

Identification of Glucagon-like Peptide-2 (GLP-2)-activated Signaling Pathways in Baby Hamster Kidney Fibroblasts Expressing the Rat GLP-2 Receptor*

(Received for publication, June 28, 1999)

Bernardo Yusta[‡], Romel Somwar[§], Feng Wang[‡], Donald Munroe[¶], Sergio Grinstein[§], Amira Klip^{||}, and Daniel J. Drucker^{‡**}

From the [‡]Department of Medicine, The Toronto General Hospital, University of Toronto, Toronto, Ontario M5G 2C4, Canada, the Departments of [§]Cell Biology and [¶]Physiology, Hospital for Sick Children, University of Toronto, Toronto, Ontario M5G 2C4, Canada, and ^{||}Allelix Biopharmaceuticals Inc., Mississauga, Ontario L4V 1P1, Canada

Glucagon-like peptide-2 (GLP-2) promotes the expansion of the intestinal epithelium through stimulation of the GLP-2 receptor, a recently identified member of the glucagon-secretin G protein-coupled receptor superfamily. Although activation of G protein-coupled receptors may lead to stimulation of cell growth, the mechanisms transducing the GLP-2 signal to mitogenic proliferation remain unknown. We now report studies of GLP-2R signaling in baby hamster kidney (BHK) cells expressing a transfected rat GLP-2 receptor (BHK-GLP-2R cells). GLP-2, but not glucagon or GLP-1, increased the levels of cAMP and activated both cAMP-response element- and AP-1-dependent transcriptional activity in a dose-dependent manner. The activation of AP-1-luciferase activity was protein kinase A (PKA)-dependent and markedly diminished in the presence of a dominant negative inhibitor of PKA. Although GLP-2 stimulated the expression of *c-fos*, *c-jun*, *junB*, and *zif268*, and transiently increased p70 S6 kinase in quiescent BHK-GLP-2R cells, GLP-2 also inhibited extracellular signal-regulated kinase 1/2 and reduced serum-stimulated Elk-1 activity. Furthermore, no rise in intracellular calcium was observed following GLP-2 exposure in BHK-GLP-2R cells. Although GLP-2 stimulated both cAMP accumulation and cell proliferation, 8-bromo-cyclic AMP alone did not promote cell proliferation. These findings suggest that the GLP-2R may be coupled to activation of mitogenic signaling in heterologous cell types independent of PKA via as yet unidentified downstream mediators of GLP-2 action *in vivo*.

The gastrointestinal mucosal epithelium contains a diverse number of specialized enteroendocrine cells that synthesize and secrete peptide hormones, frequently in a nutrient-dependent manner. Following secretion into circulation, gut-derived hormones may act in an endocrine manner by binding to receptors in tissues such as pancreas and liver, leading to the activation of signal transduction pathways and downstream

physiological events. Consistent with their location in the intestinal mucosal epithelium, enteroendocrine peptides may function in part to regulate gastrointestinal motility and nutrient digestion and absorption. For example, gastrin promotes acid secretion, whereas secretin inhibits acid secretion and promotes pancreatic exocrine secretion. Peptide hormones structurally related to secretin, such as glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1 (GLP-1),¹ stimulate glucose-dependent insulin secretion from the pancreatic beta cells, and GLP-1, unlike the glucose-dependent insulinotropic polypeptide, also inhibits gastric emptying, glucagon secretion, and food intake *in vivo* (1).

The pleiotropic actions of the glucagon/secretin/glucose-dependent insulinotropic polypeptide peptide superfamily are mediated via binding to and activation of distinct G protein-coupled receptors (GPCRs). These GPCRs are encoded by unique genes, yet are structurally related, and often share common features with respect to utilization of signaling mechanisms following ligand activation. Glucagon-related peptides regulate metabolic events, hormone secretion, and intestinal growth. For example, glucagon regulates glycogenolysis and gluconeogenesis via activation of a hepatocyte glucagon receptor (2), whereas GLP-1 stimulates glucose-dependent insulin secretion following activation of an islet beta cell GLP-1 receptor (3). Studies of glucagon and GLP-1 receptor signaling in cells expressing the endogenous receptor or in heterologous cells expressing transfected receptors demonstrate that both these peptides activate downstream signaling mechanisms coupled to the cAMP-dependent pathway (1).

In contrast to our understanding of the mechanisms underlying glucagon and GLP-1 action, much less is known about the biological activity of GLP-2, a 33-amino acid peptide located carboxyl-terminal to GLP-1 in the proglucagon precursor. GLP-2 administration to mice or rats promotes stimulation of crypt cell proliferation and inhibition of enterocyte apoptosis resulting in hyperplasia of the small bowel villous epithelium (4, 5). GLP-2 also exerts trophic effects in animal models of both small and large bowel injury such as experimental small bowel resection or chemically induced colitis (6, 7). In addition to stimulation of epithelial proliferation, GLP-2 also acutely regulates gastric emptying (8) and exerts rapid metabolic effects promoting stimulation of intestinal hexose transport

* This study was supported in part by operating Grant MT14799 from the Medical Research Council of Canada (to D. J. D., S. G., and A. K.) and the Ontario Research and Development Challenge Fund (to D. J. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** A consultant to Allelix Biopharmaceuticals Inc. To whom correspondence should be addressed: Toronto General Hospital, 101 College St., CCRW3-838, Toronto, Ontario M5G 2C4, Canada. Tel.: 416-340-4125; Fax: 416-978-4108; E-mail: d.drucker@utoronto.ca.

¹ The abbreviations used are: GLP, glucagon-like peptide; GPCR, G protein-coupled receptor; BHK, baby hamster kidney; DMEM, Dulbecco's modified Eagle's medium; CRE, cAMP-response element; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; IIE, immediate early; 8-Br-cAMP, 8-bromo-cyclic AMP; Erk, extracellular signal-regulated kinase; h, human.

within 30 min following intravenous GLP-2 infusion (9, 10).

The actions of GLP-2 are transduced via a recently isolated novel member of the glucagon/secretin GPCR superfamily. The GLP-2 receptor, isolated by expression cloning, exhibits 50% homology to the glucagon and GLP-1 receptors, is expressed in the central nervous system and gut, and has been localized to human chromosome 17 (11). Consistent with studies of glucagon and GLP-1 receptor signaling, the GLP-2 receptor is coupled to the adenylate cyclase pathway in transfected fibroblasts (11). Although several studies suggest that both the glucagon and GLP-1 receptors may be coupled to multiple signal transduction pathways, little is known about the potential for GLP-2 to activate signaling via nonadenylate cyclase-dependent mechanisms. Furthermore, unlike glucagon and GLP-1, the major action of GLP-2 involves stimulation of cell growth, and the mechanisms coupling GLP-2 receptor activation, directly or indirectly, to cell proliferation have not been examined. As intestinal cells expressing the endogenous GLP-2R have not yet been identified, we have now analyzed the actions of GLP-2 on downstream signaling pathways and cell proliferation in baby hamster kidney (BHK) fibroblasts stably transfected with the rat GLP-2 receptor.

EXPERIMENTAL PROCEDURES

Materials—Glucagon, GLP-1-(7–36)NH₂, and rat GLP-2-(1–33) were from Bachem California Inc. (Torrance, CA). Recombinant human [Gly²]-GLP-2 was a kind gift from Alielix Biopharmaceuticals Inc. (Mississauga, ON). 3-Isobutyl-1-methylxanthine, forskolin, and 8-Br-cAMP were obtained from Sigma. The protein kinase A (PKA) inhibitor H-89 was from Calbiochem.

