The Role of the Prohormone Convertases in the Post-Translational Processing of Intestinal Proglucagon

by

Savita Dhanvantari

A Thesis Submitted in Conformity with the Requirements for the Degree of Doctor of Philosophy, Graduate Department of Physiology, in the University of Toronto

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Abstract

The Role of the Prohormone Convertases in the Post-Translational Processing of Intestinal Proglucagon

Savita Dhanvantari, PhD (1997)

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Proglucagon (proG) is post-translationally processed in a tissue-specific manner to yield glucagon in the A cells of the pancreas, and glicentin, oxyntomodulin, glucagon-like peptide (GLP)-1 and -2 in the L cells of the intestine. Each of the constituent peptides of proG is flanked by pairs of basic amino acids, which are known sites for prohormone cleavage. The identification of the prohormone convertase (PC) family of processing enzymes has facilitated the study of prohormone processing. To determine the roles of PC1 and PC2, and other convertases, in the tissue-specific processing of proG, vaccinia virus (vv)-mediated infection of endocrine-derived cell lines was utilized. GH3 and AtT-20 were co-infected with vv:proG plus vv:furin, vv:PC1, or vv:PC2. Cells were also infected with vv:PAC4, vv:PC5a or vv:PC5b alone, or with vv:PC2. PC1 processed proG to glicentin and oxyntomodulin, GLP-1^{1-37/36NH2} and GLP-1^{7-37/36NH2} and GLP-2. Infection with PC2 alone or in combination with other enzymes resulted in the production of glicentin, but not glucagon. To further confirm these findings, we stably transfected the pancreatic InR1-G9 cell line with PC1 and/or antisense PC2 (ASPC2). Transfection with PC1 increased glicentin, GLP-1^{1-36NH2} and GLP-2 production, while a reduction in PC2 attenuated glicentin, but not glucagon, production. Co-regulation of PC1 and proG mRNA in the L cell was examined in an enteroendocrine cell line, GTag-Y. Stimulation of protein kinase A in GTag-Y cells increased secretion of both GLP-1^{7-36NH2} and GLP-2, however,
neither levels of proG nor PC1 mRNA were increased. Therefore, we were unable to show co-regulation of proG and PC1 due to lack of suitable models. Taken together, these studies establish a role for PC1 in the processing of proG to yield the intestinal peptides, glicentin, oxyntomodulin, GLP-1 \textsuperscript{1-36NH\textsubscript{2}} and GLP-2, and suggest that PC2 is responsible for the processing of proG to glicentin, another enzyme being required for the production of glucagon.
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<tr>
<td>8-Br-cAMP</td>
<td>8-bromo-cyclic adenosine-5',3'-monophosphate</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>AP</td>
<td>anterior pituitary</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>Arg</td>
<td>arginine</td>
</tr>
<tr>
<td>Asn</td>
<td>asparagine</td>
</tr>
<tr>
<td>Asp</td>
<td>aspartic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>C18</td>
<td>18 carbon chain</td>
</tr>
<tr>
<td>C-terminal</td>
<td>carboxy-terminal</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic-5',3'-adenosine monophosphate</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response element</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>CPE</td>
<td>carboxypeptidase E</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>F/I</td>
<td>forskolin/isobutylmethylxanthine</td>
</tr>
<tr>
<td>FRIC</td>
<td>fetal rat intestinal cells</td>
</tr>
<tr>
<td>G protein</td>
<td>GTP-binding protein</td>
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GLI  glucagon-like immunoreactivity
GLP  glucagon-like peptide
HEPES N-(2-hydroxyethyl)piperazine-N'-ethanesulfonic acid
His  histidine
IBMX isobutylmethylxanthine
IR  immunoreactivity
IRE insulin response element
IRG immunoreactive glucagon
kb kilobase
kDa kilodalton
LPH lipotrophic hormone
Lys lysine
M3 receptors mucaricin type 3 receptors
mM millimolar
mRNA messenger ribonucleic acid
MSBR massive small bowel resection
MSH melanocyte-stimulating hormone
MW molecular weight
N-glycosylated glycosylated on asparagine
N-terminal amino-terminal
ng nanogram
NIDDM noninsulin-dependent diabetes mellitus
NIL neurointermediate lobe
PBS phosphate buffered saline
<table>
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<tr>
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<th>Definition</th>
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<tr>
<td>PC</td>
<td>prohormone convertase</td>
</tr>
<tr>
<td>pg</td>
<td>picogram</td>
</tr>
<tr>
<td>PGDP</td>
<td>proglucagon-derived peptide</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol myristyl acetate</td>
</tr>
<tr>
<td>POMC</td>
<td>pro-opiomelanocortin</td>
</tr>
<tr>
<td>Pro</td>
<td>proline</td>
</tr>
<tr>
<td>proG</td>
<td>proglucagon</td>
</tr>
<tr>
<td>RIN</td>
<td>rat insulinoma</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>SS-14</td>
<td>somatostatin-14</td>
</tr>
<tr>
<td>SS-28</td>
<td>somatostatin-28</td>
</tr>
<tr>
<td>SSC</td>
<td>sodium chloride/sodium citrate</td>
</tr>
<tr>
<td>STC</td>
<td>secretin tumour cell</td>
</tr>
<tr>
<td>STZ</td>
<td>streptozotocin</td>
</tr>
<tr>
<td>TGN</td>
<td>trans Golgi network</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μM</td>
<td>micromolar</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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Chapter 1: Introduction

The proglucagon (proG) gene is expressed in the pancreas, gastrointestinal tract and brain. ProG is post-translationally processed in a tissue-specific manner to yield glucagon in the pancreatic A cell, and glicentin, oxyntomodulin, glucagon-like peptide-1 (GLP-1) and GLP-2 in the intestinal L cell. Although strong structural homology exists between glucagon, GLP-1 and GLP-2, these peptides exhibit unique biological activities. Two of these peptides, glucagon and GLP-1, play major roles in nutrient metabolism. Glucagon is the major glucose counterregulatory hormone, as it is released from the A cell during the fasting state to increase glycogenolysis and gluconeogenesis, thereby increasing blood glucose levels. In contrast, the release of GLP-1 from the L cell is contingent upon nutrient ingestion, whereupon it increases insulin secretion, thus decreasing blood glucose levels. This property of GLP-1 as an incretin has led to clinical trials examining the possible therapeutic effects of GLP-1 in the treatment of noninsulin-dependent diabetes mellitus (NIDDM). Apart from the metabolic effects of glucagon and GLP-1, GLP-2 functions as an intestinal growth factor. When injected into normal mice, GLP-2 causes an increase in intestinal epithelial cell proliferation, as evidenced by an increase in crypt-villus height. Thus, GLP-2 could potentially be used for therapy of patients with short bowel syndrome, in whom nutrient absorption is compromised.

Study of the physiology of the L cell has been the subject of research in our lab for several years. The L cell is an ‘open’ type of cell, being subject to regulation by lumenal nutrients, intestinal peptides and neurohormones. The discovery of the functions of the intestinal proG-derived peptides (PGDPs), particularly GLP-1 and GLP-2, has led to studies examining the regulation of synthesis and secretion of the intestinal PGDPs.
At the time this project was initiated, the mechanisms underlying the biosynthesis of the intestinal PGDPs were largely unknown. The then newly discovered mammalian processing enzymes, the prohormone convertases (PCs) finally allowed for the study of the post-translational processing of proG in the intestine. Herein, I present data which demonstrates a role for PC1 in the generation of the intestinal PGDPs.

1.1 The Proglucagon Gene

1.1.1 Anglerfish proglucagon

Identification of the anglerfish proG gene led to the elucidation of the post-translational processing of the proG precursor, which was instrumental in establishing a model for the processing of mammalian proG. The utilization of cell-free translation systems aided in the identification of anglerfish proG. When islet poly(A)-containing mRNA was translated in a cell-free system, two products of MW 14,500 (anglerfish proG I, APG I) and MW 12,500 (APG II) were identified as containing immunoreactive glucagon (1). Cloning of the cDNA encoding APG I revealed nucleotide sequences which contained two glucagon-related sequences arranged in tandem (2,3). Interestingly, the glucagon-like sequences within each gene were flanked by pairs of basic amino acids (Lys-Arg pairs), known sites for endoproteolytic cleavage, thus suggesting that APG I and APG II are post-translationally processed to yield glucagon and a glucagon-like peptide (GLP). Metabolic labelling experiments demonstrated that APG II is processed to 3 cleavage products: glucagon-II$^{52-80}$, GLP-II$^{89-122}$ and GLP-II$^{89-119}$ (4). It appears that GLP-II$^{89-119}$ is processed from GLP-II$^{89-122}$, and is also additionally modified through amidation at its C-terminal end (5). Analysis of the cleavage products of APG I revealed a similar pattern of processing, in that glucagon-I and GLP-I were generated, demonstrating that both
APG I and APG II are post-translationally processed at pairs of basic amino acids to generate a number of unique peptides.

1.1.2 Mammalian proG

Identification of proinsulin in 1967 (6) led to the search for the precursor to pancreatic glucagon. Glucagon-like immunoreactivity (GLI) was also characterized in the intestine (7,8), which suggested an additional site of origin for the precursor. Attempts to identify proG resulted in the isolation of a 12,000 MW protein in extracts of both pancreas and intestine (9) and several smaller MW peptides which could conceivably be contained within the larger sequence. The 12,000 MW protein was named 'glicentin', as it contained glucagon-like immunoreactivity (GLI) and was thought to consist of 100 (cent) amino acids. However, pulse-chase studies in isolated rat islets identified proG as an 18,000 MW protein (10). Cloning of the cDNA encoding hamster preproglucagon (11) revealed nucleotide sequences encoding glucagon and two glucagon-like peptides, GLP-1 and GLP-2, each flanked by pairs of basic amino acids, thus suggesting that these peptides may be products of post-translational processing. Cloning of the cDNA for human (12), bovine (13) and rat (14) proG revealed an identical organization of peptides within the precursor. The proG gene is now known to consist of 6 exons, three of which contain the sequences for glucagon, GLP-1 and GLP-2 (12,14). This structural conservation, along with a high degree of sequence conservation (90-100 %) suggests that the proG gene arose from exon duplication and divergence. In humans and rodents, expression of the proG gene is highly tissue-specific: it is found only in the A cells of the pancreas, the L cells of the intestine and select neurons of the hypothalamus and brain stem. Both the A and L cells are subject to regulation by a variety of factors, including nutrients,
endocrine peptides and neurohormones. As discussed below, several of these factors may act to alter proG gene expression, PGDP synthesis or secretion.

1.1.3 Tissue-specific control of proG gene transcription

Cell-specific control of proG gene expression is mediated through discrete cis-acting DNA elements (Fig. 1.1). Experiments which have determined the nature of these sequences have utilized gene transfer methods to transfect islet cell lines of different phenotypes with constructs encoding fragments of the proG gene 5'-flanking sequence fused to a chloramphenicol acetyltransferase (CAT) reporter gene. In order to determine the protein complexes which bind to these sequences, electro-mobility shift assays (EMSA) utilizing fragments of the proG gene 5'-flanking region were employed. Transfection of proG-CAT fusion genes into islet and non-islet cell lines showed that proG gene transcription occurred only in islet cell lines, and that expression was greater in proG-expressing cells (15). As well, a pronounced decrease in CAT activity occurred after deletion of the 5'-flanking sequence from -239 to -168 (15). These results indicated that elements within the 5'-flanking region were responsible for the islet-specific expression of the glucagon gene, and that the glucagon gene promoter may reside downstream from -168. DNase 1 footprinting analysis revealed three protected regions in the 5'-flanking sequence: G3, from -238 to -264; G2, from -174 to -192; and G1, from -52 to -100 (16).

G3 and G2 have been described as enhancer-like elements, which are defined as cis-acting DNA elements capable of increasing gene transcription from a heterologous promoter independent of distance or orientation. Both G2 and G3 are capable of increasing transcription only in islet-derived cell lines; transcription was not increased in non-islet cells, thus suggesting that G2 and G3 mediate islet-specific expression of the
proG gene (16). Interestingly, deletion of G3 or mutation of the distal half of G3 (from -258 to -243) results in a loss of the glucagon response to insulin (17), suggesting that G3 is the insulin response element (IRE) of the proG gene. Hepatocyte nuclear factor 3β (HNF 3β) binds to G2 to decrease transcription (18). Three isoforms of HNF 3β exist in islets, and may interact to regulate transcription (19). Therefore, islet-specific proteins may bind to G2 and G3 to direct proG gene expression in the islet.

Promoter elements are generally defined as DNA sequences closest to the transcriptional start site. Cellular specificity of function may also be determined by the promoter, as enhancer-stimulated transcription may be more efficient when driven from homologous, rather than heterologous, promoters. The proximal promoter element of the proG gene is the G1 region, and has been so defined based on a number of mutation and deletion studies (16,20). The mechanisms responsible for A cell-specific expression may be explained by islet-specific proteins which form complexes with G1. Interestingly, G1 contains proximal and distal AT-rich sequences characteristic of the core motif known to bind homeobox transcription factors. A number of homeobox proteins are known to activate pancreatic hormone gene expression, including the IDX-1/IPF-1/STF-1 family (21,22), the LIM-domain protein, Isl-1 (23), and a member of the caudal-related gene family, cdx 2/3 (24-26). Isl-1 binds to the distal AT-rich region of G1 (23), while cdx 2/3 binds to the proximal half of G1 (24,25), and both act to control A cell-specific proG gene expression. It is possible that cdx-2/3 may dimerize, although the nature of this dimerization has yet to be fully elucidated.

Much less is known about the elements which direct control of proG gene expression in the intestine. Transgenic mice harbouring approximately 1.3 kb of the proG 5′-flanking region fused to the SV40 large T antigen (GluTag) showed that oncoprotein
expression was directed to the brain and A cells of the pancreas, but no T antigen synthesis was observed in the intestine (27). However, when 2.2 kb of the 5'-flanking region was used in the GluTag fusion construct, transgene expression was directed to the brain, pancreas and the endocrine cells of the stomach and small and large intestine (28). Mice expressing the GluTag (-2.2 kb) fusion product exhibited hyperplasia of the proG-expressing cells in the pancreas and large intestine, and by 4 weeks of age, invasive carcinoma and metastases were evident in the large bowel (28,29). Results from these studies reveal that control of intestinal proG gene expression lies between -1.3 and -2.2 kb. This region, known as the glucagon gene upstream enhancer (GUE), was utilized to further elucidate mechanisms underlying the control of prog gene expression. Utilizing probes generated from the GUE and nuclear extracts from islet (InR1-G9) and intestinal (STC-1 and GTag-Y) cell lines, it was found that the GUE contains both positive and negative transcriptional elements in both islet and intestinal cell lines, and that these effects were mediated through protein complexes both unique to and common to both cell types (30). However, the specific sequences which target proG gene expression to the intestine remain unknown.

1.1.4 Regulation of proG gene expression

1.1.4.1 Second messenger pathways

ProG gene expression is regulated through activation of a number of second messenger pathways. Most studies have examined the roles of the protein kinase A (PKA) and PKC pathways in the modulation of pancreatic and intestinal proG gene expression. Elucidation of the pathways involved in the regulation of gene expression may lead to understanding of the mechanisms underlying the regulation of PDGP biosynthesis.
Fig. 1.1  Molecular Determinants of Proglucagon Gene Expression in the Pancreas.

The 5'-flanking region of the proG gene is depicted up to -2400 bp. The first 300 bp contain the promoter region, with the cAMP response element (CRE) delineating the 5' end of the promoter region. G1 directs proG gene expression to the A cell, while G2 and G3 are enhancer elements directing islet-specific expression of the proG gene. Within G3 is contained the insulin response element (IRE), and G1 binds the homeobox transcription factors, isl-1 and cdx 2/3. As described in Section 1.1.3, fusion constructs containing 1300 bp of the proG gene 5'-flanking region direct expression to the pancreas and brain, while those containing 2400 bp direct proG gene expression to the pancreas, brain and intestine.
The role of cAMP in the regulation of proG gene transcription has been well studied in both islet and intestinal cells and cell lines. The rat and hamster proG gene contain a cAMP response element (CRE) between -291 and -298 in the 5'-flanking sequence (11,14). However, the reported 5'-flanking sequence of the human proG gene (31) does not contain a classical CRE (5'-TGACGTCA-3'), suggesting that the results of these studies are applicable only to rodent proG gene regulation. Since the rodent proG gene contains the perfect CRE octamer sequence (TCACGTCA), it was reasoned that the PKA pathway may be important in the regulation of proG gene transcription. In order to address this question in pancreatic A cells, the hamster islet cell line, InR1-G9, was utilized in the studies described below. Initial studies suggested that the CRE of the glucagon gene may not be functional, as these cells failed to respond to agents which stimulated cAMP levels (32,33). However, when the catalytic subunit of PKA was co-transfected with a proG-CAT fusion construct containing the intact CRE (-350GLU-CAT), gene transcription was increased 4-fold (32,33), and deletion of the CRE sequence abolished this response (32). The results of these studies therefore suggest that the proG gene CRE is functional, and that the PKA pathway plays a role in regulating proG gene transcription. InR1-G9 cells may therefore harbour a defect in the cAMP signal transduction pathway. DNase 1 footprinting analysis indicated that the transcription factor CREB binds to the CRE (32), and additional nuclear proteins may associate with CREB to modulate proG gene transcription in the A cell (34), thereby providing a mechanism by which proG gene transcription, and perhaps PGDP biosynthesis, may be regulated. The results of these studies, while useful in determining the mechanisms underlying regulation of proG gene transcription, also illustrate the limitations of immortalized cell lines. In primary cultures of rat islets, glucagon mRNA levels are increased by treatment with
forskolin (33), thus providing further evidence that proG gene transcription may be regulated in a PKA-dependent manner in the pancreatic A cell.

Assessment of the role of cAMP in proG gene expression in the intestinal L cell was first studied in primary cell cultures due to the lack of suitable L cell lines. Treatment of fetal rat intestinal cell (FRIC) cultures with dibutyryl cAMP, forskolin or cholera toxin increased both proG mRNA and total PGDP levels (35,36). Consistent with these findings, treatment of a mouse enteroendocrine cell line, GTag-Y, with forskolin increased proG gene transcription (37). However, results from studies utilizing the mouse neuroendocrine cell line STC-1 showed forskolin-induced proG gene transcription in the presence and absence of the CRE (38), suggesting a cell-specific mechanism in the cAMP-mediated induction of proG gene transcription.

Utilizing the RIN 1056A islet-derived cell line, proG mRNA levels increased upon treatment with sodium butyrate (39) and the phorbol esters, phorbol myristate acetate (PMA) and dioctanolylglycerol (40). This increase was due to an increase in proG mRNA transcription, as mRNA half-lives did not decrease. Phorbol esters also increased PGDP synthesis and secretion (40), indicating that an increase in proG gene expression results in an increase in peptide production through the PKC pathway in RIN cells. Very recently, a PKC response element has been mapped to G2 of the proG gene promoter region using the islet cell line αTC2 (41), thus providing a potential mechanism by which PKC may stimulate proG gene expression in the pancreas. In contrast, proG gene expression in intestinal cells does not appear to be regulated through the PKC pathway. Treatment of fetal rat intestinal cell cultures (36) and of intestinal cell lines (37,38) with PMA failed to increase proG mRNA levels, although secretion of PGDPs was increased. These results therefore suggest that the PKC pathway mediates peptide secretion, but not
synthesis or gene transcription, in intestinal proG-expressing cells.

1.1.4.2 Extracellular Signals

The A cells of the pancreatic islets are subject to regulation by glucose and other islet hormones. In normal mice, pancreatic proG gene expression is stimulated by fasting and hypoglycemia in vivo (42,43). The effects of insulin on proG gene expression appear to be dependent upon the ambient glucose concentrations. Insulin infusion in normal mice results in hypoglycemia, thus increasing proG gene expression (43). However, insulin infusion in hyperglycemic STZ-diabetic rats results in euglycemia and a decrease in proG gene expression (42), possibly mediated through the IRE (17). As well, proG mRNA is decreased in ob/ob mice, which are hyperglycemic and hyperinsulinemic (42). Therefore, insulin may serve to decrease pancreatic proG gene expression only under conditions of hyperglycemia. ProG gene expression is also reduced by somatostatin in a cAMP-dependent manner (44), in part due to a reduction in the rate of transcription.

The location of the L cells of the intestine render them subject to regulation by a host of factors, including nutrient ingestion, intestinal peptides and neural hormones. Jejunal proG mRNA levels decrease upon fasting, and return to control levels following refeeding (45). As well, infusion of long-chain triglycerides stimulate jejunal proG mRNA levels (45). Interestingly, ileal proG mRNA levels were unaffected by nutrient status in this study. However, the possibility that ileal proG gene expression is altered by fat ingestion cannot be ruled out, as levels of proG mRNA were assessed at only one time point.

Adaptive increases in intestinal growth are associated with diabetes (46) and massive small bowel resection (MSBR) (47,48). Ileal proG mRNA levels are increased in STZ-diabetic rats, and return to normal levels following insulin treatment, thus correlating
with changes in intestinal growth (49). Following MSBR, ileal, not jejunal, proG mRNA expression increases (47,48), and this increase is independent of the presence of lumenal nutrients (50). Chronic changes in the levels of several gastrointestinal hormones accompany MSBR (51). Consistent with this, several intestinal hormones are known to increase proG mRNA, as well as PGDP, levels (36), including gastrin-releasing peptide (GRP) (52) and glucose-dependent insulinotropic peptide (GIP) (53). Taken together, these studies provide a possible hormonal mechanism by which MSBR increases proG mRNA levels in the ileum.

1.2 Post-Translational Processing of ProG

As mentioned above, the first indication that peptide hormones are synthesized from larger precursors came from studies carried out by Steiner et al (6). A model for proinsulin processing was proposed whereby mature insulin and C-peptide are generated within secretion granules through cleavage of proinsulin at pairs of basic amino acids (6). Similarly, it was proposed that proopiomelanocortin (POMC) was a precursor protein within which was contained the smaller peptides, adrenocorticotrophic hormone (ACTH), and endorphin (54). In humans and rodents, only one proG gene is expressed in the A cells of the pancreas, the L cells of the intestine and select neurons of the hypothalamus and brainstem. One mRNA is transcribed in each of these tissues (55-57), giving rise to a single preproG protein. Upon removal of the 20-aa N-terminal signal peptide, proG is transported through the Golgi stacks, apparently without acquiring any additional post-translational modifications, such as glycosylation, sulfation, amidation, acetylation or phosphorylation. Some evidence indicated that proG may be O-glycosylated within its C-terminal sequence (58). However, more recent evidence shows that no glycosylated forms
of proG are detectable in the pancreas (59). Therefore, the diversity of peptides in the pancreas and intestine is due to tissue-specific post-translational processing of the proG precursor.

1.2.1 ProG Processing in the Pancreatic A Cell

In the pancreas, proG is processed extensively at its N-terminal end to yield glucagon as the major bioactive peptide. Pulse-chase studies utilizing isolated islets (10) or pancreatic-derived cell lines (60) have determined that glucagon is detectable only after 3h of chase, indicating that the liberation of glucagon from proG is a late processing event. Other PGDPs derived from the processing of the N-terminal end include glicentin-related polypeptide (GRPP) (61) and a hexapeptide corresponding to proG 64-69 (62) known as intervening peptide 1 (IP-1). There have also been some studies reporting "GLI 9000" (9,63) or "9K peptide" (64), which consists of glucagon completely processed at its C-terminal end but with the N-terminal end remaining attached to GRPP (Fig 1.2).

The C-terminal end of proG remains relatively unprocessed as the major proG fragment (MPGF) within which are contained two glucagon-like sequences (59,65,66). More extensive analysis of the pancreatic processing products of the C-terminal end of proG indicated that MPGF corresponds to proG 72-158, and is produced in equimolar amounts to glucagon (59). As well, small amounts of glucagon-like peptide (GLP)-1, corresponding to proG 72-107 amide, were identified in extracts of human pancreas (59). One study has examined the kinetics of processing of the C-terminal end of proG in pancreatic cells (60). Generation of MPGF occurs early in the secretory pathway, while GLP-1 was detectable after only 3 h of chase, indicating that cleavage at Arg\textsuperscript{109}Arg\textsuperscript{110} is a late processing event. This study also examined the role of a member of the
prohormone convertase (PC) family of endopeptidases in the processing of proG to glucagon, the significance of which will be discussed in Chapter 5.

1.2.2 ProG Processing in the Intestinal L Cell

The intestine contains two GLI peptides, glicentin (61,67,68) and oxyntomodulin (69). Both peptides contain the sequence of glucagon and a C-terminal hexapeptide (Fig 1.2), indicating that, in the intestine, cleavage of the N-terminal portion of proG occurs at Lys\textsuperscript{70}Arg\textsuperscript{71} to generate glicentin, and also Lys\textsuperscript{31}Arg\textsuperscript{32} to yield oxyntomodulin. Processing at Lys\textsuperscript{62}Arg\textsuperscript{63} therefore occurs only in the A cells of the pancreas to liberate glucagon, thus illustrating the tissue-specific processing of the N-terminal end of proG.

That the C-terminal end of proG could be processed to yield the two glucagon-like peptides, GLP-1 and GLP-2, was first evidenced through transfection of a number of cell lines with the cDNA encoding proG and analysing the resulting peptide products by gel filtration chromatography (70). It was also demonstrated that two forms of GLP-1 were produced: full-length GLP-1 (GLP-1\textsuperscript{1-37}) and an N-terminally truncated form, GLP-1\textsuperscript{7-37}. Both forms of GLP-1 were subsequently identified in rat intestine (56), and later studies demonstrated that both forms are C-terminally amidated (66,71) to yield GLP-1\textsuperscript{1-36NH\textsubscript{2}} and GLP-1\textsuperscript{7-36NH\textsubscript{2}}. The major form of GLP-1 in human and rodent small and large intestine was identified as GLP-1\textsuperscript{7-36NH\textsubscript{2}} (64,71,72). Extracts of rat small intestine also contain mature GLP-2 (GLP-2\textsuperscript{1-33}) and intervening peptide 2 (IP-2) which corresponds to proG 111-123 (56,71,72). Additionally, IP-2 was shown to be amidated at its C-terminal end (56), leading to the speculation that IP-2 may be a biologically active peptide. Some
Fig 1.2 Peptide Products of the Tissue-Specific Post-Translational Processing of Proglucagon.

The major products of proG processing are glucagon in the A cell of the pancreas, and glicentin, oxyntomodulin, GLP-1$^{7-36NH2}$ and GLP-2 in the L cell of the intestine, as described in Section 1.2. GRPP, glicentin-related polypeptide; IP, intervening peptide; MPGF, major proglucagon fragment.
Proglucagon

Pancreas

GRPP  Glucagon  IP-1  MPGF

9K Peptide

Intestine

Glicentin  GLP-1\textsuperscript{1-37}/\textsuperscript{38NH}_2  GLP-2

Oxyntomodulin  GLP-1\textsuperscript{7-37}/\textsuperscript{38NH}_2  IP-2
studies have shown that some GLP-2 immunoreactivity may be contained within a larger peptide, which may consist of GLP-2 attached to the IP-2 moiety (56,60). However, more extensive analysis of proG 111-160 revealed that amidated IP-2 and GLP-2\textsuperscript{1-33} were the major processed products of this region in the human intestine (72). Both GLP-1 and GLP-2 appear to be secreted from the intestinal L cell in a 1:1 molar ratio (73), although this study did not distinguish between the different forms of GLP-1. The kinetics of processing of proG in the intestine have not been examined, possibly due to a lack of available intestinal cell lines. Finally, the prohormone convertase PC1 has been implicated in the processing of proG to the intestinal PGDPs (74-77), the results of which will be discussed in Chapters 2-4.

