Discovery, characterization, and clinical development of the glucagon-like peptides

Daniel J. Drucker,[1] Joel F. Habener,[2] and Jens Juul Holst[3]

[1]Lunenfeld-Tanenbaum Research Institute, Mt. Sinai Hospital, University of Toronto, Toronto, Ontario, Canada. [2]Laboratory of Molecular Endocrinology, Massachusetts General Hospital, Harvard University, Boston, Massachusetts, USA. [3]Novo Nordisk Foundation Center for Basic Metabolic Research, Department of Biomedical Sciences, University of Copenhagen, Copenhagen, Denmark.

The discovery, characterization, and clinical development of glucagon-like-peptide-1 (GLP-1) spans more than 30 years and includes contributions from multiple investigators, science recognized by the 2017 Harrington Award Prize for Innovation in Medicine. Herein, we provide perspectives on the historical events and key experimental findings establishing the biology of GLP-1 as an insulin-stimulating glucoregulatory hormone. Important attributes of GLP-1 action and enteroendocrine science are reviewed, with emphasis on mechanistic advances and clinical proof-of-concept studies. The discovery that GLP-2 promotes mucosal growth in the intestine is described, and key findings from both preclinical studies and the GLP-2 clinical development program for short bowel syndrome (SBS) are reviewed. Finally, we summarize recent progress in GLP biology, highlighting emerging concepts and scientific insights with translational relevance.

The endocrine activity of the gastrointestinal tract has been studied for more than a century, with gut hormones such as secretin emerging from the seminal studies of Bayliss and Starling (1). The concept that the gut also controlled pancreatic islet secretions was supported by experiments demonstrating that administration of crude gut extracts lowered blood glucose in animals. The development of the insulin radioimmunoassay enabled the description of the incretin effect, namely that glucose administered into the gut potentiated insulin secretion to a greater extent than isoglycemic stimulation of insulin secretion achieved through i.v. glucose administration. The first incretin hormone, glucose-dependent insulinotropic polypeptide (GIP), was isolated by John Brown in the 1970s (2). Here, we describe the discovery and characterization of the second incretin hormone, GLP-1. We highlight 3 decades of science from multiple laboratories supporting the development of GLP-1– and GLP-2–based therapies. GLP-1 is now used for the treatment of type 2 diabetes (T2D) and obesity, whereas GLP-2 is used for the therapy of short bowel syndrome (SBS).

Discovery of GLP-1

Although GIP was isolated through classical peptide purification and protein sequencing methodology, the discovery of the GLP-1 sequence stemmed from the application of recombinant DNA approaches developed in the laboratories of Stanley Cohen, Paul Berg, and Herb Boyer in the early 1970s. This remarkable new technology allowed for a rapid and accurate prediction of the amino acid sequences of proteins by the decoding of the nucleotide sequences of cloned recombinant cDNA copies of messenger RNAs. The Habener lab utilized this technology to elucidate proglucagon amino acid sequences from cDNAs and genes isolated from anglerfish in the early 1980s (3–5) and the rat proglucagon cDNA and gene sequences followed shortly thereafter (refs. 6, 7, Figure 1, and Figure 2). Corresponding proglucagon sequences from hamster, bovine, and human were identified by Graeme Bell and others in the early 1980s (8–10). These sequences revealed that glucagon and related GLP sequences were encoded by larger protein precursors, termed preprohormones (Figure 2). The anglerfish preproglucagons (Figure 2A), isolated and characterized by Lund and Goodman (3–5), were interesting as there were two different cDNAs encoded by separate (nonallelic) genes and they each contained a glucagon-related sequence, in addition to glucagon. The two anglerfish glucagon–related peptides resembled GIP, a glucoincretin hormone released from the gut into the circulation during meals, subsequently shown by Dupre and Brown in 1973 to augment glucose-dependent insulin secretion (11). Unlike the two anglerfish preproglucagons, each of which harbored glucagon and a single glucagon-related peptide, the mammalian preproglucagons all contained glucagon and two additional glucagon-related peptides, designated GLP-1 and GLP-2 (Figure 2B). Notably, the corresponding amino acid sequences of the GLP-1s in the four mammalian species were identical (12), with conservation of sequence implying as yet unknown but potentially important biological actions of GLP-1.

Collectively, these findings further supported the evolving notion at the time that small peptide hormones are synthesized in the form of larger prohormones and that the final bioactive peptides are formed posttranslationally by selective enzymatic cleavages from the prohormones (Figure 2B). Earlier studies

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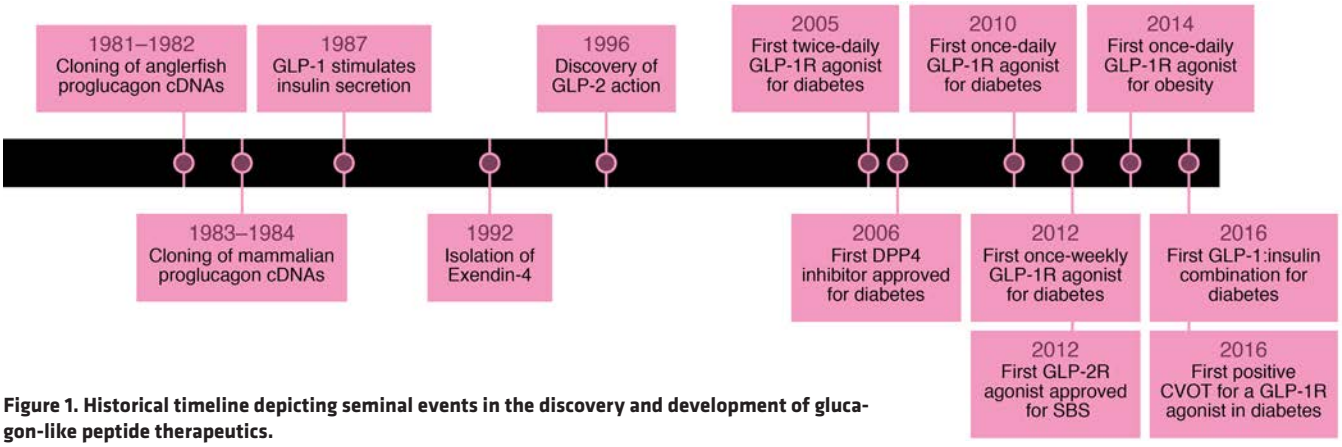

Figure 1. Historical timeline depicting seminal events in the discovery and development of glucagon-like peptide therapeutics.

had demonstrated that insulin and parathyroid hormone are synthesized as preprohormones. The hydrophobic amino terminal sequences, termed the pre- or signal sequences, are cleaved from the nascent polypeptide chains during their translation on ribosomes, leaving the prohormones as the precursor of the peptide hormones. The A and B chains of insulin and parathyroid hormone were found to be cleaved from their respective prohormones by the actions of specific endopeptidases, prohormone convertases, which cleave proteins at the sites of two consecutive basic amino acids, arginine and lysine (13). These earlier findings of prohormone convertase specificity were applied to deduce potential cleavage sites in proglucagon.

Structure-activity properties of GLP-1

Examination of the amino acid sequence of proglucagon initially presented a conundrum regarding the processes by which potentially bioactive GLP-1 peptides might be liberated from the prohormone (Figure 2B). In keeping with the rule that bioactive peptides are cleaved from prohormones at sites consisting of two basic amino acids (13), several investigators initially predicted that bioactive GLP-1 would be a peptide of 37 amino acids beginning with histidine and ending in glycine, GLP-1(1-37). However, further inspection of the prohormone sequences revealed a second single basic amino acid followed by histidine residing 6 amino acids carboxyl-proximal to the first histidine, predicting a GLP-1 peptide of 31 amino acids, GLP-1(7-37). Further, at the carboxyl-terminal region of the putative GLP-1 peptide resides a sequence RGRR predicting a prohormone convertase–directed cleavage site followed by an amidation of the penultimate arginine by a peptidylglycine α-amidating monooxygenase (14) resulting in peptides of 36 and 30 amino acids, $GLP\text{-}1(1\text{-}36)_{amide}$ and $GLP\text{-}1(7\text{-}36)_{amide}$.

The availability of the amino acid sequences of GLP-1 and GLP-2 obtained from the decoding of the nucleotide sequences based on the genetic code allowed for the chemical synthesis of the predicted peptides, examination of their biological activities, and preparation of peptide-specific antisera. Daniel Drucker joined the Habener laboratory in the summer of 1984, with the original intent of studying the molecular control of thyroid hormone biosynthesis. However, the thyroid group, led by Bill Chin, was decamping for the Brigham and Women's Hospital, and Daniel was assigned to work on the proglucagon gene. To understand whether proglucagon might be processed to yield multiple proglucagon-derived peptides (PGDPs), including GLP-1 and GLP-2, Drucker transfected a proglucagon cDNA expression vector into fibroblasts, pituitary cells, and islet cells (15). Although minimal processing was observed in fibroblasts, immunoreactive GLP-1 and GLP-2 was detected by chromatography and radioimmunoassays in medium and extracts from transfected pituitary and islet cells (15). Application of similar chromatography and radioimmunoassay techniques in studies of rat (16), pig (17), and human (18) tissues revealed distinct profiles of PGDPs in pancreas and gut (Figure 3). Of note, in addition to glucagon, the pancreas contained a large peptide with immunoreactive determinants for both GLP-1 and GLP-2 (but not glucagon), consistent with incompletely cleaved proglucagon (major proglucagon fragment [MPGF]) (Figure 3, A and C, and ref. 19). In contrast to findings in pancreas, immunoreactive GLP-1 peptides detected in gut extracts consisted entirely of smaller peptides (refs. 15 and 16, and Figure 3, B and C). These findings indicated that the cleavage of proglucagon into small GLP-1–immunoreactive peptides was more efficient in the gut compared with pancreas. These observations were also consistent with the incretin concept in which, in response to oral nutrients, glucoincretin hormones such as GIP (and subsequently GLP-1) originate from the gut and not the pancreas.

Svetlana Mojsov in the Habener laboratory detected glycine-extended and arginine-amidated isoforms of GLP-1 — as well as both the amino-terminally extended peptides GLP-1(1-37) and $GLP\text{-}1(1\text{-}36)_{amide}$ and the amino-truncated peptides GLP-1 (7-37) and $GLP\text{-}1(7\text{-}36)_{amide}$ — in extracts of pancreas (Figure 3A). Nevertheless, the abundance of these peptides was much greater in the gut (20).

Bioactivities of the GLP-1 peptides

The earliest studies of the bioactivities of GLP-1(1-37) were indecisive. One study found that the 37 amino acid peptide activated adenylyl cyclase in membranes prepared from rat pituitary and hypothalamus (21), whereas another study failed to detect any effects of the peptide on glucose and insulin in cortisone-treated rabbits

