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The HeLa Cell Glucagon-Like Peptide-2 Receptor Is Coupled to Regulation of Apoptosis and ERK1/2 Activation through Divergent Signaling Pathways

J. A. Koehler, B. Yusta, and D. J. Drucker

Department of Medicine, The Banting and Best Diabetes Centre, Toronto General Hospital, University of Toronto, Toronto, Canada M5G 2C4

Glucagon-like peptide-2 (GLP-2) regulates proliferative and cytoprotective pathways in the intestine; however GLP-2 receptor (GLP-2R) signal transduction remains poorly understood, and cell lines that express the endogenous GLP-2R have not yet been isolated. We have now identified several expressed sequence tags from human cervical carcinoma cDNA libraries that correspond to GLP-2R nucleotide sequences. GLP-2R mRNA transcripts were detected by RT-PCR in two human cervical carcinoma cell lines, including HeLa cells. GLP-2 increased cAMP accumulation and activated ERK1/2 in HeLa cells transiently expressing the cloned human HeLa cell GLP-2R cDNA. However, the GLP-2R-induced activation of ERK1/2 was not mediated through G_αs, adenylyl cyclase, or transactivation of the epidermal growth factor receptor, but was pertussis toxin sensitive, inhibited by dominant negative Ras, and dependent on $\beta\gamma$-subunits. GLP-2 also induced a significant increase in bromodeoxyuridine incorporation that was blocked by dominant negative Ras. Furthermore, GLP-2 inhibited HeLa cell apoptosis induced by LY294002 in a protein kinase A-dependent, but ERK-independent, manner. These findings demonstrate that the HeLa cell GLP-2R differentially signals through both G_αs/cAMP- and G_i/G_o-dependent pathways, illustrating for the first time that the GLP-2R is capable of coupling to multiple heterotrimeric G proteins defining distinct GLP-2R-dependent biological actions. (*Molecular Endocrinology* 19: 459–473, 2005)

THE PANCREAS AND gastrointestinal epithelium contain a diverse number of specialized endocrine cells that secrete peptide hormones regulating multiple metabolic functions including nutrient intake, absorption, and disposal (1, 2). The proglucagon gene is expressed in both pancreatic and intestinal endocrine cells (3–5) and gives rise to several distinct proglucagon-derived peptides, including glucagon and the glucagon-like peptides (GLP-1 and GLP-2). Glucagon secreted from pancreatic A cells is a key regulator of glucose metabolism whereas GLP-1 controls blood glucose through regulation of insulin and glucagon secretion, gastric emptying, and appetite (2, 6, 7).

GLP-2 acutely regulates nutrient absorption through enhancement of intestinal hexose transport (8, 9) and by inhibiting gastric acid secretion and gastric emptying (10, 11). The most striking aspect of GLP-2 activity, however, is its ability to maintain the integrity of the intestinal mucosal epithelium. Administration of GLP-2 in rodents promotes expansion of the intestinal epithelium due to stimulation of crypt cell proliferation and inhibition of enterocyte apoptosis (12–16). Furthermore, GLP-2 mitigates the severity of experimental intestinal injury by reducing apoptosis and promoting regeneration in the crypt compartment (15, 16).

The proglucagon-derived peptides exert their diverse biological effects through stimulation of specific G protein-coupled receptors (GPCRs), which in turn activate downstream signaling pathways coupled to specific G protein(s). The receptor for GLP-2 has been cloned from hypothalamic and intestinal cDNA libraries (17) and is structurally related to members of the class II glucagon/secretin receptor superfamily (18–20). Unlike the more widely distributed glucagon and GLP-1 receptors (21–23), immunocytochemical analysis revealed that the GLP-2 receptor (GLP-2R) is expressed in a highly restricted tissue-specific manner in subsets of enteroendocrine cells in the human small and large intestine (24). In contrast, GLP-2R RNA transcripts have been localized in the rodent gut to the enteric nervous system (25). Thus the proliferative and reparative effects of GLP-2 on the mucosal epithelium *in vivo* may be partly direct, by protecting cells that express the GLP-2R from apoptosis, or indirect, by

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Abbreviations: βARKct, β-Adrenergic receptor kinase; BrdU, bromodeoxyuridine; CMV, cytomegalovirus; DAPI, 4′,6-diamidino-2-phenyl-indole; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; EGFR, EGF receptor; EST, expressed sequence tag; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; GLP-2, glucagon-like peptide-2; GLP-2R, GLP-2 receptor; GPCR, G protein-coupled receptor; GSK, glycogen synthase kinase; Hsp90, heat shock protein 90; MEK, MAPK kinase; PARP, poly (ADP-ribose) polymerase; PKA, protein kinase A; PKC, protein kinase C; RSV, rous sarcoma virus; RT, reverse transcriptase.

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stimulating GLP-2R-expressing cells to release factor(s) that induce cell proliferation and/or survival.

A major limitation in the study of GLP-2 biology is the absence of cellular models for analysis of GLP-2R signaling. To date, no cell lines have been identified that express the endogenous GLP-2R. Accordingly, experiments using COS and BHK fibroblasts expressing transfected human or rat GLP-2Rs have shown that GLP-2 activates adenylyl cyclase leading to cAMP accumulation (17, 26). Activation of the transfected GLP-2R in BHK fibroblasts induced both cAMP response element (CRE)- and activator protein 1 (AP-1)-dependent transcriptional activity in a protein kinase A (PKA)-dependent manner, whereas higher (100 nM to 10 μM) concentrations of GLP-2 weakly stimulated proliferation and immediate early gene expression (26). Furthermore, GLP-2 directly inhibited apoptosis induced by the protein synthesis inhibitor cycloheximide, the phosphatidylinositol 3-kinase inhibitor LY294002, or the chemotherapeutic agent, irinotecan, in BHK cells expressing the rat GLP-2R (16, 27, 28).

Due to the lack of suitable cell lines for studying the endogenous GLP-2R, analysis of GLP-2R signaling has been focused, by necessity, exclusively on cells expressing a transfected GLP-2R. To extend our understanding of GLP-2R signaling to a model encompassing endogenous expression of the GLP-2R, we have searched for cell lines that express GLP-2R mRNA transcripts. We now demonstrate that the GLP-2R is endogenously expressed in a subset of cervical carcinoma cell lines, including HeLa cells, as well as in normal human cervix. However, endogenous GLP-2R expression in the HeLa cell line is confined to only a subset of cells. Thus, GLP-2R signaling was further analyzed in HeLa cells transiently expressing the cloned HeLa cell GLP-2R cDNA. These experiments revealed, unexpectedly, that GLP-2R coupling is not restricted to G_{α}s, but interacts with more than one G protein, resulting not only in intracellular cAMP generation but also activation of the Ras/MAPK pathway.

RESULTS

Identification of GLP-2R Expression in Human Cervical Cancer Cells and in Normal Human Cervix

Using the human GLP-2R nucleotide sequence to query the Expressed Sequence Tag (EST) Database we found several ESTs that corresponded to GLP-2R nucleotide sequences from human large cell lung cancer (GenBank accession nos. BQ051297 and BQ925506) and from a nonspecified human cervical carcinoma cell line (GenBank accession no. BI261112) cDNA libraries. To identify the specific cervical carcinoma cell line expressing the GLP-2R, we used RT-PCR to analyze RNA from 10 human cervical carcinoma cell lines obtained from the American Type Culture Collection. RNA isolated from DLD-1 cells stably expressing the human GLP-2R (DLD-1:hGLP-2R) was used as a positive control for GLP-2R expression. GLP-2R transcripts were detected in RNA from HeLa cells (Fig. 1A), but not in RNA from eight other human cervical carcinoma cell lines, including Hs 636.T [C4-I]; Hs 588.T; DoTc2 4510; C-4I; SW756; C-33 A; HT-3; and the ME-180 cell line (Fig. 1A). GLP-2R expression was also detected in the Ca Ski cervical carcinoma cell line (CRL 1550), although at greatly reduced levels compared with HeLa cells (Fig. 1A). Furthermore, GLP-2R transcripts were detected in RNA from normal human cervix (Fig. 1B). To determine whether the GLP-2R RNA transcript was translated to an immunoreactive GLP-2R protein in human cervical carcinomas, we employed a polyclonal GLP-2R antiserum (24) for immunohistochemical analysis of three human cervical adenocarcinomas. Rare groups ($<$5% of total tumor area) of focal GLP-2R-immunopositive cells were observed in two of the three human cervical tumors examined (Fig. 1C).

The GLP-2R Is Expressed in a Subset of HeLa Cells

Studies of GLP-2R signal transduction using transfected fibroblast cells (17, 26, 27) demonstrated that the GLP-2R is coupled to activation of adenylate cyclase. Surprisingly, GLP-2 induced only a modest increase in intracellular cAMP in HeLa cells (Fig. 2A). The lack of a significant GLP-2-stimulated cAMP response was not attributable to a generalized defect in HeLa cell cAMP generation or coupling of G_{α}s to adenylate cyclase, as forskolin (an activator of adenylate cyclase) and cholera toxin (which activates G_{α}s through ADP-ribosylation) markedly increased the levels of cAMP in HeLa cells (Fig. 2B). Similarly, the GLP-1R agonist, exendin-4, stimulated cAMP accumulation in HeLa cells transfected with the rat GLP-1R (Fig. 2B), demonstrating that HeLa cells contain the signaling machinery capable of coupling glucagon family receptors to cAMP generation.

