

# Ontogeny of the Glucagon-Like Peptide-2 Receptor Axis in the Developing Rat Intestine\*

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## ABSTRACT

Glucagon-like peptide-2 (GLP-2) is secreted by enteroendocrine cells in the small and large intestines and exerts intestinotropic effects in the gastrointestinal mucosal epithelium of the adult rodent. The actions of GLP-2 are mediated by the GLP-2 receptor, a new member of the G protein-coupled receptor superfamily. To ascertain whether the GLP-2/GLP-2 receptor axis is expressed and functional in the developing intestine, we have studied the synthesis of GLP-2 and the expression of the GLP-2 receptor (GLP-2R) in the fetal and neonatal rat gut. GLP-2 immunoreactivity (GLP-2-IR) was detected in the fetal rat intestine, and fetal rat intestinal cell cultures secreted correctly processed GLP-2<sup>1-33</sup> into the medium. High levels of GLP-2<sup>1-33</sup> were also detected in the circulation of 13-day-old neonatal rats ( $P < 0.001$  vs. adult). Analysis of GLP-2 receptor expression by RT-PCR demonstrated GLP-2R messenger RNA transcripts in fetal intestine and in neonatal stomach, jejunum, ileum, and colon. The levels

of GLP-2R messenger RNA transcripts were comparatively higher in the fetal and neonatal intestine ( $P < 0.05$ – $0.01$  vs. adult) and declined to adult levels by postnatal day 21. Subcutaneous administration of a degradation-resistant GLP-2 analog, h[Gly2]-GLP-2 once daily for 10 days increased stomach ( $0.009 \pm 0.0003$  vs.  $0.007 \pm 0.002$  g/g body mass, h[Gly2]-GLP-2-treated vs. controls;  $P < 0.05$ ) and small bowel weight ( $0.043 \pm 0.0037$  vs.  $0.031 \pm 0.0030$  g/g body mass;  $P < 0.05$ ). h[Gly2]-GLP-2 also increased both small ( $2.4 \pm 0.05$  vs.  $1.8 \pm 0.17$  cm/g body mass;  $P < 0.05$ ) and large bowel length ( $0.32 \pm 0.01$  vs.  $0.25 \pm 0.02$  cm/g body mass, h[Gly2]-GLP-2-treated vs. saline-treated controls, respectively;  $P < 0.05$ ) in neonatal rats. These findings demonstrate that both components of the GLP-2/GLP-2 receptor axis are expressed in the fetal and neonatal intestine. The ontogenic regulation and functional integrity of this axis raises the possibility that GLP-2 may play a role in the development and/or maturation of the developing rat intestine. (*Endocrinology* 141: 4194–4201, 2000)

THE PROGLUCAGON gene is expressed in the central nervous system, islet A cells, and enteroendocrine cells of the small and large intestines during fetal and adult life (1). In the pancreas, tissue-specific processing of proglucagon liberates glucagon, whereas the glucagon-like peptides remain incompletely processed within a larger major proglucagon fragment. In contrast, glicentin, oxyntomodulin, glucagon-like peptide-1 (GLP-1), and GLP-2 are liberated from endocrine cells of the small and large bowels (2). The processing profile of proglucagon-derived peptides (PGDPs) in the brain appears intermediate between those of pancreas and intestine, with both glucagon and GLPs detected in the hypothalamus and brainstem (3, 4).

GLP-2, located carboxyl-terminal to GLP-1 in the proglucagon molecule, appears to function as a regulator of intes-

tinal mucosal homeostasis (5). Administration of GLP-2 to normal rodents stimulates crypt cell proliferation and inhibits enterocyte apoptosis, resulting in expansion of the small bowel mucosal epithelium (6, 7). The observations that intestinal injury is associated with increased production of the intestinal PGDPs (8) taken together with the finding of increased circulating levels of bioactive GLP-2 in human patients with inflammatory bowel disease (9) provide indirect evidence supporting a role for GLP-2 as an endogenous trophic regulator of intestinal mucosal repair *in vivo*. Consistent with this hypothesis, administration of GLP-2 to rodents with experimental intestinal injury reduces weight loss and disease activity scores, enhances mucosal regeneration, and reduces mortality in the setting of both small and large bowel inflammation (10–12, 46).

Although intestinal proglucagon gene expression is detectable during fetal gut development (13, 14), the biological actions of the intestinal PGDPs during fetal and neonatal life remain unclear. As GLP-2 exhibits intestinotropic activity in adult rodents, it seems possible that GLP-2 may play a role in fetal gut development and/or possibly in the complex transition from the neonatal to the adult gut. The actions of GLP-2 are mediated by the GLP-2 receptor (GLP-2R), a recently identified novel member of the G protein-coupled receptor superfamily (15). Although GLP-2R RNA transcripts have been identified in the adult rodent gastrointestinal tract (15), the developmental onset of GLP-2R expression has not yet been reported. Furthermore, whether the

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fetal or neonatal gut is capable of processing proglucagon to bioactive GLP<sup>21-33</sup> remains unknown. To determine whether the GLP-2/GLP-2R axis is expressed and functional during rat development, we have now analyzed the coordinate expression of GLP-2 and the GLP-2R in fetal and neonatal rats.

## Materials and Methods

### Animals

Wistar rats were obtained from Charles Rivers Canada, Inc. (St. Constant, Canada). All animal experiments described in this manuscript were approved by the animal care committee of the University Health Network or the University of Toronto.