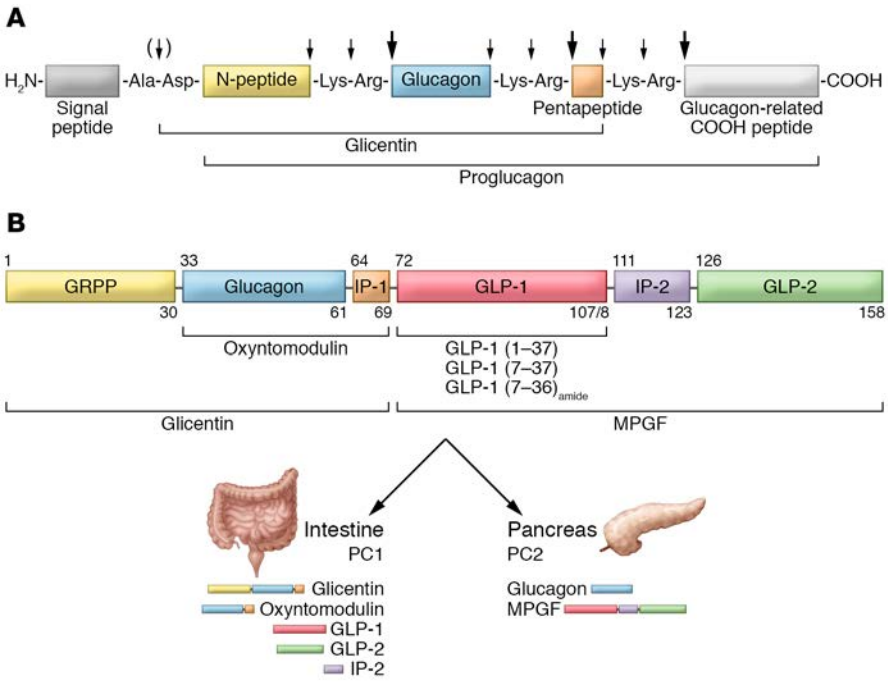

Figure 2. Structure and processing of anglerfish and human proglucagon. Representation of the structures of proglucagon cDNAs from Anglerfish (**A**) and Human (**B**), with tissue-specific liberation of individual proglucagon-derived peptides in pancreas or intestine. PC1, prohormone convertase 1; PC2, prohormone convertase 2; MPGF, major proglucagon fragment. Arrows in **A** represent sites of cleave by prohormone convertase enzymes.

(22). To determine whether the shorter GLP-1 isoforms were bioactive, Drucker incubated truncated forms of GLP-1 with multiple cell lines, examining cell growth, gene expression, and signal transduction. Remarkably, insulin-producing islet cells responded to N-terminally truncated forms of GLP-1, exhibiting increased levels of cyclic AMP accumulation within minutes of exposure to 50 pM–5 nM GLP-1(7-37) (Figure 4A). In contrast, neither glucagon, GLP-1(1-37), or GLP-1(1-36)–NH2 increased cAMP accumulation at these concentrations (23). GLP-1(7-37) also increased the levels of insulin mRNA transcripts and stimulated insulin secretion at 25 mM but not 5.5 mM glucose in studies with isolated islet cell lines (Figure 4, B and C, and ref. 23). In contrast, neither glucagon nor GLP-2 induced insulin gene expression. Furthermore, GLP-1 (7-37) had no effect on levels of angiotensin gene expression in the insulinoma cell line and did not change levels of glucagon, prolactin, and corticotropin mRNAs in islet or pituitary cell lines (23). Hence, these findings (Figure 4, A–C) published in 1987 (23) established that GLP-1(7-37) directly augments glucose-dependent insulin biosynthesis and secretion from ß cells.

