Original Article

Activation of Glucagon-Like Peptide-1 Receptor Signaling Does Not Modify the Growth or Apoptosis of Human Pancreatic Cancer Cells

Jacqueline A. Koehler and Daniel J. Drucker

Glucagon-like peptide (GLP)-1 promotes β-cell proliferation and survival through stimulation of its specific G-protein–coupled receptor; however, the potential for GLP-1 receptor (GLP-1R) agonists to promote growth and proliferation of human pancreatic-derived cells remains poorly understood. We identified five human pancreatic cancer cell lines that express the GLP-1R and analyzed cell growth and survival in response to GLP-1R activation. Although cholera toxin (an activator of Gαs) and forskolin (an activator of adenylyl cyclase) increased levels of intracellular cAMP in all cell lines, the GLP-1R agonist exendin-4 (Ex-4) increased cAMP only in CFPAC-1 cells. Conversely, Ex-4 induced extracellular regulated kinase (ERK) 1/2 activation in PL 45 cells in a GLP-1R– and epidermal growth factor receptor–dependent manner, whereas Ex-4 inhibited ERK1/2 phosphorylation in Hs 766T and CAPAN-1 cells. Ex-4 did not modulate the proliferation of these cell lines in vitro and did not inhibit apoptosis after exposure of cells to cytotoxic agents such as cycloheximide, indomethacin, LY294002, or cyclopamine. Furthermore, daily Ex-4 treatment for 4 weeks had no effect on the propagation of CFPAC-1 or PL 45 tumor cells evaluated in nude mice in vivo. Thus, acute or chronic (4 weeks) GLP-1R stimulation does not modify the growth or survival of human pancreatic cancer cells. *Diabetes* 55:1369–1379, 2006

The pancreas and gastrointestinal epithelium contain highly specialized endocrine cells that secrete peptide hormones regulating food intake, gastrointestinal motility, gastric acid secretion, and nutrient absorption, with profound implications for the control of body weight regulation and glucose homeostasis. The proglucagon gene is expressed in both islet and gut endocrine cells and gives rise to a precursor prohormone, proglucagon, that encodes multiple distinct proglucagon-derived peptides liberated after tissue-specific posttranslational processing. These peptides are predominantly synthesized in endocrine cells of the distal small bowel and colon and include glicentin, oxyntomodulin, glucagon-like peptide (GLP)-1, and GLP-2.

GLP-1 is secreted together with structurally related proglucagon-derived peptides in a nutrient-dependent manner. GLP-1 regulates blood glucose at multiple levels, including stimulation of glucose-dependent insulin secretion from islet β-cells and inhibition of glucagon secretion, gastric emptying, and appetite (1–3). Therapy with GLP-1 receptor (GLP-1R) agonists has also been associated with expansion of β-cell mass via stimulation of β-cell proliferation, promotion of islet cell neogenesis, and inhibition of β-cell apoptosis (4–9). The demonstration that GLP-1R agonists may modify the outcome of diabetes in preclinical studies has raised considerable interest in the long-term effects of these agents for the treatment of human diabetes. Furthermore, a role for endogenous GLP-1 in the control of β-cell proliferation and survival is suggested by observations that GLP-1R$^{-/-}$ mice exhibit fewer large β-cell clusters, increased susceptibility to streptozotocin-induced β-cell apoptosis (9), and an impaired ability to regenerate islet mass after partial pancreatectomy (10).

The proliferative, neogenic, and anti-apoptotic effects of GLP-1 have also been demonstrated in multiple experiments using pancreatic or fibroblast cell lines in vitro (7,8,11,12). GLP-1R agonists also modulate the growth and differentiation of pancreatic exocrine cells, including rat ARIP and human PANC-1 ductal cells, rat AR42J acinar cells, and Capan-1 cells (a human pancreatic ductal carcinoma cell line). Exposure of exocrine cells to GLP-1R agonists promotes differentiation toward an endocrine, β-cell–like phenotype exemplified by expression of β-cell genes and the ability to secrete insulin in a glucose-dependent manner (13–15).