### Tissue preparation for HPLC analysis

Fetal rat intestinal cells (19–20 days gestation) were dispersed with collagenase, hyaluronidase, and deoxyribonuclease (40, 50 and 5 mg/dl, respectively; Blend Type H, Sigma, St. Louis, MO), and placed into 60-mm culture dishes in DMEM containing 5% FBS and 4.5 g/liter glucose for 24 h. Cells were then washed and treated with medium containing 0.5% FBS, 1 g/liter glucose, and 20  $\mu$ U/ml insulin for 2 h. Cells and cell media were collected separately at the end of the incubation period (16). Tissues [whole intestine from fetal (19–20 days gestation) and postnatal day 1 (PD1) rats, 5 cm of ileum from all other rats (PD12, PD21, PD42, and adult), and fetal rat intestinal cells in culture;  $n = 3-6$  each] were homogenized in 1 N HCl containing 5% HCOOH, 1% trifluoroacetic acid (TFA), and 1% NaCl, followed by extraction of peptides and small proteins by passage through a cartridge of C<sub>18</sub> silica (Sep-Pak, Waters Corp., Milford, MA). Plasma was collected into a 10% volume of Trasylol (5000 KIU/ml)-EDTA (12 mg/ml)-diprotin A (34  $\mu$ g/ml). Two volumes of 1% TFA (pH 2.5; with diethylamine) were added to plasma, and 1 vol 10% TFA was added to the medium before extraction of the peptides on C<sub>18</sub> silica ( $n = 3-5$  each) (2, 17).

### HPLC analysis

GLP-2-related peptides were separated by HPLC using a C<sub>18</sub>  $\mu$ Bondapak column (Waters Corp.) with a 45-min linear gradient of 30–60% solvent B (solvent A = 0.1% TFA in water; solvent B = 0.1% TFA in acetonitrile), followed by a 10-min purge with 99% solvent B. The flow rate was 1.5 ml/min, and 0.3-min fractions were collected (2, 17).

### RIA

RIAs for PGDPs were conducted on dried samples using a panel of site-specific antisera. The N-terminal PGDPs (glucagon, oxyntomodulin, and glucagon) were detected using antiserum K4023 [glucagon-like immunoreactivity (GLI); Biospecific, Emeryville, CA], whereas antiserum 04A was used to detect glucagon alone [immunoreactive glucagon (IRG); Dr. R. Unger, Dallas, TX]. GLP-1-containing peptides were analyzed using an antiserum for the free C-terminal, Gly-extended form of GLP-1 (antiserum b5; Dr. S. Mojsov, New York, NY) as well as an antiserum that recognizes the amidated C-terminal end of the molecule (GLP-1<sup>7-36</sup>NH<sub>2</sub>; Affinity Research Products Ltd., Mamhead, UK). GLP-2-related peptides were detected using antiserum UTH7, which detects the midsequence amino acids 25–30 and, thus, both GLP-2<sup>1-33</sup> and the degraded form, GLP-2<sup>3-33</sup> (2, 17). Tissue protein levels were determined by the Lowry protein assay (18).

### Tissue isolation for RT-PCR analysis

Rat gastrointestinal tissues were extracted, cleaned of debris, immediately snap-frozen in liquid nitrogen, and stored at  $-80$  C until RNA was isolated. Fetal (19–20 days gestation) stomachs and the entire lengths of the fetal small and large intestines were pooled separately ( $n = 5$  each). For neonatal rats, stomachs from PD1 pups were removed and pooled, and the intestines were divided into small and large bowels and pooled ( $n = 4$  each). For PD12 rats, stomachs were removed, and RNA was prepared from each sample. Intestinal samples from PD12 rats consisted of 5 cm of jejunum (10–15 cm from stomach), 5 cm of ileum (5 cm proximal to cecum), and the entire length of the colon. For all other

age groups, separate gastrointestinal RNA preparations were obtained from rats (PD21, PD42, and adult rats) consisting of the entire stomach alone or 5-cm segments of jejunum (10–15 cm distal to the duodenum), ileum (5-cm proximal to the cecum), or colon (5–10 cm distal to cecum).

### RNA isolation and semiquantitative RT-PCR analysis

Total RNA was isolated using a modified guanidinium isothiocyanate protocol (19), and RNA integrity was assessed by agarose gel electrophoresis. For first strand complementary DNA (cDNA) synthesis, samples were treated with deoxyribonuclease I (Life Technologies, Inc., Toronto, Canada), primed with random hexamers (Life Technologies, Inc.), and reverse transcribed with Superscript II reverse transcriptase (Life Technologies, Inc.). RT<sup>-</sup> reactions were analyzed to control for genomic DNA and template contamination. PCR amplification was carried out using *Taq* DNA polymerase (MBI Fermentas). Oligonucleotide primer pairs for PCR amplification were as follows: rat GLP-2R, 5'-TTGTGAACGGGCGCCAGGAGA-3' and 5'-GATCTCACTCTCTCC-AGAATCTC-3'; rat proglucagon, 5'-GTTTACATCGTGGCTGGATTG-3' and 5'-TGAATTCCTTTGCTGCTGGC-3'; and for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-TCCACCACCCTGTTGCTGTAG-3' and 5'-GACCACAGTCCATGACATCACT-3'. Semiquantitative RT-PCR analysis of rat GLP-2R messenger RNA (mRNA) transcripts was achieved by amplifying 2  $\mu$ l dilute cDNA for 24 cycles using the indicated primer pairs. The expected PCR product for the rat GLP-2R is 1672 bp, which corresponds to the full-length rat GLP-2R cDNA. For proglucagon, 2  $\mu$ l dilute cDNA was amplified for 22 cycles, and the expected PCR product is 323 bp. For PCR amplification of GAPDH cDNA, 1  $\mu$ l dilute cDNA was amplified for 18 cycles with an expected PCR product of 452 bp. To control for nonspecific amplification, PCR reactions were also carried out in the absence of first strand cDNA. The conditions for linear PCR amplification of GLP-2R, proglucagon, and GAPDH PCR products were determined by carrying out multiple PCR reactions at varying cycle numbers (no. 8–28) and different cDNA input concentrations (0.01–2  $\mu$ l) as indicated in Fig. 3. The linear range for PCR amplification was determined by plotting the PCR product yield against either the cycle number or cDNA input amount.