The demonstration that GLP-1 directly increased cAMP levels provided conditional evidence for the existence of a Gs protein-coupled receptor in β cells. In studies of insulin secretion using the isolated perfused pancreas, Mojsov and Weir demonstrated that GLP-1(7-37) and not GLP-1(1-37) stimulated insulin secretion at concentrations as low as 50 pM (ref. 24 and Figure 5, A–C). Likewise, as outlined below, Jens Holst and colleagues showed that luminal glucose stimulated GLP-1 secretion from the perfused intestine (Figure 5D), and doses from 500 pM to 5 nM GLP-1(7-36)$_{amide}$ stimulated insulin secretion in the perfused pig pancreas (Figure 5E and ref. 25). Thus, it turned out that a cleavage in proglucagon at the single basic amino acid, arginine, and not the double basic amino acids, generated the active GLP-1 peptides, GLP-1(7-37) and GLP-1 (7-36)$_{amide}$ (Figure 5C). First in man studies reported in December 1987 by Kreymann and Bloom (26) rapidly established the insulinotropic actions of GLP-1(7-36)$_{amide}$ in human subjects. Although numerous studies have demonstrated that the amino-terminally truncated forms of GLP-1, GLP-1(7-37), and GLP-1(7-36)$_{amide}$ are active glucoregulatory hormones, no compelling bioactivities for the extended forms, GLP-1 (1-37) and GLP-1(1-36)$_{amide}$, have yet been determined. Furthermore, no distinctive biological activities have been attributable specifically to the amidated forms of GLP-1.

The view from Denmark

In Copenhagen, Jens Holst and colleagues were interested in the incretin effect and were studying the condition of postprandial reactive hypoglycemia after gastric surgery (27). This type of hypoglycemia was clearly hyperinsulinemic, yet the signal for insulin secretion was unknown. Looking for possible candidates, they were inspired by Lise Heding's work on glucagon and her identification of the immunological differences between gut and pancreatic glucagon (28). Knowing that glucagon would stimulate insulin secretion, they were interested in the numerous cells in the gut that produce immunoreactive glucagon (29). Eventually, this work led to the identification of glicentin and oxyntomodulin (Figure 2B), which both contain the full glucagon sequence, explaining the immunoreactivity in the gut (30–32).

Having identified all of the molecular components of glicentin also in the pancreas (33), they proposed that glicentin represents at least part of a common gut and pancreatic glucagon precursor, which undergoes differential processing in the two tissues, a hypothesis subsequently confirmed through identification of the human proglucagon gene by Graeme Bell and colleagues (7). However, it was also clear that proglucagon was larger than glicentin, and the interest focused on peptides contained within the MPGF representing the remainder of proglucagon (minus glicentin) (34). The early work decoding the anglerfish proglucagon cDNA by Lund and Habener (3) followed by elucidation of the hamster proglucagon cDNA by Graeme Bell (9) supported a hypothesis that cleavage of MPGF might result in liberation of the GLPs. The Holst group quickly developed radioimmunoassays for GLP-1 and GLP-2 to test this hypothesis. To their excitement, they found that MPGF was indeed differentially processed in the gut, but not in the pancreas, to yield two GLPs (ref. 17 and Figure 3C). However, using the perfused pancreas preparation, they soon realized that neither of the two GLPs used in these