1.2.3 ProG Processing in the CNS

In contrast to the wealth of information available on proG processing in the pancreas and intestine, comparatively little is known about proG biosynthesis and processing in the brain. Immunocytochemical studies have detected GLI peptides in hypothalamus, thalamus, cortex, cerebellum and brain stem (78-82) with the highest levels being detected in nuclei of the hypothalamus. HPLC analysis of GLI peptides in hypothalamic extracts revealed the presence of glicentin and oxyntomodulin (80,82,83) and the absence of pancreatic glucagon, suggesting that proG in the brain was processed in an intestinal fashion. Consistent with this, gel filtration analysis of hypothalamic extracts has demonstrated the presence of immunoreactive GLP-1, the predominant form of which is GLP-1\textsuperscript{7-36NH\textsubscript{2}} (84). Interestingly, glucagon is detectable in fetal rat hypothalamus, and constitutes a significant proportion of GLI (83). However, the GLI:IRG ratio undergoes a dramatic increase between day 21 and adult (85), indicating that a switch to primarily
intestinal-type processing of proG occurs at this developmental stage. The mechanisms underlying this switch have not been investigated, although it may be speculated that a decrease in expression of a processing enzyme may be responsible. Although both PC1 and PC2 mRNA have been detected by in situ hybridization throughout the hypothalamus (86), it is of note that PC1 mRNA expression predominates in the magnocellular neurons of the paraventricular nucleus of the hypothalamus, coincident with the immunocytochemical localization of GLI (80). Taken together, these results suggest that proG and PC1 may be synthesized within the same cell type in the hypothalamus, therefore implicating PC1 in the processing of proG in the brain to the intestinal forms of the PGDPs.

1.2.4 Post-Translational Modifications of PGDPs

In addition to processing by endopeptidases, maturation of peptides derived from prohormone precursors is completed through the action of exopeptidases which remove basic amino acids from the carboxyl terminus of the processed peptides. These enzymes, known as carboxypeptidases, have been localized to the secretory granules of several endocrine tissues, and have been implicated in the maturation of the POMC-derived peptides (87) as well as insulin (88). Indeed, prohormone processing is impaired in mice which lack carboxypeptidase E (CPE; also known as CPH) activity (89), resulting in hyperproinsulinemia. The co-localization of CPE and glucagon in the A cell (90) suggests that a requirement may also exist for CPE-mediated removal of the C-terminal basic amino acids following the processing of proG.

The production of amidated forms of GLP-1 and IP-2 implicates a role for peptidylglycine α-amidating monooxygenase (PAM) in the C-terminal amidation of these
peptides. PAM is comprised of two enzyme activities, peptidylglycine \(\alpha\)-hydroxylating monooxygenase (PHM) and peptidyl-\(\alpha\)-hydroxyglycine \(\alpha\)-amidating lyase (PAL). Both PHM and PAL activities have been co-localized with GLP-1 in the pancreatic A cell (91) and PHM has been detected in the intestinal L cell (92). Furthermore, work from our lab has shown that addition of ascorbate to the medium of fetal rat intestinal cell cultures increases the production of amidated GLP-1 (53), consistent with the known ascorbate dependency of PAM activity (93). Therefore, PAM is a likely candidate for the amidation of GLP-1 and IP-2.

1.3 Regulation of PGDP Secretion: Roles of Nutrients, Endocrine and Neural Factors

The regulation of glucagon secretion from the pancreas and PGDP secretion from the intestine have been studied extensively. Therefore, factors affecting the release of these hormones will be discussed below.

1.3.1 Glucagon

1.3.1.1 The Roles of Glucose and Insulin

It is well known that hyperglucagonemia occurs in both Type 1 and Type 2 diabetes (94). However, in long-standing diabetes, the glucagon response to hypoglycemia is defective (95). Both of these observations have led to the study of the role of glucose and insulin in the regulation of glucagon secretion.

Hyperglycemia suppresses glucagon secretion in vivo (96). The presence of the glucose sensor, glucokinase, in the A cell (97) suggests that glucose mediates a direct effect on glucagon secretion. However, high glucose levels also stimulate the release of
insulin, which also suppresses glucagon secretion in islet cell lines (98,99). It is likely that the effects of insulin on the A cell are direct, as the existence of insulin receptors on these cells has been demonstrated (99). The above studies therefore indicate that both glucose and insulin suppress glucagon secretion via direct effects on the A cell.

In the diabetic (insulin-deficient) state, plasma glucagon levels are abnormally high, and are not suppressed by the hyperglycemia of diabetes. Paradoxically, an abnormal glucagon response to hypoglycemia is observed in long-standing diabetes (95), suggesting that the chronic diabetic state results in defects in both glucagon secretion and glucose counter-regulation.

The amino acids, arginine, alanine and glutamine, all elicit glucagon release in vivo (100) and from purified A cell cultures (101), alone or in combination. Arginine is the most potent amino acid, and also exerts its effects in vivo (102). The mechanism of action is not known.

1.3.1.2 Effects of Second Messengers and Peptide Hormones

Glucagon secretion is increased by stimulation of intracellular cAMP levels, as assessed by treatment of islet cell cultures with forskolin or analogues of cAMP (33,103,104). In addition to the PKA pathway, the PKC pathway also operates in stimulating glucagon secretion from isolated rat islets (104,105) and an islet cell line (40). Both signal transduction pathways may converge to increase intracellular Ca^{2+} concentrations (103,106), although the link between glucagon secretion and intracellular Ca^{2+} concentration has not been well established.

Since the mechanism of glucagon secretion appears to operate through the PKA and PKC pathways, it is not surprising that the endocrine and neuropeptide hormones
which modulate glucagon secretion also exert their actions through these signal transduction pathways. Somatostatin (44,107,108) and GLP-1 (109-111) inhibit glucagon secretion, while the endocrine peptides, GIP (112,113), cholecystokinin (CCK) (114) and secretin (115) all positively regulate glucagon secretion. Receptors for somatostatin and GIP, but not GLP-1, have been found on the A cell (116), suggesting that the inhibitory effects of GLP-1 may be mediated indirectly through somatostatin.

1.3.1.3 Effects of the Autonomic and Central Nervous Systems

The pancreas is innervated by the parasympathetic and sympathetic nervous systems which provide cholinergic, peptidergic and adrenergic stimulation of glucagon release (117,118). The exact mechanisms by which glucagon secretion occurs via these inputs are not known.

1.3.2 Regulation of the Secretion of Intestinal PGDPs

1.3.2.1 Effects of Nutrient Ingestion

It has long been known that administration of nutrients (glucose, fats or mixed meals) into the intestine stimulates the secretion of GLI peptides (119-121). Plasma glicentin levels rise following in vivo intraduodenal administration of glucose (122) or fats (123) in pigs. In preparations of vascularly perfused, lumenally distinct rat duodenum and ileum, fat and glucose infusion increase plasma GLI levels (124,125). In humans, analysis of plasma PGDPs following an oral glucose or fat load revealed that both glicentin and GLP-1\textsuperscript{7-36NH\textsubscript{2}} (referred to as GLP-1 in this section) levels increased (126). Interestingly, the secretory response of GLP-1 to nutrients is observed approximately 20 minutes following ingestion (127-129). However, the majority of GLP-1 containing L cells are located in the
distal ileum (130,131); glucose does not reach the ileum, and ingested fats do not reach the ileum in the first 20 minutes after a meal (132,133), therefore implicating indirect effects of ingested fats on the L cell (125). However, since fats do eventually reach the ileum, direct stimulation of the L cell by fats may also increase GLP-1 release, and may be responsible for the sustained increase in plasma GLP-1 levels seen following ingestion of a mixed meal (128). In support of this hypothesis, GLI and GLP-1 secretion from fetal rat intestinal cell cultures is increased in response to long-chain, monounsaturated fatty acids (134).

To explain the acute effects of glucose and fat ingestion on GLP-1 secretion in vivo, investigations into the hormonal and neural regulation of GLP-1 secretion were carried out. In fetal rat intestinal cells, PGDP secretion is increased in a PKA-, PKC-, and Ca$^{2+}$-dependent manner (35). Intestinal regulatory peptides, most notably GIP, stimulate PGDP secretion from fetal rat intestinal cells (135), and plasma PGDP levels increase in response to intravenous GIP infusion in vivo in rats (125). A novel enteroendocrine loop therefore was proposed, whereby nutrient ingestion lumenally stimulates GIP release from the duodenum, which then acts in an endocrine fashion to increase GLP-1 secretion from the ileal L cell (124,125). This hypothesis is supported by the findings that GIP stimulates GLP-1 secretion from isolated perfused rat ileum (136) and colon (137). However, infusion of GIP may not stimulate GLP-1 release in humans (138,139). Therefore, in humans, the GLP-1 response to nutrient ingestion may also be mediated by a neural mechanism.

The intestine is innervated by both extrinsic and intrinsic nervous systems. Extrinsic innervation occurs via the vagus nerve, which sends postganglionic cholinergic fibres to the small intestine and proximal colon. The vagus may mediate its effects through the myenteric plexus, synapsing with cholinergic or peptidergic neurons. Infusion of
acetylcholine increases gut GLI secretion in dogs (140), and atropine inhibits this response, indicating that cholinergic mechanisms play a role in stimulation of gut GLI secretion. Muscarinic agonists stimulate PGDP secretion in FRIC cultures (135), and also increase GLP-1 secretion in preparations of isolated perfused rat ileum (136,141) and colon (137), possibly acting through M3-subtype receptors (142). The role of the vagus nerve in stimulating GLP-1 secretion has been further examined through performing selective vagotomies in rats. Celiac vagotomy abolished the GLP-1 response to oral glucose (143). Vagal control of fat-induced PGDP secretion was demonstrated when bilateral subdiaphragmatic vagotomy abolished the fat-induced rise in PGDP secretion in anesthetized rats (144). Therefore, the acute response of GLP-1 to nutrient ingestion may also be regulated through the vagus nerve.

As mentioned above, the vagus forms synapses with neurons within the myenteric plexus. It has been shown that nerve endings within the myenteric plexus and submucosal ganglia contain GRP immunoreactivity (145), and that GRP release is mediated through vagal stimulation (146), thus implicating a vagus-GRP mechanism in the regulation of the L cell. Indeed, in preparations of isolated perfused rat ileum, infusion of GRP or bombesin elicits a biphasic GLP-1 response (136,141) and physiological concentrations of GRP increase PGDP secretion from fetal rat intestinal cells (135). That GRP may mediate the increase in GLP-1 following ingestion of fats was demonstrated when infusion of a GRP antagonist abolished the fat-induced rise in PGDP secretion (147). However, plasma GIP levels were reduced by 60%, indicating that GRP exerts its effects independent of GIP. Therefore, in the rat, the nutrient-induced rise in GLP-1 levels may be mediated not only through the endocrine system, but also through the vagus nerve and GRPergic neurons in the intrinsic nervous system of the gastrointestinal tract.
A similar mechanism underlying regulation of GLP-1 secretion has been proposed to exist in humans (129).

1.4 Biological Functions of the PGDPs

Of the PGDPs described above, biological functions have been ascribed to glucagon, oxyntomodulin, GLP-1$^{7-36\text{NH}_2}$ and GLP-2.

1.4.1 Glucagon

The discovery of glucagon was made in 1923, upon the observation that pancreatic extracts induced hyperglycemia when administered to depancreatized dogs. The contaminating substance was named "glucagon", or mobilizer of glucose. Since then, decades of research have resulted in the elucidation of the physiological actions of glucagon, including identification of target organs, receptors and intracellular signalling pathways, and regulation of glucagon production.

It is well established that glucagon is the major glucose counterregulatory hormone (148), the others being epinephrine, cortisol and growth hormone. The major physiological target cells of glucagon are the hepatocyte and perhaps the adipocyte. The primary action of glucagon is to stimulate hepatic glucose production through glycogenolysis and gluconeogenesis, as well as through inhibition of glycogen synthesis (149). Experiments carried out in the conscious dog have demonstrated the importance of glucagon in maintaining euglycemia during fasting (150), and also show that glycogenolysis and gluconeogenesis are equally sensitive to glucagon stimulation (151). However, some controversy exists as to whether glucagon regulates lipid turnover in adipocytes. Some studies have shown that glucagon regulates free fatty acid metabolism by increasing the
rate of lipolysis (152). Glucagon receptors have been localized on adipocytes (153), suggesting direct effects of glucagon on adipocytes. These results are consistent with the known catabolic effects of glucagon, and also may provide an indirect mechanism by which the resulting increase in glycerol would provide substrate for gluconeogenesis. However, other studies have failed to demonstrate a role for glucagon in lipolysis (154). The confounding effect of insulin underscores the major difficulties in studying regulation of lipid metabolism in vivo. In addition to fuel metabolism, glucagon also exerts positive inotropic and chronotropic effects on the heart, diuretic effects on the kidney and spasmolytic effects on the digestive tract (149), although some of these effects may be pharmacological rather than physiological.

The cloning of the rat hepatic glucagon receptor (155) provided a means by which the molecular mechanisms of glucagon action could be examined. Upon binding to its receptor on the hepatocyte, glucagon exerts its effects through two intracellular signalling pathways: stimulation of adenylyl cyclase via the stimulatory G protein, Gs, resulting in activation of the PKA pathway; and increasing IP₃ production through Gq, resulting in elevation of intracellular Ca²⁺ (156). The two pathways may converge, resulting in the synergistic activation of glucose metabolic enzymes in the liver (106,157). However, the link between second messenger pathways and activation of specific hepatic enzymes is not yet clear.

1.4.2 Oxyntomodulin

A 37-aa peptide containing the sequence of glucagon was first isolated from extracts of small intestine (69) and shown to bind to membrane preparations from rat gastric fundus (158). This peptide was termed "oxyntomodulin" since it was shown to
inhibit pentagastrin-stimulated gastric acid secretion from the parietal (oxyntic) cell in a dose-dependent manner (159,160). Oxyntomodulin also decreases gastric acid secretion induced by histamine or nutrient ingestion (161,162). Interestingly, the C-terminal fragment of oxyntomodulin (oxyntomodulin<sup>19-37</sup>) appears to be equipotent in its effects (161). Since receptors for oxyntomodulin have not been identified, the effects of oxyntomodulin may be indirect. This conclusion is supported by the finding that oxyntomodulin stimulates somatostatin release from primary cultures of rabbit fundic D cells (163). Therefore, oxyntomodulin may act to increase somatostatin release, which then inhibits gastric acid secretion via a paracrine effect on the parietal cell. Some evidence also suggests that oxyntomodulin may act in conjunction with CCK, a known regulator of the D cell, to stimulate somatostatin release (163).

1.4.3 GLP-1

The identification of a glucagon-like sequence in the mammalian proG gene led to the search for a biological action for GLP-1. An early study reported that GLP-1<sup>1-36NH2</sup> weakly stimulated insulin release from isolated rat islets (164). However, the biological function of GLP-1 was firmly established through a series of studies, the first of which demonstrated that the N-terminally truncated form of GLP-1, GLP-1<sup>7-37</sup>, increased insulin secretion and gene expression in a rat insulinoma cell line (165). Subsequent studies showed that GLP-1<sup>7-37</sup> also increased insulin secretion from perfused rat (166) and pig (167) pancreas. Infusion of GLP-1<sup>7-36NH2</sup> in humans demonstrated that the insulinotropic effects of GLP-1 were glucose-dependent (138), thus establishing GLP-1<sup>7-36NH2</sup> as an incretin hormone. Both GLP-1<sup>7-37</sup> and GLP-1<sup>7-36NH2</sup> are equipotent in their insulinotropic effects (168), while full-length GLP-1 fails to elicit an insulin response when administered
at physiological concentrations (110), indicating that N-terminal truncation, and not C-terminal amidation, confers biological activity. As well, GLP-1\textsuperscript{7-36NH\textsubscript{2}} was found to be the predominant form of GLP-1 in the human intestine (138,169), and these studies showed that plasma levels of GLP-1\textsuperscript{7-36NH\textsubscript{2}} increase after ingestion of a meal. That GLP-1 accounts for about 50% of the postprandial incretin activity was clearly demonstrated when infusion of the specific GLP-1 antagonist, exendin\textsuperscript{9-39}, reduced the insulin response after a meal, and also resulted in a slight increase in blood glucose levels (170,171). Additionally, the insulin response to an oral glucose load is impaired in mice carrying a null mutation in the GLP-1 receptor (172), further illustrating the importance of GLP-1 as a physiological incretin.

The biological property of GLP-1 as an incretin has made it an attractive candidate for therapy of non-insulin dependent diabetes mellitus (NIDDM). The actions of GLP-1 are glucose-dependent, an advantage over the conventional sulfonylurea therapy. In addition to its effects on insulin secretion, GLP-1 also increases insulin gene expression (165,173) and biosynthesis (173), thus ensuring that insulin stores are not depleted. Evidence from several studies indicates that administration of GLP-1 to NIDDM patients significantly reduces the postprandial hyperglycemia normally observed, and stimulates secretion of endogenous insulin (174-180).

In addition to its insulinotropic effects, GLP-1 also decreases glucagon secretion (110,168,181) and inhibits gastric emptying (178,182) and gastric acid secretion. Since GLP-1 receptors have not been localized on the pancreatic A cell (116), the glucagonostatic effects of GLP-1 may be mediated through increasing somatostatin release from the pancreatic D cell (183). A similar mechanism of action may operate in the gastric inhibitory effects of GLP-1, as it has been shown that GLP-1 increases
somatostatin release in a dose-dependent manner from perfused rat stomach preparations (184), and that this increase is inhibited by exendin$^{9-39\text{NH}}$r2 (185). Together, these effects of GLP-1 could contribute to its glucose-lowering effects by reducing hepatic glucose production and slowing nutrient absorption. These mechanisms could also explain the reduction in postprandial glycemic excursions observed upon administration of GLP-1 to IDDM patients (186,187), who do not produce endogenous insulin.

Studies investigating a role for GLP-1 in enhancing glucose disposal and hepatic glycogen synthesis have yielded conflicting results. Consistent with the presence of putative GLP-1 binding sites on membranes of hepatocytes (188) and skeletal muscle (189), glycogenic effects of GLP-1 on liver (190) and muscle (191) have been reported. However, other studies could not substantiate these results (192). Some in vivo studies have demonstrated that GLP-1 may enhance glucose disposal in an insulin-independent manner (193,194) while others have not observed this effect (195). These contradictory results could be explained through differences in experimental technique in measuring glucose disposal in the absence of insulin. Expression of GLP-1 receptor mRNA cannot be detected in liver or skeletal muscle by Northern blot analysis (196), although, using RT-PCR, GLP-1 receptor mRNA transcripts could be detected in the liver (197). The disparities in GLP-1 receptor detection could be attributed to the expression of different types of GLP-1 receptors on hepatocytes and muscle cells. However, studies carried out on mice expressing a null mutation of the GLP-1 receptor (172) clearly demonstrated functional elimination of the GLP-1 response to glucose ingestion, indicating that only one receptor type is expressed. That these mice exhibited abnormal glucose handling upon both oral and intraperitoneal administration of glucose provides support for the hypothesis that GLP-1 may enhance glucose disposal in both an insulin-dependent and insulin-
independent manner.

A recent study identified GLP-1 as a potent satiety factor (198), as ICV administration of GLP-1 in rats sharply decreased food intake. That GLP-1 receptors are expressed in the hypothalamus (199,200) is consistent with a role for GLP-1 in the central regulation of feeding behavior. However, normal feeding behavior is observed in mice which do not express functional GLP-1 receptors (172). These results indicate that, while GLP-1 may be a mediator of satiety, other regulatory peptides may be more important within the complex model of regulation of feeding behavior.

The cloning of the GLP-1 receptor (201) and its localization on the pancreatic B cell (116) has led to the identification of intracellular signalling pathways through which GLP-1 stimulates insulin production. Not surprisingly, GLP-1 enhances the B cell response to glucose, thus rendering B cells glucose competent (202). GLP-1 potentiates the glucose-induced rise in intracellular Ca\(^{2+}\), and additionally increases cAMP levels (203,204), which results in activation of voltage-dependent Ca\(^{2+}\) channels, thus increasing insulin secretion. It has also been postulated that nonselective cation channels may be activated, resulting in a Na\(^{+}\) current which may be coupled to increased insulin secretion (205). As well, GLP-1 and glucose synergistically inhibit K\(^{-}\)-ATP channels (202), thus increasing membrane depolarization and generation of action potentials. The combination of increased Ca\(^{2+}\) and membrane electrical activity results in increased insulin secretion by GLP-1 in a glucose-dependent fashion. It has been shown that binding of GLP-1 to its receptor on the rat gastric gland or in rat brain and pituitary homogenates also results in an increase in the adenylyl cyclase-mediated signal transduction cascade (206,207). The mechanism of action of GLP-1 at other receptor sites (lung and kidney) is not known, although the stucture of the receptor implicates the activation of the PKA pathway.
1.4.4 GLP-2

Several lines of evidence support a relationship between elevated levels of plasma PGDPs and increased bowel growth. Two early reports of patients with enteroglucagon tumours and small intestinal hypertrophy suggested a correlation between PGDPs and bowel growth (208,209). Additionally, increased small bowel growth was observed in GLUTag transgenic mice (28,210), which have elevated PGDP plasma levels. Finally, propagation of proG-producing tumours in nude mice resulted in an increase in crypt:villus height of the small intestinal epithelium (211). Subsequent administration of synthetic intestinal PGDPs to normal mice resulted in the identification of GLP-2 as the PGDP responsible for intestinal growth (211).

GLP-2 acts specifically on the mucosa of the small intestine to increase crypt-villus height (211), resulting from an increase in the rate of crypt cell proliferation and a decrease in apoptosis (212). The intestinotrophic properties of GLP-2 are maintained through various regimens of dose, vehicle and route of administration (212) and in male and female mice of all ages (213). As well, this newly grown bowel is functional, as assessed through assessment of intestinal enzyme activities and through oral nutrient tolerance tests (214). The biological activity of GLP-2 is regulated through degradation by the plasma enzyme, dipeptidyl peptidase IV, and a degradation-resistant analogue of GLP-2 has been developed and shown to exhibit enhanced growth-promoting effects as compared to native GLP-2 (215,216). The growth effects of GLP-2 are consistent with reports of increased intestinal proG gene expression associated with intestinal adaptation (48,217) and massive small bowel resection (47,48). The above efforts to characterize the actions of GLP-2 in vivo may eventually lead to the use of GLP-2 as therapy for patients with short-bowel syndrome, in whom nutrient absorption is compromised (218).
Indeed, concomitant administration of GLP-2 with total parenteral nutrition (TPN) prevents the TPN-induced intestinal hypoplasia that is often a source of complications in patients with impaired gastrointestinal function (219).

The GLP-2 receptor has not yet been identified, but it is likely that it belongs to the glucagon/vasoactive intestinal peptide/secretin receptor subfamily of seven transmembrane, G protein-coupled receptors, and thus exerting its actions through increasing intracellular cAMP (206). Evidence exists for the activation of the MAP kinase pathway, which is coupled to regulation of cell growth, division and differentiation, by G protein-coupled receptors (220). It is therefore possible that GLP-2 could exert its actions through such a mechanism.

1.5 Summary

Mammalian proG is processed in a tissue-specific manner to yield a number of peptides unique to the pancreas and intestine. Novel biological functions have been ascribed to the intestinal PGDPs, GLP-1 and GLP-2, thus raising interest in the mechanisms underlying their biosynthesis. The recent discovery of the prohormone convertase family of processing enzymes facilitated the study of the processing of proG to the intestinal PGDPs.

1.6 Identification of The Prohormone Convertase Family of Processing Enzymes

As described above, bioactive peptides are generated through post-translational processing of a larger precursor. Until recently, the enzymatic activity which cleaves prohormones could only be referred to as "trypsin-like". However, within the past 8 years, a family of mammalian subtilisin-like serine proteases, coined "prohormone convertases"
have been identified, extensively characterized, and shown to cleave a number of proproteins and prohormones at pairs of basic amino acids to liberate their bioactive peptides. The following is a review of the discovery of the PCs, their biosynthesis and regulation and their role in prohormone processing.

1.6.1 KEX2 Was Identified as a Eukaryotic Processing Enzyme

One approach for the search for prohormone processing enzymes began using the yeast *Saccharomyces cerevisiae* as a model (221). It was known that *S. cerevisiae* required processing of prohormones involved in its life cycle. Pro-α-mating factor and pro-killer toxin are translated in tandem copies and must be cleaved at Lys-Arg and Arg-Arg residues to generate the mature proteins (221). This enzymatic activity was mapped to the *KEX2* locus, and was named KEX2 (for killer expression 2), since an exopeptidase activity had been named KEX1 (221). Biochemical characterization of KEX2 revealed it as a Ca\(^{2+}\)-dependent serine protease (222,223). Cloning of the *KEX2* gene (224) showed it to be related to the bacterial subtilisin family, based on sequence homology of the catalytic domains.

KEX2 is initially synthesized as proKEX2, the N-terminal prosegment of which must be removed for catalytic activity (225) via an autocatalytic, intramolecular reaction (226). Mutation of the active site His\(^{213}\) or Ser\(^{385}\), or of the P domain (Fig. 1.3), results in failure to cleave the prosegment (226), indicating the functional importance of these domains. Studies on the intracellular targeting of KEX2 showed that its C-terminal domain contains a determinant which localizes the enzyme to a late Golgi compartment (227). These structural features of KEX2 are paralleled in the mammalian PC family, as shown in Fig. 1.3.
That the functions of KEX2 and its mammalian counterpart are highly conserved was evidenced by the fact that KEX2 cleaves proinsulin to mature insulin in yeast (228), and POMC to some of its expected peptide products in heterologous cell lines (229). These results indicated that KEX2 might contain the structural and functional features of a mammalian processing enzyme. In particular, it was the conservation of structure, especially within the catalytic domain, which ultimately permitted the identification of the mammalian PC enzymes.