GLP-1 exerts its diverse biological effects through stimulation of its specific G-protein–coupled receptor (16). The GLP-1R is widely expressed and is structurally related to the class II glucagon/secretin receptor superfamily (16,17). The observation that activation of GLP-1R signaling is robustly coupled to stimulation of cell proliferation and enhanced cell survival is widely viewed as a positive aspect of GLP-1 action in the context of promoting expansion and survival of functioning β-cells (18). Nevertheless, the GLP-1R may also be expressed in the pancreatic ductal

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BrdU, 5-bromo-2′-deoxyuridine; CREB, cAMP-responsive element–binding protein; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular regulated kinase; Ex-4, exendin-4; FBS, fetal bovine serum; GLP, glucagon-like peptide; GLP-1R, GLP-1 receptor; Hsp90, heat shock protein 90; IBMX, 3-isobutyl-1-methylxanthine; PARP, poly(ADP-ribose) polymerase; PI 3-kinase, phosphatidylinositol 3-kinase; PMA, phorbol 12-myristate 13-acetate.

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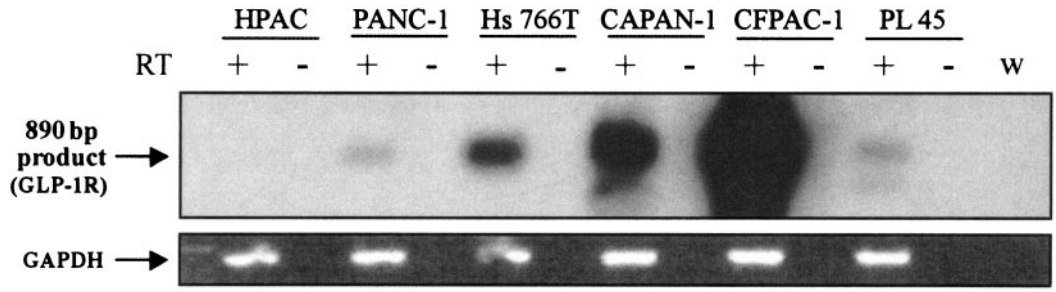

FIG. 1. GLP-1R expression in human pancreatic cancer cell lines. *A*: RT-PCR was performed using 5 μg total RNA isolated from a variety of human pancreatic cancer cell lines. PCR products were analyzed by agarose gel electrophoresis followed by ethidium bromide staining (glyceraldehyde-3-phosphate-dehydrogenase; *bottom panel*) or by Southern blot analysis (GLP-1R; *top panel*) using an internal GLP-1R–specific oligonucleotide probe. Control reactions used samples in which RNA (w) or the reverse transcriptase (RT) was omitted from the RT reaction mixture (RT−). The designations for each cell line denote the assignations for each specific pancreatic cancer cell line obtained from American Type Culture Collection: pancreatic adenocarcinoma CRL2119 (HPAC), CRL1918 (CFPAC-1), CRL2558 (PL 45), HTB 79 (CAPAN-1), carcinoma HTB 134 (Hs 766T), and CRL 1,469 (PANC-1).

epithelium, a potential precursor site for the development of pancreatic cancer (19). We have therefore studied the consequences of GLP-1R activation on the growth and survival of human pancreatic adenocarcinoma cells in short-term studies in vitro and in longer-term experiments in nude mice in vivo.

