INTESTINAL GROWTH IN MODELS OF GLUCAGON-LIKE PEPTIDE-2 OVEREXPRESSION

by

Kirk D. Fischer

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


Exogenous glucagon-like peptide-2 (GLP-2) has been shown to be a potent and specific stimulator of small intestinal mucosal proliferation in vivo. However, to date no studies have looked at the role of endogenous GLP-2 in the control of bowel growth. As experimental diabetes is associated with bowel growth, we examined the relationship between GLP-2 and intestinal growth in rats made diabetic by streptozotocin (STZ) injection, treated with or without insulin for three weeks. Compared to non-diabetic control animals, untreated diabetic rats showed increases in ileal and plasma GLP-2 (p<0.05), wet and dry small intestinal weight (p<0.01), intestinal protein (p<0.001) and lipid (p<0.05) content, villus height (p<0.001), and crypt depth (p<0.01). Thus, STZ-diabetes is associated with both increased production of GLP-2 and enhanced bowel weight, thereby suggesting a role for GLP-2 in diabetes-associated bowel growth. To develop a specific GLP-2 overexpression system, Baby Hamster Kidney cells were transfected with a cDNA construct consisting of the coding region of human proglucagon signal peptide fused to that for human GLP-2. These cells successfully transcribed the construct but failed to synthesize or secrete GLP-2 peptide. In conclusion, this study supports a role for GLP-2 as an endogenous intestinal growth factor and provides a basis for future work on the development of a GLP-2 overexpression system.
For my brother, Kai,

and my parents,

Rosemarie and Jörg Fischer.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>x</td>
</tr>
</tbody>
</table>

## Chapter 1  Introduction

### 1.1 Proglucagon

1.1.1 Posttranslational Processing in the Intestine 2

1.1.2 Secretion and Synthesis of the Intestinal PGDPs 5

1.1.3 Physiology of the Intestinal PGDPs 9

1.1.4 Metabolism of the Intestinal PGDPs 11

### 1.2 Intestinal Growth and Adaptation

11

### 1.3 Regulation of Mucosal Growth

14

1.3.1 Nutrients 17

1.3.2 Regulatory Peptides 18

1.3.2.1 PGDPs 19

1.3.2.2 Other Enteroendocrine Peptides 22

1.3.2.3 Other Endocrine Peptides 24

1.3.2.4 Luminal Peptides 26

1.3.2.5 Local Peptides 27

1.3.3 Pancreaticobiliary Secretions 28
3.1 Introduction

3.2 Materials and Methods

3.2.1 Nude Mice

3.2.2 Construction and Sequencing of Plasmid

3.2.3 Cell Culture, DNA Transfection and Clone Selection

3.2.4 RNA Analysis

3.2.5 High Pressure Liquid Chromatography and Radioimmunoassay

3.2.6 Statistics

3.3 Results

3.4 Discussion

Chapter 4 Discussion

Chapter 5 References
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Table 1
GLP-2 content in cell extracts and medium from BHK cells transfected or untransfected with hPGSP/GLP-2 after 2 and 24 hours of incubation in minimal medium.

Page 62
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Fig.</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig.1.</td>
<td>Schematic representation of tissue-specific posttranslational processing of mammalian preproglucagon to the PGDPs.</td>
<td>3</td>
</tr>
<tr>
<td>Fig.2.</td>
<td>Alignment of amino acid sequences of human and rat PGDPs.</td>
<td>4</td>
</tr>
<tr>
<td>Fig.3.</td>
<td>Confirmation of STZ-induced diabetes in rats.</td>
<td>39</td>
</tr>
<tr>
<td>Fig.4.</td>
<td>Plasma concentrations of gut GLI, N-GLP-2 and IRG in Control, STZ and STZ+I rats.</td>
<td>40</td>
</tr>
<tr>
<td>Fig.5.</td>
<td>Ileal concentrations of GLI, GLP-1(^{36NH_2}) and T-GLP-2 in Control, STZ and STZ+I rats.</td>
<td>41</td>
</tr>
<tr>
<td>Fig.6.</td>
<td>Wet weight, dry weight, protein content and lipid content of 5cm intestinal segments from Control, STZ, and STZ+I rats.</td>
<td>42</td>
</tr>
<tr>
<td>Fig.7.</td>
<td>Villus height, crypt depth, and muscle thickness in intestinal sections from Control, STZ, and STZ+I rats.</td>
<td>43</td>
</tr>
<tr>
<td>Fig.8.</td>
<td>Representative histological cross-sections of duodenum from Control, STZ and STZ+I rats.</td>
<td>44</td>
</tr>
<tr>
<td>Fig.9.</td>
<td>The hPGSP/GLP-2 fusion construct.</td>
<td>59</td>
</tr>
<tr>
<td>Fig.10.</td>
<td>Plasma GLI and small intestinal wet weight of from controls and nude mice carrying subcutaneous GLUTag or BHK-proG tumours.</td>
<td>60</td>
</tr>
<tr>
<td>Fig.11.</td>
<td>Northern blot of total RNA from untransfected BHK, GLUTag, InR1-G9, mixed transfections and a single cell clone.</td>
<td>61</td>
</tr>
<tr>
<td>Fig.12.</td>
<td>HPLC profiles of 24 hr media samples from BHK cells transfected with hPGSP/GLP-2 and media alone.</td>
<td>63</td>
</tr>
</tbody>
</table>
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
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<td>brush border membrane</td>
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<td>standard error of the mean</td>
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VAL

valine

xvi
Chapter 1 Introduction

The small intestinal mucosa is a rapidly proliferating compartment with a unique capacity for growth and regeneration. Although the details remain unclear, it appears that the regulation of intestinal epithelial growth and differentiation is complex, requiring the orchestration of many factors. These include nutritional status and the products of various endocrine, exocrine and neural secretions.

Recently, glucagon-like peptide-2 (GLP-2), one of the intestinal products of the proglucagon gene, has been found to be a potent and specific trophic factor for the small intestinal epithelium. When injected subcutaneously into mice, exogenous GLP-2 was found to stimulate both villus elongation and crypt cell proliferation in the small bowel. Subsequent studies indicate that the newly grown gut shows a normal or enhanced capacity for nutrient digestion and absorption. Such an agent may therefore be useful for the treatment of patients with gastrointestinal diseases, such as short bowel syndrome, which are characterized by a compromised functional absorptive capacity.

To date, however, no studies have examined the role endogenous GLP-2 may play in the control of intestinal epithelial proliferation. Thus, the goal of the first study was to characterize the effects of endogenous GLP-2 overexpression. It has been reported that experimental diabetes, although primarily a catabolic disease, is also accompanied by small intestinal growth. Therefore, we hypothesized that the concentrations of plasma GLP-2 would be elevated in streptozotocin-induced diabetic rats.

In nude mice, the subcutaneous passage of several proglucagon-producing cell lines results in small intestinal proliferation. However, these tumour cells secrete several
of the intestinal proglucagon-derived peptides (PGDPs) simultaneously, and, until recently, it was unclear which PGDP was responsible for the observed gut growth. Thus, it was the aim of the second study to develop a cell line that secreted high concentrations of GLP-2 and to inject these cells subcutaneously into nude mice, where they would form tumours. It was hypothesized that these tumours would secrete significant quantities of GLP-2 into the circulation and thereby induce small intestinal proliferation.

1.1 Proglucagon

1.1.1 Posttranslational Processing in the Intestine

In all mammals studied to date, a single preproglucagon gene is expressed in pancreatic A cells, intestinal L cells, and some parts of the central nervous system (1-4). This gene encodes an identical 180 amino acid preproglucagon protein in all three of these tissues. However, tissue-specific post-translational processing results in distinct populations of PGDPs in the pancreas and intestine (fig. 1). In the A cell, the major products are glucagon and the major proglucagon fragment (MPGF), while the L cell produces the following: glicentin, oxyntomodulin, glucagon-like peptide-1 (GLP-1), glucagon-like peptide-2 (GLP-2), and intervening peptide-2 (IP-2) (fig. 1). Figure 2 shows the amino acid sequences of both the human and rat intestinal PGDPs, highlighting the marked structural conservation of these peptides both within and between species. Processing of prohormone molecules to their mature peptides is achieved through the actions of the prohormone convertases (PCs). These endopeptidases cleave prohormones on the C-terminal side of pairs of basic amino acid residues [e.g. ARG-ARG\(^i\), LYS-lys\(^i\), (5)]. Within proglucagon, such sites flank glicentin,
Fig. 1. Schematic representation of the tissue-specific posttranslational processing of mammalian preproglucagon to the PGDPs. RIA, as described in section 2.2.7, detect GLI peptides (solid square), IRG (solid triangle), GLP-1<sup>37</sup> (solid circle), GLP-1<sup>x-36NH2</sup> (solid line), N-GLP-2 (open circle), T-GLP-2 (cross). N, amino-terminus; C, carboxy-terminus; SP, signal peptide; GRPP, glicentin-related pancreatic polypeptide; MPGF, major proglucagon fragment; GLP-1, glucagon-like peptide-1; GLP-2, glucagon-like peptide-2; IP-1, intervening peptide-1; IP-2, intervening peptide-2. Adapted from (7).
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<tr>
<td>hGlicentin</td>
<td>RSLQDTEKS RSIFSASQADP LSDPDQMNED KRSCHQFTS DYSKYLDSSR AQDFVWVLN TKRNKNIA</td>
<td>69</td>
</tr>
<tr>
<td>rGlicentin</td>
<td>HAPQDTEENA RSFPASQTPQ LQDPQINEQ KRSCHQFTS DYSKYLDSSR AQDFVWVLN TKRNKNIA</td>
<td>69</td>
</tr>
<tr>
<td>rOxyntomodulin</td>
<td></td>
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<tr>
<td>hOxyntomodulin</td>
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<tr>
<td>Glucagon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLP-1(7-37)</td>
<td></td>
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</tr>
</tbody>
</table>

**Fig. 2.** Alignment of amino acid sequences of human and rat PGDPs. For both glucagon and GLP-1(7-37) the human and rat sequences are identical, thus only one sequence is shown for each. The number to the right of each sequence indicates its amino acid chain length. Boxed residues denote the conservation of identical amino acids between the sequences of all eight peptides. h, human; r, rat; GLP-1, glucagon-like peptide-1; GLP-2, glucagon-like peptide-2.
oxyntomodulin, glucagon, GLP-1\(^{1-37}\), and GLP-2. Recent studies using antisense ablation (6) and vaccinia virus-mediated overexpression (7) approaches have now identified PC1 (also called PC3) as the convertase responsible for processing proglucagon to the intestinal PGDPs. This enzyme has also been shown to process GLP-1\(^{1-37}\) to GLP-1\(^{7-37}\) at a single basic amino acid (ARG\(_{\text{e}}\)) (7). Localization of PC1 using immunohistochemistry has found it to be present in L cells (8), but not in A cells (9). As PC1 does not process proglucagon to glucagon, these observations suggest it is not involved in production of the pancreatic PGDPs. In the L cell, most of the GLP-1 and IP-2 is also amidated, most likely by peptidyl glycine \(\alpha\)-amidating monooxygenase (10).

Unlike mammals, other vertebrates show a more complicated pattern of proglucagon gene expression. Initial studies describing proglucagon sequences from anglerfish (11,12) and catfish (13) pancreas, indicated that two mRNA transcripts are produced which contain only glucagon and GLP-1. These authors concluded that GLP-2 was therefore a late addition in evolution. However, Irwin and Wong (14) found that differential exon splicing generates distinct mRNA transcripts in the pancreas and intestine of both trout and chicken. The intestinal transcript encodes glucagon, GLP-1 and GLP-2, whereas only glucagon and GLP-1 are encoded by the pancreatic species. Thus, GLP-2 is indeed evolutionarily conserved, supporting the idea that it has an important biological function.

1.1.2 Secretion and Synthesis of the Intestinal PGDPs

There is no single study examining PGDP secretion in which a radioimmunoassay for each of the different peptides has been employed, as usually only one or two of the
peptides are measured. However, studies in our laboratory have shown that glicentin/oxyntomodulin and GLP-1 are released in parallel from both fetal rat intestinal cell (FRIC) cultures (15,16) and a mouse enteroendocrine cell line (17). Furthermore, Holst and colleagues have presented similar statistical correlations between secretion of GLP-1 and GLP-2 (18,19). Taken together, and in the absence of conflicting information, it is therefore assumed that all of the intestinal PGDPs are secreted in a one-to-one ratio. Thus, the following discussion refers to the secretory products of the L cell collectively as the intestinal PGDPs, regardless of which peptide was being examined in the study under discussion.

The intestinal L cell is an open-type enteroendocrine cell (20,21), and is thus exposed to luminal contents at its apical membrane and to circulating and local factors at its basolateral surface. Glucose can directly stimulate PGDP secretion from FRIC cultures *in vitro* (22) and in the rat *in vivo* (23). That L cells are responsive to glucose is consistent with the presence of the glucose sensor glucokinase in these cells (24). *In vitro* studies have shown a direct effect of mono-unsaturated long chain fatty acids (but not saturated or short/medium chain mono-unsaturated fatty acids) on PGDP secretion (16). Consistent with these findings, rats receiving intraluminal infusion of mixed fats show increases in intestinal PGDP secretion (23,25). Significant increments in plasma levels of the intestinal PGDPs are detected 15-20 minutes after nutrient ingestion (26). Since the greatest concentration of L cells is in the distal ileum and colon (20,21,25), this time course appears to be too rapid to be a result of direct stimulation by luminal contents. It was therefore suggested that an endocrine/neural proximal-distal loop exists which indirectly mediates this effect (23,25,27).

Consistent with this hypothesis, a number of endocrine hormones influence
intestinal PGDP secretion. In the rat, glucose-dependent insulinotropic peptide (GIP; formerly called gastric inhibitory peptide) stimulates PGDP release from FRIC cultures (27), perfused ileum (28) and in vivo (23). However, GIP appears to have no effect in humans (29), indicating species-specific differences in L cell regulation. Both insulin and somatostatin (SS-14 and SS-28) inhibit intestinal PGDP secretion in vitro (22,27) and in vivo (30,31). As GLP-1 stimulates secretion of all of these hormones (29,32,33), their effects appear to provide negative feedback regulation of the L cell.