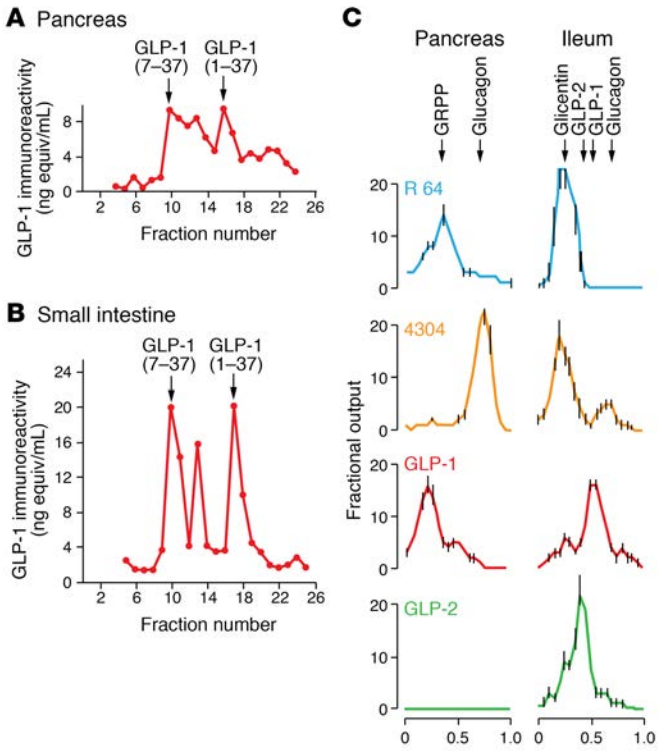

Figure 3. Processing of proglucagon and glucagon-like peptides in the pancreas and intestine. Detection of immunoreactive forms of GLP-1 in extracts from rat pancreas (**A**) and intestine (**B**) as adapted from ref. 16. Characterization of proglucagon-derived peptide immunoreactivity secreted from perfused pig pancreas and intestine (**C**) using peptide-specific antisera reveal tissue-specific posttranslational processing of the PGDPs, as outlined in ref. 17.

studies had any effect on pancreatic hormone secretion. They therefore decided to isolate the naturally occurring hormone from porcine and human and gut extracts, and they found that the naturally occurring peptide was a truncated from of GLP-1 representing proglucagon (aa 78-108) (Figure 5C) and subsequently found to be amidated, corresponding to proglucagon $78\text{-}107_{amide}$ (35). Importantly, this hormone was potently insulinotropic (Figure 5E and ref. 25), so they had also described a new incretin hormone in 1987; however, they wondered whether GLP-1 was more interesting than the already known incretin GIP, which exhibited a diminished effect on insulin secretion in patients with T2D (36). They soon found, using the perfused pancreas, that GLP-1 — in contrast to GIP — also powerfully inhibits glucagon secretion (37). Eventually, they demonstrated that during infusions of physiological amounts of GLP-1 into humans, insulin secretion would be stimulated and glucagon secretion inhibited, resulting in a decrease in hepatic glucose production (38). However, the effect was self-limiting, with the insulin-stimulating activity attenuated as plasma glucose levels started to fall, limiting the fall to 0.5–1 mmol/l.

At that time, the Copenhagen group realized that GLP-1 was extremely interesting and, in further studies, demonstrated that it strongly inhibited gastric motility and gastric and pancreatic exocrine secretion (39), consistent with an important role for this hormone as a regulator of upper gastrointestinal function. They also demonstrated that infusions of GLP-1 in humans inhibited appetite and food intake, actions subsequently exploited in the clinic to treat obesity (40). In studies published in 1993 by Michael Nauck and colleagues in Göttingen, i.v. infusion of GLP-1 completely normalized severely elevated fasting glucose concentrations in patients with long-standing T2D as a consequence of the actions of GLP-1 to stimulate insulin and inhibit glucagon secretion (41). Although GLP-1 clearly had therapeutic potential, s.c. injections of GLP-1 were disappointingly ineffective (42). The explanation was an extremely rapid metabolism and inactivation of GLP-1. With inspiration from Rolf Mentlein in Kiel, Holst and Deacon showed that the GLP-1 molecule was cleaved by the enzyme dipeptidyl-peptidase-4(DPP-4) in vivo and that inhibitors of this enzyme could completely protect the molecule (43). In fact, the circulating half-life of GLP-1 was only 1.5–2 minutes in human subjects with diabetes, and they proposed that inhibitors of DPP-4 could maintain higher levels of intact active endogenous GLP-1 for therapeutic purposes (44). Subsequent studies soon demonstrated that DPP-4–resistant GLP-1 analogues were longer-acting than native GLP-1 (45). Furthermore, inhibitors of DPP-4 completely prevented the breakdown of GLP-1 in the circulation and amplified the insulinotropic actions of GLP-1 (46). This exciting development, presented in a Perspectives article in *Diabetes* in 1998 (47), was soon followed by the development of clinically useful inhibitors, first vildagliptin and subsequently sitagliptin.

It remained to be understood whether GLP-1 receptor agonists would actually be useful for clinical diabetes therapy or whether tachyphylaxis would develop upon chronic administration. The group in Copenhagen administered synthetic GLP-1 by constant s.c. infusion for 6 weeks to a group of individuals with long-standing T2D (48). Fortunately, no tachyphylaxis was observed; GLP-1 therapy reduced fasting and mean plasma glucose by 4.3 and 5.5 mmol/l; glycated hemoglobin by 1.3 %; and body weight by 2 kg. Moreover, insulin sensitivity and β cell function, assessed by clamp studies, greatly improved. Importantly, no limiting side effects were recorded (48), providing proof of concept in 2002 for GLP-1 therapy in subjects with T2D. It was now clear that GLP-1–based therapies had tremendous potential.

The Toronto perspective: the physiology of GLPs and discovery of GLP-2

Building on the availability of cloned proglucagon gene sequences in the Habener lab (6), Daniel Drucker and Jacques Philippe pursued the analysis of the molecular control of islet α cell proglucagon gene expression in the mid 1980s (49, 50). Upon returning to Toronto in 1987, Drucker extended these studies to examine proglucagon gene expression in the intestine

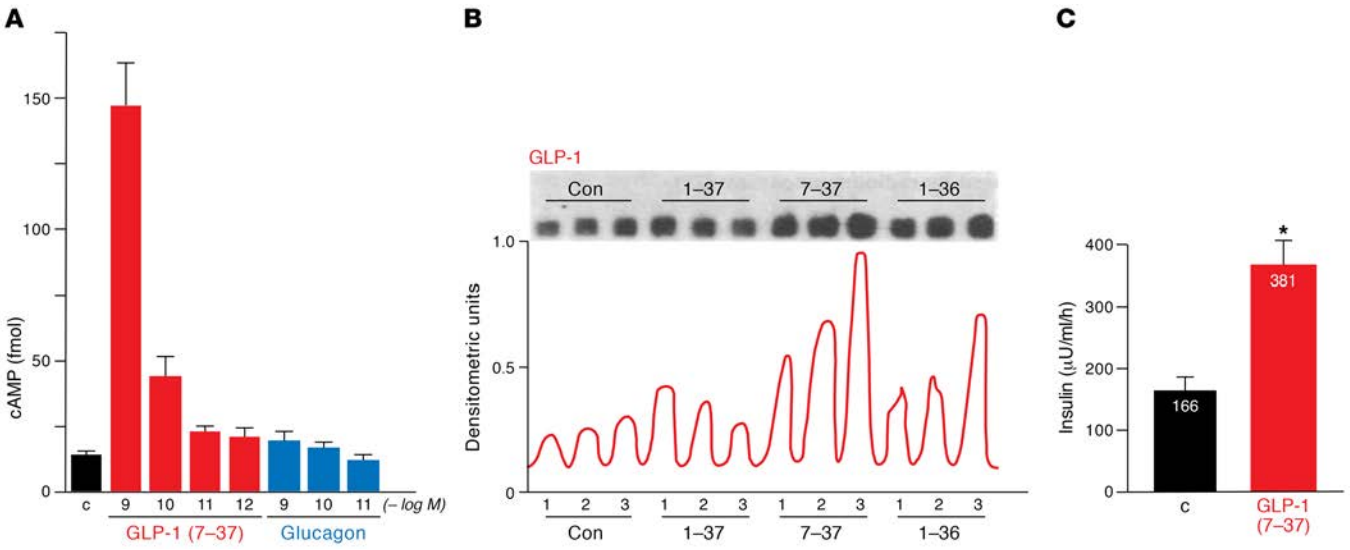

Figure 4. GLP-1 directly stimulates signal transduction and glucose-dependent insulin secretion in islet cells. GLP-1(7-37) directly stimulates cyclic AMP accumulation (**A**), insulin gene expression (**B**), and glucose-dependent insulin secretion (**C**) in a rat insulinoma cell line, as adapted from ref. 23.