The p3AP₁-luciferase (12) and pCRE/ β -galactosidase (13) reporter plasmids were gifts from C. A. Hauser (San Diego, CA) and R. D. Cone (Portland, OR), respectively. The expression plasmid MtR(AB) that encodes a dominant negative mutant of the PKA regulatory subunit (14) was a gift from G. S. McKnight (Seattle, WA). The PathDetect Elk-1 *trans*-reporting system was purchased from Stratagene (La Jolla, CA). Anti-phospho-extracellular signal-regulated kinase (Erk) antibody was obtained from New England Biolabs (Beverly, MA). Polyclonal anti-Akt1 (C-20) and anti-p70 S6 kinase antibodies, p70 S6 kinase peptide substrate, and PKA and protein kinase C inhibitor peptides were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Whatman p81 filter paper was purchased from Whatman (Tewksbury, MA). Okadaic acid and microcystin were from Biomol (Plymouth Meeting, PA). Rapamycin was from Calbiochem. Polyclonal anti-Akt2 and Akt substrate peptide (Crosstide) were purchased from Upstate Biotechnology (Lake Placid, NY). [³²P]ATP (6000 Ci/mmol) and enhanced chemiluminescence (ECL) reagents were purchased from Amersham Pharmacia Biotech. Purified phosphatidylinositol was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Oxalate-treated TLC Silica gel H plates (250 microns) were from Analtech (Newark, DE). Protein A and protein G-Sepharose were purchased from Amersham Pharmacia Biotech. All electrophoresis and immunoblotting reagents were purchased from Bio-Rad.

Cell Culture and Transfections—BHK fibroblast were grown in Dulbecco's modified Eagle's medium (DMEM, 4.5 g/l glucose) supplemented with 5% calf serum. Cells were transfected with cDNAs encoding the rat GLP-2 receptor (11) or the rat GLP-1 receptor (15) cloned in the pcDNA3.1 eukaryotic expression vector (Invitrogen, San Diego, CA) or with pcDNA3.1 alone by calcium phosphate coprecipitation. Stably transfected cell populations were selected by growth in G418 (Life Technologies, Inc.) at 0.8 mg/ml for 2 weeks. Individual cell clones were obtained by limited dilution cloning, expanded for further characterization, and maintained in DMEM with 0.5 mg/ml G418. The BHK-GLP-2R clone utilized for the present studies was representative of several G418-resistant clones that expressed the GLP-2R and gave identical results in signal transduction studies.

For transient transfection assays, BHK cells stably expressing the rat GLP-2 receptor (BHK-GLP-2R) or the empty vector pcDNA3.1 (BHK-pcDNA3) were plated in medium without G418. Cells were transfected at 60–70% confluency by calcium phosphate coprecipitation with either 10 μ g of pCRE/ β -galactosidase or 5 μ g of p3AP₁-luciferase reporter constructs plus pBluescript II (Stratagene) carrier DNA for a total of 20 μ g of DNA. In the transfections involving the PathDetect Elk-1 *trans*-reporting system, the precipitate contained 9.5 μ g of the

GAL4-luciferase reporter plasmid, 0.5 μ g of expression vector encoding the GAL4-Elk-1 chimeric *trans*-activator protein, and 10 μ g of carrier DNA or MtR(AB) expression vector. This plasmid was also used in studies of GLP-2 activation of both CRE- and AP-1-dependent activity. Four hours after transfection, cells were glycerol-shocked and incubated for 18–20 h in DMEM + 0.2% calf serum. Indicated drugs or peptides were added for 6 h in DMEM supplemented with 0.1% calf serum and 10 μ M 3-isobutyl-1-methylxanthine before harvesting cells for analysis of β -galactosidase and luciferase activities as described previously (13, 16, 17). Reporter gene activities were normalized to the protein concentration in each cell extract. Protein content was determined using a Coomassie dye assay (Bio-Rad). Data are presented as the mean \pm S.E. from a minimum of 3–4 independent transfections, each carried out in triplicate or quadruplicate.

RNA Isolation and Analysis—RNA was isolated using a modified acid-ethanol guanidinium thiocyanate method as described previously (18). For Northern blot analysis, RNA was size-fractionated in an agarose gel, transferred to a nylon membrane, and immobilized with ultraviolet light, and hybridization and washing were carried out as described previously (19).

cAMP Production Assays—BHK-GLP-2R cells were grown in 24-well plates at 37° C and treated with 10 nM h[Gly²]-GLP-2 or 20 μ M forskolin in DMEM supplemented with 0.1% calf serum and 10 μ M 3-isobutyl-1-methylxanthine. Incubations were terminated at the indicated times by the addition of chilled ethanol (65% final concentration). cAMP was measured in dried aliquots of ethanol extracts using a cAMP radioimmunoassay kit (Biomedical Technologies, Stoughton, MA), and cAMP data were normalized to the protein content/well.

Proliferation Assays—BHK-GLP-2R and BHK-pcDNA cells grown in 96-well plates were serum-starved for 24 h and then incubated for 48 h in serum-free medium in the absence or presence of h[Gly²]-GLP-2 at the indicated concentrations. Control cells were treated identically but were exposed to 5% calf serum for 48 h. Fresh medium and treatments were replaced every 24 h. At the end of the incubation period the number of viable cells in each condition was measured using the Cell-Titer 96 aqueous nonradioactive cell proliferation assay kit (Promega, Madison, WI) according to the manufacturer's suggestions.

Cytosolic Calcium—Cytosolic-free calcium was measured as described previously (20). Briefly, cells grown on 25-mm glass coverslips were loaded with Fura-2 by incubation with 2 μ M of the precursor acetoxymethyl ester for 20 min at 37° C. Fura-2 fluorescence ratio measurements were made on a Nikon Diaphot TMD microscope equipped with a Fluor 40X, 1.3 N. A. oil immersion objective and a high sensitivity photometer (D-104, Photon Technology Instruments), which was interfaced to a NEC computer with a 12 bit A/D board (Labmaster). Illumination was provided by a 100 watt xenon lamp coupled to the microscope via a rotating mirror and fiber optic assembly (Ratiomaster, PTI). The cells were alternately excited at 340 and 380 nm while recording emission at 510 nm. Photometric data were acquired at 10 Hz using the Oscar software (PTI). Ionomycin and EGTA were used to calibrate the fluorescence ratio *versus* calcium concentration.

Mitogen-activated Protein Kinase (MAPK) Phosphorylation—Prior to all experimental manipulations for analysis of kinase activity, cells were deprived of serum overnight (12 h). MAPK phosphorylation was detected as described (21). Briefly, BHK-GLP-2R cells were treated with 20 nM GLP-2 or 100 nM insulin for the indicated time periods. Cells were lysed in a solution containing 10% glycerol, 4% SDS, 115 mM Tris/HCl (pH 6.8), 10 mM dithiothreitol, 0.25 mg/ml bromophenol blue, protease inhibitors (100 μ M phenylmethylsulfonyl fluoride, 10 μ M E-64, 1 μ M pepstatin, 1 μ M leupeptin), and phosphatase inhibitors (40 mM sodium fluoride, 7.5 mM sodium pyrophosphate, 1.5 mM Na₃VO₄). Lysates were passed five times through a 25-gauge syringe to shear the DNA and boiled for 3 min. To detect MAPK phosphorylation, 30 μ g of total cellular protein were resolved by 10% SDS-polyacrylamide gel electrophoresis, electrotransferred onto polyvinylidene difluoride membranes, and then immunoblotted with phospho-specific MAPK antibody (22) (polyclonal, 1:1000 dilution). Protein was detected by the enhanced chemiluminescence method using goat anti-rabbit IgG conjugated to horseradish peroxidase (1:5000 dilution) as the secondary antibody.

