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The Glucagon-Like Peptides

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I. Introduction

IT HAS been 15 yr since the initial discovery of the glucagon-like peptides (GLPs) as potential bioactive peptides encoded in the preproglucagon gene. The GLPs and glucagon are formed by alternative tissue-specific cleavages in the L cells of the intestine, the α-cells of the endocrine pancreas, and neurons in the brain. Glucagon-like peptide-1 (GLP-1) is now known to be a potent glucose-dependent insulinotropic hormone, which has important actions on gastric motility, on the suppression of plasma glucagon levels, and possibly on the promotion of satiety and stimulation of glucose disposal in peripheral tissues independent of the actions of insulin. As a consequence of these properties, GLP-1 is under investigation as a potential treatment of diabetes mellitus. GLP-2 was recognized only recently to have potent growth-promoting activities on intestinal epithelium.

The interest in the GLPs has grown exponentially. By 1988 there were 170 publications describing the properties of the GLPs. Five years later this number grew to 426 and currently (1999) more than 1,000 publications appear in the database of the National Library of Medicine (PubMed).

Since the last comprehensive review of GLP-1 appeared in *Endocrine Reviews* in 1995 (1), many new developments have occurred and are described in this review. The purpose of this article is to emphasize the newer and what are perceived to be the more current and important aspects of the biology of the GLPs. For additional information and references, the reader is referred to several informative earlier reviews (1-12).

II. History of the Incretin Concept: Discovery of Gastric Inhibitory Polypeptide

As a result of their discovery of secretin in 1902, Bayliss and Starling (13) speculated that signals arising from the gut after ingestion of nutrients might elicit pancreatic endocrine

responses and affect the disposal of carbohydrates. In 1906 Moore *et al.* (14) postulated that the duodenum produced a 'chemical excitant' for pancreatic secretion and attempted to treat diabetes by injecting gut extracts. Zunz and Labarre (15, 16) pursued this factor and prepared an intestinal extract free of secretin activity that was able to produce hypoglycemia in dogs. Labarre (16) introduced the term 'incretin' to describe the humoral activity of the gut that might enhance the endocrine secretion of the pancreas (16). Although other investigators also reported the presence of hypoglycemic factors in duodenal extracts (17-20), Loew and colleagues (21) were unable to lower blood glucose levels in dogs with extracts of dog or hog intestinal mucosa obtained by a number of methods. Although these later extracts were tested only in fasting animals, after this report, interest in isolating an intestinal hypoglycemic factor declined.

The development of a reliable RIA for insulin in the 1960s by Yalow and Berson (22), which allowed measurements of the circulating levels of this hormone, renewed interest in the search for incretins. It was demonstrated by both immunoassay (23, 24) and bioassay (25, 26) that the action of glucose on the pancreas could not account completely for the insulin response observed in the blood. These reports demonstrated that iv glucose administration resulted in a lower plasma insulin response than when given by intrajejunal infusion, even though lower blood glucose levels were achieved by the later (Fig. 1). Perley and Kipnis (27) estimated the alimentary component to be close to 50% by subtracting from the insulin secretory response seen after oral glucose that insulin response obtained with the infusion of iv glucose, which duplicated the oral blood glucose profile.

In 1969, Unger and Eisentraut (28) named the connection between the gut and the pancreatic islets the 'enteroinsular axis.' Creutzfeldt (29) suggested that this axis encompasses nutrient, neural, and hormonal signals from the gut to the islet cells secreting insulin, glucagon, somatostatin, or pancreatic polypeptide (Fig. 2). Furthermore, Creutzfeldt (29) defined the criteria for fulfillment of the hormonal or incretin part of the enteroinsular axis as: 1) it must be released by nutrients, particularly carbohydrates, and 2) at physiological levels, it must stimulate insulin secretion in the presence of elevated blood glucose levels.