RESEARCH DESIGN AND METHODS

Tissue culture medium and serum were from Invitrogen Life Technologies (Carlsbad, CA). Forskolin, cycloheximide, indomethacin, phorbol 12-myristate 13-acetate (PMA), 5-bromo-2′-deoxyuridine (BrdU), protease inhibitor mixture (P-2714), 3-isobutyl-1-methylxanthine (IBMX), and phosphatase inhibitor cocktail I were from Sigma (St. Louis, MO). Cholera toxin, LY294002, H-89, epidermal growth factor (EGF), and AG1478 were from Calbiochem (San Diego, CA). Exendin-4 (Ex-4) was from California Peptide (Napa, CA), and cyclopamine was from Toronto Research Chemicals (Toronto, ON, Canada). Ex-4 was dissolved in PBS, EGF, and cholera toxin in water; cycloheximide in ethanol; and forskolin, LY294002, indomethacin, cyclopamine, PMA, IBMX, H-89, and AG1478 in dimethyl sulfoxide.

Cell lines, DNA plasmids, and transient transfections. Human pancreatic cancer cell lines were obtained from American Type Culture Collection (Manassas, VA) and were maintained in the recommended culture medium. CFPAC-1 cells were established from a pancreatic adenocarcinoma from a patient with cystic fibrosis (20); Capan-1 cells exhibit properties consistent with a mucin-producing ductal adenocarcinoma and were derived from a liver metastasis (21); Hs 766T cells were derived from a pancreatic lymph node metastasis (22); PL 45 cells were derived from a poorly differentiated ductal adenocarcinoma and harbor mutations in K-*ras* and p53 (23). All experiments were performed using early passage cells (up to passage 8). cDNAs encoding the human GLP-1R from pancreatic cell lines were generated by RT-PCR, subcloned into a TOPO TA cloning vector (pc4TOPO; Invitrogen Life Technologies), and sequenced. The pEGFP-C1 plasmid was purchased from Clontech-BD Biosciences (Mississauga, ON, Canada), and the cDNA encoding the rat GLP-1R was previously subcloned into the pcDNA3.1 eukaryotic expression vector (Invitrogen Life Technologies). Transient transfections were performed using Fugene reagent (Roche Diagnostics, Laval, QC, Canada), according to the manufacturer's instructions. To monitor transfection efficiency, cells were visualized with a Leica inverted fluorescent microscope to assess green fluorescent protein expression 2 days after transfection with a pEGFP-C1 plasmid (Leica Microsystems, Wetzlar, Germany).

RT-PCR and Southern blot analysis. Total RNA was isolated from cells using the guanidinium thiocyanate method. Five micrograms total RNA was reversed transcribed at 42°C for 60 min using Revert Aid H Minus cDNA synthesis kit (MBI Fermentas) and random hexamer primers (Invitrogen Life Technologies). The GLP-1R was amplified by PCR using 1/10 of the RT reaction using *Taq* polymerase (Invitrogen Life Technologies) and primer pairs 5′-TTC TGC AAC CGG ACC TTC GA-3′ and 5′-ATG AGT GTC AGC GTG GAC TTG-3′. Amplification of human GLP-1R cDNA was performed using 30 cycles at an annealing temperature of 58°C, resulting in the generation of a 890-bp product spanning bases 181-1070 (amino acids 60–357) of the GLP-1R open reading frame. Glyceraldehyde-3-phosphate-dehydrogenase cDNA amplification was performed as previously described (24).

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′-TGTATAGCACAGCCGCCCAG-3′) overnight at 48°C in hybridization buffer (5% dextran sulfate, 1 mol/l NaCl, and 1% SDS). Blots were washed in 2× sodium chloride–sodium citrate, 1% SDS for 25 min at 48°C and exposed to Kodak BioMax MS film (Eastman Kodak, Rochester, NY) at −70°C with an intensifying screen for the indicated period of time.

cAMP assays. Cells were grown in 24-well plates to ~70% confluence and were serum-starved for 3 h by washing cells with PBS, after which cells were re-incubated with Dulbecco's modified Eagle's medium (DMEM) lacking serum. For analysis of cAMP in transient transfectants, at 48 h after transfection, cells were serum-starved for 3 h and then treated with the indicated agonists in DMEM supplemented with 100 μmol/l IBMX. 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 radioimmunoassay kit (Biomedical Technologies, