Glucagon-like peptide 2 induces vasoactive intestinal polypeptide expression in enteric neurons via phosphatidylinositol 3-kinase-γ signaling

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Glucagon-like peptide 2 (GLP-2) is a unique enteroendocrine hormone; it is produced by enteroendocrine cells, and subepithelial myofibroblasts in culture. Over 1–4 h, GLP-2 stimulation of SMP increased phosphorylated Akt/Akt ratios 6.1-fold, phosphorylated ERK/ERK 2.5-fold, and p70S6K 2.2-fold but did not affect intracellular cAMP. PI3Kγ gene deletion or pharmacological blockade of PI3Kγ, mammalian target of rapamycin (mTOR), and MEK/ERK pathways blocked the increase in VIP expression by GLP-2. GLP-2 increased the expression of growth factors and their receptors in SMP cells in culture [IGF-1r (3.2-fold increase), EGF (5-fold), and ErbB2–4r (6- to 7-fold)] and ligands [IGF-I (1.5-fold), amphiregulin (2.5-fold), epiregulin (3.2-fold), EGF (7.5-fold), heparin-bound EGF (2.0-fold), β-cellulin (50-fold increase), and neuregulins 2–4 (300-fold increase) (by qRT-PCR)]. We conclude that GLP-2 acts on enteric neurons and glial cells in culture via a PI3Kγ/Akt pathway, stimulating neuronal differentiation via mTOR and ERK pathways, and expression of receptors and ligands for the IGF-I and ErbB pathways.

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METHODS

Experimental plan. The effects of GLP-2 were studied using cells derived from primary cultures of submucosal enteric neurons, which exhibited a definitive increase in VIP expression following GLP-2 stimulation. The primary end points were VIP expression and detection of the activation of intracellular signaling pathways; studies were done primarily in cultures derived from the submucosal plexus of the colon to model the effects of GLP-2 in vivo (33). To determine the signaling pathways activated by GLP-2, short-term stimulation studies were done, detecting intracellular Akt/phosphorylated (phospho) Akt, ERK/phospho-ERK, and P70S6K by Western blot. To demonstrate the relative effects of signaling pathways activated by the GLP-2 receptor, studies were done using cells isolated from mice lacking PI3Kδ or from wild-type animals subjected to pharmacological blockade, using specific blockers of PI3Kδ (AS-605240), mTOR, and MEK/ERK pathways (PD-98059). The actions of GLP-2 on growth factor receptors (epidermal growth factor (EGF) Erb-1, IGF-I, and GLP-2 itself) and ligands (IGF-I, amphiregulin, epieregulin, and heparin-bound EGF) were quantified using quantitative RT-PCR.