One hormone that clearly fits the requirements to be an incretin is glucose-dependent insulinotropic polypeptide (GIP). GIP was originally isolated as an 'enterogastrone,' or hormone secreted in response to fat or its digestive products in the intestinal lumen that inhibits gastric acid secretion (30). Brown and colleagues (31-33) isolated GIP from impure preparations of cholecystokinin (CCK) that contained acid-inhibitory activity using the canine Heidenhain pouch as a bioassay. GIP was shown to be a potent inhibitor of gastric acid and pepsin secretion and was thus originally named 'gastric inhibitory polypeptide' (34, 35). Earlier, Dupré and Beck (26) had demonstrated that a crude preparation of CCK also possessed insulinotropic activity. In 1972, Rabinovitch and Dupré (36) found that this insulinotropic action could be removed by further purification of the CCK. This observation resembled the loss of the acid-inhibitory activity reported previously by Brown and Pederson (33) during the purification of GIP from CCK and led Dupré *et al.* (37) to the hypothesis that GIP may also possess insulin-releasing capabilities. In 1973, Dupré *et al.* (37) demonstrated that a purified preparation of porcine GIP infused intravenously in humans in concert with glucose stimulated the release of significantly greater quantities of immunoreactive insulin than when the same dose of glucose was administered alone. The insulin response was sustained for the duration of the GIP infusion and was not observed during the euglycemic state (37). The glucose-dependent nature for the insulinotropic activity of GIP was later demonstrated *in vivo* in dogs (38) and humans (39) and in the perfused rat pancreas (40). Furthermore, GIP released in response to the oral ingestion

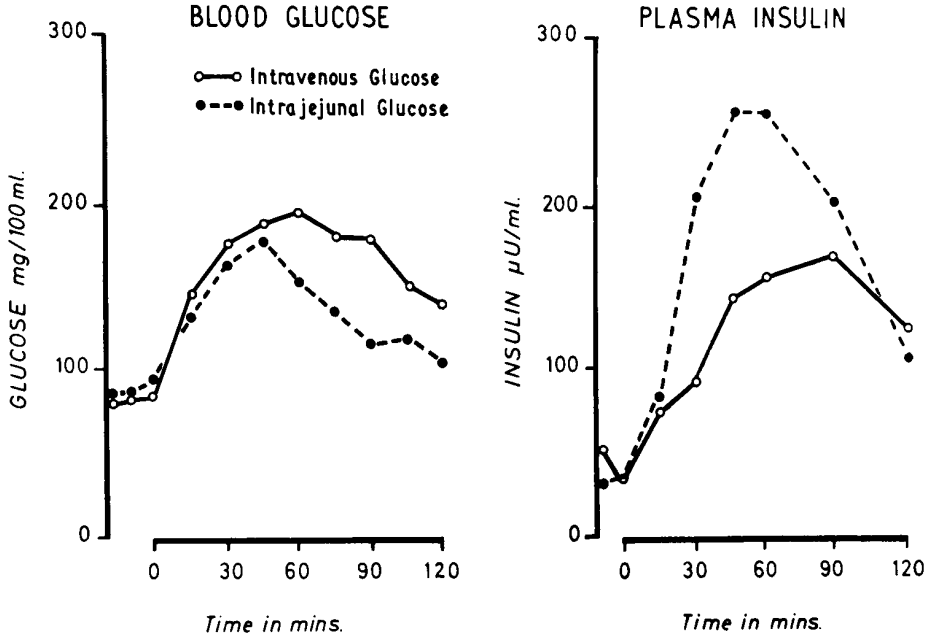

FIG. 1. Demonstration of the incretin concept. Blood glucose and insulin responses after either intravenous or intrajejunal glucose infusion in normal subjects. Although plasma glucose levels after intravenous glucose infusion were higher than those after intrajejunal glucose infusion, the latter generated a larger insulin response. Based on these results, McIntrye *et al.* (23) suggested that a humoral substance was released from the jejunum during glucose absorption, acting in concert with glucose to stimulate insulin release from pancreatic β-cells. [Reproduced with permission from N. McIntyre *et al.*: *Lancet* 2:20-21, 1964 (23) © The Lancet Ltd.].