In addition to endocrine hormones, neurotransmitters/modulators also regulate the L cell. Acetylcholine stimulates PGDP secretion from a mouse intestinal cell line (34), and in the rat both in vitro (27) and in perfused intestinal loops (35). In contrast, canine L cells either do not respond or are inhibited by acetylcholine (36). The effects of epinephrine remain unclear, as it appears to stimulate secretion in some systems but not in others (27,37). Gastrin releasing peptide (GRP), a neuropeptide found in both vagal efferent fibers and the enteric nervous system (38), stimulates PGDP secretion both in vitro in the rat (27) and in vivo in humans (39) and rats (31,40). Furthermore, infusion of a GRP antagonist abolished secretion from the L cell and diminished the GIP response when fat was simultaneously placed into the duodenum (40). Calcitonin gene-related peptide and several opioid peptides also stimulate the L cell (27,41), while both SS-14 and galanin inhibit intestinal PGDP secretion (27,31,42). Thus, a host of factors from endocrine and neural sources, influence PGDP secretion from the L cell, of which several appear to display species-specific activity.

Cell culture systems from rat (15,41,43), mouse (17,34) and dog (37), have been used to identify the intracellular signalling pathways involved in stimulating PGDP secretion. Calcium appears to be important for basal and stimulated secretion, as a
calcium channel blocker completely inhibited, while a calcium ionophore increased, PGDP release in vitro (41). Activators of the protein kinase C (PKC) pathway, such as phorbol esters, also stimulate PGDP secretion (17,34,41,43), supporting the data obtained for physiologic activators of this pathway, such as GRP (27,31,39,40). Although the intracellular mechanism by which fatty acids regulate the L cell is unknown, roles for both a fatty acid binding protein and PKC-ζ, an isoform of PKC, have been suggested (16). Another intracellular signalling cascade involved in stimulation of PGDP secretion is the protein kinase A (PKA) pathway (17,34,43,44). The direct effects of GIP on PGDP secretion in vitro are most likely mediated by this pathway.

In addition to stimulating PGDP secretion, activation of the PKA pathway also results in increased synthesis of the PGDPs by stimulating proglucagon gene transcription (17,44). These data are in agreement with the identification of a functional cyclic AMP response element (CRE) in the rat proglucagon gene promoter (44,45). This CRE is activated in a PKA-dependent fashion as well as by membrane depolarization and calcium influx (17,44-46). In a rat insulinoma-derived cell line, proglucagon gene transcription was also induced after administration of phorbol esters (47), however these agents have no effect on proglucagon transcription in either FRICs (43) or a mouse enteroendocrine cell line (17).

Most studies examining regulation of proglucagon gene expression have used the proglucagon gene transfected into various pancreatic cell lines. Analysis of the rat proglucagon 5'-flanking region has revealed that the first 300 base pairs upstream of the transcription start site contain islet-specific enhancer-like elements (48) and an insulin response element (49). Generation of a transgenic mouse, in which 1,252 bp of the rat proglucagon 5'-flanking region was fused to the coding region for SV40 large T antigen
(SV40Tag), resulted in expression of the transgene in pancreas and brain but not in the gastrointestinal tract (50). However, a second transgenic mouse, in which SV40Tag expression was driven by 2,252 kb of the rat 5′-flanking region, showed transgene expression in the stomach, small intestine and colon, in addition to expression in the pancreas and brain (51). Thus, this 1000 bp upstream region appears to contain a regulatory element which specifies intestinal proglucagon gene expression.

Therefore, a number of luminal, endocrine and neural factors, acting through diverse intracellular signalling pathways, influence the synthesis and secretion of the intestinal PGDPs.

1.1.3 Physiology of the Intestinal PGDPs

Glucagon is the primary bioactive PGDP secreted from the A cell. Its role, in the control of glycemia, as the major counter-regulator of insulin is well established (52). In contrast, the biologic actions of some of the intestinal PGDPs have only recently been established. Of these, GLP-1 has been the most extensively studied. The N-terminally truncated peptides, GLP-1⁷⁻⁴⁷/C⁶ⁿH₂ (tGLP-1) are the biologically active forms (fig. 2). The most important activity of tGLP-1 is its potent stimulation of glucose-dependent insulin secretion (32,53,54). Furthermore, tGLP-1 also stimulates insulin biosynthesis by directly increasing proinsulin gene transcription, thus replenishing intracellular stores of the hormone (53). Other actions in the pancreas include stimulation of somatostatin secretion from D cells and inhibition of glucagon secretion from A cells (32). Taken together, these actions of tGLP-1 make it a prime candidate for treating the hyperglycemia and hypoinsulinemia of patients with non insulin-dependent diabetes mellitus (54).
Extrapancreatic effects of tGLP-1 include a number of effects on the stomach, namely inhibition of both acid secretion (55) and gastric emptying (56). Recently, tGLP-1 has been reported to induce satiety following intracerebroventricular injection into rats (57). However, mice with a null mutation of the tGLP-1 receptor (GLP-1R) allele, do not show any increase in food intake or abnormalities in body weight (58). Studies examining the structure-activity relationships of tGLP-1 have revealed that the N-terminal histidine (fig. 2) is essential for bioactivity, whereas the presence or absence of the C-terminal amide does not appear to affect biological function (59).

Both glucagon and tGLP-1 mediate their physiologic effects through G protein-coupled seven transmembrane receptors linked to cAMP/PKA intracellular signalling pathways (52,60). Studies of GLP-1R gene expression indicate it is present in pancreatic islets, lung, stomach, intestine, and brain (60,61). Although the cAMP/PKA pathway appears to mediate the physiologic effects of GLP-1R on insulin synthesis and secretion (53), in heterologous cell lines it can also couple to the phospholipase C (PLC) intracellular pathway (60). The GLP-1R has been shown to undergo desensitization and internalization, probably by a phosphorylation-dependent mechanism (62).

GLP-2 has recently been found to be a potent stimulator of intestinal mucosal growth when injected into normal mice and these results are discussed in detail below [(63,64); see 1.3.2.1]. Also, a 4 hour GLP-2 infusion has been reported to enhance basolateral glucose uptake in rat small intestine (65). Of the remaining intestinal PGDPs, oxyntomodulin inhibits pentagastrin-induced gastric acid secretion both in vitro (66) and in vivo (67), while biological roles for glicentin and IP-2 remain unclear.
1.1.4 Metabolism of the Intestinal PGDPs

The biologically active peptides GLP-1<sup>7-37/36NH<sub>2</sub></sup> are metabolized to GLP<sup>9-37/36NH<sub>2</sub></sup>, the inactive forms, through the action of dipeptidylpeptidase IV [DPIV; EC 3.4.14.5], which cleaves peptides at penultimate N-terminal proline or alanine residues. Degradation of GLP-1, and of the structurally related peptide GIP, has been observed both in human serum <i>in vitro</i> (68,69) and in the rat <i>in vivo</i> (70). Recent data indicates that DPIV may also be responsible for cleaving GLP-2<sup>1-33</sup> to GLP-2<sup>3-33</sup> both <i>in vitro</i> and <i>in vivo</i> in the rat (71). The kidney appears to be a major site for metabolic clearance of both GLP-1 and GLP-2 (72).

1.2 Intestinal Growth and Adaptation

In mammals, the small intestine is composed of four layers (73). The outermost layer is the serosa, which is continuous with the peritoneum. The second layer consists of two sheets of smooth muscle; the outer longitudinal and the inner circular, collectively called the muscularis externa. The third layer is the submucosa, which contains the blood vessels and lymphatics that supply the mucosa. The innermost layer is called the mucosa, whose luminal surface presents an absorptive surface, the finger-like villi, to the luminal contents. Three sublayers make up the mucosa: the muscularis mucosa, the lamina propria, and the epithelium. Each villus is comprised of a core of lamina propria, a layer composed primarily of connective tissue but also containing immune cells, neurons and mast cells, which is clothed by a single sheet of columnar epithelial cells, the most superficial layer of the mucosa. Within the epithelium, a single villus is supplied with new cells by several crypts of Lieberkühn, located in the trough-like depressions
between villi. A thin basal lamina separates the epithelial cells from the underlying lamina propria, and the mucosa is separated from the submucosa by the muscularis mucosa.

The intestinal epithelium is composed of four main cell types (74). Goblet and Paneth cells are thought to play protective roles in the intestinal epithelium, through the secretion of mucous and anti-microbial molecules, such as lysozyme and defensins, respectively. Enteroendocrine cells play important roles in a variety of gastro-entero-pancreatic functions via secretion of hormones into the circulation from their basolateral surface. The brush border membrane (BBM) of absorptive cells contains digestive enzymes and molecular transporters. The enzymes convert peptides into amino acids and di/tripeptides, and disaccharides into monosaccharides. These smaller moieties then cross the BBM through the facilitative transporter molecules along a sodium concentration gradient, traversing the enterocyte and entering the circulation directly. In contrast, fatty acids diffuse directly into the absorptive cells across the lipid bilayer and enter the bloodstream indirectly via the lymphatic system (75).

The currently accepted model of intestinal epithelial cell renewal stipulates that a single, infrequently dividing stem cell, located near the base of each crypt, supplies a clonal population of transit stem cells. These cells, in turn, divide more frequently and ultimately give rise to all four of the fully differentiated epithelial cell types (74). A few of these undifferentiated cells migrate towards the crypt base and become Paneth cells, however, most of them migrate up the villus, simultaneously differentiating into the other three mature cell types: enteroendocrine; goblet; and absorptive cells (74). Although enteroendocrine and goblet cells are also found in the crypt, absorptive cells are only located on the villus. Cells continue to migrate up the villus until they reach its tip, at which point, they are sloughed into the lumen and removed with the fecal material. This
exfoliation event is most likely due to apoptosis or programmed cell death of mature enterocytes (76). The turnover time, after which the total differentiated cell population is replaced, is approximately 3 days in the rat (77) and 4-6 days in man (78). This entire process is highly regulated, requiring integration of the influences from many factors.

The preceding discussion describes the cyclical process of intestinal epithelial regeneration and maintenance of normal gut morphology. However, situations do arise in which this normal process is significantly altered and the intestine undergoes a process of adaptation. Intestinal adaptation can take several forms. For example, if the epithelial barrier has been breached, a mechanism called restitution is invoked which involves sealing the wound by migration of mature epithelial cells into the afflicted area (re-epithelialization), followed by an increase in crypt cell mitogenesis (79). In intestinal hypertrophy, the affected gut undergoes dilatation and augmentation of the mucosal compartment. The mucosal enlargement is due primarily to an increase in cell number (cellular hyperplasia), although an increase in cell size (cellular hypertrophy) may also be observed (80). In the small intestine, these changes result from an increase in crypt cell proliferation followed by enhanced cell migration and consequent elongation of villi.

Several animal models have been used to study adaptive intestinal hypertrophy. The most common is massive small bowel resection (MSBR), involving removal of 50-90% of the length between a point 5 cm distal to the ligament of Treitz to a point 5 cm proximal to the ileo-cecal valve (81). After resection, the following structural and functional changes are observed in the remaining bowel: dilatation, mucosal hyperplasia, and enhanced absorption (80). Other models used to study hypertrophic adaptation include: experimental diabetes, lactation, cold acclimation, and neonates receiving their first enteral meal (82,83). Furthermore, other surgical manipulations, either alone or in the
presence of MSBR, have proven to be useful in studying the potential involvement of humoral factors in the adaptive response and are discussed in detail below (see section 1.3.2).

Patients suffering from severe bowel ischemia or inflammatory bowel disease may require small intestinal resection of the affected area. Normal human small intestine has an average length between 12-20 feet (365-600 cm), and patients with greater than 75% resection are at greatest risk for severe compromise of absorptive capacity (84). The resulting condition is called short bowel syndrome (SBS), which is characterized by malabsorption, malnutrition, and possibly steatorrhea (84). As mentioned above, the bowel remaining after MSBR displays a compensatory adaptive response. Thus, intestinal-specific growth factors may be useful in the treatment of SBS.

When an animal is starved or placed on total parenteral nutrition (TPN), its intestinal architecture atrophies from lack of luminal stimuli (85,86). Essentially the reverse of hypertrophy, atrophy is characterized by a significant reduction in villus height, crypt depth and circumference.

1.3 Regulation of Mucosal Growth

Numerous factors are believed to be involved in controlling the complex process of intestinal mucosal growth. These include exogenous factors, such as luminal nutrition, as well as various internal mediators from endocrine, exocrine, neural and immune sources. Although many studies do not measure it directly, the accepted parameter indicating stimulation of intestinal growth is an increase in the crypt cell production rate (CCPR) (87). At the cellular level, small intestinal epithelial growth can be ascribed to
any of the following three mechanisms, acting alone or in combination: decreased cell cycle time, decreased crypt-to-villus transit time, or increased ratio of proliferating (crypt) to functional (villus) cells (87). An increase in villus cell life span may also lead to an increased intestinal mass and villus height (88), however this would not constitute a true increase in intestinal growth as measured by enhanced cellular proliferation.

Although the epithelium is normally the most rapidly proliferating compartment of the intestine, it is important to recognize that the other layers of the intestinal wall can also increase or decrease in size in response to various situations. For example, adaptation following surgical resection or induction of diabetes is characterized by an increase in the entire gut circumference, including the muscularis externa (80,89). In fact, within the mucosa itself, it is clear that the elongation of villi observed during adaptation cannot be accounted for simply by an increase in epithelial cell proliferation. Because of the contribution of both the epithelium and the lamina propria to villus structure, this elongation process must involve both of these mucosal compartments, although the mechanism by which this interaction is regulated is currently unknown. Some researchers have shown that the differentiation of epithelial cells can be influenced by interaction with components of the underlying lamina propria, particularly the pericryptal fibroblasts (90).

Thus, the mucosa most likely possesses an inherent control system in which local juxtacrine, paracrine and autocrine signalling mechanisms regulate its growth and regeneration (90,91). Long term intestinal cell cultures are notoriously difficult to establish due to the presence of local microbial fauna. Therefore, unlike the isolation of haematopoietic stem cells, putative gut stem cells have yet to be isolated and the field of intestinal growth regulation is still in its infancy. Accordingly, a definitive distinction between the concepts of proliferation and differentiation remains unclear in the context
of intestinal growth control. For example, if, as Galjaard et al have proposed (91), crypt cell proliferation is under the influence of feedback control by mature villus cells, then a factor that induces differentiation of a crypt cell into an absorptive cell type may thereby indirectly affect crypt cell proliferation.

Depending on the investigator, a number of endpoints have been used to demonstrate bowel growth. These include increases in wet/dry weight and length of whole or segmented intestine, protein and/or DNA content of mucosal scrapings, incorporation of tritiated thymidine or bromodeoxyuridine (BrdU) into cells or tissue slices, metaphase arrest, crypt depth and villus height. Although some of these techniques either directly measure mitosis (eg. metaphase arrest), or correlate well with such measurements (eg. villus height), many others may be misleading. For example, mucosal scrapings invariably contain submucosal components; in fact, it has been estimated that about 10% of cells in such scrapings are not epithelial (87). These non-epithelial cells would include immune cells from the lamina propria, which are well known for their high proliferative capacity, yet few researchers using mucosal scrapings have reported proper controls ruling out lymphoproliferation. Thus, observed increases in the content of mucosal DNA, RNA or protein, do not necessarily imply increased epithelial proliferation. Similarly, compartments in addition to the epithelium may contribute to perceived increases in gut weight.

The intestinal mucosa is a complex three-dimensional structure. This presents a problem when growth data is based on changes observed within two-dimensional tissue sections. A parameter such as villus height, although it correlates well with CCPR when the villi are finger-like (87), tells us nothing about changes in villus circumference or shape. Also, changes in this parameter are insensitive to potential changes in the
absolute number of villi, although it is believed that this does not change under most circumstances (87). Similarly, crypt depth reveals little about changes in crypt width or the absolute number of crypts. Furthermore, techniques measuring changes in compartment height cannot distinguish between cellular hyperplasia and cellular hypertrophy. Crypt depth, in particular, seems to be an unreliable measure of mucosal growth. For example, injection of GLP-2 into mice results in increased small intestinal wet weight, villus height, and increased proliferating cell nuclear antigen (PCNA) labelling of crypt cells, but crypt depth remains unaltered (63). Therefore, when making a case for intestinal mucosal growth, it is necessary to examine several endpoints in order to obtain a comprehensive assessment of the observed changes.

1.3.1 Nutrients

It has long been recognized that the presence of food in the intestinal lumen promotes maintenance of the gut architecture. Animals that have been starved or supplied only with TPN, display marked intestinal atrophy compared to those receiving normal luminal nutrition (85,86). The consensus appears to be that it is the chemical nature, rather than the bulk of the ingested matter, which is responsible for maintaining the mucosal architecture (92).

Local nutrition is one possible mechanism whereby nutrients stimulate mucosal growth (93). In this model, the unique location of the villus cells permits them to use fuel molecules prior to them entering the circulation. Thus, the metabolic activity of these cells increases and this is somehow translated into a proliferative effect on the crypt compartment, perhaps via a mechanism involving polyamines (see 1.3.3.2). However,
luminal infusion of isotonic solutions of galactose, methylglucoside and NaCl, all substances not metabolized by the mucosa, stimulate cell proliferation to the same extent as glucose (94). A feature that these substances share with glucose is that they are all actively transported across the epithelium. Thus, an increase in functional workload may constitute another mechanism whereby luminal nutrients influence mucosal growth.

Luminal nutrients are known to stimulate secretion of gut hormones, some of which are trophic to the intestine itself. Examples of such intestinotrophic factors include gastrin and GLP-2. In fact, the release of such factors may constitute a third mechanism by which nutrients stimulate bowel growth (see 1.3.2). Many models used to study adaptation are associated with hyperphagia. Increased intake of luminal nutrients following MSBR is essential to the adaptive response seen in resected rats (95,96). Furthermore, hyperphagia is associated with experimental diabetes, cold acclimation and lactation (83,97). It has therefore become important to distinguish between the effects of nutrients and endogenous factors during intestinal growth.

1.3.2 Regulatory Peptides

There is a large body of literature describing the trophic actions of numerous peptides on the intestinal epithelium. Methods used to study the effects of various peptides include exogenous administration in both normal and disease states, hypersecretion from ectopic tumours, and the stimulation or disruption of endogenous production. Many peptides trophic to the gut are secreted from various cell types located along the intestinal tract itself. These may be separated into the following three categories: those secreted into the lumen by exocrine glands, those produced locally by
the epithelial cells themselves, as well as neurons and immune cells, and those released from enteroendocrine cells. Products secreted from this last cell type may potentially influence intestinal growth via endocrine, paracrine or autocrine mechanisms. Besides the gut, it must be noted that other endocrine tissues also secrete peptides known to stimulate intestinal growth.

If one partner of a pair of animals in vascular parabiosis is subjected to MSBR, not only does the remaining bowel of this animal become hyperplastic, but so does the bowel of the un-operated partner (98). Similar results have been shown for twin pigs sharing a crossed-circulation (99). A Thiry-Vella fistula is a surgical manipulation achieved by creating a self-emptying loop from the intervening segment of bowel following a resection. In this preparation, the vasculature of the bypassed segment is left intact and one of its ends is anastomosed to the abdominal wall. In this model, the bypassed segment shares the hyperplastic response of the gut in continuo, both after the surgery and when the animal is fed enterally (100). Taken together these studies therefore indicate that the endocrine component of the adaptive response to resection is critical.

1.3.2.1 PGDPs

A role for the intestinal PGDPs in gut growth has been postulated since the early 1970’s, when Gleeson and coworkers described a patient presenting with a renal endocrine tumour; this tumour secreted high levels of the PGDPs and was associated with marked small bowel villus hypertrophy (101). After tumour resection, the affected bowel returned to normal. Furthermore, intraperitoneal injection of extracts from this tumour into mice resulted in macroscopic enlargement of the small intestine (102).
Follow-up work by Bloom indicated these PGDPs were of intestinal origin, so-called gut glucagon-like immunoreactivity (gGLI) (103). In 1984, a second patient carrying a tumour secreting high levels of gGLI also exhibited enlarged small intestinal villi (104). gGLI actually refers collectively to the intestinal PGDPs glicentin and oxyntomodulin (fig. 1; see also chapter 2, section 2.2.7).

There exists a large body of literature describing a correlation between gGLI and adaptive gut growth. Plasma levels of gGLI increase after small intestinal resection in both rats (83,105) and man (106). Furthermore, in rats, both tissue and plasma levels of gGLI increase in proportion to the extent of small intestinal resection (105). Patients with adaptation consequent to villus cell loss in celiac disease or tropical sprue also show significantly elevated plasma levels of the intestinal PGDPs (107). Furthermore, cold acclimated and lactating rats also exhibit elevated plasma levels of gGLI in association with bowel growth (83). Some researchers have reported an increase in ileal proglucagon gene expression after MSBR (108,109), while one abstract has reported a direct effect of partially purified gGLI (possibly glicentin) on DNA synthesis in cultured guinea-pig jejunum (110).

While studying the effects of proglucagon gene overexpression in glucagon-SV40 T antigen (GLUTag) transgenic mice, Drucker and coworkers observed that, in addition to developing proglucagon-expressing tumours and elevated plasma levels of the PGDPs, these animals also exhibited small intestinal hypertrophy [see 1.1.2, (51,63,111)]. A follow-up experiment demonstrated that this effect was not unique to GLUTag mice. In fact, subcutaneous passage of four distinct proglucagon-expressing cell lines in nude mice resulted in small bowel growth in each case (63). Although these experiments showed a causative relationship between elevated plasma concentrations of the intestinal
PGDPs and stimulation of intestinal growth, it was impossible to determine the relative contributions of each PGDP to the observed phenotype, as the plasma of these animals was characterized by a simultaneous elevation in the concentrations of several of the PGDPs.

This point was subsequently resolved after subcutaneous injection of synthetic PGDPs (glicentin, GLP-1, IP-2, GLP-2) into normal mice (63). This bioassay demonstrated that GLP-2 was the only PGDP which stimulated significant increases in small bowel wet weight, villus height, and, most importantly, crypt cell proliferation, as measured by immunohistochemical staining for PCNA (63). In the same study, glicentin also stimulated an increase in small intestinal wet weight but failed to increase either villus height or PCNA-immunopositive crypt cell staining. Prior to the elucidation of the complete proglucagon gene structure (1-4,11-13), researchers were unaware of the nature of the intestinal C-terminal glucagon-like peptides. Taken together with the evidence that the intestinal PGDPs are secreted in parallel (see 1.1.2), it is most likely that GLP-2, rather than gGLI, was the actual PGDP responsible for the growth effects observed in both the tumour-bearing patients and the experimental models described in the preceding paragraph.

Some researchers have reported that the intestinal PGDPs inhibit intestinal proliferation. One group examined the effects of GLP-1 and GLP-2 on DNA synthesis in IEC-6 cells, a rat jejunal crypt cell line. They found that neither peptide had an effect on serum-starved cells, but that GLP-2 reduced serum-induced increases in DNA synthesis in a dose-dependent manner (112). Gregor et al reported inhibition of proliferation when purified gGLI was administered to FRIC cultures (113). This group also reported that immunoneutralization of gGLI had no influence on intestinal growth in rats in vivo (113).
This work may be criticized on the basis that the molecular identity of the gGLI used in these experiments was never defined. Furthermore, in view of the evidence that GLP-2 is the intestinotrophic PGDP, it is not surprising that immunoneutralization of gGLI had no effect on bowel growth.

Finally, it should be noted that pancreatic glucagon has also been reported to influence gastrointestinal epithelial growth. When administered to cultured human embryonic intestinal epithelial cells it increased proliferation (114) and, after two days of intraperitoneal injections in rats, stimulated DNA synthesis in oxyntic gland and colonic mucosa (115). However, others found no intestinal growth effects after 7 days of glucagon infusion into both normal rats and animals with partial small bowel resection receiving TPN (116). Moreover, Lorenz-Meyer et al reported that exogenous glucagon actually reduced villus height and mucosal cell migration (117). Thus, a role for glucagon in intestinal epithelial cell proliferation is unclear.

In summary, of all the intestinal PGDPs it appears that GLP-2 is by far the most potent stimulator of small intestinal mucosal growth. Furthermore, in all studies to date, the growth promoting effects of GLP-2 appear to be specific to the intestine, suggesting that it may be a potentially useful agent in the treatment of patients suffering from compromised absorptive capacity.

1.3.2.2 Other Enteroendocrine Peptides

As with gGLI, a role for gastrin in gut growth was first surmised from a clinical observation; in this case it was noted that patients with Zollinger-Ellison syndrome displayed mucosal hyperplasia coincident with hypergastrinemia (118). Experimental
work studying the effects of gastrin on the gastrointestinal tract was initiated by Johnson and colleagues with their report that injection of exogenous pentagastrin (a gastrin analog) into fasted rats induced a stimulation of protein synthesis in oxyntic gland and duodenal mucosa \textit{in vitro} (119). Although earlier studies claimed a proliferative effect was observed in mucosa along the entire gastrointestinal tract, it is now clear that this action of gastrin is confined primarily to the oxyntic gland of the stomach and the duodenum (120). Opinions vary as to the relative contribution of gastrin to the endocrine response after MSBR. One group reported that plasma gastrin peaked at two days but remained elevated 10 days after resection, as compared to transected controls (121). Others have found that serum gastrin levels do not correlate with MSBR (122).

Some investigators have shown that gastrin stimulates mucosal proliferation in the colon (123), however the significance of this remains controversial (120). Gastrin has been shown to stimulate proliferation of several colonic cancer cell lines \textit{in vitro} (124). Finally, a recent report indicates that unprocessed progastrin is mitogenic for colonic mucosa in mice [see 3.4, (123)].

Cholecystokinin (CCK), a structural relative of gastrin is not believed to have a direct effect on mucosal growth, but most likely induces growth indirectly via stimulation of pancreaticobiliary secretion [see 1.3.3, (116)]. As bile acids have been found to stimulate L cell secretion in both man and dog (125,126), this suggests that the CCK growth response could, in fact, be mediated by the intestinal PGDPs. Exogenous neurotensin stimulates mucosal DNA and protein synthesis in both the gut \textit{in continuo} and the bypassed segment of rats with Thiry-Vella fistulas (127).

PYY is another L cell product whose plasma levels rise in models of adaptation (128). An increase in ileal PYY gene transcription occurs after MSBR, suggesting an L
cell - rather than a proglucagon-specific - response (129). While not trophic to the gut, PYY is believed to mediate the 'colonic brake' (130) effect seen in adaptation, although GLP-1 may also contribute to this by inhibition of gastric emptying (see 1.1.3).

In addition to stimulating mucosal proliferation, some enteroendocrine peptides have been shown to inhibit intestinal growth. Somatostatin infusion resulted in decreased DNA synthesis index in small intestinal mucosa of normal rats (131). This may involve a GLP-2-dependent mechanism, as somatostatin also inhibits L cell secretion in rats (31). Secretin inhibits gastrin-stimulated DNA synthesis in oxyntic gland and duodenal mucosa (132).

Therefore, many gut peptides have been reported to influence intestinal mucosal growth. In most cases, it is unclear whether these hormones mediate their effects directly or indirectly.

1.3.2.3 Other Endocrine Peptides

A number of reports suggest a role for pituitary hormones in small bowel growth. Hypophysectomized rat pups show a decreased small intestinal growth rate (133), while in adult rats, hypophysectomy results in a reduced capacity for adaptive growth in response to resection (134). The presence of growth hormone (GH) receptor expression in rat intestine suggests this hormone may play a role in intestinal growth (135). A transgenic mouse model of GH overexpression displays greater small bowel mucosal mass and jejunal villus height, but no difference in CCPR, suggesting an effect on cell lifespan rather than hyperplasia (88).

The insulin-like growth factors (IGFs) 1 and 2 are peptides sharing structural and
functional homology with insulin (136). The IGFs are produced principally by the liver, although local synthesis in many tissues, including the gastrointestinal tract (137,138), indicates these peptides may have important paracrine actions. Both IGFs have been shown to stimulate intestinal epithelial mitogenesis. This appears to be a direct effect, as receptors for both of the IGFs are expressed in the gastrointestinal epithelium (139). 

_in vivo_, subcutaneous infusion of IGF-1 results in increased CCPR in both normal rats (140) and after jejunoileal resection (141). Oral administration of IGF-1 to neonatal pigs also increases intestinal mucosal growth (142). Overexpression of IGF-1 in transgenic mice enhances small bowel mass and length, as well as increasing villus height and crypt depth (143). In addition, the mucosa of these mice also showed increased crypt cell proliferation, which contrasts with the GH transgenic mice described above (88). It is generally believed that IGF-1 mediates the growth-promoting effects of GH (136). However, the observed differences between the GH and IGF-1 transgenic mice indicate that these peptides exert independent effects on gastrointestinal (GI) epithelial growth. Further evidence for a direct effect of GH and IGF-1, as well as insulin, has been shown in cultured human duodenal mucosa (144). One report suggested that IGF-2 stimulated gut growth in adult mice, but the data was not shown (145). In any case, such an effect is probably not physiological as IGF-2 is considered to be important only in fetal and neonatal development (136). The mitogenic effects of IGF-1, IGF-2 and insulin are most likely mediated by the same receptor, namely the IGF-1 receptor (136,144).