To ascertain whether the lack of a robust cAMP response to GLP-2 was attributable to the presence of one or more mutations in the sequence of the HeLa GLP-2R, we used RT-PCR and cDNA cloning to generate multiple HeLa cell GLP-2R cDNA clones, and three clones were randomly picked for DNA sequence analysis. All three HeLa GLP-2R cDNAs contained two silent nucleotide changes at positions 510 (GA**C** to GA**T**) and 1326 (GA**A** to GA**G**) compared with the sequence originally reported (17) and deposited in GenBank (accession no. AF105367). Similarly, the lung cancer and cervical carcinoma cell line GLP-2R EST sequences in GenBank also contained both the C-T and A-G substitutions at the identical nucleotide positions (accession nos. BQ051297, BI261112, and BQ925506). As these changes do not alter the predicted open reading frame of the receptor, this finding

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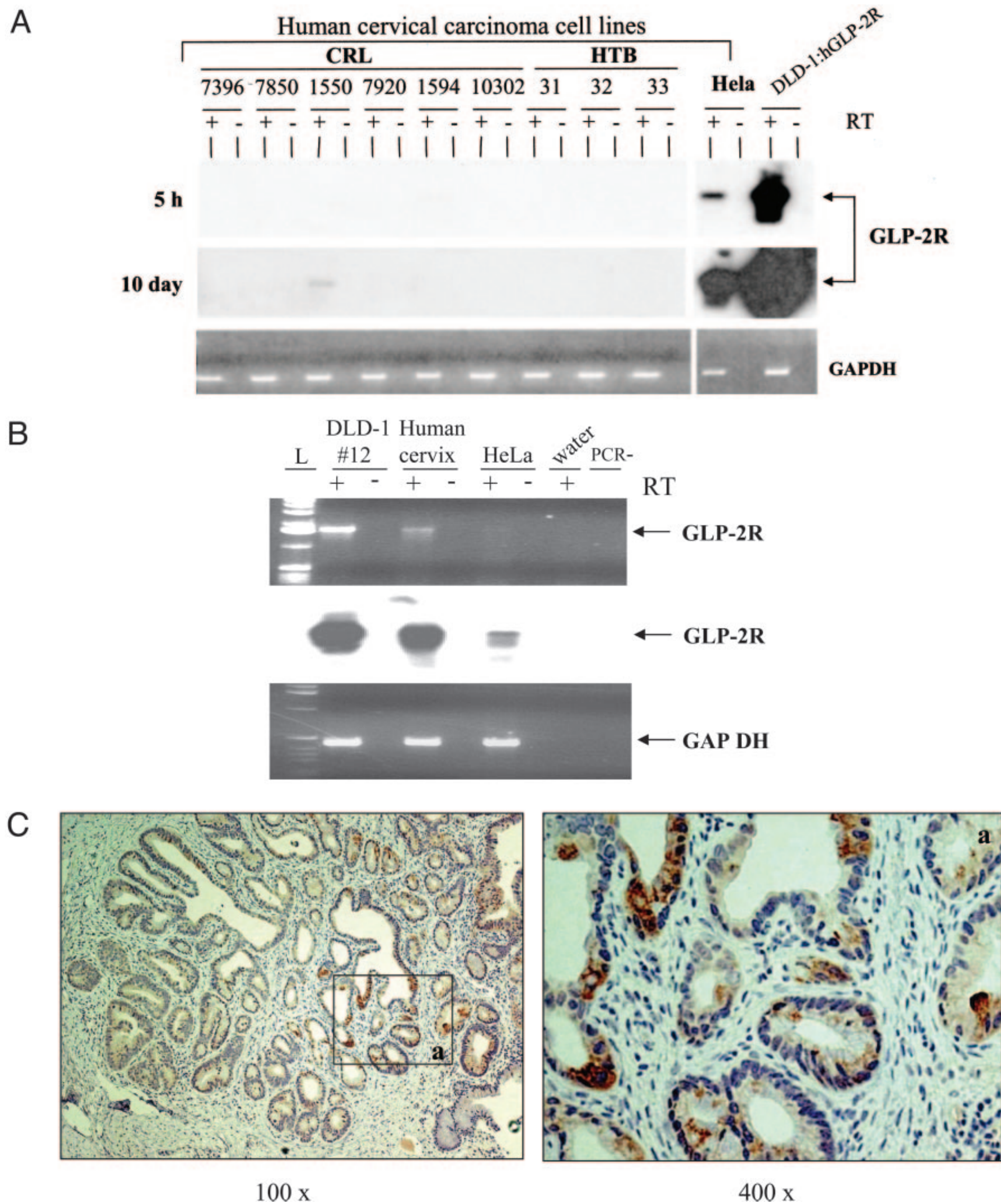

Fig. 1. The GLP-2R Is Expressed in Human Cervical Cancer Cells and in Normal Human Cervix

A, RT-PCR was performed using 5 μg total RNA isolated from a variety of human cervical carcinoma cell lines. PCR products were analyzed by agarose gel electrophoresis followed by ethidium bromide staining (GAPDH, *bottom panel*) or by Southern blot analysis (GLP-2R, *top two panels*) using an internal GLP-2R-specific oligonucleotide probe. RNA isolated from DLD-1 cells stably expressing the human GLP-2R (DLD-1:hGLP-2R) was used as a positive control for GLP-2R expression. The specificity of each reaction was monitored by control reactions using samples in which the RT was omitted from the RT reaction mixture (RT−). The CRL and HTB numbers denote the specific cervical cancer cell lines obtained from ATCC: CRL-7396 (Hs-636.T), CRL-7920 (DoTc2 4510), CRL-1594 (C-41), HTB-31 (C-33 A), HTB-32 (HT-3); adenocarcinoma: CRL-7850 (Hs 588.T), CCL-2 (HeLa); epidermoid carcinoma: CRL-1550 (Ca Ski), HTB-33 (ME-180); squamous cell carcinoma: CRL-10302 (SW 756). B, RT-PCR followed by Southern blot analysis were performed as in panel A using RNA isolated from DLD-1 cells stably expressing the human GLP-2R (DLD-1:hGLP-2R), normal human cervix, or HeLa cells. *Top and bottom panels*, Ethidium bromide staining of PCR products after amplification of GLP-2R (*top panel*) or GAPDH (*bottom panel*). *Middle panel*, Southern blot analysis using an internal GLP-2R probe. C, Localization of GLP-2R-immunopositive cells in a human cervical adenocarcinoma. Magnification (×100 and ×400) is indicated in each panel.

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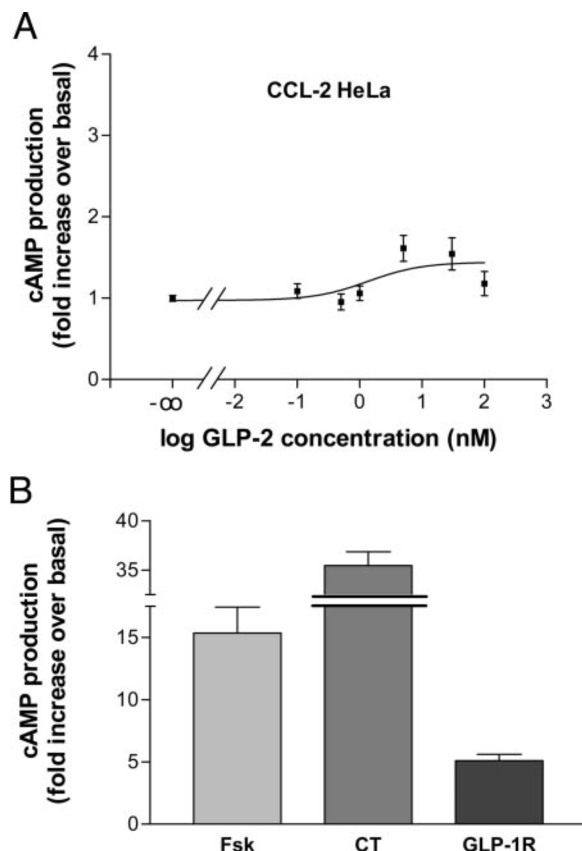

Fig. 2. Effect of GLP-2 on cAMP Production in HeLa Cells

A, cAMP production in HeLa cells after treatment with the indicated concentrations of GLP-2 for 10 min. cAMP content was measured by a RIA. Data represent mean ± SE of three independent experiments performed in triplicate and is expressed as fold increase over basal cAMP content. B, cAMP production in HeLa cells after treatment with 20 μM forskolin (Fsk) for 10 min, or 1 μg/ml cholera toxin (CT) for 4 h. HeLa cells transiently transfected with the rat GLP-1R were treated with 30 nM exendin-4 for 10 min. Data represent mean ± SD of two independent experiments performed in triplicate and is shown as a fold increase over basal.

did not explain the lack of a GLP-2-stimulated cAMP response in HeLa cells.

We hypothesized that the modest cAMP response to exogenous GLP-2 may be attributable to low level or heterogeneous GLP-2R expression in HeLa cells. To assess this hypothesis, RT-PCR was performed on RNA isolated from pools of 10 or 50 cells. Southern blot analysis detected a wide range of GLP-2R expression levels in pools of cells ranging from strong to weak to absent signals (data not shown), consistent with marked variation in the expression of the GLP-2R in HeLa cells.

The HeLa Cell GLP-2R Couples to the Adenylate Cyclase Pathway and to ERK1/2 Activation

As the GLP-2R was variably expressed in subsets of HeLa cells, we attempted to isolate clonal HeLa cell lines that differed in their level of endogenous GLP-2R expression. However, we did not detect the GLP-2R in any of the individual clones isolated, nor were we able to stably express the GLP-2R in HeLa cells. We therefore reexamined the cAMP response to GLP-2 stimulation in transient transfection experiments employing the cloned human HeLa cell GLP-2R cDNA. GLP-2 stimulated cAMP accumulation in a dose-dependent manner in HeLa cells transfected with the GLP-2R (Fig. 3A). Thus the GLP-2R cloned from HeLa cells is functional, expressed only in subsets of HeLa cells, and couples to downstream signaling pathways resulting in the accumulation of intracellular cAMP.

To determine whether the GLP-2R is coupled to signaling pathways other than adenylate cyclase and cAMP generation in HeLa cells, we assessed the phosphorylation of p44/p42 MAPK (ERK1/2) and p90Rsk (a downstream effector of ERK1/2). GLP-2 significantly induced ERK1/2 phosphorylation in GLP-2R-transfected HeLa cells in a dose- (Fig. 3B) and time-dependent (Fig. 3C) manner, with maximal activation occurring between 5 and 15 min using 20 nM GLP-2. Similarly, incubation of HeLa cells with 10% fetal bovine serum (FBS) also increased ERK1/2 and p90Rsk phosphorylation (compare lane 1 with lane 6 in Fig. 3B).