and CNS. He isolated human neonatal brainstem cDNAs encoding proglucagon, which exhibited an identical sequence to that described for islet proglucagon (51). Molecular cloning of the rat intestinal proglucagon cDNA similarly revealed an open reading frame identical to that elucidated for the rat pancreatic islet proglucagon cDNA (52). Moreover, in studies carried out in collaboration with Patricia Brubaker, forskolin, cholera toxin, and dibutyryl cyclic AMP increased the synthesis and secretion of intestinal PGDPs from primary cultures of rat intestinal cells (52). At the time, there were no differentiated GLP-1–secreting enteroendocrine cell lines suitable for studies of intestinal proglucagon gene expression. Accordingly, Ying Lee, a fellow in the Drucker laboratory, generated a transgenic mouse expressing the SV40 T antigen cDNA under the control of the proglucagon gene promoter. This transgenic mouse reproducibly developed GLP-1–secreting enteroendocrine tumors of the colon (53), enabling isolation of the first differentiated GLP-1–producing enteroendocrine L cell line in 1992, designated GLUTag cells. GLUTag cells were easily propagated ex vivo; secreted immunoreactive GLP-1, glicentin, oxyntomodulin, and GLP-2 in response to cyclic AMP analogues (54, 55); and resembled primary cultures of nonimmortalized gut endocrine cells in regard to their response to a battery of secretagogues (56).

Two unexpected observations were made during isolation of GLUTag cells. First, mice harboring s.c. GLUTag cell tumors exhibited a marked reduction of pancreatic islet α cell mass (57). Second, mice with s.c. GLUTag, InR1-G9, or RIN1056A glucagon-producing tumors all exhibited marked enlargement of the small bowel. These findings led Drucker to reinvestigate the link between glucagon-producing tumors and gut growth, first reported in a human subject studied at the Hammersmith hospital in 1970 by Dowling and colleagues in London (58). A series of simple experiments from the Drucker lab published in 1996 identified GLP-2 as the PGDP with the most potent intestinotrophic activity in mice (58). Remarkably, although immunoreactive GLP-2 had been detected in intestinal extracts of various species (refs. 16, 59, and Figure 3), no previous biological activity had yet been identified for GLP-2 in vivo.

The actions of GLP-2 to stimulate small bowel growth were rapid, detectable within days, and associated with increased crypt cell proliferation (58). Surprisingly, when similar doses of GLP-2 were administered to rats, intestinal growth was not significantly increased, although an increase in crypt plus villus height was observed (60). With hindsight, these findings reflected the importance of DPP-4 for the degradation of GLP-2, more evident in rats than in mice. Subsequent studies in the Drucker lab demonstrated that native GLP-2 robustly increased small bowel growth in Fischer 344 rats with an inactivating mutation in the *Dpp4* gene (60). Furthermore, a GLP-2 analogue with a single amino acid substitution [Gly2]-GLP-2 exhibited substantial resistance to DPP-4 cleavage and robust intestinotrophic activity in normal rats in vivo (60). Hence, the importance of DPP4 for cleavage of both GLP-1 and GLP-2 became evident very early in the study of the GLPs.

The identification of GLP-2 as an intestinal growth factor spurred a series of experiments examining the actions of GLP-2 in the context of experimental gut injury. GLP-2 administration was generally associated with preservation of gut mucosal structure and function in the setting of chemical, radiation, or surgically induced intestinal injury in preclinical studies (61–63). Notably, GLP-2 also rapidly increased nutrient absorption in normal rodents (64) and in animals with surgical gut resection mimicking SBS (65). Excitingly, the findings in animals were soon extended to humans with SBS. In a pilot study carried out around 2000–2001, Jeppesen and colleagues demonstrated that native GLP-2 administered twice daily for 35 days increased nutrient absorption, energy absorption, and weight gain in human subjects with SBS (66). These findings, namely expansion of intestinal mucosal surface area coupled with

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