In the human fetus, IGF-1 mRNA is expressed in the lamina propria and submucosa (138), but immunoreactive peptide is found in the epithelial cells (146). Taken together, these observations could support a paracrine mechanism of action for IGF-1 in gastrointestinal growth, although a study combining both in situ hybridization and
immunohistochemistry is required.

Thus, a number of endocrine hormones are capable of stimulating intestinal growth. Although circulating GH and the IGF-1 are general somatotrophins and stimulate the growth of many tissues, it appears that local expression of growth factors, such as IGF-1, may be important in paracrine control of mucosal proliferation.

1.3.2.4 Luminal Peptides

Epidermal growth factor (EGF), or its human homolog urogastrone, has been reported to stimulate mucosal proliferation after both intraluminal and intravenous administration in rats (147,148). Urogastrone is synthesized and secreted from the submandibular salivary glands and the duodenal Brunner's glands in the human gastrointestinal tract (149). Although secreted into the lumen, receptors for EGF are located exclusively on the basolateral membranes of enterocytes (150). Also, normal plasma levels of EGF never reach those required for stimulation of mucosal mitogenesis. Taking these two facts into consideration, it has been proposed that the physiologic role of EGF is to act as a “surveillance” factor, capable of stimulating mitogenesis via its receptor only if the mucosal barrier has been breached (151). This idea is supported by the description by Wright and colleagues (152) of what is believed to be a novel EGF-secreting cell lineage found at the borders of mucosal ulcerations in biopsies from patients with various inflammatory bowel diseases.

Trefoil peptides, named for their compact clover leaf-like structure which makes them highly protease-resistant, are secreted into the lumen from various sites along the GI tract. Three members of this family have been reported to date; pS2, pancreatic
spasmolytic peptide, and intestinal trefoil factor (ITF). These peptides stimulate epithelial cell migration during restitution but have no effect on epithelial cell proliferation (153). The novel ulcer-associated cell lineage described above also contains high levels of the trefoil peptides (154), and mice lacking ITF display a delayed healing response to mucosal ulceration induced by acetic acid (155).

Thus, it appears that the primary role of the luminal peptides may be in epithelial restitution following damage to the mucosal barrier and stimulation of mitogenesis may be a secondary effect.

1.3.2.5 Local Peptides

This section discusses peptides expressed by the epithelial cells themselves as well as those produced by local neurons, immune cells and mesenchymal cells. Transforming growth factor α (TGFα) is only 35% identical to EGF but signals its mitogenic effects through the EGF receptor (EGFR), as there is no distinct receptor for TGFα (153). Interestingly, within the intestinal mucosa, TGFα mRNA and protein levels are highest in the villus cells and lowest in the crypt compartment. Because the expression of TGFα is greater than that of EGF in the human fetal intestinal epithelium, it has been suggested that TGFα, rather than EGF, is the physiologic ligand for EGFR in the gastrointestinal tract (156).

TGFβ is structurally distinct from the EGF/TGFα ligand family and has growth inhibitory effects on intestinal epithelial cells (90). In the intestine, TGFβ expression is highest in crypt cells and lowest in villus cells; the converse of the expression pattern seen for TGFα. TGFβ is also a potent stimulator of mucosal cell migration and may play
a role in epithelial restitution (157). Another local cytokine found in the gut mucosa is interleukin-11 (IL-11). Most likely produced by lymphocytes in the lamina propria, IL-11 increases CCPR and villus height when administered after radiation/chemotherapy in mice (158) and after 90% MSBR in rats (159).

Basic fibroblast growth factor (FGF) modulates proliferation and migration in several intestinal epithelial cell lines (160) and when administered orally, accelerates the healing of duodenal ulcers in rats (161). Keratinocyte growth factor (KGF), another member of the FGF family, and its receptor are both expressed in epithelia along the normal rat GI tract. Administration of exogenous KGF to rats results in selective induction of goblet cell lineages in a dose-dependent fashion (162).

Exogenous bombesin, whose mammalian homolog is the neuropeptide GRP, induces modest increases in mucosal weight and DNA and protein content in rats fed either an elemental diet or treated with methotrexate (163). As GRP stimulates intestinal PGDP secretion, at least in the rat [see 1.1.2; (31)], the intestinotrophic effect of this enteric plexus neuropeptide may be mediated by GLP-2.

1.3.3 Pancreaticobiliary Secretions

Diversion of pancreaticobiliary secretions by transplanting duodenal papillae to the ileum results in ileal hypertrophy but duodenal atrophy, suggesting that these secretions are trophic to the mucosa of both normal (164) and resected (165) intestine. These secretions may also account for the proximal-distal gradient of villus height found along the aboral intestinal axis of normal animals. Interestingly, bile acids alone can stimulate secretion of the intestinal PGDPs (125,126), suggesting that GLP-2 may be involved in
mediating the trophic effects of these secretions.

1.3.4 Cellular Mechanisms of Intestinal Adaptation

1.3.4.1 Polyamines

Polyamines are ubiquitous molecules involved in metabolism and DNA, RNA and protein synthesis in all cells (166). Ornithine decarboxylase (ODC) catalyses the rate-limiting step in their synthesis and the activity of this enzyme is often used as a marker of cell proliferation in models of intestinal adaptation. Luk and Baylin have reported a significant correlation between increases in CCPR and villus height with upregulation of ODC activity following MSBR in the rat (167). A subsequent study showed that treatment of resected rats with α-difluoromethyl ornithine, a specific irreversible ODC inhibitor, prevented the adaptive epithelial hyperplasia usually seen after this operation (168). Conversely, suppression of diamine oxidase activity, the key enzyme for polyamine degradation, results in greater adaptive mucosal hyperplasia than that which occurs spontaneously after resection (169). Thus, polyamines appear to be integral to the adaptive response to small bowel resection.

1.3.4.2 Signal Transduction Pathways

Insulin and IGF-1 are believed to exert their mitogenic effects through receptor tyrosine kinases (RTKs) linked via insulin receptor substrate-1 (IRS-1) to the Ras/MAPK (mitogen activated protein kinase) and phosphatidylinositol 3-kinase (PI3K) intracellular signalling pathways (170,171), whereas growth hormone signals through a Jak/STAT
(Janus kinase/signal transducers and activators of transcription) pathway (172). The RTK and Jak/STAT signalling cascades have for some time been considered the classical pathways involved in mediating the mitogenic effects of extracellular growth factors (173). However, only recently has it become clear that seven transmembrane G protein-linked receptors may also be involved in mitogenic signalling (173,174). That G protein-coupled signalling is important in intestinal mitogenesis is supported by the observation of colon adenocarcinoma in mice lacking a $\alpha_2$ subunit (175). To date, gastrin is the only gut peptide known to mediate its mitogenic effects directly through a G protein-coupled receptor, namely the gastrin/CCK$_6$ receptor (176), which is linked to a pertussis toxin-sensitive G-protein (177). Gastrin has also been shown to stimulate phosphorylation of IRS-1 and its association with PI3K (178). Wang et al (179) have reported that gastrin stimulates expression of the protooncogene c-myc in a rat crypt cell line through a mechanism involving polyamines. Thus, it appears that intestinal growth factors can signal their mitogenic effects through a number of different intracellular signalling pathways. The GLP-2 receptor has not been identified and it is therefore unknown whether GLP-2 mediates its proliferative effects directly or indirectly, a topic discussed further in chapter 4.

1.4 Hypothesis and Experimental Rationale

Exogenous GLP-2 has been shown to be a potent stimulator of small intestinal epithelial proliferation. However, to date no studies have looked at the role of endogenous GLP-2 in bowel growth. Thus, the aim of the first project in this study was to examine GLP-2 levels in a physiologic model of gut growth, namely the intestinal
adaptation that accompanies streptozotocin-induced diabetes in rats. We hypothesized that the plasma concentration of GLP-2 would be elevated in these rats, indicating that GLP-2 may be responsible for the observed bowel adaptation.

As GLP-2 appears to be specific to the gastrointestinal tract, it may be an excellent candidate for treatment of patients with compromised intestinal function, such as those suffering from short bowel syndrome. The proglucagon gene encodes several bioactive peptides and, in vivo, overexpression of this gene results in a complex phenotype in which one cannot distinguish between the effects of the various PGDPs. Thus, the rationale for the second project in this study was to engineer a cell line that secreted, as its primary product, high concentrations of GLP-2. We hypothesized that after subcutaneous injection into nude mice, these cells would form tumours that would continue to secrete elevated levels of GLP-2 and thus induce small bowel growth.
2.1 Introduction

A number of reports have described an increase in gut weight in rodent models of poorly controlled diabetes (82,97,180). This appears paradoxical in view of the general catabolic phenotype of this disease, however it may constitute a mechanism to compensate for a perceived state of starvation. Intestinal growth is apparent as early as nine days following induction of diabetes and persists for the period of metabolic derangement (97,181); the changes are reversible with insulin treatment (181,182). During the early growth phase, the predominant characteristics are increased mucosal mass and enhanced DNA synthesis in the crypts (82,182). Independent studies have shown elevated levels of both plasma and tissue intestinal PGDPs in diabetes. In rats, streptozotocin (STZ) treatment induces elevation of plasma gGLI (183), as well as increases in the levels of ileal GLP-1 (184). In view of the recent demonstration that GLP-2 is trophic to the intestinal epithelium [see 1.1.4; (63,64)], we hypothesized that GLP-2 may be increased in diabetes and may play a role in diabetes-associated intestinal growth. To further study the relationship between GLP-2 and intestinal growth, we have now examined the synthesis and secretion of GLP-2 in STZ-induced diabetic rats as a model of gut adaptation.

2.2 Materials and Methods

2.2.1 Induction of Diabetes
Adult male Wistar rats (320-380 g; Charles River Canada Ltd., St. Constant, Quebec, Canada) were made diabetic on day 0 by injection of STZ (65 mg/kg; Sigma Chemical Co., St. Louis, MO, USA) into a lateral tail vein. Age-matched control animals (Controls) were injected with vehicle (0.9% saline) only (n=7-8 rats per group). STZ-treated rats were given 5% glucose in their drinking water for the first 24 hours to counter any initial hypoglycemia. After injection, rats were housed individually in wire-bottom metabolic cages and permitted access to standard rodent chow and water *ad libitum* for three weeks. On day 1 STZ-treated animals were paired according to urine glucose and volume and then divided into two groups. One group (STZ + I) received daily injections of porcine NPH insulin (1-3 Insulin Units, subcutaneously; Eli Lilly Co., Indianapolis, IN, USA) at 1700 hours on day 1 and thereafter. Insulin dose was adjusted daily based on urine glucose, urine volume and body weight. The second group (STZ) remained untreated. In a second series of experiments, vehicle and STZ-injected animals (n=4 per group) were sacrificed on day 4.

2.2.2 Plasma and Tissue Collection

Rats were sacrificed by decapitation on day 21 or day 4 after STZ or vehicle injection. Trunk blood was collected into chilled tubes containing a 10% volume of 5000 KIU/ml Trasylol (Miles Canada, Etobicoke, Canada): 1.2 mg/mL EDTA: 0.1 mM Diprotin A (Sigma Chemical Co., St. Louis, MO, USA), and plasma was collected after centrifugation at 4 °C. The entire small intestine from pylorus to ileo-cecal junction was removed, flushed with ice cold saline, and weighed. It was then divided into four equal pieces representing duodenum, proximal and distal jejunum, and ileum. Sections for
analysis were collected from the identical anatomical position (as measured in centimeters from the pylorus and cecum) along the length of the small intestine.

2.2.3 Intestinal Protein and Lipid Content

Wet weight was determined by weighing 5 cm segments from each small intestinal region (i.e. duodenum, proximal and distal jejunum, and ileum). These same segments were then lyophilized for at least 12 hours and reweighed to obtain dry weight. They were then individually reconstituted overnight with a volume of distilled water equal to the difference between wet and dry weight. The reconstituted segments were subjected to lipid extraction following the method of Folch (185). In brief, tissues were homogenized in 20 volumes of chloroform:methanol (2:1). An additional 4 volumes of methanol was added and the segments homogenized again. Following centrifugation at 4 °C, supernatant and pellet were separated, dried in vacuo and weighed. Total protein content of the resuspended pellets was determined by Lowry protein assay (186).

2.2.4 Intestinal Morphometry

2 cm segments from each of the four small bowel regions were fixed in 10% buffered formalin, dehydrated and embedded in paraffin. Cross-sectional slices 5 μm thick were stained with hematoxylin and eosin. Micrometric quantitation was achieved using a Leitz microscope connected to a Mertz square-based micrometer (Leica Q500MC Image Processing and Analysis System, Leica Cambridge Ltd, Cambridge, UK). A minimum of 10 to a maximum of 70 villus-crypt units per section in 2-4 sections per
region per rat were analyzed in a blinded fashion. Villus height, crypt depth and muscle thickness (including submucosa) were recorded as \( \mu m \pm \text{SEM} \), averaged for each intestinal region.

2.2.5 RNA Analysis

5 cm of distal ileum was homogenized in 5 mL guanidium isothiocyanate (ICN, Montreal, Quebec, Canada). Following phenol-chloroform extraction and ethanol purification, RNA was stored in DEPC water at -70 \( ^{\circ} \)C until analysis, as previously described (187). Total RNA was size-fractionated on a 1% agarose-formaldehyde gel. The gel was stained with ethidium bromide to assess RNA integrity and migration. The RNA was transferred to a nylon membrane using the downward alkaline blotting method (188) and cross-linked to the membrane by UV irradiation. Blots were prehybridized and hybridized in Denhardt’s (200 \( \mu g/mL \) each of polyvinyl pyrrolidine, bovine serum albumin and Ficoll), 4X sodium citrate salt solution (SSC, 0.6M NaCl and 0.06M Na citrate), 200 \( \mu g/mL \) herring sperm DNA, 40% deionized formamide in 0.014M Tris-HCl, pH 7.4. The cDNA probes for rat proglucagon and 18S (loading control) mRNA transcripts have been described previously (43). Probes were labelled with \(^{32}P\)dCTP to a specific activity of 8x10^8 cpm/\( \mu g \) using a Random Priming kit (Boehringer Mannheim, Laval, Quebec, Canada), and blots were hybridized with 1x10^6 cpm/ml of labelled probe for 16-24 hours at 42 \( ^{\circ} \)C. Final washing conditions were 0.2% SSC plus 0.2% sodium dodecyl sulphate at 65 \( ^{\circ} \)C. Autoradiography was carried out using Kodak X Omat film (Eastman Kodak, Rochester, NY, USA) at 70 \( ^{\circ} \)C.
2.2.6 Peptide Extraction

Ileal peptides and plasma for PGDP RIAs were extracted using reversed phase adsorption techniques, as described previously (7,183,189). In brief, 5 cm distal ileum was homogenized in 10 mL extraction medium [1 M HCl containing 5% formic acid, 1% trifluoroacetic acid (TFA), and 1% NaCl] at 4 °C. Plasma for GLP-2 RIA was acidified by addition of 2 volumes 1% TFA (pH adjusted to 2.5 with diethyl amine). Peptides and small proteins were then collected by adsorption to octadecylsilyl silica (C\textsubscript{18} Sep-Pak, Waters Associates, Milford, MA, USA), followed by elution with 80% isopropanol containing 0.1% TFA. Extracts were stored at -70 °C before analysis.