Enteric neuronal and glial cultures. Cells were isolated from rat colon or mouse ileum; protocols were approved by the University of Calgary Animal Care Committee, following the Canadian Council on Animal Care guidelines. Using previously described methods, primary cultures of submucosal neurons were prepared (5, 16, 31). In brief, animals (rats and mice aged 2–3 mo) were anesthetized using halothane by inhalation (Halocarbon Products, River Edge, NJ) and then exsanguinated. The colon or ileum was removed and placed in iced Krebs solution (133 mM NaCl, 4.7 mM KCl, 1.0 mM MgCl2, 1.4 mM NaHCO3, 2.5 mM CaCl2-2H2O, and 7.8 mM glucose, pH 7.2). The segment was opened along the mesenteric border and gently pinned mucosal side up to a solid Sylgard-coated dish. The mucosa was trimmed of fat and lymphoid tissue, and adherent proteins were removed using aseptically prepared calcium- and magnesium-free Hanks’ balanced salt solution (Gibco). The exposed submucosa was then removed, separating it from the rapid-lying cells such as fibroblasts or glial cells. After 30 min, the digested tissue was triturated and then centrifuged at 12000 g. After 40 min, the digested tissue was Triturated and then centrifuged at 12000 g. After 40 min, the digested tissue was triturated and then centrifuged at 12000 g. The supernatant was recovered in primary culture medium with 10% fetal bovine serum for 24 h, changed to primary culture medium containing 10% fetal calf serum, and incubated for 1 h with the secondary antibodies. Neurons were detected using either mouse anti-β-III-tubulin (1:500; R & D Systems, Minneapolis, MN) or mouse anti-HuC/D (1:200; Molecular Probes, Eugene, OR), and glial cells were detected with mouse anti-glial fibrillary acidic protein (GFAP) (1:250; Chemicon, Billerica, MA). Smooth muscle cells were detected with rabbit anti-α-smooth muscle actin (1:100; Abcam, Cambridge, MA) antibody and fibroblasts with mouse anti-anti-human β-3 tubulin (1:500; R & D Systems, Minneapolis, MN) or mouse anti-HuC/D (1:200; Molecular Probes, Eugene, OR), and glial cells were detected with mouse anti-glial fibrillary acidic protein (GFAP) (1:250; Chemicon, Billerica, MA). Smooth muscle cells were detected with rabbit anti-α-smooth muscle actin (1:100; Abcam, Cambridge, MA) antibody and fibroblasts with mouse anti-anti-human β-3 tubulin (1:500; R & D Systems, Minneapolis, MN) or mouse anti-HuC/D (1:200; Molecular Probes, Eugene, OR), and glial cells were detected with mouse anti-glial fibrillary acidic protein (GFAP) (1:250; Chemicon, Billerica, MA). Smooth muscle cells were detected with rabbit anti-α-smooth muscle actin (1:100; Abcam, Cambridge, MA) antibody and fibroblasts with mouse anti-anti-human β-3 tubulin (1:500; R & D Systems, Minneapolis, MN) or mouse anti-HuC/D (1:200; Molecular Probes, Eugene, OR), and glial cells were detected with mouse anti-glial fibrillary acidic protein (GFAP) (1:250; Chemicon, Billerica, MA). Smooth muscle cells were detected with rabbit anti-α-smooth muscle actin (1:100; Abcam, Cambridge, MA) antibody and fibroblasts with mouse anti-anti-human β-3 tubulin (1:500; R & D Systems, Minneapolis, MN) or mouse anti-HuC/D (1:200; Molecular Probes, Eugene, OR), and glial cells were detected with mouse anti-glial fibrillary acidic protein (GFAP) (1:250; Chemicon, Billerica, MA).

Regulation of differentiation. The primary stimulus throughout the experiments was the addition of exogenous GLP-2 to the medium. GLP-2 (1–33 human; American Peptide, Sunnyvale, CA) was dissolved in sterile water and diluted to a final concentration in medium of 10−8 M [optimal concentration for proliferation (15, 38)]. On days 10 and 11, in the 0.1% FBS medium, cells were treated with GLP-2 10−8 M or control medium for 1–28 days. Preliminary studies that demonstrated a robust increase in VIP expression by 24 h were done (data not shown).

Immunohistochemistry and identification of cell populations. After completion of the stimulation phase, cells were fixed in 0.1 M PBS containing 4% paraformaldehyde for 30 min at room temperature and then washed three times with PBS. To control for variations in plating cell numbers, comparisons were always made between wells grown from the same tissue preparation with and without added GLP-2. Typically, cells were labeled with primary antiserum, washed three times with PBS, and incubated for 1 h with the secondary antibodies. Neurons were detected using either mouse anti-β-III-tubulin (1:500; R & D Systems, Minneapolis, MN) or mouse anti-HuC/D (1:200; Molecular Probes, Eugene, OR), and glial cells were detected with mouse anti-S-100 (1:500; Sigma) and anti-γ-III-glial fibrillary acidic protein (GFAP) (1:250; Chemicon, Billerica, MA). Smooth muscle cells were detected with rabbit anti-α-smooth muscle actin (1:100; Abcam, Cambridge, MA) antibody and fibroblasts with mouse anti-anti-human β-3 tubulin (1:1,000; Millipore, Billerica, MA). VIP expression was determined by double-labeling with anti-HuC/D or β-tubulin, and VIP-expressing neurons were detected with rabbit anti-vasoactive intestinal peptide (1:600; EuroDiagnostics, Malmo, Sweden), neuronal nitric oxide synthase (nNOS) with rabbit anti-nNOS (1:250; EuroDiagnostics). GLP-2 receptor was detected using goat anti-GLP2R (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) antibody. Donkey anti-mouse IgG conjugated to Alexa 488 (1:100; Jackson ImmunoResearch Laboratories, West Grove, PA) or goat anti-rabbit Cy3 (1:1,000; Jackson ImmunoResearch Laboratories) was used to