After PCR amplification, PCR products were separated by gel electrophoresis on a 1% (wt/vol)-agarose gel and transferred onto a nylon membrane (GeneScreen, Life Technologies, Inc.), and blots were hybridized overnight with a <sup>32</sup>P-labeled internal cDNA probe for the rat GLP-2R (15), rat proglucagon (20), or rat GAPDH (21) in a hybridization buffer containing formamide. Membranes were washed stringently at 65 C in 0.1  $\times$  SSC (standard saline citrate) and 0.1% SDS. Membranes were then exposed to a phosphor screen (Molecular Dynamics, Inc., Sunnyvale, CA), and PCR products were visualized and quantified densitometrically using a STORM 840 phosphorimager (Molecular Dynamics, Inc.) and ImageQuant software (version 5.0, Molecular Dynamics, Inc.).

### GLP-2 administration to neonatal rats

h[Gly2]-GLP-2 was a gift from NPS Allelix Corp. (Mississauga, Canada). Pregnant Wistar rats were acclimatized to the animal facilities and delivered pups 1 week after arrival. Within 24 h of birth, all pups in one litter ( $n = 14$  pups) were injected sc with h[Gly2]-GLP-2 (5  $\mu$ g in a total volume of 100  $\mu$ l dissolved in PBS), and all pups in a second litter ( $n = 11$ ) were injected sc with saline (100  $\mu$ l) alone. Pups in both litters were injected once a day at approximately 1700 h for a total of 10 days. On day 11, approximately 20 h after the last injection, the neonatal rats were killed by CO<sub>2</sub> anesthesia. Gastrointestinal tissues (stomach, jejunum, ileum, and colon) were removed, cleaned, and weighed as previously described (6, 7, 22, 23). Tissue samples collected for histological examination were fixed overnight in 10% neutral-buffered formalin, paraffin embedded, and counterstained in hematoxylin and eosin.

### Statistics

Area under the curve for HPLC peaks was determined as the sum of the immunoreactivity under the peak. Differences between groups were determined by Student's unpaired *t* test or ANOVA using *n-1 post-hoc* comparisons, as appropriate, on an SAS system (SAS Institute, Inc., Cary, NC).

### Results

The control of proglucagon processing is complex, and the developmental control of proglucagon processing in the intestine has not been extensively studied. To ascertain whether intestinal processing of proglucagon was comparable in fetal, neonatal, and adult rat intestine, we analyzed the intestinal profile of PGDPs using antisera directed against glicentin/oxyntomodulin, glucagon, GLP-1, or GLP-2. The fetal rat intestine contained readily detectable levels of GLI (glicentin/oxyntomodulin;  $P < 0.001$  vs. adult levels), with insignificant amounts of glucagon detected in the same extracts (Fig. 1). The content of intestinal GLI increased progressively in the neonatal rat and reached adult levels by PD21. Consistent with the known profile of proglucagon processing in the intestine (1, 24), the two principal bioactive forms of GLP-1, GLP-1<sup>7-36</sup>NH<sub>2</sub> and GLP-1<sup>7-37</sup>, were detected in all intestinal extracts analyzed (Fig. 1), with a progressive increase in the levels of both molecular forms of GLP-1 from fetal to adult life. Similarly, the changes in immunoreactive intestinal GLP-2 paralleled the pattern observed for both GLI and GLP-1, with relatively lower levels in newborn intestine ( $P < 0.001$  vs. adult) increasing to adult levels by PD21.

To determine whether GLP-2 is correctly processed and secreted by enteroendocrine cells of the developing rat intestine, we analyzed levels of circulating GLP-2 by HPLC and RIA. With this protocol, the levels of both bioactive GLP-2<sup>1-33</sup> and its circulating degradation product, GLP-2<sup>3-33</sup>, are de-

termined as a reflection of total GLP-2 secretion (9, 17, 25, 26). Plasma levels of GLP-2 in PD12 rats were 8-fold higher than those in adult rats ( $P < 0.001$ ), and then declined progressively to lower levels in older animals (Fig. 2). The small amount of plasma available from fetal rats precluded assessment of circulating GLP-2 in these animals. Accordingly, to ascertain whether the fetal rat enteroendocrine cell exhibits the capacity for processing and secretion of bioactive GLP-2<sup>1-33</sup>, we assessed whether immunoreactive forms of GLP-2 were detectable in fetal rat intestinal cell cultures (27). Both GLP-2<sup>1-33</sup> and GLP-2<sup>3-33</sup> were detected in the medium from fetal rat intestinal cultures (Fig. 2B). Furthermore, GLP-2<sup>1-33</sup> and GLP-2<sup>3-33</sup> were also detected in fetal rat intestine cell extracts (Fig. 2B). Taken together, these findings clearly demonstrate that fetal and, by inference from plasma studies, neonatal rat intestines exhibit the capacity to process proglucagon into bioactive GLP-2<sup>1-33</sup>, which is then secreted into the circulation.