Immunoprecipitation and Assay of p70 S6 Kinase Activity—p70 S6 kinase activity was determined as described previously (23). BHK-GLP-2R cells grown in 6-well plates were incubated with 20 nM h[Gly²]-GLP-2, washed twice with ice-cold phosphate-buffered saline, and lysed in 1 ml of lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 20 mM β -glycerophosphate, 10 mM EDTA, 10 mM sodium pyrophosphate, 100 mM NaF, 1 mM Na₃VO₄, 1 mM dithiothreitol, 10 mM okadaic acid, and 1% (v/v) Nonidet P-40) containing a mixture of protease inhibitors (1 μ M leupeptin, 1 μ M pepstatin A, 10 μ M E-64, and 200 μ M phenylmethylsul-

fonyl fluoride). After 15 min of slow agitation and centrifugation (15,000 × *g* for 15 min), the supernatant was subjected to immunoprecipitation. p70 S6 kinase was immunoprecipitated using 250 μg of total protein and 1 μg of a rabbit polyclonal p70 S6 kinase antibody. The p70 S6 kinase immunocomplex was washed three times with wash buffer (50 mM Tris acetate, pH 8, 50 mM NaF, 5 mM sodium pyrophosphate, 5 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 mM EDTA, 1 mM EGTA, 10 mM okadaic acid, 0.1% (v/v) β-mercaptoethanol) including all the protease inhibitors used above and twice with kinase buffer (20 mM 4-morpholinepropanesulfonic acid, pH 7.2, 25 mM β-glycerophosphate, 5 mM EGTA, 2 mM EDTA, 20 mM MgCl₂, 2 mM Na₃VO₄, and 1 mM dithiothreitol in a final volume of 50 μl of kinase buffer containing 1 μM protein kinase A and protein kinase C inhibitor peptides, 0.2 mM S6 peptide, and 0.25 mM Mg-[γ-³²P]ATP at 30°C for 10 min. Aliquots (30 μl) were transferred onto Whatman p81 filter papers and washed 3 times for 15 min with 175 mM phosphoric acid. ³²P incorporated into the S6 peptide was measured by liquid scintillation counting.

Immunoprecipitation and Assay of Akt Protein Kinase Activity.—Immunoprecipitation of Akt and kinase assay was performed as described (23) with modifications. Anti-Akt antibodies were pre-coupled to a mixture of protein A- and protein G-Sepharose beads by incubating 2 μg of antibody/condition with 20 μl of protein A-Sepharose (100 mg/ml) and 20 μl of protein G-Sepharose (100 mg/ml) for a minimum of 2 h. The anti-Akt-bead complexes were washed twice with ice-cold phosphate-buffered saline and once with ice-cold lysis buffer. Akt was immunoprecipitated by incubating 200 μg of total cellular protein with the anti-Akt-bead complex for 2–3 h under constant rotation (4°C). The Akt1 immunocomplex was isolated and washed 4 times with 1 ml of wash buffer (25 mM HEPES, pH 7.8, 10% glycerol (v/v), 1% Triton X-100 (v/v), 0.1% bovine serum albumin (v/v), 1 M NaCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μM microcystin, 100 mM okadaic acid) and twice with 1 ml of kinase buffer (50 mM Tris/HCl, pH 7.5, 10 mM MgCl₂ and 1 mM dithiothreitol). This was then incubated under constant agitation for 30 min at 30°C with 30 μl of reaction mixture (kinase buffer containing 5 μM ATP, 2 μCi of [γ-³²P]ATP, and 100 μM Crosslide). Following the reaction, 30 μl of the supernatant were transferred onto Whatman p81 filter paper and treated as described above for p70 S6 kinase assay.

Actin Staining.—To stain F-actin, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline for 20 min at room temperature and then permeabilized using 0.1% Triton X-100 in phosphate-buffered saline. Permeabilization was followed by incubation with a 1:1000 dilution of rhodamine phalloidin (Molecular Probes) for 45 min at room temperature. The samples were then washed extensively and mounted using Dako mounting medium. Samples were visualized by epifluorescence on a Leica DM-IRB microscope, and images were acquired with a MicroMax 2 cooled charge-coupled device camera (Princeton Instruments) using WinView software and a PC compatible computer.

RESULTS

The initial characterization of GLP-2R signaling was carried out in COS cells transiently transfected with the GLP-2R (11). As cell lines that express an endogenous GLP-2R have not yet been identified, we chose to establish *in vitro* models for reproducible analysis of GLP-2 action by generating BHK fibroblast clones that stably expressed the rat GLP-2 receptor. BHK cells were transfected with an expression vector containing the full-length rat GLP-2R coding sequence under the control of the cytomegalovirus promoter in the pcDNA3.1 expression vector. Following selection with the antibiotic G418, surviving clones were expanded and characterized for GLP-2R expression. Several BHK-GLP-2R cell lines were identified that expressed the GLP-2R and responded identically to GLP-2. A representative clone, hereafter referred to as BHK-GLP-2R, was chosen for more detailed analysis of GLP-2-dependent signal transduction. Studies were carried out with either native rat GLP-2 or h[Gly2]-GLP-2, a protease-resistant GLP-2 analogue recently shown to exhibit greater *in vitro* and *in vivo* stability compared with the native peptide (24).

As analysis of GLP-2R signaling in transiently transfected COS cells suggested GLP-2 activated the adenylate cyclase pathway (11), we initially analyzed the GLP-2-dependent activation of a cAMP-dependent reporter gene, CRE-β-galactosidase, in transfected BHK-GLP-2R cells. The activity of this

reporter gene has been shown to correlate, in a linear manner, with accumulation of intracellular cAMP (13). Both rGLP-2 and h[Gly2]-GLP-2, from 0.01 to 20 nM, increased β-galactosidase activity in BHK-GLP-2R cells (Fig. 1A). Furthermore, the level of β-galactosidase induction following transfection of CRE-β-galactosidase and incubation with GLP-2 was similar in magnitude to that obtained by treating the cells with either forskolin or 8-bromo-cyclic AMP, two well characterized activators of the adenylate cyclase pathway (Fig. 1B). In contrast, the structurally related peptides glucagon and GLP-1 did not stimulate β-galactosidase activity in BHK-GLP-2R cells (Fig. 1A). Furthermore, GLP-2 had no effect on the activity of a cotransfected CRE-β-galactosidase reporter gene in control cells stably expressing the parental expression vector pcDNA3.1 (Fig. 1C). To verify that the activation of CRE-dependent β-galactosidase activity reflected the accumulation of intracellular cAMP following GLP-2 stimulation, we compared the levels of cAMP in BHK-GLP-2R cells at various time points after incubation of cells with either 10 nM h[Gly2]-GLP-2 or 20 μM forskolin. The relative magnitude and kinetics of intracellular cAMP accumulation were comparable from 10 to 360 min following exposure of cells to either reagent (Fig. 1D). Furthermore, the EC₅₀ for stimulation of CRE-dependent β-galactosidase activity was ~0.06 nM, identical to the value reported for GLP-2-stimulation of cAMP accumulation in 293-EBNA cells expressing the rat GLP-2 receptor (11).

As the structurally related peptides glucagon and GLP-1 stimulate AP-1-dependent signaling pathways (25, 26), we next ascertained whether activation of the GLP-2 receptor was also coupled to AP-1-dependent transcriptional activation. BHK-GLP-2R cells were transfected with a reporter gene containing three tandemly linked AP-1 sites adjacent to a luciferase reporter gene. h[Gly2]-GLP-2, at concentrations of 0.01–20 nM, stimulated a 3–4-fold induction of AP-1-dependent luciferase activity in BHK-GLP-2R cells (Fig. 2A) but not in BHK cells stably transfected with the expression vector alone (BHK-pcDNA3.1, Fig. 2B). Induction of AP-1-directed luciferase activity was also observed with activators of the adenylate cyclase pathway such as forskolin and 8-bromo-cyclic AMP (Fig. 2C); however, the relative magnitude of induction with these PKA activators was less than that observed for h[Gly2]-GLP-2 alone (Fig. 2C, *p* < 0.05). Similarly, exposure of BHK-GLP-2R cells to 10% fetal calf serum significantly activated AP-1-directed luciferase activity (Fig. 2C). Taken together, these findings establish the sensitivity and specificity of GLP-2-induction of adenylate cyclase and AP-1-dependent pathways in BHK-GLP-2R cells *in vitro*.

