration method for the treatment of Type 2 diabetes patients.
The development of more stable GLP-1 analogs or GLP-1 receptor agonists is also receiving much attention at present. Although there are many variations on this theme, ranging from peptide agonists to nonpeptidergic agonists, it is evident that complete knowledge of the structural determinants that confer GLP-1's activity is essential to further progression of the drug development process. An example can be taken from somatostatin. An analogue to somatostatin has been developed based on the amino acids considered to constitute the pharmacophore (24). Sandostatin, an octapeptide sharing a 4 amino acid homology with the natural compound has a plasma half-life of approximately 2 hours. It is commonly used in the treatment of symptomatic neuroendocrine tumors. Although somatostatin has a host of actions including inhibition of insulin secretion, and inhibition of pancreatic enzyme secretion due to the presence of somatostatin receptors in the pancreas, the anticipated side effects have been infrequent and transient in nature.

1.10 Thesis Aim

Both GIP and GLP-1 are potent physiological incretins and as such have received much attention since their discovery. In particular, GLP-1 is being pursued as a potential therapeutic agent for the treatment of Type 2 diabetes due to its antidiabetogenic actions in these patients. Many studies have been aimed at understanding the physiology and molecular biology of these peptide hormones. In keeping with these studies, the common goal of the studies undertaken here are to contribute to the design and development of pharmacological agents for use in treating Type 2 diabetes.

The aim of the first project was to characterize the relative contributions of GIP and GLP-1 to insulin biosynthesis in mice with a targeted disruption of the GLP-1 receptor gene (GLP-1R/- mice). Since both GIP and GLP-1 make up the El axis, the absence of GLP-1 signaling in these mice will allow the study of the GIP component of the El axis. Although previous studies have demonstrated the contributions of GIP and GLP-1 by using GIP antisera and GLP-1 antagonist, exendin(9-39), the results are still in question due to the limitations of the antisera and the interaction of exendin(9-39) with the GIP receptor.
The characterization of the functional determinants of the GLP-1 molecule is a prerequisite for the design of more stable and effective GLP-1. Thus the aim of the second project was to characterize the critical functional determinants of GLP-1 by comparing structure/function information from three GLP-1-like peptides from the clawed frog, *Xenopus laevis*.

The aim of the third project was to localize activation regions in a GIP/GLP-1 chimeric receptor. Based on the premise that the GLP-1 and GIP receptors share approximately 45% sequence identity and have a common intracellular signaling pathway GIP/GLP-1 receptor chimeras were generated in an attempt to localize functional domains of the receptors. Two chimeric receptors, named CH-2 and CH-3 differ by only 19 amino acids at the membrane proximal N terminal domain but demonstrate different GIP-induced activation. Examination of these receptors may reveal residues critical for the activation of the GIP receptor. An understanding of the activation mechanisms of these receptors will complement studies on the hormone structures and aid in the development of agonists.

**1.10.1 Specific Objectives**

(1) To characterize the effects of loss of GLP-1 function on pancreatic hormones and to understand the relative physiological roles of GLP-1 and GIP on the pancreas of mice with a targeted disruption of the GLP-1 receptor gene.

(2) To characterize the biological activities (binding to, and activation of human GLP-1 receptor) of novel GLP-1-like peptides encoded by the *Xenopus* proglucagon gene in an attempt to further the knowledge of the residues important for biological function of GLP-1 by correlating structure/function data.

(3) To gain further knowledge of activation mechanism of the GIP receptor using site-directed mutagenesis studies of a GIP/GLP-1 chimeric receptor.
CHAPTER 2 CHARACTERIZATION OF THE ENTERO-INSULAR AXIS IN GLP-1R-/− MICE

2.1 Introduction

The relative importance and physiological potency of GIP and GLP-1 as candidate incretins remains controversial. Acute antagonism of GLP-1 action in rodents has been shown to diminish the incretin effect and increase blood glucose, results consistent with the concept that GLP-1 is a major component of the enteroinsular axis (52,316). In contrast, blockade of the GIP receptor in rats has been associated with a significant reduction in glucose-stimulated insulin secretion, suggesting that GIP may be the dominant incretin in vivo (317). Additional complications arise due to the limitations of studies involving the GLP-1 receptor antagonist, exendin(9-39) and GIP antisera. To better understand the relative physiological importance of GLP-1 for long-term glucose control and food intake, mice with a targeted disruption in the GLP-1 receptor (GLP-1R) gene were generated by Drs L.A. Scrocchi and D.J. Drucker (270).

2.1.1 GLP-1R-/− Mice

GLP-1R-/− mice were generated by using a homologous recombination approach (270). In brief, a mouse GLP-1 receptor gene fragment was cloned by hybridization to rat GLP-1 R cDNA from a genomic mouse ES cell 129 I Dash library. A targeting vector was constructed by replacing exons coding for the sequence from transmembrane 1 to 3 of the receptor with a PGK-neo cassette in the same transcription orientation along with 4.8 and 3.5 kb of GLP-1R sequences 5’ and 3’ to the PGK-neo cassette, respectively. The linearized construct was electroporated into ES cell line R1. Positive and negative selection was used to enrich for homologous recombination. Clones were analyzed by Southern blot and PCR for recombination events. Positive clones were aggregated with CD1 morulae to generate germ line chimeras. GLP-1R+/− mice from separate litters were mated to obtain mice homozygous for the GLP-1R mutation. The disruption of the GLP-1 receptor gene is presumed to affect the proper processing and
trafficking of the receptor to the cell surface as well as functional activity, although mRNA transcripts of the disrupted gene have been found (318).

The homozygous deletion was not lethal and the mice developed with no visible defective phenotype. Although GLP-1 is postulated to be a satiety factor, GLP-1R-/- mice gained weight normally (270), suggesting that GLP-1 signaling is not required for central control of satiety. GLP-1R-/- mice exhibit mild fasting hyperglycemia, and exhibit increased levels of blood glucose with diminished levels of circulating insulin after an oral glucose challenge (270), indicating a physiological role for GLP-1 in glycemic control, and in particular, a role for GLP-1 signaling in the regulation of glucose-stimulated insulin secretion. Since both GLP-1 and GIP comprise the enteroinsular axis, mice with a targeted disruption of one of the components of the enteroinsular axis provide a unique opportunity to further our knowledge of the axis.

The relative biological importance of GLP-1 and GIP still remains to be clarified. These mice were found to be moderately glucose intolerant but not severely diabetic (270). This may be attributed to compensation by other insulin regulatory systems. In particular, the second component of the enteroinsular axis, GIP, may be involved.

2.1.2 GIP Component in GLP-1R-/- Mice

The modest glucose intolerance (Figure 7a) of the GLP-1 R-/- mice coupled with the presence of functional GIP receptors on the β cells suggested that loss of GLP-1 action may be associated with compensatory changes in β cell response to the second incretin GIP. Therefore circulating GIP in response to oral glucose, as well as the insulin response to GIP from isolated perfused pancreas were measured by Dr. R.A. Pederson (319). Serum GIP levels in GLP-1R-/- mice were significantly elevated 30 minutes following an oral glucose load (369 ± 40 pM vs 236 ± 28 pM, GLP-1R-/- vs +/- mice respectively, p < 0.05, Figure 7b). The observation that glucose induced GIP secretion is enhanced in GLP-1R-/- mice suggests that the activity of the intestinal GIP-secreting K cell is upregulated after disruption of GLP-1 action; however, the mechanism underlying increased K cell secretion remains unknown. The specific intestinal cell types that express GLP-1 receptors have not yet been identified. Accordingly, whether GLP-1 acts directly on the intestinal K cell to regulate GIP secretion remains unknown. Alternatively, enhanced GIP release in GLP-1R-/- mice
Figure 7: Glucose tolerance in GLP-1R-/ and CD1 control mice. 

a) Plasma glucose levels following an oral glucose load in GLP-1R-/ (hatched bars) and control CD1 (solid bars) mice, 8-12 weeks of age. Values are expressed as mean ± SEM of n = 21 measurements for each time point per group. * = p < 0.05 vs controls.

b) Plasma GIP levels following an oral glucose load in the same groups of mice studied in a). n = 20 at t = 0 and 30 min, n = 5-6 at t = 60 min, * = p < 0.05 vs controls.

might be secondary to the reduced insulin response that follows enteric glucose challenge. It was previously demonstrated that insulin reduces glucose-dependent GIP secretion in the rat (320). Furthermore, the results of several additional studies in rodents and humans have raised the possibility that GIP secretion may be regulated in part by insulin (321,322). Accordingly, the enhanced GIP secretion seen in GLP-1R-/- mice may have resulted in part from defective feedback inhibition of insulin-regulated GIP release in the GLP-1R-/- mice. A complementary explanation for enhanced GIP secretion derives from observations of GLP-1-mediated regulation of gastric emptying (272). The relative magnitude of GIP, but not GLP-1, release appears to be greater after intraduodenal glucose perfusion compared with oral glucose loading in normal human subjects (323). It thus remains theoretically possible that increased transit of and more rapid exposure to glucose in the duodenum and proximal jejunum of GLP-1R-/- mice might augment the amplitude of the K cell secretory response, leading to increased GIP secretion in vivo.

Upon stimulation of the pancreas with GIP in the presence of elevated glucose, an enhanced insulin secretory response was observed in the GLP-1R-/- mice compared to control mice (Figure 8), while pancreatic perfusion of GLP-1 confirmed the complete functional loss of GLP-1 receptors, as no insulin response was seen in GLP-1R-/- mice (Figure 9). Because both the GLP-1 and GIP receptors are coupled to cAMP-dependent signaling pathways in the β cell (235), disruption of GLP-1 receptor signaling may be associated with a new enhanced threshold for the glucose-dependent response to GIP receptor activation. The specific mechanisms that might account for enhanced β cell GIP sensitivity remain unknown, but may include increased GIP receptor expression and/or signaling. Taken together, these results indicate a compensatory mechanism in the GLP-1R-/- mice whereby upregulation of the GIP component of the EI axis occurs due to the loss of GLP-1 signaling and action (319). However these findings do not explain the persistence of mild glucose intolerance coupled with diminished levels of circulating insulin following a glucose load.

These observations may be explained by investigating the relative contributions GLP-1 and GIP to pancreatic insulin levels since both peptides are known to stimulate insulin biosynthesis. In addition, since both GLP-1 and GIP stimulate insulin gene transcription, it is of interest to determine how insulin mRNA levels are affected in GLP-
Figure 8: Insulin secretion from the isolated perfused pancreas in response to GIP in the presence of high glucose. Values are expressed as mean ± SEM of n = 6 mice for each group, 8-10 weeks of age. The mean integrated insulin response is shown in the inset. ** = p ≤ 0.005 vs control mice. From: Pederson RA, Satkunarajah M, McIntosh CHS, Scrocchi LA, Flamez D, Schuit F, Drucker DJ, and Wheeler MB: Enhanced glucose-dependent insulinotropic polypeptide secretion and insulinotropic action in glucagon-like peptide-1 receptor -/- mice. Diabetes 47: 1046-1052, 1998.
Perfusion of the Isolated Perfused Pancreas with GLP-1

16.6 mM Glucose
1 nM GLP-1

Figure 9: Insulin secretion from the isolated perfused pancreas in response to GLP-1 in the presence of high glucose. Values are expressed as mean ± SEM of n = 4 for each group. The mean integrated response is indicated in the inset. * = p < 0.05 vs control mice. From: Pederson RA, Satkunarajah M, McIntosh CHS, Scrocchi LA, Flamez D, Schuit F, Drucker DJ, and Wheeler MB: Enhanced glucose-dependent insulinotropic polypeptide secretion and insulinotropic action in glucagon-like peptide-1 receptor -/- mice. Diabetes 47: 1046-1052, 1998.
1R/- mice. The glucose-lowering action of GLP-1, particularly in diabetic patients, has been attributed to the glucagon-suppressing action of this peptide (46,249). It has been speculated that this action may be indirect via somatostatin secretion (324). These findings provide the rationale for comparing pancreatic glucagon and somatostatin content of GLP-1R/- and control mice.

2.2 Hypothesis

GLP-1 has been shown to be the major contributor to the EI axis, yet GLP-1R/- mice exhibit only mild glucose intolerance due to upregulation of the GIP component of the axis. We hypothesized that abnormalities in the pancreatic islet hormone levels may explain the persistence of mild diabetes in these mice.

2.3 Specific Aims

2.3.1 Measurement of the levels of pancreatic islet hormones: insulin, glucagon, and somatostatin in control and GLP-1R/- mice to determine the relative contributions of GLP-1 and GIP to the levels of these hormones.

2.3.2 Characterization of pancreatic insulin mRNA levels in control and GLP-1R/- mice to determine the relative contributions of GLP-1 and GIP.