2.2.7 Assays

Aliquots of ileal and plasma extracts were dried in vacuo before assay. RIAs for glucagon-like immunoreactivity used antiserum K4023 (Biospacific, Emeryville, CA, USA) which is directed toward the midsequence of glucagon and recognizes glucagon, glicentin and oxyntomodulin (GLI peptides). Immunoreactive glucagon (IRG) used antiserum O4A (R.H. Unger, Dallas, TX, USA) which recognizes the free carboxy terminal end of glucagon and therefore detects glucagon but not glicentin or oxyntomodulin (7,183,189). Plasma gutGLI equals GLI minus IRG and thus represents plasma glicentin plus oxyntomodulin. Ileal GLP-1 immunoreactivity was assessed using anti-GLP-1\textsuperscript{7-36NH2} antiserum (Affinity Research, Nottingham, UK) which recognizes the C-terminally amidated forms GLP-1\textsuperscript{1-36NH2} and GLP-1\textsuperscript{7-36NH2} (7,189). Total immunoreactive GLP-2 (T-GLP-2) in the ileum was measured using antiserum UTTH-7, which recognizes GLP-2\textsuperscript{25-30} and therefore detects GLP-2, proglucagon and the major proglucagon fragment (GLP-2
attached to GLP-1) (Dr. P.L. Brubaker, personal communication). Plasma N-terminal immunoreactive GLP-2 (N-GLP-2) was assessed using antiserum #92160 (a gift from Dr. J.J. Holst, Copenhagen, Denmark) which detects bioactive GLP-2\textsuperscript{1-33} but does not crossreact with degradation products, such as GLP-2\textsuperscript{33-33} (Dr P.L. Brubaker, personal communication). Ileal protein content was determined by Lowry protein assay (186). An insulin radioimmunoassay kit (Linco, St. Charles, MO, USA), with rat insulin as a standard, was used to determine plasma insulin levels. Plasma glucose values were determined by the glucose oxidase method (Glucose Autoanalyzer 2, Beckman Instruments, Fullerton, CA, USA).

2.2.8 Statistics

All data are expressed as the mean ± SEM. Statistical significance of data from animals after 3 weeks of treatment was assessed by ANOVA with n-1 custom hypothesis tests. Statistical significance of data from animals after 4 days of treatment was assessed by an unpaired t test between untreated diabetics (STZ) and controls. All statistics were analyzed using a SAS program for IBM computers (Statistical Analysis Systems, Cary, NC).

2.3 Results

Three weeks after STZ injection, the body weight of rats not receiving exogenous insulin was 27±2% lower than that of vehicle-injected controls (P<0.001; fig. 3). Insulin treatment partially prevented this weight loss, maintaining body weight at 92±3 % of
controls (P<0.05). Untreated diabetics were profoundly hypoinsulinemic (P<0.001) and this loss of insulin was accompanied by hyperglycemia compared to controls (P<0.001). Blood glucose appeared to have been poorly controlled by daily insulin injection, however it should be noted that these rats were not fasted at the time of sacrifice and had received their last insulin injection 18 hours prior to sacrifice.

Plasma concentrations of gutGLI (i.e. glicentin plus oxyntomodulin) in untreated diabetics were elevated to 177±15% those of controls (P<0.01; fig. 4). A parallel increase was observed for plasma N-GLP-2 (191±32%; P<0.05). IRG levels in plasma were also slightly greater in uncontrolled diabetes (P<0.05). Insulin replacement prevented the increments in plasma levels of all the PGDPs. Interestingly, there was a highly significant correlation between the concentrations of gutGLI and N-GLP-2 in plasma (r=0.86, P<0.001).

Distal ileum was sampled for PGDP content because it contains the greatest population of proglucagon-expressing L cells (20,21). Ileal concentrations of GLI and GLP-1 increased to 160±13% and 137±13% of controls, respectively, in untreated diabetics (P<0.05-0.001; fig. 5), a change that was prevented by insulin treatment. A similar trend was detected in ileal T-GLP-2 content, rising to 144±31% of controls in STZ rats and remaining at control levels with insulin treatment. Analysis of total RNA from ileum showed increased levels of proglucagon mRNA transcripts in STZ animals (1.5-fold increase by laser densitometry; data not shown). Proglucagon gene expression in STZ+I animals was not different from controls.

The weight of the entire small intestine was significantly increased in untreated diabetes. Wet weight of STZ-diabetic bowel was 73±18% (P<0.01) and dry weight was 76±20% (P<0.001) greater than that of control animals (fig. 6). With insulin treatment,
Fig. 3. Confirmation of STZ-induced diabetes in rats. Body weight, plasma insulin (IRI) and plasma glucose were determined in Control (solid bar; n=7), untreated-diabetic (STZ; open bar; n=8) or insulin-treated diabetic (STZ+I; hatched bar; n=8) Wistar rats after 3 weeks of treatment. * P<0.05 ** P<0.01 *** P<0.001 vs Controls.
Fig. 4. Plasma concentrations of gutGLI, N-GLP-2 and IRG in Control (solid bar; n=4), STZ (open bar; n=4) and STZ+l (hatched bar; n=4) rats after 3 weeks of treatment. Inset: plasma concentrations of N-GLP-2 in Control (solid bar; n=4) and STZ (open bar; n=4) rats after 4 days of treatment. * P<0.05 ** P<0.01 *** P<0.001 vs Controls.
Fig. 5. Ileal concentrations of GLI, GLP-1\textsuperscript{36NH\textsubscript{2}} and T-GLP-2 in Control (solid bar; n=7), STZ (open bar; n=8) and STZ+I (hatched bar; n=8) rats after 3 weeks of treatment. * P<0.05 ** P<0.01 *** P<0.001 vs Controls.
Fig. 6. Wet weight, dry weight, protein content and lipid content of 5cm intestinal segments from Control (solid bar; n=4), STZ (open bar; n=4), and STZ+I (hatched bar; n=4) rats after 3 weeks of treatment. Prox. Jej. = proximal jejunum. * P<0.05 ** P<0.01 *** P<0.001 vs Controls.
Fig.7. Villus height, crypt depth, and muscle thickness in intestinal sections from Control (solid bar; n=4), STZ (open bar; n=4), and STZ+I (hatched bar; n=4) rats after 3 weeks of treatment. * P<0.05 ** P<0.01 *** P<0.001 vs Controls.
Fig. 8. Representative histological cross-sections of duodenum from Control, STZ and STZ+I rats after 3 weeks of treatment (the bar represents 500 \( \mu \text{m} \)).
diabetic small intestinal wet and dry weight were not different from control values. Macromolecular analysis of the small intestine indicated that the observed weight increase was due to overall increases in both the protein (P<0.001) and lipid (P<0.05) content of diabetic gut (fig. 6).

Morphometry of bowel sections revealed major changes in the mucosal compartment in untreated diabetes (fig. 7). In duodenum from STZ rats, villus height was 51±6 % greater in STZ-diabetic rats compared to non-diabetic control animals (P<0.001) and this correlated strongly with plasma N-GLP-2 concentrations (r=0.78, P<0.01). Duodenal crypt depth was also augmented slightly in untreated diabetic animals (117±5% of controls; P<0.05). Insulin treatment prevented these increases in villus height and crypt depth. No differences in thickness of the underlying submucosal and muscle layers were detected in diabetic animals. Figure 8 depicts the changes observed in cross-sections of duodenum.

In a second series of experiments, rats were sacrificed 4 days after STZ or vehicle treatment. In untreated diabetics, plasma levels of N-GLP-2 were significantly increased to 92±57% above controls (P<0.05; fig. 4). Small intestinal wet weights of 4 day diabetic rats were not different from those of controls (data not shown).

2.4 Discussion

Substantial evidence supports a role for the intestinal PGDPs in gut growth (83). Small bowel hypertrophy was described in two patients carrying proglucagon-expressing tumours which secreted the intestinal PGDPs (101,104). Tumour resection resulted in
normalization of the affected bowel, and subsequent injection of the tumour cells into normal mice stimulated intestinal growth in these animals (83). Recent work in our laboratory has shown that exogenous GLP-2 is a highly specific stimulator of intestinal epithelial growth (63). The present study is the first to demonstrate increased concentrations of both plasma and tissue GLP-2 that correlate with intestinal growth and adaptation to STZ-diabetes. Furthermore, both the elevation in GLP-2 levels and the bowel growth response were prevented by insulin therapy. Interestingly, the increments in plasma levels of GLP-2 preceded any changes in intestinal weight, thereby providing further evidence for a relationship between GLP-2 and the induction of intestinal growth in diabetes.

The significantly augmented mucosal compartment observed in the present study is consistent with previous reports which describe elongated villi and enhanced crypt depth as the primary structural changes associated with experimental diabetic intestine (181,190). Stereologic studies indicate these changes are due to crypt and villus cell hyperplasia as well as crypt, but not villus, cell hypertrophy (181). Thus, the villus:Crypt cell ratio is unaltered in STZ-diabetes (181). Increased activity of ODC and increased polyamine content have been associated with the hyperplasia of STZ-diabetes (191). Treatment with difluoro-α-methylornithine (an ODC inhibitor) prevented the hyperplasia in these rats and delayed epithelial growth in control animals.

Small intestinal morphology of mice treated with exogenous GLP-2 shows elongated villi but no change in crypt depth, although there is increased mitogenesis in the latter compartment (63,64). This differs somewhat from data presented here, in which intestinal adaptation to STZ-diabetes in rats was associated with increases in both villus height and crypt depth. It may be that the uni-dimensional parameter of crypt depth does
not accurately reflect the true activity of this dynamic three-dimensional compartment. Nonetheless, the two models share the feature of elongated villi and the present study is the first to have demonstrated a significant correlation between plasma levels of endogenous N-GLP-2 and villus height.

Insulin treatment of the diabetic animals prevented the increments in both plasma PGDP concentrations and intestinal growth. The effect of insulin on bowel growth is in agreement with other reports (181,182), and it has been shown that insulin prevents the hyperplasia of both the villus and crypt cells, but has no effect on the crypt cell hypertrophy seen in untreated diabetics (181). The data presented here is the first demonstration that the elevated plasma N-GLP-2 concentrations seen in STZ-diabetes are prevented by treatment of diabetic rats with exogenous insulin. A previous report showed that levels of ileal proglucagon mRNA transcripts from insulin-treated diabetic rats were no different from those of controls (183). In addition to confirming this observation, the current study showed that the levels of ileal proglucagon mRNA from untreated diabetics were, on average, 1.5-fold greater than those of controls (data not shown). Although a direct effect of insulin on the intestinal L cell has not been demonstrated, insulin has been shown to inhibit gutGLI release both in vitro (22) and in vivo (30). However, the current study, as in all in vivo studies, cannot distinguish between the effects of insulin, glucose or other metabolic abnormalities on the regulation of intestinal PGDP biosynthesis and secretion in diabetes.

Untreated diabetic rats eat 2-3 times as much as controls and it has been suggested that this hyperphagia accounts for the observed intestinal growth seen in these animals (97). However, diabetic rats pair-fed diets isocaloric with those of controls still exhibit significantly greater small bowel mass and enhanced crypt cell DNA synthesis.
This indicates that at least part of the growth response of intestinal adaptation in diabetes is independent of increased nutrient consumption. Nevertheless, the mechanism by which hyperphagia stimulates intestinal growth is unclear. The present study supports the idea that an increased nutrient load may stimulate release of hormones trophic to the intestine. The combination of hyperphagia and increased gastric emptying (192) observed in diabetic rats, results in a greater nutrient load being delivered to the small intestine. As luminal nutrients, including fat and carbohydrate, are physiologic L cell secretagogues in vivo (25,26,193), this provides a possible mechanism whereby increased luminal nutrients stimulate release of GLP-2 into the circulation thereby exerting a trophic action on the intestinal epithelium.

Release of gastrin, a hormone trophic to the mucosa of stomach and proximal small intestine (119,194), is also stimulated by nutrient intake. Three weeks after induction of diabetes, plasma and antral gastrin concentrations are 1.5 to 2-fold higher in ad libitum fed diabetic rats compared to non-diabetic controls (195). The increase in duodenal villus height and crypt depth observed in the present study could therefore be due in part to the action of gastrin. However, as the mitogenic effects of gastrin in the small intestine are specific to the duodenum (120), elevated plasma levels of this hormone cannot account for the increased growth response observed in more distal regions of the bowel. Furthermore, after massive small bowel resection, serum gastrin levels do not always correlate with the observed intestinal growth in the non-resected remnant (122).

Other humoral factors, such as growth hormone (GH) and insulin-like growth factor-1 (IGF-1), may also play roles in adaptation of the gut to diabetes. In STZ-diabetes, plasma levels of GH are reported to increase (196), but those of IGF-1, a
molecule believed to be an important mediator of GH action, are depressed to approximately 50% those of non-diabetic controls (197,198). Given the general catabolic state of diabetic animals, it is unlikely that fluctuations in plasma concentrations of general somatic growth factors like GH and IGF-1 are responsible for the organ-specific changes observed in experimental diabetes. It is more likely that molecular candidates for promoting intestinal adaptation would be paracrine/autocrine regulators and/or tissue-specific trophic hormones such as gastrin and GLP-2.