Table 1. Primer sequences

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<th>Reverse Primer (5’-3’)</th>
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EGF, epidermal growth factor; HB-EGF, heparin-binding EGF-like factor; TGF-α, transforming growth factor-α; GLP-2R, glucagon-like peptide-2 receptor; EGF-R, EGF receptor; IGF-IR, IGF-I receptor.
Fig. 1. Vasoactive intestinal polypeptide (VIP) expression in cells derived from the submucosal plexus following glucagon-like peptide-2 (GLP-2) stimulation. The effects of GLP-2 on cells isolated from submucosal ganglia prepared from rat colon were studied in vitro. The submucosal neurons were isolated by microdissection, separated, and cultured for 8 days, being treated with cytosine arabinoside over the last 3 days. Medium was replaced with 0.1% FCS with or without added GLP-2 (10^{-6} M), and cells were studied by immunohistochemistry after 24 h. Labeled cells were imaged using a confocal microscope (Olympus FV1000) and Olympus Fluoview version 3.0 software. A, top: staining for HuC/D (in green). A, middle: staining for VIP (red). A, bottom: merged images. Arrows indicate nuclei. Scale bar, 50 μm. B: effects of GLP-2 on the proportion of cells expressing VIP. Nos. of cells costaining for VIP, as a proportion of all nuclei detected by 4,6-diamidino-2-phenylindole (DAPI) staining, in 10 high-powered fields from 8 coverslips/determination. Data are means ± SE; n = 8. ***P < 0.01 vs. controls (CTL).
detect the primary antibodies. Cells were washed with PBS and mounted onto slides with Fluorosave mounting medium.

**Cell signaling studies.** To determine the requirement for PI3K-induced activation of Akt and downstream activation of mTOR and MEK/ERK by the GLP-2 receptor, cells isolated from the submucosal plexus were prepared as described above and treated with $10^{-8}$ M GLP-2 or control medium. After 0, 15, 30, 60, or 120 min, cells were collected on ice in immunoprecipitation buffer (RIPA or Tris buffer containing 1% Triton X-100). Cells were lysed, mixed, and centrifuged ($120 \times g$ for 5 min at 4°C). Protein content was determined using the Lowry assay. Samples were denatured by boiling for 5 min. Equivalent amounts of protein were loaded on 10% SDS polyacrylamide gel and separated at 100 V for 1.5 h at 4°C. Membranes were blocked with 5% BSA for 1 h and then probed with the following primary antibodies: phospho-Akt (Ser473, 1:750), total Akt (1:1,000), mTOR (phosphorylated mTOR; Ser2448, 1:500), total mTOR (1:1,000), phospho-70SK6 (Thr389, 1:1,000), total p70S6K1 (1:500), phospho-ERK1/ERK2 (1:3,000), total ERK1/2 (1:1,000), and actin (1:2,000) (all antibodies raised in rabbit; Cell Signaling, Danvers, MA). The membrane was washed with PBS and then incubated with anti-rabbit horseradish peroxidase IgG secondary (1:2,000). This was then developed with a commercial horseradish peroxidase detection system (Amersham Biosciences, Piscataway, NJ), scanned, and analyzed using Quantity One software (Bio-Rad).

The functional effects of the blockade of these pathways were determined by preparing identical sets of cultures from rat colonic tissue pretreated with a nonselective PI3K inhibitor (LY-294002, 50 μM; Calbiochem, Billerica, MA), the selective PI3Kγ inhibitor