The induction of AP-1-luciferase activity by both forskolin and 8-Br-cAMP suggested that GLP-2 might activate AP-1 activity via PKA-dependent mechanisms. To examine this possibility, the CRE-β-galactosidase and AP1-luciferase reporter genes were transfected into BHK-GLP-2R cells in the presence or absence of a cDNA encoding a dominant negative inhibitor of PKA, MtR(AB) (14). The GLP-2-dependent induction of β-galactosidase activity was reduced by 80% in the presence of the cotransfected PKA inhibitor (Fig. 3). Similarly, the forskolin induction of CRE-β-galactosidase was reduced by ~80% in similar experiments, consistent with the results of previous studies (14). Furthermore both the GLP-2- and forskolin-dependent activation of AP-1-luciferase activity were also significantly reduced in the presence of the PKA inhibitor MtR(AB) (Fig. 3, *p* < 0.001–0.005). Similar results were also obtained with the PKA inhibitor H89 (data not shown). However, whereas the forskolin induction of AP-1 activity was eliminated in the presence of PKA inhibition, a small but detectable GLP-2-induction of AP-1 luciferase was still observed in the pres-

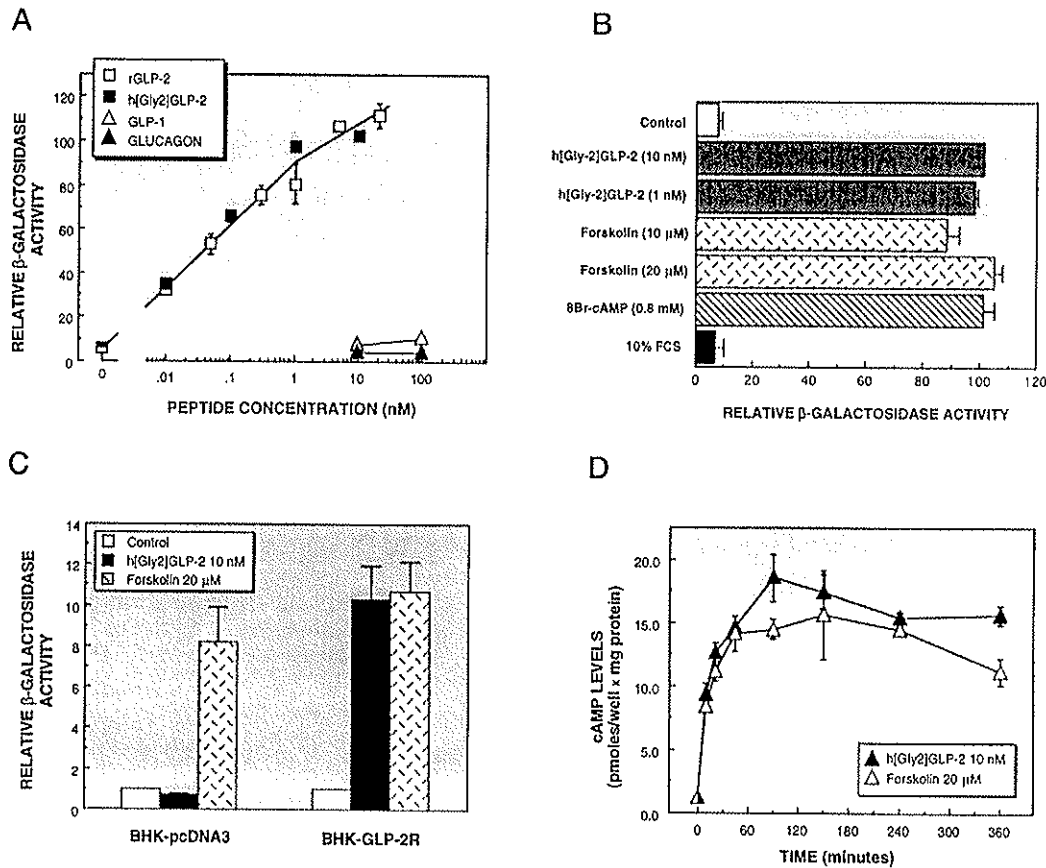


Fig. 1. A, induction of CRE-dependent transcription by GLP-2 in BHK cells stably expressing the rat GLP-2 receptor. BHK cells stably expressing the rat GLP-2 receptor (BHK-GLP-2R) were transiently transfected by the calcium phosphate coprecipitation method with the pCRE/ β -galactosidase reporter plasmid. Cells were left untreated or were treated for 6 h with h[Gly2]-GLP-2, rat GLP-2, GLP-1-(7-36)NH₂, or glucagon at the indicated concentrations and then assayed for β -galactosidase activity. Reporter gene activity was expressed as a percentage of the β -galactosidase activity obtained after incubation of the transfected cells with 10 nM h[Gly2]-GLP-2 following normalization for protein. Data represent mean \pm S.E. from three separate experiments. B, activation of CRE-dependent β -galactosidase activity by h[Gly2]-GLP-2, forskolin, 8-Br-cAMP, or fetal calf serum (FCS) in BHK-GLP-2R cells. Cells were transiently transfected with pCRE/ β -galactosidase and treated for 6 h with h[Gly2]-GLP-2, forskolin, 8-Br-cAMP, or fetal calf serum, at the indicated concentrations. Cell lysates were then assayed for β -galactosidase activity, and reporter gene activity was normalized as in Fig. 1A. Data are mean \pm S.E. ($n = 5-7$). C, specificity of GLP-2 action in BHK cells. BHK cells stably transfected with the pcDNA3.1 expression vector or rat GLP-2 receptor were transfected with the pCRE/ β -galactosidase expression vector and treated with h[Gly2]-GLP-2 or forskolin for 6 h following which cell extracts were prepared for analysis of β -galactosidase activity. Reporter gene activity is expressed as fold induction versus untreated cells. Data are mean \pm S.E., $n = 3$ experiments. D, stimulation of cAMP accumulation in BHK-GLP-2R cells. BHK-GLP-2R cells were treated with h[Gly2]-GLP-2 or forskolin in the presence of 10 μ M 3-isobutyl-1-methylxanthine. cAMP content was measured at the indicated times by radioimmunoassay. Data represent mean \pm S.D. of triplicate determinations.

ence of Mtr(AB). These findings suggest the existence of alternate pathways independent of PKA for induction of AP-1 activity. Consistent with the existence of these alternate pathways, the serum induction of AP-1-dependent luciferase activity was not diminished by co-transfection with the PKA inhibitor plasmid (Fig. 3).

As activation of the AP-1 pathway is frequently associated with stimulation of cell proliferation, the finding that GLP-2 activated AP-1-dependent transcriptional activity suggested that GLP-2 receptor signaling might be directly coupled to cell proliferation. Accordingly, we examined whether GLP-2 activates a generalized pattern of immediate early gene expression associated with stimulation of cell proliferation. Serum-deprived BHK-GLP-2R cells were exposed to h[Gly2]-GLP-2 or serum following which immediate early (IE) gene expression was analyzed by Northern blotting. h[Gly2]-GLP-2 at a concentration of 100 nM induced the expression of *c-fos*, *c-jun*, *junB*, and *zif/268* in quiescent BHK-GLP-2R cells, although the relative magnitude of mRNA induction was clearly much greater following serum stimulation for all 4 IE genes examined (Fig. 4). In contrast, 1 nM h[Gly2]-GLP-2 was much less effective in

stimulating IE gene expression (Fig. 5) despite near maximal activation of CRE- β -galactosidase and AP-1-luciferase activities with 1 nM h[Gly2]-GLP-2 (Figs. 1 and 2). These findings imply that GLP-2-stimulated increases in cAMP are not likely responsible for stimulation of IE gene expression in BHK-GLP-2R cells.