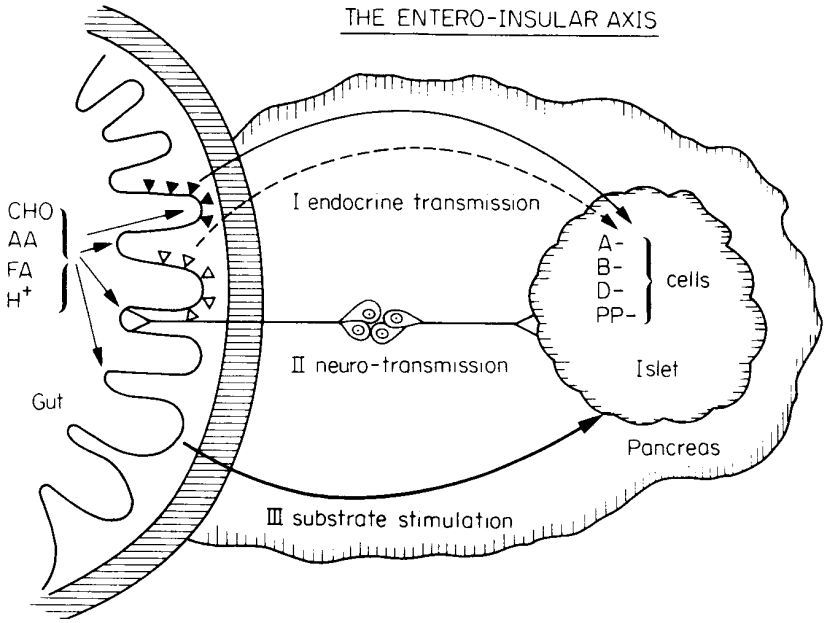

FIG. 2. The enteroinsular axis. After ingestion of nutrients, hormone secretion from different cell types of the pancreatic islets [A (α), B (β), D (δ), PP] may be modified by one or more modalities of: I, endocrine transmission; II, neurotransmission; and III, direct substrate stimulation. [Reproduced with permission from W. Creutzfeldt: *Diabetologia* 16:75-85, 1979 (29) © Springer-Verlag].

of fat yielded no increase in plasma insulin levels unless intravenous glucose was also administered (41-43). The glucose dependency of GIP-stimulated insulin secretion appeared to provide an important safeguard against inappropriate stimulation of insulin release during a high-fat, low-carbohydrate meal. The recognition of this additional important physiological function of GIP led to the alternate designation glucose-dependent insulinotropic polypeptide (GIP) (44).

In accordance with the roles of GIP as an enterogastrone and an incretin, immunoreactive GIP cells have been located in the upper small intestine of ruminants (45), humans, pigs, dogs (46), and rats (47). In the gastrointestinal tract of dog and man, immunoreactive GIP is present in cells predominantly in the midzone of the duodenal villi and, to a lesser extent, in the jejunum (48). Levels of GIP rise several fold shortly after ingestion of a meal containing fat (41-43) or glucose (38, 49, 50). It appears glucose may act directly at the level of the GIP-secreting K cells to stimulate GIP release (51, 52).

Studies employing GIP antisera to immunoneutralize endogenous GIP indicated that intestinal hormones other than GIP contribute substantially to the incretin effect (53, 54). These findings were supported by the observation that insulinotropic activity remained in intestinal extracts after removal of GIP by immunoadsorption (55). Finally, a major contribution to the incretin effect from the lower gastrointestinal tract was shown in studies of patients after varying degrees of resection of the small intestine (56). The incretin effect of oral glucose correlated positively to the total length of residual small bowel rather than to an integrated release of GIP. Patients with preserved ileal residues had much larger incretin effects than patients with no ileal residues, despite equal integrated increases in plasma GIP, findings indicative of the presence of incretins other than GIP in the ileum (56).

III. Discovery of GLP-1

In the interim between the discovery in the 1970s of GIP as an important intestinal incretin hormone to the actual discovery of GLP-1, it was suspected that there must be a second incretin hormone in addition to GIP (54-56). The ushering in of the era of recombinant DNA technology in the late 1970s provided the means necessary for the identification of the 'missing' incretin hormone. In the early 1980s, the cloning of cDNAs encoding the preproglucagons from pancreata of the anglerfish was accomplished (57, 58). The anglerfish was found to have two separate nonallelic preproglucagon genes, I and II, both encoding a glucagon and a glucagon-related peptide (GRP) (58). Notably, the glucagon-related peptide encoded in the anglerfish, preproglucagon-I, located carboxy proximal to the sequence of glucagon, bore a strong homology to the sequence of GIP, leading Lund *et al.* (57) to suggest that the anglerfish GRP-1 may be an intestinal incretin hormone. In support of this supposition Lund *et al.* (59) showed that similar preproglucagon mRNAs were expressed in the anglerfish pancreas and intestine, a finding that strongly supported the prediction that GRP could be an incretin hormone. Subsequently, preproglucagon mRNAs were cloned from human (60) and rat (61) gut and shown to be identical in sequences to the mRNAs in pancreas.