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**Fig. 2.** Identity of cells in primary cultures derived from the submucosal plexus and the effects of GLP-2 stimulation. Primary cultures of cells from the submucosal neuronal plexus were prepared from rat colon, as outlined in Fig. 1. A: cell identification by immunohistochemistry. Coverslips were stained with antibody against the neuronal nuclear marker HuC/D (green), the glial marker glial fibrillary acidic protein (GFAP; green), α-smooth muscle actin (α-SMA; red), or the fibroblast marker TE-7 (red) and costained with the nuclear stain DAPI (blue). Double-labeled cells are marked by arrows. Scale bar, 100 μm. B: effects of GLP-2 on cells derived from the submucosal plexus in culture. Cell types detected by immunohistochemical labeling after 24 h of stimulation with 0.1% FCS (controls) or 0.1% FCS + GLP-2 (10^-8 M). Values are the proportion of cells labeled by the antibody as %total cells (detected by DAPI staining) from 10 high-powered fields from 8 coverslips/determination. Data are means ± SE; n = 8. *P < 0.05 vs. controls; ***P < 0.001 vs. controls.
AS-605240 (50 μM; Calbiochem), the ERK1/2 inhibitor PD-98059 (50 μM, Calbiochem) or mTOR inhibitor rapamycin (50 μM, Sigma), a or control carrier for 1 h and then stimulated with GLP-2 (10^{-8} M) or control medium for 24 h, and VIP expression was quantified as described above (27). To further demonstrate the role of PI3Kα in mediating GLP-2 activity, similar submucosal plexus cell preparations were made from PI3Kα^{-/-} [deletion of p110 subunit (2, 28)] or wild-type mice and stimulated for 7 days with control medium or medium + GLP-2 (10^{-8} M), quantifying VIP expression in HuC/D expressing neurons by immunohistochemistry. To examine the effects of GLP-2 on other regulatory signaling pathways, short-term stimulation studies were done using cells from rat colon stimulated with GLP-2 (10^{-8} M) or control protein; to follow the time course of genes induced, cells were lysed at 30 min, 60 min, 2 h, 6 h, and 24 h (n = 6/time point). Real-time quantitative reverse-transcribed PCR (qRT-PCR) was done on the mRNA isolates from each time point for the expression of the genes for GLP-2R, IGF-I receptor, EGF receptor, ErbB2, ErbB3, and ErbB4 receptors, and the ligands EGF, IGF-1, amphiregulin, epiregulin, β-cellulin, heparin-binding EGF-like factor (HB-EGF), and neuroregulins 1–4. Total RNA was extracted using TRizol (Invitrogen, Carlsbad, CA), following the manufacturer’s instructions, and then reverse transcribed using Superscript-II reverse transcriptase (Invitrogen). Sequence amplification was performed using Express SYBR Green master mix with ROX (Invitrogen) in an ABI 7900 real-time PCR thermocycler detection system. All primer sequences were obtained from the University of Calgary DNA Core, Calgary, AB, Canada (Table 1). Samples were run in duplicate with a resulting threshold cycle (C_T) being determined, using 18S ribosomal RNA as the endogenous control. Data are expressed as a relative fold change that was calculated using the ΔΔC_T method.

To examine the involvement of cAMP in the actions of GLP-2, the effect of GLP-2 stimulation on intracellular cAMP levels was quantified. Using the methods described above, with medium containing 10 μM 3-isobutyl-1-methylxanthine (Sigma), cells were treated with GLP-2 (10^{-8} M) or control; 20 μM forskolin (Calbiochem) was used as a positive control (n = 8 wells/group). Cells were treated for 0–10–30–60 min, washed with Hanks’ solution, and lysed in 0.1 N HCl, and the lysates were frozen and centrifuged, with the supernatant for cAMP analyzed using a commercial kit (Biomedical Technologies). Briefly, this is an enzyme immunoassay that measures the amount of cAMP using a competitive binding of cAMP and an alkaline phosphate derivative of cAMP to a specific bound amount of antibody on a microplate. The cell culture medium containing cAMP was mixed with cAMP-alkaline phosphatase tracer and cAMP antiserum and added to the wells already coated with cAMP-specific antibody. After incubation overnight, the wells were washed and reagents added to produce a enzymatic color reaction with the materials bound to the plates read at 405–410 nm absorbance. The relative bindings were calculated against a standard curve to determine picomoles per milliliter of cAMP that was then normalized to total protein in each sample.

Statistics. All data are means ± SE. Data were analyzed using Prism Software (version 4.1). Comparisons were made using Student’s t-test or ANOVA, followed by Bonferroni’s post hoc testing, as appropriate for parametric data or Kruskal-Wallis testing was used for non parametric data, with a P value of <0.05 considered significant.