To assess whether GLP-2 induction of IE gene expression was associated with increased cell proliferation, BHK cells stably transfected with the expression vector pcDNA3.1 or BHK-GLP-2R cells were incubated with 10–100 nM h[Gly2]-GLP-2 for 48 h, and cell proliferation was analyzed using a CellTiter cell proliferation assay. h[Gly2]-GLP-2 had no effect on cell proliferation in BHK cells transfected with the pcDNA3.1 expression vector alone. In contrast, a small but significant stimulation of cell proliferation was observed following incubation with BHK-GLP-2R cells with 100 nM, but not 10 nM h[Gly2]-GLP-2, a dose that significantly increases both CRE- and AP-1-dependent activities (Fig. 6, $p < 0.05$ for 100 nM GLP-2 versus control). Furthermore, activators of the PKA pathway such as 8-Br-cAMP failed to stimulate cell proliferation in BHK-GLP-2R cells (Fig. 6), providing further evidence

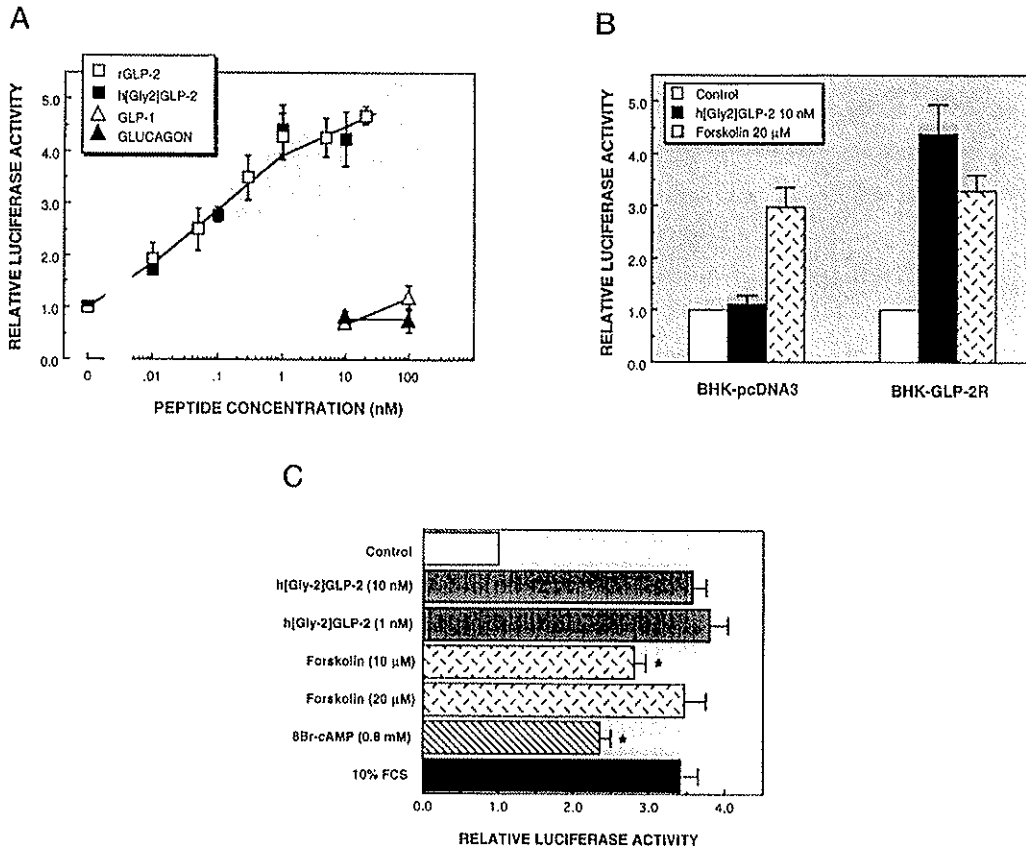


FIG. 2. A, induction of AP-1-dependent transcription by GLP-2 in BHK cells stably expressing the rat GLP-2 receptor. BHK cells stably expressing the rat GLP-2 receptor (BHK-GLP-2R) were transiently transfected by the calcium phosphate coprecipitation method with the p3AP1-luciferase reporter plasmid. Cells were treated for 6 h with h[Gly2]-GLP-2, rat GLP-2, GLP-1-(7-36)NH₂, or glucagon at the indicated concentrations and then assayed for luciferase activity. Relative luciferase activity is expressed as fold induction *versus* the untreated control following normalization for protein. Data shown represent mean \pm S.E. ($n = 3$ separate experiments). B, specificity of GLP-2 action in BHK cells. BHK cells stably transfected with the pcDNA3.1 expression vector or rat GLP-2 receptor were transfected with the p3AP1-luciferase expression vector and treated with h[Gly2]-GLP-2 or forskolin for 6 h following which cell extracts were prepared for analysis of luciferase activity. Reporter gene activity is expressed as in A. Data are mean \pm S.E. for $n = 3$ separate experiments. C, activation of AP-1-dependent transcriptional activity by h[Gly2]-GLP-2, forskolin, 8-Br-cAMP, or fetal calf serum in BHK-GLP-2R cells. Cells were transiently transfected with p3AP1-luciferase and treated for 6 h with h[Gly2]-GLP-2, forskolin, 8-Br-cAMP, or fetal calf serum (FCS) at the indicated concentrations. Cell lysates were then assayed for luciferase activity and reporter gene activity was normalized as in A. Data are mean \pm S.E. ($n = 5-7$). *, $p < 0.05$, experimental *versus* h[Gly2]-GLP-2.

for a dissociation between activation of mitogenic pathways and GLP-2-stimulation of adenylate cyclase *in vitro*.

The results of these studies suggest that stimulation of GLP-2R signaling activates not only PKA and AP-1 but likely additional pathways linked to the activation of IE genes and cell growth. Analysis of glucagon receptor signaling in BHK cells expressing a transfected glucagon receptor suggested that glucagon may signal via intracellular calcium influx ostensibly because of generation of inositol 1,4,5-trisphosphate upon activation of phospholipase C (26). To assess whether GLP-2 is similarly capable of activating phospholipase C, cytosolic calcium was measured in BHK-GLP-2R cells. As shown in Fig. 7, free cytoplasmic calcium remained unaltered when the cells were stimulated with h[Gly2]-GLP-2. The sensitivity of the assay and responsiveness of the cells were verified by the subsequent addition of bradykinin, which as reported earlier (27), induced a cytosolic calcium transient in BHK cells (Fig. 7). As was the case for GLP-2R cells, pools of BHK cells expressing the GLP-1 receptor failed to respond to the cognate ligand GLP-1 with a rise in calcium (data not shown). These findings imply that unlike activated glucagon receptors, engagement of GLP-1 and GLP-2 receptors by their respective ligands does not promote measurable stimulation of phospholipase C, at least in BHK cells.

We next analyzed signaling pathways that are known to mediate cellular proliferation and cell survival by other growth factors, in particular, the MAPK Erk1, Erk2, and ribosomal p70 S6 kinase. The MAPKs, a family of serine/threonine kinases, are phosphorylated on tyrosine and threonine residues, and phosphorylation of these sites is used as a measure of kinase activation. BHK GLP2-R cells were stimulated for 2-10 min with 20 nM h[Gly2]-GLP-2; cells were lysed, and an equal amount of protein was resolved by SDS-polyacrylamide gel electrophoresis and immunoblotted with a phospho-specific MAPK antibody, which recognizes both isoforms of MAPK (Erk1 and Erk2). As illustrated in Fig. 8A, 20 nM h[Gly2]-GLP-2 did not increase the phosphorylation of either Erk1 or Erk2. Instead, a reduction in the basal phosphorylation was observed. Consistent with these findings, h[Gly2]-GLP-2 alone did not activate Elk-1 activity in a cotransfection assay (Fig. 8B) and actually inhibited serum-stimulated Elk-1 activity (Fig. 8B). However, intriguingly, a small but significant stimulation of Elk-1 activity by GLP-2 was observed following cotransfection with the PKA inhibitor cDNA, Mtr(AB) (Fig. 8B). These findings, using both analysis of Erk1/2 kinase activity and Elk-1 transcriptional activation, are consistent with PKA- and h[Gly2]-GLP-2-dependent inhibition of Raf1 (28) leading to down-regulation of Erk1/2 activity in BHK-GLP-2R cells.

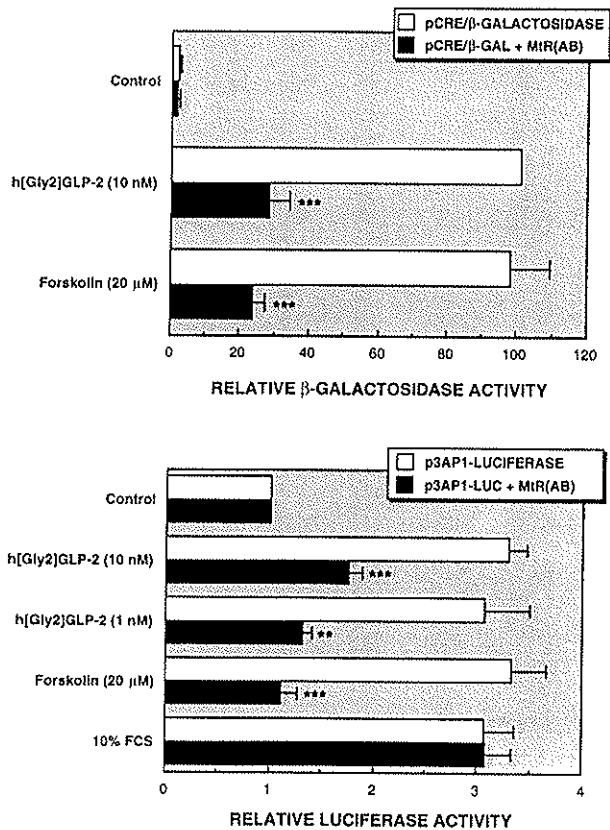


FIG. 3. Effect of PKA inhibition on the GLP-2-induced transcriptional activation of pCRE/ β -galactosidase and p3AP₁-luciferase reporter plasmids in BHK-GLP-2R cells. BHK-GLP-2R cells were transiently transfected with pCRE/ β -galactosidase or p3AP₁-luciferase reporter plasmids either alone or in combination with a dominant negative PKA mutant expression plasmid (Mtr(AB)). Cells were treated for 6 h with h[Gly2]-GLP-2, forskolin, or fetal calf serum at the indicated concentrations and then assayed for reporter gene activities. β -galactosidase activity was normalized as in Fig. 1A, and luciferase activity was normalized as per Fig. 2A. Data are mean \pm S.E. ($n = 4-6$). Statistical analysis was performed using the Student's t test. **, $p < 0.005$ and ***, $p < 0.001$, respectively, treatment alone versus treatment plus Mtr(AB). FCS, fetal calf serum.

To determine whether activation of the GLP-2R stimulated ribosomal p70 S6 kinase (p70 S6 kinase) activity, we utilized an *in vitro* kinase assay. BHK-GLP-2R cells were stimulated for 5–15 min with 20 nM h[Gly2]-GLP-2. Cells were lysed; p70 S6 kinase was immunoprecipitated, and kinase activity was determined. GLP-2 stimulated a rapid and transient increase in p70 S6 kinase activity (Fig. 9A). The stimulation at 5 min was 1.60 ± 0.28 -fold above basal ($n = 8$, $p < 0.05$, Student's paired t test). Although not statistically significant, there was still elevated p70 S6 kinase activity after 10 min ($n = 8$, $p > 0.05$, Student's paired t test). Activity returned to basal level by 15 min ($n = 5$). In comparison, a 10-min insulin (100 nM) challenge elicited a 4-fold increase in p70 S6 kinase activity. Stimulation of p70 S6 kinase activity by both agonists was prevented by pretreatment with 20 nM rapamycin, a known inhibitor of the activation of this kinase (data not shown). It is currently believed the activation of p70 S6 kinase by growth factors is mediated by the serine/threonine kinase Akt (also referred to as protein kinase B). One of the main cellular functions of Akt is the prevention of apoptosis. As GLP-2 stimulates epithelial proliferation and inhibits apoptosis (5), we utilized an *in vitro* kinase assay to determine if GLP-2 altered the activity of Akt. BHK cells stably expressing the GLP-2 receptor were stimulated for 5 min with 20 nM h[Gly2]-GLP-2. Cells were lysed;

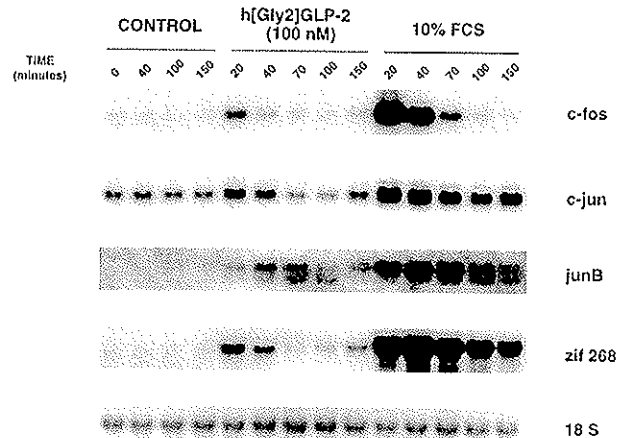


FIG. 4. Northern blot analysis of immediate early gene expression in BHK-GLP-2R cells. BHK-GLP-2R cells were incubated in DMEM plus 0.1% serum for 24 h, following which fresh medium was added containing 0.1% serum (control), 10% fetal calf serum (FCS), or h[Gly2]-GLP-2. RNA was isolated in triplicate from BHK-GLP-2R cells at various time points following addition of either serum or peptide. The *c-fos*, *c-jun*, *junB*, and *zif 268* RNAs were analyzed using specific cDNA probes as described previously (25). The 18 S RNA was assessed by ethidium bromide staining of the gel prior to transfer to the nylon membrane. The Northern blots shown depict representative experiments ($n = 3$).

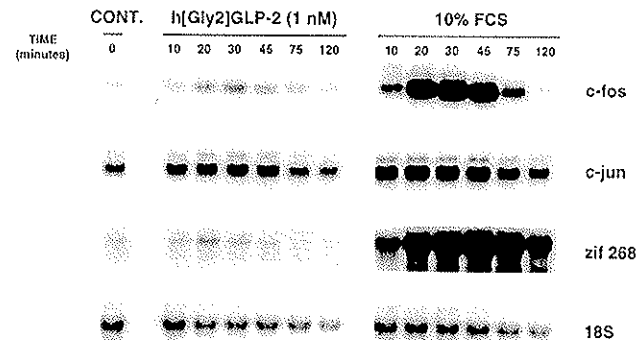


FIG. 5. Northern blot analysis of immediate early gene expression in BHK-GLP-2R cells following 1 nM h[Gly2]-GLP-2. BHK-GLP-2R cells were incubated in DMEM plus 0.1% serum for 24 h following which fresh medium was added containing 0.1% serum (control), 10% fetal calf serum (FCS), or h[Gly2]-GLP-2. RNA was isolated in triplicate from BHK-GLP-2R cells at various time points following the addition of either serum or peptide. The *c-fos*, *c-jun*, *zif 268*, and 18 S RNAs were analyzed as described above. The Northern blots shown depict representative experiments ($n = 3$).

Akt1 was immunoprecipitated, and kinase activity was determined. These results are illustrated in Fig. 9B. Unlike the results obtained with p70 S6 kinase, GLP-2 was unable to activate Akt1 ($n = 3$; control, 1.00 ± 0.00 ; GLP2, 1.03 ± 0.14). In contrast, a 5-min insulin-like growth factor-1 (10 nM) treatment of these cells resulted in a 1.67 ± 0.04 -fold increase in Akt1 activity ($n = 3$, $p < 0.05$, Student's paired t test). In preliminary experiments GLP-2 was also without effect on the activation of Akt2 (data not shown). These results suggest that Akt is not responsible for the modest activation of p70 S6 kinase by GLP-2 in BHK-GLP-2R cells.

Actin redistribution is often associated with activation of cellular proliferation, as well as upon stimulation of adenylate cyclase. To study the effects of GLP-2 on the distribution of F-actin, cells treated with or without the hormone were fixed and stained using labeled phalloidin. As shown in Fig. 9C, untreated cells show numerous well developed stress fibers in addition to a rim of cortical actin. In contrast, the length and number of stress fibers were drastically reduced in cells treated

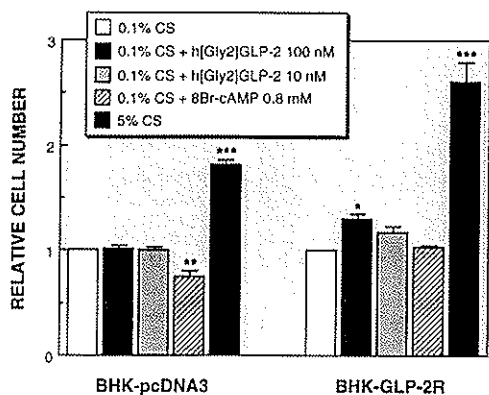


FIG. 6. Analysis of cell proliferation in BHK cells containing the stably integrated pcDNA3.1 plasmid (BHK-pcDNA3) or the identical plasmid directing expression of the rat GLP-2 receptor (BHK-GLP-2R). Cells were serum-deprived for 24 h and then incubated for 48 h in DMEM plus 0.1% calf serum alone or supplemented with h[Gly2]-GLP-2, 5% calf serum, or 8-Br-cAMP. The number of viable cells was quantified using a nonradioactive cell proliferation assay. Cell number is expressed as the fold increase versus the DMEM plus 0.1% serum control group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, experimental versus 0.1% calf serum (CS) control.

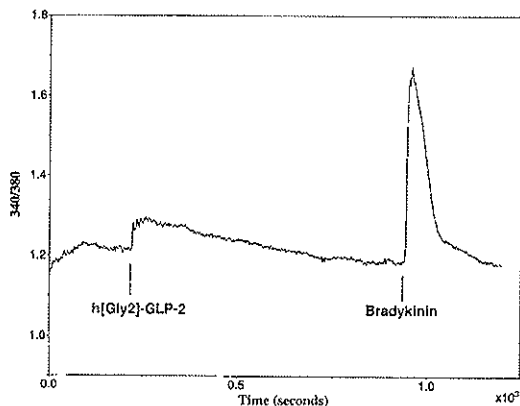


FIG. 7. Effects of h[Gly2]-GLP-2 and bradykinin on cytosolic-free calcium in BHK-GLP-2R cells. Cells were loaded with Fura-2 by incubation with the precursor acetoxymethyl ester and used for ratio microfluorimetry as detailed under "Experimental Procedures." Where indicated, the cells were stimulated with 10 nM h[Gly2]-GLP-2 and subsequently with 1 nM bradykinin. Abscissa, time in seconds. Ordinate, ratio of fluorescence with excitation at 340 and 380 nm. Emission was recorded at 510 nm. Representative of four similar experiments.

with GLP-2, whereas the amount and distribution of cortical actin was not greatly affected.

DISCUSSION

The glucagon receptor is coupled to activation of multiple signal transduction pathways, including stimulation of adenylate cyclase, production of inositol phosphates, and activation of protein kinase C activity in liver cells (29–31). Similarly, activation of glucagon receptor signaling in BHK cells expressing a transfected glucagon receptor leads to activation of adenylate cyclase and a phospholipase C-dependent increase in intracellular-free Ca^{2+} (26). Furthermore, GLP-1 receptor signaling has been extensively analyzed using both islet cell lines and cells expressing a transfected GLP-1 receptor (3, 32, 33). As cell lines that express the endogenous GLP-2 receptor have not yet been reported, we analyzed GLP-2R signaling in BHK cells expressing a transfected GLP-2 receptor. BHK cells were selected for these studies as they do not express either the proglucagon encoding GLP-2 or the GLP-2R gene, and they have previously been used for studies of glucagon receptor signaling

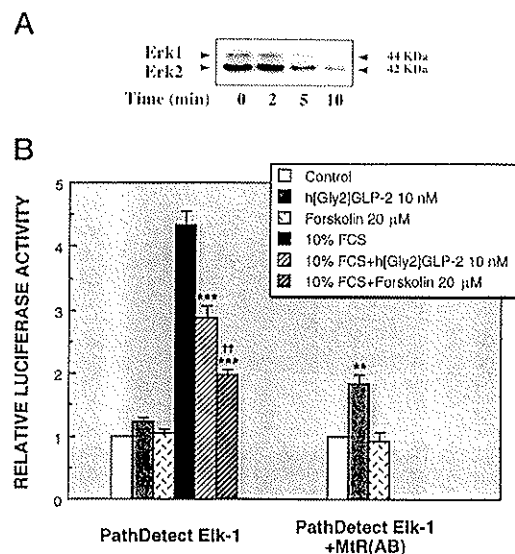


FIG. 8. A, effect of GLP-2 on MAPK activation. BHK cells stably expressing the GLP-2 receptor were stimulated for the time periods indicated with 20 nM h[Gly2]-GLP-2. Cells were lysed; 50 μ g of total cellular protein were resolved by 10% SDS-polyacrylamide gel electrophoresis and then immunoblotted with a phospho-specific MAPK antibody. This antibody recognizes dual phosphorylated Erk1 and Erk2. A representative immunoblot is shown. Similar results were obtained in seven independent experiments. B, effect of GLP-2 on Elk-1-mediated transcriptional activation in BHK-rGLP-2R cells. BHK-GLP-2R cells were cotransfected with the GAL4-Elk-1 expression vector and the GAL4-luciferase reporter plasmid (PathDetect Elk-1 *trans*-reporting system) alone or in combination with the dominant negative PKA mutant expression plasmid, Mtr(AB). Cells were treated for 6 h with h[Gly2]-GLP-2, forskolin, or fetal calf serum (FCS) alone or combined as indicated and then assayed for luciferase activity. Data are normalized as indicated in the legend of Fig. 2A and represent mean \pm S.E. ($n = 3-5$). Statistical analysis was performed using the Student's *t* test. +, $p < 0.005$, 10% FCS plus forskolin versus FCS plus h[Gly2]-GLP-2; **, $p < 0.005$, control versus h[Gly2]-GLP-2 in the presence of Mtr(AB); ***, $p < 0.001$, experimental versus 10% FCS alone.

(26). Although our initial report describing the cloning and preliminary characterization of GLP-2R signaling using transient transfection techniques demonstrated that GLP-2 activates a cAMP-dependent pathway (11), we have now extended these findings by demonstrating that GLP-2 also activates an AP-1-dependent pathway, likely indirectly via PKA. Nevertheless, the observation that activation of PKA alone was not sufficient to account for induction of IE gene expression and cell proliferation prompted us to assess additional pathways potentially downstream of GLP-2R activation.