1.6.2 Furin, the First Mammalian Processing Enzyme

A search of databases for sequences homologous to KEX2 resulted in identification of a sequence corresponding to the human gene fur (227). Comparison of the sequences for KEX2 and the fur gene product, named furin, revealed significant structural conservation, particularly of the active site residues within the catalytic domain, and of the C-terminal domain (227), suggesting that furin may be a mammalian processing enzyme. The presence of a hydrophobic transmembrane domain followed by a charged, hydrophilic cytoplasmic tail (227) is unique among the mammalian PC family. Furin has a ubiquitous distribution, as assessed by Northern blot analysis (230), and cleaves a large number of constitutively expressed proteins, including viral glycoproteins, toxins, receptors, plasma proteins, and hormones and growth factors (231). The consensus site for endoproteolytic cleavage by furin is an Arg-X-Lys/Arg-Arg motif (232). More extensive analysis of the furin cleavage site indicated that the absolute requirements for furin-mediated cleavage are the presence of Arg at position -1 and a basic residue at position -4, while basic residues at position -2 and -6 have an additive effect on the activity of furin (233).
1.6.3 Identification of PC1/3 and PC2

In 1990, two laboratories, working independently but using similar approaches, identified two distinct cDNA sequences which were structurally related to KEX2 and furin. Smeekens and Steiner (234) screened a human insulinoma cDNA library with a PCR-derived cDNA probe. The degenerate oligonucleotides used as primers for the PCR reaction were designed from the highly conserved active site of KEX2 and the bacterial subtilisins. Isolation of the corresponding full-length cDNA clone revealed an open reading frame which encoded a protein structurally homologous to KEX2, particularly within the catalytic domain. This protein was named PC2, as furin was the first PC to be identified. The active site Ser in the PC2 sequence aligned exactly with that from KEX2 and furin, indicating that PC2 was a serine protease with similar functions.

Using a similar approach based on the concept of sequence conservation around the active sites of serine proteases, Seidah et al (235,236) isolated two distinct cDNA clones from a mouse pituitary cDNA library. One sequence was identical to the PC2 sequence identified by Smeekens and Steiner (234), and the other was designated PC1. A full-length sequence identical to PC1 was also subsequently identified in the mouse corticotroph-derived AtT-20 cells by Smeekens et al (237), but was termed PC3, as it was the third mammalian prohormone convertase identified. However, throughout this chapter, this enzyme will be referred to using the original designation, PC1. Sequence alignment showed that mouse PC1 has 55%, 64% and 47% amino acid identity with mouse PC2, human furin and KEX2, respectively, within the catalytic domains (236,237) (Fig 1.3). In particular, the major sites of sequence identity were comprised of the amino acids surrounding the catalytic triad Asp$^{140}$, His$^{181}$ and Ser$^{355}$ (236). Other sites of sequence identity include Asn$^{282}$, which is replaced by Asp$^{285}$ only in PC2, and is associated with
development of the oxyanion hole in bacterial subtilisins (238). As well, a tripeptide sequence, Arg-Gly-Asp, is present in PC1, PC2 and furin, but not in KEX2 or subtilisin BPN' (236). This motif is a recognition sequence by cell surface integrins implicated in cell-cell adhesion (239). However, significant structural differences exist between the C-terminal domains of KEX2, furin, PC1 and PC2 (Fig. 1.3). Unlike furin or KEX2, PC1 and PC2 contain a putative amphipathic helical structure within their C-terminal segments which may function in targeting to secretion granules (240,241). Structural comparisons of all the known PCs, as well as KEX2 and furin, are depicted in Fig 1.3, and are described at the end of this section.

The tissue distribution of PC1 and PC2 is unique among the PC family, in that the expression of these enzymes to be restricted to endocrine and neural cells and tissues (236,242). Both PC1 and PC2 have been localized within the secretory granules in pancreatic islet cells (243), the anterior and neurointermediate lobes of the pituitary (242), endocrine cells of the heart (244) and the neuropeptide-rich regions of the CNS (86,245). As well, PC1 and PC2 each exhibit distinct tissue and cell distributions (236,242), thus suggesting that tissue-specific expression of PC1 and PC2 may be responsible for the tissue-specific processing of several prohormones. This will be further explored in later sections.

1.6.4 Identification of Other Members of the PC Family

1.6.4.1 PACE4

Screening of human liver- and kidney-derived cDNA libraries resulted in the identification of PACE4 (for paired amino acid converting enzyme 4) (246). Differential splicing of PACE4 results in the generation of several isoforms (246-248). Like furin,
PACE4 has a ubiquitous distribution, being expressed in most cells and tissues (230). Interestingly, only one isoform of PACE4 is detected within the pancreatic islet cells (249). Using *in situ* hybridization, the expression of PACE4 mRNA within the CNS is detected in both neuronal and non-neuronal cells (250). In fact, rat PACE4, compared to human PACE4, exhibits higher tissue-specificity of expression, being highly expressed in the anterior pituitary, and various regions of the brain and heart (251). In the anterior pituitary, PACE4 expression may be regulated by thyroid status (251), suggesting that, in the rat, PACE4 may play a role in the processing of peptide hormones.

1.6.4.2 PC4

Identification of the fifth member of the prohormone convertase family of mammalian serine proteases, PC4, was made through screening of a mouse testis cDNA library with probes derived from the catalytic domain of furin, PC1 and PC2 (252). Structurally, PC4 contains similar domains to those found in both PC1 and PC2, with a C-terminal domain intermediate in size between that of PC1 and PC2. Alternate splicing of the rat PC4 gene results in the generation of five isoforms (253,254). Using *in situ* hybridization, it has been shown that PC4 expression is restricted to pachytene spermatocytes and round spermatids during the early stages of spermiogenesis (253,255), suggesting that PC4 may have a specific physiological function in reproduction. Co-localization of PC4 and proenkephalin expression suggests a possible enzyme-substrate relationship, although this has yet to be shown.

1.6.4.3 PC5/6

A sixth member of the PC family was identified by two different groups by
screening an adrenocortical-derived cDNA library, and called PC5 (256), and mouse intestinal and brain cDNA libraries, and designated PC6 (257). Throughout this chapter it will be referred to as PC5. Alternative splicing of the PC5 gene results in the generation of two isoforms, PC5a and PC5b (258), the primary distinguishing feature being the presence of a transmembrane domain in PC5b (Fig. 1.3). PC5 is most highly expressed in neuronal cells of the brain (250), throughout the small intestine (256,257), and the adrenal gland (256,259). That PC5 has been co-localized with renin within the zona glomerulosa of the adrenal gland suggests that it could play a role in the activation of a local renin-angiotensin system in the adrenal cortex (259). Very recently, PC5a has been immunocytochemically co-localized with glucagon and PC2 within the secretory granules of pancreatic A cells (260). Interestingly, PC5a and PC5b appear to be sorted to different intracellular compartments (260), with PC5a being targeted to secretory granules, while PC5b is retained within the Golgi. Thus, PC5a may play a role in the processing of prohormones, while PC5b, like furin, may participate in the processing of constitutively expressed proteins.

1.6.4.4 PC7

The cDNA sequence for rat PC7 was very recently identified by screening total RNA obtained from rat anterior pituitary and human BEN cells (261). The amino acid sequence for PC7 predicts a protein structure very closely resembling that of furin (262). Northern blot analysis revealed that PC7 is expressed in a wide variety of tissues, and most highly expressed in the colon and lymphoid tissues (261). Although intracellular localization has not yet been determined, it is predicted that PC7, like furin resides primarily in the Golgi. Thus, PC7 may function in the processing of constitutively
expressed proteins.

1.6.5 Summary

Structural comparison of KEX2, furin, and the known members of the PC family indicates organization into discrete domains (263) (Fig 1.3). Each enzyme contains an N-terminal signal peptide, followed by a pro-segment, removal of which is required for enzyme activation, a catalytic domain containing the highly conserved active site Asp, His and Ser, and a structural domain sometimes referred to as homoB or P domain, within which is contained the Arg-Gly-Asp sequence. After the P domain, each enzyme has a unique C-terminal sequence. Furin, PACE4, PC5a (256,257) and PC5b (258) all contain, to varying degrees, a domain containing Cys-rich repeats, the function of which is unknown. KEX2, furin and PC7 are the only enzymes in which a transmembrane domain and hydrophilic cytoplasmic tail have been identified. The C-terminal segments of PC1 and PC2 contain a putative amphipathic helical structure which may function in targeting to secretion granules (240,241). Differential splicing of PACE4 results in multiple isoforms differing in their N- or C-termini (247,248) and similarly, diverse forms of PC4 also exist which differ in their C-terminal structures (253,254).

1.7 Biosynthesis and Regulation of Furin, PC1 and PC2

The presence of a putative pro-region within the structure of the prohormone convertase enzymes led to the hypothesis that these processing enzymes may themselves be synthesized as inactive pro-proteins. That a site for pro-peptide cleavage (Arg-Ser-Lys-Arg, RSKR) is present at the junction of the pro- and catalytic domains further suggested that the maturation of the PCs occurs through removal of the N-terminal
Structural Domains of the Prohormone Convertases.

Schematic representation of the various domains of the mammalian PCs and their comparison to the yeast processing enzyme, KEX2 (top) and the bacterial subtilisin (Sub) family of processing enzymes (bottom). The catalytic triad of Asp (D), His (H) and Ser (S) are indicated within the catalytic domain, as well as the conserved Asn (N). This residue is replaced by Asp in PC2. The indicated transmembrane domain within PC7 is putative, as it contains a hydrophobic stretch of amino acid residues, and has not yet been characterized. y, yeast; h, human; m, mouse; r, rat.
yKex2
hFurin
mPC1/3
mPC2
rPC4
hPACE4
rPC5/6
rPC7
Sub BPN'

Signal Peptide
Prosegment
Catalytic
P-domain
Ser/Thr
Transmembrane
Cytosolic
Cys Repeats
Amphipathic
RGD Sequence
pro-region. Several studies have now characterized the biosynthesis, processing and enzymatic activities of furin, PC1, PC2, and, more recently, PC5.

1.7.1 Furin

Furin is initially synthesized as a 104 kDa precursor. Pulse-chase studies have shown that processing is completed within 10-40 minutes of translation (264,265), suggesting that processing of profurin occurs in the ER. Mutation of the active site Asp or Ser inhibit proregion cleavage, and this inhibition cannot be rescued by expression of wild-type furin in trans (266), indicating that proregion removal occurs in the ER, and is an autocatalytic, intramolecular event.

Furin has been localized to the Golgi (264,267), and the means by which mature furin is localized to the Golgi has been extensively studied. Like KEX2 (227), the cytoplasmic tail of furin contains determinants for Golgi localization (264,268). Two motifs within the cytoplasmic tail may function to target furin to the Golgi, as well as in the internalization from the cell surface: a strongly hydrophilic region containing seven acidic amino acids and a tyrosine-based sequence which is similar to the motif present in other TGN proteins (268,269). The localization of furin to the Golgi appears to involve casein kinase II-mediated phosphorylation of serine residues within the acidic region of its cytoplasmic tail (270,271).

Within the TGN, furin processes proproteins which are targeted to the constitutive secretory pathway, although overexpression of furin can result in the processing of prohormones which are normally sorted to the regulated secretory pathway (272,273). Overexpression of furin in endocrine-derived cells may function in cell dedifferentiation by causing a switch in the sorting of prohormones from the regulated to the constitutive
secretory pathway (274). Additionally, expression of PCs which function within the regulated secretory pathway is decreased. Analysis of the 5'-flanking region of the gene encoding furin revealed the presence of multiple promoters which contain sequences characteristic of housekeeping genes (275), consistent with promoter regions of various Golgi-associated proteins.

1.7.2 PC1

1.7.2.1 Mechanisms Underlying the Biosynthesis of PC1

The biosynthesis of PC1 has been studied extensively. Pulse-chase analysis was used to investigate processing of endogenous PC1 in AtT-20 cells (276) or vaccina virus-infected PC1 in a number of cell lines (277,278). Various molecular weight forms of PC1 of 87-88 kDa, 80-83 kDa and 66-67 kDa were detected, corresponding to pro-PC1, N-terminally truncated PC1, and N- and C-terminally truncated PC1, respectively. Removal of the pro-segment occurs through cleavage at the RSKR83 motif, as assessed through N-terminal microsequencing (277,279) and mutational analysis (280,281). Prosegment removal, as well as C-terminal truncation, occurs through an autocatalytic, intramolecular reaction, since rendering the enzyme inactive through mutation of the active site (240,280,281) inhibits the processing of proPC1 to 87 kDa PC1, and this inhibition could not be rescued through overexpression of catalytically active PC1 or furin. Studies on the kinetics of proPC1 processing demonstrate that the 83 kDa PC1 is detected after 20 min of chase (276,278,279,282), while the 66 kDa form is detectable only after 2-3 h of chase (276,282). These results indicate that cleavage of proPC1 to PC1 occurs in the ER, while generation of the 66 kDa form takes place in a late compartment, most likely within the secretory granules. The processing of pro-PC1 or the 83 kDa form of PC1 does not
appear to be Ca\(^{2+}\)- or pH-dependent (281,283). However, the enzymatic properties of the 83 kDa and the 66 kDa forms of PC1 are pH- and Ca\(^{2+}\)-dependent, in accordance with the intracellular compartment in which they are produced (279,284,285). Therefore, the pH and Ca\(^{2+}\) requirements for enzymatic activity of PC1 are more stringent than those for processing.

A model for the processing and activation of proPC1 can therefore be proposed. Following signal peptide removal, 87 kDa proPC1 is processed to the 83 kDa form by removal of the N-terminal prosegment. This form is transported through the Golgi and is catalytically active, and may therefore process some prohormones early in the secretory pathway. The C-terminal end targets PC1 to the secretory granule (240), whereupon C-terminal truncation yields a 66 kDa form of PC1, which would be responsible for the processing of prohormones later in the secretory pathway.

1.7.2.2 Regulation

PC1 is the only prohormone convertase for which the promoter region has been extensively characterized. Analysis of the mouse PC1 promoter region indicated the presence of several putative transcriptional regulatory elements, including an AP-1 binding site, suggesting regulation by the PKC pathway, and a CRE, suggesting regulation through the PKA pathway. Analysis of the 5'-flanking region of the human PC1 gene revealed the presence of two putative CREs, CRE-1 and CRE-2, which are closely spaced (286). Deletion analysis indicated that both elements could direct a cAMP-mediated response to hormonal stimulation. Subsequently, electromobility shift assays identified three protein complexes, two of which bound CRE-1 (287). One of these protein complexes appeared to contain CREB-1 and ATF-1, and both transcription factors were shown to activate PC1
gene expression (287), thus indicating a potential mechanism by which processing enzymes and their substrates may be co-regulated by the same intracellular signalling pathway. As well, PC1 mRNA transcript levels are regulated by treatment of cells with PMA (288), indicating a role for the PKC pathway in regulating PC1 expression.

1.7.3 PC2

The kinetics of processing and enzymatic activation of PC2 follows a different path from that of PC1. The maturation of PC2 alone will first be discussed, followed by the role of its molecular chaperone, 7B2, in the process of maturation and activation.

Several studies have determined that proPC2 is initially synthesized as a 75 kDa protein, acquires several N-glycosylated moities during transport through the Golgi (278,281,289,290), and is processed through removal of the N-terminal prosegment to generate a 68 kDa, catalytically active form of PC2 within the secretory granule (289,291). The mechanism of prosegment removal appears to be autocatalytic and intermolecular (189,291,292). Cleavage occurs in a late secretory compartment, as the 68 kDa form is detectable only after 14-16 hours of chase in Xenopus oocyte translation systems (281,289), and after 3 hours of chase in endocrine and non-endocrine cells (240,278,290,291). The C-terminal domain of PC2 may serve as a sorting domain for targeting to secretory granules (241,291). Both proPC2 processing and PC2 bioactivity are pH- and Ca\(^{2+}\)-dependent (281,293-295), consistent with the known site of action of PC2 within the secretory granule compartment.

1.7.3.1 The Role of 7B2 in the Maturation and Activation of PC2

In addition to hormones and neuropeptides, secretory granules in endocrine cells
also contain one or more chromogranin/secretogranin proteins (296). These proteins, known collectively as the granins, are widely distributed in a variety of endocrine cells and neurons, and have been used as markers for such cells (297). It is thought that the granins play a role in the sorting of proteins to the regulated secretory pathway, and in the biogenesis of secretory granules (296,298). One of the members of the granin family is 7B2, which has been co-localized with insulin and glucagon in the secretory granules of the B and A cell, respectively (299,300).

7B2 has recently been found to associate with PC2 to modulate its activity (301) and to prevent its premature activation (302). Interestingly, the pro-form of 7B2 contains two discrete domains: the N-terminal domain bears significant sequence similarity to the chaperonin 60 subclass of molecular chaperones, while the C-terminal domain bears some sequence similarity to the potato inhibitor 1 family of protease inhibitors (302). Pro7B2 binds to proPC2 in the ER (303), and the nature of the interaction appears to involve the oxyanion hole Asp \textsuperscript{309} residue of proPC2 (304) and a Pro-rich helical structure within the C-terminal domain of 7B2 (305). The function of the N-terminal domain of 7B2 is not clear, since this domain appears to be cleaved from 7B2 prior to proPC2 maturation (306,307). However, the 21-kDa C-terminal domain of 7B2 (CT-peptide) is a potent inhibitor of PC2 activity (308). In particular, a \textsuperscript{171}-Lys\textsuperscript{172} site within the CT-peptide appears important for the ability of 7B2 to inhibit PC2 activity, since mutation of this site strongly diminished the inhibitory effect (309). Dissociation of the PC2-CT-peptide complex may involve two steps: cleavage of the CT-peptide at Lys\textsuperscript{171}-Lys\textsuperscript{172} by PC2, followed by removal of the Lys-Lys pair by CPE (310). This process takes place in the secretory granules, thus ensuring that PC2 is activated within the appropriate intracellular compartment. The presence of large amounts of intact CT peptide within the secretory
granule (310) suggests that it may continue to serve a regulatory function in some capacity.

1.7.4 Differential Sorting of PC5a and PC5b

Only a single study has investigated the biosynthesis and regulation of PC5 (260). ProPC5a (126 kDa) is processed to 117 kDa PC5a through removal of the prodomain, as confirmed through N-terminal microsequence analysis. As well, a 65 kDa C-terminally truncated form of PC5a is produced late in the secretory pathway. That PC5a is routed to the secretory granule compartment was shown through immunocytochemical co-localization with ACTH in AtT-20 cells, and with glucagon and PC2 in pancreatic A cells. In contrast, PC5b is initially synthesized as a membrane-bound form which is processed late in the secretory pathway, and is located in the TGN. Only PC5a is subject to cAMP-dependent regulation, further suggesting that it is routed to the regulated secretory pathway. Investigations into the biosynthesis of PACE4, PC4, or PC7 have not yet been conducted.

1.8 The Roles of Prohormone Convertases in Prohormone Processing

Since the identification of the PC enzymes, their functions in the processing of prohormones have been well studied. In particular, the roles of PC1 and PC2 in prohormone processing have been extensively documented, as expression of these PCs are restricted to endocrine and neural tissues. The following is a discussion of the roles of PC1 and PC2 in the post-translational processing of POMC, proinsulin and prosomatostatin, as well as other prohormones, and finally, proglucagon.
1.8.1 PC1 and PC2 Process POMC in a Tissue-Specific Fashion

It has long been known that pro-opiomelanocortin (POMC) is processed to ACTH and β-LPH in the anterior pituitary (AP), and to α-melanocyte-stimulating hormone (α-MSH) and β-endorphin in the neurointermediate lobe (NIL) (311,312). It has now been established that the tissue-specific expression of PC1 and PC2 is responsible for the differential processing of POMC in the AP and NIL.

Northern blot analysis (313) and in situ hybridization (242,314) revealed a predominance of PC1 expression in the AP, and of PC2 expression in the NIL. At the cellular level, POMC and PC1 mRNA co-localize in the AP, while POMC and PC2 mRNA colocalize in the NIL (242), indicating that both enzyme and substrate are expressed in the same cell type within the pituitary. Initial studies on the roles of PC1 and PC2 in POMC processing utilized vaccinia virus-mediated co-infections of POMC and PC1 or PC2 in cell lines (313,315), followed by HPLC analysis of the resulting peptide products. Consistent with their tissue distributions, PC1 processes POMC to yield ACTH and β-LPH, while PC2 cleaves POMC to β-endorphin (313,315). Both PC1 and PC2 act in a concerted manner to produce γ-LPH and β-endorphin from β-LPH (315).

The results of these studies were extended by other studies which utilized stable transfection systems to further elucidate the mechanisms of POMC processing. The cell line of choice for these studies are AtT-20 cells, derived from mouse corticotrophs, which express POMC and PC1 (313,316), and process POMC to primarily ACTH and β-LPH. Stable expression of antisense constructs to PC1 (316) or PC2 (317) resulted in a disappearance of ACTH-related peptides, thus confirming roles for PC1 and PC2 in the production of ACTH and smaller peptides. That PC1 and PC2 cleave POMC in a sequential fashion to yield peptides unique to the NIL was shown by stably transfecting
AtT-20 cells with PC2, which increased the production of β-endorphin and smaller ACTH-related peptides (318). Pulse-chase (318) and temperature block (319) experiments indicated that production of ACTH and β-LPH by PC1 occurs in the Golgi, while production of β-endorphin and smaller peptides by PC2 occurs later in the secretory granules. However, POMC is processed by recombinant PC1 in vitro to most of the known POMC-derived peptides produced in vivo by both PC1 and PC2 (320). These results indicate that a redundancy may exist in processing enzyme utilization, and also that PC1-mediated processing of POMC is subject to certain regulatory mechanisms in vivo.

Finally, POMC and PC1 and PC2 expression may be co-regulated within melanotrophs. PC1, PC2 and POMC mRNA levels in the NIL are increased by haloperidol, a dopamine receptor antagonist, and are decreased by bromocryptine, a dopamine receptor agonist (242). Furthermore, both POMC and PC2 mRNA transcripts are increased in the NILs of black background-adapted toads (321), again illustrating co-regulation of enzyme and substrate.

1.8.2 PC1 and PC2 in Proinsulin Processing

Proinsulin is converted to mature insulin through cleavage at pairs of basic amino acids separating the B chain/C peptide junction and the C peptide/A chain junction. PC1 cleaves proinsulin primarily at the B chain/C peptide site (322), while PC2 cleaves at the C peptide/A chain junction (323). Low levels of PC1 are associated with a slower rate of processing at the B chain/C peptide junction (324), confirming the role for PC1 in processing at this site. Interestingly, mutations of amino acid residues adjacent to, but not within, the PC1 cleavage site also inhibited processing at this site (325), indicating that
contextual sequences may also mediate cleavage site specificity. Vaccinia virus-mediated infection of PC1, PC2 and proinsulin in the constitutively-secreting COS-7 cells could not mimic the in vivo proinsulin conversion events (326). However, expression of proinsulin with PC1 or PC2 in cells with a regulated secretory pathway results in the production of mature insulin (325,327). These latter findings suggest that, as for POMC, a redundancy in processing enzyme utilization exists.

Both PC1 and PC2 have been detected in pancreatic B cells through immunoblot analysis (328) and immunocytochemistry (326,329). Additionally, both PC1 and PC2 have been detected in immature secretory granules in pancreatic B cells (243), providing further evidence that both enzymes may process proinsulin. Interestingly, PACE4C expression has also been detected in pancreatic B cells (249). However, it is not known in what capacity PACE4C may function regarding the processing of proinsulin.

The actions of a carboxypeptidase are required to complete proinsulin processing through removal of flanking basic amino acid residues. CPE activity has been identified in insulin secretory granules (88), and mice harbouring a deficiency in CPE activity show aberrant processing of proinsulin, resulting in severe hyperproinsulinemia leading to diabetes and obesity (89).

The co-regulation of proinsulin and PC expression by glucose has been examined in isolated islets and B cell-derived cell lines. Glucose-dependent regulation of PC1, but not PC2 biosynthesis in concert with proinsulin biosynthesis was demonstrated in isolated rat islets (330). The lack of regulation of PC2 by glucose may have been due to the fact that all islet cell types are known to express PC2 (329) and therefore any changes in B cell PC2 may have been masked. This hypothesis is supported by findings that both PC1 and PC2 biosynthesis are regulated by glucose in islets obtained from ob/ob mice (331),
which contain a significantly greater proportion of B cells than regular islets. As well, glucose levels regulate expression of PC1 and PC2 mRNA together with proinsulin mRNA in βTC3 cells (332), and glucose-dependent translational control of PC1, PC2 and proinsulin is observed in MIN6 cells (333). Therefore, expression of both PC1 and PC2 may be regulated in a glucose-dependent fashion in parallel with proinsulin expression in the pancreatic B cell.

1.8.3 Processing of Prosomatostatin by the PCs

Prosomatostatin (PSS) is post-translationally processed at a pair of basic amino acids to generate SS-14 and at a single Arg to generate SS-28. Transfection of PSS into AtT-20 cells results in the production of SS-14, the secretion of which is stimulated by 8-Br-cAMP (334,335), suggesting that endogenous PC1 is processing PSS within the regulated secretory pathway. As well, co-expression of PC2 and PSS in AtT-20 cells (336) or GH4C1 cells (337) results in the production of SS-14. Expression of furin (335,338) or PACE4 (336) results in the processing of PSS to SS-28, indicating that SS-28 is liberated in Golgi. While the generation of a peptide hormone in the Golgi is an unusual event, this phenomenon has also been described for proparathyroid hormone (339).

1.8.4 Other Prohormones

PC1 and PC2 have been directly implicated in the processing in a number of other prohormones, including progastrin (340,341), proenkephalin (342,343), pro-neuropeptide Y (344), proCCK (345,346) and pro-thyrotropin-releasing hormone (347). Interestingly, prodynorphin is cleaved by PC1 at a single Arg residue both in vivo and in vitro, indicating
that PC1 is capable of cleaving prohormones at single basic amino acids (348). These
studies have utilized vaccinia virus- and adenovirus-mediated infection, stable
transfection, and antisense constructs, showing that involvement of PC1 and PC2 in
prohormone processing may be examined using a variety of approaches.

1.9 Roles of the Prohormone Convertases in the Processing of ProG: Rationale
   for the Hypothesis.

   As discussed above, tissue-specific processing of POMC occurs as a consequence
   of the differential expression of PC1 and PC2 in the anterior pituitary and the
   neurointermediate lobe. At the time that this project was initiated, it was only known that
   PC2 was expressed in the pancreatic islet (237,326), and therefore could be implicated
   in the processing of proG to glucagon. It was not known if intestinal endocrine cells
   expressed any PC enzymes. However, since the distributions of PC1 and PC2 were
   known to be neuroendocrine-specific (236), I reasoned that it was likely that PC1 and/or
   PC2 could be expressed in the L cells of the intestine. Therefore, differential expression
   of PC enzymes could be responsible for the tissue-specific processing of proG, in a
   similar fashion to that of POMC.

   1.9.1 Hypothesis

   PC1 and PC2 are key enzymes in the tissue-specific post-translational processing
   of proG to glicentin, oxyntomodulin, GLP-1 and GLP-2 in the L cells of the intestine, and
to glucagon in the A cells of the pancreas.
1.9.2 Aims and Objectives

The aim of this project was to identify the roles of PC1 and PC2 in the processing of proG. This project consisted of three studies to address the hypothesis.

1. Both non-endocrine (constitutive) and endocrine (regulated) cell lines were infected with vaccinia virus vectors encoding the cDNAs for proG and/or either PC1 or PC2. As the study progressed, more PCs were identified and the intracellular events leading to PC1 and PC2 biosynthesis became more defined. The study was therefore expanded to investigate the roles of other known PCs and molecular chaperones, either alone or in combination, in proG processing.

2. The roles of PC1 and PC2 in the processing of proG were further examined through the use of antisense and overexpression strategies to change the processing phenotype of a pancreatic-derived cell line to that of an intestinal cell.

