Intestinal response to growth factors administered alone or in combination with human [Gly^2]glucagon-like peptide 2

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Drucker, Daniel J., Lorraine DeForest, and Patricia L. Brubaker. Intestinal response to growth factors administered alone or in combination with human [Gly^2]glucagon-like peptide 2. *Am. J. Physiol.* 273 (*Gastrointest. Liver Physiol.* 36): G1252–G1262, 1997.—The control of intestinal epithelial growth is regulated by interactions of growth factors in various cellular compartments of the small and large bowel. Little information is available on the intestinal growth response to combinations of growth factors. We studied the intestinotrophic properties of a dipeptidyl peptidase IV resistant glucagon-like peptide 2 (GLP-2) analog, human [Gly^2]GLP-2 (h[Gly^2]GLP-2), as well as of epidermal growth factor (EGF), long [Arg^3]insulin-like growth factor I (LR^{3}IGF-I), [Gly^1]IGF-II, and human growth hormone (hGH), administered by subcutaneous injection alone or in combination in mice. At the doses tested, h[Gly^2]GLP-2 was the most potent agent for increasing small and large bowel mass. Mice treated with h[Gly^2]GLP-2 and either GH or IGF-I exhibited greater increases in histological parameters of small intestinal growth than did mice treated with h[Gly^2]GLP-2 alone. Administration of all five growth factors together induced significant increases in crypt plus villus height and in small and large bowel length and weight. The results of these experiments define regional differences in both the cellular targets and relative activities of intestinotrophic molecules and raise the possibility that selective growth factor combinations may be useful for enhancement of intestinal adaptation in vivo.

protein; bowel; gut; adaptation; mouse

THE CAPACITY OF THE MUCOSAL epithelium of the small intestine for rapid renewal and adaptation after injury or resection has focused interest on the factors important for regulation of epithelial proliferation. The control of crypt cell proliferation and apoptosis and expansion of the enterocyte mass leading to intestinal growth are regulated via a complex interplay of nutrients, pancreatic and biliary secretions, and both locally derived and circulating growth factors. Studies of hypophysectomized rodents demonstrating a reduction in the mass and rate of cellular proliferation in the small bowel (41) and of transgenic mice with growth hormone (GH) excess and increased bowel mass support the importance of GH as an intestinotrophic molecule (34). Consistent with the intestinotrophic properties of GH, insulin-like growth factor I (IGF-I) has also been shown to increase crypt cell proliferation, leading to increased intestinal mass in both mice and rats (15, 18, 39). The observation that IGF-I, but not GH (34), stimulates crypt cell proliferation suggests that the effects of GH in the bowel may not be completely dependent on IGF-I action. Although IGF-II may display weak trophic properties in the gastrointestinal tract (40), its role as a growth factor for adult small intestine has not been extensively studied.

Peptides that signal through the epidermal growth factor (EGF) receptor, EGF and transforming growth factor-α (TGF-α), are also known to be trophic for the small intestinal epithelium. Disruption of EGF receptor signaling via homologous recombination results in mice with abnormal hemorrhagic and distended intestines, with shorter and fewer villi and a thinner muscle layer (17). EGF, whether administered orally or injected parenterally, is trophic for the intestinal mucosa and stimulates crypt cell proliferation in vivo (16, 21, 35). TGF-α is expressed locally in the intestinal epithelium (2) and stimulates intestinal epithelial proliferation (21), and increased TGF-α expression in the villus epithelium occurs consequent to intestinal injury in rats (9).

The proglucagon-derived peptides (PGDPs) are produced in enteroendocrine L cells in both the large and small bowel, with the highest density of cells localized to the terminal ileum. Several lines of evidence suggest a link between gut-derived PGDPs and small bowel growth. Increased proglucagon gene expression and circulating levels of the PGDPs are observed subsequent to the induction of intestinal injury or resection (3, 4, 14, 30). Our observation (10) that mice implanted with subcutaneous glucagonomas developed small bowel growth led to the identification of glucagon-like peptide 2 (GLP-2) as the PGDP with intestinotrophic activity. GLP-2, a 33-amino acid peptide located COOH-terminal to GLP-1 in the proglucagon sequence, is cosecreted along with glicentin, oxyntomodulin, and GLP-1 from L cells of the bowel.

The increasing number of growth factors with intestinotrophic activity raises the possibility that one or more of these peptides may be useful for regeneration of intestinal epithelium in human diseases characterized by intestinal insufficiency in vivo. Administration of GH and glutamine, in combination with a specialized diet, to patients with short bowel syndrome appears to enhance intestinal adaptation (6), and EGF administration to an infant with necrotizing enterocolitis was associated with intestinal regeneration and clinical recovery (27). The majority of studies examining growth factor activity in the bowel have utilized a single trophic agent, and there are surprisingly few data available regarding the comparative efficacy of two or more growth factors for stimulation of intestinal growth. Furthermore, little comparative information exists on the relative changes in specific intestinal cellular compartments that ensue after treatment with various growth factors in the same animal model. To understand the relative biological activities of GLP-2 in the

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intestine, we have analyzed the gastrointestinal tract of mice treated with a novel degradation-resistant GLP-2 analog alone or in combination with EGF, IGF-I, IGF-II, or GH in vivo.

METHODS

Experimental protocol. Six-week-old female CD1 mice (~22–25 g; obtained from Charles River Canada) were housed in plastic-bottom wire-lid cages, maintained on a 12:12-h light-dark cycle, and allowed access to chow and water ad libitum throughout the 10–14 day treatment period. Each treatment group consisted of four mice housed together, for a total of 11–12 experimental groups. Peptides were administered by subcutaneous injection twice a day. Mice were fasted for ~14 h before being killed by CO_2 anesthesia. Body weight measurements were made on *days 4*, *8*, *12*, and *15*. Mice were killed on *day 15*. The length of the small and large bowel was measured under constant tension. Blood was taken via cardiac puncture at the time of death, and tissues were removed for histological analysis and measurement of enzyme activity, bowel mass, and PGDP content, as previously described (5, 32, 33). All animal experiments were performed in accordance with the local animal welfare guidelines of the Toronto Hospital Animal Care Committee.

Peptides and reagents. For the experiments shown in Fig. 1, 12 separate treatment groups were established as follows: *group 1*, control [phosphate-buffered saline (PBS) injected bid]; *group 2*, 2.5 μg native rat GLP-2 bid; *group 3*, 2.5 μg human [Gly^2]GLP-2 bid (h[Gly^2]GLP-2); *group 4*, 40 μg native IGF-I bid; *group 5*, 25 μg/day human GH (hGH; Humatrope); *group 6*, 40 μg long [Arg^3]IGF-I bid (LR^3IGF-I); *group 7*, 40 μg native IGF-I bid and 2.5 μg GLP-2 bid; *group 8*, 25 μg/day GH and 2.5 μg GLP-2 bid; *group 9*, 40 μg LR^3IGF-I bid and 2.5 μg GLP-2 bid; *group 10*, 40 μg native IGF-I bid and 2.5 μg h[Gly^2]GLP-2 bid; *group 11*, 25 μg/day hGH and 2.5 μg h[Gly^2]GLP-2 bid; and *group 12*, 40 μg LR^3IGF-I bid and 2.5 μg h[Gly^2]GLP-2 bid.