In contrast to the glucagon-stimulated increase in intracellular-free calcium observed in studies of hepatocytes and transfected BHK cells (26), we did not detect any significant change in intracellular-free calcium following incubation of BHK-GLP-2R cells with GLP-2. However, the positive calcium response observed with bradykinin in the same experiments demonstrates that the necessary proteins required for coupling of related GPCRs to intracellular calcium influx are functional in BHK-GLP-2R cells. The lack of a GLP-2-stimulated calcium response implies that the glucagon and GLP-2 receptors exhibit functional differences perhaps mediated by heterologous expression of G proteins differentially coupled to various receptors leading to Ca^{2+} influx. Although GLP-1 has also been reported to increase levels of intracellular Ca^{2+} in some studies (15, 34), only a small fraction of beta cells responded to GLP-1 with an increase in intracellular Ca^{2+} in similar experiments (35). Moreover, we did not observe increases in intracellular Ca^{2+} influx in BHK cells stably transfected with the GLP-1R, and other investigators have failed to demonstrate GLP-1-

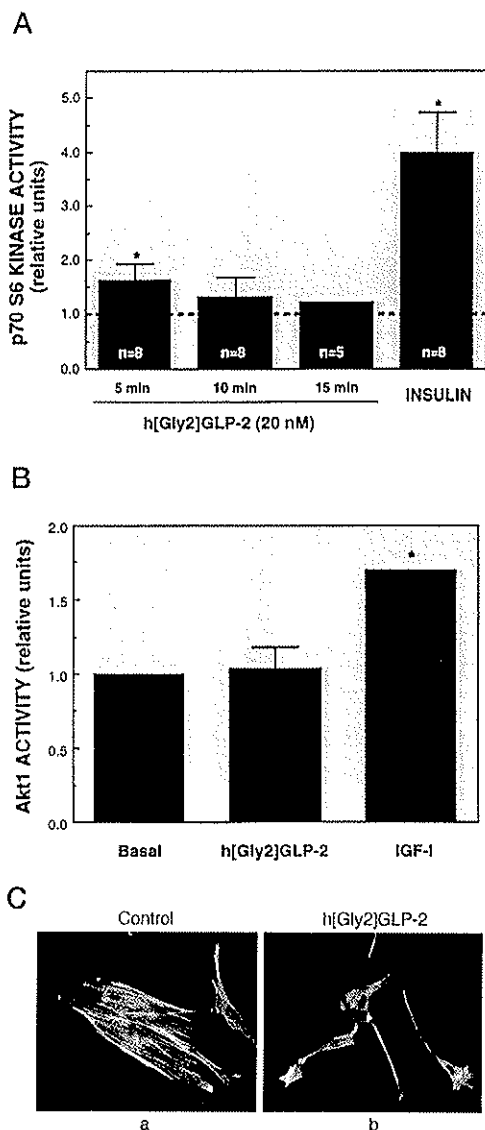


FIG. 9. A, effect of GLP-2 on p70 S6 kinase activity. BHK cells stably expressing the GLP-2 receptor (BHK-GLP-2R cells) were stimulated for the time periods indicated with 20 nM h[Gly2]-GLP-2 or with 100 nM insulin for 10 min. Cells were lysed; p70 S6 kinase was immunoprecipitated, and an *in vitro* kinase assay was used to determine kinase activity. Results represent the mean \pm S.E. of 5–8 experiments. *, $p < 0.05$, significantly different from basal, Student's paired *t* test. B, effect of GLP-2 on Akt1 kinase activity. BHK-GLP-2R cells were stimulated for 5 min with 20 nM h[Gly2]-GLP-2 or with 10 nM insulin-like growth factor-1 (IGF-1). Cells were lysed; Akt1 was immunoprecipitated, and an *in vitro* kinase assay was used to determine kinase activity. Results represent the mean \pm S.E. of three experiments. *, $p < 0.05$, significantly different from basal, Student's paired *t* test. C, distribution of F-actin in control and h[Gly2]-GLP-2-treated cells. BHK-GLP-2R cells treated with vehicle (a) or with 20 nM h[Gly2]-GLP-2 (b) for 20 min were fixed, permeabilized, and stained with rhodamine phalloidin as described under "Experimental Procedures." Representative epifluorescence images are shown.

stimulated increases in intracellular Ca^{2+} in islet cells or fibroblasts transfected with the GLP-1 receptor, despite observing responses to carbachol or thrombin in the same experiments (32). Taken together, our data clearly demonstrate lack of GLP-2R signaling coupled to a Ca^{2+} influx in fibroblasts, but do not exclude the possibility that GLP-2 might stimulate Ca^{2+} influx in nontransformed intestinal cells expressing the endogenous GLP-2R *in vivo*.

The principal consequence of GLP-2 administration to rats

and mice *in vivo* is hyperplasia of the intestinal mucosal villous epithelium (36). Intestinal regulatory peptides such as gastrin that signal through GPCRs have been shown to stimulate GPCR-mediated *fos* expression, serum response element-dependent transcriptional activity, and Erk2 and Elk-1 activity, leading to the stimulation of cell growth in rat acinar AR42J cells (37). Similarly, GLP-1, the peptide most structurally related to GLP-2, potentiated glucose-stimulated immediate early gene expression in beta cells and increased pancreatic islet cell proliferation following administration to mice *in vivo* (25, 38). Whether the growth-promoting effects of GLP-2 in intestinal cells are direct, via coupling of the GLP-2R to mitogenic signaling pathways, or indirect remains unclear. GLP-2 was also found to induce a pronounced redistribution of F-actin in BHK cells. The precise mechanism underlying this effect remains to be defined, but the findings are consistent with the reported inhibition of Rho when phosphorylated by PKA (39). The inhibition is also compatible with the earlier notion that PKA directly phosphorylates and inactivates myosin light chain kinase (40), although this view is currently disputed.

Although it is not currently known whether GLP-2 directly stimulates cell proliferation in the intestinal epithelium, considerable evidence links activation of GPCR signaling to stimulation of growth factor-dependent pathways and cell proliferation. For example, pituitary adenylyl cyclase-activating peptide stimulates Erk1/2 activity via protein kinase C in a Ras-independent, mitogen-activated protein kinase/Erk kinase-dependent manner in PC 12 cells (41). Similarly, the GPCR ligands endothelin-1, lysophosphatidic acid, and thrombin stimulate tryptophan phosphorylation of *neu* and the epidermal growth factor receptor, demonstrating that mitogenic growth factor receptors may be transactivated via cross-talk from GPCR signaling (42). These findings, taken together with studies demonstrating GLP-1-dependent cell proliferation (38), raise the possibility that peptide hormone receptors such as the GLP-2R may also be directly coupled to mitogenic pathways in distinct cell types.

Our findings in BHK-GLP-2R cells demonstrate that relatively high concentrations of GLP-2, such as 100 nM, are needed for induction of immediate early gene expression and stimulation of cell proliferation. These actions are unlikely because of the activation of PKA- or AP-1-dependent pathways alone, as the EC_{50} for stimulation of signal transduction coupled to activation of CRE- and AP-1-directed transcriptional activity and cAMP accumulation was ~ 0.06 nM. In contrast, 10 nM GLP-2, which significantly stimulates PKA- and AP-1-dependent reporter genes, had no effect on cell proliferation, and 1 nM GLP-2 did not stimulate IE gene expression. Furthermore, the growth-promoting effects of GLP-2 in BHK cells could not be directly linked to activation of specific growth-related kinase pathways. For example, GLP-2 inhibited Erk1 and Erk2 activity, failed to stimulate Akt1 activity, and had only a modest and transient effect on p70 S6 kinase activity in BHK-GLP-2R cells. These findings imply that one or more as yet unidentified pathways coupled to IE gene expression and mitogenic stimulation are activated, independent of PKA and AP-1, by GLP-2 in BHK-GLP-2R cells.

An important question raised by our findings is whether the circulating concentrations of GLP-2 *in vivo* are sufficient to achieve stimulation of cell proliferation on target cells expressing the endogenous intestinal GLP-2R. Although the concentrations of intact circulating GLP-2-(1–33) in humans and rats are generally in the range from 50–150 pM (43–45), the concentration of liberated GLP-2-(1–33) in the intestinal mucosa has not yet been precisely determined. Nevertheless, we did not observe effects on cell growth and IE gene expression at 1–10

nM concentrations of GLP-2, clearly higher than the peak circulating levels of GLP-2 observed in the postprandial state *in vivo*. Indeed, 10 nM h[Gly²]-GLP-2 actually inhibited Erk1/2 activity and reduced serum-stimulated Elk-1 activity in BHK-GLP-2R cells. Hence, although our data suggest that relatively high concentrations of GLP-2 are capable of stimulating mitogenic pathways in BHK fibroblasts, whether the endogenous GLP-2R is directly coupled to stimulation of cell proliferation in the intestinal mucosa by physiological levels of GLP-2 requires further analysis in future studies.

Acknowledgments—GLP-2 is the subject of a licensing agreement between D. J. D., Allelix Biopharmaceuticals Inc, the University of Toronto, and the Toronto General Hospital.

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