Glucagon-Like Peptides: Regulators of Cell Proliferation, Differentiation, and Apoptosis

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Peptide hormones are secreted from endocrine cells and neurons and exert their actions through activation of G protein-coupled receptors to regulate a diverse number of physiological systems including control of energy homeostasis, gastrointestinal motility, neuroendocrine circuits, and hormone secretion. The glucagon-like peptides, GLP-1 and GLP-2 are prototype peptide hormones released from gut endocrine cells in response to nutrient ingestion that regulate not only energy absorption and disposal, but also cell proliferation and survival. GLP-1 expands islet mass by stimulating pancreatic β-cell proliferation and induction of islet neogenesis. GLP-1 also promotes cell differentiation, from exocrine cells or immature islet progenitors, toward a more differentiated β-cell phenotype. GLP-2 stimulates cell proliferation in the gastrointestinal mucosa, leading to expansion of the normal mucosal epithelium, or attenuation of intestinal injury in experimental models of intestinal disease. Both GLP-1 and GLP-2 exert antiapoptotic actions in vivo, resulting in preservation of β-cell mass and gut epithelium, respectively. Furthermore, GLP-1 and GLP-2 promote direct resistance to apoptosis in cells expressing GLP-1 or GLP-2 receptors. Moreover, an increasing number of structurally related peptide hormones and neuropeptides exert cytoprotective effects through G protein-coupled receptor activation in diverse cell types. Hence, peptide hormones, as exemplified by GLP-1 and GLP-2, may prove to be useful adjunctive tools for enhancement of cell differentiation, tissue regeneration, and cytoprotection for the treatment of human disease. (Molecular Endocrinology 17: 161–171, 2003)
EFFECTS OF GLP-1 ON CELLULAR DIFFERENTIATION AND PROLIFERATION

\(\beta\)-Cell Differentiation and Exocrine Cell Lines

The actions of GLP-1 are mediated by the glucagon-like peptide-1 receptor (GLP-1R), a 463-amino-acid member of the G protein-coupled receptor (GPCR) superfamily (22). GLP-1R is expressed on islet \(\beta\)-cells (9, 22), and GLP-1R activation leads to activation of cAMP and both protein kinase A-dependent and -independent actions (23–25). In addition to enhancement of proinsulin biosynthesis and insulin secretion in islet cells, GLP-1 agonists induce features of cellular differentiation in exocrine cell lines. Exposure of rat AR42J cells to GLP-1 for 24–48 h leads to an initial increase in levels of cAMP and cellular proliferation, followed by cessation of proliferation and expression of the islet hormones, insulin, glucagon, and somatostatin, in up to 50% of cells (26). Furthermore, GLP-1 treatment also induced expression of mRNAs for glucose transporter-2 (GLUT-2) and glucokinase, in association with the capacity to release insulin in a glucose-dependent manner (26). A similar set of experiments was carried out using two pancreatic ductal cell lines, rat ARIP and human PANC-1 cells (27). GLP-1 or exendin-4 (a potent lizard GLP-1R agonist) treatment for 72 h of ARIP cells, which express endogenous pancreatic duodenal homeobox-1 (PDX-1), promoted cell aggregation, with some heterogeneity in the number of cells responding (27). GLP-1 treatment also induced insulin expression in a majority of ARIP cells. Native PANC-1 cells that do not express PDX-1 did not differentiate into insulin-producing cells in response to GLP-1; however, transfection with PDX-1 was sufficient for establishing GLP-1-dependent differentiation into insulin-producing cells (27). Furthermore, PANC-1 cells transfected with PDX-1 exhibited increased expression of the endogenous GLP-1R.

The importance of PDX-1 and hepatocyte nuclear factor-3\(\beta\) for GLP-1R-dependent cell differentiation has also been examined in Capan-1 cells, derived from a human pancreatic ductal carcinoma, which express the GLP-1R. A subset, approximately 10% of Capan-1 cells, normally contain insulin and/or glucagon immunopositivity, and after several days of exposure to 0.1 nM exendin-4, approximately 40% of the cells exhibited immunopositivity for insulin and/or glucagon (28). Treatment of Capan-1 cells with exendin-4 increased MAPK activity but not cell proliferation and was associated with increased cAMP accumulation and enhanced expression of glucokinase, GLUT-2, BET2/NeuroD, hepatocyte nuclear factor-3\(\beta\) (Foxa2), and PDX-1. Exendin-4 promoted the nuclear localization of PDX-1, which was abolished after coadministration of the protein kinase A (PKA) inhibitor H-89 (28).