Glucagon, GLP-1, and GLP-2 exert their actions through unique G protein-coupled receptors (15, 28, 29). In contrast to the glucagon and GLP-1 receptors, the GLP-2 receptor is expressed in a highly tissue-specific manner, predominantly in the gastrointestinal tract (15). To determine whether the GLP-2 secreted from the fetal and neonatal rat intestine is potentially bioactive, we assessed the expression of the GLP-2 receptor and proglucagon genes in different regions of the rat gastrointestinal tract by semiquantitative RT-PCR. We have previously used this methodology to assess the

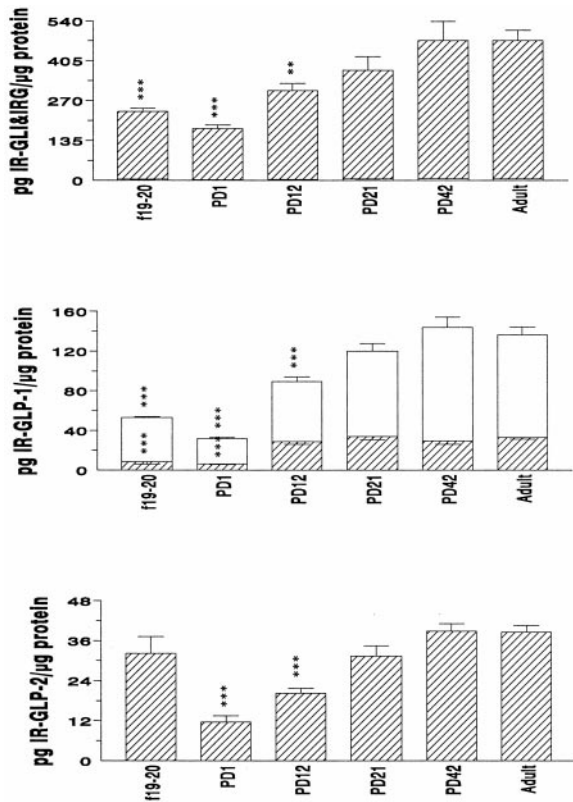


FIG. 1. PGDP levels were determined in intestinal extracts from rats of different developmental stages. *Upper panel*, GLI (▨) and IRG (□). *Middle panel*, GLP-1<sup>7-36</sup>NH<sub>2</sub> (□) and GLP-1<sup>7-37</sup> (▨). *Bottom panel*, GLP-2 (▨).  $n = 3-6$ . \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  (vs. adult levels).

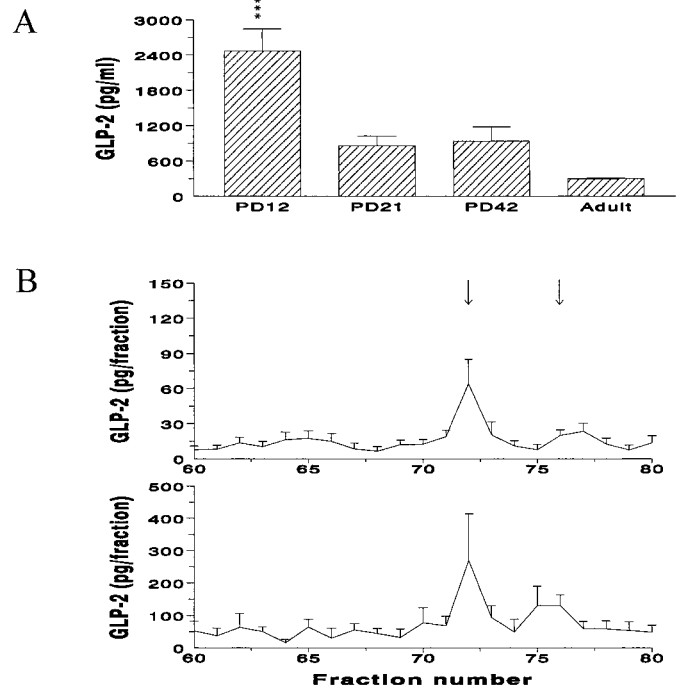


FIG. 2. A, Plasma GLP-2 from rats of different developmental stages was analyzed by HPLC and RIA, and the area under the curve was quantitated ( $n = 3$ ). \*\*\*,  $P < 0.001$  vs. adult levels. B, HPLC analysis of GLP-2-immunoreactive peptides secreted into the media (*upper panel*) and contained in the cells (*lower panel*) of fetal rat intestine cell (FRIC) cultures ( $n = 5$ ). The arrows indicate the elution positions of standard GLP-2<sup>1-33</sup> (fraction 72) and GLP-2<sup>3-33</sup> (fraction 76).

tissue-specific and developmental expression of both the glucagon and GLP-1 receptors in the mouse (30). To verify that GLP-2R mRNA transcripts could be detected and assessed semiquantitatively, we analyzed the relationship among input cDNA, PCR cycle number, and the relative levels of intestinal GLP-2R mRNA transcripts. The data clearly show a linear relationship between the relative levels of GLP-2R mRNA transcripts and PCR cycle number (Fig. 3A). Similar results were obtained for analysis of intestinal rat proglucagon and GAPDH mRNA transcripts in comparable experiments (Fig. 3A). Furthermore, the relative levels of PCR products for all three transcripts exhibited a linear relationship between product abundance and input cDNA (Fig. 3B). A representative PCR analysis from experiments demonstrating this relationship is shown in Fig. 3C.