3. Finally, as the literature indicated that PC1 expression could be co-regulated along with its prohormone substrates, the co-regulation of PC1 and proG gene expression was investigated using an intestinal L cell-derived cell line.
CHAPTER 2

Role of the Prohormone Convertases in the Processing of Proglucagon in the Intestine: Vaccinia Virus-Mediated Overexpression of Proglucagon and the PCs*.

*This chapter is based on an article which appeared in the literature as:
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The work described in this chapter was done entirely by me with the exception of preparation of the recombinant vaccinia virus constructs and subsequent infections of cell lines, which was carried out by Dr. N.G. Seidah, Montreal, QC.
2.1 Introduction

Proglucagon (proG) is post-translationally processed in a tissue-specific manner in pancreatic A cells and intestinal L cells. The constituent peptides within proG are flanked by pairs of basic amino acids, a known consensus site for cleavage by endopeptidases. The A cells release glucagon as the main product of proG processing, while the L cells produce glicentin, oxyntomodulin, glucagon-like peptide-1 (GLP-1) and GLP-2 (Fig 1.2). GLP-1 is further processed through N-terminal truncation at a single arginine to liberate the biologically active form of the peptide, GLP-1\(^{7-37}\). Both GLP-1\(^{1-37}\) and GLP-1\(^{7-37}\) may be amidated at their C-terminal end (71) with no effect on the biological activity of truncated GLP-1 (168).

The biological activities of glucagon and GLP-1 are well known, while those of the other PGDPs are not known or have just recently been elucidated. Glucagon is the major glucose counter-regulatory hormone, increasing hepatic glucose production in response to low plasma glucose levels. The biological activity of glicentin is not known, while oxyntomodulin functions as an enterogastrone by inhibiting gastric acid secretion from the parietal cells of the stomach, and by decreasing gastric motility (160,349). However, it is the functions of GLP-1 and GLP-2 which have most recently been the focus of interest.

Of the total GLP-1 immunoreactivity in the small intestine, approximately 80% corresponds to GLP-1\(^{7-36NH2}\) (71,350), and plasma levels of this peptide increase 6-fold after a meal (169). Infusion of either form of truncated GLP-1 in normal individuals or subjects with non-insulin-dependent diabetes mellitus (NIDDM) increases plasma insulin levels and lowers the postprandial rise in plasma glucose (174,351). This property of truncated GLP-1 as a physiologic incretin has led to clinical trials to determine if
hyperglycemia in NIDDM may be controlled through administration of GLP-1.

The mature form of GLP-2 is produced by removal of the C-terminal pair of basic amino acids (Lys^{159}-Lys^{160}) through a carboxypeptidase B-like activity, and is the major form in the human intestine (72). The biological function of GLP-2 as an intestinal growth factor has recently been reported (211). Administration of GLP-2 to normal mice results in a 100% increase in intestinal weight, which is entirely due to increased epithelial cell growth. Recent studies indicate that the newly grown bowel is physiologically functional (214). GLP-2 may therefore be useful for patients with short bowel syndrome, in whom nutrient absorption is compromised.

Recently, a family of mammalian proteases has been identified and shown to cleave prohormones to produce bioactive peptides. The known members of the prohormone convertase (PC) family are furin (352), PC1 (also known as PC3) and PC2 (234-237), PC4 (253), PACE4 (246), PC5a (also known as PC6a) (256), PC5b (also known as PC6b) (258) and, most recently, PC7 (261). Northern blot analysis and \textit{in situ} hybridization have demonstrated highly specific localization of PC1 and PC2 to neuro/endocrine cells and tissues (235,236). Except for the testis-specific PC4, all other PCs have a widespread distribution (230,261).

PC1 and PC2 are known to process several prohormones to liberate bioactive peptides. In parallel with the tissue-specific distribution of PC1 and PC2 in the pituitary, pro-opiomelanocortin (POMC) is processed in a tissue-specific manner (313,318). PC1 and PC2 have also been implicated in the processing of islet as well as intestinal prohormones. Roles for PC1 and PC2 have been elucidated in the processing of proinsulin (243,326) and pro-pancreatic polypeptide (353), while PC1, PC2 and PACE4 have all been implicated in the processing of prosomatostatin (329,335,336). Transfection
of progastrin (354), pro-neuropeptide Y (355) or pro-peptide YY (356) into cells expressing PC2 also resulted in processing to mature peptides. Finally, it has been suggested that PC2 cleaves proglucagon to glucagon in an A cell-derived cell line (60). These results are consistent with the expression of PC2 mRNA and protein in the A cell (243,249,328,329). However, a glucagonoma-derived cell line which processes proglucagon to glucagon and GLP-1 was found to express neither PC1 nor PC2 (357). These investigators were also unable to detect PC1 and PC2 in sections of rat intestine. Nonetheless, other studies have localized PC1 mRNA transcripts to the ileum (230), the cell population of which includes the GLP-1-producing L cells (131). Indeed, PC1 and proglucagon have been co-localized in the intestinal L cell by immunocytochemistry (358).

Another convertase, PC5, has been detected in an A cell-derived cell line (359) and in the ileum (230), although the role for this, and other convertases such as PACE4 and PC5/6b in the processing of proG has not yet been examined.

In order to elucidate a definitive role for both PC1 and PC2, as well as for other convertases, in the pancreatic and intestinal processing of proG, we utilized a vaccinia virus system to infect a number of constitutive and regulated cell lines with recombinant proG and the known PCs. We show that PC1 processes proG to all of the intestinal peptides: glicentin, oxyntomodulin, GLP-1$^{1-37}$, GLP-1$^{7-37}$ and GLP-2, while the other PCs, including PC2, produce only glicentin. These results implicate PC1 as the enzyme responsible for the production of the biologically active peptides, oxyntomodulin, GLP-1$^{7-37/7-36NH_2}$ and GLP-2.
2.2 Materials and Methods

Cell Cultures

Baby hamster kidney cells which stably express the rat proglucagon gene under the control of the mouse metallothionein promoter (BHK-proG) were a gift from Dr. D.J. Drucker (Toronto, Canada), and were maintained in culture as previously described (70). GH₃ and GH₄C₁ cells (derived from a rat somatomammotroph tumor) were obtained from American Type Culture Collection (ATCC, Maryland) and were maintained in Ham’s F10 Nutrient Solution (Gibco, Life Technologies, MD) containing 12.5% (v/v) fetal bovine serum (FBS) and 2.5% (v/v) horse serum. AtT-20 cells (derived from mouse corticotroph tumor) were also obtained from ATCC and were maintained in Dulbecco’s Minimal Essential Medium (DMEM, Gibco) containing 10% (v/v) FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. All cells were maintained at 37°C in 5% CO₂/95% air with constant humidity.

Vaccinia Virus Infections

The purified recombinant vaccinia virus (vv) preparations of mouse PC1 (vv:mPC1) and vv:mPC2 were constructed using the full-length cDNA inserts of mouse PC1 (236) and mouse PC2 (235) as reported previously (313). The recombinant vv:furin was prepared using the cDNA for human furin (gift from Dr. A. Rehemtulla, Genetics Institute) (313). The recombinant vv:PACE4, vv:PC5a and vv:PC5b were prepared as reported previously (313) using the full-length cDNAs for human PACE4 (246), mouse PC5a (256) and mouse PC5b (258). Recombinant vv:proglucagon (vv:proG) was constructed as reported previously (313) using the full-length cDNA insert of rat proglucagon (a gift from Dr. D.J. Drucker, Toronto, Canada). Recombinant vv:prodynorphin was prepared as
previously reported (348). Recombinant preparations of mouse pro-7B2 (vv:7B2), human chromogranin A (vv:CgA), mouse chromogranin B (vv:CgB) and rat secretogranin II (vv:SgII) were constructed as reported previously (313) using full-length cDNA inserts for CgA (NG Seidah, unpublished) CgB (360), SgII (361) and 7B2 (362). The vaccinia virus recombinant preparations of vv:furin, vv:PC1, vv:PC2, vv:PACE4 and vv:PC5a have been shown to be reproducibly expressed at high levels when infected in a number of cell lines (336,361). vv:7B2 and vv:SgII have also been shown to be expressed at high levels when infected in AtT-20 and GH₄C₁ cells (303,361).

When cells reached 70-80% confluence in 10 cm dishes, medium was removed and the cells washed in PBS-M (PBS: 2.7 mM KCl, 1.5 mM KH₂PO₄, 137 mM NaCl, 4.2 mM Na₂HPO₄; and M: 1 mM MgCl₂). The cells were infected with a minimal volume of cold PBS-MB [PBS-M plus 0.01% (v/v) bovine serum albumin (BSA)] containing the vaccinia virus preparation which was first sonicated and vortexed six times for 10 s. All co-infections were performed as described previously (313) at a multiplicity of infection of 1 plaque-forming unit per cell for each recombinant virus. Following the appropriate infection period, the inoculum was replaced with complete minimal essential medium (BHK and AtT-20) or Ham’s F10 nutrient mixture (GH₃ and GH₄C₁), and cells were incubated overnight (17 h) at 37°C. Cells were subsequently washed with 4 mL of medium plus 0.001% (w/v) BSA and finally incubated with 4 mL of medium plus 0.001% (w/v) BSA at 37°C. After 4 h of incubation, the medium was removed, centrifuged at 200 x g for 5 min and stored at -70°C. Cells were washed with PBS-DMEM, removed from the plate with trypsin, pelleted and stored at -70°C. Each experiment was repeated 2-6 times, and mean values are shown.
Transient Transfection of PC12/PC2 Cells.

PC12/PC2 cells were obtained from Dr. N.G. Seidah, and were originally generated by Dr. S.A. Tooze (London, UK) (363). The expression vector containing full-length rat proglucagon under the control of the mouse metallothionein promoter was obtained from Dr. D.J. Drucker (Toronto, ON). Cells were plated at a density of 40% confluency in 60 mm dishes and were cultured for 2 days. The cells were transfected with 5 μg DNA and 25 μL Lipofectin (Gibco) according to the manufacturer's instructions. After 12 h, transfection medium was removed and replaced with culture medium. Twenty-four hours after transfection, expression of the proG gene was induced by incubation of the cells for 24 h in experimental medium (DMEM, 1.0 g/l glucose, 0.5% (v/v) FBS) containing 70 μM Zn²⁺.

Peptide Extraction

Cells were homogenised twice in 10 ml of extraction medium [1 N HCl containing 5% (v/v) formic acid, 1% (v/v) trifluoroacetic acid (TFA), and 1% (w/v) NaCl]. Media was acidified through addition of 1 ml 1% (v/v) TFA. Cells and media were passed twice through a C18 silica cartridge (C18 SepPak, Waters Associates, Milford MA), and adsorbed peptides were eluted with 4 ml 80% (v/v) isopropanol/0.1% (v/v) TFA and stored at -70°C, as previously described (35,210,350,364,365). We have previously reported greater than 88% recovery of the proG-derived peptides (PGDPs) using these methods (35,364).

High Pressure Liquid Chromatography (HPLC)

PGDPs contained in media and cell extracts were separated on the basis of
hydrophobicity using a Waters Associates Liquid Chromatography System and a C18 μBondapak column (Waters Associates, Milford, MA) as previously described (210,350,365). Glicentin, oxyntomodulin, glucagon, GLP-2 and proglucagon were separated using a 45 min linear gradient of 25-62.5% (v/v) Solvent B [Solvent A: 1% (v/v) TFA, buffered with diethylamine to pH 2.5; Solvent B: 80% (v/v) acetonitrile], followed by a 10 min purge at 99% (v/v) Solvent B. The flow rate was 1.5 ml/min, and fractions were collected every 0.3 min. The peptide content of fractions 1-60 was below the limits of detection of the RIAs utilized and is therefore not reported. Analyses were performed on two experiments, one representative example of which is shown.

Full-length and truncated forms of GLP-1 were separated using a 30 min linear gradient of 45-68% (v/v) Solvent B [Solvent A: 0.1% (v/v) phosphoric acid, 0.3% (v/v) triethylamine, buffered with NaOH to pH 7.0; Solvent B: 60% (v/v) acetonitrile, 40% (v/v) Solvent A], followed by a 10 min purge at 99% (v/v) Solvent B (210,350,365). The flow rate was 1.0 ml/min, and fractions were collected every min. Analyses were performed on two experiments, one representative example of which is shown.

Separation of peptides on the basis of molecular weight was carried out using two Protein Pak 125 gel permeation columns (Waters Associates, Milford, MA) in series with a 30 min isocratic gradient of 50% (v/v) Solvent B [Solvent A: 0.1% (v/v) TFA; Solvent B: 80% (v/v) acetonitrile in 0.1% (v/v) 0.1% TFA]. The flow rate was 1 ml/min and fractions were collected every 0.3 min. Calibration was achieved by injection of a mixture of BSA, soybean trypsin inhibitor (both from Sigma, St. Louis, Missouri), cytochrome C (Boehringer Mannheim, Mannheim, Germany), aprotinin (Trasylol, Miles Canada Inc., Etobicoke, Canada), [¹²⁵I] glucagon (Biospecific, Emeryville CA) and Na[¹²⁵I] (Amersham, Oakville, Canada).
Radioimmunoassays (RIA)

Aliquots of cell extracts, media and HPLC fractions were dried in vacuo prior to RIA. Two antisera were used to distinguish proglucagon and its N-terminally derived peptides (glicentin, oxyntomodulin, and glucagon) as previously described (35,210,350,364,365). (i) Antiserum K4023 (Biospacific) recognizes the mid-sequence of glucagon, thus detecting the glucagon-like immunoreactive (GLI) peptides, proglucagon, glicentin, oxyntomodulin, and glucagon (Fig. 1.2). It must be noted that proG levels are underestimated in this RIA, since proG does not dilute in parallel in the GLI assay (data not shown). (ii) Antiserum 04A (Dr. R.H. Unger, Dallas TX) recognizes the free carboxy terminal end of glucagon, thereby measuring immunoreactive glucagon (IRG). The detection range of both assays was 4-400 pg/tube.

Processed forms of GLP-1 (GLP-1\textsuperscript{1-37}, GLP-1\textsuperscript{7-37}, GLP-1\textsuperscript{1-36NH\textsubscript{2}} and GLP-1\textsuperscript{7-36NH\textsubscript{2}}) were detected using two different antisera, as previously reported (210,350,365). (i) The b5 antiserum (a gift from Dr. S. Mojsov, New York, NY) recognizes the C-terminally glycine-extended forms of GLP-1, GLP-1\textsuperscript{1-37} and GLP-1\textsuperscript{7-37} (71). (ii) C-terminally amidated forms of GLP-1 (GLP-1\textsuperscript{1-36NH\textsubscript{2}} and GLP-1\textsuperscript{7-36NH\textsubscript{2}}) were detected using a GLP-1\textsuperscript{1-36NH\textsubscript{2}} antiserum (Affinity Research, Nottingham, UK). The detection ranges for the GLP-1\textsuperscript{1-37} and GLP-1\textsuperscript{1-36NH\textsubscript{2}} assays were 1-160 and 3-800 pg/tube, respectively.

Processed forms of GLP-2 were detected (366) using antiserum 92160 (gift from Dr. J.J. Holst, Copenhagen, Denmark) which recognizes the free N-terminus of GLP-2 (P. Brubaker, personal communication). The detection range of the GLP-2 assay was 10-2000 pg/tube.

Immunoreactive α-MSH (IR-α-MSH) was measured in AtT-20 cells using an RIA kit (Peninsula Laboratories, Belmont, CA).
RNA Extraction and Analysis

Cells were grown to 80% confluency in 10 cm dishes and homogenized in guanidinium isothiocyanate (ICN). RNA was phenol-chloroform extracted and ethanol purified twice, and stored in DEPC-H₂O at -70°C until analysis, as described previously (326). Twenty µg of total RNA was size-fractionated on a 1% (w/v) agarose-formaldehyde gel, and the gel was stained with ethidium bromide to assess the integrity and migration of the RNA. The RNA was subsequently transferred to a nylon membrane (Schleicher and Schuell, Keene, NH) using the downward alkaline blotting method (367), and cross-linked to the membrane with a 4-min exposure to UV light. Blots were prehybridized and hybridized with 1X Denhardt's (200 µg/ml each of polyvinyl pyrrolidine, BSA and Ficoll), 4X SSC (1X SSC is 0.15 M NaCl and 0.015 M Na citrate), 200 µg/ml herring sperm DNA, 40% deionized formamide, and 0.014 M Tris-HCl, pH 7.4. The full-length rat proglucagon probe was a gift from Dr. D.J. Drucker (Toronto, ON). Probes were labelled with [³²P]dCTP to a specific activity of 8X10⁸ cpm/µg using a Random Priming kit (Boehringer Mannheim), and blots were hybridized with 1X10⁶ cpm/ml of labelled probe for 16-24 h at 42°C. Final washing conditions were 0.2X SSC/0.2% SDS at 65°C. Autoradiography was carried out using Kodak X-Omat film (Eastman Kodak, Rochester, NY) at -70°C.
2.3 RESULTS

Processing of ProG to Glicentin, Oxyntomodulin and Glucagon

BHK Cells

Total cellular immunoreactivity of GLI and IRG was measured in all cell lines before HPLC analysis. In order to determine the roles of PC1 and PC2 in the processing of proglucagon, BHK-proG cells were used initially. These cells stably express, but do not process, proG (70). After induction of the metallothionein promoter by incubating cells with 70 μM Zn²⁺ for 24 h, proG mRNA levels increased 4-5 fold, indicating that the promoter was still functional in these cells (Fig 2.1A). As well, cellular GLI increased 2.5-fold (p<0.001) and GLI secretion increased 7-fold (p<0.001) upon Zn²⁺ treatment (Fig 2.1 B,C). That these cells secrete through a constitutive pathway was demonstrated by the fact that, over a 4-hour period, 95% of total GLI was released into the medium. HPLC analysis of GLI peptides in cell extracts revealed one small heterogenous peak at fraction 79, which was identified as glicentin, and one large peak eluting at fraction 132 (Fig 2.2A). In the medium, one large peak of GLI was seen at fraction 136 (Fig 2.2B), indicating that this peptide was secreted. This peak was tentatively identified as proglucagon, as it was recognized by the K4023 antiserum as previously reported (60) and its late elution is in keeping with the predicted hydrophobic nature of proG (368). Identification of this peak as proG was confirmed through additional analysis of the corresponding fractions by high-pressure gel permeation chromatography (Fig 2.3). ProG eluted with an apparent Mₐ of greater than 13,500, in keeping with the predicted Mₐ of 18,000 (10).

Upon vv:infection, BHK-proG cells produced GLI and IRG in a ratio of 32:1 (Fig 2.4A). However, this ratio decreased in the medium (Fig 2.4B), indicating that most of the
Fig 2.1 Induction of proG mRNA expression and GLI production in BHK-proG cells.

BHK-proG cells were treated with 70 μM Zn$^{2+}$ for 12 h, after which cells were analysed for proG mRNA (A), secreted GLI (B) and cellular GLI (C). Values are means ± SEM (n=8).
Fig. 2.2 Processing of proG in BHK-proG cells.

HPLC analysis of GLI in BHK-proG cell extracts (A) and medium (B). Elution positions of glicentin (a), oxyntomodulin (b), and glucagon (c) are shown. Results are representative of 4 independent analyses.
Fig 2.3 Identification of fraction 132 as proglucagon.

Fractions 130-134 from HPLC analysis of medium from BHK-proG cells were further analysed by high-pressure gel permeation chromatography. Letters indicate elution positions of standard molecular weight markers, as follows: BSA, $M_r=65$ kDa (a), soybean trypsin inhibitor, $M_r=20$ kDa (b), cytochrome c, $M_r=13.5$ kDa (c), aprotinin, $M_r=6.5$ kDa (d), and glucagon, $M_r=3.5$ kDa (e). The second peak appears to elute with a molecular weight of $>13,500$. 
Fig 2.4 Production of N-terminal PGDPs in BHK-proG cells infected with furin, PC1 or PC2.

BHK-proG cells were treated with 70 µM Zn$^{2+}$ to induce proG gene expression, then infected with the indicated PCs. RIAs for glucagon-like immunoreactivity (GLI) and immunoreactive glucagon (IRG) were carried out in cell extracts (A) and 4h secretion medium (B). Results are means of 2-6 experiments. NI, non-infected cells; wt, cells infected with wild-type virus; Fur, vv:furin.
IRG produced was secreted. Infection of BHK-proG cells with the wild-type virus appeared to decrease total GLI production by 49% compared with non-infected cells, a phenomenon which has also been observed upon infection of BSC-40 cells with vv:prorenin (277). Identification of the GLI peptides by HPLC in cell extracts revealed that, as compared to the wild-type infection, both vv:PC1 and vv:furin processed proG to glicentin, while vv:PC2 liberated only insignificant amounts of glicentin (Fig 2.5). The small peak eluting at fraction 75 has not been identified, but may represent oxidized glicentin. Consistent with the lack of IRG in whole cell extracts, no glucagon could be detected. HPLC analysis of the medium also indicated that vv:furin and vv:PC1 processed proG to glicentin (Fig 2.6). Additionally, a small peak of oxyntomodulin was observed, indicating that this peptide is also produced but is secreted. Upon infection with vv:PC2, a larger peak of glicentin was observed in the medium as compared with cell extracts, indicating that this peptide was produced by PC2 and secreted. However, despite the presence of IRG in the medium, HPLC analysis failed to reveal a glucagon peak (Fig 2.6). These results indicated possible roles for furin, PC1 and PC2 in proG processing.

**GH₃ Cells**

It is now known that PC2 requires a secretory granule environment to best exert its actions (281). Therefore, a rat somatotroph-mammotroph-derived cell line, GH₃, was employed in order to examine proG processing, since it has been shown that these cells secrete in a regulated manner, and have an appropriate intracellular environment for processing (369). As well, GH₃ cells are known to express PC2 endogenously (230). Upon vv:infection with proG and the various enzymes, cellular levels of total GLI peptides were variable (Fig 2.7A). However, the ratio of GLI:IRG was approximately 40:1, and was
Fig. 2.5  Processing of proG by furin, PC1 and PC2 in BHK-proG cells.

HPLC analysis of GLI in BHK-proG cell extracts after infection of indicated PCs. Elution positions of glicentin (a), oxyntomodulin (b), and glucagon (c) are shown. Results are representative of 2 independent experiments.
Fig. 2.6 PGDPs secreted from BHK-proG cells infected with furin, PC1 and PC2.

HPLC analysis of GLI in 4h medium of BHK-proG cells after infection of indicated PCs. Elution positions of glicentin (a), oxyntomodulin (b), and glucagon (c) are shown. Results are representative of 2 independent experiments.
N-terminal PGDP immunoreactivity in GH3 cells infected with PCs.

Total cellular GLI and IRG immunoreactivity in GH3 cells after infection with vv:proG and the indicated PCs. Cells were infected with each PC alone or in combination with PC2 (A) or with furin or PC1 alone or in combination with PACE4 (B). Results are means from 2-6 experiments. D, vv:dynorphin.
not altered upon infection of any of the PCs, indicating that significant amounts of glucagon were not produced. HPLC analysis of GLI peptides indicated that processing of proG by the endogenous PCs alone liberated only glicentin (Fig 2.8A). However, vv:PC1 processed proG to both glicentin and oxyntomodulin, when infected alone or in combination with vv:PACE4. Glucagon was not produced by PC1. In contrast, infection of GH₃ cells with the other known PCs resulted in the production of glicentin only, but no oxyntomodulin or glucagon was produced. Since vv:PC2 alone did not produce glucagon as suggested by others (60), we hypothesized that PC2 may be acting with another convertase to liberate glucagon. Therefore, the effects of co-infection with vv:proG and vv:PC2 alone or in combination with vv:PACE4, vv:PC5a and vv:PC5b on proG processing were also examined (Fig 2.7A). In order to explore the effects of other enzyme combinations on vv:proG processing, GH₃ cells were infected with vv:furin, vv:PC1 or vv:PACE4 alone or in combination (Fig 2.7B). The number of virus infections was controlled for by including vv:prodynorphin in the infections, as appropriate. Analysis of cell extracts indicated that none of these enzymes or enzyme combinations increased production of IRG relative to GLI. As well, the presence of vv:dynorphin did not appear to affect production of total GLI or IRG (Fig 2.7B vs A). HPLC analysis confirmed the absence of glucagon in that the processing profiles obtained from infection with various enzyme combinations (Fig 2.8B,C) did not differ from those found for each PC alone.

One remarkable observation is that no enzyme or enzyme combination resulted in the production of glucagon, whether expressed in a constitutive (BHK) or regulated (GH₃) cell line. In light of these results, we co-infected GH₃ cells with PC2 in combination with the chromogranin family of molecular chaperones, CgA, CgB, SgII or 7B2, since it has been shown these proteins may play roles in the proper targeting of peptide
hormones, as well as the PCs, to secretory granules (296,370). It is now well established that 7B2 is a specific molecular chaperone for PC2, assisting in proper folding and inhibiting premature activation of PC2 in the secretory pathway (306). However, in the presence of vv:PC2, the amounts of IRG produced did not differ from those found in other experiments, indicating that the presence of chromogranins or 7B2 did not affect the actions of PC2 (Fig 2.9A). In order to establish the role of PC2 in combination with PC1 on proG processing, GH3 cells were infected with vv:proG, vv:PC1, vv:PC2 and vv:7B2 (Fig 2.9B). This combination of enzymes also did not result in the production of IRG. Since expression of PC5a had been detected in A cells of the pancreas, and co-localized with glucagon by immunocytochemistry (N.G. Seidah, pers. comm.), GH3 cells were co-infected with vv:proG and vv:PC5a alone, in combination with vv:PC2, or in combination with vv:PC2 and vv:7B2. Again, this combination of enzymes failed to produce IRG. Since the ratio of PC2:7B2 was 1:1 in the above experiments, we co-infected GH3 and GH4C1 cells with vv:PC2 and vv:7B2 in a ratio of 1:2 to increase the likelihood of PC2:7B2 association (Fig 2.10). GH4C1 cells were included in the experiment since, at the time, it was reported that no endogenous 7B2 expression could be detected in these cells (N.G. Seidah, personal communication). Again, an insignificant amount of IRG was produced, indicating that PC2 is unable to produce glucagon even in the presence of excess 7B2.

**AtT-20 Cells**

As no glucagon could be detected in GH3 cells upon vv:enzyme infection in the absence or presence of chromogranins, we also investigated actions of the PCs on proG processing in AtT-20 cells, a heterologous regulated cell line known to express PC1
Fig. 2.8. N-terminal processing of proG in GH3 cells.

HPLC identification of N-terminal PGDPs produced by GH3 cells infected with various PCs. Cells were infected with vv:proG alone or in combination with furin, PC1 or PC2 (A); with PACE4 alone or in combination with furin, PC1 or PC2 (B); or with PC5a or PC5b alone or in combination with PC2 (C). Elution positions for glicentin (a), oxyntomodulin (b), and glucagon (c) are shown. GLI is shown as a heavy line, and IRG as a light line. Results are representative of 2-6 experiments.
Fig 2.9  Effects of the granin family of molecular chaperones on glucagon production in GH3 cells.

Total GLI and IRG in GH3 cells infected with PC2 alone or in combination with chromogranin A (CgA), chromogranin B (CgB), secretogranin II (SgII) or 7B2, (A); and in GH3 cells infected with the indicated PCs in the absence or presence of 7B2 (B). Values are means of 2 experiments.
Fig 2.10 Stoichiometric effects of 7B2 and PC2 on proG processing in \( \text{GH}_3 \) and \( \text{GH}_4\text{C}_1 \) cells.

\( \text{GH}_3 \) (A) and \( \text{GH}_4\text{C}_1 \) cells (B) were infected with PC2 alone or in combination with 7B2 in a 1:2 molar ratio. Cell extracts were subsequently measured for GLI and IRG. Values are means of 2 experiments.
A

GH₃ Cells

GLI

IRG

IR-peptide (ng/dish)

PC2

PC2+7B2

B

GH₄C₁ Cells

GLI

IRG

IR-peptide (ng/dish)

PC2

PC2+7B2
As for GH3 cells, insignificant amounts of IRG relative to GLI were detected following infection of vv:proG with the various PCs alone (Fig 2.11). Small amounts of IRG were detected in cells infected with vv:PC2 alone or in combination with vv:PACE4, vv:PC5a or vv:PC5b. Infection with vv:proG alone, or with vv:PC2 or vv:furin yielded similar processing profiles to those obtained in GH3 cells, with the exception that small amounts of oxyntomodulin were observed in each profile (Fig 2.12A). However, vv:furin increased the processing of proG to glicentin, as compared to processing by endogenous PC1 alone, or with vv:PC2. Processing profiles from AtT-20 cells infected with vv:PC2 alone or in combination with other PCs revealed only an insignificant peak of glucagon (Fig 2.12B); the profiles did not differ from those obtained with each enzyme alone. Finally, in order to establish the role of PC2 in combination with PC1 on proG processing, AtT-20 cells were infected with vv:proG, vv:PC1, vv:PC2 and vv:7B2, (Fig 2.13A). In contrast to results obtained with GH3 cells, IRG was detectable in AtT-20 cells, however, the ratio of GLI:IRG produced did not differ from that found for proG alone. Also, the role of PC5 in combination with PC2 and 7B2 was examined. Again, the amount of IRG produced did not differ from that produced by infection of AtT-20 cells with vv:proG alone.

To verify that the enzymes were functional, production of immunoreactive-α-melanocyte-stimulating hormone (IR-α-MSH) from endogenous POMC was also determined in AtT-20 cells (Fig 2.13B). This cleavage is known to be mediated by the concerted actions of PC1 and PC2 (313,318). Compared to cells infected with vv:proG alone, co-infection with vv:PC2 increased IR-α-MSH production 5-fold, and co-infection with vv:PC1, vv:PC2 and vv:7B2 increased production of IR-α-MSH 80 times over that produced by vv:PC2 alone. Interestingly, co-infection of AtT-20 cells with vv:PC2 and vv:PC5 increased generation of IR-α-MSH 2.5-fold compared to infection with vv:PC5 alone, and the presence of
Fig 2.11  Total N-terminal immunoreactivity in AtT-20 cells.

AtT-20 cells were infected with proG alone or in combination with the indicated PCs, either alone or in combination with PC2. Values are means of 2-4 experiments.
Processing of proG to N-terminal PGDPs in AtT-20 cells.

HPLC identification of N-terminal PGDPs produced in AtT-20 cells infected with vv:proG alone or in combination with furin or PC2 (A), or with other PCs alone or in combination with PC2 (B). Elution positions for glicentin (a), oxyntomodulin (b), and glucagon (c) are shown. GLI is shown as a heavy line, and IRG as a light line. Results are representative of 2 experiments.
Fig 2.13 Total GLI and IRG (A) and endogenous α-MSH immunoreactivity (B) in AtT-20 cells infected with PCs and/or 7B2.

Total cellular GLI and IRG (A) and α-MSH immunoreactivity (B) was measured in AtT-20 cells after infection with the indicated PCs and/or vv:7B2. Values are means of 2 experiments.
vv:7B2 increased production by almost 10 times that achieved with vv:PC2 and vv:PC5 without 7B2.

**Processing of ProG to GLP-1**

No GLP-1 immunoreactivity was detected in BHK-proG cells (data not shown). In contrast, RIA of GH₃ cell extracts for free C-terminal GLP-1₁⁻³⁷/₃⁶NH₂ immunoreactivity indicated that only furin or PC1 liberated GLP-1 from proG (Fig 2.14). No other enzyme or enzyme combination produced GLP-1 from proG. HPLC analyses indicated that both vv:furin and vv:PC1 produced all known forms of GLP-1 (Fig 2.15A,B); 1-37, 1-36NH₂, 7-37 and 7-36NH₂. The presence of the amidated forms of GLP-1 (35% to 80% of the total immunoreactive peptide) is consistent with the expression of peptidylglycine-α-amidating monooxygenase (PAM) in these cells (371). Both GLP-1₁⁻³⁷ and GLP-1₁⁻³⁶NH₂ were the primary products of processing by PC1. As well, co-infection with the various molecular chaperones and vv:PC2 did not increase production of total GLP-1 immunoreactivity (Fig 2.14C). Co-infection with vv:PC1, vv:PC2 and vv:7B2 resulted in the generation of GLP-1, 80% of which was amidated (Fig 2.14D). However, the presence of PC2, PC5a and 7B2 did not affect the production of GLP-1, again indicating that only PC1 is responsible for the processing of proG to GLP-1.

In AtT-20 cells, infection of proG alone resulted in the production of significant amounts of GLP-1 immunoreactivity, indicating that the endogenous PC1 processed proG to GLP-1. Upon co-infection of vv:proG and the various enzymes, variable amounts of GLP-1 were detected (Fig 2.16). Up to 67% of the GLP-1 was amidated, consistent with the presence of PAM activity in this cell line (372). In order to determine the effects of endogenous PC1 alone on proG processing, HPLC analysis was carried out on cells
Fig 2.14 Production of GLP-1 in GH$_3$ cells infected with PCs and/or molecular chaperones.

GH$_3$ cells were infected with proG alone or in combination with various PCs as indicated (A,B) or with PC2 or PC5a alone or together with the granin family of molecular chaperones (C,D). Total free C-terminal GLP-1 immunoreactivity was assessed by RIA. Unamidated GLP-1 IR is shown in the solid box, amidated GLP-1 IR is shown in the empty box. Values are means of 2-6 experiments.
Fig 2.15  Processing of proG to GLP-1 in GH₃ cells.

HPLC identification of the peptides in GH₃ cells co-infected with vv:proG and vv:furin (A) or vv:PC1 (B). Elution positions of synthetic peptides are shown as follows: GLP-1¹-3⁷ (a), GLP-1²-3⁶NH₂ (b), GLP-1⁷-3⁷ (c) and GLP-1⁷-3⁶NH₂ (d). GLP-1⁵-3⁷ immunoreactivity is shown as a solid line, and GLP-1⁵-3⁶NH₂ immunoreactivity is shown as a dotted line. Results are representative of 3 experiments.
**vv:furin**

(A)

- IR-peptide (ng/fraction)
- Fraction #

**vv:PC1**

(B)

- IR-peptide (ng/fraction)
- Fraction #
Production of GLP-1 in AtT-20 cells infected with PCs and/or molecular chaperones.

AtT-20 cells were infected with proG alone or in combination with various PCs as indicated (A) or with PC2 or PC5a alone or together with the granin family of molecular chaperones (B). Total free C-terminal GLP-1 immunoreactivity was assessed by RIA. Unamidated GLP-1 IR is shown in the solid box, amidated GLP-1 IR is shown in the empty box. Values are means of 2 experiments.
Fig 2.17  Processing of proG to GLP-1 in AtT-20 cells.

HPLC analysis of GLP-1 peptides in AtT-20 cells infected with vv:proG alone (A) or with vv:furin (B). Elution positions of synthetic peptides are shown as follows: GLP-1^{1-37} (a), GLP-1^{1-36NH2} (b), GLP-1^{7-37} (c) and GLP-1^{7-36NH2} (d). GLP-1^{x-37}-IR is shown as a solid line, and GLP-1^{x-36NH2}-IR is shown as a dotted line. Results are representative of 2 experiments.
which were infected with vv:proG alone. The resulting processing profile revealed that, while all four forms of GLP-1 could be detected, the full-length forms were predominant (Fig 2.17A). As well, infection of AtT-20 cells with vv:furin resulted in the production of all four forms of GLP-1 (Fig 2.17B).

**Processing of ProG to GLP-2**

RIA of GH₃ cell extracts for free N-terminal GLP-2 immunoreactivity indicated that infection with vv:PC1, and, to a lesser extent, vv:furin, liberated GLP-2 from proG (Fig 2.18A,B). Production of GLP-2 by PC1 or furin was significant in the presence of other enzymes and molecular chaperones, however, no other enzyme or combination of enzymes processed proG to GLP-2 (Fig 2.18C,D). HPLC analysis of GLP-2 immunoreactive peptides demonstrated the existence of several peaks (Fig 2.19). While one of the peaks corresponded to the elution position of synthetic rat GLP-2, the major peak eluted much later (fractions 130-133). We speculate that this peak may represent GLP-2-Lys₁⁵⁹-Lys₁⁶⁰, which would be more hydrophobic than fully processed GLP-2 in our system, resulting in a later elution position. Infection of AtT-20 cells with vv:proG alone or with various PCs resulted in the production of variable amounts of GLP-2 (Fig 2.20), as expected from the expression of endogenous PC1 in these cells. However, infection with vv:PC5 alone or in combination with vv:PC2 or vv:7B2 decreased GLP-2 production to background levels. Identification of GLP-2 peptides by HPLC was not carried out on AtT-20 cell extracts.

**Processing of ProG in PC12/PC2 Cells**

In order to assess the processing of proG in the presence of PC2 only and in the
Production of GLP-2 in GH₃ cells infected with PCs and/or molecular chaperones.

GH₃ cells were infected with proG alone or in combination with various PCs as indicated (A,B) or with PC2 or PC5a alone or together with the granin family of molecular chaperones (C,D). Total free N-terminal GLP-2 immunoreactivity was assessed by RIA. Values are means of 2-6 experiments. ND, value not determined.
Fig 2.19  Processing of proG to GLP-2 in GH₃ cells.

HPLC identification of GLP-2 immunoreactive peptides in GH₃ cells after infection with vv:proG and vv:furin (A) or vv:PC1 (B). The elution position for synthetic rat GLP-2 is indicated by the arrow. Results are representative of 2 experiments.
A

vv: furin

B

vv: PC1
Production of GLP-2 in AtT-20 cells infected with PCs and/or molecular chaperones.

AtT-20 cells were infected with proG alone or in combination with various PCs as indicated (A) or with PC2 or PC5a alone or together with the granin family of molecular chaperones (B). Total free N-terminal GLP-2 immunoreactivity was assessed by RIA. Values are means of 2 experiments.
absence of other PCs, proG was transiently transfected into PC12 cells overexpressing PC2. These cells do not express any other PC, except furin (230), which does not process proG, as the present study demonstrates. Cells were transfected with the cDNA containing proG, the expression of which is under the control of the metallothionein promoter, and proG expression was induced by incubation of cells with 70 μM Zn²⁺ for 24 h. Cell extracts were then assessed for the presence of GLI and IRG. In wt PC12 cells, the ratio of GLI:IRG produced was approximately 2.4:1, and in cells expressing PC2, the ratio was approximately 1.7:1 (Fig 2.21). This difference was not significant, indicating that PC2 alone is unable to process proG to glucagon.
Fig 2.21 Glucagon is not produced in PC12 cells stably transfected with PC2.

Wild-type PC12 cells and PC12/PC2 cells were transiently transfected with proG. Twenty-four hours after induction of proG gene expression by 70μM Zn$^{2+}$, cells and medium were assayed for GLI and IRG production. Values are means ± SEM (n=6).
2.4 DISCUSSION

PC1 Processes ProG to the Intestinal PGDPs.

The results of the present study are the first to demonstrate a definitive role for PC1 in the processing of proG to all of the intestinal PGDPs: glicentin, oxyntomodulin, GLP-1\(^{1-37/36NH_2}\), GLP-1\(^{7-37/36NH_2}\) and GLP-2 (Fig 2.22). In GH\(_3\) cells infected with vv:proG and vv:PC1 and in AtT-20 cells (which express PC1 endogenously) infected with vv:proG alone, proG was processed to the intestinal PGDPs. No other enzyme or combination of enzymes processed proG in this fashion. These findings are consistent with the known expression of PC1 in the ileum (230), the predominant site of proG-expressing L cells (373), and with its absence from A cells (60,249,328,329,359). The recent finding of co-localization of GLP-1 and PC1 in the human L cell provides additional evidence for a role of PC1 in the tissue-specific post-translational processing of proG (358). Finally, it has also been very recently demonstrated that recombinant PC1 processes proG to glicentin, oxyntomodulin, and GLP-1 \textit{in vitro} (74). Somewhat unexpectedly, while glicentin, oxyntomodulin, GLP-1 and GLP-2 were consistently processed from proG in GH\(_3\) cells, oxyntomodulin, GLP-1 and GLP-2 were liberated to a lesser extent in the AtT-20 cells. As proG is overexpressed in the AtT-20 cells under the control of vaccinia virus, while the endogenous PC1 is compartmentalized within secretory granules (282), this lack of PC1 relative to proG may contribute to the smaller yield of oxyntomodulin, GLP-1 and GLP-2. A requirement for sequential processing of PC1 through the Golgi and secretion granules (374) may also account for the differences in processing observed between GH\(_3\) and AtT-20 cells. PC1 is first synthesized as a 87 kDa protein which undergoes autocatalytic processing to an 84 kDa active form in the endoplasmic reticulum, whereupon it is transported to the Golgi where it begins to exert its actions (278,281,282,285). This form
is then further processed to a 66 kDa form in secretory granules (276,282). The processing of POMC to ACTH and β-LPH was significantly reduced when PC1 was prevented from entering secretory granules (282), thus indicating that each form of PC1 may process prohormones differently. In keeping with their lack of a regulated secretory pathway, BHK-proG cells infected with vv:PC1 did not process proG to oxyntomodulin or GLP-1. We therefore speculate that PC1-mediated processing of proG to glicentin occurs in the Golgi, while liberation of the other intestinal PGDPs may require secretory granules. Consistent with this hypothesis, pulse-chase studies in pancreatic islets and αTC1-6 cells have established that proG is processed initially to glicentin (45 min), followed by oxyntomodulin (100 min) (10,60); GLP-1 is not detected until 3 hr of chase (60).

In the present study, we show for the first time that PC1 is responsible not only for cleavage at pairs of basic amino acids (Lys31-Arg32, Lys70-Arg71, Arg109-Arg110, and Arg124-Arg125) but also for the processing of proG at a single basic amino acid residue, Arg77, to yield the biologically active form of GLP-1, GLP-17-37NH2 (Fig 2.22). The profile obtained with PC1 in GH3 cells is similar to that of the intestinal L cell (169). Interestingly, the efficiency of proG processing to GLP-1 appeared to be reduced as compared to that of glicentin and oxyntomodulin in the same cells (83 ng/dish GLI as compared to 11 ng/dish GLP-1 in vv:PC1-infected cells). These findings suggest that the cleavages at Arg109Arg110 and Arg77 are less efficient than at Lys70Arg71 (Fig 2.22). As well, infection of AtT-20 cells with vv:proG alone resulted in the generation of predominantly full-length forms of GLP-1 and smaller amounts of the truncated forms. The apparent inefficiency of cleavage at Arg77 suggests that endogenous PC1 in these cells was overwhelmed by the vv-induced overexpression of proG. Previous studies have not examined the processing of proG to the different forms of GLP-1 (60,74), however, PC1 has also been
implicated in the processing of prodynorphin at a single Arg (348). Additionally, we show that up to 62% of the total GLP-1 produced is amidated, consistent with the presence of PAM in GH3 (371) and AtT-20 cells (372). PAM consists of two activities: peptidylglycine α-hydroxylating monooxygenase (PHM) and peptidyl-α-hydroxyglycine α-amidating lyase (PAL). PAM activity has been characterized throughout the rat gastrointestinal tract (375) and PHM has been co-localized with GLP-1 in intestinal L cells (92). As well, addition of ascorbate to the medium of fetal rat intestinal cell cultures increases the ratio of amidated:non-amidated GLP-1 (53), consistent with the known ascorbate-dependency of PAM (376). Therefore, PAM is a likely candidate for the amidation of GLP-1. We conclude that PC1 is responsible for the production of bioactive GLP-1\textsuperscript{7-37}, and that PAM may play a role in the amidation of GLP-1 (Fig. 2.22).

An additional novel finding of the present study is the processing of proG by PC1 to several forms of GLP-2, identified as GLP-2 and a putative GLP-2-Lys\textsuperscript{159}-Lys\textsuperscript{160}. In contrast, amino acid sequence analysis of porcine intestinal extracts indicated that GLP-2 is the major peptide (72). Previous studies of proG processing have not characterized the molecular forms of GLP-2 liberated from proG (60,74). Our study is therefore the first to demonstrate production of fully processed GLP-2 by PC1. Interestingly, our finding of a major additional peak of GLP-2 (which we have tentatively identified as GLP-2-Lys-Lys) suggests that processing at this site by endogenous carboxypeptidase E (CPE) is particularly inefficient. Similarly, in RIN m5F cells, \textit{vv:POMC} is processed to a Lys-Lys-extended form of β-endorphin (377). POMC is fully processed to β-endorphin upon mutation of the C-terminal Lys to Arg, however, in keeping with the known substrate specificity of CPE (378). As well, the overproduction of proG in our cells may have overwhelmed the capacity of endogenous CPE, thus also contributing to the inefficiency
of processing at the C-terminal basic amino acids of GLP-2.

Infection of BHK-proG cells with vv:furin resulted in the production of glicentin, but not oxyntomodulin or GLP-1. In contrast, vv:furin processed proG to glicentin, GLP-1 and GLP-2, but not oxyntomodulin in GH₃ and AtT-20 cells. Glicentin was produced by every enzyme in this study, indicating that the cleavage site for the liberation of glicentin from proG (Lys⁷⁰-Arg⁷¹) is easily accessible. The liberation of GLP-1 by vv:furin in GH₃ and AtT-20, but not BHK-proG cells, indicates that furin may act in a manner similar to that of PC1 when overexpressed in cells with a regulated secretory pathway. However, vv:PC1 produced oxyntomodulin, while vv:furin did not, suggesting that this particular cleavage site (Lys³¹-Arg³²) may be specific for PC1. Consistent with this, none of the other enzymes tested produced oxyntomodulin. Furin is expressed in most cells, including BHK cells (230). Endogenous furin in BHK-proG cells did not, however, liberate glicentin from proG. Our results therefore suggest that furin may not normally process proG, and provide additional evidence that intestinal PGDPs are produced by the actions of PC1 alone.

**PC2 Processes ProG to Glicentin, but not Glucagon**

It has been suggested that PC2 plays a role in proG processing, based upon its co-localization with glucagon in A cells (249,329), and its expression in A cell-derived cell lines (60,328,359). In the present study, however, infection of GH₃ and AtT-20 cells with vv:PC2 resulted in liberation of glicentin, but not glucagon, from proG. Our results are the first to demonstrate the production of glicentin by expression of PC2 in a cell system; however, our results are similar to those of a very recent *in vitro* study (74), in which proG was processed to glicentin and oxyntomodulin, but not glucagon, by immunopurified PC2. Pulse-chase studies have indicated that glicentin may be a precursor to glucagon in the
A cell (10,60). Consistent with these results, expression of antisense PC2 mRNA in \( \alpha \)TC1-6 cells resulted in the reduction of both glicentin and glucagon (60). Therefore, while the available evidence suggests that PC2 cleaves proG to glicentin, there is no conclusive data demonstrating a role for PC2 in the production of glucagon. Finally, it is well established that activation of PC2 occurs late in the secretory pathway (281,293). The inefficient processing by PC2 in the constitutively-secreting BHK-proG cells is consistent with the requirements of secretion granules for the appropriate maturation and processing of PC2 (281,290,293). Using the vaccinia virus model, it has been shown that vv:PC2 is appropriately processed in the endocrine-derived \( \text{GH}_4 \text{C}_1 \) cells, even after 6 hr of chase, to its 66 kDa form, (278) which is present only in secretion granules (281,282). Moreover, in this study, vv:PC2 significantly increased endogenous POMC processing to IR-\( \alpha \)-MSH in AtT-20 cells, while failing to process vv:proG to glucagon in the same cells. Since the cleavage of POMC to \( \alpha \)-MSH is a late event in the secretory pathway (313,318), it therefore appears that vv:PC2 was processed and transported correctly to secretion granules in our system. Thus, these findings are consistent with those of others (74) and suggest that the production of glucagon in the pancreatic A cell requires another factor or enzyme in addition to PC2.

Recently, it has been proposed that a member of the granin family, the neuroendocrine protein 7B2, is a molecular chaperone which interacts specifically with PC2 in the secretory pathway (302,303). 7B2 mRNA is expressed in pancreatic islets, insulinomas and A cell-derived cell lines, and has been immunocytochemically localized to secretory granules in A cells (230,299). The 27 kDa pro7B2 binds to pro-PC2 in the endoplasmic reticulum, and may facilitate the maturation of PC2 (306), while the C-terminal region of 7B2 inhibits the enzymatic activity of PC2 (309). 7B2 therefore
stabilizes the activity of PC2 through inhibition of pro-PC2 processing until the enzyme reaches the trans Golgi network or secretory granules, and through enhancing the stability and lifespan of active PC2 (303,304). It has been shown that, in the vaccinia virus model, vv:7B2 is correctly processed and targeted to the regulatory secretory pathway, and associates with vv:PC2 (303,370). The stabilization of PC2 activity by 7B2 was confirmed in our system, as 7B2 greatly increased PC2-mediated processing of endogenous POMC to IR-α-MSH in AtT-20 cells. PC2-mediated processing of POMC can also be enhanced by 7B2 in vitro (379). Therefore, we hypothesized that co-infection of vv:7B2 with vv:PC2 would permit processing of proglucagon to glucagon. However, the co-expression of 7B2 and PC2 in GH₃ cells did not result in the production of glucagon, even when 7B2 was expressed in a 2:1 ratio with PC2. Furthermore, enhancement of PC2 activity through co-infection of other members of the granin family was also examined, since CgA, CgB and SgII have all been localized to the A cell (380), and are thought to direct peptide hormones into secretory granules (296,297). Our results show that co-infection of GH₃ cells with vv:PC2 and vv:CgA, vv:CgB or vv:SgII also did not result in the production of glucagon by PC2. Therefore, we conclude that PC2 processes proG to glicentin, and that another factor may be required for the production of glucagon. This factor could be an as yet unknown molecular chaperone, or possibly another enzyme.

**Roles of Other PCs in ProG Processing**

In order to examine roles of other PCs, alone or in combination with PC2, in the processing of proG to its derived peptides, GH₃ and AtT-20 cells were infected with vv:PACE4, vv:PC5a or vv:PC5b alone, or with vv:PC2. The role of PC4 was not examined since this convertase is expressed only in the testis (230), and is therefore not a
candidate for the processing of proG. Infection of AtT-20 cells with v:PC2 did not result in the production of glucagon. Since AtT-20 cells endogenously express high levels of PC1 (230), these results demonstrate that a combination of PC1 and PC2 does not liberate glucagon from proG. Consistent with these findings, co-infection of GH3 and AtT-20 cells with v:PC1 and v:PC2 also did not result in the production of glucagon. These results are in agreement with those obtained in vitro (74). Until very recently (303,336), the roles of the other convertases in prohormone processing had not been examined. In the present study, PACE4 was found to be capable of producing glicentin. However, in combination with either PC1 or PC2, PACE4 had no further effect on proG processing. Similarly, PC5a and PC5b liberated glicentin, but had no further effect on PC2-mediated processing. None of these enzymes liberated glucagon, GLP-1 or GLP-2. PACE4 may not be a relevant enzyme in proG processing, since PACE4 has recently been localized almost exclusively to the pancreatic B cell (249) and is not expressed in the ileum (230). In contrast, PC5a has been detected in proG-expressing αTC1-6 cells (359). A full analysis of the tissue distribution of PC5b has not yet been done. Thus, when taken with our data, these findings suggest that PACE4, PC5a and PC5b do not play major roles in the tissue-specific processing of proG, and that PC2 does not cleave proG to glucagon in the presence of any of these enzymes.

Conclusions

In conclusion, we show that PC1 is the sole enzyme responsible for the processing of proG to the intestinal peptides, glicentin, oxyntomodulin, GLP-1\textsuperscript{1-37/36NH\textsubscript{2}}, GLP-1\textsuperscript{7-37/36NH\textsubscript{2}} and GLP-2. Our results therefore suggest that the tissue-specific processing of proG is due to the differential distribution of PC1 and another convertase. A role for PC2 in the
processing of proG to glucagon, alone or in the presence of PACE4, PC5a, or PC5b, or the granins CgA, CgB, SgII or the PC2-binding protein 7B2, could not be demonstrated. Our findings open the possibility that regulation of the activity of PC1 in the intestinal L cell could lead to the enhanced production of GLP-1^{7-37/7-36NH2}, a potent insulinotropic peptide of current interest for therapy in Type II diabetes.

As this study was being prepared for publication, a report was published which demonstrated that PC1 processes hamster proG to glicentin, oxyntomodulin, GLP-1^{11-37/1-36NH2} and GLP-1^{7-37/7-36NH2} in AtT-20 cells (75), consistent with the results of the present study. However, in contrast to our findings, Rouillé et al (75) were unable to demonstrate a role for PC1 in the processing of proG to truncated forms of GLP-1 in vitro, and suggested a requirement for an additional enzyme in this cleavage. Processing of proG to GLP-1^{7-37/7-36NH2} was not examined in the in vitro studies of Rothenberg et al (74). The results of these studies therefore suggest that the actions of PC1 in vitro do not fully mimic those of PC1 in vivo. Evidence was also provided by Rouillé et al (75) for a role for PC2 in the processing of proG to glucagon in AtT-20 cells which had been stably transfected with PC2. This finding was not corroborated in vitro (74) or by the present study. As well, a study published back-to-back with the present study described the effects of antisense-mediated inhibition of PC2 on proG processing (76). Despite a 90% reduction in PC2 protein levels, glucagon production was not altered, although glicentin production decreased significantly. These results therefore provide further evidence that PC2 alone does not process proG to glucagon, but requires another factor to mediate this cleavage.

Although the vaccinia virus system has proved to be a useful model in determining the role of PC1 in proG processing, it is known that the virus may have deleterious effects
Fig 2.22 Proposed actions of the prohormone convertases in the tissue-specific processing of proG.