In experimental diabetes, small intestinal functional adaptation accompanies the observed morphological changes. Protein and mRNA levels of both the apical sodium-dependent glucose luminal transporter (SGLT-1) and the basolateral facilitative glucose transporter GLUT-2 are significantly elevated in enterocytes from diabetic rats as compared to non-diabetic and insulin-treated controls (199). A previous study showed that diabetic rats also exhibit an enhanced ability to absorb 3-O-methylglucose in both jejunum and ileum (200). In the context of the present study, it is interesting that Cheeseman and Tsang have reported enhanced basolateral glucose uptake following acute 4 hour infusion in rats (65). Recent work indicates that GLP-2-treated mice display an increase in \textit{in vitro} disaccharidase activity that is proportional to the increase in mucosal growth (201). However, these researchers also reported no change in the ability of these mice to absorb glucose \textit{in vivo}. Furthermore, the small intestine of GLP-2-treated mice actually showed decreased levels of both SGLT-1 and GLUT-2 mRNA transcripts (201). Thus, besides stimulating intestinal proliferation, GLP-2 may also be involved in modulating carbohydrate metabolism and transport, although the latter remains controversial.

In summary, these data are the first demonstration that plasma and tissue
concentrations of GLP-2 are upregulated in a physiologic model of intestinal adaptation. Our results provide strong correlative evidence linking the synthesis and secretion of the intestinal PGDPs, including GLP-2, to the adaptive response of the small bowel in STZ-induced diabetes in rats. As GLP-2 is the major intestinal PGDP shown to have significant trophic effects on the bowel (63), our data strongly implicate GLP-2 as the mediator of small intestinal growth in uncontrolled diabetes.
Chapter 3  A GLP-2 Overexpression System

3.1 Introduction

One method of studying the importance of a molecule for a certain biological effect is to overexpress it in vivo. For example, transgenic mice overexpressing IGF-1 and GH have been very useful in studying the roles of these molecules in intestinal growth [see 1.3.2.3; (88,143)]. Unlike the genes encoding these hormones, the proglucagon gene encodes several peptides, whose individual biological activities are only now coming to light (see section 1.1.3). In both transgenic mice overexpressing the proglucagon gene (111) and nude mice carrying subcutaneous proglucagon-expressing tumours (63,202,203), a simultaneous increase in the plasma levels of all measured PGDPs was found. In addition to PGDP hypersecretion, these two models both exhibit significant small intestinal growth. Thus, in these models it was impossible to determine the relative contributions of the individual peptides to the observed phenotype. However, it has recently been demonstrated that when synthetic rat intestinal PGDPs are injected into mice, GLP-2 is the PGDP with the most potent effect on intestinal epithelial cell growth (63). The observation that injection of extracts from PGDP-producing human tumours induced small bowel growth in mice (see 1.3.2.1), together with the high degree of conservation between rat (4) and human (3) GLP-2 sequences, suggested that human GLP-2 could also stimulate growth of mouse small intestine. Thus, the current experiment was designed to examine the effects of overexpressing human GLP-2 in vivo.

To this end, a construct consisting of the cDNA encoding human proglucagon signal peptide (hPGSP) fused to that encoding human GLP-2<sup>1-33</sup> was inserted into an
expression vector and subsequently transfected into BHK fibroblasts. It was hypothesized that the transfected cells would synthesize and secrete GLP-2 in significantly high quantities, and that subcutaneous transplantation of these cells into nude mice would result in tumour formation, elevation of GLP-2 plasma concentrations and consequent induction of small intestinal growth.

3.2 Materials and Methods

3.2.1 Nude Mice

Six week old female Swiss nude mice (22-28 g; Charles River Canada Ltd., St. Constant, Quebec, Canada) were divided into groups (n=5/group) and injected subcutaneously with BHK-proG cells [4.8 \times 10^6 cells per mouse; BHK cells transfected with the rat preproglucagon gene under control of the metallothionein promoter, (204)], GLUTag cells [7.25 \times 10^6 cells per mouse; derived from a neuroendocrine intestinal carcinoma in SV40-proglucagon transgenic mice (see 1.1.2); (17)], or saline (Controls). After 5 weeks significant tumour growth was observed and the animals were sacrificed, blood collected by cardiac puncture, tumours excised and the entire small intestine removed, cleaned of contents and then weighed. Plasma obtained from centrifugation of blood samples was aliquoted and subjected to RIA for GLI (see chapter 2, section 2.2.7).

3.2.2 Construction and Sequencing of Plasmid

Partial cDNAs encoding the 20 amino acid (aa) human proglucagon signal peptide
(hPGSP) and GLP-2<sup>1-33</sup> were obtained from a human neonatal brainstem λgt11 cDNA library subcloned into the Bluescript plasmid following digestion with the restriction enzyme EcoRI, as previously described (205). Both hPGSP and GLP-2 cDNAs were amplified by polymerase chain reaction (PCR; fig. 9A). Primer 1 (P1 = 5'-ttggatccagaacagcagaaatga-3'; for each primer reported, the italics indicate the portion of the sequence available for hybridization and the underline denotes the restriction enzyme cleavage recognition site), corresponding to the 5' end of hPGSP and a portion of the 5' untranslated region, was designed to encode a cleavage site for the restriction enzyme BamHI upstream of the hPGSP coding region. Primer 2 (P2 = 5'-ggcatgttgccagctgccttgaccag-3'), corresponding to the 3' end of hPGSP, contained a cleavage site for the restriction enzyme NlaIII downstream of the hPGSP coding region. Similarly, primer 3 (P3 = 5'-cgcagatgtgctgctgctgctgctgtc-3'), corresponding to the 5' end of GLP-2, encoded an NlaIII cleavage site upstream of the peptide coding region. Finally, a recognition site for the restriction enzyme XhoI was inserted downstream of the GLP-2 coding sequence in primer 4 (P4 = 5'-tgctcggtatattctctgtcagtga-3'), which corresponded to the 3' end of GLP-2 and some of the 3' untranslated region. All PCR primers were obtained from ACGT, Toronto, Ontario, Canada; 10X PCR buffer was made as previously described (206); 10X deoxynucleotide triphosphates were obtained from Boehringer Mannheim, Laval, Quebec, Canada; and Taq polymerase was obtained from the Hospital for Sick Children Biotechnology Centre, Toronto, Ontario, Canada. DNA amplification was performed in a Perkin Elmer Cetus thermal cycler (Perkin Elmer Cetus Instruments, Mississauga, Ontario, Canada) for 30 cycles of denaturation (94°C, 30s), annealing (50°C, 30s) and extension (72°C, 30s). hPGSP was amplified using P1 and P2, while GLP-2 was amplified using P3 and P4. After amplification, the PCR products were
precipitated overnight in 2 volumes 100% ethanol, 0.5 volumes 7.5M ammonium acetate and 5 μg oyster shell glycogen at -20°C. The products were then digested with Nlalll at 37°C for 2 hours and then ligated to one another with T4 DNA ligase (New England Biolabs, Mississauga, Ontario, Canada) overnight at 15°C. The ligation products were separated by electrophoresis on a 2% agarose gel and the desired band of ~200bp was excised with a clean razor blade and purified using a GeneClean II kit (Bio101, Vista, CA, USA). This product was then amplified by PCR using P1 and P4 and the conditions described above. After PCR, the fusion product was digested for 2 hours with BamHl followed by a 20 hour Xhol digestion, both at 37 °C. Following precipitation and purification, as described above, the fusion product was ligated into the BamHl and Xhol sites of the expression vector pcDNA 3.1(+) (Invitrogen, San Diego, CA, USA), between the cytomegalovirus promoter and a bovine growth hormone 3' polyadenylation sequence. This construct was then transformed into competent DH5α Escherichia coli and positive clones selected by plating onto ampicillin-treated agar. DNA mini preparations were digested with RNAse and precipitated with polyethylene glycol. Samples were sequenced using the Sequenase Version 2.0 DNA Sequencing Kit (Amersham Canada Ltd., Oakville, Ontario, Canada), followed by polyacrylamide gel electrophoresis. Transformations, and preparation, precipitation and sequencing of DNA followed previously described standard protocols (206). The final construct was called pcDNA3.1-B5. All restriction enzymes were obtained from New England Biolabs, Mississauga, Ontario, Canada.
3.2.3 Cell Culture, DNA Transfection and Clone Selection

Baby Hamster Kidney (BHK) fibroblasts were obtained from the American Type Tissue Collection, Rockville, Maryland, USA, and maintained in monolayer in Dulbecco’s modified Eagle’s medium (DMEM; 4.5 g/L glucose; Canadian Life Technologies, Burlington, Ontario) supplemented with 5% calf serum (Canadian Life Technologies, Burlington, Ontario) and penicillin/streptomycin under an atmosphere of 5% CO₂. Cells were grown in 10cm culture dishes to ~70% confluency and then transfected with pcDNA3.1-B5 using the calcium phosphate precipitation method, as described previously (206). Stable transfectants were selected by supplementing the culture medium with 800 μg/mL G-418 sulfate (Canadian Life Technologies, Burlington, Ontario) over a period of 6-8 weeks. Single cell clones were obtained by dilution cloning into 96 well culture plates.

InR1-G9 cells [subcloned from a BK virus-induced hamster insulinoma, (207)], GLUTag cells, and BHK-proG were maintained in culture as previously described (17,47,203,204,208,209).

3.2.4 RNA Analysis

Cells were grown to confluency in 10 cm dishes and total RNA was isolated (see 2.2.5) from three distinct transfections (in duplicate), a single cell clone (in singlicate), untransfected BHK cells (in duplicate), InR1-G9 cells (in singlicate), and GLUTag cells (in singlicate). cDNA encoding the hPGSP/GLP-2 fusion peptide, obtained by digesting pcDNA3.1-B5 with BamHI and Xhol, was used to probe the blot (see 2.2.5 and fig. 9A).
3.2.5 High Pressure Liquid Chromatography and Radioimmunoassay

Transfected BHK cells were grown to confluency in 10 cm dishes, washed twice with 1X phosphate buffered saline and cultured in DMEM containing 0.1% calf serum. Two and 24 hours later, the medium was collected in 0.2% TFA and the cells were homogenized in 5 mL extraction medium (see 2.2.6). DMEM spiked with 0.1% calf serum was used as a negative control. Both cell extracts and media were passed through a C18 Sep Pak column (Waters Associates, Milford, MA, USA; see 2.2.6) prior to separation based on relative hydrophobicity using high pressure liquid chromatography (HPLC). The HPLC system consisted of two Model 510 pumps, two Series 440 UV detectors (measuring absorbance at 214 and 280 nm), a C18 Guard Pak, and a C18 µBondpak column (Waters Associates, Milford, MA, USA) as previously described (183). Based on levels of immunoreactive GLP-2 detected in a preliminary RIA for total immunoreactive GLP-2 (antiserum UTTH-7, see 2.2.7; table 1), appropriate volumes of sample to load onto the HPLC column were calculated. For the HPLC profiles in figure 12, 8 mL (= 1 dish) of 24 h media from BHK cells transfected with hPGSP/GLP-2 (Transfected) or fresh medium (Control) was diluted 1/8 with Solvent A [0.1% (v/v) TFA (Reagent Grade; Fisher Scientific, Fair Lawn NJ, USA)] to dilute out the sample solvent [80% isopropanol/0.1% TFA (see section 2.2.6)]. For each sample, the column was pre-equilibrated with 15% Solvent B [99.9% (v/v) acetonitrile (HPLC grade; Mallinckrodt, Mississauga, Ontario)/0.1% (v/v) TFA] prior to loading the sample onto the column. Peptides were separated using a 45 min linear gradient of 30-60% (v/v) Solvent B, followed by a 10 min purge with 99% Solvent B. The flow rate was 1.5 mL/min, and 450 µL fractions were collected every 18 s into 12 × 75 mm polystyrene tubes using a Gilson Model 203 microfraction collector.
These fractions were dried in vacuo and stored at -20°C for RIA. The fractions were probed for GLP-2-like immunoreactivity using the UTTH-7 antiserum (see section 2.2.7). The elution positions of immunoreactive GLP-2 peptides were determined by comparison with the elution position of the internal standard \( ^{125}\text{I}-\text{TYR}_{34}\)-rat GLP-2 peptide and with the known elution position of human GLP-2 (Dr. P. L. Brubaker, personal communication).

3.2.6 Statistics

All data are expressed as the mean ± SEM. Statistical significance was assessed by ANOVA with n-1 custom hypothesis tests using a SAS program for IBM computers (Statistical Analysis Systems, Cary, NC).

3.3 Results

To determine whether BHK fibroblasts would be suitable for use as a vehicle for the fusion construct, nude mice carrying either GLUTag or BHK-proG tumours were examined. Plasma GLI was significantly elevated in both mice carrying GLUTag tumours (698±229% of controls; P<0.001), and those with BHK-proG tumours (444±51% of controls; P<0.001; fig. 10). Previous studies indicate that GLUTag cells process proglucagon to both glucagon and the intestinal PGDPs (7), while BHK-proG cells do not process proglucagon to any of the PGDPs (7,204). In keeping with the processing characteristics of these two cell lines, the small intestinal wet weight of mice carrying GLUTag tumours was significantly greater than that of controls (128±5% of controls; P<0.01; fig. 10), whereas no difference was observed between the small intestinal wet
weight of mice carrying BHK-proG tumours and controls. These data indicate that GLUTag cells secreted intestinotrophic PGDPs, while BHK-proG cells did not secrete any factors trophic to the small intestine. Since the latter did not secrete any factors that would interfere with the bowel growth assay, it was concluded that this cell type could serve as a vehicle for the fusion construct. Furthermore, these data show that hypersecretion of unprocessed proglucagon did not stimulate small intestinal growth.

Sequencing of the hPGSP/GLP-2 fusion construct confirmed that the correct construct had been prepared (fig. 9B). Total RNA from BHK cells transfected with the hPGSP/GLP-2 fusion construct was analysed by Northern blot, using the construct as probe (fig. 11). A band of ~500 bp (estimated size of transcript based on sum of the nucleotide bases downstream of the transcriptional start site, as shown in fig. 9B) was detected in both mixed populations and a single cell clone. This indicated that the construct was being transcribed in transfected BHK cells. A band of ~1.4kb (estimated size based on comigration with 18S ribosomal RNA) was detected in both InR1-G9 and GLUTag cells, indicating the expression of endogenous proglucagon in these two cell lines and showing that the human probe was capable of hybridizing with both hamster and mouse proglucagon. Untransfected BHK cells did not contain either proglucagon or the hPGSP/GLP-2 fusion construct.