To further assess the functional consequences of GLP-2-induced ERK1/2 phosphorylation, HeLa cells were cotransfected with the GLP-2R, a luciferase reporter plasmid (pFR-luc), and a *trans*-activator plasmid (pFA2-Elk-1) (PathDetect Elk-1 *trans*-reporting system). As Elk-1 is a downstream effector of ERK1/2, activation of ERK1/2 should phosphorylate and activate the Elk-1 fusion protein resulting in transcription of the luciferase gene and an increase in luciferase activity. GLP-2 stimulated an approximately 3-fold increase in luciferase activity in GLP-2R-transfected HeLa cells cotransfected with the luciferase/Elk-1 plasmids (Fig. 3D), demonstrating that GLP-2 stimulates both ERK1/2 phosphorylation and activation, leading to induction of downstream transcriptional activity.

To determine whether the effect of GLP-2 on ERK1/2 phosphorylation/activation was due merely to overexpression of the GLP-2R in HeLa cells, we titrated the amount of the GLP-2R expression vector transfected into HeLa cells and monitored ERK1/2 phosphorylation and cAMP accumulation. Moreover, we indirectly measured the relative level of GLP-2R expression by examining luciferase activity in cells that were transfected with a similarly titrated luciferase expression plasmid under the control of the same promoter [cytomegalovirus (CMV)] as the GLP-2R. As shown in Fig. 4, decreasing the amount of the luciferase reporter plasmid transfected into HeLa cells by 40-fold (50 ng), while keeping the total amount of DNA transfected constant (2 μg), markedly reduced the level of luciferase activity (Fig. 4C). Transfection efficiencies were similar in each titration experiment as monitored by visualizing the number of green fluorescent protein (GFP)-positive cells (data not shown). In contrast to findings observed after titration of the luciferase cDNA, both the level of ERK1/2 phosphorylation and cAMP accumulation induced by GLP-2 were rela-

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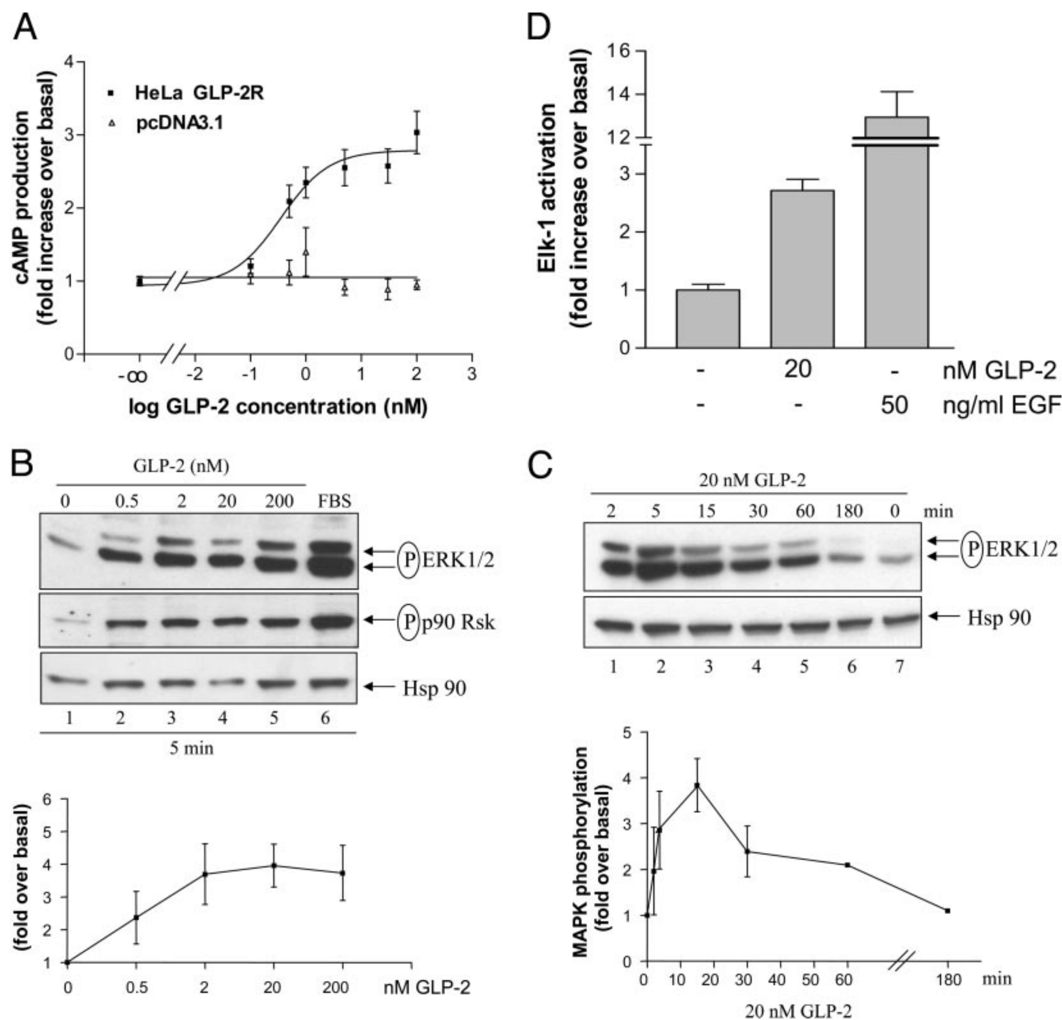

Fig. 3. Transient Transfection of the Cloned HeLa Cell GLP-2R into HeLa Cells Activates Adenylate Cyclase and p42/44 MAPK in a GLP-2-Dependent Manner

A, HeLa cells were transfected with pcDNA3.1, or the human GLP-2R cloned from HeLa cells expressed from pcDNA3.1 (HeLa GLP-2R). Two days after transfection, cells were treated with the indicated concentrations of GLP-2 for 10 min. cAMP content was measured by a RIA. Data represent mean ± SD of three independent experiments performed in triplicate. B and C (*top panels*), GLP-2R-transfected HeLa cells were serum starved overnight and stimulated for 5 min with increasing amounts of GLP-2 or 10% FBS as indicated (panel B) or stimulated with 20 nM GLP-2 for the indicated times (panel C). After stimulation, cells were lysed and equal amounts of whole-cell lysate were immunoblotted using primary antibodies against phosphorylated/activated p90Rsk, phosphorylated/activated ERK1/2, or Hsp90 as a loading control. Shown are representative blots from three independent experiments. B and C (*bottom panels*), The intensity of the ERK2 signal after treatment with GLP-2 was quantified by densitometry, corrected by the intensity of the Hsp90 signal, and expressed relative to the values for serum-starved control cultures. Data are means ± SD from three independent experiments. D, Effect of GLP-2 on ELK-1-mediated transcriptional activation. HeLa cells were cotransfected with the GLP-2R, pFR-Luc (reporter) plasmid, and the pFA2-Elk-1 (fusion *trans*-activator) plasmid. After serum starvation overnight, cells were incubated with or without 20 nM GLP-2 or 50 ng/ml EGF for 5.5 h. Luciferase activity was measured from cell lysates and is shown as the fold increase over basal (serum-starved) activity. Data are means ± SD from three independent experiments.

tively preserved after a similar 40-fold reduction in the amount of GLP-2R cDNA transfected into HeLa cells (Fig. 4, A and B). Furthermore, progressively titrating downward the amount of transfected GLP-2R cDNA by 200-fold (10 ng) reduced, but did not abolish, GLP-2-induced ERK1/2 phosphorylation or cAMP production (Fig. 4), suggesting that activation of either of these signaling pathways by GLP-2 is not simply a consequence of massive overexpression of the transfected GLP-2R.

cAMP Accumulation and Epidermal Growth Factor Receptor (EGFR) Transactivation Do Not Account for GLP-2-Stimulated ERK1/2 Activation

Several GPCRs (29, 30), including the related GLP-1R (31), activate the MAPK/ERK1/2 cascade by stimulating receptor tyrosine kinases such as the EGFR (referred to as "transactivation") or through activation of cAMP-dependent guanine-nucleotide exchange factors for Ras

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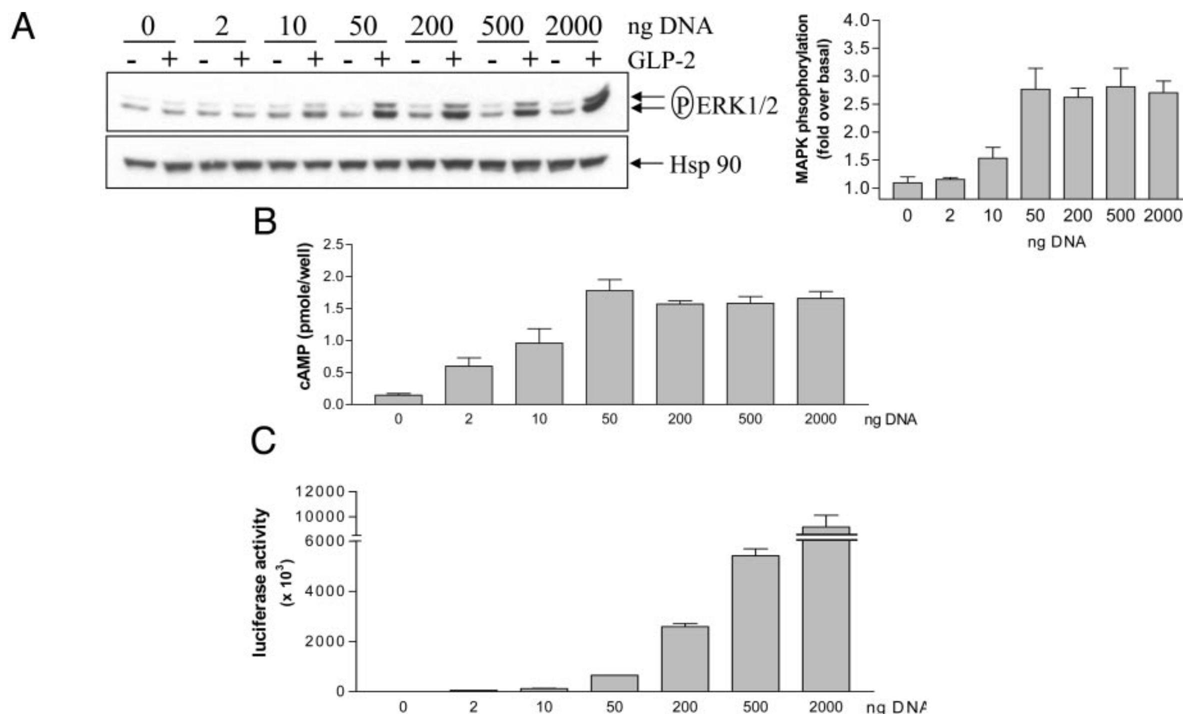

Fig. 4. Decreasing the Amount of the GLP-2R cDNA Transfected into HeLa Cells Does Not Abolish GLP-2-Induced ERK1/2 Phosphorylation or cAMP Accumulation

A (*left panel*), HeLa cells were transiently transfected with the indicated amounts of the cloned HeLa GLP-2R cDNA expressed from pcDNA3.1 [total DNA used in each transfection remained constant (2 μg)]. One day after transfection, cells were serum starved overnight before treatment with or without 20 nM GLP-2 for 5 min. Cells were lysed, and equal amounts of whole-cell lysate were immunoblotted using primary antibodies against phosphorylated/activated ERK1/2, or Hsp90 as a loading control. Shown are representative blots from three independent experiments. A (*right panel*), The intensity of the ERK2 signal was quantified by densitometry, corrected by the intensity of the Hsp90 signal, and expressed as a fold increase over serum-starved levels. Data are means ± SD from three independent experiments. B, HeLa cells were transfected as in panel A. Two days after transfection, cells were treated with the indicated concentrations of GLP-2 for 10 min. cAMP content was measured by a RIA. Data represent mean ± SD of three independent experiments performed in triplicate. C, HeLa cells were transfected with the indicated amounts of a CMV-luciferase plasmid [again, total DNA used in each transfection remained constant (2 μg)]. Cells were lysed 2 d after transfection, and luciferase activity was measured.

or Rap (32, 33). To determine whether cAMP accumulation was responsible for ERK1/2 activation by GLP-2 in HeLa cells, ERK1/2 phosphorylation was analyzed after stimulation with 20 μM forskolin or treatment with cholera toxin. Although both forskolin and CT increased intracellular cAMP (Fig. 2B), neither treatment induced the phosphorylation of ERK1/2 (Fig. 5A, lanes 1 and 2). Moreover, treatment with the cell-permeable cAMP analog, 8-bromo-cAMP, did not stimulate ERK1/2 phosphorylation (data not shown), suggesting that neither activation of $G_{\alpha}s$ nor increased levels of cAMP were sufficient to activate ERK1/2 in HeLa cells. Similarly, the specific EGFR inhibitor, AG1478, had no effect on GLP-2-induced ERK1/2 phosphorylation (Fig. 5A, lanes 4 and 5) but completely abolished ERK1/2 activation by EGF (Fig. 5A, lanes 6 and 7), demonstrating that EGFR transactivation is not required for ERK1/2 activation by GLP-2 in HeLa cells.

To identify additional signaling molecules important for the GLP-2-mediated stimulation of ERK1/2, we examined the effects of different kinase inhibitors on ERK1/2 activation. Phosphorylation of ERK1/2 by GLP-2 was completely blocked by the MAPK kinase (MEK) inhibitor PD98059 (Fig. 5B, lane 3). Conversely, the PKA inhibitor H89 and the PI-3 kinase inhibitor LY294002 did not significantly inhibit ERK1/2 phosphorylation by GLP-2 (lanes 2 and 5, respectively), demonstrating that MEK, but not PKA or PI-3 kinase, mediates ERK1/2 activation by GLP-2.

GLP-2-Induced ERK1/2 Activation Is Mediated through G_i/G_o and Ras

Several GPCRs are known to activate the Ras/Raf/MAPK pathway by coupling to the G_i or G_q subfamily of heterotrimeric G proteins (34). To identify the G proteins responsible for coupling the GLP-2R to ERK1/2 activation, HeLa cells were pretreated with pertussis toxin before stimulation with GLP-2. As shown in Fig. 6A, pertussis toxin, which blocks G_i/G_o protein-mediated signaling by locking these G proteins in the GDP-bound state through ADP ribosylation of G_i/G_o α-subunits, abolished ERK1/2 phosphorylation by GLP-2 (Fig. 6A, lane 2), but had no effect on

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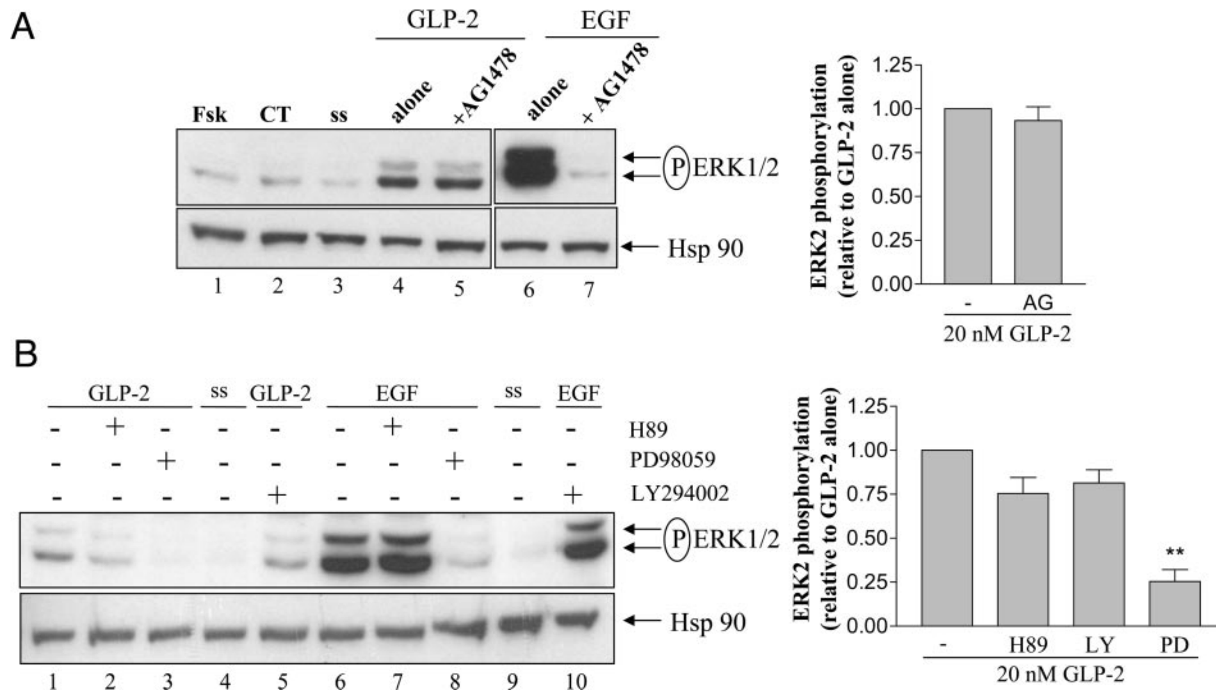

Fig. 5. cAMP Accumulation, PKA Activation, and EGFR Transactivation Do Not Account for GLP-2-Stimulated ERK1/2 Activation in HeLa Cells

A (*Left panel*), GLP-2R-transfected HeLa cells were serum starved (ss) overnight (lane 3) before treatment with 20 μM forskolin for 5 min (lane 1) or 1 μg/ml cholera toxin for 4 h (lane 2), or were pretreated for 1 h with 1 μM AG1478 before stimulation with 20 nM GLP-2 for 5 min (lanes 4 and 5). As a control for EGFR activation, cells were pretreated with or without AG1478 before stimulation with 50 ng/ml EGF for 5 min (lanes 6 and 7). After treatment, equal amounts of whole-cell lysate were immunoblotted using antisera against phosphorylated ERK1/2 or Hsp90. Shown are representative blots from three independent experiments. B (*Left panel*), Western blot analysis of GLP-2R-transfected HeLa cells after stimulation with GLP-2 or EGF in the presence or absence of H-89, PD98059, or LY294002. Serum-starved (ss) cells (lanes 4 and 9) were pretreated with 10 μM H-89 (lanes 2 and 7), 50 μM PD98059 (lanes 3 and 8), or 50 μM LY294002 (lanes 5 and 10) for 1 h before stimulation with 20 nM GLP-2 (lanes 1–3 and 5) or 50 ng/ml EGF (lanes 6–8 and 10) for 5 min and immunoblotted as in panel A. Shown are blots representative of four independent experiments. A and B (*Right panels*), ERK2 phosphorylation levels after treatment with GLP-2 in the presence or absence of kinase inhibitors or the EGFR antagonist AG1478. The intensity of the ERK2 signal was quantified by densitometry, corrected by the intensity of the Hsp90 signal, and expressed relative to the values of GLP-2 alone-treated cells. Data are mean ± SD from three (panel A) or four (panel B) independent experiments. **, $P < 0.001$ (GLP-2 alone *vs.* pretreatment with inhibitor before stimulation with GLP-2).

EGF-mediated ERK1/2 activation (Fig. 6A, lane 7). Furthermore, β-adrenergic receptor kinase (βARKct), which sequesters $\beta\gamma$-subunits thereby inhibiting G$\beta\gamma$-mediated signaling (35, 36), inhibited GLP-2-, but not EGF-induced ERK1/2 phosphorylation (Fig. 6A, lane 4). Similarly, phosphorylation of ERK1/2 by GLP-2 was inhibited by dominant negative Ras (Fig. 6B, lane 3). Thus, the GLP-2R activates the Ras/Raf/MAPK pathway by coupling to G_i/G_o, and this activation is mediated, at least in part, by $\beta\gamma$-subunits of G proteins.

Recently the GnRH receptor has been shown to display an agonist-induced dose-dependent switch in its coupling to particular G proteins (37). To ascertain whether the same process regulates GLP-2R coupling to specific G proteins, cAMP accumulation after exposure to increasing concentrations of GLP-2 was assessed in GLP-2R-transfected HeLa cells. However, we did not observe a dose-dependent inhibition of cAMP production with increasing concentrations of GLP-2 up to 20 μM (Fig. 7) as has been observed with the GnRH receptor (37). Next we hypothesized that if GLP-2R coupled to G_i, then pretreatment with pertussis toxin should increase GLP-2-mediated cAMP production by alleviating the inhibitory effect of G_i on adenylate cyclase. Nevertheless, no significant increase in cAMP accumulation was detected at various concentrations of GLP-2 in the presence of pertussis toxin (Fig. 7). Taken together, the data suggest that either the GLP-2R couples to Go rather than to G_i, or that coupling to G_i does not lead to GLP-2R-dependent inhibition of adenylate cyclase in HeLa cells.

GLP-2 Increases Bromodeoxyuridine (BrdU) Incorporation in GLP-2R-Transfected HeLa Cells

Because GLP-2R activation leads to ERK1/2 activation in HeLa cells, we assessed whether GLP-2 also stimulated DNA synthesis by examining BrdU incorporation in GLP-2R-transfected HeLa cells (Fig. 8A).

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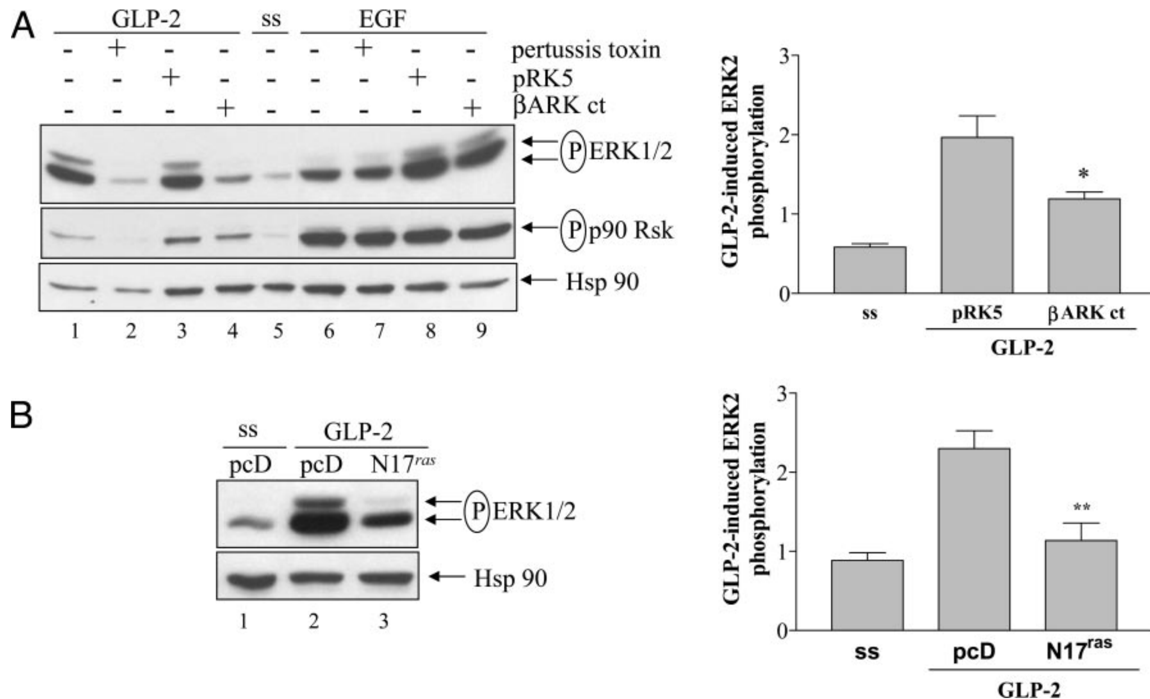

Fig. 6. GLP-2R-Stimulated ERK1/2 Activation in HeLa Cells Is Mediated through G_i/G_o, $\beta\gamma$ Subunits and the Small G-Protein Ras

A (*Left panel*), HeLa cells transfected with the GLP-2R were either cotransfected with pRK5 (lanes 3 and 8) or βARKct (lanes 4 and 9) or were treated with 400 ng/ml pertussis toxin (lanes 2 and 7), and cells were examined after serum starvation overnight (ss, lane 5) and after stimulation with 20 nM GLP-2 (lanes 1–4) or 50 ng/ml EGF (lanes 6–9) for 5 min. Cell lysates were immunoblotted against phosphorylated ERK1/2, phosphorylated p90Rsk, or Hsp90 as a loading control. A (*Right panel*), ERK2 phosphorylation levels after treatment with GLP-2 in cells transfected with βARKct. The intensity of the ERK2 signal was quantified by densitometry and corrected by the intensity of the Hsp90 signal. Data are means ± SD from four independent experiments. *, $P < 0.05$ (pRK5 *vs.* βARKct). B (*left panel*), HeLa cells were cotransfected with the GLP-2R and pcDNA3.1 (pcD, lanes 1 and 2) or a dominant negative Ras $N17^{ras}$ encoding plasmid ($N17^{ras}$, lane 3), serum starved overnight (lane 1), and stimulated with 20 nM GLP-2 (lanes 2 and 3) for 5 min. After stimulation, cells were lysed, and equal amounts of whole-cell lysate were immunoblotted against phosphorylated/activated ERK1/2 or Hsp90 as a loading control. B (*right panel*), ERK2 phosphorylation levels after treatment with GLP-2 in cells transfected with $N17^{ras}$. The intensity of the ERK2 signal after treatment was quantified by densitometry and corrected for the intensity of the Hsp90 signal. Data are means ± SD from three independent experiments. **, $P < 0.001$ (pcDNA3.1 *vs.* $N17^{ras}$).

Although cells were cultured in serum-free media for 48 h before stimulation with GLP-2 or FBS, the basal level of BrdU-positive cells remained relatively high at approximately 37% in control cells incubated in the presence of BrdU for 7 h (ss: Fig. 8A, *lower panel*). Similar rates of BrdU accumulation were also observed in cells pulsed with BrdU for 30 min or 2 h, suggesting that HeLa cells have a high intrinsic rate of DNA synthesis even under serum-starved conditions. The number of BrdU-positive cells was significantly increased to approximately 45% after stimulation with either 20 nM GLP-2 or 10% FBS (Fig. 8A), demonstrating that both GLP-2 and FBS stimulate DNA synthesis in serum-starved HeLa cells. Furthermore, transfection of the dominant negative Ras cDNA significantly attenuated BrdU incorporation induced by either GLP-2, 10% FBS, or 50 ng/ml EGF (Fig. 8B). These findings demonstrate that the effect of GLP-2 on DNA synthesis is mediated through the Ras/Raf/MAPK pathway.

GLP-2 Protects GLP-2R-Transfected HeLa Cells from LY294002-Induced Apoptosis

GLP-2 administration to mice significantly reduced chemotherapy- or indomethacin-induced intestinal injury by decreasing apoptosis in the intestinal crypt cell compartment (15, 16). Moreover, GLP-2 directly inhibited apoptosis induced by cycloheximide, LY294002, and irinotecan in BHK-GLP-2R cells (16, 27, 28). To examine whether activation of the GLP-2R in HeLa cells was also coupled to antiapoptotic pathways, cells were transiently cotransfected with the hGLP-2R and a Rous sarcoma virus-β-galactosidase expression plasmid, and β-galactosidase activity, as an indicator of viable transfected cells, was measured after a 24-h incubation in the presence or absence of LY294002 (Fig. 9A). Incubation with LY294002 alone induced clearly visible loss of cells paralleled by a significant quantitative reduction in β-galactosidase activity. In contrast, treatment with 20 nM GLP-2 (data

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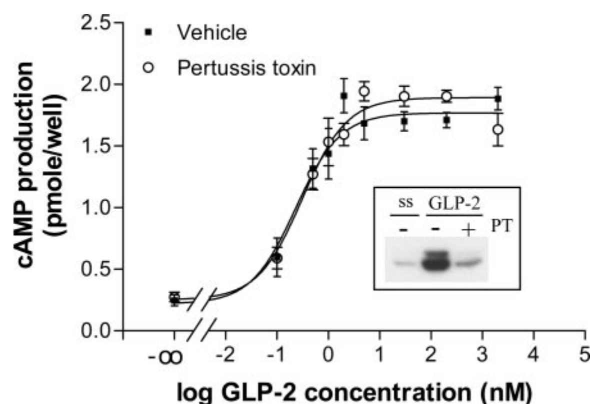

Fig. 7. Pertussis Toxin Treatment Does Not Modulate GLP-2R-Induced cAMP Accumulation in GLP-2R-Transfected HeLa Cells

GLP-2R-transfected HeLa cells were treated in the presence (○) or absence (■) of 400 ng/ml pertussis toxin for 24 h before stimulation with 0.1 nM, 0.5 nM, 1 nM, 2 nM, 5 nM, 30 nM, 200 nM, or 2 μM GLP-2 for 10 min. cAMP content was measured by a RIA. Data represent mean ± SE of four independent experiments performed in triplicate. Pertussis toxin inhibition of GLP-2R-induced ERK1/2 phosphorylation was used to confirm the toxin action in every experiment. A representative blot is shown in the *inset*.

not shown), 200 nM GLP-2, 10 μM forskolin, or 5% FBS significantly prevented cell death induced by LY294002 (Fig. 9A).

As GLP-2R activation in HeLa cells leads to increased levels of intracellular cAMP and ERK1/2, we assessed whether GLP-2-induced cell survival was mediated through PKA- or ERK1/2-dependent pathways. Both H89 and PD98059 alone reduced β-galactosidase activity in HeLa cells (Fig. 9A), suggesting that basal levels of both PKA and MAPK, respectively, are important for cell survival. The cytoprotective effects observed with GLP-2 and forskolin after exposure to LY294002 were markedly reduced by H89 (Fig. 9A). Conversely, the MEK inhibitor PD98059 did not attenuate the protective effects of GLP-2 on cell survival (Fig. 9A), demonstrating that ERK1/2 is not required for the antiapoptotic actions of GLP-2.

To determine whether the reduced cell survival in LY294002-treated cells was associated with activation of apoptotic pathways, we examined the levels of intact poly (ADP-ribose) polymerase (PARP) and glycogen synthase kinase (GSK)-3β phosphorylated at Ser9 (28). Treatment of GLP-2R-transfected HeLa cells with LY294002 alone reduced the levels of phosphorylated, catalytically inactive GSK-3β and intact uncleaved PARP (a downstream substrate of active executioner caspases) (Fig. 9B, lane 2). Conversely, cells cotreated with LY294002 and GLP-2, or to a greater extent with forskolin, exhibited increased levels of phosphorylated GSK-3β and reduced cleavage of PARP (Fig. 9B). Similarly, cleavage of PARP was attenuated, and GSK-3β was phosphorylated on Ser9 in cells cotreated with FBS (Fig. 9B, lane 5). Taken together, these results demonstrate that GLP-2 attenuates HeLa cell apoptosis induced by LY294002 in a PKA-dependent manner.

DISCUSSION

Observations that GLP-2 enhances the reparative response to mucosal injury have engendered interest in the therapeutic potential of GLP-2 for the treatment of patients with intestinal failure (38, 39). Previous studies using receptor-specific antisera or *in situ* hybridization have localized GLP-2R expression to human gut endocrine cells and murine enteric neurons, respectively (24, 25). The demonstration that the GLP-2R is endogenously expressed in the HeLa human cervical carcinoma cell line, in Ca Ski cells, as well as in normal human cervix, together with the detection of rare GLP-2R-immunopositive cells in human cervical tumors, extends GLP-2R expression to cells beyond the gastrointestinal tract and provides a new model for the analysis of GLP-2R signaling pathways. Furthermore, data from EST and SAGE (serial analysis of gene expression) database searches confirm the presence of the GLP-2R in a variety of tumor cell lines and tissues. This observation becomes increasingly relevant if GLP-2 is to be used therapeutically because the functional consequences of GLP-2R activation in cancer cell lines such as HeLa cells have not yet been characterized.

Experiments using COS and BHK cells demonstrated coupling of the transfected rat and human GLP-2R to adenylyl cyclase (17, 26). In contrast, activation of the endogenous HeLa cell GLP-2R induced a weak cAMP response, whereas a more robust augmentation of GLP-2-stimulated cAMP production was observed after transfection of the cloned HeLa cell GLP-2R. These findings, taken together with RT-PCR data demonstrating variable expression of the GLP-2R in subsets of pooled HeLa cells, suggest that the modest HeLa cell cAMP response to GLP-2 likely reflects the variable and relatively low level of endogenous HeLa GLP-2R expression.

Our results demonstrate, for the first time, that the GLP-2R couples to G_i/G_o to activate the Ras/MAPK pathway and that the GLP-2-stimulated engagement of ERK1/2 is mediated, at least in part, by $\beta\gamma$-subunits (Fig. 9). The demonstration that neither H89 nor LY294002 blocked GLP-2-mediated ERK1/2 activation and that independent increases in cAMP levels (with forskolin or cholera toxin) failed to activate ERK1/2, indicates that neither cAMP/PKA nor phosphatidylinositol 3-kinase mediate GLP-2-stimulated ERK1/2 activation. Furthermore, the inability of H89 to block GLP-2-stimulated ERK1/2 phosphorylation demonstrates that PKA is not essential for GLP-2R coupling to different G proteins in HeLa cells as has been observed with the β2-adrenergic receptor (β2AR) in human embryonic kidney 293 cells (40) and with the

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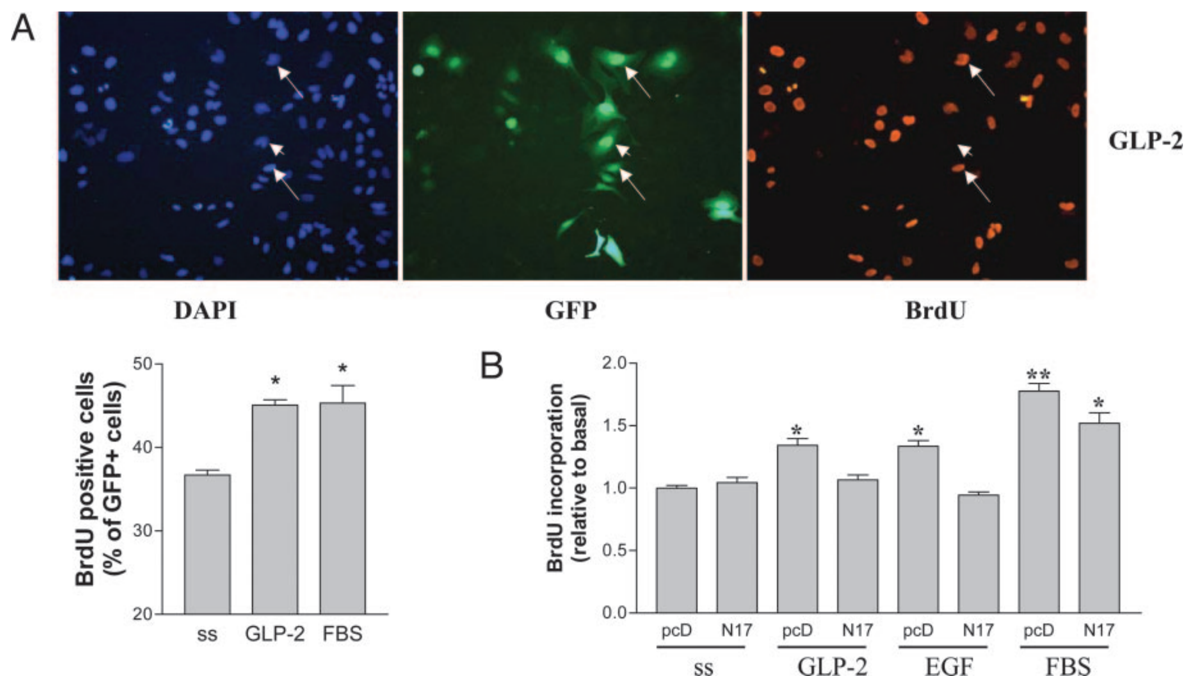

Fig. 8. GLP-2 Increases BrdU Incorporation in GLP-2R-Transfected HeLa Cells

A (*top panels*), HeLa cells were cotransfected with the GLP-2R and pEGFP-C1 (to detect transfected cells) serum starved for 48 h, and then incubated for 7 h with 10 μM BrdU in the absence or presence of 20 nM GLP-2 or 10% FBS. After treatment, slides were subjected to indirect immunofluorescence directed against BrdU and counterstained using DAPI. Representative GFP^{+} (transfected) cells (*middle panel*) that were (→) or were not (▶) stained for BrdU (*right panel*) are indicated. Magnification ×200. A (*bottom panel*), The number of BrdU-positive cells after treatment, as indicated, is shown as a percentage of the number of cells cotransfected with GLP-2R and pEGFP-C1 (GFP^{+}). For each treatment, 300–400 GFP^{+} cells were counted in each experiment and assessed for BrdU incorporation. Data shown are the means ± SD from two independent experiments. *, $P < 0.05$ (control *vs.* stimulation with GLP-2 or FBS). B, HeLa cells were cotransfected with the GLP-2R and either pcDNA3.1 or a dominant negative Ras N17^{ras} encoding plasmid. After serum starvation for 24 h, cells were stimulated with 20 nM GLP-2, 50 ng/ml EGF, or 10% FBS for 16 h. BrdU (10 μM) was then added to cells for an additional 6 h, and BrdU incorporation was assessed by ELISA. Data represent mean ± SD of three independent experiments performed in triplicate. *, $P < 0.05$; **, $P < 0.001$ (serum starved *vs.* treatment with either GLP-2, EGF, or FBS).

engagement of G_s and G_i by the vasoactive intestinal peptide receptor in pancreatic acinar cells (41).

Although GLP-1 receptor activation promotes EGFR transactivation in islet cell lines (31, 42), AG1478 failed to inhibit ERK1/2 phosphorylation by GLP-2. Hence the EGFR does not play a role in GLP-2-induced ERK1/2 activation in HeLa cells. Nevertheless, GPCR signaling is capable of transactivating growth factor receptors in HeLa cells, as lysophosphatidic acid (LPA)-induced EGFR phosphorylation and ERK1/2 activation through the generation of hydrogen peroxide (43, 44). Protein tyrosine phosphatases, which negatively regulate receptor tyrosine kinases, are transiently inactivated by hydrogen peroxide and other reactive oxygen species, which results in receptor tyrosine kinase activation (45). Thus, it remains possible that GLP-2R stimulation could lead to production of reactive oxygen species resulting in the activation of receptor tyrosine kinases, other than the EGFR, that may mediate ERK1/2 activation.

Because we did not observe any differences in levels of GLP-2-stimulated cAMP with or without pertussis toxin, we cannot definitively conclude whether the GLP-2R couples to G_i or G_o in HeLa cells. Similar results have been observed in studies of the calcitonin receptor. Although pertussis toxin inhibited ERK phosphorylation by calcitonin (46), it had little effect on calcitonin-induced cAMP accumulation in human embryonic kidney 293 cells (47). Interestingly, inhibition of protein kinase C (PKC) decreased calcitonin-induced cAMP production, an effect that was reversed by pretreatment with pertussis toxin, suggesting that PKC suppresses inhibition of adenylate cyclase by $G_{\alpha i}$ (47). As phospholipase Cβ can be activated by $\beta\gamma$-subunits released from G_i (48), it will be interesting to test whether GLP-2 stimulation also induces PKC activation, or whether PKC similarly suppresses $G_{\alpha i}$-mediated inhibition of adenylate cyclase in HeLa cells.