PC1 produces the intestinal PGDPs, glicentin, oxyntomodulin, GLP-1\(^{1-37}\), GLP-1\(^{7-37}\) and GLP-2, while PC2 produces glicentin. The presence of GLP-1\(^{1-36NH_2}\) and GLP-1\(^{7-36NH_2}\) indicates that PAM may be responsible for the amidation of GLP-1.
on cellular machinery. The advantage of using this system is that infection efficiency is almost 100% (381), such that every cell infected will express the protein of interest. As well, the virus commandeers the cell’s secretory machinery, thus ensuring that the proteins of interest are properly processed and secreted. However, this advantage can also be a disadvantage, since synthesis of the cell’s endogenous proteins may be compromised. This is evident in a number of studies which have found that peptides processed late in the secretory pathway are not produced in a vaccinia virus system, whereas they are detectable in a stable or transient transfection system. Since the processing of proG to glucagon is a late event in the secretory pathway, we were concerned that glucagon may not be produced in the vaccinia virus system. We therefore utilized PC12 cells that had been stably transfected with PC2 (PC12/PC2) to clarify the role of PC2 in the production of glucagon. When PC12/PC2 cells were transfected with proG, however, production of IRG did not differ from that produced by wt cells, thus corroborating the data obtained from the vaccinia virus studies. In light of the available evidence suggesting a role for PC2 in the production of glucagon (60) and the fact that PC2 is highly expressed in the A cells of the pancreas, we elected to further explore a possible role for PC2 in the processing of proG to glucagon. We first examined three glucagon-producing cell lines for PC1 and PC2 expression, and correlated PC expression with production of pancreatic and intestinal PGDPs. InR1-G9 cells are derived from a BK virus-induced hamster insulinoma (382) and we (64) and others (383) have shown that these cells process proG in a pancreatic fashion, the major PGDP being glucagon. RIN 1056A cells are derived from rat insulinoma (384) and process proG to both pancreatic and intestinal peptides (64,384). STC-1 is a neuroendocrine cell line derived from mouse secretinoma, and processes proG to both pancreatic and intestinal PGDPs (64). We have
shown that only PC2, and not PC1, mRNA is detected in InR1-G9 cells, whereas the other cell lines expressed both PC1 and PC2 (64). Therefore, PC1 expression can be correlated to production of intestinal PGDPs and PC2 expression to glucagon production. Since proG processing in the InR1-G9 cell line mirrors that which is found in the pancreatic A cell, and expresses PC2, but not PC1 mRNA, we utilized InR1-G9 cells in the next study to develop stable in vivo cell models in which to further examine the roles of PC1 and PC2 in proG processing.
CHAPTER 3

Role of the Prohormone Convertases in the Processing of Proglucagon in the Intestine: Antisense and Overexpression Studies.

The work described in this chapter was done entirely by me with the exception of the prohormone convertase radioimmunoassays, which were carried out by Dr. B. D. Noe, Atlanta, GA.
Tissue-specific post-translational processing of proglucagon (proG) results in the production of a diversity of peptides in the pancreas and intestine. Glucagon is the major product of processing in the A cell of the pancreas, while glicentin, oxyntomodulin, glucagon-like peptide-1 (GLP-1) and GLP-2 are the proG-derived peptides (PGDPs) produced in the L cell of the intestine. N-terminal truncation of GLP-1$_{1-37/36}$ at a single arginine (Arg$^7$, Fig 1.2) renders GLP-1 biologically active, as GLP-1$_{7-37/36}$NH$_2$. The physiological functions of many of the intestinal PGDPs have been determined. The function of glicentin is not known, while oxyntomodulin inhibits pentagastrin-stimulated gastric acid secretion in vivo (159,160). The insulinotropic effects of truncated GLP-1 have been well documented (174,351), and the results of these studies have led to clinical trials to determine if hyperglycemia in Type II diabetes may be controlled through administration of GLP-1. Finally, the biological function of GLP-2 has just been recently elucidated (211). Administration of GLP-2 to normal mice results in a 100% increase in intestinal weight, thus implicating GLP-2 as an intestinal growth factor. Therefore, GLP-2 could potentially be used as therapy for patients with short bowel syndrome in whom nutrient absorption is compromised (218).

The identification of a family of mammalian enzymes, known as prohormone convertases (PCs) has facilitated the study of the cellular mechanisms underlying the synthesis of peptide hormones. Of the PCs thus far described, PC1 (also known as PC3) and PC2 are found exclusively in neuroendocrine cells, and their roles in the processing of several prohormones have been elucidated (318,326,336). Using a variety of approaches, we (77) and others (74,75) have shown that PC1 is responsible for the processing of proG to all of the intestinal proG-derived peptides. In particular, we have
shown that PC1 produces GLP-1$^{7-37/36NH_2}$ and GLP-2. In contrast, while Rouille et al (75) also found that PC1 processed proG to GLP-1$^{1-37/36NH_2}$, they attributed the cleavage at Arg$^{77}$ (Fig 1.2) to an unidentified endopeptidase activity. Nonetheless, when taken together with the immunocytochemical localization of PC1, and not PC2, in the intestinal L cell (358), the available data strongly suggest that PC1 produces the intestinal proG-derived peptides.

In contrast to PC1, the role of PC2 in the processing of proG remains controversial. While PC2 has been co-localized with glucagon in pancreatic A cells (243,385), studies attempting to demonstrate glucagon production by PC2 have yielded conflicting results. Using recombinant vaccinia virus to infect a number of cell lines, we have shown a role for PC2 in the production of glicentin, but not glucagon, both in the absence and presence of other PCs and the chromogranin family of molecular chaperones (77). Combining immunopurified PC2 and proG in vitro also resulted in the production of glicentin, but not of glucagon (74), and antisense deletion of PC2 in a pancreatic cell line (αTC1-6) decreased the generation of glicentin, with little effect on glucagon production (76). Interestingly, using a similar approach, Rouillé et al (60) showed that antisense deletion of PC2 resulted in a decrease in both glicentin and glucagon production. Additionally, vaccinia virus-infected proG is processed to glucagon in AtT-20 cells stably expressing PC2, while no glucagon is produced upon infection of wild-type cells (75), thus suggesting a role for PC2 in the processing of proG to pancreatic glucagon.

In order to confirm the role of PC1 in the processing of proG in vivo, and to clarify the role of PC2, we utilized a well-known pancreatic cell line, InR1-G9, to provide a stable model of proG processing. InR1-G9 cells are derived from a BK virus-induced hamster
insulinoma (382), and express large amounts of proG mRNA relative to other pancreatic hormone mRNA transcripts (350). The pattern of proG processing is consistent with the pancreatic origin of the cells. InR1-G9 cells produce mainly glucagon, along with much smaller amounts of glicentin, oxyntomodulin and GLP-1-36NH2 (64,383). We have also shown that these cells express PC2, but not PC1, mRNA (64). We therefore hypothesized that: a) overexpression of PC1 in InR1-G9 cells would result in the increased production of the intestinal proG-derived peptides; b) antisense-mediated deletion of PC2 would decrease glicentin, and perhaps glucagon, production; and c) a combination of PC1 overexpression and PC2 inhibition would convert the pancreatic processing phenotype of InR1-G9 cells to an intestinal processing phenotype. Our findings confirm a role for PC1 in the production of the intestinal proG-derived peptides, and again describe a role for PC2 in the production of glicentin, but not glucagon.
3.2 Materials and Methods

Plasmids

The cDNAs encoding full-length mouse PC1 in the expression vector pRc/CMV and full-length mouse PC2 in the vector Bluescribe were obtained from Dr. N.G. Seidah (Montreal, PQ). The expression vector pZeo SV was obtained from Invitrogen (San Diego, CA). The cDNA encoding antisense PC2 (ASPC2) in pZeo was constructed by excising a 470 bp fragment, which contained the translation start site at position 61, from the full-length mouse PC2 by digestion with the restriction enzymes BssHII (5') and KpnI (3') (Boehringer Mannheim Canada, Laval, QC). The fragment was then ligated using T4 DNA ligase (Boehringer Mannheim) in the reverse orientation into pZeo to generate the pZeo/ASPC2 cDNA. Correct orientation of the fragment was confirmed through restriction digestion.

Stable Transfection of InR1-G9 Cells with PC1 or ASPC2

Wild-type (wt) InR1-G9 cells (gift from Dr. D.J. Drucker, Toronto, ON) were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, Life Technologies Inc., Grand Island, NY) containing 10% (v/v) FBS and 4.5 g/L glucose. Cells were transfected with vector controls, pRcCMV/PC1 or pZeo/ASPC2 using the calcium phosphate method (386). Briefly, cells were plated at approximately 30% confluency in 10 cm dishes 16 h prior to transfection. Cells were incubated with 10 µg DNA in 1 mL 0.1X Tris-EDTA containing 2X HBS (HEPES-buffered saline: 280 mM NaCl, 10 mM KCl, 1.5 mM Na₂HPO₄.2H₂O, 12mM dextrose, and 50mM HEPES, pH 7.05) and 2 M CaCl₂ for 4 h at 37°C. Cells were then subjected to a 3 minute glycerol shock [15% (v/v) glycerol in HBS], washed in PBS and cultured for 60 h to allow for expression of transfected DNA. On day
3 after transfection, cells were trypsinized and plated at a dilution of 1:2 in culture medium containing 800 μg/mL G418 (Sigma Chemical Co., Oakville, ON) and/or 250 μg/mL Zeocin (Invitrogen). The cells were maintained in the selection medium for 3-4 weeks before isolation of individual clones. InR1-G9/PC1 clones were screened for PC1 mRNA expression and GLP-1 secretion, and two clones (A and D) were selected for full characterization, as described in Results. InR1-G9/ASPC2 cells grew as a mixed population. InR1-G9/PC1/ASPC2 clones were screened for PC1 and PC2 mRNA expression and GLI:IRG ratios, and one clone (H7) was selected for full characterization, as described in Results.

**RNA analysis**

RNA extraction and analysis was carried out as described in Chapter 2. cDNA probes for mouse PC1 and mouse PC2 were generated by excision of the full-length fragment from the Bluescribe vector (gifts from Dr. N.G. Seidah, Montreal, PQ), and the full length rat proglucagon and actin probes were gifts from Dr. D.J. Drucker.

**Peptide Extraction**

Extraction of PDGPs was carried out as described in Chapter 2. Extraction of PC1 and PC2 protein was carried out as described previously (76). Briefly, cells were scraped in 0.5 M sodium phosphate buffer with 9 mM EDTA, pH 10, at 4°C, and subsequently sonicated in 5x10 sec bursts. Extracts were then freeze-thawed once in a dry ice-acetone bath and a 65°C water bath, centrifuged at 12 000 x g for 10 min, and stored at -70°C.
High Pressure Liquid Chromatography (HPLC)

PGDPs contained in cell extracts were separated on the basis of hydrophobicity as described in Chapter 2. Equal amounts of GLI, GLP-1 and GLP-2 were loaded onto each column in order to normalize the relative amounts of peptides measured.

Radioimmunoassays (RIA)

RIAs used to identify various PGDPs were carried out as described in Chapter 2. Presence of PC1 and PC2 protein was assessed by RIA as previously described (74,76), using antisera which recognize the N-terminus of the catalytic domain of both PCs (74). The detection ranges were 10-1280 pg for the PC1 assay and 5-1500 pg for the PC2 assay. Total cellular protein levels were determined using the Lowry protein assay (387).

Statistics

Optical density readings for PC2 mRNA transcripts and total cellular immunoreactivity (IR) of PC2 were assessed for differences between means by analysis of variance with unpaired Student’s t test. Total cellular immunoreactivities (IR) of GLI, GLP-1 and GLP-2 were not assessed for statistical significance, as these data were not normalized. Peak areas generated by HPLC/RIA for each of the PGDPs (glicentin, oxyntomodulin, glucagon, GLP-1$^{1-36NH_2}$, GLP-1$^{7-36NH_2}$ and GLP-2) were quantified for 3 independent experiments. Peak areas of GLP-1$^{17-37}$ were not quantified since little or no GLP-1$^{17-37}$ was detected by HPLC. Differences between means were assessed by analysis of variance with Tukey multiple comparisons tests. All data were log$_{10}$-transformed prior to analysis in order to minimize variability. All statistical manipulations were performed using SAS software for IBM computers (Statistical Analysis Systems,
Cary, NC).
3.3 Results

**Assessment of PC1 and PC2 Expression**

After selection of InR1-G9/PC1 clones with G418, twenty-four individual clones were screened for secretion of GLP-1. Eight clones were chosen for assessment of PC1 mRNA expression, four (A-D) of which secreted high amounts of GLP-1 (31-40 ng/dish) relative to wt cells (10 ng/dish) and four (E-H) of which secreted low amounts of GLP-1 (8-9 ng/dish). Fig 3.1 shows expression of proglucagon, PC2, PC1 and actin mRNA in wt InR1-G9 cells and the eight selected clones. Both proglucagon and PC2 were expressed in wt and PC1-expressing clones, although the observed levels were surprisingly variable. In agreement with previous results (64), PC1 mRNA could not be detected in wt cells. All eight selected clones were found to express PC1 mRNA transcripts at high levels. Two clones, A and D, were selected for characterization of proG processing, as levels of PC1 mRNA appeared to be highest in these cells. Since two transcripts of approximately 3 kb and 5 kb were detected, both were quantitated relative to those of actin, and were shown to be highly expressed relative to wt InR1-G9 cells.

InR1-G9/ASPC2 clones and vector-transfected controls were selected in Zeocin, while InR1-G9/PC1/ASPC2 clones and PC1/pZeo controls were selected in G418 and Zeocin. After selection, InR1-G9/ASPC2 cells were grown as mixed cell populations, while 26 PC1/ASPC2 clones were screened for GLI:IRG ratios to assess glucagon production. Four clones which showed the highest GLI:IRG ratios (indicating a reduction in glucagon production) were chosen for assessment of PC1 and PC2 mRNA expression. Fig 3.2 shows expression of proglucagon, PC2, PC1 and actin mRNA transcripts in wt, InR1-G9/ASPC2 and InR1-G9/PC1 cells, and InR1-G9/PC1/ASPC2 clones. As expected, proG was highly expressed in all cell lines, while PC1 mRNA was detectable only in cells...
Fig. 3.1  Expression of PC1 mRNA in InR1-G9/PC1 Cells.

InR1-G9 cells were stably transfected with PC1 and eight clones (A-H) were selected for assessment of PC1 expression. Blots were probed for glucagon, PC2, PC1 and actin mRNA, and one representative blot is shown (top). Both PC1 mRNA transcripts were quantified by densitometric scanning in clones A and D (bottom). Values are means $\pm$ SEM (n=3).
Fig. 3.2 Expression of PC2 mRNA in InR1-G9/ASPC2 and InR1-G9/PC1/ASPC2 Cells.

InR1-G9 cells were stably transfected with ASPC2 alone (AS) or together with PC1, and four clones (H3, H5, H7, H12) were selected for assessment of PC2 expression. Blots were probed for glucagon, PC2, PC1 and actin mRNA, and one representative blot is shown (top). PC2 mRNA transcripts were quantified by densitometric scanning in cells transfected with the indicated constructs (bottom). Values are means ± SEM (n=3). *, p<0.05; ***, p<0.001.
Fig 3.3 Immunoreactive PC1 and PC2 levels in all InR1-G9 cell lines.

InR1-G9 cells transfected with the indicated constructs were assessed for presence of PC1 and PC2 immunoreactivity by RIA, as described in Methods. Values are means ± SEM (n=3). *, p<0.05. ND, not detectable.
transfected with PC1. PC2 was also detectable in all cells, however, the levels were decreased by 62±9% (p<0.001) in InR1-G9/ASPC2 cells compared to control cells, and by 37±9% (p<0.05) in InR1-G9/PC1/ASPC2 clones compared to wt cells.

Concentrations of PC1 and PC2 protein were assessed by RIA (Fig 3.3). In good agreement with mRNA levels, PC1 protein was undetectable in wt cells and vector-transfected controls, while PC1 protein was expressed in InR1-G9/PC1 cells and in cells expressing both PC1 and ASPC2. Unexpectedly, however, PC1 was also detected in ASPC2 cells. PC2 protein levels were detectable in all cells, again in good agreement with mRNA levels. PC2 levels were decreased by 63±17% (p<0.05) in InR1-G9/ASPC2 cells compared to transfected controls. Surprisingly, InR1-G9/PC1/ASPC2 cells appeared to express very high levels of PC2 (Fig 3.3).

Production of Glicentin, Oxyntomodulin and Glucagon.

Effects of PC1 Overexpression

As indicated above, two PC1-expressing clones (A and D) were selected for characterization of PGDP production. Table 3.1 shows total cellular immunoreactivity for all PGDPs in all InR1-G9 cells. Since the data were expressed as ng/dish, normalization occurred upon HPLC separation of N-terminal PGDPs, when equal amounts of GLI (100 ng) were loaded onto the column.

Identification of GLI and IRG peptides by HPLC revealed that wild-type InR1-G9 cells produced glicentin and oxyntomodulin in a ratio of 1:3, and that most of the GLI produced eluted as glucagon (Fig 3.4). This profile mirrors that which is found in normal pancreatic A cells (388) and is in agreement with previous results (64,383). Overexpression of PC1 caused an increase in the production of glicentin (p<0.01) and
Table 3.1  Total Cellular Immunoreactivities (IR) of PGDPs in All InR1-G9 Cell Lines.

Cell extracts of InR1-G9/PC1 cells (A) and cells transfected with ASPC2 or both constructs (B) were assessed for the presence of GLI, IRG, GLP-1\textsuperscript{x-37}, GLP-1\textsuperscript{x-36NH2} and GLP-2 IR. All values shown are expressed as ng IR-peptide/dish, and are means ± SEM (n=3).
### A

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>GLI</th>
<th>IRG</th>
<th>x-37</th>
<th>x-36NH₂</th>
<th>GLP-2</th>
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<tbody>
<tr>
<td>WT</td>
<td>765±71</td>
<td>721±127</td>
<td>23±1</td>
<td>40±4</td>
<td>27±3</td>
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<td>18±1</td>
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<td>62±9</td>
</tr>
<tr>
<td>PC1 D</td>
<td>817±24</td>
<td>799±112</td>
<td>28±1</td>
<td>81±12</td>
<td>70±6</td>
</tr>
</tbody>
</table>

### B

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>GLI</th>
<th>IRG</th>
<th>x-37</th>
<th>x-36NH₂</th>
<th>GLP-2</th>
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<tr>
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<td>205±3</td>
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<td>82±14</td>
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<td>98±8</td>
<td>2±1</td>
<td>7±1</td>
<td>132±24</td>
</tr>
<tr>
<td>PC1/pZeo</td>
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<td>266±7</td>
<td>18±2</td>
<td>60±10</td>
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<td>276±12</td>
<td>14±2</td>
<td>42±4</td>
<td>172±15</td>
</tr>
</tbody>
</table>
oxyntomodulin (p<0.05) relative to wt cells, and increased the ratio of glicentin:oxyntomodulin to 1:1. Glucagon production was not significantly altered in InR1-G9/PC1 cells.

**Effects of Antisense-Mediated Inhibition of PC2 and Co-Expression of PC1 and ASPC2**

Identification of N-terminal PGDPs by HPLC showed that antisense-mediated reduction of PC2 severely attenuated production of glicentin (p<0.05) compared to control cells, while processing to oxyntomodulin did not change (Fig 3.4). Identification of the first GLI peak as oxyntomodulin was made on the basis of its known elution position at fraction 82 relative to elution of the internal control (210,365). However, in each experiment, significant immunoreactivity was observed in a single peak that spanned from fractions 78-84; thus the possibility that glicentin may be contained within this peak cannot be ruled out. Glucagon production in InR1-G9/ASPC2 cells decreased significantly when compared with wt cells (from 39±5 to 19±2 ng/peak, p<0.05), but not compared to vector-transfected controls. Transfection of both PC1 and ASPC2 resulted in a processing profile similar to that of wild-type cells; no significant changes in N-terminal PGDP production were observed.

**C-Terminal Processing**

**Effects of PC1 Overexpression**

RIA of free C-terminal GLP-1 IR showed that up to 75% of the total GLP-1 produced was amidated (Table 3.1), indicating the presence of the amidating enzyme PAM (376) in these cells. Analysis of cell extracts by HPLC showed that, in wt cells, the
Fig 3.4  Identification of GLI and IRG Peptides in All InR1-G9 Cell Lines.

Cell extracts were obtained from wild-type (WT) InR1-G9 cells, and cells transfected with vector alone (pZeo) or with the indicated constructs. After determination of total cellular immunoreactivity (Table 3.1), equal amounts of GLI (100 ng) were loaded onto the HPLC column for normalization of values. Cell extracts were analysed for the production of glicentin (a), oxyntomodulin (b) and glucagon (c) by HPLC. GLI is shown as a solid line, IRG as a dotted line. Each panel is representative of 3 experiments.
major form of GLP-1 produced was GLP-1$^{7-36\text{NH}_2}$, little or no GLP-1$^{1-37}$, GLP-1$^{1-36\text{NH}_2}$ or GLP-1$^{7-37}$ was detected (Fig. 3.5). Overexpression of PC1 appeared to increase processing to GLP-1$^{1-36\text{NH}_2}$, although the difference between peak areas was not significant. Processing to GLP-1$^{7-36\text{NH}_2}$ was not altered in InR1-G9/PC1 cells, compared to wt cells.

It must be noted that that the RIA utilized for GLP-1$^{x-36\text{NH}_2}$ underestimates total immunoreactive GLP-1$^{x-36\text{NH}_2}$ in cell extracts, as the yield of GLP-1$^{x-36\text{NH}_2}$ following HPLC analysis of GTag-Y cell extracts far exceeds the apparent amount loaded onto the column (37). This phenomenon appears to be specific to the GLP-1$^{x-36\text{NH}_2}$ RIA, and we speculate that some factor present in cell extracts may be interfering with the RIA.

Despite detection of significant GLP-2 IR in wt cells (Table 3.1), no GLP-2 peaks were observed after HPLC (Fig. 3.6). In contrast, one large peak of GLP-2 IR was seen after transfection of PC1 into InR1-G9.

Effects of Antisense-Mediated Inhibition of PC2 and Co-Expression of PC1 and ASPC2

HPLC identification of GLP-1 peptides revealed that production of GLP-1$^{7-36\text{NH}_2}$ increased significantly ($p<0.05$ vs pZeo control) upon transfection of ASPC2 alone (Fig 3.5). Although GLP-1$^{7-36\text{NH}_2}$ levels appeared to increase in InR1-G9/PC1/ASPC2 cells, the difference in peak area was not significant compared with wt cells, but was significantly increased ($p<0.01$) compared to vector-transfected controls.

HPLC analysis showed that little or no GLP-2 was present in either wt or control cells or in cells expressing ASPC2 alone, while, as expected, one large GLP-2 peak was present in InR1-G9/PC1/ASPC2 cells (Fig 3.6).
Cell extracts were obtained from wild-type (WT) InR1-G9 cells, and cells transfected with vector alone (pZeo) or with the indicated constructs. After determination of total cellular immunoreactivity (Table 3.1), equal amounts of GLP-1 were loaded onto the HPLC column for normalization of values. Cell extracts were analysed for the production of GLP-1(1-37) (dotted line) and GLP-1(1-36NH2) (solid line). Elution positions of synthetic standards, GLP-1(1-37) (a), GLP-1(1-36NH2) (b), GLP-1(7-37) (c) and GLP-1(7-36NH2) (d) are shown. Each panel is representative of 3 experiments.
Identification of GLP-2 in All InR1-G9 Cell Lines.

Cell extracts were obtained from wild-type (WT) InR1-G9 cells, and cells transfected with vector alone (pZeo) or with the indicated constructs. After determination of total cellular immunoreactivity (Table 3.1), equal amounts of GLP-2 were loaded onto the HPLC column for normalization of values. Cell extracts were analysed for the production of GLP-2 by HPLC. Elution of synthetic standard, rat GLP-2$^{1-33}$, is indicated by (a). Each panel is representative of 3 experiments.
Summary of Changes in ProG Processing in all InR1-G9 Cells

Fig 3.7 shows a summary of the changes observed in proG processing upon overexpression of PC1 (compared to wt cells), ASPC2 (relative to vector-transfected cells) and in InR1-G9/PC1/ASPC2 cells relative to vector-transfected controls. In InR1-G9/PC1 cells, production of glicentin, oxyntomodulin, GLP-1\textsuperscript{1-36NH\textsubscript{2}} and GLP-2 increased. Expression of ASPC2 decreased glicentin, but not glucagon, production. Interestingly, generation of GLP-1\textsuperscript{7-36NH\textsubscript{2}} increased in InR1-G9/ASPC2 cells compared to control cells. Finally, GLP-1\textsuperscript{7-36NH\textsubscript{2}} and GLP-2 production were increased in cells expressing both PC1 and ASPC2; production of all other PGDPs did not change.
Fig. 3.7 Summary of Changes in ProG Processing Upon Overexpression of PC1 and/or Antisense-Mediated Reduction in PC2 in InR1-G9 Cells.

Production of all PGDPs in InR1-G9 cells expressing PC1 (top), ASPC2 (middle) or both constructs (bottom) were expressed as percent change from wt or control (pZeo-transfected) cells. Values are means of differences in peak areas ± SEM (n=3). *, p<0.05; **, p<0.01.
3.4 DISCUSSION

In the present study, the pancreatic cell line InR1-G9 was utilized to provide a stable in vivo cell model with which to study the roles of PC1 and PC2 in proG processing. We sought to convert the pancreatic processing phenotype of these cells to an intestinal processing phenotype by overexpression of PC1 and antisense deletion of PC2. We created a number of stably transfected cell lines and identified known PGDPs, including all four forms of GLP-1, as well as GLP-2. In contrast, other studies describing the roles of PCs in proG processing utilized transiently transfected cell lines (60,76), at most distinguished between 2 forms of GLP-1 (74,75), and did not identify GLP-2. Ours is also the first study to describe changes in proG processing through stable overexpression of PC1.

Overexpression of PC1 in InR1-G9 Cells

In agreement with previous work from our lab, we were unable to detect PC1 mRNA transcripts in wt InR1-G9 cells. However, the mouse PC1 probe used in this and previous studies cannot detect PC1 expression in hamster brain tissue (data not shown), therefore these cells may be expressing PC1 that is undetectable with our cDNA probe. Our results are in contrast to those of a previous study in which PC1 mRNA expression was detected in InR1-G9 cells using a PCR-derived cDNA probe from mouse corticotroph cells (389). However, the PCR-derived probes may have detected non-specific bands, or alternatively, the InR1-G9 cells used may have developed a different phenotype from those employed in the present study. In agreement with our mRNA data, however, we also showed that protein levels of PC1 were not detectable in wt InR1-G9 cells using a highly sensitive and specific RIA. We therefore conclude that PC1 is not expressed in the
InR1-G9 cells employed in this study. That PC1 is not expressed in InR1-G9 cells is consistent with its pancreatic glucagon-producing phenotype. PC1 cannot be co-localized with glucagon by immunocytochemistry in adult mouse islets (329), and cannot be detected by Western blot of non-B cells from islets (328). However, some controversy exists as to whether PC1 is expressed in an A cell-derived cell line, αTC1-6. Some investigators have failed to detect PC1 expression by Northern (60) and Western (359) blot, while others have detected PC1 by Western (328), albeit in much smaller amounts than PC2. Still others have detected PC1 in αTC1-6 cells by Northern blot, as well as through immunocytochemistry and RIA (74,76). The results from the above studies emphasize the need for caution in extrapolating results obtained from cell lines to normal tissues.