RESULTS

GLP-2 induces enteric neuronal expression of VIP. To establish the ability of this model of cells isolated from the submucosal plexus to replicate the actions of GLP-2 in vivo in inducing the expression of VIP, we first examined the effects of exogenous GLP-2 on the neuronal cell phenotype. GLP-2 significantly increased the proportion of cells colabeling with the neuronal markers HuC/D and VIP (controls: 22 ± 5% vs. GLP-2 treated: 40 ± 6%, coexpressed HuC/D and VIP; Fig. 1, A and B). As a comparator, GLP-2 treatment also affected nNOS expression (controls: 9 ± 4% vs. GLP-2: 18 ± 2% coexpressed HuC/D and nNOS; n = 6 preparations, P < 0.05). Having established the ability to replicate the effects of GLP-2 seen in vivo to increase the expression of VIP in neurons, the identity of the responding cells in this system was probed (Fig. 2A). The

Figure 3. Expression of the GLP-2 receptor on primary cell cultures derived from the submucosal plexus. Primary cell cultures derived from the submucosal enteric plexus were prepared as outlined in Fig. 1. Coverslips were stained with antibody against the neuronal nuclear marker HuC/D (green), the glial marker GFAP (green), α-SMA (green), or the fibroblast marker TE-7 (green) and colabeled with antibody against the GLP-2 receptor (red) and the nuclear stain DAPI (blue). Double-labeled cells are marked by arrows. Scale bar, 25 μm.
The initial preparation of cells isolated and dissociated from the submucosal plexus consisted of 17% neurons, as detected by HuC/D labeling, 14% enteric glial cells (GFAP+/H11001−), 62% smooth muscle cells (α-smooth muscle actin+/H11001−), and 5% fibroblasts (TE-7−). The proportion of cells expressing neuronal and glial markers was unchanged by treatment with control serum but increased after 24 h of treatment with GLP-2 (Fig. 2B). Longer-term studies out to 28 days, which showed a continued increase in the proportion of cells expressing neuronal markers (HuC/D) vs. glial markers (GFAP), were done (day 14; ratio of neurons to glial cells, 14:1).

The identity of the cells that are capable of responding to GLP-2 was further defined, examining for the presence of the GLP-2 receptor, as detected by immunohistochemistry (Fig. 3). The typical surface staining pattern for the GLP-2 receptor was seen in 55% of neurons (identified by costaining with HuC/D) and 24% of glial cells (costaining for GFAP), but not in smooth muscle cells (α-SMA) or fibroblasts (TE-7). Similar results were seen when double-labeling for neurons was done with anti-β-tubulin and for glial cells with anti S-100 (38%).

Signaling pathways activated by endogenous GLP-2 receptor stimulation. Having recapitulated our in vivo findings in this cell culture model, we wanted to establish the mechanism. The cellular signaling pathways predicted to be activated by the GLP-2 receptor are intracellular PI3K, recruited with kinase activation at the inner cell membrane, which then acts via phosphatidylinositol-3,4,5-triphosphate phosphorylation to activate Akt. This was examined by performing Western blots for p-Akt and cAMP accumulation. Primary cultures of cells from the submucosal enteric neuronal plexus were prepared from rat colon, as outlined in Fig. 1, and treated with control medium or GLP-2 (10−8 M) for the times indicated. A: time course of phospho-Akt expression; Western blots of representative gels taken from lysates of cells exposed to GLP-2 for the times indicated and probed sequentially with antibodies against phospho-Akt, total Akt (t-Akt), and actin, with stripping and restaining of the original gel. Blots from a single gel are rearranged for clarity. Graph represents phospho-Akt/t-Akt normalized to controls, with actin used a loading control. B: effects of phosphatidylinositol 3-kinase (PI3K) inhibition. Cultured cells were pretreated for 1 h with the nonselective PI3K inhibitor LY-294002 (50 μM), the selective PI3K inhibitor AS-605240 (50 μM), or the ERK1/2 inhibitor PD-98059 (50 μM) and then stimulated with GLP-2 (10−8 M) for 15 min. Representative Western blots of phospho-Akt, t-Akt, and actin staining are presented, and blots from a single gel are arranged for clarity. Results of phospho-Akt/t-Akt normalized to controls are presented. Methods are as outlined in A; n = 6. C: cultured cells were treated with GLP-2 or control solvent vehicle for the times indicated. Positive controls were treated with forskolin (20 μM), cells lysed at the times indicated, and cAMP levels determined. Data are means ± SE; n = 8/condition. *P < 0.05 vs. controls; ***P < 0.0001 vs. controls; †P < 0.05 vs. GLP-2 treated.
The secondary signals that may have a role in regulating proliferation and differentiation of enteric neurons and glia were studied by quantifying the effects of GLP-2 on receptors and ligands of the EGF and IGF-I family, which have been implicated previously in the trophic effects of GLP-2. As shown in Fig. 7A, there was an increase in the expression of the gene for the GLP-2 receptor itself and the IGF-I receptor, with greater effects on the expression of the ERB family of receptors, with a fivefold increase in the EGF receptor and a six- to sevenfold increase in the Erb-2, Erb-3, and Erb-4 receptors. For the ligands of the ErbB family, there were two- to threefold increases in mRNA expression for ampiregulin, epiregulin, and HB-EGF ligands at the early time points of 30 and 60 min after GLP-2 was added (Fig. 7B). There were more significant seven- to 50-fold increases in the expression of EGF, TNFα, and β-cellulin mRNAs, which occurred over the 1- to 2-h time point post-GLP-2 stimulation. Finally, a truly remarkable 300-fold increase in neuregulin 4 expression beginning at 60 min after GLP-2 exposure and peaking at 24 h was noted. There were equally dramatic increases in expression of the neuregulins 1 and 3, but only at the 24-h time point (Fig. 7B).