Although previous studies using RT-PCR analysis failed to detect GLP-2R mRNA transcripts in a variety of intestinal epithelial cell lines, including Caco-2 and T84 cells (24), GLP-2 produced a dose-dependent (10 nM to 10 μM) increase in [^{3}H]thymidine incorporation in both of these cell lines, suggesting the possible existence of an, as yet unidentified, second GLP-2R (49–51). In addition, GLP-2 induced DNA synthesis in

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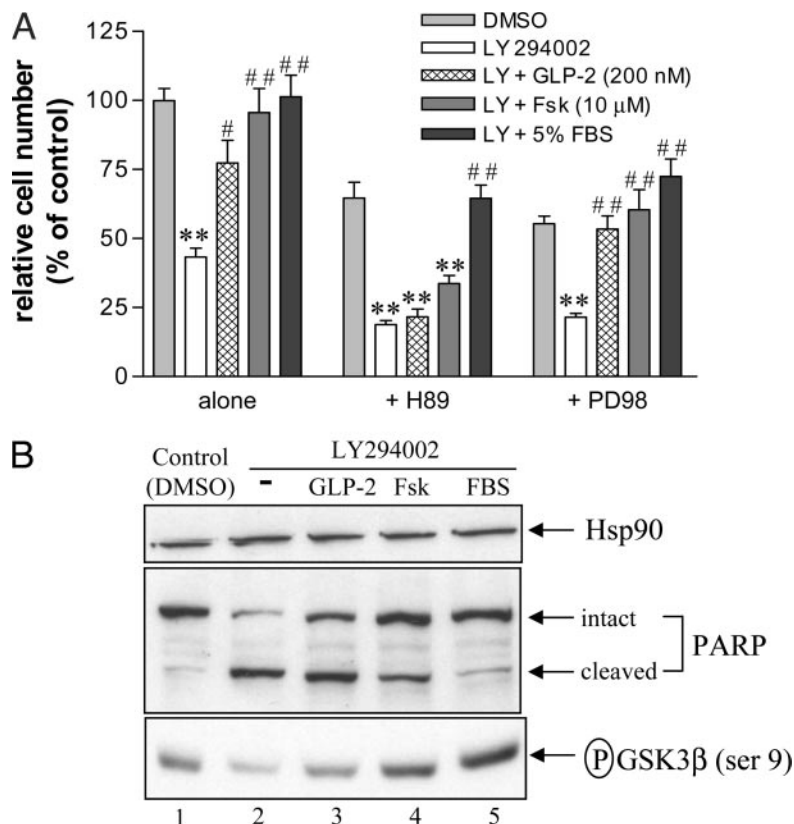

Fig. 9. GLP-2 Protects GLP-2R-Transfected HeLa Cells from LY294002-Induced Apoptosis

A, Serum-starved HeLa cells, transiently transfected with the GLP-2R and a RSV-β-galactosidase expression plasmid, were treated with 50 μM LY294002 (LY) for 24 h with or without 200 nM GLP-2, 10 μM forskolin (Fsk), or 5% FBS in the presence of vehicle, the PKA inhibitor H-89 (10 μM), or the MEK inhibitor PD98059 (50 μM). β-Galactosidase activity (as an indicator of transfected cell number) was determined from cell lysates and is shown as a percentage of the average value obtained from analysis of vehicle alone-treated cells. Data shown are the means $\pm$ SD from three independent experiments, each performed in triplicate. #, $P < 0.05$; ##, $P < 0.001$ (LY294002 + either GLP-2, forskolin or FBS *vs.* LY294002 alone). **, $P < 0.001$, treatment with LY294002 +/−GLP-2, or forskolin or FBS *vs.* control (DMSO). B, Serum-starved GLP-2R-transfected HeLa cells were treated with DMSO alone (lane 1), or 50 μM LY294002 (lanes 2–5) in the presence of 200 nM GLP-2 (lane 3), 10 μM forskolin (lane 4), or 5% FBS (lane 6). After a 24-h incubation period, attached and floating cells were combined, lysed, and immunoblotted against Hsp90 as a loading control, PARP, or phosphorylated (inactive) GSK-3β at Ser9. Data shown are representative of three independent experiments.

cultured rat astrocytes expressing the GLP-2R in a dose-dependent manner with maximal [^{3}H]thymidine incorporation being observed at 10 nM (52). Similarly, we have demonstrated a modest, but significant, increase in BrdU incorporation in transiently transfected HeLa cells after GLP-2 (20 nM) stimulation. Furthermore, both ERK1/2 phosphorylation and BrdU incorporation induced by GLP-2 were blocked by dominant negative Ras, indicating that the effect of GLP-2 on DNA synthesis is mediated through activation of the Ras/Raf/MAPK pathway.

Consistent with the potent antiapoptotic actions of GLP-2 in the rodent gastrointestinal epithelium (15, 16), and in BHK cells *in vitro* (27, 28), both GLP-2 and forskolin increased HeLa cell survival after treatment with LY294002. Furthermore, GLP-2 inhibited cleavage of PARP and stimulated GSK-3β phosphorylation, consistent with previous findings in BHK cells (28). Although caspase-9 may potentially be inhibited by ERK/MAPK through phosphorylation at Thr 125 (53), inhibition of ERK1/2 activation by PD98059 had no effect on the GLP-2-dependent attenuation of LY294002-induced cell death; hence the prosurvival effect of GLP-2 is independent of ERK1/2. Conversely, H89 abolished the cytoprotective effects of GLP-2, indicating that the antiapoptotic effect of GLP-2 in HeLa cells is regulated through a cAMP-dependent pathway, in agreement with data obtained in studies of BHK cells (28).

In summary, we have identified a human cell line that endogenously expresses the GLP-2R, albeit at low levels, providing a new model for analysis of GLP-2R-activated signaling pathways in human cells. Although the GLP-2R is coupled to a cAMP- and PKA-dependent pathway in HeLa cells, the concomitant activation of ERK1/2 is not mimicked by elevation of cAMP, implying the existence of separate GLP-2R-activated pathways coupled to cell proliferation and cell survival

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(Fig. 10) distinct from those previously defined in BHK cells (26–28). Hence, analysis of GLP-2R signaling in HeLa cells may provide new information revealing how the GLP-2R engages diverse pathways coupled to cAMP generation, MAPK activation, and cell survival.

MATERIALS AND METHODS

Materials

Tissue culture medium, serum, and G418 were from Invitrogen Life Technologies, Inc. (Carlsbad, CA). Forskolin, protease inhibitor mixture (P-2714), and phosphatase inhibitor cocktail I were from Sigma (St. Louis, MO). LY294002, pertussis toxin, cholera toxin, H-89, PD98059, EGF, and AG1478 were from Calbiochem (San Diego, CA). Human GLP-2 was from Peninsula Bachem (King of Prussia, PA). GLP-2 was dissolved in PBS, pertussis toxin, EGF, and cholera toxin in water and forskolin, LY294002, H-89, PD98059, and AG1478 in dimethyl sulfoxide (DMSO). Total cellular RNA from human cervix was from Ambion, Inc. (Austin, TX). Ribonuclease inhibitor, deoxyribonuclease I, deoxynucleotide triphosphates, and ribonuclease H were from MBI Fermentas (Burlington, Ontario, Canada).

DNA Plasmids and Transient Transfections

cDNAs encoding the rat GLP-1 receptor or the human HeLa GLP-2R (HeLa GLP-2R), derived from RT-PCR, were initially subcloned into a TOPO TA cloning vector (Invitrogen Life Technologies) and subsequently subcloned into the pcDNA3.1 eukaryotic expression vector (Invitrogen Life Technologies). The dominant negative mutant $p21^{N17ras}$ cDNA in pcDNA3.1 was obtained from the Guthrie cDNA Resource Centre (www.cdna.org). The mammalian expression vectors pRK5 and pRK5 containing the sequence encoding the C-terminal fragment of βARKct were a kind gift from Dr. Robert Lefkowitz, and the pEGFP-C1 plasmid was purchased from CLONTECH (BD Biosciences, Mississauga, Ontario, Canada). The luciferase reporter plasmid (pFR-luc) and the *trans*-activator plasmid (pFA2-Elk-1) were included in the PathDetect Elk-1 transreporting system purchased from Stratagene (La Jolla, CA). Transient transfections in HeLa cells were performed using Fugene reagent (Roche Diagnostics, Laval, Quebec, Canada) according to the manufacturer's instructions. To monitor transfection efficiency, 2 d after transfection with a rous sarcoma virus (RSV)-β-galactosidase expression plasmid, cells were fixed for 1 h at room temperature in a 2% paraformaldehyde solution containing 0.1 M piperazine-N, N′-bis(2-ethanesulfonic acid) (pH 6.9), 2 mM $MgCl_2$, 1.25 mM EGTA, washed three times with PBS, and incubated at 37 C in X-Gal solution (0.2% X-Gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 0.1 $MgCl_2$ in PBS) until staining was evident. Alternatively, cells were transfected with the pEGFP-C1 plasmid and visualized 2 d later with a Leica inverted fluorescent microscope (Leica Microsystems, Wetzlar, Germany).

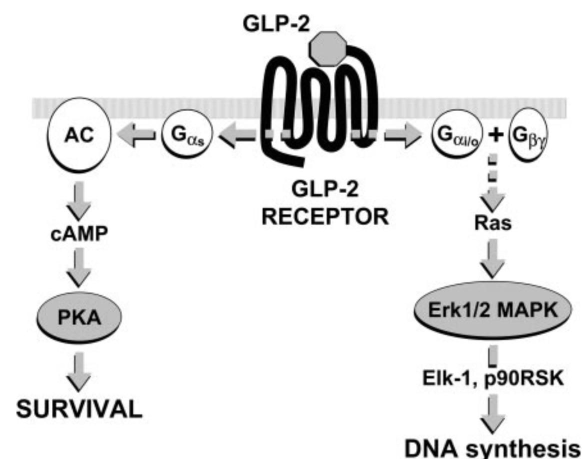

Fig. 10. Model for GLP-2R Signaling Pathways in HeLa Cells

GLP-2R activation engages a cAMP- and PKA-dependent pathway coupled to inhibition of apoptosis and also engages a G_i/G_o, Gβγ-dependent pathway that couples to activation of the Ras/ERK1/2 pathway and induces DNA synthesis.

Cell Lines

Human cervical cancer cell lines were obtained from American Type Culture Collection (Manassas, VA) and were maintained in DMEM supplemented with 10% FBS. The DLD-1 colon cancer cell line stably expressing the human GLP-2R (DLD-1:hGLP-2R) in pcDNA3.1 (54) was cultured in DMEM containing 5% FBS and 0.5 mg/ml G418.