2.4 Materials and Methods

2.4.1 Materials

All reagents and chemicals were from Sigma Chemical Co. (St. Louis, MO) or Gibco BRL (Life Technologies, Burlington, ON). Agarose was from Bioshop (Burlington, ON). [α-32P] dCTP was obtained from Mandel (NEN, Guelph, ON). Random Primers Labeling kit was from Gibco BRL. Insulin RIA kit specific to rat insulin was from Linco (St. Charles, MO). Glucagon RIA kit was from Inter Medico (Markham, ON). Protein determination reagent and ethidium bromide were from Bio-Rad (Hercules, CA). Bovine serum albumin for protein standards (10 μg/μL 100X purified)
was from New England Biolabs (Mississauga, ON). TE Midi SELECT® -D, G-50 columns for oligonucleotide purification were from 5 Prime 3 Prime (Boulder, CA). Autoradiography film (XAR 5) was from KODAK (Rochester, NY).

2.4.2 Animals

Mice were handled in accordance with ethical guidelines set forth by the Division of Comparative Medicine, University of Toronto. Male control CD1 mice used in this study were obtained from Charles River Canada Ltd. (St. Constant, QC). CD1 +/- mice were between 5 to 16 weeks of age and matched to male GLP-1R-/- mice. The GLP-1R-/- mice homozygous for a null mutation in the GLP-1 receptor (270) were obtained from Dr. D.J. Drucker's lab at the University of Toronto. The colony was expanded and maintained at the University of British Columbia (Vancouver, BC) in accordance with ethical guidelines set forth by the University of British Columbia Animal Care Committee and then shipped back to the University of Toronto. All mice used in experiments described below had access to standard rodent chow and water ad libitum. Before surgical procedure, mice were anesthetized with 6.5 mg/Kg Somnotol (MTC Pharmaceuticals, Cambridge, ON) administered intraperitoneally.

2.4.3 Solutions

10X MOPs buffer (3-[N-Morpholino]propanesulfonic acid, containing 0.4 M MOPs pH 7.0, 0.1 M Na Acetate, 10 mM EDTA), 20X SSC solution (sodium chloride—sodium citrate, 0.6 M NaCl and 0.06 M Na citrate), and 50X Denhardt’s (0.01g/mL each of polyvinylpyrrolidine, BSA, and Ficoll) and all other solutions were made according to the instructions in Molecular cloning: A laboratory manual, by Sambrook et al (332).

2.4.4 Protein Extraction

The pancreas was carefully dissected from its attachments to the spleen and intestine and homogenized in 3mL of iced 2M acetic acid with a Polytron® Homogenizer (Brinkmann Instruments Inc., Toronto, ON) (325). The homogenate was boiled at 100 °C for approximately 10 minutes (with occasional mixing). The samples were placed on ice for 10-15 minutes and then centrifuged at 21000 rpm for 30 minutes at 4 °C (Beckman J2-MI centrifuge). The supernatant was removed and the
centrifugation repeated. The supernatants were pooled and aliquoted into 400 µL quantities in microfuge tubes and stored at –80 °C until RIAs were performed.

### 2.4.5 Radioimmunoassays

Insulin RIAs were performed with a kit from Linco according to the manufacturer's instructions. This kit is based on the RIA developed by Morgan et al (326). The insulin antibody is specific to rat insulin and rat insulin was used as standards. The insulin antibody shows 100 % cross-reactivity with mouse insulin.

Glucagon was measured by a RIA kit according to the manufacturer's instructions. The double antibody glucagon procedure is based on methods outlined by Nishino et al (327,328). The sensitivity of the assay is 13 pg/mL, and the antisera shows 0 % crossreactivity with GLP-1, GLP-2, and GIP, and 0.1 % crossreactivity with oxyntomodulin. Glucagon RIAs were carried out by the Banting and Best Core Labs (University of Toronto).

RIA for total somatostatin-like immunoreactivity (SLI) was performed with synthetic somatostatin-14 as a standard, using an antiserum that recognizes somatostatin-14 (pancreatic form) and somatostatin-28 equally (329). Somatostatin RIAs were carried out by Ms. Connie Chisholm in Dr. Greenberg's lab (University of Toronto).

### 2.4.6 Protein Assay

The values for insulin, glucagon and somatostatin immunoreactivity were normalized to total protein content in pancreatic extracts. The protein assay was carried out with a dye reagent from Bio-Rad according to the manufacturer's specifications. The Bio-Rad protein assay reagent is based on the Bradford method of protein quantification (330). Bovine serum albumin was made up to a range of concentrations (140, 120, 110, 100, 90, 80, 75, 60, 50, and 40 µg/µL) to produce a standard curve. Both standards and samples were allowed to react with the dye and protein content was quantified by measuring the optical density at a wavelength of 595 nm.
2.4.7 RNA Extraction

Total cellular RNA was isolated by the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction (331). The pancreas was carefully dissected from its attachments to the spleen and intestine, quickly immersed in 8 mL of guanidinium thiocyanate solution and immediately homogenized with a Polytron® Homogenizer. To this homogenate, 800 μL of 2M sodium acetate pH 5.2, 8 mL water saturated phenol, and 1.6 mL chloroform:isoamyl alcohol (24:1) were added. The homogenate was shaken vigorously by hand, aliquoted into two round-bottom tubes and incubated on ice for 15-30 minutes. The samples were then centrifuged at 8000 rpm for 30 minutes 4 °C (Beckman J2-MI centrifuge). The upper aqueous layer (containing RNA) was transferred to a new tube. 100 μL of 1M acetic acid and 2 mL of 95 % ethanol were added. The sample was mixed by inverting and placed at ~20 °C for ~1 hour to allow the RNA to precipitate. The samples were then centrifuged at 8000 rpm for 30 minutes at 4 °C to pellet the precipitated RNA. The supernatant was poured out leaving a pellet. The pellet was washed twice in 5 mL 75 % ethanol. The RNA pellet was allowed to air dry (to evaporate all the ethanol) and resuspended in ~1 mL DEPC-treated water. The absorbance at 260 nm (UV range, DU-64 spectrophotometer, Beckman Instruments Inc, Fullerton, CA) was measured to quantitate the RNA. The RNA was aliquoted into 100 μL amounts in microfuge tubes and kept at ~80 °C. The integrity of the isolated RNA was verified by running ~1 μg of RNA on a 1.2 % agarose gel stained with ethidium bromide.

2.4.8 Northern Blot Analysis

Northern blotting was performed on total pancreatic RNA as previously described (118).

**Electrophoresis:** RNA samples were run on a denaturing 1.2 % agarose-formaldehyde gel containing ethidium bromide, 1X MOPS buffer, and 6 % formaldehyde. 25 μg of total RNA from each animal was dried in a speed vac and reconstituted in 18 μL of sample loading buffer. The loading buffer was made up on the day of the experiment and contained 6% formaldehyde, 50 % formamide, 1X MOPS buffer, 10 % glycerol, and bromophenol blue for colour. Before loading the RNA was denatured by heating at 65 °C for 5-8 minutes and mixed with a pipet tip. The gel was
run at 100 volts for the first 10 minutes and then at 120 volts for the next 90 minutes on a standard electrophoresis apparatus (Fisher Brand). The running buffer (1X MOPS buffer) was circulated after the first 10 minutes.

Transfer to Nylon Membrane: The transfer apparatus was assembled as standard (332) and the RNA was transferred to neutral nylon membrane (Amersham) overnight in 20X SSC by capillary transfer. The following day the transfer apparatus was disassembled and the membrane was washed in 1X SSC. The membrane was allowed to air dry on top of a Whatman blotting paper for about 30 minutes. It was then placed in-between cellophane and the RNA was cross-linked by exposure to UV light (Fisher Scientific) (2 minutes exposure per side).

Preparation of $[^{32}P]$ Labeled Probes: The insulin probe was made from mouse insulin cDNA corresponding to the coding sequence (~350 bp, synthesized by PCR using primers directed to the mouse insulin sequence). The probe for 18S rRNA was made from 18S cDNA (18 S cDNA subcloned into pBR322 vector (Pharmacia Biotech, Baie d'Urfé, QC) was kindly provided by Dr. D.J. Drucker) (118). The probes were labeled by the instructions provided in the Random labeling kit. The cDNA was boiled at 100 °C for 5 minutes to denature the DNA. The DNA was then placed in a 37 °C water bath for 30 seconds and spun down for 15 seconds to bring down all the condensation. Label mix which contained 2 μL dATP, 2 μL dGTP, 2 μL dTTP, 15 μL of random primers mix, 5 μL of $[^{32}P]$ dCTP and 1 μL of Klenow fragment (enzyme) was added to the DNA. The contents were mixed with a pipet tip and the reaction was left to proceed for 1 hour at room temperature. After an hour, 100 μL of water was added to the reaction and the probe was purified with a G-50 nucleotide purification column to remove unincorporated dNTPs and $[^{32}P]$ dCTP. The radioactivity of the purified probes were measured by counting 2 μL of the probe in 5 mL of scintillation cocktail ($\beta$-scintillation counter, Canberra Packard, Mississauga, ON).

Prehybridization and Hybridization: The hybridization solution contained 5X SSC, 5X Denhardt's solution, 50 % formamide, and 0.5 % SDS. Prehybridization was carried out with 12 mL hybridization solution supplemented with 100 μg of denatured salmon sperm (denatured by boiling at 100 °C for 5 minutes) for ~ 2 hours at 42 °C. Hybridization was carried out with 12 mL of hybridization solution containing 100 μg of denatured salmon sperm and either denatured insulin or 18S probe (12 X 10$^6$ cpm)
overnight at 35 °C. After hybridization with insulin probe, the blots were washed thoroughly and re-hybridized with 18S probe.

**Wash:** After overnight hybridization, blots were washed in 1X SSC, 0.1 % SDS at 60 °C for 30 minutes. After the wash, the membrane was placed in between cellophane and mounted on cardboard.

**Autoradiography:** KODAK XAR 5 mat film was used for autoradiography. The blots were placed in a cassette with film and intensifying screen and kept at −80 °C. After an appropriate amount of exposure time (keeping the exposure to the linear portion of the film) the film was developed using an automatic developer (KODAK, Rochester, NY).

### 2.4.9 Densitometry

The film was scanned into a computer using Desk Scan 2 program and a constant size area was used to convert the intensity of the bands to pixels using NIH Image Quant program (118). The density of the bands in the 18S probed blots and the insulin probed blots were compared. For each sample, the insulin density was expressed as a percentage of the 18S density (which was considered as 100 % for each sample).

### 2.4.10 Statistical Analysis

The Student’s unpaired t-test was used to compare results obtained from control versus GLP-1R-/- mice (InStat software, San Diego, CA). A $P$ value $\leq 0.05$ was considered significant.

### 2.5 Results

#### 2.5.1 Pancreatic Insulin Content

The same protein extracts (from individual animals) were used to determine levels of all pancreatic hormones, which were expressed relative to total pancreatic protein levels in order to normalize for differences in pancreatic mass between animals. GLP-1R -/- mice have been shown to have a decreased insulin secretory response after a glucose challenge, suggesting that pancreatic insulin content may be lower in these
mice compared to controls. In addition, GLP-1 is known to have a stimulatory effect on insulin biosynthesis as does GIP, although the relative contributions of each are unknown. Therefore pancreatic insulin content was measured in GLP-1R-/− mice. Pancreatic insulin levels expressed relative to total protein were 77.4 ± 9.1 pmole/mg protein in GLP-1R-/− mice versus 121.3 ± 10.3 pmole/mg protein in +/+ CD1 controls, $p < 0.005$ (Figure 10). Thus GLP-1R-/− mice exhibited a 36 % decrease in pancreatic insulin content compared to CD1 controls. This result indicates that despite increased circulating GIP levels and increased GIP sensitivity at the β cell, GIP is not able to completely compensate for the loss of GLP-1 action in GLP-1R-/− mice.

2.5.2 Pancreatic Insulin mRNA Levels

To determine whether the decreased insulin content of GLP-1R-/− mice may be explained by diminished insulin mRNA levels, Northern blotting followed by densitometric analysis was performed on total pancreatic RNA (Figure 11). In keeping with insulin protein levels, insulin mRNA levels were also decreased in GLP-1R-/− mice compared to controls with GLP-1R-/− mice exhibiting a 34 % reduction in insulin transcript levels compared to CD1 controls. Thus, although both GLP-1 and GIP are known to stimulate insulin gene transcription, the loss of GLP-1 signaling is not compensated for, by GIP. Therefore the insulin mRNA levels may explain in part, the diminished pancreatic insulin protein levels.

2.5.3 Pancreatic Glucagon Content

GLP-1 has also been shown to inhibit glucagon biosynthesis and secretion through direct and indirect mechanisms (e.g. via somatostatin) (249,333). Pancreatic glucagon levels expressed relative to total protein were 2.8 ± 0.2 pmole/mg protein in GLP-1R-/− mice versus 2.6 ± 0.2 pmole/mg protein in +/+ CD1 controls, $p = 0.4$, (Figure 12), demonstrating no appreciable differences between the two groups of mice.