GLP-1 agonists also enhanced islet cell differentiation in the \(\beta\)-cell line \(\beta\)lox5, an immortalized cell line derived from human islet \(\beta\)-cells infected with a retrovirus encoding SV40 T antigen. Treatment of \(\beta\)lox5 cells expressing transfected PDX-1 with the GLP-1 agonist exendin-4 resulted in induction of insulin gene expression; however, exendin-4 alone, in the absence of PDX-1 expression, had no effect on cell differentiation despite inducing expression of the cAMP response element binding protein (29).
endin-4 treatment of cells in vitro before transplantation under the kidney capsule was required for induction of C-peptide secretion from engrafted cells, continuous treatment with endin-4 in vivo did not result in C-peptide secretion, suggesting that sustained administration of endin-4 was not invariably associated with enhanced β-cell function or survival (29).

β-Cell Differentiation and Fetal or Adult Islet Precursors

Several studies have used fetal islet cell precursors to examine whether exposure to GLP-1R agonists is associated with enhanced differentiation of previously immature islet precursors. Incubation of fetal porcine islet-like cell clusters (ICCs) with 100 nM GLP-1 for periods ranging from hours to several days resulted in increased glucose-stimulated insulin secretion, with increased formation of β-cells from undifferentiated cells, in association with increased numbers of PDX-1+ cells (30). Furthermore, transplantation of GLP-1-treated ICCs into severe combined immunodeficient mice resulted in increased numbers of β-cells that appeared functionally mature as assessed by subsequent studies of glucose-induced insulin release in vivo (30). Endin-4 induced PDX-1 expression but did not increase the number of insulin-immunopositive cells in human ICCs prepared from 16- to 24-wk gestation human fetal pancreata (31). After transplantation of the human ICCs under the kidney capsule of athymic rats, a 10-d treatment with endin-4 was initiated commencing 48 h after transplantation. Eight weeks after ICC transplantation, the human ICCs from endin-4-treated rats exhibited glucose-stimulated insulin secretion, whereas rats implanted with ICCs in the absence of endin-4 administration did not exhibit increases in human C peptide after glucose challenge (31). Hence, these results suggest that administration of GLP-1 agonists promotes differentiation of functional β-cells both in vitro and in vivo.

GLP-1Rs have also been detected, using both immunocytochemistry and RT-PCR, on a subpopulation of nestin-immunopositive cells, designated nestin-positive islet-derived progenitor (NIP) cells, within human pancreatic islets and ducts (32, 33). GLP-1 increased intracellular calcium in nestin-positive cells at basal (5.6 mM) but not high (20 mM) glucose, and these actions were blocked by the GLP-1R antagonist endin(9–39). Expansion of NIP cultures for 7–12 d in the presence of GLP-1 promoted changes in cell morphology and the appearance of insulin-immunopositive cells in 5–30% of NIP cultures, effects that were also blocked by the GLP-1R antagonist endin(9–39). Furthermore, approximately 30% of NIP clones treated with endin-4 exhibited increased insulin secretion. Transfection of rat PDX-1 into long-term NIP cultures enhanced GLP-1 responsivity as assessed by insulin promoter activity. Intriguingly, NIP clones express the proglucagon gene when they approach confluence and secrete GLP-1 into the culture medium, raising the possibility that under some conditions, GLP-1 may act in an autocrine or paracrine manner, to regulate islet cell differentiation (33).