RT-PCR and Southern Blot Analysis

Total RNA was isolated from cells using the guanidinium thiocyanate method (55). Total RNA (5 μg) was reversed transcribed at 42 C for 60 min using Revert Aid H Minus first-strand cDNA synthesis kit (MBI Fermentas) and random hexamer primers (Invitrogen Life Technologies). The GLP-2R was amplified by PCR using one tenth of the reverse transcriptase (RT) reaction using Taq polymerase (Invitrogen Life Technologies) and the primer pairs 5′-TTG TGA AGG TGC ACG AGG AA-3′ and 5′-CAC CCT AGA TCT CAC TCT CT-3′. Amplification of human GLP-2R cDNA was performed using 30 cycles at an annealing temperature of 56 C resulting in the generation of a 1672-bp product spanning the entire GLP-2R open reading frame. GAPDH (glyceraldehyde-3-phosphate-dehydrogenase) cDNA amplification was performed as previously described (24). DLD-1 cells stably transfected with the human GLP-2R (17) were used as a positive control for GLP-2R RT-PCR experiments.

For analysis of HeLa cell GLP-2R expression, HeLa cells were counted and serially diluted in PBS such that each PCR tube contained either 10 or 50 cells in a total volume of 5 μl. Cells were lysed in first-strand cDNA synthesis buffer (50 mM Tris 8.3, 75 mM KCl, 3 mM $MgCl_2$, 0.5% NP-40, 20 U Ribonuclease inhibitor, 10 mM dithiothreitol, 1 mM deoxynucleotide triphosphates, 100 ng random hexamer primers), and the lysate was then heated to 65 C for 2 min followed by RT using 200 U Superscript II (Invitrogen Life Technologies). Amplification of the GLP-2R or GAPDH was carried out as described above using 50 rounds of PCR.

For Southern blot analysis, PCR reaction products were electrophoresed in a 1% agarose gel, capillary transferred to a Nytran membrane (Schleicher & Schuell, Keene, NH), and UV cross-linked using a Bio-Rad UV chamber (Bio-Rad Laboratories, Richmond, CA). After prehybridization for 1 h at 48 C, membranes were hybridized with a ^{32}P-labeled oligonucleotide probe (5′-AGA ATG GGT GGA TGT CCT AC-3′) overnight at 48 C in hybridization buffer (5% dextran sulfate, 1 M NaCl, 1% sodium dodecyl sulfate. Blots were washed in 2× standard saline citrate, 1% sodium dodecyl sulfate for 25 min at 48 C and exposed to Kodak X-Omat film (Eastman Kodak, Rochester, NY) at −70 C with an intensifying screen for the indicated period of time.

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cAMP Assays

HeLa cells grown in 24-well plates to approximately 70% confluence were serum starved for 3 h by washing cells with PBS after reincubation with DMEM lacking serum. For analysis of cAMP in transient transfectants, 48 h after transfection, cells were serum starved for 3 h. Cells were then treated with the indicated agonists in DMEM supplemented with 10 μM 3-isobutyl-1-methylxanthine. Incubations were terminated after 10 min by the addition of ice-cold ethanol (65% final concentration). cAMP was measured from dried aliquots of ethanol extracts using a cAMP RIA kit (Biomedical Technologies, Stoughton, MA).

Cell Survival Assay

To quantitate apoptosis in cells expressing the GLP-2R, HeLa cells were cultured in six-well dishes until 50–60% confluency. Cells were then cotransfected with the cloned HeLa cell GLP-2R in pcDNA3.1 and a RSV-β-galactosidase expression plasmid in a 2:1 molar ratio. Approximately 24 h later, cells were maintained for 16 h in DMEM lacking serum before apoptosis induction by exposure to LY294002 (50 μM final) in the same media for 24 h. Control cultures were subjected to the same manipulations as treated cells, but in the absence of drugs. At the end of the incubation period, adherent cells were collected, and β-galactosidase activity was determined as previously described (27). In this assay, the amount of β-galactosidase activity in intact adherent cells indicates the number of viable transfected cells, whereas the loss of β-galactosidase activity reflects cell death and elimination of the transfected cells (28, 56).

SDS-PAGE and Western Blot Analysis

Assessment of p44/42 MAPK and p90Rsk Activation. Cells grown in six-well dishes were transfected with either the hGLP-2R cDNA alone or cotransfected with pRK5, pRK5 βARKct, pcDNA3.1, or pcDNA3.1 p21^{N17ras} in a 1:2 molar ratio. Twenty-four hours after transfection, cultures were maintained in DMEM lacking serum for 16 h followed by treatment in the presence or absence of GLP-2 or 10% FBS. The plates were then placed on ice, the reaction was stopped by washing the cells with ice-cold PBS, and cells were then lysed in TX-100 lysis buffer (1% Triton X-100; 50 mM Tris, pH 8.0; 150 mM NaCl) supplemented with protease (1:20 dilution) and phosphatase (1:100 dilution) inhibitor cocktails and 500 μM sodium orthovanadate. Cell lysates were prepared and 20 μg of cell lysate were used for Western blot analysis as described (27, 28). Antibodies to p90Rsk phosphorylated on Ser 381 or p44/42 MAPK phosphorylated on Thr 202 and Tyr 204 were from Cell Signaling Technology (Beverly, MA) and were used at 1:1000 dilutions to detect catalytically active forms of the kinases. A primary antibody against heat shock protein 90 (Hsp90) (Transduction Laboratories, Inc., San Diego, CA) was used as a loading control at a 1:2000 dilution.

Apoptosis Assessment in CCL-2 HeLa Cells. After transfection with the GLP-2R, cells were maintained in DMEM lacking serum for 16 h followed by treatment with DMSO alone or LY294002 (50 μM final) in the presence or absence of 200 nM GLP-2, 10 μM Fsk, or 5% FBS for 24 h. Adherent cells were then collected and combined with detached, floating cells in the medium and lysed in radioimmune precipitation assay buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate in PBS) supplemented with protease (1:20 dilution) and phosphatase (1:100 dilution) inhibitor cocktails and 500 μM sodium orthovanadate. Cell lysates were analyzed by Western blotting using 30 μg total protein. Antibodies against GSK-3β phosphorylated on residue Ser 9 were purchased from Cell Signaling Technology (Beverly, MA) and used at a 1:1000 dilution and PARP from PharMingen at a 1:2500 dilution. Densitometry was performed on blots exposed on Biomax MR film (Eastman Kodak Co.) using a ScanJet 3p scanner (Hewlett-Packard Co., Palo Alto, CA) and NIH Image Software.

Luciferase Assay

The Elk-1/luciferase reporter assay was performed using the PathDetect Elk-1 transreporting system (Stratagene, La Jolla, CA) according to the manufacturer's instructions using HeLa cells cultured in six-well dishes. Briefly, 5 h after the initial transfection with pFR-luc, pFA2-Elk-1, and pcDNA3.1 HeLa GLP-2R, cells were cultured in media containing 0.5% FBS for approximately 26 h followed by overnight serum starvation. The following morning, cells were stimulated with or without 20 nM GLP-2 or 50 ng/ml EGF for 5.5 h. Cells were then lysed in luciferase lysis buffer (50 mM Tris/2-(N-morpholino) ethanesulfonic acid, pH 7.8; 0.1% Triton X-100, 1.0 mM dithiothreitol) and cleared by centrifugation and luciferase activity was analyzed as described elsewhere (57). For titration experiments, cells were transfected with 2 ng to 2 μg of a CMV-luciferase expression plasmid (total DNA in each transfection was 2 μg). Cells were lysed 2 d after transfection and luciferase activity was monitored.

BrdU Incorporation

HeLa cells were cultured in four-well chamber slides (VWR, Mississauga, Ontario, Canada) and were transfected with the HeLa GLP-2R and pEGFP-C1 in a 1:2 molar ratio. Five hours after the initial transfection, cells were cultured in serum-free media for 48 h. Cells were then stimulated with or without 20 nM GLP-2 or 10% FBS for 7 h in the presence of 10 μM BrdU (Roche Diagnostics). Cells were then fixed in PBS containing 1% paraformaldehyde and 0.01% Tween-20 for 2 h followed by permeabilization using 0.05% Triton X-100 in PBS for 10 min. Slides were then treated for 30 min at 37 C with 50 U deoxyribonuclease (Roche Diagnostics), blocked for 10 min in PBS containing 0.5% BSA, 0.1% Tween-20, and subjected to indirect immunofluorescence using anti-BrdU (clone BU-1; Upstate Biotechnology, Inc., Lake Placid, NY) at a 1:25 dilution, and Cy3-conjugated Affinity Pure Donkey antimouse IgG (Jackson ImmunoResearch Laboratories Inc. (West Grove, PA) at a 1:3000 dilution. Nuclei were counter stained using 4′,6-diamidino-2-phenyl-indole (DAPI: Sigma-Aldrich Canada Ltd, Oakville, Ontario, Canada) at a 1:50,000 dilution. Slides were visualized with a Leica DMR fluorescent microscope (Leica Microsystems, Wetzlar, Germany) using Leica filter cubes N3, GFP and A for Cy3, GFP, and DAPI visualization, respectively. Images were captured using a Leica DC300F camera and IM500 software.

Alternatively, Hela cells were cultured in 96-well plates, transfected with the Hela GLP-2R and pcDNA3.1 or a dominant negative Ras N17^{ras} encoding plasmid in a 1:2 molar ratio. Five hours after the initial transfection, cells were cultured in serum-free media for 24 h before stimulation with 20 nM GLP-2, 50 ng/ml EGF, or 10% FBS for 16 h. BrdU (10 μM) was then added to cells for an additional 6 h, and BrdU incorporation was assessed using a BrdU Cell Proliferation ELISA kit (Roche Diagnostics).

Tissue Source and Immunocytochemistry

Histological sections of cervical carcinomas were obtained from the Department of Surgical Pathology, Mount Sinai Hospital (Toronto, Ontario, Canada), and immunocytochemistry was performed using GLP-2R antisera as previously described (24). Nonspecific immunopositivity was assessed using preimmune serum as well as omission of primary antiserum.

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Statistical Analysis

Data were analyzed using ANOVA, and group comparisons were done using Bonferroni's multiple comparison post test.

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Address all correspondence and requests for reprints to: Dr. Daniel J. Drucker, Toronto General Hospital, Banting and Best Diabetes Centre, 200 Elizabeth Street, MBRW4R-402, Toronto, Canada M5G 2C4. E-mail: d.drucker@utoronto.ca.
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