2.5.4 Pancreatic Somatostatin Content

GLP-1 is a potent stimulator of somatostatin release (251,254), however the effects on its biosynthesis are unknown. In GLP-1R-/− mice, somatostatin content was approximately 20 % higher compared to +/+ mice (Figure 13). Pancreatic somatostatin
Pancreatic Insulin Content

Figure 10: Pancreatic insulin content expressed relative to total pancreatic protein. Values are expressed as mean ± SEM of n = 36 mice for each group, 12-14 weeks of age. ** = p < 0.005 vs CD1 +/+ mice.
Figure 11: a) Northern blot analysis of pancreatic RNA from GLP-1R-/- mice and CD1 mice for insulin transcript. 18S serves as loading control. Each lane represents RNA isolated from a single animal. b) Densitometric analysis of the northern blot shown in a), n = 12 for each group, **p < 0.002 vs CD1 +/+ mice.
Pancreatic Glucagon Content

Figure 12: Pancreatic glucagon content is expressed relative to total pancreatic protein. Values are expressed as mean ± SEM of n = 36 mice for each group, 12-14 weeks of age. Total pancreatic glucagon was not significantly different between the two groups, $p = 0.4$ vs CD1 +/+ mice.
Pancreatic Somatostatin Content

Figure 13: Pancreatic somatostatin content is expressed relative to total pancreatic protein. Values are expressed as mean ± SEM of n = 36 mice for each group, 12-14 weeks of age. *** = p < 0.001 vs CD1 +/+ mice.
levels expressed relative to total protein were 6.1 ± 0.3 pmole/mg protein in GLP-1R-/ mice versus 4.9 ± 0.2 pmole/mg protein in +/+ CD1 controls, p < 0.001.

2.6 Discussion

The predominant actions ascribed to GLP-1 have been glucose-dependent insulin release and insulin-independent glucose lowering actions (239,240,262). The latter include suppression of glucagon secretion (248,249), inhibition of gastric emptying (273) and insulin-like actions on peripheral glucose uptake (280,281). Other studies attribute the effects of GLP-1 on glucose disposal, as exclusively the result of the effect of this peptide on the glucoregulatory hormones insulin and glucagon (282). In keeping with these actions of GLP-1, the sites of GLP-1 receptor expression include: the pancreatic islets, lung, brain, intestine, liver, kidney, adipose tissue, skeletal muscle, stomach and heart (193,195,196,197,199,277). The observation that GLP-1R-/ mice exhibit glucose intolerance following an oral glucose challenge with diminished levels of circulating insulin, clearly demonstrates that GLP-1 is essential for blood glucose control in vivo (270).

Although the GLP-1R-/ mice do not express a functional pancreatic GLP-1 receptor, they do express a functional GIP receptor (Figures 8 and 9). In view of the fact that knockout mouse models often show less dramatic phenotypes or no phenotype at all due to compensation by a redundant pathway, it was of interest to investigate the plasticity in the EL axis in these mice. Both GIP secretion and insulinotropic action of GIP were found to be enhanced in GLP-1R-/ mice (319), changes that likely compensate in part, for the absence of GLP-1 action at the β cell (319). This upregulation attests to the importance of GLP-1 to glucose homeostasis in normal physiology. In spite of the upregulation of the GIP component of the enteroinsular axis which partially compensates for the lack of insulinotropic GLP-1 action (319), the mice are moderately glucose intolerant. The compensation by GIP is not sufficient to restore glucose-stimulated insulin secretion to normal, further emphasizing the importance of GLP-1 as a physiologically essential incretin.

To better understand the contributing factors to the glucose intolerance in GLP-1R-/ mice, the levels of the pancreatic hormones were investigated, since both GLP-1
and GIP stimulate insulin biosynthesis and insulin gene transcription (218,246). In addition, GLP-1 is known to inhibit glucagon secretion and stimulate somatostatin secretion. However, the effects of GLP-1 and GIP on the pancreatic hormones are further complicated by intra-islet paracrine interactions. A schematic of these interactions is shown in Figure 14. All studies were carried out with male mice, since male GLP-1R/- mice exhibit enhanced glucose intolerance compared to female mice (270). Insulin hormone content in pancreatic cells was found to be significantly lower in GLP-1R/- mice compared to +/- mice (Figure 10). If both GLP-1 and GIP are equipotent at stimulating insulin biosynthesis, insulin levels would be expected to be the same for GLP-1R/- and CD1 +/- mice. However in spite of the increased GIP levels and increased insulinotropic action of GIP, loss of GLP-1 action at the pancreatic P cells of GLP-1R/- mice results in diminished insulin levels. The study by Flamez et al, however, demonstrated no difference in pancreatic insulin content between the two groups of mice (318). The only differences between the present study and the study by Flamez et al, are: i) the age of the mice, 5-16 weeks in the former and 10 weeks old, respectively, and ii) normalization of insulin levels to total pancreatic protein or wet weight of pancreas, respectively. The age may explain in part, the difference in pancreatic insulin content between the two studies, since older mice may exhibit more pronounced changes in glucose metabolism.

As GLP-1 stimulates proinsulin gene expression, likely via a direct effect on insulin gene transcription (218,233), we hypothesized that disruption of P cell GLP-1 signaling may be associated with a reduction in the levels of pancreatic proinsulin gene expression. However GIP is also known to stimulate proinsulin gene transcription. In addition to increasing insulin gene transcription, GLP-1 has also been shown to inhibit insulin mRNA degradation in a insulin-secreting cell line, RIN 1046-38 (256). The reduced levels of postprandial proinsulin mRNA transcripts and insulin content in fed GLP-1R/- mice is in keeping with a role for GLP-1 in the regulation of proinsulin biosynthesis in vivo. Consistent with these findings, administration of GLP-1 to Wistar rats for 48 hours reversed the age-related deterioration in glucose tolerance and increased both islet insulin content and pancreatic proinsulin mRNA (334). However, recent studies by Scrocchi et al, with GLP-1R -/- mice presented contrasting results in which no differences in pancreatic insulin mRNA content were observed between the
Interactions between Incretins and Pancreatic Hormones

Hormone Secretion

Figure 14: Interactions between GLP-1, GIP and the pancreatic hormones. Effects on hormone secretion and gene regulation are shown. Effects on insulin by the GLP-1 and GIP are only in the presence of elevated glucose. ‘+’ indicates stimulatory effect and ‘-’ indicates inhibitory effect. (→) indicates direct effects, while (--) indicates indirect effects. ? indicates unclear effects.
two groups (335). However an important difference between the two studies is that Scrocchi et al, performed studies on fasted mice, whereas in the present study fed mice were used. GLP-1 is predominantly considered important for control of blood glucose in the postabsorptive state. However GLP-1 infusion has been shown to lower fasting plasma glucose in healthy normal subjects (312) and patients with Type 2 diabetes (247,336), and this reduction in fasting glucose is generally attributable to improved cell function. Clearly more studies should be undertaken to explain the contrasting results seen in insulin levels in GLP-1R/- mice.

GLP-1 has been shown to inhibit glucagon secretion both via a direct effect and an indirect effect. GLP-1's effect may be via other pancreatic hormones. There are GLP-1 receptors on somatostatin secreting δ cells and somatostatin has an inhibitory effect on glucagon secretion. Therefore the inhibition of glucagon secretion by GLP-1 may be mediated via somatostatin. No significant differences were detected in the pancreatic tissue content of glucagon between GLP-1R/- and CD1 +/- mice. Consistent with the present findings, Scrocchi et al, detected no significant changes in the tissue content of pancreatic glucagon mRNA and intestinal proglucagon mRNA or glucagon-like immunoreactivity in GLP-1R/- mice or +/- mice (335). Furthermore, the similar pancreatic glucagon levels between the two groups of animals is consistent with, and may explain the result seen in a previous study where no significant differences were detected in the levels of circulating plasma glucagon levels, both before and after an oral glucose tolerance test (OGTT) (270). GLP-1 stimulates the secretion of somatostatin. At present it is not known whether GLP-1 signaling affects the gene transcription or biosynthesis of somatostatin. However, pancreatic somatostatin levels were significantly higher in GLP-1R/- mice. The increased somatostatin levels, if associated with increased secretion, may contribute to the diminished insulin mRNA levels (Figure 14).

In conclusion, these results define a definitive role for GLP-1 in the regulation of insulin gene transcription and biosynthesis in vivo. The effects on glucagon and somatostatin biosynthesis by GIP and GLP-1 await further study. Future studies aimed at elucidating the effects on gene transcription and mRNA stability of glucagon and somatostatin by the incretin hormones will help to explain the protein levels seen for these hormones.
CHAPTER 3  BIOLOGICAL ACTIVITIES OF XENOPUS
GLP-1-LIKE PEPTIDES

3.1 Introduction

The potent insulinotropic effects of GLP-1, coupled with its ability to stimulate
insulin biosynthesis, has propelled it to the forefront of diabetes research as a
candidate therapeutic agent for Type 2 diabetes. However its limited action due to its
susceptibility to DP IV cleavage has prompted a search for more stable and effective
GLP-1 analogs. Naturally these studies have included the characterization of the
structural determinants of GLP-1 that confer its biological activity. Information regarding
the specific amino acids that contribute to its interaction with the GLP-1 receptor and
the subsequent activation of the receptor will be useful in the design of potent analogs.
Studies involving alanine scans of the GLP-1 molecule have provided insight into critical
amino acids. However these studies do not provide information as to amino acids
substitutions that may result in more effective GLP-1 molecules. The Xenopus
proglucagon gene which was found to encode three novel GLP-1-like peptides presents
a unique opportunity to study evolutionarily distant GLP-1 molecules that may possess
similar or better biological activity to that of mammalian GLP-1.

3.1.1 Xenopus Proglucagon

The Xenopus proglucagon gene was cloned by Dr. D.M. Irwin, by PCR
amplification of pancreatic and intestinal cDNA with degenerate primers directed
towards the conserved N-terminal sequences of vertebrate glucagon, GLP-1, and GLP-
2. The glucagon primer was the sense primer while those to GLP-1 and GLP-2 were
antisense. Xenopus pancreatic cDNA library was also screened. Two distinct but very
similar proglucagon cDNA sequences were found (Figure 15). This was expected
since Xenopus laevis is a tetraploid species. The Xenopus proglucagon gene I was
found to encode three GLP-1-like peptides in addition to the predicted glucagon and
GLP-2 sequences (Figure 15) (69). This was surprising since other mammalian
species possess only one GLP-1 sequence (Figure 16). The Xenopus proglucagon II
sequence does not contain the GLP-2 sequence. However, variants of Xenopus
**Predicted Amino Acid Sequence of Xenopus Proglucagon**

| XPGI | ATGCGCTGAAAGGAAGAGGAGCATTATCTATCGGAGGATTTGAGAAGAGCAAGAAG | 60 |
| XPGI | GCCAAGGAGGATCCCAATTTGCTGAAAGGAGGAGCATTATCTATCGGAGGATTTGAGAAGAGCAAGAAG | 120 |
| XPGII | CTTATCTATCGGAGGATTTGAGAAGAGCAAGAAG | 180 |
| XPGII | GLP-1C |
| XPGII | GLP-2 |
| XPGII | GLP-2 |
| XPGII | GLP-2 |
| XPGII | STOP |

**Figure 15:** The nucleotide sequence of *Xenopus* proglucagon cDNAs I and II are shown, with the predicted amino acid sequence of *Xenopus* proglucagon I and II (xPGI and xPGII) shown above the cDNA sequences. The DNA sequence is numbered from the 5' end of the longest cDNA, whereas the amino acid sequence is numbered from the predicted N-terminus of proglucagon. Predicted glucagon and full-length glucagon-like peptides are underlined. Potential proteolytic processing sites that yield C-terminally truncated GLP-1-like peptides are indicated by an arrow with a question mark. Dashes are gaps introduced to yield maximal alignment. *Xenopus* proglucagon II cDNAs do not encode GLP-2, and some proglucagon I cDNAs had a homologous deletion, removing the GLP-2 sequence, due to alternative splicing. From: Irwin DM, Satkunarajah M, Wen Y, Brubaker PL, Pederson RA, Wheeler MB: The *Xenopus* proglucagon gene encodes novel GLP-1-like peptides with insulinootropic properties. *Proc Natl Acad Sci USA* 97: 7915-7920, 1997.
Comparison of Human and Xenopus Proglucagon Gene Products

Human Proglucagon

<table>
<thead>
<tr>
<th>Signal</th>
<th>GRPP</th>
<th>Glu</th>
<th>IP-1</th>
<th>GLP-1</th>
<th>IP-2</th>
<th>GLP-2</th>
</tr>
</thead>
</table>

Xenopus Proglucagon

<table>
<thead>
<tr>
<th>Signal</th>
<th>GRPP</th>
<th>Glu</th>
<th>GLP-1A</th>
<th>GLP-1B</th>
<th>GLP-1C</th>
<th>GLP-2</th>
</tr>
</thead>
</table>