When PC1 expression was assessed in InR1-G9/PC1 cells, two distinct mRNA transcripts of approximately 3 and 5 kb were detected. Several studies have also detected the existence of two PC1 mRNA transcripts in tissues (314,341,390) and cell lines (391) of approximately 3 and 5 kb. Only one study has characterized multiple PC1 transcripts, and attributed a difference of 40-400 bp to different polyadenylation sites (390). However, the 2 kb difference seen in other studies, as well as in the present study, is too large to be accounted for by different polyadenylation sites.

In our study, overexpression of PC1 resulted in significantly increased production of glicentin, oxyntomodulin and GLP-2, as predicted from our previous work (77). It must be noted that this increase in glicentin and oxyntomodulin did not appear to be accompanied by a concomitant decrease in glucagon production. However, since the amounts of glicentin and oxyntomodulin are very low in wt cells, an increase of about 5 to 6 ng/peak would translate into a significant increase (up to 100%; Fig 3.7), while a
similar change in glucagon production would not translate into a change of comparable magnitude. As well, the fact that oxyntomodulin is produced in wt InR1-G9 cells suggests that very low levels of PC1 may be present, albeit undetectable using Northern blot analysis and RIA. No significant increase in peak areas of GLP-1\(^{1-36\text{NH}_2}\) or GLP-1\(^{7-36\text{NH}_2}\) could be found. While the GLP-1 processing profile was reproducible for InR1-G9/PC1 cells, the total amount of peptide within each peak was variable, thus explaining the failure to achieve statistical significance. Nonetheless, examination of the GLP-1 processing profiles in InR1-G9/PC1 cells suggests that production of GLP-1\(^{1-36\text{NH}_2}\) was increased compared with wt cells, while processing to GLP-1\(^{7-36\text{NH}_2}\) did not change. These results are in contrast to those of our previous study, in which we have shown that co-expression of vv:PC1 and vv:proG in GH\(_3\) cells results in the production of both full-length and truncated forms of GLP-1, while expression of vv:proG alone does not result in the production of significant amounts of GLP-1 IR (77). However, the fact that very little C-terminal GLP-1 IR could be detected in GH\(_3\) cells infected with vv:proG alone indicated that cleavage was not occurring at Arg\(^{109}\)-Arg\(^{110}\) (Fig 1.2), but did not preclude cleavage at Arg\(^{77}\) by another, unknown enzyme. This potential cleavage product would be an N-terminally truncated form of MPGF (proG 77-158) which is undetectable in our system since we did not have an antibody at that time which recognizes the mid-sequence of GLP-1. It is therefore possible that PC1 mediates processing of proG at Lys\(^70\)-Arg\(^{71}\) and Arg\(^{109}\)-Arg\(^{110}\) to yield GLP-1\(^{1-36\text{NH}_2}\), and that another enzyme cleaves GLP-1 at Arg\(^{77}\) to yield GLP-1\(^{7-36\text{NH}_2}\)

It has been shown that PC1 can process prohormones at a single basic amino acid (348). However, the kinetics of processing of the C-terminal end of proG have not been determined. Therefore, it is not known if GLP-1\(^{1-36\text{NH}_2}\) acts as a precursor for GLP-1\(^{7-36\text{NH}_2}\),
such as occurs for the production of α-melanocyte-stimulating hormone (αMSH) from
adrenocorticotropic hormone (ACTH) (318). Alternatively, the two peptides may be
produced through independent cleavage events, as may be the case for the generation
of SS28 and SS14 from prosomatostatin (336). The increased ratio of full-length:truncated
GLP-1 in InR1-G9/PC1 cells suggests that GLP-1[1-36NH₂] is not being further processed by
PC1 to the truncated peptide. It has been shown that PC1 is unable to produce GLP-1[7-
36NH₂] from GLP-1[1-36NH₂] in vitro (75). Furthermore, it is likely that the actions of PC1 in vitro
may mimic those of PC1 in vivo, since PC1 mediates the processing of prodynorphin at
a single Arg both in vivo and in vitro with equal efficiency (348). Thus, GLP-1[1-36NH₂] may
not be a precursor to GLP-1[7-36NH₂] in the model studied. It is also possible that the
relatively large amounts of GLP-1[1-36NH₂] produced by overexpression of PC1 is
overwhelming an endogenous Arg⁷⁷ processing activity in InR1-G9 cells. Thus, PC1 may
act preferentially at the Lys⁷⁰Arg⁷¹ cleavage site, and not at Arg⁷⁷ as suggested by our
previous study (77).

This is the first study to demonstrate the production of fully processed GLP-2 by
PC1 in vivo. Through HPLC analysis of GLP-2 IR, a single GLP-2 peptide was observed
in InR1-G9/PC1 cells, which eluted in the same position as synthetic rat GLP-2[1-33]. This
extends the observations from our previous study (77), in which an incompletely
processed form of GLP-2 was detected in GH₃ cells co-infected with vv:proG and vv:PC1.
We had speculated that this GLP-2 peak represented GLP-2-Lys¹⁵⁹-Lys¹⁶⁰, as the
overexpression of proG may have overwhelmed the capacity of endogenous
carboxypeptidase E (CPE). Since InR1-G9 cells express proG endogenously,
overexpression of proG relative to CPH did not occur in the present study, thus ensuring
appropriate levels of both substrate and enzyme to permit complete processing of GLP-2.
**Antisense-Mediated Reduction of PC2**

Our previous study (77) showed that co-expression of \( \text{vv:proG} \) and \( \text{vv:PC2} \) in a number of heterologous cell lines did not result in the production of glucagon. Although we had shown that \( \text{vv:PC2} \) was bioactive in our system by demonstrating that \( \text{vv:PC2} \) could cleave POMC to \( \alpha\text{MSH} \) in \( \text{AtT-20} \) cells (77), the liberation of glucagon from proG is a very late processing event (60), and it is known that vaccinia virus infection may interfere with some late events. Production of \( \gamma\text{Lys-MSH} \) from POMC is seen after 3 h of chase in \( \text{AtT-20} \) cells stably expressing PC2 (318), but is not observed in a vaccinia virus system (315). As well, pro-nerve growth factor is not processed by \( \text{vv:PC2} \), but is processed in \( \text{PC12} \) cells stably expressing PC2 (392). Therefore, while the vaccinia virus system provided a useful model of proG processing, we predicted that the InR1-G9 stable cell model utilized in the present study might provide a more appropriate intracellular environment for the production of glucagon.

Transfection of InR1-G9 cells with ASPC2 resulted in a 62% decrease in PC2 mRNA levels, and a 63% decrease in PC2 immunoreactivity. Some studies employing antisense-mediated deletion of PCs report complete deletion of PC expression (316,317,393), while others do not (76,343-345). Of particular importance, studies which utilized cells stably transfected with ASPC2 show concomitant alterations in PC2 expression and propeptide processing. \( \text{GH}_3 \) cells co-transfected with POMC and ASPC2 expressed almost no PC2 protein and very little production of ACTH-related peptides (317). Expression of both ASPC2 and proenkephalin in RIN5f cells resulted in a decrease, but not abolition, of PC2 expression, and proenkephalin processing was altered in a small but significant fashion (343). These two studies utilized a similar ASPC2 construct, which consisted of a 480 bp fragment spanning the translation start site, and we therefore
designed a similar construct from mouse PC2 cDNA. It may be possible that complete abolition of PC2 in InR1-G9 cells was not realized since the mouse ASPC2 construct may not have cross-reacted fully with the endogenous hamster PC2. However, the PC2 cDNA fragment used as a probe in our Northern blots hybridized with endogenous PC2 mRNA in wt InR1-G9 cells, indicating that the mouse and hamster PC2 mRNAs may be similar in sequence. As well, other studies have utilized rat antisense PC cDNA to transflect mouse-derived cells (316,345) with complete or partial inhibition of endogenous PC expression. It is therefore possible to achieve antisense-mediated inhibition of PC expression with cDNA constructs derived from a species other than that of the host cell. Since the InR1-G9/ASPC2 cells were not single cell clones, it is also possible that the antisense construct completely abolished PC2 expression in some cells and not in others. Therefore, the aim of this part of the study was to correlate a decrease in the level of PC2 expression with changes in proG processing.

Expression of ASPC2 in InR1-G9 cells resulted in the virtual disappearance of glicentin, but glucagon production did not change compared to vector-transfected controls. Although glucagon production decreased relative to wt InR1-G9 cells, it must be noted that glucagon production also decreased in vector-transfected controls, despite the fact that neither PC2 mRNA nor protein levels changed. Therefore, a reduction in PC2 expression in InR1-G9/ASPC2 cells correlated with a reduction in glicentin, but not glucagon, production. These results are in agreement with those of Rothenberg et al (76), who showed that a 90% decrease in PC2 protein levels decreased glicentin, but not glucagon, production. The fact that glucagon was still produced in the absence of glicentin indicates that glucagon may be cleaved from oxyntomodulin. This appears likely given the presence of significant amounts of oxyntomodulin in wt InR1-G9 cells (64,383) (and the
present study), as well as in normal A cells (388). Indeed, it appears that glicentin is undetectable in rat and human pancreas, leading to the conclusion that glucagon may be produced from oxyntomodulin (388). However, pulse-chase studies which positively identify oxyntomodulin as a precursor to glucagon have not been carried out.

Interestingly, an increase in GLP-1$^{7-36\text{NH}_2}$ production was seen in InR1-G9/ASPC2 cells. Since we (77) and others (60,74-76) have not shown a role for PC2 in the generation of GLP-1, and since PC2 immunoreactivity is not found in the L cells of the ileum (358), PC2 is not a candidate enzyme for GLP-1$^{7-36\text{NH}_2}$ production. It is of note that the observed increase in GLP-1$^{7-36\text{NH}_2}$ production correlates with an apparent detection of immunoreactive PC1. However, this correlation is difficult to explain since no GLP-2 IR was detected in InR1-G9/ASPC2 cells. We have shown, in this and the previous study, that PC1 expression and GLP-2 production are quite convincingly correlated. This lack of GLP-2 production in particular leads us to conclude that the PC1 IR detected does not represent bioactive PC1. Furthermore, our data has shown that PC1 IR is undetectable in both wt InR1-G9 cells and vector-transfected controls. Therefore, the PC1 antiserum utilized does not cross-react with endogenous PC2 or furin in InR1-G9 cells, consistent with the previously reported characterization of the antiserum (76), nor does it exhibit non-specific binding upon transfection with vector alone. We have not analysed wt InR1-G9 cells for the presence of other PCs, such as PC5a, which has recently been shown to co-localize with glucagon and PC2 in pancreatic A cells (260). The reduction in PC2 expression in InR1-G9/ASPC2 cells may lead to increased expression of another endogenous enzyme, such as PC5a, with which the PC1 antiserum may cross-react.
Co-Transfection of PC1 and ASPC2 in InR1-G9 Cells

Immunocytochemical studies have shown that the intestinal L cells express PC1, but not PC2 (358). Therefore, we hypothesized that the pancreatic processing phenotype of InR1-G9 cells could be converted to an intestinal processing phenotype by overexpressing PC1 and deleting PC2. Both PC1 mRNA and protein were expressed in these cells, although not to as great an extent as observed in InR1-G9/PC1 cells. However, although PC2 mRNA levels were reduced, PC2 protein levels actually appeared to increase compared to wt and vector-transfected controls. The mechanism for this increase in PC2 protein upon transfection of an antisense-coding fragment is not known. The amount of bioactive PC2 represented by the increased IR PC2 levels is not known, since the antibody used in the RIA recognizes both the pro-form and the N-terminally truncated form of PC2 (B. Noe, personal communication). Because PC2 protein levels were not decreased in InR1-G9/PC1/ASPC2 cells, it is difficult to explain the processing phenotype of these cells.

In contrast to the reduction in glicentin production observed in InR1-G9/ASPC2 cells, glicentin levels did not decrease in cells transfected with both PC1 and ASPC2 compared to control cells. As well, glicentin production increased in InR1-G9/PC1/ASPC2 cells as compared with cells transfected with ASPC2 alone (from 3±1 ng/peak to 8±1 ng/peak, p<0.05). These results may confirm a role for PC1 in the generation of glicentin from proG. However, this does not preclude a role for PC2 in the production of glicentin in InR1-G9/PC1/ASPC2 cells, since an increase in PC2 IR was observed. Our results are corroborated by those of another study showing that antisense-mediated reduction of either PC1 or PC2 in αTC1-6 cells resulted in a reduction in glicentin production (76). It therefore appears that, in InR1-G9 cells, there also exists a redundancy in processing
enzyme utilization, as has been proposed for αTC1-6 cells (76). These results confirm a role for both PC1 and PC2 in the processing of proG to glicentin.

In contrast to the unexpected increase in production of the full-length form of GLP-1 observed in InR1-G9/PC1 cells, generation of GLP-1\textsuperscript{7-36NH\textsubscript{2}} appeared to increase in InR1-G9/PC1/ASPC2 cells. These results are difficult to explain in light of the results obtained in InR1-G9/PC1 cells, but are consistent with those seen in InR1-G9/ASPC2 cells. However, production of GLP-2 increased to the same extent as in InR1-G9/PC1 cells, again confirming a role for PC1 in the production of GLP-2. The increases observed in the levels of glicentin and GLP-2 is not surprising in view of the fact that PC1 is overexpressed in these cells. We conclude that expression of both PC1 and ASPC2 in InR1-G9 cells did not convert the pancreatic processing phenotype of these cells to an intestinal processing phenotype.

**Conclusions**

In summary, overexpression of PC1 in InR1-G9 cells resulted in increased production of glicentin, oxyntomodulin, GLP-1\textsuperscript{1-36NH\textsubscript{2}} and GLP-2. Processing to GLP-1\textsuperscript{7-36NH\textsubscript{2}} did not change, suggesting that another, unknown enzyme may mediate the production of the truncated peptide. Reduction of PC2 levels through expression of antisense PC2 resulted in a decrease in glicentin production, but glucagon production was not altered. Taken together with the results of our previous study (77), these results confirm a role for PC1 in the processing of proG to the intestinal PGDPs, glicentin, oxyntomodulin, GLP-1\textsuperscript{1-36NH\textsubscript{2}} and GLP-2. A role for PC2 in the production of glicentin was also confirmed, however, we were again unable to show a role for PC2 in the liberation of glucagon from proG. We therefore conclude that PC2 generates glicentin, and another, unidentified
enzyme may be responsible for the production of glucagon.

In the previous two studies, we have shown that PC1 plays a role in processing proG to the intestinal PGDPs. Several studies have shown that PC1 and PC2 mRNA and protein levels can be regulated by a number of second messengers and neuropeptides in concert with their prohormone substrates and derived peptides. In the next study we therefore sought to determine whether PC1 mRNA transcript levels are co-regulated along with proG mRNA transcripts by agents which are known to enhance proG gene transcription and PGDP synthesis in the intact L cell.
CHAPTER 4

Regulation of Proglucagon and PC1 Expression in an Intestinal L Cell Line.
4.1 Introduction

Proglucagon (proG) is expressed in the A cells of the pancreas and the L cells of the intestine. The post-translational processing of proG results in the production of peptides which are unique to each tissue: glucagon is the major product of processing in the A cell, while glicentin, oxyntomodulin, glucagon-like peptide-1 (GLP-1) and GLP-2 are the proG-derived peptides (PGDPs) produced in the L cell. N-terminal truncation of GLP-1-1-36NH2 at a single arginine (~Arg77, Fig 1.2) renders GLP-1 biologically active, as GLP-1-7-36NH2. The physiological functions of most of the intestinal PGDPs have been determined. Oxyntomodulin inhibits pentagastrin-stimulated gastric acid secretion in vivo (159,160), while the insulinotropic effects of truncated GLP-1 have been well documented (174,351); the results of the latter studies have led to clinical trials to determine if hyperglycemia in Type II diabetes may be controlled through administration of GLP-1. The biological function of GLP-2 has just been recently elucidated (211). Administration of GLP-2 to normal mice results in increased intestinal weight due to an increase in epithelial cell proliferation, thus implicating GLP-2 as an intestinal growth factor. Therefore, GLP-2 could potentially be used as therapy for patients with short bowel syndrome in whom nutrient absorption is compromised (218).

The identification of a family of enzymes, known as prohormone convertases (PCs) has resulted in the elucidation of the cellular mechanisms which operate during peptide hormone synthesis. Of the PCs thus far described, PC1 and PC2 are found exclusively in neuroendocrine cells, and their roles in the processing of several prohormones have been elucidated. It is known that pro-opiomelanocortin (POMC) is processed in a tissue-specific fashion by PC1 and PC2 to yield different peptides in the anterior and neurointermediate lobes of the pituitary (313,318). As well, proinsulin is processed in a
sequential fashion by PC1 and PC2 to generate mature insulin and C-peptide (326). Finally, we (77) and others (74,75) have shown that PC1 processes proG to the intestinal PGDPs.

In addition to determining the roles of PC1 and PC2 in the processing of prohormones, it has been demonstrated that expression of PC1 and PC2 are coordinately regulated with expression of their prohormone substrates. For example, expression of POMC mRNA in the neurointermediate lobe of the pituitary is co-regulated with both PC1 and PC2 mRNA in response to dopaminergic agents (242,314,316). This co-regulation appeared to be tissue-specific, as parallel changes in PC1 expression were not detected in the anterior pituitary (242,316). However, using AtT-20 cells, which are derived from mouse anterior lobe corticotrophs, POMC and PC1 mRNA levels were co-regulated by dopaminergic agents (316), indicating that changes in corticotroph PC1 mRNA may have been masked in primary cultures of anterior pituitary cells by the presence of different cell types which may also express PC1. As well, glucose co-regulation of both proinsulin and PC1, but not PC2, biosynthesis has been demonstrated in rat pancreatic islets (330). However, in islets obtained from obese (ob/ob) mice (331), as well as in the insulin-producing cell line MIN6 (333), glucose regulated the biosynthesis of both PC1 and PC2 in parallel to proinsulin production, also indicating that changes in PC2 expression may not be detected in a heterogenous cell population. In light of these results, we were interested to determine if proG and PC1 expression could be co-regulated in the intestinal L cell.

The mouse PC1 gene contains a cAMP response element (CRE) in its promoter region (394) and levels of PC1 mRNA transcripts can be regulated by agents which increase intracellular cAMP levels (288). The rat proG gene also contains a CRE in its
5'-flanking region (16), and the mechanisms underlying PKA-dependent regulation of proG gene expression in A cells have been well documented (32-34). Control of intestinal proG gene expression has been less well studied. In primary cultures of fetal rat intestine, PGDP secretion, together with levels of intestinal proG mRNA transcripts, are regulated in a cAMP-dependent manner (35,36). Expression of PC1 has been immunocytochemically detected in several intestinal endocrine cell types, including the human intestinal L cell (358), and also has been detected in rat intestine through Northern blot analysis (230). Since PC1 is also expressed in a variety of intestinal cells including the L cell, fetal rat intestinal cell cultures, of which the L cell population comprises approximately 1% (364), may not provide the most appropriate model in which to examine co-regulation of PC1 and proG expression. Therefore, in order to establish a correlation between regulation of proG and PC1 mRNA expression in proG-expressing L cells, we obtained a novel enteroendocrine cell line, known as GLUTag (37) or GTag-Y.

GTag-Y cells were derived from L cell tumors originating in the large bowel of SV40-large T antigen transgenic mice (28,365), and highly express proG mRNA (37). Although these cells also process proG to both pancreatic and intestinal PGDPs, the major form of GLP-1 produced is GLP-1\(^{7-36}\text{NH}_2\) (37), consistent with normal mouse intestine (64). Synthesis and secretion of the PGDPs, as well as proG gene transcription, are regulated in a cAMP-dependent manner in GTag-Y cells (37), consistent with the known responses in normal L cells (35,36). Given their molecular and cellular phenotype, GTag-Y cells may provide a useful model with which to examine co-regulation of proG and PC1 gene expression. Therefore, we hypothesized that proG and PC1 mRNA levels would be co-regulated in a cAMP-dependent manner in the enteroendocrine cell line GTag-Y. To this end, cAMP levels in GTag-Y cells were elevated through treatment with
forskolin/isobutylmethylxanthine or with the intestinal endocrine peptide, glucose-dependent insulinotropic peptide (GIP). GIP is a known regulator of the intestinal L cell (125,135) and increases intracellular cAMP levels (203,395) through a G protein-coupled, seven transmembrane receptor (396). Our results are in contrast to those previously obtained using GTag-Y cells (37), in that proG mRNA transcript levels did not increase upon elevation of cAMP. As well, levels of PC1 mRNA transcripts did not change in response to cAMP.
4.2 Materials and Methods

GTag-Y Cells

The mouse large intestine enterendocrine cell line GTag-Y was obtained from Dr. D.J. Drucker (Toronto, ON), and maintained in DMEM containing 10% (v/v) FBS. Cells were passaged 1:3 every 5 days, and were grown to 60% confluency in 100 mm plates for each experiment. Cells were treated with 10 μM forskolin /10 μM IBMX (F/I, Sigma Chemical Co., Oakville ON) or with 10·M human GIP (Bachem Inc., Torrance, CA). After 2h of treatment, media were removed for analysis of GLP-1 x-36NH2 and GLP-2 secretion by RIA. Following replacement of the treatment media, cells were incubated for a further 22h before assessment of proG and PC1 mRNA levels.

Radioimmunoassay

Since most of the GLP-1 produced in GTag-Y cells is amidated (37), cells were assessed for presence of GLP-1 x-36NH2 immunoreactivity only. The RIA for GLP-1 x-36NH2 was carried out as described in Methods, Chapter 2.

GLP-2 immunoreactivity was detected using an antiserum, UTTH-7, which was developed in our laboratory. UTTH-7 recognizes the mid-sequence of GLP-2 (Dr. P.L. Brubaker, personal communication) and thus detects MPG and proG in addition to GLP-2. The detection limits were 10-2000 pg/tube.

RNA Analysis

Total RNA was extracted and analysed as described in Methods, Chapter 2. Blots were probed with full-length rat proG and full-length mouse PC1, as described in Methods, Chapter 2. Equal loading of lanes was confirmed with the cDNA probe for 18s
ribosomal RNA (obtained from Dr. D.J. Drucker).
4.3 RESULTS

Secretion of GLP-1 and GLP-2

Treatment of GTag-Y cells with F/I is known to increase PGDP synthesis and secretion (37). We measured GLP-1*36NH2 and GLP-2 secretion as an indicator of the secretory response of GTag-Y cells to F/I and GIP treatment, which served as a positive control. Treatment of cells with F/I increased GLP-1 secretion 3.5-fold (p<0.001, Fig. 4.1), and GIP treatment increased GLP-1 secretion 2.5-fold (p<0.05). Secretion of GLP-2 in response to cAMP-dependent secretagogues was also measured, since it has been shown that GLP-1 and GLP-2 are released in parallel from the ileum (73). Secretion of GLP-2 increased 8-fold upon treatment with F/I (p<0.001) and 4-fold upon treatment with GIP (p<0.001) (Fig 4.1). These results therefore indicate that the GTag-Y cells used in this study are responsive to both F/I and GIP treatment.

Expression of ProG and PC1 mRNA.

It has been shown previously that proG gene transcription is regulated in a cAMP-dependent manner in GTag-Y cells (37). However, no significant change in proG mRNA transcript levels were detected upon treatment with either F/I or GIP (Fig 4.2). As well, levels of PC1 mRNA transcripts did not change significantly in response to F/I or GIP treatment (Fig 4.2).
Fig 4.1 Secretion of GLP-1 and GLP-2 from GTag-Y Cells Occurs in a cAMP-Dependent Manner.

GTag-Y cells were grown to 60% confluency in 100 mm dishes and treated with vehicle alone (Control), 10μM each of forskolin and IBMX (F/I) or 10⁻⁶ M GIP for 2 h. Media was then removed for analysis of immunoreactive GLP-1₈₋₃₆NH₂ (upper panel) and GLP-2 (lower panel) by RIA. Values are means ± SEM (n=3). *, p<0.05; **, p<0.01; ***, p<0.001.
Fig 4.2  Expression of proG and PC1 mRNA Transcripts in GTag-Y Cells in Response to cAMP Stimulation.

GTag-Y cells were treated with vehicle alone, forskolin/IBMX or GIP (as described in Fig. 4.1) for a total of 24 h, after which total RNA was analysed for proG or PC1 expression. Equal loading of lanes was assessed through probing with 18s ribosomal RNA. Bottom panel shows quantitation of proG and PC1 mRNA relative to 18s RNA. Values are means ± SEM (n=3).
4.4 DISCUSSION

In the present study, we hypothesized that expression of proG and PC1 mRNA transcripts are co-regulated in a cAMP-dependent manner in an enteroendocrine L cell line. This hypothesis was made based on several findings. First, we have previously shown that PC1 processes proG to the intestinal PGDPs. Second, PDGP synthesis and secretion and proG gene expression are regulated in a cAMP-dependent manner in primary cultures of fetal rat intestine (36) as well as in GTag-Y cells (37). Third, the PC1 gene contains a CRE in its promoter region, and PC1 gene expression is regulated by agents which increase intracellular cAMP levels (288). Finally, expression of PC1 has been shown to be regulated in parallel to expression of other prohormone substrates, including proinsulin (330,331,333) and POMC (242,314,316), suggesting that PC1 and proG expression may be co-regulated in a similar fashion.

In the present study, we have shown that GLP-1 secretion from GTag-Y cells is regulated by agents that increase intracellular cAMP levels, as well as by an intestinal peptide, GIP, which is known to act through a seven-transmembrane, G protein-coupled receptor (396) to increase intracellular cAMP levels (203,395). Previous studies with GTag-Y cells have also demonstrated that forskolin/IBMX treatment increases GLP-1 secretion (37). The results of the present study indicate that the cAMP-dependent secretory response remains intact in these cells. The experiments in the present study also provide the first evidence that the intestinal peptide GIP can stimulate GLP-1 secretion in these cells. GIP is an endocrine peptide synthesized in the duodenal K cell (397), and is released upon nutrient ingestion (127); as well, its activity as an incretin hormone has been well established (171,176,398). In addition to its effects on the pancreas, GIP also exerts effects on the intestinal L cell. In fetal rat intestinal cell
cultures, GIP increases intestinal PGDP secretion at physiological doses and in a dose-dependent manner (135), indicating that GIP may be an endocrine regulator of the L cell. A novel enteroendocrine loop has been proposed to exist in vivo in the rat intestine, whereby nutrient ingestion increases GIP release from the duodenum, which then acts as a signal to stimulate GLP-1 secretion prior to arrival of nutrients into the ileum (125). The fact that GIP also increased GLP-1 secretion in GTag-Y cells in the present study establishes the GTag-Y cell line as a useful model with which to study regulation of intestinal peptide secretion.