**DISCUSSION**

The findings of the present study describe a mechanism for the previously observed GLP-2-induced differentiation of a VIP-expressing phenotype in enteric submucosal neurons in vivo. Using a model of a primary culture of cells derived from the submucosal plexus, GLP-2 stimulated primarily a neuronal phenotype, increasing VIP expression and acting via...
GLP-2 induces VIP expression in enteric neurons

**Fig. 6. Effects of pharmacological antagonists or gene knockout of intracellular signaling pathways on GLP-2-induced VIP expression.**

A: effect of pharmacological blockers of intracellular signaling. Primary cultures of cells from submucosal ganglia from mouse ileum from wild-type and PI3Kγ−/− animals were isolated and grown on coverslips, as outlined in Fig. 1, culturing cells for 14 days prior to study. Wells were treated with control medium or medium + GLP-2 (10−8 M) for 7 days. The images and the corresponding counts of cells costaining for VIP and HuC/D with each condition is shown. Scale bar, 50 μm. Data are means ± SE; n = 6–8/group. ***P < 0.001 vs. controls; †P < 0.001 vs. GLP-2 treated.

B: effect of PI3Kγ knockout (KO). Primary cultures of cells from submucosal ganglia from mouse ileum from wild-type and PI3Kγ−/− animals were isolated and grown on coverslips and pretreated for 1 h with the selective PI3K inhibitor AS-605240 (50 μM), the ERK1/2 inhibitor PD-98059 (50 μM) or the mTOR inhibitor rapamycin (50 μM) or solvent vehicle, followed by additional GLP-2 (10−8 M) or CTL (GLP-2) for 24 h. The proportion of cells costaining for VIP and HuC/D with each condition is shown. WT-CTL (Veh), WT-GLP-2 (Veh), PI3Kγ−/− CTL (Veh), PI3Kγ−/− GLP-2 (Veh).

**Wild type**

Wild type

**GLP-2**

GLP-2

**CTL**

Vehicle

**GLP-2**

GLP-2

**PI3Kγ−/−**

PI3Kγ−/−

**GLP-2**

GLP-2

**Fig. 6.**

The trophic effects of GLP-2 on the intestine generally, and the small intestinal mucosa specifically, have been well described. However, it has been a challenge to determine the specific cell types GLP-2 stimulates and the associated intracellular signaling pathways involved. Initial work following the identification of the receptor focused on studies using transfected GLP-2 receptor expression (21, 40). The present results are the first to examine the actions of GLP-2 on isolated enteric neurons, a major effector pathway for GLP-2 in the intestine (33, 34). The refinement of this model of primary culture of cells from the submucosal plexus greatly facilitated the examination of the pathways involved in GLP-2 activity. The diversity and robustness of the response to GLP-2 of this cell population was somewhat surprising. It supports the notion that the ENS is plastic; in this system the cells responded by rapidly changing phenotype (expressing VIP). There may also have been a proliferative effect; this will be the subject of further investigation. The identity of the majority of the cells that expressed VIP after responding to the GLP-2 stimulus as neurons is clear; this was confirmed using antibodies for the pan-neuronal markers HuC/D and β-tubulin at each step of the effects of GLP-2 on differentiation (9, 11). Similar demonstrations of GLP-2 activation and regulation of neuronal phenotype have been shown in vivo in the ENS and in cultures of embryonic hippocampal neurons (31, 33). However, a similar GLP-2-linked Akt-activated pathway was demonstrated in cultured astrocytes from the CNS (37, 38). In the present study, enteric glial cells were also shown to specifically express the GLP-2 receptor, whereas the other main constituents of the cell preparation, smooth muscle cells and the fibroblasts, did not. Thus, enteric glial cells, or possibly a pluripotent progenitor cell, which can differentiate into a neuronal phenotype (13, 14), likely respond to GLP-2 and may have undergone phenotypic changes with some expression of VIP. However, the majority of the cells responding and expressing VIP were of a classical neuronal phenotype identified by immunohistochemistry and morphology. Prolonged stimulation with GLP-2 did not drive an increase in the proportion of enteric glial cells in culture, even out to 28 days, but rather increased the proportion of cells with a neuronal phenotype, some of which are nNOS immunoreactive. Further studies are required to determine the extent to which the GLP-2-induced enteric neuronal differentiation seen in the present study may occur in vivo, how this might be affected by inflammation, and how enteric glial and neuronal progenitor cells may contribute to this (14). The fact that nNOS expression was enhanced in these cultures, but not in vivo, suggests that some changes may be specific to the culture model, and their validity would need to be assessed by direct comparison, as we have done for VIP.

Focusing on the intracellular signaling pathways involved, the present studies confirm the signaling events suggested in previous work done using intestinal myofibroblasts, CNS-derived astrocytes, and hippocampal cell models (15, 31, 37). In these systems, increased cAMP was not a constitutive element in the GLP-2 response (15, 31, 37), which was confirmed in the present study. Collectively, these results show that stimulation of the GLP-2 receptor activates PI3Kγ, which then leads to the phosphorylation of Akt. In the cells stimulated in the present study, the phosphorylation of Akt led to activation of mTOR and ERK1/2, as shown by the increased production of P70S6K following GLP-2 stimulation and the phospho-ERK/ERK ratio, which is similar to observations in CNS astrocyte and hippocampal neuronal cell systems (31, 37). In

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the present study, the specific involvement of these pathways in regulating differentiation was further confirmed using pharmacological blockers and a knockout model. In enteric neurons treated with PI3Kγ, ERK, or mTOR inhibitors and in cells derived from animals with a specific deletion of PI3Kγ, the increase in the proportion of cells expressing VIP with GLP-2 activation was eliminated. Blockade of PI3Kγ also eliminated the downstream increase in p-Akt, p-ERK, and P70S6K. Both
ERK/MEK and mTOR pathways appear to be required for differentiation to occur; blockade of either ERK phosphorylation or mTOR resulted in a loss of the differentiation effects of GLP-2 stimulation (Fig. 8). The present results contrast somewhat with the previous description of the role of PI3Kγ in the trophic actions of GLP-2 in the murine small intestine; in this study, Anini et al. (2) used mice with a knockout of PI3Kγ activity and found that in the knockout animals there was increase in basal intestinal epithelial crypt proliferation rates, with a further GLP-2-induced increase. However, there was a loss of GLP-2-induced mucous cell enhancement and of GLP-2-induced cAMP production by the intact jejunum in the knockout animals. The suggestion from this was that PI3Kγ was not required for GLP-2 stimulation of mucosal growth but may be required in other aspects of signaling. This same group subsequently showed that PI3Kγ activity is required for GLP-2 to stimulate increased IGF-I transcription in the myofibroblast, which is thought to be the major regulator of the proliferative effects of GLP-2 in the intestine (15). It is not clear how the GLP-2-induced increase in crypt proliferation seen in vivo was mediated in the PI3Kγ-knockout animals; however, in the present study, we used the same model to examine the requirement for PI3Kγ signaling in mediating the GLP-2-induced expression of VIP in the ENS-derived cells and found that the PI3Kγ−/− animals showed no response. Further direct study of the effects of this pathway specifically on enteric neuronal and myofibroblast signaling and the more global effects of GLP-2 is required to clarify the links between the intracellular signaling by GLP-2 and the physiological responses of the intestine.