Cleavage at single basic residues
- K109
- K151
- R189

Cleavage at paired basic residues
- R114, R115
- K153, K154
- K191, R192

Figure 16: Structures of the human and Xenopus proglucagons. The human gene encodes glucagon, GLP-1 and GLP-2. The Xenopus proglucagon gene encodes glucagon, GLP-2 and three novel GLP-1-like peptides named xenGLP-1A, xenGLP-1B, and xenGLP-1C. Putative prohormone cleavage sites are indicated on the Xenopus gene, either at single basic residues or paired basic residues. K -- lysine, R -- arginine.
proglucagon I exist in which the GLP-2 sequence is missing due to alternative mRNA splicing. The GLP-1-like peptides were named xenGLP-1A, xenGLP-1B and xenGLP-1C. Comparison of the xenGLP-1A, B and C sequences to all members of the glucagon-like family of peptides revealed that they are most similar to the GLP-1 sequence (Table 1). This structural similarity to GLP-1 suggested that they may also possess GLP-1-like activity. Although the structure and the function of glucagon is highly conserved across all species studied thus far (338), those of GLP-1 appear to be more variable. The major metabolic functions of glucagon (hyperglycemic, glycogenolytic, gluconeogenic, and lipolytic) are well known both in fish and in mammals (337,338). In all mammals studied thus far, GLP-1 acts at the level of the pancreatic β cell to potentiate insulin secretion. In contrast, in fish GLP-1 acts directly upon the liver with glucagon-like activity, and is metabolically active, enhancing fluxes through glycogenolysis, gluconeogenesis, and lipolysis (339, 340,341,342). It was observed that in salmon and trout, fish GLPs in physiological doses affect liver metabolism, enhancing gluconeogenesis (342). In fish GLP-1 does not have an insulinotrophic action due to absence of GLP-1 receptors in the pancreas, although fish GLP-1 is insulinotrophic in mammals. In amphibians (e.g., frogs such as Xenopus) the actions of GLP-1 are more consistent with those seen in mammals (339). Thus, amphibians are one of the more evolutionarily distant species from mammals that utilize GLP-1 as an insulinotropic hormone. The present study is twofold, first to determine whether the biological activities of these Xenopus GLP-1's are conserved relative to mammalian GLP-1 and the second is to make comparisons between the structure/function data to characterize functionally critical resides.

Due to the difficulty of purification of endogenous peptides from tissue coupled with the possibility of peptide cleavage during the purification process, Xenopus GLP-1 peptides were commercially synthesized for this study. The N-terminal and C-terminal ends of the peptides were based on previously published GLP-1 peptide sequences and the locations of potential prohormone convertase processing sites. PCs cleave at paired basic residues and sometimes at single basic residues (81). The exposed C-terminal basic amino acids are usually removed by carboxypeptidase E enzyme, unless the exposed basic residue is preceded by a proline (343). However the Xenopus peptides were synthesized without the actions of carboxypeptidase E taken into
## Number of Amino Acid Differences Between the Glucagon-like Peptides

<table>
<thead>
<tr>
<th></th>
<th>Glucagon</th>
<th>GLP-1A</th>
<th>GLP-1B</th>
<th>GLP-1C</th>
<th>GLP-2</th>
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<tr>
<td>Human glucagon</td>
<td>0</td>
<td>15</td>
<td>17</td>
<td>16</td>
<td>15</td>
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<tr>
<td><em>Xenopus</em> glucagon</td>
<td>0</td>
<td>15</td>
<td>17</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>Bullfrog glucagon</td>
<td>1</td>
<td>16</td>
<td>17</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td><em>Amphiuma</em> glucagon</td>
<td>3</td>
<td>15</td>
<td>17</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>Human GLP-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Xenopus</em> GLP-1A</td>
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<td>9</td>
<td>8</td>
<td>9</td>
<td>17</td>
</tr>
<tr>
<td><em>Xenopus</em> GLP-1B</td>
<td>15</td>
<td>0</td>
<td>10</td>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td><em>Xenopus</em> GLP-1C</td>
<td>17</td>
<td>10</td>
<td>0</td>
<td>6</td>
<td>18</td>
</tr>
<tr>
<td>Bullfrog GLP-1</td>
<td>16</td>
<td>11</td>
<td>6</td>
<td>0</td>
<td>17</td>
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<td>14</td>
<td>10</td>
<td>9</td>
<td>5</td>
<td>16</td>
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<tr>
<td>Human GLP-2</td>
<td>18</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>20</td>
</tr>
<tr>
<td><em>Xenopus</em> GLP-2</td>
<td>15</td>
<td>15</td>
<td>18</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>Bullfrog GLP-2</td>
<td>14</td>
<td>15</td>
<td>18</td>
<td>17</td>
<td>8</td>
</tr>
<tr>
<td><em>Amphiuma</em> GLP-2</td>
<td>15</td>
<td>16</td>
<td>17</td>
<td>17</td>
<td>8</td>
</tr>
</tbody>
</table>

**Table 1:** Number of amino acid differences between the *Xenopus* glucagon-like peptide sequences and other glucagon-like peptide sequences. The *Xenopus* GLP-1-like peptides are most similar to human GLP-1 (numbers indicated in bold) than to human glucagon or GLP-2. From: Irwin DM, Satkunarajah M, Wen Y, Brubaker PL, Pederson RA, Wheeler MB: The *Xenopus* proglucagon gene encodes novel GLP-1-like peptides with insulinotropic properties. *Proc Natl Acad Sci USA* 97: 7915-7920, 1997.
account and thus some of the Xenopus peptides have a C-terminal basic residue. Xenopus GLP-1-like peptides were synthesized based on the two possible PC cleavage sites (shown in Figures 15 and 16) to yield both full-length and truncated versions, (with deleted C-terminal basic residues only for full-length peptides). Although prohormone processing in mammals is most common at the paired basic residues, Xenopus peptides based on single basic residues were also synthesized because these peptides were similar in length to mammalian GLP-1(7-36)amide.

3.2 Hypothesis

Given the high degree of conservation of amino acids in the N- and C-terminus of the Xenopus GLP-1-like peptides compared to human GLP-1, they are expected to bind and activate the human GLP-1 receptor.

3.3 Specific Aims

3.3.1 To assess the ability of the Xenopus GLP-1-like peptides to interact with the human GLP-1 receptor using competitive displacement assays.

3.3.2 To measure the ability of the Xenopus GLP-1-like peptides to activate the human GLP-1 receptor by measuring the production of the second messenger cAMP.

3.4 Materials and Methods

3.4.1 Materials

Dulbecco's modified Eagle's media (DMEM), fetal bovine serum (FBS), L-glutamine, and penicillin/streptomycin were from Gibco BRL (Life Technologies Inc., Burlington, ON). Tissue culture plates were from Corning Inc. (Corning, NY). Bovine serum albumin (BSA), G418 (geneticin), and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma Chemical Co. (St. Louis, MO). cAMP kit was from Biomedical
Technologies (Stoughton, MA). [$^{125}$I]GLP-1(7-36)NH$_2$ was kindly provided by Dr. P.L. Brubaker (University of Toronto, Toronto, ON).

### 3.4.2 Peptides

The peptides were commercially synthesized and purified (>80% pure) by the Sheldon Biotechnology Centre (Montreal, QC). The sequences for the peptides are as follows: xenGLP-1A(1-37) (HAEGTFTSDV TQQLDEKAAK EFIDWLINGG PSKEIS), xenGLP-1A(1-32) (HAEGTFTSDV TQQLDEKAAK EFIDWLINGG PS), xenGLP-1B(1-32) (HAEGTYTNDV TEYLEEKA AK EFIEWLI KGK PK), xenGLP-1B(1-30) (HAEGTYTNDV TEYLEEKA AK EFIEWLI KGK), and xenGLP-1C(1-30) (HAEGFTNDM TNYLEEKA AK EFVGLWLR). Human GLP-1(7-36)NH$_2$ was from Bachem (Torrance, CA). All peptides were made to 10$^{-4}$ M concentrations, lyophilized and stored at -80°C. The peptides were diluted to the appropriate concentrations on the day of the experiment.

### 3.4.3 Cell Culture

A CHO-K1 cell line (R7 cell line) permanently expressing the recombinant human GLP-1 receptor was used for this study (Bmax = ~2.3 X 10$^4$ ± 0.4 X 10$^4$ receptors per cell) (266). Briefly, the cells were generated by stable transfection of the human GLP-1 receptor cDNA subcloned into the pcDNAneo expression vector. Transfected clones were selected by G418 exposure. Cells were maintained in DMEM supplemented with 10% FBS, 100 units/mL penicillin/100 µg/mL streptomycin, and 2 mM L-glutamine and kept under G418 selection (800 mg/mL). Cells were kept at 37 °C in 95% O$_2$/5% CO$_2$.

### 3.4.4 Iodination of Human GLP-1(7-36)NH$_2$

Human GLP-1(7-36)NH$_2$ was radioiodinated by the chloramine-T method as previously described (82). The [$^{125}$I]GLP-1(7-36)NH$_2$ product was purified by reverse phase adsorption to a C-18 Sep-pak column (Waters Associates, Milford, MA) and had a specific activity of approximately 125-250 µCi/µg.
3.4.5 Binding Analysis

Binding assays were performed as previously described (227,344). CHO cells expressing human GLP-1 receptors (R7 cells) were washed twice with phosphate buffered saline (PBS) and the cells were recovered from plates with 2mM EDTA in PBS. Cells were then washed with binding buffer (DMEM containing 0.4% glucose and 0.5% BSA, pH 7.4). Cells (~1 X 10^6/tube) were incubated for 45 minutes at 37 °C with radiolabeled tracer, [¹²⁵I]GLP-1(7-36)NH₂, and unlabeled Xenopus GLP-1-like peptides at concentrations ranging from 10⁻¹² to 10⁻⁶ M in a final volume of 200 µL. All the peptides were prepared on the day of the assay from lyophilized concentrated stocks with binding buffer. After incubation the cell suspensions were centrifuged for 5 minutes at 12000 rpm and the supernatant was removed. The cell pellets were re-suspended in 200 µL of PBS and the radioactivity associated with the pellet was measured in a gamma-counter (Cobra II® Gamma Counter, Canberra Packard, Mississauga, ON).

3.4.6 Measurement of cAMP Production

cAMP production was stimulated and measured as previously described (54). R7 cells were passaged into 24 well plates (~1 X 10^5/well) and cultured for an additional 48 hours. The cells were then washed with 1mL PBS and preincubated for 30 minutes at 37 °C in 0.5 mL assay buffer (DMEM containing 0.4% glucose, 0.5% BSA, pH 7.4). This was followed by a 30 minute stimulation period with varying concentrations (10⁻¹² to 10⁻⁶ M) of each of the peptides in a final volume of 500 µL of stimulation buffer (DMEM containing 0.4% glucose, 0.5% BSA, pH 7.4 and 1 mM IBMX, a phosphodiesterase inhibitor). Stimulation with each concentration was performed in triplicate. At the end of the stimulation period, the plates were placed on ice and each well was washed in 1 mL of PBS. 500 µL of cold 80 % ethanol (to rupture the cells) was added to each well and the cells were scraped with cell scraper. The cells in ethanol were transferred to microfuge tubes and kept at -20 °C until a RIA was set up. The lysate was spun for 5 min at ~1200 rpm following which the supernatant, containing cAMP was taken for RIA. cAMP-immunoreactivity was measured by radioimmunoassay, according to the instructions provided (Biomedical Technologies, Stoughton, MA).
3.4.7 Data Analysis and Statistics

Binding and cAMP data were analyzed using the nonlinear regression analysis software PRISM (Graphpad Software, San Diego, CA). Competitive displacement binding was fit to a one-site competition model and cAMP dose-response data was fit to variable slope sigmoidal curves. IC₅₀ (binding studies) and EC₅₀ (cAMP experiments) values from experiments are expressed as the mean ± SEM from at least 6 (binding assays) or 3 (cAMP assays, each performed in triplicate) independent experiments. Statistical analysis was performed using analysis of variance (ANOVA) followed by the Tukey Kramer post-test (InStat software, San Diego, CA), comparing values for each *Xenopus* peptide to values obtained for human GLP-1. A *P* value ≤ 0.05 was considered significant.