For all other experimental results shown, 11 separate treatment groups were established as follows: *group 1*, control (PBS injected bid); *group 2*, 2.5 μg h[Gly^2]GLP-2 bid; *group 3*, 1 μg EGF bid; *group 4*, 25 μg LR^3IGF-I bid; *group 5*, 40 μg [Gly^1]IGF-II bid; *group 6*, 25 μg hGH bid (Humatrope); *group 7*, 2.5 μg h[Gly^2]GLP-2 bid and 1 μg EGF bid; *group 8*, 2.5 μg h[Gly^2]GLP-2 bid and 25 μg LR^3IGF-I bid; *group 9*, 2.5 μg h[Gly^2]GLP-2 bid and 40 μg [Gly^1]IGF-II bid; *group 10*, 2.5 μg h[Gly^2]GLP-2 bid and 25 μg hGH bid; and *group 11*, 2.5 μg h[Gly^2]GLP-2 bid, 1 μg EGF bid, 25 μg LR^3IGF-I bid, 40 μg [Gly^1]IGF-II bid, and 25 μg hGH bid.

[Gly^2]GLP-2 and rat GLP-2 were obtained as a custom synthesis from California Peptide Research and were 97% pure by high-performance liquid chromatography. EGF was obtained from Allelix Biopharmaceuticals (Allelix Biopharmaceuticals, Mississauga, ON, Canada). LR^3IGF-I, native IGF-I, and [Gly^1]IGF-II were from GroPep (Adelaide, Australia). hGH (Humatrope) was from Eli Lilly (Toronto, ON, Canada). Peptides were dissolved according to the manufacturer's suggested protocol, and all peptide injections were carried out using a U-100 insulin syringe in a total volume of 0.5 ml PBS. All other chemicals and reagents were obtained from Sigma Chemical (St. Louis, MO) or Baxter Travenol Canada (Toronto, ON, Canada).

Histology. Sections of the small and large bowel were taken for histological analysis. Three segments of the small bowel were obtained from each mouse: the proximal jejunum (8 cm distal to the pylorus), the distal jejunum (18 cm distal to the pylorus), and the distal ileum (10 cm before the cecum). The large bowel was taken immediately distal to the cecum. The tissues were fixed in 10% buffered Formalin for <24 h and embedded in paraffin using standard techniques. We cut 4- to 6-μm cross sections and stained them with hematoxylin and eosin and performed intestinal micrometry using the Leica Q500MC Image Analysis system as previously described (10, 32). At least 10 well-oriented sections (for each mouse) from each intestinal region were used to determine villus height and crypt depth.

Radioimmunoassay. Five-centimeter sections of ileum were homogenized in 1 N HCl containing 5% HCOOH, 1% trifluoroacetic acid, and 1% NaCl, and peptides were extracted by reverse-phase adsorption to a C18 Sep-Pak (Waters Associates, Milford, MA). PGDPs were detected by radioimmunoassay to glucagon-like immunoreactivity (GLI) as previously described (8). Total protein content of the extract was determined by Lowry protein assay.

Maltase assay. Five centimeters of duodenum were homogenized in sodium phosphate buffer, pH 7.0. Maltase activity was determined as previously described (5). In brief, intestinal homogenates were incubated with maltose for 1 h at 37°C, after which glucose production from the maltose was determined using the glucose oxidase method. Total homogenate protein content was determined by Lowry protein assay.

Statistics. Statistical analysis was carried out by analysis of variance with $n - 1$ custom hypotheses tests using the Statistical Analysis Systems program for IBM personal computers. For mice treated with only one growth factor, analyses were performed comparing the PBS-injected control group with the growth factor-injected mice. For mice treated with two or more growth factors, analysis was carried out comparing h[Gly^2]GLP-2-treated mice with animals treated with h[Gly^2]GLP-2 plus one or more growth factors. For descriptive purposes, "additive" denotes a result equal to the sum of two or more individual separate results and "synergistic" denotes a result greater than simply the sum of two or more individual values.

RESULTS

To determine whether combinations of growth factors might produce additional increases in bowel mass above that obtained with a single growth factor alone, we initially treated mice with either native rat GLP-2, an analog of human GLP-2 (h[Gly^2]GLP-2), hGH, native human IGF-I, or an analog of IGF-I (LR^3IGF-I). Additional groups of mice were treated with either rat GLP-2 or h[Gly^2]GLP-2 together with either GH, native IGF-I, or LR^3IGF-I. The effects of these growth factors on small and large bowel weight are shown in Fig. 1. The greatest increase in small bowel mass in mice treated with a single agent was observed in the group treated with h[Gly^2]GLP-2 ($P < 0.001$). Remarkably, a statistically significant increase in large bowel mass was also detected with h[Gly^2]GLP-2 ($P < 0.05$). Furthermore, the combination of either IGF-I, LR^3IGF-I, or GH and h[Gly^2]GLP-2 produced a greater increase in large bowel mass than found in mice treated with h[Gly^2]GLP-2 alone ($P < 0.05$–0.001; Fig. 1). Normalization of the data to body weight demonstrated that the increase in large bowel mass observed in the mice treated with combinations of growth factors was still statistically significant ($P < 0.05$–0.01).

To elucidate in greater detail the comparative effects of growth factor stimulation in the mouse intestine,

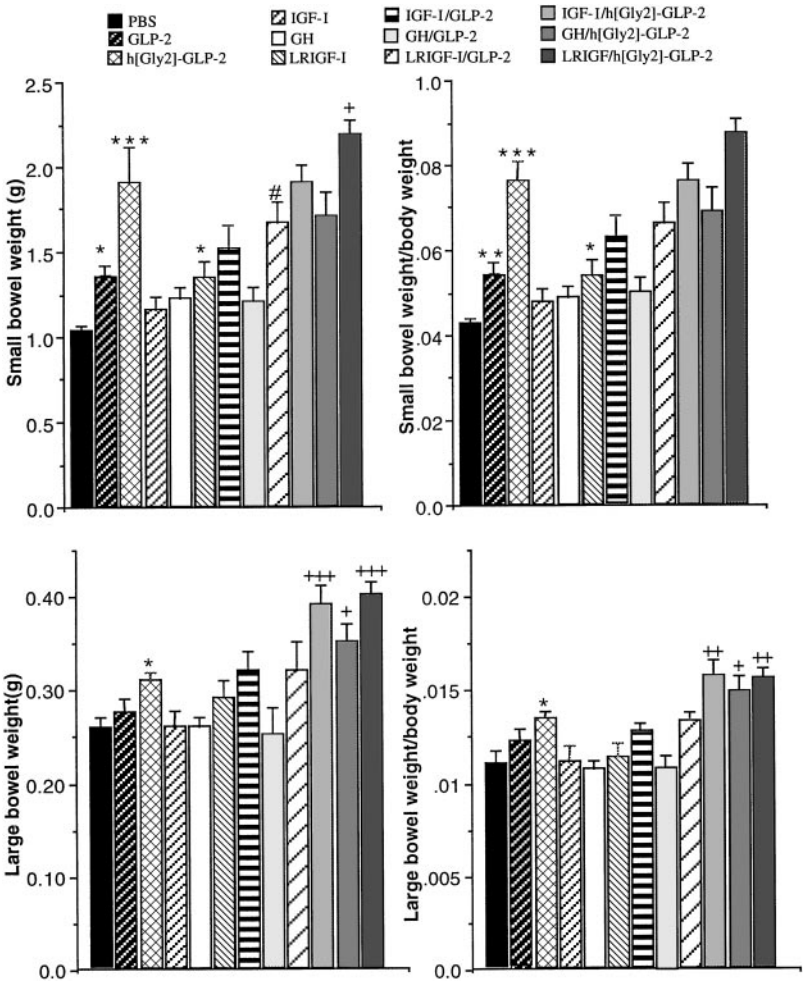

Fig. 1. Small and large bowel weight in grams or normalized to body weight in mice treated with growth factors for 10 days. Data are expressed as means ± SE. PBS, phosphate-buffered saline; GLP-2, glucagon-like peptide 2; h[Gly^2]GLP-2, human [Gly^2]GLP-2; IGF-I, insulin-like growth factor I; GH, growth hormone; LRIGF-I, long [Arg^3]IGF-I (LR^{3}IGF-I). *$P < 0.05$, **$P <$ 0.01, and ***$P < 0.001$ for PBS-treated vs. single growth factor-treated mice. #$P < 0.05$ for GLP-2-treated vs. GLP-2 + LR^{3}IGF-I-treated mice. +$P < 0.05$, ++$P < 0.01$, and +++$P < 0.001$ for h[Gly^2]GLP-2-treated vs. h[Gly^2]GLP-2 + 1 or more growth factor-treated mice.

groups of mice were treated with h[Gly^2]GLP-2, EGF, LR^{3}IGF-I, a position 1-substituted IGF-II analog ([Gly^1] IGF-II), and hGH, at concentrations of peptides previously used to induce intestinal growth in vivo (1, 26, 31). Additional groups of mice were treated with h[Gly^2]GLP-2 and either EGF, IGF-I (LR^{3}IGF-I), [Gly^1] IGF-II, or hGH, and a separate group of mice was treated with a mixture of all five peptide growth factors. For descriptive purposes below, LR^{3}IGF-I and [Gly^1]IGF-II are referred to as IGF-I and IGF-II, respectively. Mice treated with PBS injections alone for 14 days (control) had a mean body weight gain (Fig. 2*D*) of 1.35 ± 0.5 g vs. 2.6 ± 0.8 and 3.0 ± 0.6 g for h[Gly^2]GLP-2- and IGF-I-treated animals, respectively ($P < 0.05$ for IGF-I-treated mice). In experiments with a single agent, GH-treated mice exhibited the greatest change in weight (3.3 ± 0.3 g, $P < 0.01$; Fig. 2*D*). Mice treated with either EGF or IGF-II had the smallest weight gain (Fig. 2*D*), whereas the greatest increase in weight was observed in mice treated with all five peptide growth factors (6.1 ± 0.7 g, $P < 0.001$).

Small bowel mass increased after treatment with h[Gly^2]GLP-2, IGF-I, and GH (Fig. 2*A*). The greatest increase in small bowel weight was observed in the h[Gly^2]GLP-2-treated mice ($P < 0.001$, h[Gly^2]GLP-2 vs. control). In contrast, mice treated with EGF or IGF-II did not exhibit increases in small bowel weight. Administration of h[Gly^2]GLP-2 together with either IGF-I, GH, IGF-II, or EGF did not produce additive or synergistic increases in small bowel weight. Nevertheless, mice treated with h[Gly^2]GLP-2 and either IGF-I or GH exhibited greater increases in small bowel mass than mice treated with h[Gly^2]GLP-2 alone ($P < 0.05$). The greatest increase in small bowel weight (>2-fold increase compared with PBS-injected mice) was detected in mice treated with all five growth factors ($P <$ 0.001; Fig. 2*A*). As several of the growth factors are known to produce generalized increases in organ size and body mass (Fig. 2*D*), the small bowel data were normalized for body weight (Fig. 2*B*). The results demonstrate that of the mice treated with a single agent, only mice treated with h[Gly^2]GLP-2 exhibited a significant increase in small bowel weight relative to change in body mass ($P < 0.001$). Interestingly, the increase in bowel mass in mice treated with all five growth factors was still statistically significant (compared with h[Gly^2]GLP-2 alone) after normalization to body weight ($P < 0.05$). For comparative purposes, the relative change in total kidney weight is shown in Fig. 2*C*. In contrast to the marked increase in small bowel weight, no increases in kidney weight were observed in the h[Gly^2]GLP-2-treated mice.

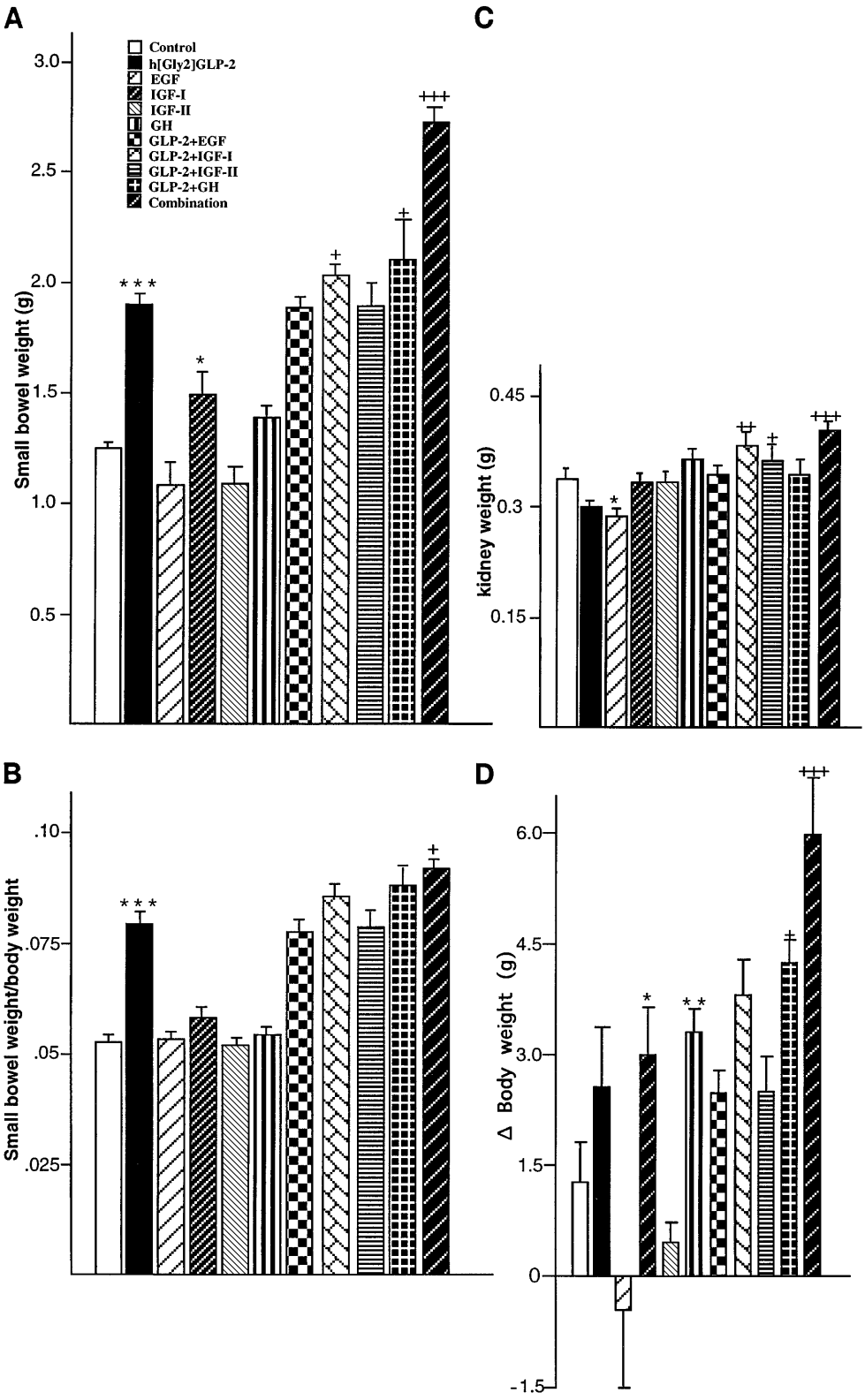

Fig. 2. *A* and *B*: small bowel weight in grams or normalized to body weight in mice treated with growth factors for 14 days. Data are means ± SE; $n = 4$ mice/treatment group. Combination, mice treated with all 5 growth factors. IGF-I is LR^3IGF-I; IGF-II is [Gly^1]IGF-II. *C*: kidney weight (both kidneys) after 2 wk in growth factor-treated mice. *D*: change in body weight over a 2-wk period in growth factor-treated mice. *A-D*: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ for PBS-treated vs. single growth factor-treated mice. $+P < 0.05$, $++P < 0.01$, and $+++P < 0.001$ for h[Gly^2]GLP-2-treated vs. h[Gly^2]GLP-2 + 1 or more growth factor-treated mice.