Shortly after the discovery of anglerfish GRP, the preproglucagon cDNAs of mammals were cloned (62-64) as well as the human gene (65). It became clear that the anglerfish GRP-I is a homolog of the GLP-1s encoded in the mammalian preproglucagons, which were subsequently proven to be potent insulinotropic incretins. There was, however, some uncertainty regarding the identification of the bioactive isoform of GLP-1 that had true insulinotropic actions. Based on the amino acid sequence of the mammalian preproglucagons, the sites that would be predicted for posttranslational

processing into peptide hormones were somewhat ambiguous. At the time it was generally believed that the yet-to-be-identified prohormone convertases (PCs) that enzymatically split prohormones into bioactive peptides required two adjacent basic amino acids, combinations of arginine, and lysine. The GLP-1 sequence begins with a histidine as the amino-terminal residue, as do most of the peptide hormones in the glucagon-related superfamily of hormones (Fig. 3). In the preproglucagon sequence, the first histidine is preceded by two basic amino acids, Lys-Arg, followed by four residues, another single basic residue, arginine, and a second histidine. The thinking at that time was that the putative bioactive peptide that would theoretically be cleaved from the preproglucagon during posttranslational processing would be at the Lys-Arg yielding a peptide of 37 or 36 amino acids, depending on whether the C-terminal glycine was present or absent and whether the penultimate C-terminal arginine was amidated in the absence of the C-terminal glycine. Thus, the 1-37 and 1-36 GLP-1 peptide isoforms were the first to be synthesized and tested for biological activity. The results of the experimental testing were disappointing. One report questioned whether GLP-1 had any relevant activity: 'How glucagon-like is glucagon-like peptide?' (66), as it had no effect on plasma glucose and insulin levels when administered to rabbits. Another report showed a weak stimulation of insulin secretion in cultured rat pancreatic islets at superpharmacological doses (25 nM) of GLP-1(1-36)amide and suggested that an N-terminally truncated peptide, GLP-1(7-36)amide, may be more active (67), as was suggested for GLP-1(7-37), an N-terminally truncated form of GLP-1(1-37) (67). These ideas were based upon alignment of the sequence of GLP-1 with the other members of the glucagon superfamily of peptide hormones (see Fig. 3), which revealed that the best alignment was with the histidine at position 7, and not position 1 of GLP-1 (12, 63, 67). In 1986 it was discovered that GLP-1 was indeed further N-terminally truncated by posttranslational processing in the intestinal L cells (68, 69). In contrast to GLP-1(1-37), GLP-1(7-37) and (7-36)amide were found to be potent insulinotropic hormones in the isolated perfused pancreas of rats (70) and pigs (71), and in humans (72). Further, it was suggested that the weak insulinotropic actions of GLP-1(1-37) at micromolar concentrations were probably artefactual due to a 0.1% level of nonspecific cleavage of GLP-1(1-37) to GLP-1(7-37) by nonspecific cathepsins in the serum-implemented tissue culture media (73). At present it is well established that the GLP-1 isoforms GLP-1(7-37) and GLP-1(7-36)amide are the bioactive insulinotropic peptides derived from preproglucagon in the intestine and the hind brain. The functions of the lesser GLP-1 isoforms GLP-1(1-37) and GLP-1(1-36)amide remain unknown.

IV. Structures of GLPs and Family of Glucagon-Related Peptides

The GLPs belong to a larger family referred to as the glucagon superfamily of peptide hormones. These hormones are classified within this family based on their considerable sequence homology, having anywhere from 21% to 48% amino acid identity with glucagon. Included in this family are: glucagon, GLP-1(7-37) and -(7-36)amide, GIP, exendin-3 and -4, secretin, peptide histidine-methionine amide (PHM), GLP-2, helospectin-1 and -2, helodermin, pituitary adenyl cyclase-activating polypeptides (PACAP)-38, and -27, PACAP-related peptide (PRP), GH-releasing factor (GRF), and vasoactive intestinal polypeptide (VIP) (Fig. 3). These peptide hormones are produced in the gut, pancreas, and the central and peripheral nervous systems and exhibit a wide variety of biological actions in which several act as neurotransmitters. Notably, even peptide hormones that are co-encoded within the same precursor, such as the peptide hormones derived from the cleavages of preproglucagon, differ significantly in the physiological processes that they regulate. For example, the major function of glucagon is to maintain blood glucose levels during fasting, whereas GLP-1 functions primarily during feeding to stimulate insulin release and to lower blood glucose levels. On the other hand, GLP-2 appears to regulate the growth of intestinal epithelial cells.

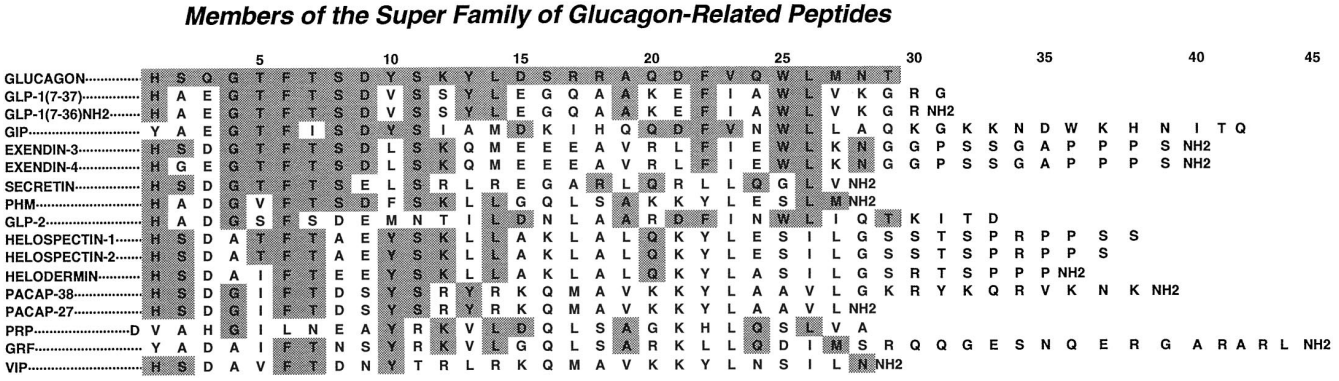

FIG. 3. Amino acid sequences of the members of the superfamily of glucagon-related peptides. Sequences include human glucagon, human GLPs, human GIP, exendins *(Heloderma horridum)*, human secretin, human peptide histidine methionine (PHM), helospectins *(Heloderma horridum)*, helodermin *(Heloderma suspectum)*, human PACAP, human PACAP-related peptide (PRP), human GRF, and human VIP. Residues identical to those of glucagon in the same position are *shaded*. *Standard single letter abbreviations* are used for amino acids (IUPAC-IUB Commission on Biochemical Nomenclature): A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

Exendin-3, exendin-4, helospectin-1, helospectin-2, and helodermin were all isolated from lizard (*Heloderma*) venom. They are potent secretagogues of the exocrine pancreas (74). Helodermin shares 53% and 42% homology with human PACAP and VIP, respectively, and has high affinity for the VIP_2 receptor (75). Exendin-4 is 53% homologous to mammalian GLP-1 and acts as a high-affinity agonist on the GLP-1 receptor (76, 77). Exendin-4 and GLP-1 may interact with specific receptors yet to be identified in guinea pig pancreatic acini tissue (78). In contrast, the amino-terminally truncated form of exendin-3(9-39) is a potent antagonist of GLP-1 actions (76, 77). Lizard helodermin, exendin, VIP/PHI, PACAP, and glucagon/GLP-1 cDNAs have been cloned, revealing that separate genes exist for these peptides (79, 80). To date, no evidence has been uncovered for the existence of mammalian homologues of the lizard helodermin or exendin (79, 80). It appears that helodermin and exendin-4 are not the evolutionary precursors to mammalian PACAP/VIP or GLP-1 but represent a distinct family of peptides. It seems likely that the high-affinity and biological activities of helodermin and exendin-4 on the mammalian VIP_2 and GLP-1 receptors, respectively, are a result of convergent evolution (80).

It is proposed that the proglucagon gene arose by the duplication of an ancestral gene approximately 800-1,000 million years ago (81). The structural organization of the genes of the glucagon superfamily of peptide hormones suggests that the ancestral gene consisted of four exons, which encoded the 5′-untranslated region of the mRNA, the signal peptide, the hormone and the 3′-untranslated region of the mRNA, respectively (82). The glucagon superfamily of hormones may have arisen by duplication and amplification of this basic gene, followed by a further duplication and amplification of the exon encoding the glucagon hormone domain to generate the multiple GLPs observed in preproglucagon (82). Based on statistical analysis of DNA sequences of the preproglucagon genes from bovine, human, hamster, and anglerfish, Lopez *et al.* (83) postulated that the two anglerfish genes arose from gene duplication approximately 160 million years ago (83). Furthermore, this analysis suggested that the GLP-2 sequence originated by duplication of the glucagon or GLP-1 sequence before the earliest divergence of fish (83). However, until recently, it was believed that GLP-2 was not expressed in either fish or birds (11, 84). Irwin and Wong (85) discovered that, unlike pancreatic proglucagon of fish and birds, the intestinal proglucagon does contain the sequence of GLP-2. Therefore, fish and bird proglucagon mRNAs from pancreas and intestine have different 3′-ends that are due to alternative mRNA splicing (85). The recent cloning of the frog (*Xenopus*) proglucagon cDNAs revealed the presence of three distinct GLP-1 peptides in addition to glucagon and GLP-2 (86). It has been postulated that the first exon duplication event resulting in the appearance of glucagon and GLP occurred at least 405-800 million years ago (81, 83). A duplication of the GLP-containing exon, giving rise to GLP-1 and GLP-2, may have occurred between 365 (divergence of mammals and amphibians) and 405 (divergence of cartilaginous fish and tetrapods) million years ago (81). The amino acid sequences of the preproglucagon genes are highly conserved among mammals (Fig. 4), and the products derived from proglucagon, glucagon (Fig. 5), and GLP-1 (Fig. 6) are highly conserved throughout the evolution of animal species. The amino acid sequence of glucagon is highly conserved during the evolution of tetrapods (3 substitutions between salamander and human), even more than the sequences of either GLP-1 (7 substitutions) or GLP-2 (15 substitutions). The high degree of conservation of the glucagon and GLP sequences during evolution indicates the importance of the physiological processes regulated by these hormones.

The conservation of GLP-1 also reflects the fact that essentially the entire amino acid sequence of GLP-1 is required for full biological activity. Removal of the N-terminal histidine (= GLP-1 8-37) results in drastic loss of receptor binding and insulinotropic activity (87-90). The positive charge of the imidazole side chain of the histidine residue appears to be crucial for GLP-1 actions (91). Likewise, N-terminal truncation of this histidine from the related insulinotropic peptide exendin-4(1-39) reduces agonist activity by approximately 10-fold (92). Notably, N-terminal truncation of exendin-4 by two residues yields a peptide that binds with the same affinity as full-length exendin but antagonizes GLP-1 action (92). In contrast, an N-terminal truncation of GLP-1 by two residues reduces binding affinity to approximately 1% that of the intact molecule (92, 93). Also, addition of an amino acid to the N terminus of GLP-1(6-37) also reduces biological activity (87, 89). Truncation at the C terminus also reduces the biological activity of GLP-1 considerably (87, 88, 90, 93). Substitution in the N-terminal part of the GLP-1 molecule with the corresponding glucagon residues impaired the affinity for the GLP-1 receptor only moderately whereas exchanges in the C-terminal portion of GLP-1 decreased the affinity for the GLP-1 receptor more than 100-fold (94). In contrast, the binding affinity of GLP-1 to its receptor is more sensitive to GIP-like changes in the N-terminal region than to changes in the C-terminal region (95).

Another approach to understanding the structure-activity relationships of GLP-1 has been obtained from studies of peptide analogs in which individual amino acids are substituted. These studies revealed that the residues in positions 1 (His), 4 (Gly), 6 (Phe), 7 (Thr), 9 (Asp) 22 (Phe), and 23 (Ile) are important for the binding affinity and biological activity of GLP-1 (96-98). Two-dimensional nuclear magnetic resonance of GLP-1 in a membrane-like environment (a dodecylphosphocholine micelle) revealed that GLP-1 consists of an N-terminal random coil segment (residues 1-7), two helical segments (7-14 and 18-29), and a linker region (15-17) – a structure similar to that observed for glucagon (99). Thus far, attempts to generate smaller active fragments of GLP-1 that retain potent insulinotropic activity have failed (89, 98, 100).

V. Tissue Distribution of the Expression of GLPs

A. Pancreatic α-cells

Pancreatic α-cells were discovered in 1907 as histologically distinct cells from the β-cells of the islets of Langerhans (101). It was not until 1962 that α-cells were shown by immunofluorescence staining studies to be the source

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