β-Cell Proliferation

The signal transduction mechanisms activated by the GLP-1R coupled to islet cell proliferation have been studied in immortalized mouse βTC-9 and rat INS-1 cells (34). Glucose and GLP-1 synergistically increased the expression of immediate early genes, including c-fos, c-jun, JunB, zif-268, and nur-77 in islet INS-1 cells, and these effects were markedly attenuated by the L-type Ca2+ channel blocker nifedipine (34). GLP-1 also increased thymidine incorporation, phosphatidylinositol 3-kinase (PI-3K) activity, and PDX-1 DNA binding in a dose-dependent manner, and these actions were blocked by the PI-3K inhibitors wortmannin and LY294002 (35). GLP-1 together with glucose increased levels of PDX-1, GLUT-2, glucokinase, and insulin mRNAs in INS-1 cells and GLP-1R activation increased the nuclear translocation of PDX-1 and enhanced PDX-1 binding to insulin promoter elements in RIN1046–38 islet cells (36). Furthermore the proliferative effects of GLP-1 were not confined to INS-1 cells but were also demonstrated in primary rat islet cell cultures (35).

Analysis of specific signal transduction pathways activated by GLP-1 using INS-1 cells, MIN6 cells, and normal rat β-cells demonstrated increased ERK 1/2, p38 MAPK, and protein kinase B activities in association with nuclear translocation of the atypical protein kinase C (PKC) ζ isoform in both INS-1 cells and in normal rat β-cells (37, 38). Functional evidence implicating a role for PKCζ in GLP-1-stimulated islet cell proliferation derives from observations that a dominant negative PKCζ protein attenuated, whereas expression of a constitutively active PKCζ mutant stimulated, islet cell proliferation (37).

The importance of GLP-1 for stimulation of islet cell proliferation was originally demonstrated in lean 20-d-old normoglycemic mice (Umea +/+ ) after 2 d of GLP-1 administration (39). Similarly, once daily administration of endin-4 stimulated islet neogenesis and β-cell proliferation in normal rats whereas daily administration of endin-4 for 10 d after partial pancreatectomy decreased blood glucose and stimulated pancreatic regeneration in rats via enhancement of islet neogenesis and β-cell proliferation (40). Remarkably, glucose tolerance remained significantly improved even several weeks after cessation of endin-4 treatment (40). GLP-1 agonists also stimulated β-cell proliferation, expansion of β-cell mass, and islet neogenesis in both young and old Wistar rats, in association with increased islet PDX-1 expression and islet insulin content (41). Treatment of neonatal Goto-Kakizaki rats with GLP-1 or endin-4 from d 2–6 resulted in stimulation of β-cell neogenesis and proliferation as measured by 5-bromo-2′-deoxyuridine labeling, with persistent expansion of β-cell mass detected at 2 months.
GLP-2 and Stimulation of Intestinal Epithelial Proliferation

Although the GLP-2 sequence is highly conserved in mammalian proglucagon genes, initial characterization of anglerfish pancreatic islet cDNAs revealed the presence of sequences encoding GLP-1 but not GLP-2, suggesting that the biological activity of GLP-2 may be unimportant in certain species (7). Later studies demonstrated that alternative RNA splicing gives rise to GLP-2-containing mRNA transcripts in fish, chicken, and lizards (8, 49), and daily GLP-2 administration was subsequently shown to promote crypt cell proliferation leading to expansion of the intestinal mucosal epithelium in mice (50).

The histological consequences of repeated GLP-2 administration are most evident in the small intestinal epithelium, which exhibits elongated villi due predominantly to enhanced crypt cell proliferation and decreased enterocyte apoptosis (51, 52). GLP-2-treated murine enterocytes appear longer and exhibit increased numbers of microvilli (53). The proliferative effects of GLP-2 have been demonstrated in the small bowel of mice, rats, pigs, and humans after exogenous peptide administration (50, 54–56). Furthermore, GLP-2 is also weakly mitogenic for cells in the stomach and colon (57, 58).

The proliferative effects of exogenous GLP-2 contribute to intestinal epithelial regeneration in the setting of small-bowel enteritis (59–63) and colitis (64) and after major small-bowel resection (56, 65, 66). Increased circulating levels of GLP-2 are associated with development of small-bowel hyperplasia in experimental rodent diabetes (67), and immunoneutralization of GLP-2 reduced small-bowel epithelial proliferation in diabetic rats (68). The presence of enteral nutrients is not required for the trophic effects of GLP-2, as exogenous GLP-2 enhances the mass of the small-bowel epithelial mucosa in normal or tumor-bearing rats maintained on parenteral nutrition (69, 70).