As the biological actions of GLP-2 may exhibit developmental and region-specific differences in distinct gastrointestinal compartments (5), we studied the ontogeny of GLP-2R expression in the stomach and both small and large intestines. GLP-2R transcripts were detected in RNA isolated from fetal, neonatal (PD1 and PD12), weaned (PD21 and PD42), and adult rat stomachs and in both small and large intestines at all ages examined (Fig. 4). In the stomach, the relative levels of GLP-2R mRNA transcripts decreased slightly from fetal to adult levels, but levels were not sig-

nificantly different in animals of different ages (data not shown). The relative levels of jejunal GLP-2R mRNA were higher in fetal and neonatal rats than those in adult rats ( $P < 0.001$ ), reaching adult levels by PD21. The relative abundance of proglucagon mRNA transcripts resembled the pattern obtained for GLP-2R RNA in the jejunum, with comparatively higher levels observed in fetal and neonatal jejunum ( $P < 0.01$  vs. adult), followed by a decline to adult levels in PD21 jejunum (Fig. 4). The developmental expression of the GLP-2R in the ileum was similar to that observed in the jejunum, with higher levels of GLP-2R mRNA transcripts detected in fetal gut and PD1 and PD12 ileum ( $P < 0.05$  vs. adult), followed by a decline to lower levels in older animals. In contrast, proglucagon mRNA transcripts were lower in the fetus and PD1 ileum, increased markedly by PD12 ( $P < 0.05$  vs. adult), then decreased to lower levels in the ileum of older animals (Fig. 4). In the colon, the levels of GLP-2R mRNA transcripts were most abundant at PD12 ( $P < 0.001$  vs. adult), followed by a progressive decrease to adult levels at PD12. In contrast, proglucagon gene expression in the colon was not significantly different at various stages of rat development.

The concomitant expression of GLP-2 and GLP-2R in neonatal rat gastrointestinal tissues prompted us to determine whether the GLP-2/GLP-2R axis was functional in the gas-