3.5 Results

3.5.1 Binding Properties of the Xenopus GLP-1 Peptides

To examine the interaction of the *Xenopus* GLP-1 peptides with the human GLP-1 receptor, competitive binding-displacement assays were performed on R7 cells which stably express the human GLP-1 receptor. A total of five peptides were chosen for the study—xenGLP-1A(1-32), xenGLP-1A(1-37), xenGLP-1B(1-30), xenGLP-1B(1-32), xenGLP-1C(1-30), representing both full-length and C-terminally truncated peptides. Human GLP-1(7-36)NH₂ was used as a control with each *Xenopus* peptide response compared to it. Binding-displacement curves for each of the truncated peptides, represented by at least six independent experiments, are shown in Figure 17. A one-site competition model was used to draw the curves that best described the data. Specific binding is expressed as a percentage of binding at 10⁻¹² M of peptide (B₀, zero binding). As shown in Figure 17, xenGLP-1A(1-32), xenGLP-1B(1-30), and xenGLP-1C(1-30) were capable of efficiently displacing labeled hGLP-1 from the human GLP-1 receptor. The apparent IC₅₀ values (half-maximal inhibitory capacity) for all five *Xenopus* peptides is in nanomolar range, comparable to human GLP-1 (Table 2). Full-length xenGLP-1A(1-37) and xenGLP-1B(1-32) gave similar results to the corresponding truncated peptides. In contrast, in a previous study, the structurally related peptide, glucagon, achieved less than 50 % displacement at 10 μM with the
Competitive Displacement from the Human GLP-1 Receptor

Figure 17: Displacement of $[^{125}\text{I}]h\text{GLP-1}(7-36)\text{NH}_2$ from CHO cells stably expressing the human GLP-1 receptor, by each of the Xenopus GLP-1-like peptides; comparing human GLP-1 to the Xenopus peptides. All data were fit to a one-site competition model and are representative of $n \geq 6$ independent experiments. The IC$_{50}$ values are summarized in Table 2.
### Summary of Binding and Activation Data

<table>
<thead>
<tr>
<th>Peptides</th>
<th>hGLP-1 (7-36)NH₂</th>
<th>xenGLP-1A (1-37)</th>
<th>xenGLP-1A (1-32)</th>
<th>xenGLP-1B (1-32)</th>
<th>xenGLP-1B (1-30)</th>
<th>xenGLP-1C (1-30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC₅₀ (nM)</td>
<td>4.4 ± 1.0</td>
<td>12.7 ± 4.3</td>
<td>17.5 ± 4.7*</td>
<td>2.9 ± 0.7</td>
<td>1.1 ± 0.4**</td>
<td>43.4 ± 10.5†</td>
</tr>
<tr>
<td>EC₅₀ (nM)</td>
<td>0.6 ± 0.2</td>
<td>2.0 ± 0.9</td>
<td>8.6 ± 3.5§</td>
<td>1.1 ± 0.3</td>
<td>0.17 ± 0.02</td>
<td>18.8 ± 0.4‡</td>
</tr>
</tbody>
</table>

**Table 2:** Summary of binding data and cAMP responses for *Xenopus* GLPs. IC₅₀ values represent the half-maximal displacement of labeled GLP-1 from the human GLP-1 receptor by the peptides. EC₅₀ values indicate the half-maximal cAMP dose-response upon stimulation by each of the peptides. IC₅₀ values represent the mean ± SEM of at least 6 independent experiments. EC₅₀ values represent the mean ± SEM of at least three independent experiments, each performed in triplicate. * p < 0.02 vs hGLP-1(7-36)NH₂, ** p < 0.02 vs hGLP-1(7-36)NH₂, † p < 0.004 vs hGLP-1(7-36)NH₂, § p < 0.003 vs hGLP-1(7-36)NH₂, ‡ p < 0.0001 vs hGLP-1(7-36)NH₂.
human GLP-1 receptor (82). Thus these *Xenopus* peptides exhibit high affinity binding to the human GLP-1 receptor. XenGLP-1B(1-30) was found to achieve an apparent IC₅₀ at significantly lower peptide concentrations than human GLP-1, xenGLP-1A(1-32), or xenGLP-1B(1-30) (p < 0.02 vs hGLP-1), indicating more affinity for the human GLP-1 receptor. XenGLP-1C(1-30) demonstrated the highest IC₅₀ value, 43.4 nM ± 10.5 nM vs 4.4 nM ± 1.0 nM for human GLP-1 (p < 0.004), and thus has the lowest affinity for the human GLP-1 receptor of all the peptides tested. These data indicate that all five *Xenopus* peptide bind to the human GLP-1 receptor, leading to the suggestion that they may also efficiently activate the human GLP-1 receptor.

### 3.5.2 Effects of *Xenopus* GLP-1s on Second Messenger Activation

Studies have shown that the GLP-1 receptor can couple to at least two G-protein-coupled signaling pathways, adenylyl cyclase and phospholipase C (227). However, studies with the endogenous receptor expressed in CHO cells suggest that cAMP is the main second messenger and along with free cytosolic calcium, triggers insulin secretion (228,230). Hence, a measure of cAMP response is a good indication of both GLP-1 receptor activation and insulinotropic ability of the peptides. To determine the level of activation of the GLP-1 receptor and the adenylyl cyclase system, cAMP assays were performed on R7 cells. Variable-slope sigmoidal dose-response curves best describe the data obtained for the cAMP responses. Responses for the three truncated peptides, xenGLP-1A(1-32), xenGLP-1B(1-30), and xenGLP-1C(1-30) are shown in **Figure 18**. The apparent EC₅₀s (half-maximal excitatory capacity) for cAMP accumulation for all five peptides are summarized in **Table 2**. These data demonstrate a reasonable correlation between apparent IC₅₀ values and half-maximal cAMP responses for each of the peptides, with xenGLP-1B and human GLP-1 demonstrating similar potency. In addition, xenGLP-1B(1-30) produced a significantly lower cAMP response at a 1 μM concentration of peptide compared with the other peptides (p < 0.05) strongly suggesting that it may be desensitizing the receptor at this supraphysiological dose. XenGLP-1C(1-30) produced the least response of the peptides tested with an apparent EC₅₀ of 18.8 nM ± 0.4 nM vs 0.6 nM ± 0.2 nM for human GLP-1 (p < 0.0001). Thus, a similar trend is seen with the cAMP results as with the binding data. Taken together the binding and cAMP data indicate that all five
cAMP Response Upon Stimulation of the Human GLP-1 Receptor

Figure 18: Stimulation of cAMP production in CHO cells expressing the human GLP-1 receptor by human GLP-1 and the Xenopus peptides, (n ≥ 3 independent experiments (performed in triplicate) for each peptide). Data were fit to fixed-slope dose-response curves. The EC50 values are summarized in Table 2.
Xenopus GLP-1s are able to bind and activate the human GLP-1 receptor, thus possessing similar biological activity as human GLP-1.

3.6 Discussion

Characterization of the Xenopus proglucagon cDNAs (69) revealed that the mRNAs could potentially encode three GLP-1-like peptides (xenGLP-1A, xenGLP-1B, and xenGLP-1C, Figure 16). All three Xenopus GLP-1-like sequences were found to be most similar to mammalian GLP-1 (Table 1). All three Xenopus GLP-1-like sequences are equally similar to the GLP-1 sequence from a divergent amphibian lineage, the Amphiuma (345) while xenGLP-1C was most similar to a GLP-1 that was identified in bullfrog (346). Taken together, these observations raised the possibility that the Xenopus GLP-1s may possess insulinotropic properties. Thus xenGLP-1A, -1B, and -1C were tested for their ability to: (i) bind, and (ii) activate the recombinant human GLP-1 receptor expressed in CHO cells. XenGLP-1B was superior to all the peptides tested with respect to binding, displaying a greater affinity for the receptor than hGLP-1. In keeping with the binding, xenGLP-1B was at least as potent as hGLP-1 in stimulating cAMP production. Thus, despite an average of nine amino acid differences between each of the Xenopus peptides and hGLP-1 sequences, their biological activities have been conserved. In addition to binding and activating the receptor in vitro, all five Xenopus peptides were potent at stimulating insulin secretion from the isolated perfused rat pancreas (69). Thus the biological activities of these GLP-1-like peptides has been conserved.

Previous studies with alanine substituted analogs of mammalian GLP-1 identified residues 7, 10, 12, 13, 15, 28, and 29 as being sensitive to substitution with alanine (134,135). The N-terminal residues were important for receptor interactions, whereas substitution of C-terminal residues resulted in conformational changes such that the peptide could not be recognized by the receptor (134). Alanine substitutions of the remaining residues or replacement of homologous residues in glucagon if alanine was originally in GLP-1 did not significantly alter GLP-1 function as measured by receptor binding (134,135).
The *Xenopus* peptides differ from hGLP-1 at positions 6, 8, 10, 11, 12, 13, 15, 16, 17, 29, 24, 27, and 28 (position 1 of the *Xenopus* peptides corresponds to position 7 in mammalian GLP-1) (Figure 19). Only one residue, 23, had previously been identified as crucial for GLP-1 function. Replacement of valine-23 with alanine resulted in a peptide that could not be recognized by the GLP-1 receptor (134,135). Instead of valine at position 23, all three *Xenopus* peptides have isoleucine, a very conservative substitution. Thus this conservative substitution does not radically alter the conformation of the GLP-1-like peptides as a alanine substitution did (both valine and isoleucine are hydrophobic amino acids). The results also indicate that changes in the side chains at many positions in GLP-1 do not dramatically alter the activity of GLP-1. These were similar to results found with fish GLPs (347).

There are both conservative and non conservative amino acid differences between the mammalian and *Xenopus* peptides. Examples of conservative change is serine11 to threonine and non-conservative are tyrosine 13 to glutamine (Figure 19). However both these amino acids were found to not be important for the biological activity of the peptide. In the case of xenGLP-1B, its superior ability to bind and activate the GLP-1 receptor compared to hGLP-1 suggests that one of the unique replacements at positions 6 (phenylalanine to tyrosine), 12 (serine to glutamic acid), or 30 (arginine to lysine) may be responsible for the increased biological activity. With xenGLP-1A and -1C less effective than hGLP-1 at binding and activating the receptor, these findings suggest that changes at position 12 (serine to glutamine or asparagine, respectively) may result in lower biological activity. Taken together these observations strongly indicate that position 12 and particularly the charge of the amino acid at this position may have a role in ligand-receptor interactions. In two recently isolated GLP-1-like peptides from the cane toad, *Bufo marinus*, positions 6 and 12 also have similar substitutions, with tyrosine 6 in Bufo 37 and phenylalanine 6 in Bufo GLP-32 (132). At position 12, Bufo 37 has glutamine, a residue similar to asparagine in xenGLP-1C. Ligand-receptor interactions seem to involve hydrogen bonds, ion pairs and hydrophobic contacts (189) and explains the importance of side chains.

These sequence comparisons indicate possible amino acid substitutions that may result in better mammalian GLP-1 analogs. Future studies should be aimed at making single amino acid substitutions of mammalian GLP-1 with divergent residues in
### AMINO ACID

**POSITION** | 1 | 10 | 20 | 30 | 35
---|---|---|---|---|---
**Human GLP-1** | HAEGT | FTCDE | SSYE | GQAAK | EFlAWE | LVKGR
**xenGLP-1A** | ----- | ----- | TQQ-D | EK--- | ---D- | -IN-G | PSKEI | IS
**xenGLP-1B** | ----- | Y-N-- | TTE--- | EK--- | ---E- | -I--K | PK
**xenGLP-1C** | ----- | --N-M | TN--- | EK--- | --VG- | -I--- | PK
**Bufo GLP-37** | ----- | Y-N-- | TQF-- | EK--- | ---D- | -L--I | PKRQR | LS
**Bufo GLP-32** | ----- | -----M | T-F-- | EK--- | --VD- | -I--- | PK
**Exendin-4** | -G--- | -----L | -KQM-- | EE-VR | L--E- | -KN-G | PSSGA | PPPS

**Figure 19:** Comparison of *Xenopus, Bufo mariuns*, and human glucagon-like peptide amino acid sequences. The sequence of the lizard peptide exendin-4 is also shown. The predicted amino acid sequences of human GLP-1 are shown in single letter code with differences in *Xenopus* GLP-1s, *Bufo* GLPs and exendin-4 indicated. Dashes (-) denote amino acid identity. Numbers indicate positions on *Xenopus* GLP-1s, *Bufo* GLPs and exendin sequences, with number 1 corresponding to 7 in mammalian GLP-1. Adapted from: Irwin DM, Satkunarajah M, Wen Y, Brubaker PL, Pederson RA, Wheeler MB: The *Xenopus* proglucagon gene encodes novel GLP-1-like peptides with insulinotropic properties. *Proc Natl Acad Sci USA* 97: 7915-7920, 1997 and Conlon JM, Abdel-Wahab YHA, O'Harte FPM, Nielsen PF, and Wittaker J: Purification and characterization of insulin, glucagon, and two glucagon-like peptides with insulin-releasing activity from the pancreas of the toad, *Bufo marinus*. *Endocrinology* 139: 3442-3448, 1998.
xenGLP-1B to further localize specific amino acid residues that are responsible for the increased receptor binding affinity seen for xenGLP-1B. This study has furthered our knowledge of the structure/function relationship of GLP-1 residues. Furthermore, the study revealed that despite an average of 9 residue differences between the predicted *Xenopus* peptides and human GLP-1, their insulinotropic activities have been remarkably preserved.
CHAPTER 4 LOCALIZATION OF AN ACTIVATION DOMAIN OF THE GIP RECEPTOR

4.1 Introduction

4.1.1 Ligand-Binding and Receptor Activation

GIP and GLP-1 belong to the secretin/glucagon/VIP family of peptides. This class of peptide hormones is 30-42 amino acids long while the N-terminal segments of their respective receptors are 116-147 amino acids long (183). In recent years evidence has emerged to support the role of the N-terminal domain in ligand-receptor interactions and ligand binding. For example, chimeric receptor and mutagenesis studies of the glucagon receptor revealed that certain amino acids in the N-terminal region alter binding affinity (348). In the glucagon receptor, the asparagine126 to lysine137 region of the 145-amino acid N-terminal segment and EC loop 1 are crucial for ligand binding (349). The N-terminal domain is primarily responsible, but not sufficient for high affinity ligand binding as the extracellular (EC) hydrophilic loops and transmembrane (TM) segments appear to be required for calcitonin binding (350). Chimeric receptor studies with GIP/GLP-1 receptors have localized the ligand binding domain of the GIP receptor to the N-terminal segment, although, the N-terminal is not sufficient for receptor activation (208).