The GLP-2 receptor (GLP-2R) and cell proliferation

The molecular cloning of the cDNA encoding the GLP-2R has enabled delineation of mechanisms coupling GLP-2R activation to intestinal cell proliferation. The GLP-2R is structurally related to the glucagon and GLP-1 receptors and is coupled to cAMP generation in cells expressing a transfected human or rat GLP-2R (71, 72). Exogenous GLP-2 increased AP-1-dependent transcriptional activity and immediate early gene expression and weakly stimulated cell proliferation in BHK fibroblasts expressing a stably transfected rat GLP-2R (71, 72). The GLP-2R is expressed in a highly tissue-specific manner predominantly in the gastrointestinal tract (71, 73). A combination of immunocytochemistry and in situ hybridization experiments has localized GLP-2R expression to human enteroeendocrine cells and murine enteric neurons (73, 74). In the murine gut, GLP-2 stimulates division of columnar, and not mucous progenitor cells, in association with activation of nuclear c-fos expression in enteric glia, followed by subsequent fos activation in crypt cells that do not directly express the GLP-2R (74). Hence, the GLP-2-dependent stimulation of intestinal epithelial proliferation in vivo appears indirect (Fig. 2) and is regulated by as yet unidentified downstream mediators of GLP-2 action (13, 75).
mass, respectively, has fostered studies directed at determining whether these peptides exert their effects via stimulation of cell proliferation alone or via both enhanced proliferation and decreased apoptosis (Fig. 2). Although the number of detectable apoptotic islet and intestinal epithelial cells is normally low in uninjured normal tissue, induction of experimental islet or intestinal injury leads to increased numbers of apoptotic cells in the endocrine pancreas and gut epithelium. Treatment of rat islets with the GLP-1 analog NN2211 reduced cytokine-induced apoptosis \textit{in vitro} (76), and GLP-1 increased cell survival and reduced caspase activation in BHK fibroblasts expressing a transfected GLP-1R (77). Similarly, treatment of mice with exendin-4 reduced β-cell apoptosis induced by STZ, whereas GLP-1R−/− mice exhibit increased susceptibility to STZ-induced β-cell apoptosis (77). Furthermore, exendin-4 directly reduced the extent of apoptotic cell death in purified rat β-cells exposed to a combination of cytotoxic cytokines, consistent with a direct action for β-cell GLP-1R signaling in promoting resistance to cellular apoptosis (77).

The antiapoptotic properties of GLP-1 agonists have been demonstrated in Zucker diabetic rats and db/db mice. A 2-d continuous infusion of recombinant GLP-1 was associated with a marked increase in islet size and β-cell mass, formation of new islet-like clusters, and extraislet insulin-positive cells (78). GLP-1-treated rats exhibited increased numbers of Ki-67-positive cells in both the endocrine and exocrine pancreas, with aggregates of mitotic cells detected in association with small and medium-sized islets. GLP-1-treated rats also exhibited reduced numbers of apoptotic cells in the exocrine parenchyma. Remarkably, the percentage of apoptotic β-cells in this study was found to be greater than 20% and was significantly reduced in rats treated with GLP-1 (78). Treatment of normoglycemic db/db mice with daily exendin-4 for 2 wk prevented the progression to hyperglycemia, in association with increased β-cell mass, enhanced numbers of 5-bromo-2′-deoxyuridine+ islet cells, and reduced numbers of Tunel+ apoptotic β-cells (79). Exendin-4-treated rats also exhibited increased levels of pancreatic Akt1 and p44 MAPK and reduced expression of activated caspase-3 (79).

The antiapoptotic actions of GLP-1 agonists have been demonstrated in cultured fetal rat hippocampal neurons that exhibit GLP-1-dependent increases in cAMP formation. Both GLP-1 and exendin-4 significantly reduced the extent of glutamate-induced cell death in short-term cultures of hippocampal neurons (80). Furthermore, both GLP-1 and exendin-4 reduced depletion of choline acetyltransferase immunoreactivity, a marker for cholinergic neurons in the basal forebrain, after administration of ibotenic acid (80). Hence, the demonstration that GLP-1R activation reduces cell death in transfected fibroblasts, islet β-cells, and neurons suggests that direct coupling to antiapoptotic signaling pathways may represent a generalized feature of GLP-1R action in diverse cell types (Fig. 3).