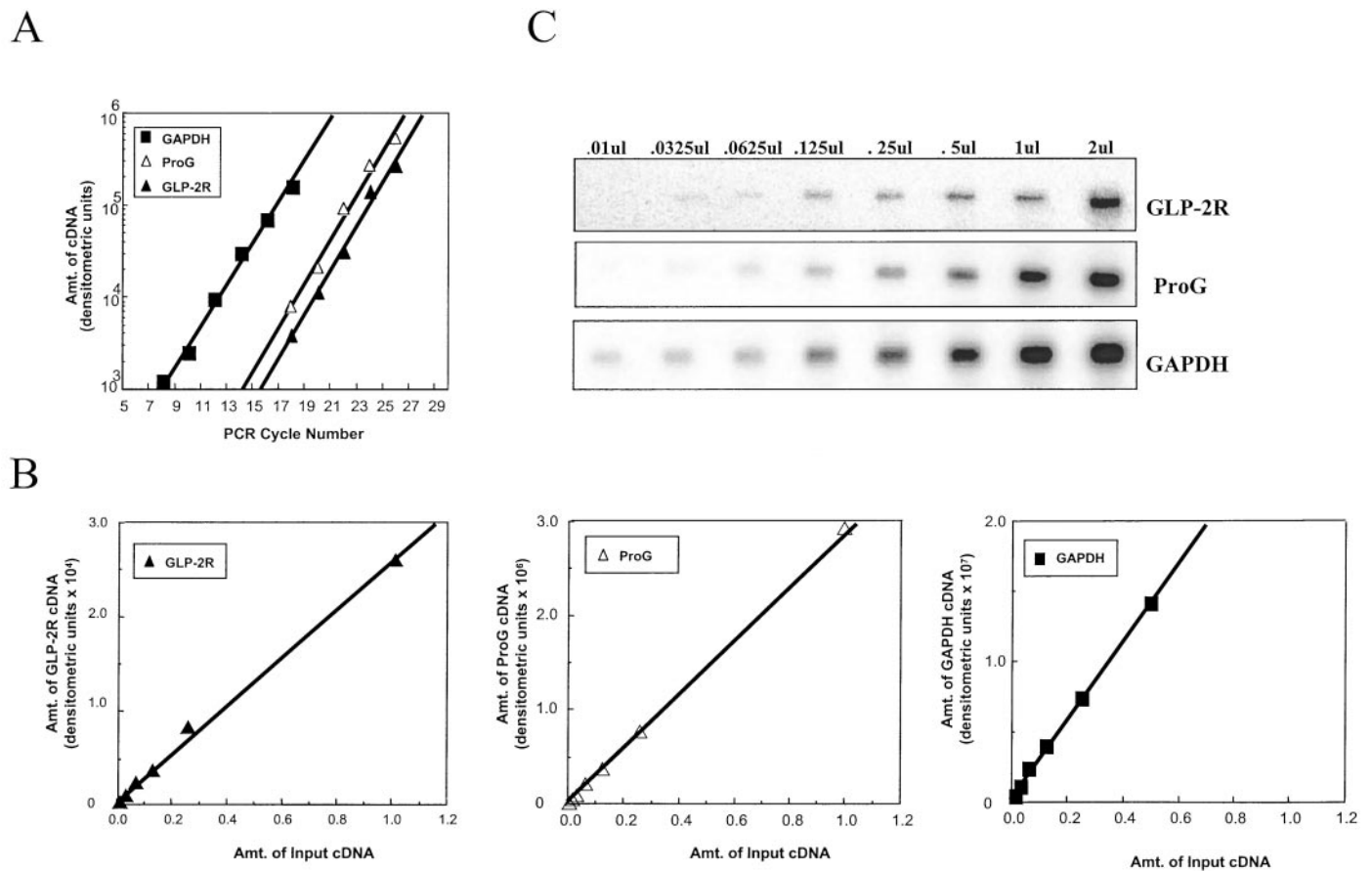


FIG. 3. Analysis of GLP-2R, proglucagon, and GAPDH RNA transcripts by RT-PCR. A, Semiquantitative relationship between PCR cycle number and amounts of PCR products for GLP-2R, Proglucagon and GAPDH RNA transcripts. B, Linear relationship between input cDNA and PCR product over a range of input cDNA levels for GLP-2R, proglucagon, and GAPDH RNA transcripts. C, Representative Southern blot analysis of RT-PCR reactions carried out over a range of cDNA concentrations for GLP-2R, proglucagon, and GAPDH.

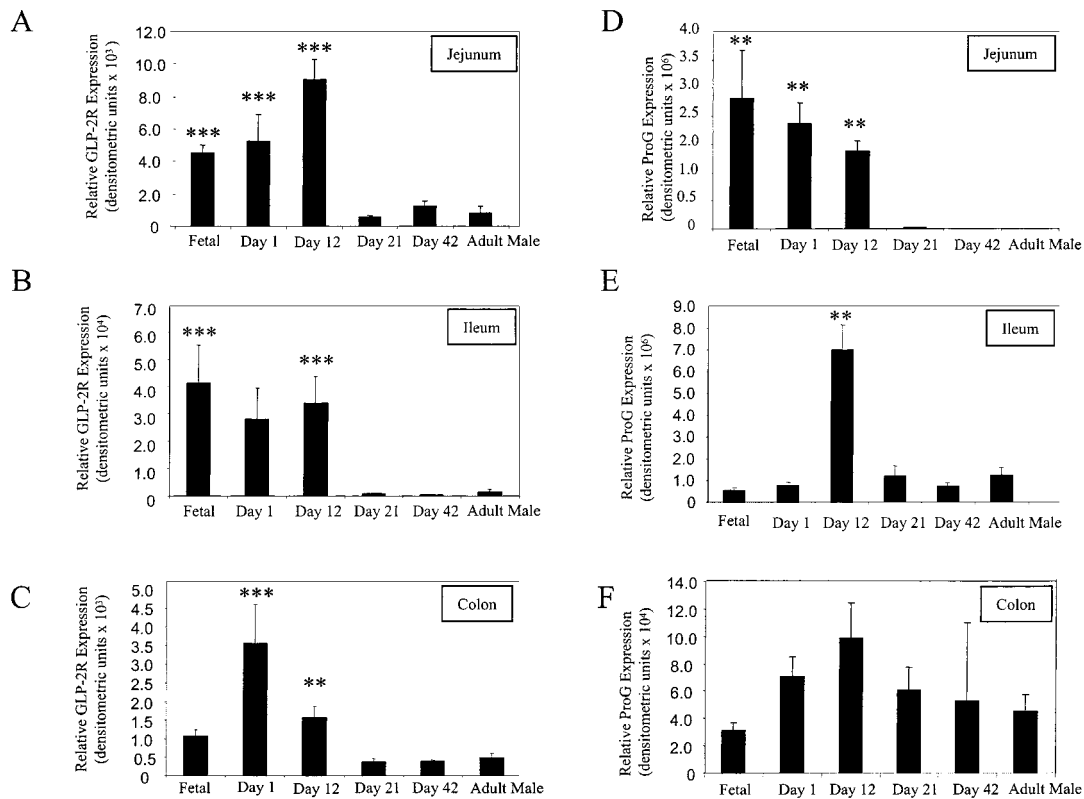


FIG. 4. Relative levels of GLP-2 receptor and proglucagon mRNA transcripts in developing rat gastrointestinal tissues. Tissue samples were collected as described in *Materials and Methods* from fetal (embryonic days 19/20), PD1, PD12, PD21, PD42, and adult rats. Day 1 rat intestinal samples were dissected between the small and large bowels. The relative expression of GLP-2R (A–C) and proglucagon (D–F) mRNA transcripts was expressed relative to the densitometric intensity of the GAPDH signal in the same PCR analysis. The ratios are expressed as the mean  $\pm$  SEM. Densitometry values were quantified using the Molecular Dynamics, Inc., PhosphorImager and ImageQuant. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  (vs. levels in adults).

traintestinal tract of the neonatal rat. Accordingly, separate litters of rats were injected once daily with either saline or h[Gly2]-GLP-2, a degradation-resistant human GLP-2 analog (25) for 10 days (Fig. 5). Analysis of intestinal tissues demonstrated a significant increase in stomach and small bowel weight and small bowel length in the h[Gly2]-GLP-2-treated rats ( $P < 0.05$ ; Fig. 5). The crypt and villus compartment appeared similar in control and h[Gly2]-GLP-2-treated rats (Fig. 5, E and F). In contrast, no increase in colon weight, but an increase in colon length, was detected after h[Gly2]-GLP-2 administration. These findings demonstrate that activation of the GLP-2/GLP-2 receptor axis is coupled to increased intestinal growth in the neonatal rat intestine.