Based on structural homology of the receptors and the fact that they act through common or similar G proteins, it was hypothesized that the superfamily of seven TM receptors must have a similar molecular activation mechanism. However this assumption is giving way to a new model where there is no requirement for a common active site. It is now suggested that peptides could activate their receptors merely by stabilizing a active conformation through interactions in the exterior parts of the receptor (351). So the receptor would exist in equilibrium between an inactive (R) and an active (R*) conformation capable of activating G proteins. It is widely accepted that there are cooperative interactions among the ligand, receptor, and G protein (352). This is referred to as the ‘ternary complex model’. This model has recently been extended to accommodate the observation that many receptors can activate G proteins in the absence of agonist and that mutations in different structural domains of the receptor
can enhance this agonist-independent activity (353,354). The high basal activity observed for many ligand-activated GPCRs, suggests that the energy barrier between R and R* is surmountable in the absence of agonist. Transitions between R and R* can occur in the absence of agonists. Agonists bind preferentially to the R* conformation and thereby shift the equilibrium and increase the proportion of receptors in the R* state. This may explain instances of constitutive activity of receptors. If mutations occurred near the regions of the receptor thought to be directly involved in G protein interaction, constitutive activity is easily explained. However mutations in EC parts of the receptor have also led to constitutive activity (180). Coughlin and coworkers showed that chimeric substitutions in the C-terminal part of the EC loop 2 caused a dramatic increase in basal signaling activity in the human thrombin receptor (180).

4.1.2 Model of Receptor Binding and Activation

Lefkowitz, Costa, and coworkers have proposed the ‘allosteric ternary complex model’ of seven TM receptor activation and binding (352,353) (Figure 20, modified model) whereby ligands exert their effect merely by selecting and stabilizing pre-formed receptor conformations and thereby shifting the equilibrium towards either an active or an inactive form. ‘Allosteric’ refers to allosteric competitive antagonism, where the ligands compete for the whole receptor by binding in a mutually exclusive mode to two different sites presented in distinct receptor conformations (355,356). This is in contrast to the classical view of isosteric competitive antagonism where two ligands compete for the same binding site (355,356). An extension of this model contains three possible scenarios for binding and subsequent activation of the incretin receptors by the endogenous ligand (A1, A2, A3 in Figure 20). In the model, the receptors can exist in two states, inactive (R) and active (R*). In the active state, the receptor is stabilized by a change in conformation that allows an interaction with and activation of G protein. The incretin hormones are postulated to interact with the EC N-terminus domain. After this initial interaction, there are several models that could then explain activation and second messenger stimulation. In A1 the physical interaction of the ligand and receptor is able to initiate a cascade of conformational alterations that are transduced through the TM segments to the region involved in G protein interaction, namely the third IC loop. In A2, the ligand initially binds the NT, upon which the NT domain shifts, allowing
Model of Receptor Binding and Activation

Figure 20: Model of receptor binding and activation for GPCRs. The receptor is in equilibrium between an inactive, R, and active state, R*. The inactive state is favoured due to stabilizing interactions. Ligand binding, stabilizes the receptor molecule in the active conformation and allows further conformational changes which allow the G protein to interact with the receptor, forming a ternary complex. Adapted from: Schwartz TW, Gether U, Schambye HT, and Hjorth SA: Molecular mechanisms of action of non-peptide ligands for peptide receptors. Curr Pharmaceut Design 1: 325-342, 1992.
the peptide to interact with receptor elements distal to the NT, leading to the formation of a high affinity complex that stabilizes the receptor in its R* state. A3 is similar to A2, however in this case instead of the ligand interacting with the EC loops, the NT domain itself interacts and causes activation of the receptor. In addition the receptor can be stabilized by an agonist, X, in A4, while antagonists would bind to and stabilize an inactive conformation of the receptor. In the case of A1, A2, or A3, it is evident that the region at the base of the NT domain, the ‘hinge’ region could be important for receptor activation. Either this region may allow for transduction of conformational changes as in A1 or in the case of A2 and A3, may be important to allow the NT to move and interact with other regions of the receptor. In the latter case, flexibility and length of the NT 'base' will be important to allow proper spatial configuration. In support of the importance of this NT base to receptor activation, is evidence from two chimeric GIP/GLP-1 receptors.

4.1.3 GIP/GLP-1 Receptor Chimeras

Chimeric receptor approaches have been useful in delineating regions important for ligand binding and signal transduction. Chimeric receptors of two highly homologous receptors provide a means to study structural determinants without grossly affecting the structure of the receptor. GIP/GLP-1 receptor chimeras are useful because both receptors share approximately 45 % overall sequence identity (207) and likely signal via identical intracellular mechanisms (54,193,207).

GIP and GLP-1 are homologous peptides that are recognized by likewise homologous but highly selective receptors. GIP and other members of the glucagon peptide family, including VIP, PACAP did not influence GLP-1 receptor binding, indicating high specificity of ligand binding to the GLP-1 receptor (190,357). In addition, these two receptors share the same signal transduction pathways, involving activation of adenylate cyclase. It was therefore, hypothesized in a previous study by Gelling et al that distinct regions of the two receptors could be exchanged with retention of binding and signaling properties. The high ligand specificity of the GIP and GLP-1 receptors enabled the generation of chimeric receptors to try to uncouple binding from activation. Two GIP/GLP-1 receptor chimeras generated by Gelling et al (208), were called CH-3 (1-151 amino acids of the rat GIP receptor spliced to 162-463 of the human GLP-1
receptor) and, CH-2 (1-132 of the rat GIP receptor spliced to 144-463 of the human GLP-1 receptor). Comparison of the two chimeric receptors revealed interesting findings (Figure 21). The CH-3 chimera was able to bind GIP and be activated (although compared to wildtype rat GIP receptor, it had a cAMP dose-response, EC\textsubscript{50} value ~ 30 % to that of wildtype) (208). However, the CH-2 which has a shorter GIP receptor component lost all ability to be activated by GIP although it was able to bind GIP. These observations indicate that the 19 amino acid extension of the GIP receptor component into the putative TM region is responsible for the uncoupling of binding to activation. In the present study, the difference between these two chimeric receptors was investigated by making mutants of the CH-3 receptor in which the divergent residues were systematically exchanged with those found in CH-2 chimera sequence.

### 4.2 Hypothesis

We propose that we can identify residues of the GIP receptor that facilitate receptor activation located in the first transmembrane region or membrane proximal amino terminal region.

### 4.3 Experimental Strategy and Specific Aims

To determine which residues of the CH-3 chimera confer activation, alignments of the rat GIP and human GLP-1 receptors (Figure 22) were performed and the amino acids that differ between the CH-3 and CH-2 chimeras were changed to corresponding residues in the CH-2 sequence. There are various alignments of the two receptors possible, based on homology of residues, or hydrophobicity of amino acids. Based on three alignments, six residues were chosen for mutation. The corresponding amino acid in CH-2 that was similar in property/charge to the one in CH-3 was not substituted (e.g. Val to Leu, or Ile to Leu, etc.)

#### 4.3.1 The following mutants were generated by site-directed mutagenesis:

- CH-3 GIP/GLP-1 Chimera (considered as "wild type" or control)
- CH-3: Deletion of Glutamate 132, Arginine 133 from CH-3
Figure 21: Comparison of two GIP/GLP-1 chimeric receptors. Comparison of CH-2 and CH-3 chimeras. CH-2 is made up of 1-132 amino acids of GIP receptor and 144-463 amino acids of GLP-1 receptor. CH-3 is made up of 1-151 amino acids of the GIP receptor and 162-463 amino acids of the GLP-1 receptor. CH-3 contains a 19 amino acid extension of the GIP receptor into the putative transmembrane domain. Although CH-2 chimeric receptor can bind GIP, it is not activated by ligand binding. For activation, extension of the GIP receptor component into the plasma membrane, chimeric CH-3, is required. Adapted from: Gelling RW, Wheeler MB, Xue J, Gyomorey S, Nian C, Pederson RA, and McIntosh CHS: Localization of the domains involved in ligand binding and activation of the glucose-dependent insulinotropic polypeptide receptor. *Endocrinology* 138: 2640-2643, 1997.
GIP and GLP-1 Receptor Protein Alignments

Alignment # 1

Membrane spanning region

human GLP-1 Rc: \( F - - L Y I I Y T V G Y A L S F S A L V I A S A \)
rat GIP Rc: \( I L - E R I Q V Y T V G Y S L A I L L A L L \)

Alignment # 2

Membrane spanning region

human GLP-1 Rc: \( F L Y - - - I I Y T V G Y A L S F S A L V I A S A \)
rat GIP Rc: \( I L - E R I Q V Y T V G Y S L A I L L A L L \)

Alignment # 3

Membrane spanning region

human GLP-1 Rc: \( F - L Y I I Y T V G Y A L S F S A - L V I A S A \)
rat GIP Rc: \( E R I Q V Y T V G Y S L S L A I L L A L L \)

residue 132  residue 151

Figure 22: Three possible amino acid sequence alignments of the rat GIP and human GLP-1 receptors. Amino acid swaps were made on the CH-3 receptor to corresponding residues on the CH-2 chimeric. The mutations are shown in bold lettering. The splice site of CH-3 is at residue 151, that of CH-2 is at 132. The shaded region indicates the sequence of the CH-3 chimera.
CH-3: Glutamine 135 Tyrosine
CH-3: Serine 143 Alanine
CH-3: Leucine 146 Phenylalanine
CH-3: Alanine 147 Serine
CH-3: Threonine 148 Alanine

4.3.2 The altered receptors were analyzed by competitive radioligand displacement assays to evaluate ligand binding.

4.3.3 Ligand-induced activation of the receptors was assessed by measuring cAMP production upon stimulation with $10^{-8}$ M GIP.

4.4 Materials and Methods

4.4.1 Materials

Chameleon Double-Stranded, Site-Directed Mutagenesis Kit (catalog # 200509) and pBluescript II KS (-) were from Stratagene (San Diego, CA). Plasmid DNA miniprep and midiprep kits were from Qiagen® (Santa Clarita, CA). Expression vector pcDNA3(+) was from Invitrogen (San Diego, CA). Primers were from ACGT Corp. (Toronto, ON). Restriction enzymes were from Gibco BRL (Life Technologies, Burlington, ON) or New England Biolabs (Mississauga, ON). T7 Polymerase sequencing kit and DEAE-Dextran were from Pharmacia Biotech (Baie d'Urfé, QC). Chloroquine was from Sigma Chemical Co. (St. Louis, MO). cAMP RIA kit was from Biomedical Technologies (Stoughton, MA). Dimethyl sulphoxide (DMSO) was from Caledon Laboratories Ltd. (Georgetown, ON). Trayslol (aprotinin) was from Bayer Inc. (Etobicoke, ON). Porcine GIP(1-42) was from Bachem (Torrance, CA). $[^{125}]$GIP(1-42) was kindly provided by Dr. R.A. Pederson (University of British Columbia, Vancouver, BC). Tissue culture media and reagents were from Gibco BRL (Life Technologies Inc., Burlington, ON).
4.4.2 Primer Design

Primer oligonucleotides for mutagenesis that contained a mutation or deletion of one or more amino acids were designed according to the primer design specifications in the mutagenesis kit, briefly:

1) both the mutagenic and selection primers annealed to the same strand of plasmid
2) primers were between 25 and 45 bases in length and had a melting temperature ($T_m$) between 40 and 44 °C. The $T_m$ was calculated by assigning 2 °C for adenosine (A) or thymine (T) bases and 4 °C for cytosine (C) or guanine (G) bases.
3) the mismatched portions were in the middle of the primer with approximately 10 to 15 bases of wild type sequence on either side.
4) the primers had a minimum GC content of 40 % and terminated in one or more C or G bases.

4.4.3 Generation of Receptor Mutants

CH-3 GIP/GLP-1 chimeric receptor was constructed by introducing a Nhe I restriction enzyme site at nucleotide 480 of the human GLP-1 receptor cDNA (Leucine to Isoleucine substitution at amino acid 161) (208). This allowed ligation of the N-terminal Hind III/Nhe I fragment of rat GIP receptor to the carboxyl terminal Nhe I/Xba I fragment of human GLP-1 receptor. The chimeric CH-3 GIP/GLP-1 receptor was cloned into the pBluescript II KS(-) for mutagenesis. Using this vector construct as a template, mutants (described in section 3.3.1 were generated with the Chameleon Double-Stranded, Site-Directed Mutagenesis kit (modified from a method described by Deng and Nickoloff, (358)). An overview of the protocol is shown in Figure 23. Clones were screened by DNA sequencing (Sanger Dideoxy method using the T7 sequencing kit from Pharmacia Biotech). Receptors which had incorporated the desired mutation were then subcloned into the Hind III/Xba I site of the expression vector pcDNA3(+). These positive clones were grown up and plasmid DNA was extracted using the Qiagen miniprep kit. The clones were then sequenced to confirm the existence of the mutation. Positive clones were transformed into XL1-Blue competent cells, a single colony was picked the following day and grown overnight in LB media. Large scale DNA preps
Site-directed Mutagenesis Protocol

Gene in pBSKS II (+) plasmid with a unique restriction site for selection and a target site for mutation.