Considerable experimental evidence from animal studies \textit{in vivo}, and experiments with transfected cells \textit{in vitro} links activation of GLP-2R signaling to attenuation of apoptotic pathways. The nonsteroidal antiinflammatory agent indomethacin induces crypt compartment apoptosis in the murine small- and
large-bowel epithelium, and pretreatment of mice with a GLP-2 analog markedly reduced mortality, decreased the extent of mucosal injury, and suppressed the appearance of apoptotic cells in the gut epithelium (59). Furthermore, administration of the cytotoxic chemotherapeutic agents irinotecan or 5'-fluorouracil produces significant epithelial damage and apoptosis in a position-dependent manner along the crypt-to-villus axis that was markedly attenuated by pretreatment with a potent GLP-2 agonist (81). Exogenous GLP-2 infusion also reduced epithelial apoptosis in the small bowel of premature parenterally fed piglets (55).

Heterologous cells that express a transfected GLP-2R exhibit enhanced survival after external injury in the presence of GLP-2, which reflects decreased cellular apoptosis. BHK-GLP-2R cells exposed to cycloheximide exhibit reduced viability, morphological features of apoptosis and DNA laddering, and reduced viability; these parameters are markedly attenuated after incubation with GLP-2 agonists (82). Furthermore, GLP-2 reduced activation of caspase-3, caspase-8, and caspase-9, decreased poly(ADP-ribose) polymerase cleavage, and reduced mitochondrial cytochrome c release in BHK-GLP-2R cells in vitro (82). The antiapoptotic effects of GLP-2 in the setting of cycloheximide-induced injury were cAMP dependent yet protein kinase A independent. Similarly, GLP-2 increased cell survival after cycloheximide in the presence of the phosphatidylinositol 3-kinase, and MAPK inhibitors LY294002 and PD98054, respectively (82).

The direct antiapoptotic effects of GLP-2 in BHK cells do not require activation of the survival kinase Akt, p90Rsk, or p70 S6 kinase, as GLP-2 reduced caspase activation and cytochrome c release after LY294002 in the absence of Akt, p90Rsk, or p70 S6 phosphorylation. GLP-2R activation in BHK-GLP-2R cells is coupled to inhibition of glycogen synthase kinase 3 (GSK-3) through phosphorylation of Ser21 in GSK-3α and Ser9 in GSK-3β in a PI-3K-independent, PKA-dependent manner. GLP-2 also reduced the magnitude of LY294002-induced mitochondrial localization of the proapoptotic Bcl family Bad and Bax proteins and stimulated Bad phosphorylation at Ser155 in a PI-3K-independent, PKA-dependent manner (83). Although the antiapoptotic properties of GLP-2 have not yet been directly demonstrated in gut endocrine cells or enteric neurons, the structurally related pituitary adenylate cyclase activating peptide (PACAP) promotes neuronal survival via cAMP-dependent mechanisms in sympathetic neurons (84).