Interestingly, the results from these different studies suggest that the intracellular pathways stimulated by GLP-2 may diverge after Akt activation. From the foregoing, in enteric neurons and CNS astrocytes and hippocampal neurons, ERK/MEK and mTOR are implicated as important intracellular signaling pathways following GLP-2 stimulation. In contrast, in isolated intestinal myofibroblasts in culture, the ratio of p-ERK1/2 to t-ERK1/2 did not change with GLP-2 stimulation, and the primary action was an increase in IGF-1 mRNA expression (15). In the present study, both the proximal signals of mTOR and ERK1/2 and the more downstream IGF-1 and ErbB systems were activated with GLP-2 stimulation; in addition, the degree of GLP-2-increased IGF-1 ligand transcription and the time course were similar to that described in previous studies using myofibroblasts and neurons (15, 31). The increase in the IGF-1 receptor expression is greater than seen previously in astrocytes, whereas in contrast myofibroblasts showed a reduction in IGF-1 receptor expression with GLP-2 stimulation. Overall, the results suggest that the IGF-1 axis is likely relevant in controlling some aspects of the proliferative and differentiation response seen with GLP-2 stimulation in both neurons and myofibroblast signaling but may be regulated differently in the different cell types.

Further evidence of the specificity of the GLP-2 response in enteric neurons is shown by the remarkable increases in the ErbB family of receptors and ligands following GLP-2 stimulation. The observed increases in amphiregulin, EGF, HB-EGF, and epiregulin are similar to previously described effects of GLP-2 in intact intestinal tissues (41). However, the increases in the ErbB–4 receptor, and especially the very high levels of expression of β-cellulin and the Nrg family of ligands, are novel. These findings, coupled with the observed GLP-2 effects on cell phenotype, suggest a link between the Akt, ERK/MEK, and mTOR signaling initiated by GLP-2 and ongoing modulation by ErbB-mediated effects on neuronal function (3, 39). ErbB-Nrg signaling has been shown to be involved in the regulation of neural crest cell development, which ultimately forms the ENS (4); a corollary of this would be that GLP-2 might be involved in regulating the development of the ENS. In support of this idea, GLP-2 has been shown to have a high level of expression in the immature gut; indeed, in human infants and mouse models, levels of both the hormone...
and receptor peak in the later phases of gestation, when intestinal development is maximal (1, 3, 19). This will require more direct study to validate.

Of considerable potential importance is that the potent effects of GLP-2 on the growth and neurotransmitter profile of the enteric neurons in vitro suggest a role for GLP-2 in regulating the ENS phenotype in the mature state in vivo. The relative role of the different receptor/ligand combinations stimulated in the present study is unclear, but the early responses of the EGF-related ligands and the EGF receptor are likely important; similar patterns of expression have been described with increases in cell division and differentiation in the intestinal mucosa (30). GLP-2 may be an intermediary induced by nutrient availability and regulating the function and phenotype of the enteric neuronal system. Further work is needed to explore these relationships and the potential role these systems may have in regulating ENS form and function.

The implications for this will depend on the cumulative effect of GLP-2 stimulation within the different plexuses of the bowel. In the present study, we have focused on the submucosal plexus. The present evidence would suggest that high levels of endogenous nutrients would increase the proportion of neurons expressing VIP over time. Given the normal functions of VIP as a promoter of secretion and as an anti-inflammatory mediator, the net effect might be anticipated to be an increase in some aspects of basal secretion, reduction in permeability, and a decrease in the inflammatory status of the bowel. These observations fit at least in part the observed effects of GLP-2 in inflammatory states (12, 34). The converse effects of GLP-2 in inflammatory states (12, 34). The converse


dependence, it may be that this change in transmitter expression may be signaled by a reduction in ongoing endogenous GLP-2 levels as a consequence of the lack of enteral stimulation. These results are relevant in linking the activity of the nutrient-related signaling of GLP-2 with the activity and overall function of the ENS, especially in the submucosal plexus. This may be important in both physiological as well as pathophysiological states of intestinal function.

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REFERENCES

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