Plasmids are denatured and primers are annealed.

The new mutant DNA strand is extended and ligated during incubation with dNTPs and enzyme mix.

Digest with the selection restriction enzyme to linearize the remaining parental plasmid.

Transformed into XLmutS competent cells and grown in liquid culture.

DNA from the pool of transformants using a miniprep procedure.

A second digest is performed with the selection restriction digest.

DNA is transformed into XL1-Blue competent cells and colonies are screened for the desired mutation.

Figure 23: Overview of the double-stranded, site-directed mutagenesis protocol. The Chameleon Double-Stranded Site-Directed Mutagenesis kit from Stratagene was used. The method required the use of two primers, one with the mutation and a second selection primer that mutates a restriction enzyme site on the vector in order to allow for selection. Figure adapted from Stratagene manual provided with mutagenesis kit.
were obtained from these grow-ups using Qiagen midiprep kit. The DNA was quantitated by measuring optical density at 260 nm (DU-64 spectrophotometer).

4.4.4 Cell Culture and Transfections

COS-7 cell (originally obtained from New England Medical Center, Boston, MA) was used for all transfections and assays. Cells were maintained in HG DME supplemented with 10 % fetal bovine serum, 100 units/mL penicillin/100 µg/mL streptomycin, and 2 mM L-glutamine at 37 °C in 95% O₂/5% CO₂. The schedule was for transfections and assays was:

Day 1: ~3 X10⁶ cells were seeded to 10 cm plate.
Day 2: 5/10 µg of DNA was transfected using the DEAE-Dextran transfection method for cAMP assay/binding assay, respectively.
Day 3: For cAMP Assays, cells were passaged to 6 well plates
        For binding assays, cells were passaged to 10 cm plates
Day 5:  cAMP assay or binding assay

Plasmid DNA containing the mutant receptors was mixed with 4.3 mL HG DME, 0.5 mL Nu-serum and 0.18 mL of a DEAE-dextran-chloroquine solution (1 mL of a 1.29 % chloroquine solution plus 9 mL of 1.22 % DEAE-dextran solution) (282). The DNA-transfection media mixture was poured onto the 10 cm plated cells and the plates were placed in the 37 °C tissue culture incubator for approximately 4 hours. Cells were then shocked for 90 seconds with 10 % Dimethyl sulphoxide diluted with HG DME media. Cells were then maintained in culture media until the assays were performed.

4.4.5 Radioligand Binding-Displacement Assay

COS-7 cells expressing the mutant receptors were washed twice with phosphate buffered saline (PBS) and recovered from plates with 2mM EDTA in PBS. Cells were centrifuged and the cell pellets were resuspended in appropriate volume of KRB buffer (containing 115 mM NaCl₂, 5 mM KCl, 24 mM NaHCO₃, 2.5 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES buffer, 0.1 % BSA) supplemented with 40 KIU/UL/mL of Trasylol (a polypeptide proteinase inhibitor). Cells were incubated for 45 minutes at 37 °C with radiolabeled tracer [¹²⁵I]GIP and unlabeled porcine GIP at concentrations from 10⁻¹² to 10⁻⁹M in a final volume of 200 µL. All the peptides were prepared on the day of the
assay from lyophilized concentrated stocks. After incubation the cell suspensions were centrifuged at 12000 rpm for 5 minutes and the supernatant was aspirated. The cell pellets were resuspended in 200 µL PBS and the radioactivity associated with the pellet was measured in a gamma-counter (Cobra II® Gamma Counter, Canberra Packard, Mississauga, ON).

4.4.6 Measurement of cAMP Response

COS-7 cells expressing the mutant receptors (in 6-well plates) were washed with 1mL PBS and pre-incubated for 30 minutes at 37 °C in 1 mL assay buffer (HG DME containing 0.4% glucose, 0.1% BSA). This was followed by a 30 minute stimulation period with $10^{-8}$ M GIP in a final volume of 1 mL stimulation buffer (HG DME containing 0.4% glucose, 0.1% BSA and 1 mM IBMX, a phosphodiesterase inhibitor). At the end of the stimulation period, the plates were placed on ice and each well was washed in 1 mL of PBS. 1 mL of cold 80 % ethanol was added to each well and the cells were scraped off the wells. The cells in ethanol were transferred to microfuge tubes and kept at -20 °C until a RIA was set up. cAMP-immunoreactivity was measured by radioimmunoassay kit according to the manufacturers instructions.

4.4.7 Data Analysis and Statistics

Binding and cAMP data were analyzed using the nonlinear regression analysis software PRISM (GraphPad Software, San Diego, CA). Competitive displacement binding was fit to a one-site competition model. $IC_{50}$ (binding studies) values are expressed as the mean ± SEM from 3 independent experiments. cAMP measurements upon stimulation by GIP were for each ‘mutant’ CH-3 receptor were expressed as a percentage of values for control CH-3 receptor. The cAMP values represent the mean ± SEM of 3 independent experiments, each one performed in triplicate. Statistical analysis was performed using ANOVA followed by the Tukey Kramer post test (InStat software, San Diego, CA), comparing each altered CH-3 chimeric to wildtype CH-3 chimeric receptor. A $P$ value $\leq 0.05$ was considered significant.
4.5 Results

4.5.1 Ligand-Binding

To assess the ability of the CH-3 chimeric mutants to interact with GIP, competitive radioligand binding-displacement assays were performed with [\(^{125}\text{I}\)]GIP (Figure 24). As expected, the mutants are not significantly different from the wildtype CH-3 receptor (which serves as the control). However all mutants, including CH-3 control, were less efficient at ligand binding compared wild type rat GIP receptor (IC\(_{50}\) values shown in Table 3). All substituted CH-3 receptors exhibited IC\(_{50}\) values of ~ equal order of magnitude to CH-3 chimera (Table 3). The expression levels of the mutant receptors are also comparable to the CH-3 control with no significant differences in Bmax values (Table 3). Binding of these receptors to [\(^{125}\text{I}\)]GLP-1 was not performed since Gelling et al demonstrated that both the CH-3 and CH-2 receptors did not bind GLP-1 (208).

4.5.2 Receptor Activation

Because the CH-2 chimera is not activated by GIP binding, substitution of residues in CH-3 with corresponding amino acids from the CH-2 receptor is expected to cause loss of activity for the mutant CH-3 receptors. The second messenger cAMP was used as a measure of receptor activation since both the GIP and GLP-1 receptors have been shown to couple to adenylate cyclase through a stimulatory G protein. Second messenger activation was measured upon stimulation with 10\(^{-8}\) M GIP. This concentration was chosen since previous studies indicated this concentration to be close to the IC\(_{50}\) value for binding (208). cAMP production in Figure 25 shows that some of the mutants are activated by GIP. The Leu146Phe and Thr148Ala substituted chimeras demonstrated a trend towards higher activation although the results were not significant. A summary of the cAMP responses for the altered CH-3 receptors, expressed relative to CH-3 control are presented in Table 3. The CH-3 mutant in which residues Glu132 and Arg133 were deleted shows a dramatic decrease in cAMP production upon stimulation with 10\(^{-8}\) M GIP. This mutant showed ~ 70 % reduction in the cAMP response compared to CH-3 receptor (the control) (Table 3, \(p < 0.05\)). The Gln135Tyr mutant’s cAMP response was reduced by ~ 93 % compared to the CH-3 control, \(p < 0.01\). Bmax values reveal that these two mutants have the same level of
receptor expression as the CH-3 chimera (Bmax values of 2905 ± 816 for CH-3 versus 2957 ± 953 for Deletion Glu132, Arg133 mutant and 2368 ± 641 for Gln135Tyr mutant, p > 0.05). Taken together these observations suggest that the difference in function between CH-2 and CH-3 chimeras could be explained by residues 132, 133 and 135 of the GIP receptor sequence. However, these residues may also have additive effects to produce the response seen for CH-2 chimera (208).
Figure 24: Binding Characteristics of altered CH-3 chimeric receptors. The top panel shows specific binding in cpm. The bottom panel shows specific binding expressed relative to maximal binding of radioligand. The data represents mean ± SEM of at least 3 independent experiments. The IC$_{50}$ values are summarized in Table 3.
### Summary of Binding and Activation Data

<table>
<thead>
<tr>
<th>Receptors</th>
<th>Binding</th>
<th>Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC50 (nM)</td>
<td>Bmax (cpm)</td>
</tr>
<tr>
<td>Wildtype ratGIP Rc</td>
<td>37.7 ± 11.0</td>
<td>9738 ± 2238</td>
</tr>
<tr>
<td>CH-3 chimeric</td>
<td>183.5 ± 72.4</td>
<td>2905 ± 816</td>
</tr>
<tr>
<td>Del Glu132,Arg133</td>
<td>166 ± 106</td>
<td>2957 ± 953</td>
</tr>
<tr>
<td>Gln135Tyr</td>
<td>519 ± 231</td>
<td>2368 ± 641</td>
</tr>
<tr>
<td>Ser143Ala</td>
<td>593 ± 352</td>
<td>3212 ± 831</td>
</tr>
<tr>
<td>Leu146Phe</td>
<td>241 ± 115</td>
<td>2978 ± 633</td>
</tr>
<tr>
<td>Ala147Ser</td>
<td>1080 ± 735</td>
<td>3819 ± 1331</td>
</tr>
<tr>
<td>Thr148Ala</td>
<td>775 ± 557</td>
<td>4246 ± 1396</td>
</tr>
</tbody>
</table>

Table 3: Summary of binding data and cAMP responses for GIP/GLP-1 chimeric receptors. IC₅₀ values represent the half-maximal displacement of labeled GLP-1 from the chimeric receptors by unlabeled GIP. Data are presented as the mean ± SEM of ≥ 3 independent experiments for binding or cAMP analysis. * p < 0.05, and ** p < 0.01 vs CH-3 chimeric.
Figure 25: Second messenger activation upon stimulation of GIP/GLP-1 receptor chimeric, CH-3 and mutants. Response to $10^{-8}$ M GIP is measured and expressed as a percentage of the CH-3 value to normalize responses from different sets of experiments. Values represent mean $\pm$ SEM of $\geq 3$ independent experiments (performed in triplicate). * $p < 0.05$ vs CH-3 mutant (control), ** $p < 0.01$ vs CH-3 mutant (control).
4.6 Discussion

Gelling et al, reported the importance of the base of the EC N-terminal region and neighboring portion of the TM 1 segment for cAMP activation for a GIP/GLP-1 chimeric receptor (208). This requirement for signaling is similar to the secretin receptor, whereas only the EC NT is required for high affinity binding and signaling in the VIP receptor. The difference between the CH-2 and CH-3 chimeras is a 19 amino acid region between the base of the NT and N-terminal of TM1 (Figure 21). However the overall length of both receptors compared to the wildtype rat GIP receptor and to each other are approximately equal (CH-2 is 451 amino acids long, CH-3 is 452 amino acids long, and the wildtype rat GIP receptor is 455 amino acids long). CH-2 is a chimera in which the splice site between the two wild-type receptors occurs at a crucial point: the beginning of the putative TM domain. In some receptors this is an important structural region because it stabilizes the TM α helix and may provide flexibility for movement of the helix vertically through the plasma membrane lipid bilayer (359,360). This may account for the loss of activity seen in CH-2. The splice site for the CH-3 receptor is in the interior of the putative TM 1. These observations indicate that this region of the receptor may be important for one of two reasons: it may disrupt a conformation that is conducive to ligand-activated activation or it may be important for signal transduction or movement of the TM domain of the molecule during the activation process.