### Discussion

The roles, if any, of glucagon and GLP-1 during fetal development are not known. The proglucagon gene is expressed in the fetal pancreas and intestine (13, 14), and both glucagon and GLP-1 are synthesized in the fetal pancreas and intestine, respectively (4, 31). The results of previous studies have detected GLP-2R expression in adult rats (15); however, the developmental ontogeny of GLP-2R expression in the developing gut has not yet been examined. The data presented here establish that the developing rat intestine is capable of synthesizing, secreting, and responding to the enterotrophic peptide GLP-2. These findings are in agreement

with recent studies demonstrating nutrient-dependent secretion of GLP-2 in the neonatal pig (32). As the GLP-2R is also expressed in the fetal and neonatal rat gastrointestinal tract, these findings raise the possibility that GLP-2 may play one or more roles during intestinal development and differentiation. Although the GLP-1 receptor is widely expressed in multiple tissues during murine development (30), whether GLP-1 plays a metabolic role in the fetus remains unknown. However, GLP-1 receptor<sup>-/-</sup> mice develop normally (33), precluding a major role for GLP-1 in the control of pattern formation or organ development. Similarly, although the glucagon receptor is expressed in the fetal liver (30), glucagon action, as assessed by adenylate cyclase stimulation, is markedly attenuated in fetal hepatocytes and develops postnatally (34).

Developmental analysis of enteroendocrine cell differentiation has established the presence of glucagon-immunoreactive fetal L cells in both small and large intestines (35–37). Although proglucagon-immunoreactive cells and proglucagon RNA transcripts are first detected by fetal day 14 in the rat gut (13), a major up-regulation in the levels of L cell density and intestinal proglucagon mRNA transcripts occurs between fetal days 17 and 18. Intriguingly, an intestinal profile of glucagon-like immunoreactive peptides is also first detected in the developing rat intestine between fetal days 17–19 (4, 38), suggesting that the molecular machinery re-