**GPCRs, PEPTIDE HORMONES, AND ENGAGEMENT OF APOPTOTIC PATHWAYS**

Several peptide hormones structurally related to the glucagon-secretin superfamily exert either pro- or anti-apoptotic actions in diverse cell types (Table 1). Glucose-dependent insulinotropic peptide exerts both proliferative and antiapoptotic actions in the immortalized INS-1 islet cell line (85), and PACAP promotes neuronal survival in cerebellar neurons (86). Vasoactive intestinal peptide (VIP) reduces apoptosis in ovarian follicles (87), and both VIP and PACAP reduced...
thymocyte apoptosis induced by glucocorticoid withdrawal (88) and inhibited Fas ligand expression and NF-κB activation in T lymphocytes in a cAMP-dependent manner (89). Similarly, antiapoptotic actions have been detected after activation of CRH (90), FSH (91), adrenomedullin (92), gastrin (93), TSH (94), and substance P/neurokinin-1 (95) receptors, whereas receptors for angiotensinogen 1 (96), opioids (97), calcitonin gene-related peptide (98), natriuretic peptide(s) (99), PTH (100), and somatostatin (101) have been linked to enhanced apoptosis in diverse cell types. Hence, control of cell survival via peptide hormone-activated GPCR signaling (Table 1) may be an increasingly recognized mode of action of specific regulatory peptides in diverse cell types.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Cell/Tissue</th>
<th>Action</th>
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<tbody>
<tr>
<td>CCK</td>
<td>Pancreas</td>
<td>Increased acinar apoptosis (106, 107)</td>
</tr>
<tr>
<td>CRH</td>
<td>Hypothalamic neurons</td>
<td>Reduced amyloid β-associated apoptosis (108)</td>
</tr>
<tr>
<td>Gastrin</td>
<td>Pancreatic cells, tumors</td>
<td>Reduced (AR42J) (93) or increased (109) apoptosis</td>
</tr>
<tr>
<td>GIP</td>
<td>Islet cells</td>
<td>Reduced β-cell apoptosis (85)</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Islet cells, neurons</td>
<td>Reduced β-cell and hippocampal neuronal apoptosis (77, 80)</td>
</tr>
<tr>
<td>GLP-2</td>
<td>Gut epithelium</td>
<td>Decreased crypt and villous apoptosis (59, 81)</td>
</tr>
<tr>
<td>Neutensin</td>
<td>Cancer cells</td>
<td>Reduced apoptosis (110)</td>
</tr>
<tr>
<td>PTH/PTHrP</td>
<td>Bone, diverse cell types</td>
<td>Suppression or activation of apoptosis (100, 111, 112)</td>
</tr>
<tr>
<td>PACAP</td>
<td>Neuroendocrine cells, T cells</td>
<td>Reduction of apoptosis (113, 114)</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>Cancers, neuroendocrine cells</td>
<td>Induction of apoptosis (115, 116)</td>
</tr>
<tr>
<td>VIP</td>
<td>Neurons, lymphocytes</td>
<td>Neuroprotection, lymphocyte survival (88, 117)</td>
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CCK, Cholecystokinin.

UNANSWERED QUESTIONS AND FUTURE RESEARCH DIRECTIONS

The available evidence clearly indicates that activation of GLP receptors (Figs. 2 and 3), and more broadly, related classes of GPCRs, may be important determinants of cell survival, particularly in the setting of tissue injury. For example, adrenergic receptor signaling modifies cardiomyocyte survival in a ligand- and receptor-specific manner (102, 103), and vasoactive peptides including endothelin, angiotensin II, VIP, atrial natriuretic peptide, and adrenomedullin exert both trophic and either pro- or antiapoptotic actions through specific subclasses of GPCRs (104). Considerable evidence implicates progressive β-cell failure as an inevitable feature accompanying the progression of type 2 diabetes in affected human subjects (105). Accordingly, strategies directed at enhancing islet neogenesis and β-cell proliferation, and/or preservation of existing β-cell mass via reduced susceptibility to apoptosis may prove to be useful in preventing loss of β-cell function in the diabetes clinic. Whether chronic therapy with GLP-1 agonists will prove useful for enhancement or preservation of β-cell mass in patients with type 2 diabetes will require long-term clinical studies and/or improvements in our currently limited ability to assess β-cell mass in human subjects. Similarly, it remains unclear whether GLP-1 agonists may enhance cell preservation and reduce β-cell apoptosis in the setting of islet transplantation. Furthermore, the signal transduction pathways activated by the GLP-1R in human ducts or β-cells that transduce proliferative or antiapoptotic signals have not yet been determined. In contrast to the direct actions of GLP-1 on islet cell survival, the trophic and antiapoptotic actions of GLP-2 leading to expansion of the intestinal epithelial mucosa are largely indirect (Fig. 3), and the identity of the specific downstream mediators that convey GLP-2-activated mitogenic and cytoprotective signals to the stomach and small and large intestinal epithelium remain unknown. Furthermore, the emerging clinical use of long acting GLP analogs, with inherent proliferative and antiapoptotic actions, suggests that ongoing surveillance of tissues such as the pancreatic ductal (GLP-1) or colonic (GLP-2) epithelium appears prudent. Given the emerging interest in the therapeutic use of GLP analogs for the treatment of diabetes and intestinal disease, a more detailed understanding of the cellular pathways coupling GPCR signaling to control of cell proliferation and survival seems warranted.

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