This study is the first to demonstrate the secretion of GLP-2 immunoreactivity from the intestinal L cell in response to cAMP and GIP. Both GLP-1 and GLP-2 showed similar secretory responses, however, there was a two-fold difference in the amount of immunoreactive GLP-2 as compared to GLP-1-x-36NH2 in unstimulated cells, and this difference increased upon treatment with either F/I or GIP. It has been shown that both GLP-1 and GLP-2 are secreted in a 1:1 molar ratio from perfused pig ileum in response to glucose or gastrin-releasing peptide (GRP) (73), therefore indicating that GLP-1 and GLP-2 are secreted in equimolar amounts by the same stimuli. It has been suggested previously that the RIA utilized for GLP-1-x-36NH2 underestimates total immunoreactive GLP-1-x-36NH2 in cell extracts, as the yield of GLP-1-x-36NH2 following HPLC analysis of GTag-Y cell extracts far exceeds the apparent amount loaded onto the column (37) (and Chapter 3). The reason for this phenomenon is unknown, although it can be speculated that some factor present in cell extracts may be interfering with the RIA. Nonetheless, our results indicate that both GLP-1 and GLP-2 are secreted in parallel by the same stimuli. We therefore conclude that the release of both GLP-1 and GLP-2 from GTag-Y cells may be mediated by peptides acting through a cAMP-dependent mechanism.
In contrast to the results previously obtained with GTag-Y cells, in which proG gene transcription was activated in a cAMP-dependent manner (37), we were unable to demonstrate regulation of proG mRNA expression in response to treatment with forskolin/IBMX. These results are all the more puzzling since the cells were treated under the same conditions as in the previous study. The proG gene contains a CRE within its 5'-flanking region (16), and studies utilizing islet cell lines have shown that proG gene expression is enhanced through binding of the CRE binding protein (CREB) to the CRE (34). A similar mechanism may also operate in the intestinal L cell, as stimulation of cAMP levels increases steady-state proG mRNA levels in FRIC cultures (35,36) and enhances activation of proG gene transcription in GTag-Y cells (37). Recent evidence suggests that activation of PC1 gene expression may also occur through a CREB-mediated mechanism. PC1 mRNA levels increased in response to IBMX treatment in neuropeptide-expressing cell lines (288). Additionally, it has been shown that the human PC1 gene contains two CREs (CRE-1 and CRE-2), and that mutation of one or both elements attenuates forskolin-induced transcriptional activity (286). As well, it has been very recently demonstrated that CREB binds to CRE-1 to enhance PC1 promoter activity in neuroendocrine cells (287). Taken together, the results of the above studies provide for a molecular mechanism of coordinate regulation of proG and PC1 gene expression.

However, the results of the present study do not support such an hypothesis.

Since the cAMP-dependent secretory response appears to be intact in the GTag-Y cells utilized in the present study, as indicated by the increases in GLP-1 and GLP-2 secretion, our results suggest the development of a possible defect in the transcriptional component of the PKA pathway of GTag-Y cells. Other cell lines have been shown to be defective in components of the PKA signaling pathway. For example, proG gene
expression in InR1-G9 cells does not respond to increases in intracellular cAMP (32,33). The cAMP response was restored upon transfection of the catalytic subunit of PKA (32,33), thus suggesting that InR1-G9 cells may not express PKA, or express a defective form of PKA. Since we were able to demonstrate a secretory, but not transcriptional, response to cAMP stimulation in GTag-Y cells, we postulate that a defect may lie downstream of PKA, possibly affecting CREB. If CREB is indeed expressed in our GTag-Y cells, it is possible that CREB is not being phosphorylated in response to forskolin treatment, thereby failing to activate proG and PC1 gene transcription. It is well known that phosphorylation of CREB at Ser133 is critical to activation of cAMP-dependent gene transcription (399). Inhibition of CREB phosphorylation through mutation of Ser133 (399), or dephosphorylation of CREB by protein phosphatase 1 (400), abolishes PKA-mediated transactivation of gene expression. Therefore, to assess both the presence of CREB and the phosphorylation of CREB in response to forskolin treatment, immunoblot analysis of GTag-Y cell extracts should be carried out, utilizing anti-CREB and anti-phospho-CREB antibodies to detect both total and phosphorylated CREB. If CREB is phosphorylated on Ser133, then assessment of proper dimerization and binding of CREB to the CRE of the proG and PC1 gene in GTag-Y cells may be required to locate the defect within the PKA-dependent transcriptional apparatus of GTag-Y cells. As well, it has been shown that CREB-binding protein, CBP (401), may be a co-activator of CREB-mediated transcription (402). Expression of CBP in GTag-Y cells can be assessed through immunoblot analysis, and binding of CREB to CBP can be determined by using [32P]CREB to detect CBP.

Another possible explanation for the lack of transcriptional response to cAMP in GTag-Y cells may lie with the expression of other modulatory transcription factors, such as CREM (403) or ATF-1 (404), such that the abundance of CREB-CREM or CREB-ATF-
1 heterodimers relative to CREB homodimers may be increased. Such heterodimers can bind to DNA but cannot mediate a cAMP response (403,405). Indeed, it has been shown that cells which contain a significant amount of CREB homodimers are cAMP-responsive, while cells which express a relatively large number of CREB-ATF-1 heterodimers fail to mount a transcriptional response to cAMP (405). Additionally, the expression of ATF-1 appears to be increased in dedifferentiated cells (406). It is known that GTag-Y cells are dedifferentiated to a certain extent, as they produce large amounts of glucagon (37). Expression of ATF-1 in GTag-Y cells could be assessed through immunoblot analysis, while expression of CREB-ATF-1 heterodimers could be determined through immunoprecipitation studies, utilizing anti-phospho-ATF-1 antibodies for immunoprecipitation and anti-phospho-CREB antibodies for detection. Similar experiments could be carried out to detect CREB-CREM heterodimers, the presence of which also may account for the lack of transcriptional response to cAMP observed in GTag-Y cells.

In summary, we utilized the enteroendocrine cell line GTag-Y to examine the hypothesis that proG and PC1 expression are co-regulated in the intestinal L cell. Both GLP-1 and GLP-2 secretion were increased in a cAMP-dependent manner, indicating that the secretory response was intact in these cells. Additionally, GIP increased both GLP-1 and GLP-2 secretion, thus establishing the GTag-Y cell line as a model of L cell secretory responses. However, we failed to observe a concomitant increase in proG or PC1 mRNA levels, indicating that GTag-Y cells may harbour a defect within a transcriptional component of the PKA pathway. We speculate that the defect may lie within the transcription factor CREB, or that dedifferentiation of this cell line may have allowed for expression of other modulatory transcription factors, thus rendering GTag-Y cells transcriptionally unresponsive to cAMP.
CHAPTER 5: Discussion

5.1 Role of the Prohormone Convertases in Proglucagon Processing

The tissue-specific processing of proG leads to the production of a variety of peptides unique to the pancreas and intestine. Each of the PGDPs within proG are flanked by pairs of basic amino acids, and are liberated through cleavage at these sites. The exception to this rule is the generation of truncated GLP-1, which occurs through processing at a single basic amino acid. The search for the endopeptidases responsible for the processing of prohormones led to the discovery of a family of serine proteases related to the bacterial subtilisin family, the prohormone convertases (PCs). At the time that this project began, the only known convertases were furin, PC1 (also known as PC3) and PC2. The expression of PC1 and PC2 exclusively in neuroendocrine cells and tissues implicated these enzymes in the processing of prohormones. Therefore, the focus of this project was to determine the roles of PC1 and PC2 in the processing of proG. Several correlative approaches have since been utilized that suggest roles for PC1 and PC2 in proG processing. The localization of PC1 with GLP-1 immunoreactive cells in the ileum (358) and the identification of PC2 immunoreactivity in pancreatic A cells (249) implicated PC1 and PC2 in the processing of proG to the intestinal and pancreatic PGDPs, respectively. Stable transfection of different endocrine cell lines with proG showed that oxyntomodulin was produced in AtT-20 cells, which express PC1, and glucagon was produced in GH3 cells, which express PC2 (389). We (64) and others (60,74,75,359) have shown that PC1 mRNA is expressed in cells which process proG to the intestinal PGDPs, while PC2 mRNA is expressed in cells which produce glucagon. In the pancreatic-derived αTC1-6 cell line, PC2 mRNA (60) and immunoreactive PC2 (359) are expressed at high levels, and PC2 has been immunocytochemically co-localized with glucagon within the
same secretory granules (74). However, a transplantable glucagonoma-derived cell line, MSL-G-AN, was reported to process proG to both pancreatic and intestinal PGDPs, but does not express either PC1 or PC2, as determined by Northern blot (357). Therefore, some controversies still exist as to the exact functions of PC1 and PC2 in the tissue-specific processing of proG, highlighting the need for direct studies, as we (77) (and Chapter 3) and others (60,74-76) have carried out.

5.1.1 PC1 and the Production of the Intestinal PGDPs

Most investigators now agree on the role of PC1 in the generation of the intestinal PGDPs. In vitro, recombinant PC1 processes proG to glicentin, oxyntomodulin and GLP-1 (74). Expression of antisense PC1 message in αTC1-6 cells results in a 40% reduction in immunoreactive PC1 levels and correlates with a decrease in glicentin, and to a lesser extent, oxyntomodulin, glucagon and GLP-1 production (76). However, the molecular forms of GLP-1 were not identified, and GLP-2 production was not assessed in either of these studies. In Chapter 2, we showed that v:v:PC1 processes v:v:proG to glicentin, oxyntomodulin, GLP-1\(^{7-36NH_2}\) and GLP-2 in GH\(_3\) and AtT-20 cells, providing the first evidence that PC1 could produce the bioactive form of GLP-1 as well as GLP-2 (77). We concluded that PC1 processes proG to all of the intestinal PGDPs, and could cleave proG at Arg\(^{77}\) to yield the truncated, bioactive form of GLP-1. However, Rouillé et al (75) were unable to generate GLP-1\(^{7-36NH_2}\) by incubating GLP-1\(^{1-36NH_2}\) with recombinant PC1 in vitro, suggesting that another enzyme may be responsible for the production of GLP-1\(^{7-37/36NH_2}\).

In Chapter 3, we show that stable transfection of PC1 into InR1-G9 cells increases the production of the intestinal PGDPs, glicentin, GLP-1\(^{1-36NH_2}\) and GLP-2. However, the production of GLP-1\(^{7-36NH_2}\) was not augmented, suggesting that PC1 cleaves proG to full-
length, but not truncated, GLP-1. Taken together, the results described above implicate PC1 in the processing of proG to the intestinal PGDPs glicentin, oxyntomodulin, GLP-1$^{37-36\text{NH}}_2$ and GLP-2, while the role of PC1 in the production of GLP-1$^{7-37-36\text{NH}}_2$ remains to be clarified. Since PC1 could not cleave GLP-1$^{1-36\text{NH}}_2$ at Arg$^7$ in vitro (75), this also suggests that GLP-1$^{1-36\text{NH}}_2$ may not serve as a precursor for GLP-1$^{7-36\text{NH}}_2$, and may not present the proper conformation required for cleavage by PC1. As well, the actions of PC1 in vitro may not fully mimic the actions of PC1 in vivo. Further studies are required to examine the role of PC1 in the generation of GLP-1$^{7-36\text{NH}}_2$, such as expression of antisense PC1 in intestinal cell lines, the use of specific PC1 inhibitors, or the generation of PC1 knockout mice.

5.1.2 PC2 and the Production of Glucagon

That PC2 may be implicated in the processing of proG to glucagon was first postulated using the pancreatic cell line, αTC1-6 (60). Expression of antisense PC2 in the A cell-derived cell line, αTC1-6, resulted in a decrease in PC2 expression which correlated with a decrease in both glicentin and glucagon production (60). In a later study, this group demonstrated that proG was processed to glucagon in AtT-20 cells stably transfected with PC2 (75). Based on these findings, it was concluded that PC2 was the enzyme which processed proG to glucagon in the A cell of the pancreas. In contrast, Rothenberg et al (76) found that a 90% reduction in immunoreactive PC2 levels in αTC1-6 cells correlated with a decrease in glicentin, but not glucagon, production. These results corroborate those from an earlier study (74), which showed that immunopurified PC2 processed proG in vitro to glicentin, but not glucagon. Therefore, the results obtained from the latter two studies strongly suggest that PC2 processes proG to glicentin, and
may require another enzyme to produce glucagon. As well, the fact that glucagon production could remain unchanged while glicentin production decreased also suggests that glicentin may not be required as an immediate precursor to glucagon. The results described in Chapters 2 and 3 also support the conclusion that PC2 does not play a major role in the processing of proG to glucagon. We have shown that vv:PC2 processes vv:proG to glicentin, but not glucagon, in both GH₃ and AtT-20 cells (77). Furthermore, vv:PC2 was unable to process vv:proG to glucagon in the presence of other known PCs or its molecular chaperone, 7B2 (77). The lack of glucagon production in these experiments could not be attributed to lack of PC2 bioactivity, as endogenous POMC in AtT-20 cells was processed to α-MSH in the presence of vv:PC2, consistent with the role of PC2 in the processing of POMC (318). However, infection of cells with vaccinia virus may cause disruptions within the secretory pathway, thus potentially interfering with the proper compartmentalization of proG and PC2. In order to overcome this problem, pancreatic-derived InR1-G9 cells were therefore utilized to establish a stable in vivo model. Expression of antisense PC2 in these cells reduced both PC2 mRNA and immunoreactive PC2 levels by 60%, and correlated with a decrease in glicentin, but not glucagon, production. To summarize, Rouillé et al (60,75) have concluded that PC2 processes proG to glucagon in the A cell. In contrast, work from our lab (77) (and Chapter 3), together with studies carried out by Rothenberg et al (74,76) indicate that PC2 processes proG to glicentin, and may require another enzyme to produce glucagon. Thus, while PC2 is necessary in the processing of proG to glucagon in the pancreas, it is not clear if the actions of PC2 alone are sufficient. Recently, the generation of mice with a null mutation in the gene encoding PC2 has been reported (407). The PC2⁻ mice are viable and show little or no glucagon production. However, the complete proG
processing profile in these mice has not yet been characterized. Thus, it is not known if ablation of PC2 also resulted in the abolition of glicentin production, as shown previously in antisense studies (60,76) and Chapter 3. Interestingly, histological examination of the pancreas revealed that A cell hyperplasia was evident. These results indicate that PC2 may be important in A cell development as well as in proglucagon processing in the pancreas.

5.1.3 Other Processing Enzymes Which May Function in the Processing of ProG

We have shown that other members of the PC family, namely PACE4, PC5a and PC5b, process proG to glicentin (Chapter 2). However, a number of enzymes belonging to families other than the PC family of serine proteases have also been implicated in the processing of various prohormone substrates, including thiol, aspartyl and metalloendoproteases. The novel cysteine protease, prohormone thiol protease (PTP), is expressed in adrenal chromaffin cells and cleaves proenkephalin amino-terminal to paired basic residues (408). PTP processes proenkephalin with a much greater efficiency than do PC1 and PC2 together (409), although both PC1 and PC2 have been implicated in the processing of proenkephalin in vivo (342). In contrast, POMC appears to be a poor substrate for PTP, and is preferentially cleaved by PC1 and PC2 (409,410). In addition, a 70-kDa aspartic protease, known as POMC-converting activity (PCE) has been identified in secretory granules of the pituitary neurointermediate lobe, and cleaves POMC to yield ACTH and β-endorphin (409). Similarly, the yeast aspartic protease, YAP3p, belongs to the same family as PCE and cleaves POMC to neurointermediate lobe peptides (411). A YAP3-like processing activity has been localized to secretion granules
of pituitary corticotrophs (412), strongly suggesting that this aspartic protease activity plays a role in POMC processing. Finally, a member of the metalloendopeptidase family, N-arginine dibasic convertase (NRD) may also function as a prohormone processing enzyme (413). However, it is not known if any of the above processing enzymes are expressed in the pancreatic A or intestinal L cell. Therefore, whether these enzymes may be candidates for the biosynthesis of the PGDPs remains to be established. Nonetheless, the above studies outline the importance of determining all endogenous processing activities within a cell type in order to provide a mechanism for the post-translational processing of a particular prohormone substrate. Therefore, expression of other families of processing enzymes in the A and L cells should be determined and their effects on proG processing should be examined in vivo in order to clarify the mechanisms underlying the production of the PGDPs, and of glucagon and GLP-1 \( ^{7-36}_{\text{NH2}} \) in particular.

Two arginine-specific serine protease enzymes have been isolated and characterized from intestinal mucosa. One was characterized as membrane-bound (414) and the enzyme activity was estimated to reside in the membrane compartment, although the precise subcellular location was not determined. Interestingly, this protease cleaves a number of prohormone substrates in vitro, including neoendorphin at a single Arg residue. Another endopeptidase was isolated from the secretory granule fraction of rat intestinal mucosa and shown to cleave prosomatostatin at a single Arg to generate SS28 (415,416). It is tempting to speculate that this enzyme activity is equivalent to that of PC1, and that it may also generate GLP-1 \( ^{7-36}_{\text{NH2}} \). However, it is not known if this processing activity is present in proG-expressing L cells. To date, the only processing enzyme known to be localized within the L cell is PC1 (358). Again, as above, all processing activities within the L cell must be examined to determine the enzyme responsible for the
generation of GLP-1$^{7-36\text{NH}_2}$. The three models which are currently available are the fetal rat intestinal cell culture (FRIC) (35), the intestinal-derived STC-1 cells, and the L cell-derived cell line, GTag-Y (37). While FRICs have been shown to be a reliable model of the L cell (135,417), cell type heterogeneity is a problem. As described in Chapter 4, GTag-Y cells, although entirely comprised of L cells, are dedifferentiated and produce large amounts of glucagon in addition to GLP-1$^{7-36\text{NH}_2}$, thereby rendering them unsuitable for the study of processing activities. Thus, approaches to establish cell cultures enriched in the L cell population may be necessary in order to determine the enzyme activity which generates GLP-1$^{7-36\text{NH}_2}$.

5.2 Clinical Aspects of PC1 and PC2 Function

A number of studies have attempted to link abnormalities in prohormone processing with aberrant PC expression. Expression of PC1 and PC2 have been demonstrated in a number of human neuroendocrine tumours (358,418-420), suggesting that detection of PC expression may be utilized as a marker of neuroendocrine tumours, or perhaps in defining the degree of differentiation of tumour cells. Treatment of human small cell lung carcinoids with protease inhibitors results in a decrease in both PC1 and PC2 mRNA levels together with a suppression of cell growth (421). These results suggest that PCs within a given tumour may be a potential target for tumour-suppressing drugs.

Two studies have attempted to correlate mutations in the genes encoding PC1 or PC2 with the abnormal proinsulin processing sometimes observed in NIDDM (422,423). The specific genetic mutations in the PC1 and PC2 genes found could not be associated with abnormal processing of proinsulin, and are thus unlikely to contribute to the development of NIDDM in the subjects studied. However, these findings do not rule out
the possibility that defects in PC1 or PC2 could result in abnormal prohormone processing leading to disorders in hormone production. Indeed, one such example is described below.

Two reports have been published which describe a patient whose phenotype is associated with abnormalities in proinsulin and POMC processing, both of which were attributed to a deficiency in PC1 expression (424,425). The subject was extremely obese in childhood, and exhibited impaired glucose tolerance, hypogonadotrophic hypogonadism, and elevated levels of plasma proinsulin, des-64,65 proinsulin and POMC. Further genetic analysis of PC1 in this subject has revealed the presence of two mutations (425). The first mutation is a Gly$^{483}$-Arg substitution, which causes PC1 to be incorrectly processed, thus rendering the protein biologically inactive. The second mutation causes PC1 mRNA to be aberrantly spliced, such that exon 5 is skipped, resulting in the creation of a premature stop codon within the catalytic domain. Therefore, the subject lacks the active PC1 enzyme, effectively constituting a "knock-out" of PC1. HPLC analysis of the subject’s serum GLP-1 levels has been carried out in our lab, and suggests that both fasting and postprandial GLP-1$^{7-36NH2}$ levels are normal. However, serum proG levels are abnormally high, suggesting the existence of a compensatory mechanism within the intestinal L cell to maintain GLP-1 production. We speculate that L cell proG gene expression may be increased in response to the deficiency in PC1, along with increased expression of an alternate processing enzyme, such as furin, to produce adequate levels of GLP-1$^{7-36NH2}$. Taken together with the subject’s abnormal processing of proinsulin and POMC, this case provides the first clinical evidence of a deficiency in PC1 which causes disorders in prohormone processing.

Additionally, there exists a mouse model of obesity and diabetes due to a genetic
defect in a prohormone processing pathway. The fat/fat mouse harbours a mutation in the gene encoding CPE, leading to impaired proinsulin processing and subsequent development of diabetes and obesity (89). Although hyperproinsulinemia occurs in some cases of NIDDM (426), whether a similar mutation in the CPE gene exists in some humans with NIDDM remains to be established. It has also been shown that the processing of some neurohormones is also impaired in these mice (427), which may explain the obese phenotype. I would also predict that the intestinal PGDPs will be incompletely processed, leaving the C-terminal basic amino acid pairs intact. It is known that the C-terminal end of GLP-1 contains determinants for receptor binding (334,428); it is therefore likely that the presence of the C-terminal Arg^{109}-Arg^{110} of GLP-1 may result in reduced receptor binding, thereby attenuating GLP-1-mediated incretin effects on the B cell, consistent with the phenotype of mice lacking the GLP-1 receptor (172). In Chapter 2, we showed that infection of vv:proG and vv:PC1 resulted in the production of GLP-2 which was incompletely processed, possibly due to the overwhelming of endogenous CPE, and the fact that the C-terminal Lys^{159}Lys^{160} is a poor substrate for CPE (77). It is predicted that the fat/fat mice will also produce incompletely processed GLP-2, with the C-terminal Lys^{159}Lys^{160} intact. Due to the strong structural homology with GLP-1^{7-36NH2}, it is also possible that binding to the GLP-2 receptor may be affected. As GLP-2 is now known to be an intestinal growth factor (211), these mice may exhibit less elaborate intestinal epithelium, or may show decreased post-traumatic intestinal healing.

Very recently, it has been suggested that human monocyte-derived macrophages could be utilized for the clinical genetic diagnosis of convertase disorders (429). This study showed that differentiated macrophages express PC1, PC2 and CPE, and therefore may be used as a non-surgical source for genetic studies. Therefore, genetic defects
leading to alterations in prohormone processing as described above could be studied using macrophages as a source of genetic material.

5.3 Possible Clinical Applications Derived from this Project

The identification of PC1 as the enzyme responsible for the generation of the intestinal PDGPs may have some important clinical applications for the treatment of NIDDM. As mentioned in Chapter 1, administration of GLP-1\(^{7-36\text{NH}_2}\) to NIDDM patients reduces the postprandial glycemic excursion by increasing insulin secretion. However, the route of administration must be optimized in order for the therapy to be viable. Presently, administration of GLP-1 through subcutaneous injection has achieved the greatest success. Treatment through injection reduces patient compliance, however, and oral administration of GLP-1 is not possible due to peptide degradation in the gastrointestinal tract. Therefore, alternative treatment modalities based on an understanding of the role of PC1 in the generation of GLP-1 are suggested.

The development of a B-cell delivery system has been pioneered to establish a treatment for NIDDM patients. Non-pancreatic neuroendocrine cell lines have been engineered to sense glucose and release insulin (430). An endocrine cell type was chosen since an appropriate intracellular compartment would be provided for the proper processing of proinsulin to mature insulin, as well as the targeting of insulin to the regulated secretory pathway. Eventually such an engineered cell, which would effectively represent an artificial B cell, could be encapsulated and introduced to a suitable transplantation site within the body as a treatment for NIDDM. Similarly, cells could also be engineered to overproduce GLP-1\(^{7-36\text{NH}_2}\) as a possible treatment for NIDDM. If PC1 is the enzyme responsible for the production of GLP-1\(^{7-36\text{NH}_2}\), then overexpression of PC1
could ensure production of bioactive GLP-1 from such a system. A similar approach could also be utilized to develop a system which produces GLP-2. Thus, this could possibly represent a first step towards engineering an artificial L cell.

5.4 Future Directions

A number of outstanding questions have been left unresolved by the studies presented in this thesis. These questions are: 1) what are the enzymes responsible for the production of glucagon in the pancreatic A cell; 2) what is the role of PC1 in the generation of GLP-1\(^{7-36}\text{NH}_2\); and 3) are proG and PC1 expression co-regulated in the intestinal L cell?

Although the focus of this thesis was the processing of intestinal proG, the production of pancreatic glucagon was also explored. As discussed in section 5.1, a number of approaches have been utilized to examine the role of PC2 in the production of glucagon, with differing results. Most remarkably, the expression of antisense PC2 message in the \(\alpha\)TC1-6 cell line yielded completely different results in two separate studies (Section 5.1). Although the cell line used in these studies were identical in source, one study revealed that these cells now express PC1 in equimolar amounts to PC2 (76); thus, the phenotype of this cell line is different from normal A cells, which express PC2 and almost no PC1. In order to resolve the question of the role of PC2 in the production of glucagon, islet cells could be sorted using flow cytometry to provide cultures enriched in A cells (431). Sorted A cells could then be transfected with an antisense construct to PC2 to assess the role of PC2 in the production of glucagon in A cells, in lieu of using cell lines. As well, given the discrepancy in results utilizing the same cell line, it is possible that an enzyme activity which processes proG to glucagon is present in one
strain of αTC1-6 cells but not the other. Identification of a unique enzyme could be carried out by differential display of mRNA, followed by cloning of unique cDNA product(s) and purification of the recombinant protein(s). As well, it is possible to determine the expression of other processing enzymes, such as YAP3 or PTP (as discussed in Section 5.1.3), in the A cell. The effects of these novel processing enzymes on proG processing could be examined using *in vitro*, stable transfection and antisense methods as described in Section 5.1.

The role of PC1 in the generation of GLP-1^7-36NH2_ is uncertain, as results from vaccinia virus (77), *in vitro* (75) and stable transfection studies (Chapter 3) have differing results. In order to resolve this question, the intestinal cell line GTag-Y, which expresses PC1 and processes proG to GLP-1^7-36NH2_ (Chapter 4), could be utilized for pulse-chase experiments in order to establish precursor-product relationships between the full-length and truncated forms of GLP-1. Additionally, GTag-Y cells could be transfected with an antisense construct to PC1 and used for pulse-chase experiments in order to establish the role of PC1 in the production of GLP-1^7-36NH2_.

Finally, a new model could be developed for the study of proG and PC1 co-regulation. The existing models include FRICs, which are composed of a heterogeneous population of cells, and STC-1 and GTag-Y cell lines, which exhibit abnormal transcriptional responses to cAMP (52) (and Chapter 4). The most effective means by which PC1 and proG co-regulation can be studied may be through L cell-enriched primary cell cultures. Centrifugal elutriation has been used to enrich the L cell population of canine intestinal L cells by up to 20-fold (432). However, neither dog proG nor PC1 have yet been described, while it is known that both rat proG and PC1 contain CREs in their respective 5'-flanking regions. Therefore, cells derived from rat intestine could be enriched
in L cells through centrifugal elutriation. The resulting cell cultures could be employed in order to study PKA-dependent regulation of proG and PC1 mRNA levels, as well as co-regulation of PGDP and PC1 protein levels. Additionally, this method could be used to purify intestinal cells obtained from *in vivo* experiments designed to increase intestinal proG gene expression. Such experiments would include changes in nutrient status or inducing intestinal adaptation. I predict that changes in proG mRNA levels will be paralleled by changes in levels of PC1 mRNA transcripts, and that both intestinal PGDP production and PC1 protein levels will increase upon stimulation of PKA, nutrient ingestion or intestinal adaptation.
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