To further localize the specific residues that may contribute to the differing activities of these two chimeric receptors, individual amino acids in the CH-3 receptor were substituted with corresponding amino acids in CH-2. All the altered CH-3 receptor isoforms were similar to the CH-3 receptor in terms of ability to bind the ligand, GIP. This is indicative of a preserved binding domain that does not include the 19 residue region that is different between CH-2 and CH-3. However, the decreased affinity compared to wild type rat GIP receptor binding, may be explained by either a difference in the NT conformation or distal binding sites. The previous study with these chimeras revealed that they were both incapable of binding GLP-1 (208). This indicates that the NT domain of the GLP-1 receptor is crucial for high affinity GLP-1 binding, although the EC loops are also involved in a complex interaction. These receptors do however possess high affinity GIP binding (208). The present results indicate that positions 132,
133 and 135 are crucial for receptor activation by GIP. Although these results do not confirm which of the possible ligand-NT-receptor interactions are involved (Figure 20, A1, A2, or A3), they do indicate the importance of the region at the base of the NT domain which lies before the start of the first membrane spanning region. Different types of amino acids e.g., hydrophobic, charged, etc, play different roles in the structure and function of membrane proteins (360). Acidic amino acids (glutamate and asparagine) are often found at the amino-termini of helices while basic amino acids (lysine, histidine, arginine) are located near the carboxyl-termini of helices (360). These charges may neutralize the helix dipole and, in doing so, can participate in stabilization of the helix (359). Glutamate is the strongest helix former (361) and is often found within the amino-terminal section of helices. The location of negative charges near the amino-termini of helices can neutralize the helix dipole. Thus deletion of glutamate 132 may serve to destabilize the N terminus of the first transmembrane helix and in doing so causes a loss in receptor activation.

As a positively charged residue, arginine is usually found on protein surfaces. It is a basic residue with a side chain that has five hydrogen bond donors within its guanidinium moiety, and plays a major role in stabilizing protein structures by forming hydrogen bond networks and salt links with acidic side-chains (360). Thus deletion of arginine 133 may also lead to destabilization of the first transmembrane domain. In the case of glutamine to tyrosine at position 135, tyrosine functions as a consummate amphipathic residue that can be positioned or perhaps move between the aqueous and lipid phase. Glutamine is hydrophilic but uncharged. Thus this mutant may be more conducive to vertical movement through the lipid bilayer. However the charged residues, glutamate and arginine at position 132 and 133 may be the limiting factor to this movement. For the serine 143 to alanine mutation and the alanine 147 to serine mutation, alanine has small side chains and would allow close packing of a helical segments (362). Alanine is a very common residue in helical segments and is classified as a strong helix former (361). It is a generic amino acid with a small, unreactive methyl side-chain that is found either in protein interiors or on the protein surface (363). Leucine and phenylalanine, along with isoleucine and valine are classified among the most nonpolar in most hydrophobicity scales. Thus the leucine to phenylalanine...
substitution at position 146 is conserved and as the results indicate, does not drastically affect the activation capability of the receptor compared to CH-3 wild type receptor.

Although the results do not confirm a mode of activation, the loss of activity due to certain amino acid substitutions indicates strongly of the importance of these residues in interactions with either the NT, ligand or TM domains that may interact with TM 1. Scenario A2 and A3 may be tested by elongating the N-terminus at the base where it connects to the first TM domain. This would affect the interaction of the N-terminal-ligand with the EC loops due to a change in spacing between the NT and the EC loops. Clearly, these two chimeric receptors provide interesting results and insight into the possible mechanism of action of the receptors. The residues glutamate 132, arginine 133, and glutamine 135 are critical for GIP receptor activation and these resides should be targeted for mutagenesis on the wild type rat receptor to confirm their importance.
The studies presented here have addressed three issues with respect to the incretin hormones: i) their roles in the enteroinsular axis, ii) the functional domains critical for activity of GLP-1, and iii) the domain involved in GIP induced receptor activation. The common goal of these studies is to help in the development of pharmacological analogs and/or mimetics of GLP-1 and GIP, since both hormones are insulinotropic, with GLP-1 in particular being a candidate therapeutic agent for Type 2 diabetes.

Characterization of the enteroinsular axis of GLP-1R−/− mice reveals the complexity of the axis and the plasticity associated with abrogation of GLP-1 signaling. This plasticity is exemplified by upregulation of the GIP component, which serves to compensate for the loss of the GLP-1 component. We have shown this compensation to include both increased GIP secretion in response to oral glucose and increased insulinotropic action of GIP at the pancreas. The increased sensitivity to GIP is at the level of the pancreatic islets since this effect is observed in studies with isolated islets from GLP-1R−/− mice, that lack any neural connection to the intestine. The increased sensitivity may be due to increased numbers of GIP receptors expressed at the cell surface, to increased coupling to G proteins, or increased sensitivity of the β cell intracellular machinery. The latter case may be envisioned by a situation where both GLP-1 receptor and GIP receptor compete for the same signal transduction molecules; the absence of GLP-1 receptor protein may allow greater interaction of the GIP receptor with the same number of signaling molecules. Future studies examining the levels of GIP receptor expression will give insight into the mechanism of the increased sensitivity to GIP in GLP-1R−/− mice. This knowledge will add to our understanding of the dynamics involved in pancreatic β cell function. The studies outlined here clearly place GLP-1 in a key role for glucose homeostasis and for maintaining the state of the pancreatic insulin content.

Present research on GLP-1 is aimed at determining the specific functional determinants that confer its biological activity, i.e., the determinants involved in binding to the GLP-1 receptor as well as activation of the receptor. The consequence of these
studies are to design analogs that possess the insulinotropic activity of GLP-1 but without its undesirable properties. It is important to realize though that GLP-1 receptor expression has been reported in the stomach, brain, kidney, lung and heart. The receptors in these tissue appear to be the product of the same gene as in the pancreatic islets. Therefore any agonist produced towards the pancreatic GLP-1 receptor will likely bind to the extra-pancreatic receptors, leading to side effects. Thus the design of agonists will have to take into account these consequences.

Characterization of the *Xenopus* peptides revealed several residues where alterations are tolerated, with some apparently increasing affinity. The existence of three novel GLP-1-like peptides on the proglucagon gene of *Xenopus* raises questions as to how these peptides originated and the purpose of having three such homologous peptides. Exon duplication most likely explains the existence of these similar peptides. Could there be three GLP-1 receptor-like receptors for each of the peptides and if so are there differences in tissue expression of the receptors as well as the peptides? The peptides themselves have not yet been isolated *in vivo*. Isolation of these peptides from *Xenopus* will provide information as to the exact PC cleavage sites. Further investigation of these peptides should reveal whether they possess other properties besides being insulinotropic. The existence of three GLP-1-like peptides in this species and more recently the purification of two GLP-1-like peptide in the cane toad, *Bufo marinus*, certainly provides a good rationale to look for more than one GLP-1 receptor isoform in these organisms. If more than one receptor exists, it could help to delineate structurally and functionally important residues in the human GLP-1 receptor through comparative studies. For a physiologist, it remains of primary importance to isolate purified peptides from a particular animal species and to test them preferentially in the same animal species in physiological concentrations. Thus studies are aimed at isolating and cloning GLP-1-like receptors in *Xenopus laevis*. These studies may lead to the identification of agents that preferentially associate with a given receptor, perhaps a pancreatic/glucoincretin receptor.

It was anticipated that these *Xenopus* GLP-1-like peptides may represent more stable analogues of human GLP-1 and may be used in therapy. Salmon calcitonin is a good example of the use of a peptide from a evolutionarily distant species, in humans, since it is more potent than mammalian calcitonin (364). Although the *Xenopus*
peptides are better than or equipotent to hGLP-1 in insulinotropic activity as exhibited by perfused pancreas studies (67), the fact that they have similar sequences in the first 6 residues of the peptides, especially at the site of DP IV cleavage (penultimate alanine) means that they are most likely susceptible to DP IV cleavage as is hGLP-1. This consensus sequence was also found in the Bufo GLPs. The degradation of these peptides may be important for proper biological function. It may also indicate something about proteolytic enzymes in evolutionarily distant species and the conserved nature of their functions.

To generate agonists to the receptors, information regarding the binding and activation domains/regions of the receptors is also valuable. The best way to gain structural knowledge would be to crystallize the receptor alone and in the presence of the ligand. However determining the crystal structures of membrane proteins is difficult and could take years. The most feasible method at present is to use site-directed mutagenesis studies as well as chimeric receptor analysis to determine the amino acids or regions of the receptor that are involved in binding or activation of G proteins. This approach was undertaken to localize an activation domain of the GIP receptor to three residues amino-terminal to the first transmembrane domain. The complexity of peptide structure and peptide-peptide interactions makes the interpretation of mutagenesis data difficult. If a particular amino acid, when mutated, results in a change in activity, it could either be important for conferring structure and conformation or it may be directly involved in peptide interactions. One method used to distinguish between the two possibilities is to make parallel mutations in both the ligand and receptor. For example in the β-adrenergic receptor this approach led to the discovery of aspartate 113 in TM 3 that is directly involved in a specific interaction with the amine function of the ligand (189).

GPCRs are involved in the response to hormones and neurotransmitters, and are the principal signal transducers for the senses of light and smell. Additionally mutations have been observed that involve a wide spectrum of hereditary and somatic diseases and disorders from cancer to infertility. These mutant receptors are incapable of binding ligand or generating normal signals, constitutively generate signals, or are not appropriately expressed on the cell surface. The importance of GPCRs to cellular functions and their roles in disease warrant a complete understanding of the
mechanism of ligand binding and activation. This knowledge will aid in the
development of agonists and antagonists for these receptors. This study demonstrates
the importance of TM 1 to GIP receptor activation. Future studies should be aimed at
completing the model for the mechanism of receptor binding and activation. To this end,
studies should also be aimed at determining the biophysical properties of the TM
domains during receptor binding and activation. For example photo-labeled peptides
can be used to probe binding sites of the receptor that may become exposed as the
ligand-receptor interaction proceeds.
CHAPTER 6 CONCLUSIONS

Enteroinsular Axis of GLP-1R-/− Mice
1. In GLP-1R-/− mice, pancreatic insulin levels were decreased compared to CD1 controls, indicating an important role for GLP-1 in determining steady-state insulin levels.

2. Steady-state levels of insulin mRNA in the pancreas of GLP-1R-/− mice are decreased compared to control mice, and may account for the decreased insulin protein levels.

3. Pancreatic glucagon levels were not significantly different between GLP-1R-/− and control mice.

4. The pancreatic level of somatostatin in GLP-1R-/− mice is increased compared to CD1 controls.

Biological Activities of Xenopus GLP-1-like Peptides
5. Despite an average of nine amino acid differences between each of the Xenopus GLP-1-like peptides and human GLP-1, they possess similar biological activity to that of human GLP-1.

6. XenGLP-1B has a glutamate instead of serine at position 12 and possesses increased biological activity. XenGLP-1A has glutamine at position 12 while xenGLP-1C has asparagine at position 12, both of which are negatively charged. Thus the charge at position 12 may have a role in ligand-receptor interactions.

7. The conservative substitution of isoleucine for valine at position 23 confirms that this position is likely important for peptide activity since alanine substitution of mammalian GLP-1 at the corresponding position (position 29) renders it inactive.
*Activation Domain of the GIP Receptor*

8. Glutamate 132, arginine 133, and glutamine 135 play a critical role in the activation of the GIP/GLP-1 receptor chimera, CH-3.
CHAPTER 7   REFERENCES


80. Ørskov C, Holst JJ, Knuhtsen S, Baldissera FGA, Poulsen SS, Nielsen OV: Glucagon-like peptides GLP-1 and GLP-2, predicted products of the glucagon gene, are secreted separately from the pig small intestine, but not pancreas. *Endocrinology* 119: 1467-1475, 1986


83. Patzel C, Schiltz E: Conversion of proglucagon in pancreatic alpha cells: The major endproducts are glucagon and a single peptide, the major proglucagon fragment, that contains two glucagon-like sequences. *Proc Natl Acad Sci USA* 81: 5007-5012, 1984


85. Rouillé Y, Westermark G, Martin SK, Steiner DF: Proglucagon is processed to glucagon by prohormone convertase PC2 in αTC1-6 cells. *Proc Natl Acad Sci USA* 91: 3242-3246, 1994


204. Graziano MP, Hey PJ, Strader CD: The amino terminal domain of the glucagon-like peptide-1 receptor is critical for subtype specificity. *Recept Chann* 4: 9-17, 1996


207. Usdin TB, Mezey E, Button DC, Brownstein MJ, Bonner TI: Gastric inhibitory polypeptide receptor, a member of the secretin-vasoactive intestinal polypeptide receptor family, is widely distributed in peripheral organs and the brain. *Endocrinology* 133: 2861-2870, 1993


220. Thorens B: Recent studies on the GLP-1 receptors. Digestion 54: 341-347, 1993


238. Muller MK, Demol P, Goebell H, Fladrich G, Brown JC: Natural purified porcine gastric inhibitory polypeptide (GIP) stimulates exocrine pancreas secretion due to


246. Fehmann HC, Göke B. Characterization of GIP(1-30) and GIP(1-42) as stimulators of proinsulin gene transcription. Peptides 16: 1149-1152, 1995


285. Beck B, Max JP: Gastric inhibitory polypeptide enhancement of the insulin effect on fatty acid incorporation into adipose tissue in the rat. Regul Pept 7: 3-8, 1983


304. Koch BD, Schonbrunn A: The somatostatin receptor is directly coupled to adenylate cyclase in GH4C1 pituitary cell membranes. *Endocrinology* 114: 1784-1790, 1984


311. Knudsen LB, Pridal L: Glucagon-like peptide-1-(9-36) amide is a major metabolite of glucagon-like peptide-1-(7-36)amide after *in vivo* administration to


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