Whereas mouse small bowel weight increased after treatment with h[Gly^2]GLP-2, IGF-I, or GH, but not EGF or IGF-II, histological assessment of small bowel villus height (Fig. 3) in growth factor-treated mice demonstrated region-specific increases in the height of the mucosal villus epithelium in all groups of growth factor-treated mice (Fig. 3). Whereas GH produced a statistically significant increase ($P < 0.05$) in villus height in the proximal and distal jejunum, GH alone did not induce significant changes in villus height in the ileum. IGF-I induced a significant increase in villus height only in the distal jejunum ($P < 0.05$). In con-

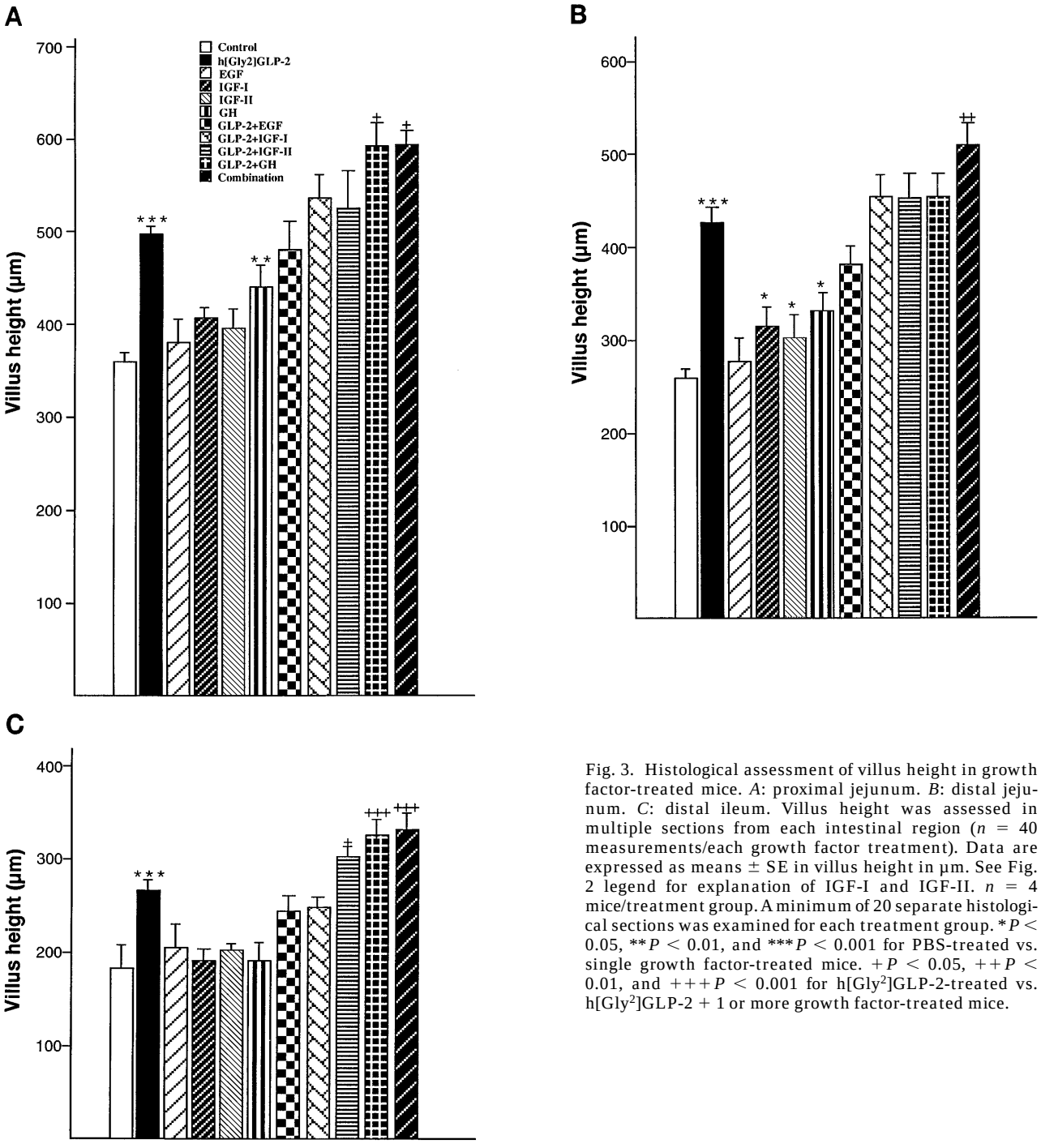

Fig. 3. Histological assessment of villus height in growth factor-treated mice. *A*: proximal jejunum. *B*: distal jejunum. *C*: distal ileum. Villus height was assessed in multiple sections from each intestinal region (n = 40 measurements/each growth factor treatment). Data are expressed as means ± SE in villus height in μm. See Fig. 2 legend for explanation of IGF-I and IGF-II. n = 4 mice/treatment group. A minimum of 20 separate histological sections was examined for each treatment group. *$P <$ 0.05, **$P < 0.01$, and ***$P < 0.001$ for PBS-treated vs. single growth factor-treated mice. +$P < 0.05$, ++$P <$ 0.01, and +++$P < 0.001$ for h[Gly^2]GLP-2-treated vs. h[Gly^2]GLP-2 + 1 or more growth factor-treated mice.

trast, h[Gly^2]GLP-2-treated mice exhibited statistically significant increases in the villus height of the proximal and distal jejunum and the distal ileum ($P < 0.001$, control vs. h[Gly^2]GLP-2-treated mice; Fig. 3). The increase in villus height observed in mice treated with h[Gly^2]GLP-2 and GH was also significantly greater than that observed for h[Gly^2]GLP-2 alone in the proximal jejunum ($P < 0.05$; Fig. 3*A*) and distal ileum ($P < 0.001$; Fig. 3*C*) but not in the distal jejunum (Fig. 3*B*). Mice treated with a combination of all five growth factors exhibited the greatest increase in mucosal villus height, which was statistically significant (compared with h[Gly^2]GLP-2 alone) in all regions of the small intestine ($P < 0.05$–0.001; Fig. 3). Taken together, these observations illustrate that various regions of the small bowel epithelial mucosa exhibit differential sensitivity to the trophic effects of growth factors administered alone or in combination in vivo.

The relative changes in small bowel crypt depth after growth factor administration are shown in Fig. 4. Only h[Gly^2]GLP-2 alone induced a significant increase in crypt depth in the small intestine. Consistent with the changes observed for villus height, the combination of h[Gly^2]GLP-2 and GH produced significant increases in

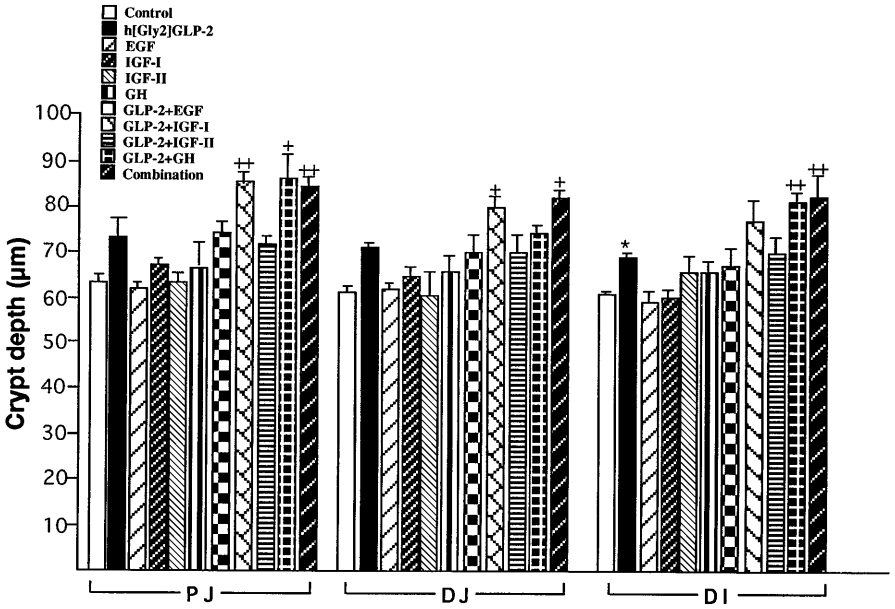

Fig. 4. Crypt depth in growth factor-treated mice. PJ, proximal jejunum; DJ, distal jejunum; DI, distal ileum. n = 4 mice/treatment group. A minimum of 20 separate histological sections was examined for each treatment group. See Fig. 2 legend for explanation of IGF-I and IGF-II. *$P < 0.05$ for PBS-treated vs. single growth factor-treated mice. +$P <$ 0.05 and ++$P < 0.01$ for h[Gly^2]GLP-2-treated vs. h[Gly^2]GLP-2 + 1 or more growth factor-treated mice.

crypt depth in the proximal jejunum and distal ileum compared with mice treated with h[Gly^2]GLP-2 alone. In contrast to the relative changes observed for villus height, the combination of h[Gly^2]GLP-2 and IGF-I produced additive increases in jejunal crypt depth that were statistically significant ($P < 0.05$–0.01; Fig. 4). These observations provide further evidence in support of region-specific differences in the intestinal response to growth factor activity.

Of the mice injected with a single growth-promoting agent, only h[Gly^2]GLP-2 significantly increased large bowel weight ($P < 0.05$; Fig. 5*A*), consistent with the data shown in Fig. 1. Remarkably, mice treated with EGF or IGF-II exhibited a statistically significant decrease in large bowel weight ($P < 0.05$). A significant increase in large bowel weight was observed in mice treated with all five growth factors ($P < 0.001$; Fig. 5*A*), and this increase remained significant (compared with h[Gly^2]GLP-2) even after normalization for the increase in body weight ($P < 0.05$; Fig. 5*B*). A similar profile of changes in large bowel crypt depth was observed after growth factor administration (Fig. 5*C*), with an increase in mice treated with h[Gly^2]GLP-2 ($P < 0.001$) and a decrease observed in mice treated with EGF or IGF-II (Fig. 5*C*). The combination of h[Gly^2]GLP-2 plus EGF resulted in a smaller increase in crypt depth compared with mice treated with h[Gly^2]GLP-2 alone. An increase in colon crypt depth was also observed in mice treated with all five growth factors ($P < 0.05$), consistent with the data obtained for large bowel weight.

Only GH produced a significant increase in small bowel length. The combination of h[Gly^2]GLP-2 plus either IGF-I or GH produced an increase in small bowel length that was greater than the increase observed after h[Gly^2]GLP-2 alone ($P < 0.05$–0.01; Fig. 6*A*). None of the growth factors alone produced a significant increase in large bowel length; however, as was the case for small bowel length, administration of h[Gly^2]GLP-2 plus GH or IGF-I or administration of all five growth factors together was associated with a significant increase in length of the large bowel compared with mice treated with h[Gly^2]GLP-2 alone ($P < 0.05$–0.001; Fig. 6*B*).

In contrast to the trophic effects detected in the villus epithelium and crypts, mice treated with subcutaneous growth factor injection did not exhibit significant increases in the muscularis layers of the jejunum or colon (Fig. 7). Significant decreases in jejunal muscle thickness were observed in mice treated with EGF ($P < 0.001$), and decreases in colon muscle thickness were detected in mice treated with EGF, IGF-I, IGF-II, and GH (Fig. 7).

The results of previous studies demonstrated increased maltase expression in the duodenum of GLP-2-treated mice; however, correction for increased protein content resulted in comparable amounts of maltase activity in control and GLP-2-treated mice (5). Similarly, no significant changes in duodenal maltase activity were detected after growth factor administration (Fig. 8). Analysis of ileal GLI content, a reflection of intestinal PGDP synthesis, demonstrated no significant induction of GLI synthesis after 2 wk of growth factor administration (Fig. 9). No suppression of GLI was detected in mice treated with h[Gly^2]GLP-2, strongly suggesting that GLP-2 itself does not mediate inhibition of intestinal PGDP biosynthesis. In contrast, mice treated with EGF alone exhibited a reduction in ileal GLI (46% of control, $P < 0.05$).

DISCUSSION

The results of experiments using the 33-amino acid native rat GLP-2 molecule have demonstrated that this peptide is intestinotrophic in mice, with increases in small bowel crypt cell proliferation detected after a single GLP-2 injection (32). Subsequent studies (11) demonstrated that comparable doses of native rat and

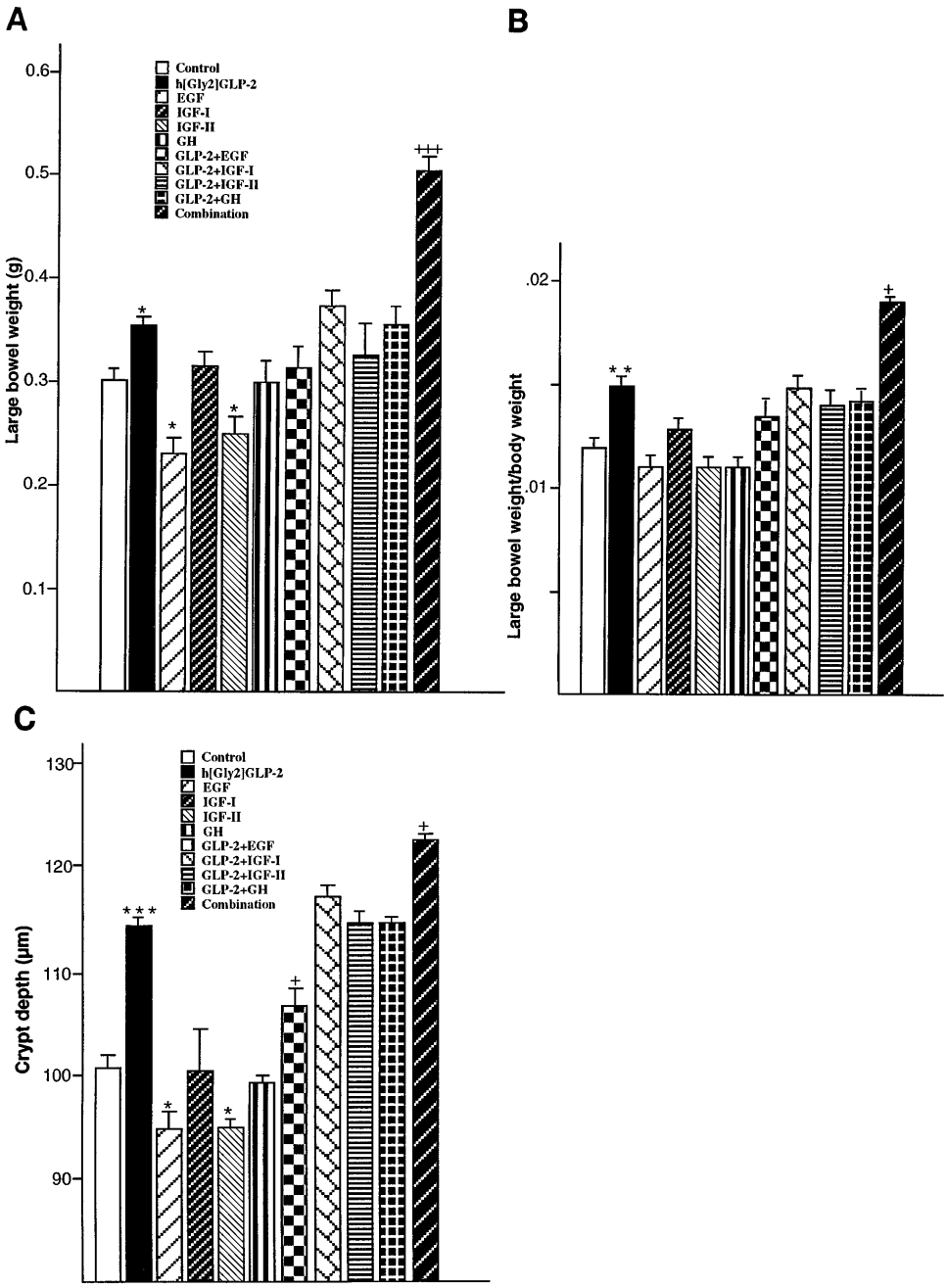

Fig. 5. *A*: large bowel weight in growth factor-treated mice. The large bowel was excised (not including the appendix) and cleaned, and data shown are means ± SE. *B*: large bowel weight normalized to body weight in growth factor-treated mice. *C*: crypt height expressed as means ± SE in μm of colonic mucosa in growth factor-treated mice. $n = 4$ mice/treatment group. A minimum of 20 separate histological sections was examined for each treatment group. See Fig. 2 legend for explanation of IGF-I and IGF-II. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ for PBS-treated vs. single growth factor-treated mice. $+P < 0.05$ and $+++P < 0.001$ for h[Gly^2]GLP-2-treated vs. h[Gly^2]GLP-2 + 1 or more growth factor-treated mice.

human GLP-2 are less intestinotrophic in vivo in rats compared with GLP-2 derivatives containing position 2 substitutions that confer resistance to degradation by the enzyme dipeptidyl peptidase IV (DPP-IV). The data presented here extend these observations and demonstrate that h[Gly^2]GLP-2 also exhibits intestinotrophic properties in mice. Native GLP-2 consistently induced a 1.3–1.5-fold induction of small bowel weight in 10- to 14-day experiments (32, 33). The data reported here with the DPP-IV-resistant analog h[Gly^2]GLP-2 (using doses of peptide identical to that utilized in experiments with native GLP-2, 0.1 mg/kg twice daily) demonstrate a 1.8-fold induction of small bowel weight in a comparable experiment. These observations suggest that on a molar basis, the DPP-IV-resistant analog of GLP-2, h[Gly^2]GLP-2, is also more potent than native GLP-2 in mice in vivo, in keeping with recent studies of the relative potency of this analog in rats (11).

Analysis of mice treated with low doses of native rat GLP-2 did not demonstrate marked changes in large bowel weight using doses of peptide sufficient to induce an increase in small bowel mass (32, 33). Nevertheless, mice treated with native GLP-2 for 10 days did exhibit a statistically significant increase in protein content of the mouse colon, consistent with a direct effect of GLP-2 on the large bowel (32). The data reported here clearly extend these observations by demonstrating that mice treated with h[Gly^2]GLP-2 exhibit a statistically significant increase in both large bowel weight and epithelial crypt depth, strongly implicating a role for GLP-2 in the

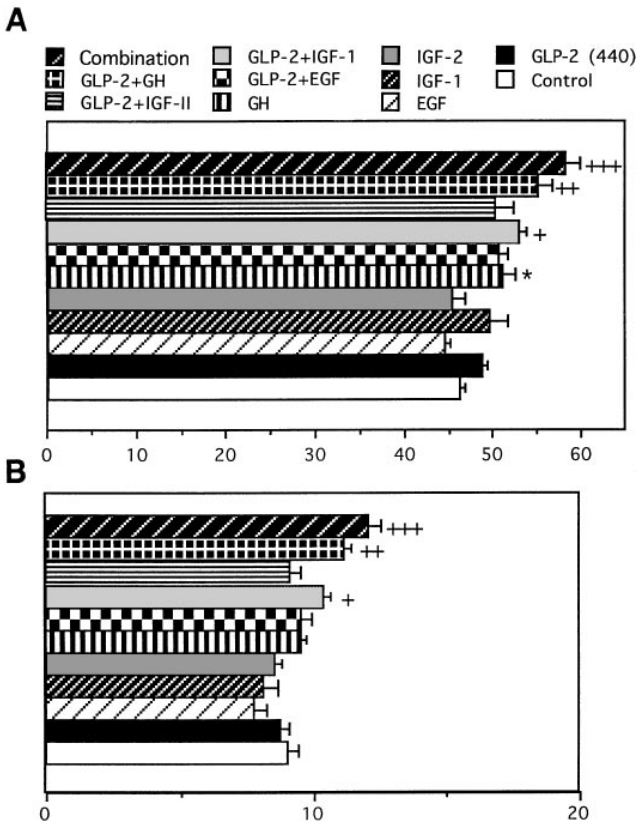

Fig. 6. Small (*A*) and large (*B*) bowel length (means ± SE in cm) in growth factor-treated mice. GLP-2, h[Gly^2]GLP-2. n = 4 mice/treatment group. See Fig. 2 legend for explanation of IGF-I and IGF-II. *$P < 0.05$ for PBS-treated vs. single growth factor-treated mice. +$P < 0.05$, ++$P < 0.01$, and +++$P < 0.001$ for h[Gly^2]GLP-2-treated vs. h[Gly^2]GLP-2 + 1 or more growth factor-treated mice.

regulation of both small and large bowel growth. As the receptor mediating the action of GLP-2 in the intestine has not yet been identified, whether GLP-2 acts directly or indirectly in the intestine remains unknown. Nevertheless, the presence of GLP-2-secreting L cells throughout the bowel is consistent with the hypothesis that locally produced GLP-2 may directly contribute to regulation of both small and large bowel growth and regeneration in vivo.