Table 1 shows the levels of immunoreactive GLP-2 from cell extracts and medium after both 2 and 24 hours of serum-deprivation, as well as GLP-2 levels in fresh medium. These data indicate that BHK cells transfected with hPGSP/GLP-2 neither contained nor secreted levels of GLP-2 above those observed for untransfected BHK cells or fresh medium. Figure 12 shows that, after 24 hours of incubation in minimal medium, the HPLC profile for transfected cell medium was essentially no different from that of the
Fig. 9. The hPGSP/GLP-2 fusion construct. (A) Schematic representation of the PCR cloning strategy. Primers 1-4 (P1-P4) are described in section 3.2.2. (B) Schematic representation of the coding region after insertion into pcDNA 3.1. Box: determined nucleotide and predicted amino acid sequences of the fusion construct. hPGSP, human proglucagon signal peptide (hatched bar); GLP-2, human glucagon-like peptide-2 (solid bar); G, guanine; A, adenine; T, thymine; C, cytosine; P<sub>CMV</sub>, cytomegalovirus promoter; polylinker, multiple cloning site; UT, untranslated region. The arrow at +1 indicates the putative transcriptional start site. The numbers beneath the boxes indicate the approximate number of nucleotide bases from the transcriptional start site. Amino acids are reported in the standard three letter code (see abbreviations).
Fig.10. Plasma GLI and small intestinal wet weight of control (n=5) and nude mice carrying subcutaneous GLUTag (n=5) or BHK-proG tumours (n=5). ** P<0.01 *** P<0.001 vs Controls.
Fig. 11. Northern blot of 8μg total RNA from untransfected BHK (lanes 1-2), GLUTag (lane 3), InR1-G9 (lane 4), mixed transfection 1 (lanes 5-6), mixed transfection 2 (lanes 7-8), mixed transfection 3 (lanes 9-10), and a single cell clone (lane 11).
Table 1. Immunoreactive GLP-2 content in cell extracts (cells) and medium (8mL/dish) from confluent 10 cm dishes of BHK cells transfected with the hPGSP/GLP-2 construct (BHK+) and untransfected BHK cells (BHK-) after 2 and 24 hours of incubation in DMEM supplemented with 0.1% calf serum. Medium alone was also assayed for GLP-2 content (Fresh Medium).

<table>
<thead>
<tr>
<th></th>
<th>Cells (ng/dish)</th>
<th>Medium (ng/8mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2h</td>
<td>24h</td>
</tr>
<tr>
<td>BHK+ (n=3)</td>
<td>4.5±0.7</td>
<td>5.6±0.9</td>
</tr>
<tr>
<td>BHK- (n=1)</td>
<td>4.0</td>
<td>5.3</td>
</tr>
<tr>
<td>Fresh Medium (n=2)</td>
<td></td>
<td>4.4-4.5 (ng/8mL)</td>
</tr>
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Fig. 12. HPLC profiles of 24 hr media samples from BHK cells transfected with hPGSP/GLP-2 (Transfected) and media alone (Control). The arrow indicates the elution position of the internal rat GLP-2 standard.
negative control.

Taken together, these data indicate that, under basal conditions, transfected cells neither synthesized nor secreted GLP-2-like peptides. Furthermore, the high levels of GLP-2 immunoreactivity observed in the HPLC profiles for 8 mL of media (fig. 12) indicates that the preliminary RIA (table 1) underestimated the actual GLP-2 levels present in the samples; similar discrepancies have been observed for GLP-1 RIAs (17). These data also suggest that 0.1% calf serum alone contains some serum element, possibly GLP-2 or perhaps bovine serum albumin, that cross-reacts strongly with the UTTH-7 antiserum.

As the cells transfected with the hPGSP/GLP-2 fusion construct did not contain or secrete quantities of GLP-2 peptide above the levels observed for the negative controls, we did not proceed with injecting the transfected cells into nude mice.

3.4 Discussion

The posttranslational processing pattern of GLUTag tumours to glicentin, oxyntomodulin and GLP-1, has been previously described, but this study did not assay for immunoreactive GLP-2 peptides (202). However, preliminary studies indicate GLUTag cells do contain immunoreactive GLP-2 (Dr. P.L. Brubaker, personal communication). Together with the observation that mice harboring GLUTag tumours also have significantly enlarged small bowels (63), this provides further evidence that these tumours also secrete GLP-2. BHK cells transfected with proglucagon do not process this molecule to the PGDPs, and the available data indicate that these cells secrete unprocessed proglucagon in a constitutive fashion (7,204).
Data presented here are the first demonstration that elevated plasma concentrations of intact proglucagon had no effect on small intestinal wet weight *in vivo*. This is important in view of a recent study suggesting that unprocessed progastrin is trophic to colonic mucosa *in vivo* (123). This report described two strains of progastrin transgenic mice; one of which processed progastrin normally, while the other was incapable of processing progastrin to its mature peptides and thus had progastrin as the major circulating form (123). Interestingly, both strains of mice displayed an increased BrdU labeling index in their colonic mucosa, as compared to wildtype littermates. It is unknown if this effect of progastrin is direct or mediated by a mature gastrin peptide, which may have been liberated as a result of local progastrin processing by the target tissue. A precedent for such a mechanism occurs in the liver, where glucagon is processed to miniglucagon, a peptide believed to mediate the effects of glucagon on the heart (52). Indeed, a direct effect of progastrin would be intriguing, since the general consensus is that prohormones are biologically inactive precursor molecules. As mice carrying BHK-proG tumours did not show enhanced bowel mass, it was also concluded that BHK cells do not secrete any other factors that would interfere with the gut growth assay and were thus a suitable vehicle for GLP-2 overexpression *in vivo*.

Although BHK cells transfected with the hPGSP/GLP-2 fusion construct transcribed this construct into messenger RNA (mRNA), they failed to synthesize or secrete significant quantities of GLP-2 peptide. In order to develop hypotheses as to the nature of the defect in the current system, it is necessary to review the currently accepted model for the synthesis, trafficking, and secretion of normal secretory proteins (for example, hormones) in mammalian cells. After transcription and splicing, mRNA is targeted to a ribosome on the cytosolic surface of the endoplasmic reticulum (ER). Here the mRNA
is translated into protein and this protein is simultaneously translocated into the lumen of the ER (210). The newly translated N-terminal portion of the native protein comprises a hydrophobic signal sequence (the "pre" portion of a preprohormone), which permits targeting of the polypeptide to the translocon, a protein complex spanning the ER membrane (211). Delivery to the ER membrane is mediated by a cytosolic protein called the signal recognition particle (SRP), which recognizes and binds the signal peptide. A component of the translocon serves as a signal recognition particle receptor and facilitates docking of the SRP with the translocon complex (210). The latter is a large protein complex, whose primary function is to serve as a pore through which secretory proteins are extruded into the ER lumen (211). Although the translocation event requires a core of proteins used exclusively for this purpose, other proteins in the complex serve various accessory functions. For example, signal peptidase, a translocon component located on the luminal side of the ER membrane, is the enzyme that cleaves the signal peptide from secretory proteins, once they have entered the ER (211). Following translocation and signal peptide cleavage, the protein (or prohormone) is folded into its proper conformation by various chaperones and isomerases, some of which may be components of the translocon complex. Once folded the protein is then shuttled to the Golgi apparatus.

The consensus among investigators in this field is that all cells can secrete proteins in a constitutive fashion, but only endocrine, exocrine and neural cells have a distinct pathway for regulated secretion (212,213). In such cells, sorting into either the constitutive or regulated secretory pathways is achieved in the trans-Golgi compartment. If sorted into the latter, the protein is encapsulated into a dense core secretory granule (SG), which releases its contents only when the cell is stimulated by a secretagogue.
The SG is the compartment in which the prohormone convertases (see 1.1.1) cleave prohormones into their constituent peptides. Some endocrine peptides may undergo further modifications within the SG, such as amidation or acetylation. If the protein is sorted into the constitutive or default pathway, it is shuttled to the plasma membrane in a constitutive vesicle. Regardless of which pathway is used, the protein is ultimately expelled from the cell via an exocytotic mechanism (212).

Such is the sequence of events for normal secretory proteins. What happens if the protein is in some way abnormal, if, for instance, the protein was mutated and thus unable to fold properly? It has been known for some time that the cell actively screens out and destroys defective proteins. Although it is unclear exactly how a cell identifies defective proteins, it appears as if this screening process occurs at an early step along the trafficking route, most likely at the level of the ER. Although substantial evidence indicates protein degradation occurs within its lumen (214), a growing body of evidence supports the idea that the ER may play an even more active role in disposing of unwanted proteins (215). In this regard, the translocon appears to be the key player. Crowley and coworkers were the first to describe the translocon as a gated, aqueous channel through which hydrophilic proteins may traverse the hydrophobic membrane (216). Others have provided evidence that trafficking through the pore need not be unidirectional. In fact, retrograde transport of proteins from the ER lumen to the cytosol appears to be one step in an alternative mechanism for disposing of defective proteins. Once in the cytosol, the offending protein acquires a polyubiquitin tag and is subsequently shuttled to the proteasome complex for degradation (217).

It may be that the process of screening out defective proteins starts even before the proteins reach the ER lumen. For example, Crowley et al (216) have presented
evidence suggesting that the translocon is a gated channel. Furthermore, they found that successful translocation through this channel is dependent on the chain length of the native protein. The model proposed by this group went as follows: a protein must be a minimum of ~70 aa long, including the signal peptide, in order to mediate opening of the putative gate on the lumenal side of the translocon pore (216). Failure to initiate opening of the pore presumably results in retrograde expulsion of the protein into the cytosol, where it is targeted for degradation by the proteasome (211,215,217). Although it awaits confirmation by an independent experimental approach, this is the only study that has reported the requirement of a minimum native chain length for translocation across the ER membrane. Thus, the functional translocating apparatus is a complex consisting of the ribosome, newly translated polypeptide and translocon, the regulation of which is highly dependent on the biophysical and structural interactions between these constituent components (210).

Data presented here indicates that BHK cells transfected with the hPGSP/GLP-2 fusion construct successfully transcribed the construct, but failed to synthesize or secrete any GLP-2 peptide. This suggests that the defect in the current system lies somewhere after transcription. The preceding discussion has shown us that the translation of secretory proteins is intimately related with translocation into the ER, which is in turn dependent on the physico-chemical properties of the newly synthesized proteins themselves. In the present study, translation of the DNA encoding the fusion construct, would have resulted in a peptide with a total length of only 53 aa (i.e. 20 aa hPGSP plus 33 aa hGLP-2). This peptide would have been 17 aa shorter than the 70 aa minimum described by Crowley et al (216). Therefore, one potential reason for the failure of the current system to synthesize and secrete GLP-2 is that the engineered peptide was too
short to pass through the translocon pore. Failure of the peptide to mediate opening of the putative lumenal gate would most likely have resulted in it being tagged with polyubiquitin and subsequently degraded by the cytosolic proteasome complex.

One way to test this hypothesis would be to insert a spacer peptide between hPGSP and GLP-2. Such a spacer would have to be chosen carefully to avoid hydrophobic sequences with an affinity for membrane insertion. Perhaps the ideal candidate would be a second GLP-2 molecule. This would also circumvent the potential complications of introducing a heterologous spacer peptide into the system. However, whereas the present construct was purposely designed with only a single cleavage site for signal peptidase, adding a second GLP-2 would necessitate insertion of a second cleavage site. Furin is a ubiquitous endoprotease found in the trans-Golgi network of many cell types, including BHK (218), that cleaves proteins on the C-terminal side of the consensus sequence ARG-X-LYS/ARG-ARG+ (219). Thus, insertion of a furin cleavage site between the two GLP-2 molecules might resolve this dilemma. Alternatively, since PC1 is responsible for processing proglucagon to GLP-2 (see section 1.1.1), one could insert a consensus site (ARG-LYS+) for this enzyme between the two GLP-2 molecules. In BHK cells, this site would most likely be cleaved by furin as well (7). Since BHK cells do not process proglucagon to GLP-2 (204), it is unlikely that these cells contain PC1. Thus, this enzyme would also have to be transfected into these cells, adding an extra step to the engineering scheme.

In the present study, a large portion of the native proglucagon molecule was absent from the engineered construct. Even if the peptide had crossed the ER membrane, the loss of important secondary and tertiary structure may have resulted in misfolding and made the peptide susceptible to proteolytic degradation. There is some
evidence that, in addition to the signal peptide, which targets proteins to the ER, proteins encode other sorting signals within their native sequences. Most of the sorting signals identified to date are primary sequence motifs. For example, the KDEL motif is responsible for keeping proteins within the ER lumen, and similarly, the SKYQRL sequence designates localization to the trans-Golgi network (212). The existence of a similar motif, permitting sorting into the regulated secretory pathway at the TGN, has been speculated for some time. However, it is only recently that a somewhat convincing model has been proposed (220). Initially, Cool et al (221) examined the sorting of proopiomelanocortin (POMC) and found that the first 26 aa of this prohormone comprise a putative sorting motif for the regulated secretory pathway (RSP). This motif differs from previously reported sorting signals in that it is the secondary structure, and not the primary sequence, which appears to be responsible for providing the sorting information. These 26 aa form a hairpin loop structure which is maintained by two internal disulfide bonds. This structure appears to be unique to POMC and thus does not constitute a universal sorting signal for the RGS (220). Recently, this group have identified carboxypeptidase E as a putative sorting receptor that directly binds the 26 aa POMC motif and facilitates sorting to the RSP in Neuro2a cells [a neuroendocrine cell line (220)]. In this study, these researchers demonstrated that both POMC and proinsulin bound secretory granule membranes from bovine pituitary (220). However, they did not determine if proinsulin actually bound the putative receptor directly. Nonetheless, it is tempting to speculate that other prohormones may contain sorting information similar to that described for POMC.

It remains possible that native proglucagon possesses structural sorting information which is absent in the engineered peptide. Given that the RSP sorting signal for POMC
was located in the N-terminal region of the prohormone, it may be that the hPGSP/GLP-2 fusion peptide, which lacks all of proglucagon except the extreme C-terminal peptide (i.e. GLP-2), was incapable of being sorted into the proper secretory apparatus. It is equally likely that the sorting information in proglucagon is located elsewhere in the molecule. In fact, the hPGSP/GLP-2 construct may contain the proper signal, and the fault may be in the choice of cell line transfected. For instance, BHK cells do not have an RSP and therefore might also lack the putative sorting receptor. Thus, transfection of this construct into a neuroendocrine cell line would allow testing of this hypothesis. However, the data presented here indicates a complete lack of GLP-2-like peptide in transfected BHK cell extracts, even though these cells contained significant levels of hPGSP/GLP-2 mRNA transcripts. Thus, the data support the view that the defect of the current system occurs after transcription, perhaps during co-translational ER translocation or soon thereafter. Had the peptide reached the Golgi, but was incapable of being sorted to the RSP, cell extracts might have tested positive for GLP-2-like immunoreactivity.