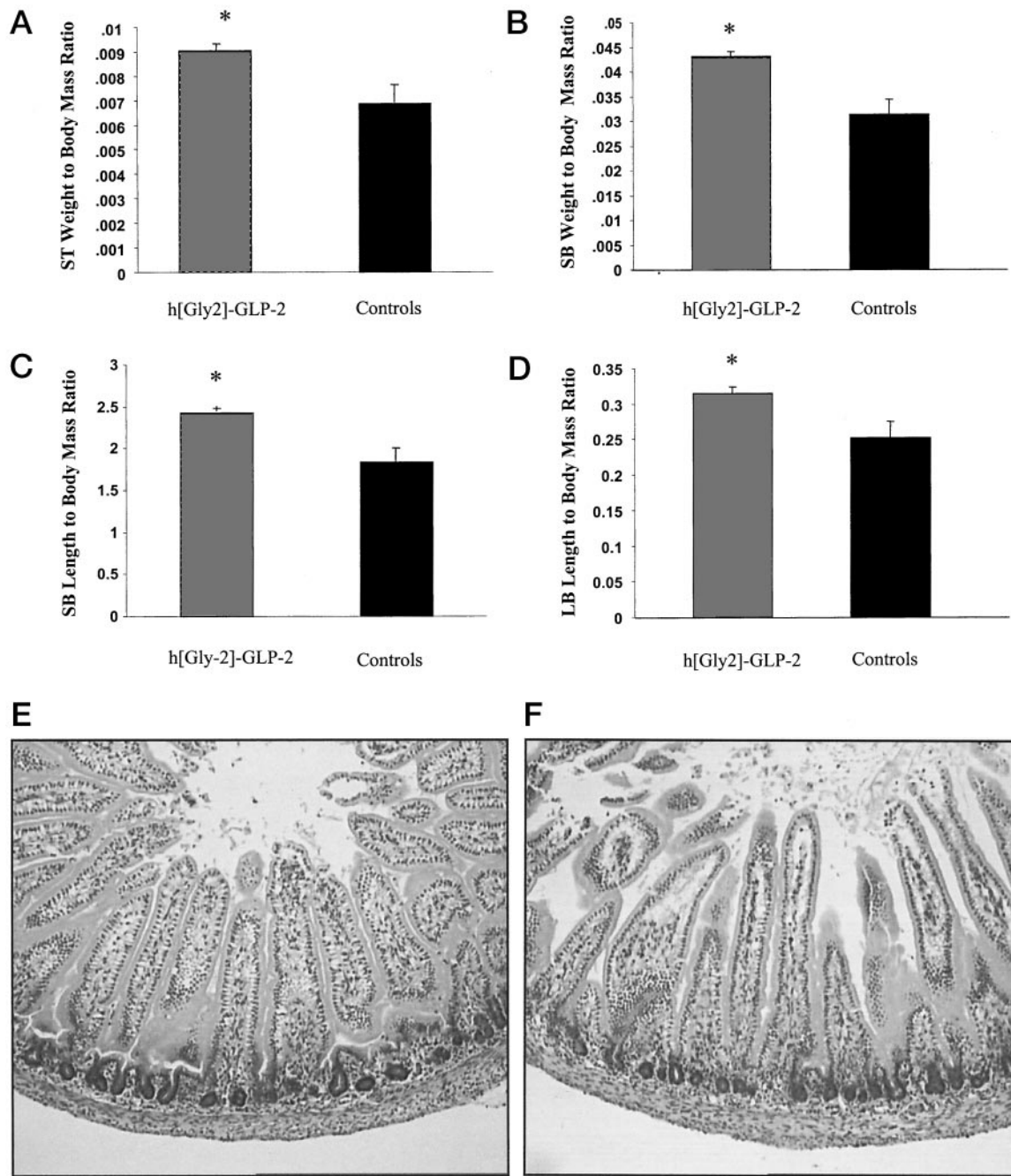


FIG. 5. Effect of daily h[Gly2]-GLP-2 administration in neonatal rats. Rat pups were administered either h[Gly2]-GLP-2 (5  $\mu$ g) or saline sc once daily for 10 days. On day 11 rats were killed for assessment of intestinal lengths and weights, which are expressed as a ratio after normalization to the body mass of each rat (in grams). The ratios are expressed as the mean  $\pm$  SEM (n = 14 for h[Gly2]-GLP-2 -treated and n = 11 for saline-treated groups for all parameters measured). A, For stomach weights: \*,  $P < 0.01$ , h[Gly2]-GLP-2 vs. saline-treated controls. B, For small bowel weights: \*,  $P < 0.001$ , h[Gly2]-GLP-2 vs. saline-treated controls. C, For small bowel lengths: \*,  $P < 0.001$ , h[Gly2]-GLP-2 vs. saline-treated controls. D, For large bowel lengths: \*,  $P < 0.01$ , h[Gly2]-GLP-2 vs. saline-treated controls. E and F, Histological sections from proximal jejunum of neonatal rats treated for 10 days with h[Gly2]-GLP-2 (E) or saline (F). ST, stomach; SB, small bowel; LB, large bowel.

quired for intestinal processing of proglucagon may be highly regulated at this stage of intestinal development. Consistent with these findings, prohormone convertase 1/3, the enzyme required for liberation of GLP-1 and GLP-2 from proglucagon (2, 39–41), is developmentally regulated and expressed in the intestine of the fetal rat (42). The finding that bioactive GLP-2<sup>1–33</sup> is synthesized in and secreted from fetal

rat intestinal endocrine cells taken together with the detection of GLP-2R mRNA transcripts in fetal intestine, raise the possibility that the GLP-2/GLP-2R axis may also be functional during the period of rapid intestinal development *in utero*.

GLP-2 administration to adult rats and mice promotes crypt cell proliferation and inhibits apoptosis, leading to

expansion of the mucosal epithelium (7, 23, 25); however, the putative action of GLP-2 in the fetal or neonatal gut has remained unclear. Although we have demonstrated the presence of both bioactive GLP-2 and GLP-2R mRNA transcripts in the rat fetal gut, our data do not allow us to make specific inferences about the biological role, if any, of GLP-2 during fetal intestinal development. We recently determined that *pax6*<sup>SEY<sup>neu</sup> mice exhibit a marked deficiency of intestinal GLP-2-producing L cells and a greater than 95% reduction in intestinal proglucagon mRNA transcripts (43). Nevertheless, these mutant proglucagon-deficient mice exhibit apparently normal intestinal development (43), strongly suggesting that normal levels of intestinal GLP-2 are not essential for development of the fetal murine intestine.</sup>

The relative levels of GLP-2R expression were comparatively greater in fetal and neonatal rat intestines and declined to lower levels in older animals. Whether these differences reflect changes in GLP-2R transcription and/or RNA stability or developmental differences in the numbers of GLP-2R-positive cells cannot presently be determined, as the cellular localization of GLP-2R expression in the rat intestine has not yet been reported. As the developing intestine undergoes a complex series of molecular changes in the growth, differentiation, and function of the intestinal epithelium in response to enteral nutrition (44), it seems likely that gut peptides such as GLP-2 that are secreted in a nutrient-dependent manner may contribute to the development and maturation process of the neonatal intestinal epithelium *in vivo*. Although previous experiments demonstrated that GLP-2 is trophic to the mucosal epithelium of 4- to 5-week-old mice (7), the data presented here extend the window of intestinotropic GLP-2 action to the immediate neonatal period in the developing rat gut. Whether GLP-2 is essential for one or more aspects of intestinal adaptation in the transition from the neonatal to adult intestinal epithelium awaits the development of specific GLP-2 antagonists or a GLP-2R knockout mouse.

As the fetal and neonatal intestinal epithelium remains comparatively immature, exhibits defective barrier function, and continues to develop postnatally (44), it remains highly susceptible to external injury. Indeed, premature infants are prone to the development of necrotizing enteritis, a disease characterized by necrotizing infection and destruction of the intestinal mucosa that frequently requires surgical intervention and prolonged hospitalization in the neonatal intensive care unit (45). Although GLP-2 reduces bacterial infection (46) and decreases mucosal epithelial permeability in adult mice, whether GLP-2 exhibits similar actions in the premature developing human gut remains unknown. Nevertheless, our demonstration that the rat intestinal GLP-2/GLP-2R axis is present and functional during the neonatal period suggests that a role for GLP-2 in the prevention or treatment of neonatal intestinal injury be examined in future experiments.

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