Histological analysis of mouse small bowel after growth factor treatment demonstrated a reasonable correlation between the relative increase in small bowel weight and increases in villus height. Although EGF and IGF-II treatments were not associated with increased small bowel weight, analysis of villus height in EGF- and IGF-II-treated mice did reveal small increases in villus height, consistent with the known trophic effects of these molecules. Despite the lack of evidence for synergism with respect to changes in small bowel mass after treatment with combinations of h[Gly^2]GLP-2 and other growth factors, an additive increase in villus height was detected in the distal ileum of mice treated with h[Gly^2]GLP-2 and GH or IGF-II (Fig. 3*C*). Furthermore, the additive changes in villus height raise the possibility either that a submaximal dose of h[Gly^2]GLP-2 was utilized or, as is highly likely, that h[Gly^2]GLP-2 may exert its effects through receptor signaling pathways that are distinct from those used by other growth factors.

The results of our studies suggest that under the present experimental conditions, h[Gly^2]GLP-2 is at least as potent an intestinal growth factor as the other intestinal growth factors tested. However, it must be recognized that we did not carry out these studies using a range of different peptide concentrations or different peptide delivery systems. It is therefore entirely pos-

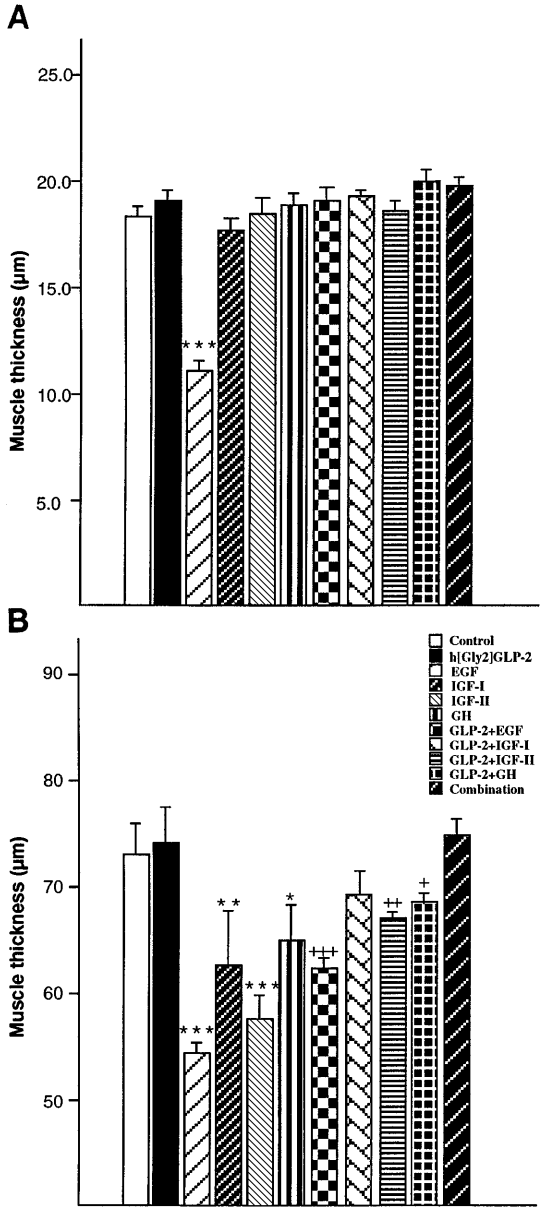

Fig. 7. Histomorphometric analysis of muscle layers from jejunum (circular and longitudinal muscle layers) (*A*) and colon (muscularis externa, circular, and longitudinal muscle layers) (*B*) of growth factor-treated mice. Data are means ± SE; n = 4 mice/treatment group. A minimum of 20 separate histological sections was examined for each treatment group. See Fig. 2 legend for explanation of IGF-I and IGF-II. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ for PBS-treated vs. single growth factor-treated mice. +$P < 0.05$, ++$P < 0.01$, and +++$P < 0.001$ for h[Gly^2]GLP-2-treated vs. h[Gly^2]GLP-2 + 1 or more growth factor-treated mice.

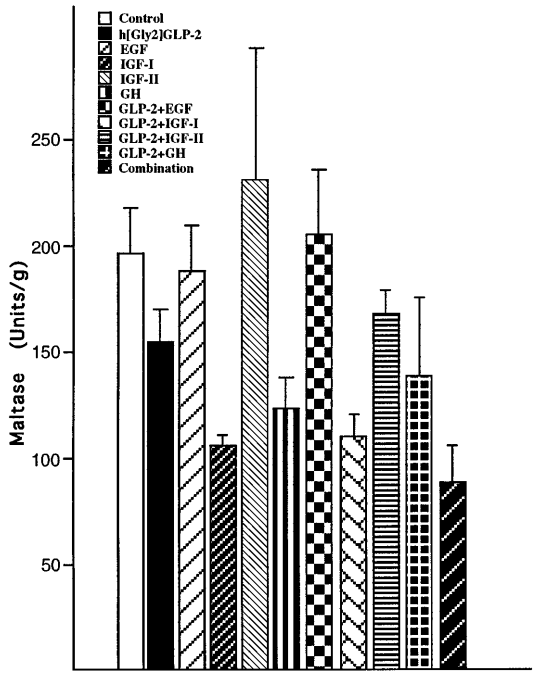

Fig. 8. Maltase activity in duodenum of growth factor-treated mice. Data (means ± SE) are expressed as units of enzyme activity/g protein; n = 4 mice/treatment group. See Fig. 2 legend for explanation of IGF-I and IGF-II.

sible that had we used larger amounts of EGF, GH, or IGF-I, the comparative results obtained may have been quantitatively different. Furthermore, it is possible that for other growth factors, intermittent or continuous intravenous administration, rather than intermittent subcutaneous administration, may produce greater biological effects.

The results of our studies did not reveal significant changes in small bowel mass after administration of either EGF or IGF-II. Previous studies of the intestinotrophic properties of EGF in mice and rats utilized doses of 10–150 $\mu g \cdot kg^{-1} \cdot day^{-1}$ (21, 24, 37). As the dose used in our study was 80 $\mu g \cdot kg^{-1} \cdot day^{-1}$, one possible explanation for the lack of trophic effects with EGF may simply relate to the amount of bioactive EGF administered to mice after subcutaneous injection. Remarkably, the large bowel of EGF-treated mice was smaller than in PBS-injected controls, with significant decreases in the colon crypt depth and muscle layer detected in EGF-treated animals. Consistent with these observations, a decrease in cell production rates in the colonic crypts of EGF-treated mice was observed in mice treated with 10 μg/kg EGF every 8 h (1); however, rats treated with a higher dose of EGF (600 $\mu g \cdot kg^{-1} \cdot day^{-1}$) exhibited a reduction in mucosal damage and inflammation after chemically induced colitis (22). These observations suggest that the trophic effects of EGF in the bowel are likely to be species and dose dependent.

There are few experimental data demonstrating the intestinotrophic activity of IGF-II in the small bowel. Although mice expressing an IGF-II transgene exhibited increased growth of the appendix and colon, no changes in the mass of the duodenum and small bowel were reported (38). Mice treated with IGF-II for 14 days did not exhibit increases in the mass of the small or large bowel (7). However, small increases in jejunal villus height were detected in the present study in IGF-II-treated animals. These observations suggest that the intestinotrophic effects of IGF-II, at the dose studied here, are comparatively modest, and the significance of IGF-II in the regulation of intestinal growth in the adult remains unclear.