Thus, transfection of a hPGSP/GLP-2 fusion construct into BHK fibroblasts resulted in successful transcription into mRNA, but failure to synthesize and secrete levels of immunoreactive GLP-2 peptide above those detected in 0.1% serum supplemented medium. The preceding discussion has detailed several hypotheses concerning the possible defects in the current system and suggested experiments to test these ideas.
Chapter 4 Discussion

Since the report that a patient carrying an intestinal PGDP-secreting tumour displayed coincident small intestinal hypertrophy, it has been speculated that one or more of the intestinal PGDPs is(are) trophic to the small intestine (see 1.3.2.1). Recently, a seminal experiment demonstrated that exogenous GLP-2 is a potent stimulator of intestinal growth in mice (63). In this study, the observed increase in small intestinal wet weight was shown to be primarily the result of villus elongation, with no significant changes observed in either crypt depth or thickness of the muscle layer (63). The second important feature observed in the guts of these mice was an increase in crypt cell proliferation (63). These researchers recently published a second report confirming their earlier observations (64). Furthermore, a recent report by another group provides an independent demonstration that treating mice with exogenous GLP-2 results in significant intestinal growth (222). Although these investigators did not examine histological changes, they did report increases in the wet weight, as well as DNA and protein content of jejunum, ileum and colon. Thus, a role for exogenous GLP-2 as a potent intestinal growth factor has been firmly established. However, studies examining a role for endogenous GLP-2 have not been published. Therefore, the data presented in chapter two of this thesis is the first demonstration that a physiologic model of gut adaptation, i.e. STZ-induced diabetes in rats, is associated with elevated plasma and tissue levels of GLP-2 in vivo.

Previous studies have shown that subcutaneous passage of proglucagon-expressing cell lines in nude mice results in small bowel mucosal proliferation and villus
elongation (63). At least two of the cell lines used in this study, namely InR1-G9 (208) and RIN 1056A (223), are known to process proglucagon to the intestinal PGDPs and have been shown to secrete GLP-2. Similarly, GLUTag cells contain immunoreactive GLP-2 (Dr. P.L. Brubaker, personal communication) and mice with subcutaneous GLUTag tumours, display small intestinal hypertrophy [(63) and chapter 3 of this thesis]. Thus, the nude mouse experiments are in keeping with the notion that GLP-2 is the primary intestinotrophic PGDP. However, all of the tumours in these studies also secreted other PGDPs (63), making it impossible to actually dissect the effects of the various peptides [see 3.1, (202,203)]. Therefore, in order to examine the effects of specifically overexpressing GLP-2 in vivo, chapter 3 describes an attempt to engineer BHK fibroblasts to synthesize and secrete high concentrations of GLP-2. Although the BHK cells transfected with the hPGSP/GLP-2 construct (see 3.2.2) successfully expressed construct mRNA, they failed to synthesize or secrete immunoreactive GLP-2 in significant quantities. Had these cells been competent GLP-2 secretors, they would then have been injected into nude mice subcutaneously, working under the hypothesis that the elevated plasma levels of GLP-2 would induce bowel growth. Although the primary object of chapter 3, i.e. engineering a GLP-2-secreting cell line, was unsuccessful, other data presented in this chapter contributes to our understanding of the role of proglucagon in intestinal growth. Specifically, data presented here is the first demonstration that an increase in the plasma levels of unprocessed proglucagon is not trophic to the small intestine in vivo. This is important in view of the recent observation that elevated plasma levels of unprocessed progastrin are trophic to the colonic epithelium in mice in vivo (123).

Although not reported for the original GLP-2-treated mice (63), the two most recent
studies describing the effects of treating mice with exogenous GLP-2 both suggest that GLP-2 induces a greater trophic response in the jejunum as compared to the ileum (64,222). Thus, these data may indicate a differential regional effect of GLP-2 in the small intestine. This differential sensitivity to GLP-2 may reflect regional differences in expression of the putative GLP-2 receptor, a hypothesis that cannot be examined until this receptor is identified. Alternatively, these regional differences may reflect the inherent differences in the size of the mucosal architecture along the aboral axis of the small bowel [(93); see also fig. 6]. In this case, putative GLP-2 receptors would not need to exhibit a differential regional expression, but could be evenly distributed along the gastrointestinal tract. Another possibility can be conceived in view of the notion that a concentration gradient of pancreaticobiliary secretions is responsible for the observed aboral architectural gradient (see 1.3.3). That is, it may be that GLP-2 exerts its effects indirectly by stimulating these secretions.

Interestingly, the trophic response to IGF-1 (see 1.3.2.3) infusion in rats is also greater in jejunum compared to ileum (141). Although it is well established that the proglucagon-expressing L cells are distributed along the entire aboral axis, with the highest concentrations found in distal ileum and colon (20,21,25), a detailed examination of the distribution of IGF-1-positive cells within the intestine has not been published. However, the available data indicate the latter are found along the entire length of the gastrointestinal tract (137,224). Expression of both the proglucagon and IGF-1 genes is influenced by nutritional status, particularly in the jejunum. In rats following a 72 h fast, the levels of both jejunal proglucagon (225) and IGF-1 (226) mRNA transcripts are significantly lower than those in ad libitum fed controls, and refeeding returns transcript levels to those seen in controls. A similar trend is observed for the plasma levels of IGF-
1 (226), GLP-1 and gGLL (225). However, no changes were observed in the abundance of ileal proglucagon mRNA transcripts after fasting (225).

Taken together, these data indicate that the products of these two genes may be involved in mediating at least some of the effects of nutrient-induced mucosal growth. Furthermore, the observation that the peptide plasma levels reflect the changes in jejunal gene expression, support a humoral role for both GLP-2 and IGF-1 in nutrient-stimulated intestinal growth. However, it is possible that these factors may also act through a local mechanism to stimulate mucosal growth in vivo. That IGF-1 stimulates epithelial growth via both local and humoral mechanisms, is supported by observations that both intraluminal (142) and subcutaneous (140) infusion of the peptide result in a trophic response. However, in transgenic mice overexpressing IGF-1, it is impossible to distinguish between the enterotrophic effects of circulating and local IGF-1, as both are significantly elevated in these animals (143). The parallelism between the activities of the jejunal proglucagon and IGF-1 genes may indicate a link between these two pathways. It is possible that GLP-2 may act to stimulate the local IGF-1 system, which in turn exerts a direct mitogenic effect on crypt cells.

Although the receptor for GLP-2 has not been cloned, the high degree of homology that GLP-2 shares with glucagon and GLP-1 [fig. 2; (1-4)], as well as the homology between the glucagon and GLP-1 receptors (52,60), suggests that the putative GLP-2 receptor may also be a G protein-coupled seven transmembrane receptor. In keeping with this hypothesis, GLP-2 has been shown to stimulate adenylate cyclase in rat pituitary and hypothalamic homogenates (227), as well as in preparations of isolated rat crypt cells (228). It is interesting to speculate that GLP-2 may act through a mechanism analogous to that of GLP-1. That is, as GLP-1 stimulates the synthesis and secretion of insulin from
the pancreatic beta cell, so GLP-2 may act as a secretagogue for the insulin-like molecule IGF-1, which may, in turn, exert its mitogenic action in a local paracrine manner within the intestine. Alternatively, it is now known that G protein-coupled receptors are capable of mediating mitogenic effects directly, although the intracellular details remain unclear. For example, in many cell types an increase in intracellular cAMP inhibits mitogenesis, but in others it is found to activate the Ras/MAPK pathway [see 1.3.4.2, (174)]. Thus, it is possible that GLP-2 may exert its intestinotrophic effects directly.

In studies not described in this thesis, an attempt was made to determine whether GLP-2 has direct effects on crypt cell proliferation. IEC-18 cells, a rat crypt cell line (229), were therefore incubated in DMEM (containing 0.1% calf serum) and treated with either 5% calf serum or a range of GLP-2 concentrations (10^-14-10^-8 M). The cells were then assayed for incorporation of tritiated thymidine. Although the cells showed a well-defined dose response to serum, GLP-2 appeared to be ineffective in stimulating thymidine incorporation. Another group reported similar findings using IEC-6 cells, a rat jejunal crypt cell line, whereby GLP-2 had no effect on serum-starved cells, but reduced serum-induced increases in DNA synthesis in a dose-dependent manner (112). This lack of effect of GLP-2 on the proliferation of serum-starved cells may suggest that the putative receptor is not expressed in these cells or, even if it is, that the cells actively degraded the exogenous peptide.

DPIV is a prime candidate for mediating GLP-2 degradation (see 1.1.4) and, although predominantly expressed by mature enterocytes, it may be present in the IEC cell lines. However, this enzyme is abundant in serum (see 1.1.4), suggesting one possible explanation for the observed inhibitory effect of GLP-2 on serum-induced mitogenesis (112). It may be that increased levels of serum DPIV were responsible for
converting GLP-2\textsuperscript{1-33} to GLP-2 \textsuperscript{3-33} and that this degradation product then acted as an antagonist by competing with bioactive GLP-2\textsuperscript{1-33} for binding to the putative GLP-2 receptor, thereby inhibiting DNA synthesis. One precedent for this is that GLP-1-(9-36)-amide, the N-terminally truncated metabolite of GLP-1-(7-36)-amide, can act as an antagonist of the pancreatic GLP-1 receptor (230). One important area for future study will be to define which parts of the GLP-2 molecule are critical for effective structure-activity relationships. The design of GLP-2 analogs which are resistant to DPIV degradation or function as superagonists may be important for potential clinical use of this peptide in the treatment of diseases such as short bowel syndrome.

Another interpretation of the IEC data is that GLP-2 does not stimulate crypt cell mitogenesis directly, but may induce differentiation of these cells to a mature phenotype and thus promote proliferation indirectly by a feedback mechanism \textit{in vivo} (see 1.3). This idea could be tested by examining the expression of differentiation state-specific genes and cell morphological phenotype both before and after treatment with GLP-2. In fact, a recent study has shown that the small intestines of GLP-2-treated mice display an upregulation in the activities of brush border membrane enzymes which parallels the observed increase in villus height (201). Such studies could be expanded to include markers for the other epithelial cell types, as was done in one study examining the effects of exogenous KGF administration (162). This report established that, in addition to stimulating crypt cell proliferation, treating rats with KGF selectively induced goblet cell lineages, but either had no effect or decreased the other three cell lineages [(162); see section 1.2].

Several peptides trophic to normal intestine have also been shown to ameliorate the effects of experimentally induced mucosal ulceration. For example, pretreatment with
EGF protected against the erosions typically observed following trinitrobenzenesulfonic acid/ethanol (TNBS)-induced colitis in rats (231). Similarly, oral administration of FGF accelerated the healing of duodenal ulcers in rats (161). One drawback of using molecules such as EGF or FGF to treat gastrointestinal ulceration is that such growth factors are not specific for the gut. Thus, if administered systemically they will most likely stimulate proliferation, and perhaps tumour formation, in other tissues of the body. As GLP-2 is an intestine-specific growth factor that does not appear to affect any other tissues (63), it may be a prime candidate for treatment of gastrointestinal diseases characterized by erosion of the functional mucosa.

In the initial phase of this thesis, we adapted a model of TNBS-induced ileitis from hamsters (232), in order to determine whether GLP-2 was capable of alleviating the effects of mucosal ulceration. In this model, the distal ileum of an anaesthetized mouse was exteriorized through an abdominal incision and TNBS injected into its lumen. Unfortunately, the model was not reproducible in the mouse, leading to perforation or, alternatively, having no effect at all, and the project was therefore discontinued. However, the most popular experimental model of inflammatory bowel disease is chemically-induced ulcerative colitis, induction of which does not require abdominal surgery but simply intrarectal infusion of TNBS (231). As GLP-2 may also be trophic to the colon (222), it would be useful to examine the effects of administering this peptide in a model of colitis.

Future studies should focus on the mechanism of GLP-2 action. One important direction is to identify the putative GLP-2 receptor. A number of strategies could be employed to achieve this end. For example, it is likely that the GLP-2 receptor is related to the glucagon and GLP-1 receptors, which share a high degree of structural and functional homology (see 1.1.4). This assumption could be used to advantage by
designing degenerate PCR primers based on the sequences of these two receptors. Alternatively, a biochemical approach could be employed. For example, radiolabelled GLP-2 could be infused into a rat and various tissues examined by autoradiography for labelling in vivo. Once a tissue exhibiting high GLP-2 binding capacity is identified, then use of a GLP-2 molecule attached to a photosensitive crosslinking moiety can be used to ‘capture’ the receptor, followed by affinity chromatography, using an antibody directed against GLP-2, to purify the receptor-GLP-2 complex. This complex could then be dissociated and the putative receptor sequenced.

In the absence of a cloned receptor, it will be necessary to design experiments to elucidate the physiological mechanism of GLP-2 action. For example, as the IEC cell lines proved ineffective for establishing a direct mitogenic response to GLP-2, perhaps short term primary cultures of enterocytes could be prepared as a more physiologically relevant model in which to examine such an effect. Both intestinal cell lines and primary cultures could be used to study potential non-mitogenic effects of GLP-2, for example induction of second messenger systems or differentiation markers (as described above). In addition to increasing crypt cell proliferation [see 1.3.2.1, (63)], a very recent study indicates that GLP-2 also decreases villus cell apoptosis (233). Thus, it will be necessary to determine if either of these effects is direct and establish the causative relationship between them.

In conclusion, the demonstration that GLP-2 is a potent and specific stimulator of intestinal epithelial growth is relatively recent. However, research to date indicates that this molecule may be an ideal candidate for clinical situations characterized by compromised absorptive capacity, such as short bowel syndrome or inflammatory bowel disease. Information about the mechanism of GLP-2 action remains scarce and more
work is required before it can be satisfactorily characterized. Data presented in this thesis: (i) support a role for endogenous GLP-2 in small intestinal growth *in vivo*; and (ii) provide a basis for future work on the development of a specific GLP-2 delivery system.
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IMAGE EVALUATION
TEST TARGET (QA-3)

1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5

150mm
6"