In contrast to the weak intestinotrophic effects of IGF-II, several studies have clearly shown that treatment of rodents with IGF-I or its analogs at doses of 1.5–3.2 $mg \cdot kg^{-1} \cdot day^{-1}$ leads to increased mass of the small and large intestine (20, 36). The IGF-I analog utilized here, LR^3IGF-I, contains a glutamine to arginine substitution at position 3 and a 13-amino acid NH_2-terminal extension from porcine GH, rendering the hybrid IGF-I analog ~10 times more potent than native IGF-I in vivo (25). LR^3IGF-I administered by continuous subcutaneous infusion to neonatal rats at doses of 2–12.5 $\mu g \cdot g^{-1} \cdot day^{-1}$ for 6.5 days increased both small bowel weight and length, crypt depth and villus height, and thickening of the muscularis externa (25). The concentration of LR^3IGF-I used in our studies was ~2 $\mu g \cdot g^{-1} \cdot day^{-1}$, and although not strictly comparable due to differences in the experimental models under study, the effects of LR^3IGF-I on intestinal

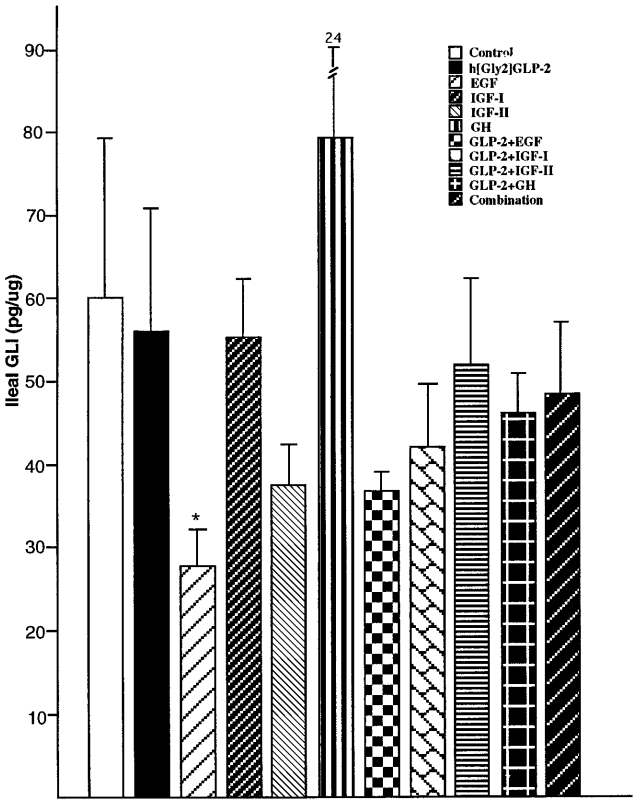

Fig. 9. Glucagon-like immunoreactivity (GLI) in ileum of growth factor-treated mice. Data are expressed as pg GLI/μg protein. The SE for GH-treated mice was 24; n = 4 mice/treatment group. See Fig. 2 legend for explanation of IGF-I and IGF-II. *$P < 0.05$ for PBS-treated vs. single growth factor-treated mice, respectively.

growth in mice reported here appear to be consistent with results of previous studies using continuous infusion delivery systems in rats (23, 25, 26).

The effect of growth factors on disaccharidase activity in the small bowel appears to be somewhat variable and region specific. After 80% jejunoileal resection in rats, IGF-I treatment was associated with normal levels of sucrase, maltase, and leucine aminopeptidase in the duodenojejunal remnant, despite increased DNA and protein content reflecting ongoing mucosal adaptation (36), and IGF-I transgenic mice exhibited increased small bowel growth but normal intestinal disaccharidase activities (18). Subcutaneous administration of GH to rats after small bowel resection enhanced maltase activity in the ileum but had no additional effect on the growth of the small bowel mucosa (19). Short-term subcutaneous infusion of EGF into rabbits after small bowel resection did not result in enhancement of intestinal maltase activity; however, rabbits infused with EGF for 3 wk did exhibit increased intestinal maltase activity (28). In contrast, rabbits treated with EGF following 50–60% midjejunal enterectomy exhibited normal levels of enzyme activity after normalization for increased protein content of the mucosa (28).

Analysis of intestinal disaccharidase activity in mice treated with rat GLP-2 for 10 days has previously demonstrated no significant changes in duodenal maltase, lactase, or sucrase activity after normalization of enzyme activity for the increased protein content of the mucosa (5). Similarly, we did not detect significant changes in duodenal maltase activity in mice treated with the human GLP-2 analog h[Gly^2]GLP-2. Taken together, the available information indicates variability in the response of intestinal maltase activity to growth factor administration, and at least part of this variability may be attributable to the animal model and region of the intestine under study, as well as the dose and duration of growth factor administration.

The analysis of intestinal GLI in growth factor-treated mice was prompted by the observation that mice carrying subcutaneous proglucagon-producing tumors exhibit high levels of circulating PGDPs and suppression of endogenous pancreatic proglucagon gene expression (12). These findings suggest that one or more circulating PGDPs might inhibit expression of the proglucagon gene in the pancreas or intestine, leading to a reduction in PGDP synthesis. However, the PGDP responsible for this inhibitory effect has not yet been identified. Analysis of mice treated for 10 days with native rat GLP-2 did not demonstrate any diminution in the levels of intestinal or pancreatic PGDPs or proglucagon mRNA (5). The data reported here extend these findings by demonstrating that mice treated for a longer period of time with the more potent peptide h[Gly^2]GLP-2 exhibited normal levels of GLI in the ileum. As current knowledge suggests that intestinal PGDPs such as glicentin, GLP-1, and GLP-2 are synthesized and cosecreted from the enteroendocrine cell in a coordinated and equimolar fashion, it seems unlikely that treatment with GLP-2 or its analog is associated with inhibition of endogenous PGDP biosynthesis.

The observation that endocrine peptides and epithelial growth factors display intestinotrophic activity raises the possibility that one or more of these hormones or growth factors may function in part in an indirect manner, perhaps through stimulation of GLP-2 biosynthesis and secretion. Examination of PGDP content in growth factor-treated mice did not reveal evidence for a significant stimulation of intestinal PGDP synthesis. In contrast, mice treated with EGF and IGF-II exhibited a small decrease in intestinal PGDP content, raising the possibility that these growth factors may have direct inhibitory effects on the intestinal L cell. Functional receptors for IGF have been reported on glucagon-producing cell lines, and IGF-I, but not IGF-II, inhibits islet glucagon gene transcription (13). However, effects of IGFs on intestinal proglucagon gene expression or PGDP biosynthesis have not previously been reported. Furthermore, whether GLP-2 exerts its intestinotrophic effects in part via induction of other intestinal growth factors remains unknown.

Although we did not observe synergistic changes in small or large bowel growth after treatment of mice with h[Gly^2]GLP-2 and either EGF, IGF-I, IGF-II, or GH, the combination of h[Gly^2]GLP-2 with GH or IGF-I or the combined administration of all five growth factors together resulted in a greater degree of bowel growth than that observed with h[Gly^2]GLP-2 alone, even after correction for changes in body weight. At the present time, strategies for enhancing bowel regeneration in human subjects with intestinal compromise have not yet been optimized, and few studies have been performed that examine the consequences of combinatorial growth factor administration on parameters of bowel growth in vivo. The observation that enhancement of both small and large bowel growth can be achieved through the selective use of combinatorial therapy suggests that further study of the mechanism of action and effects of various intestinotrophic factors, administered as single agents or in combination with other molecules (using varying dosing regimens and routes of administration), may be useful for design of optimal strategies that facilitate intestinal adaptation in vivo.

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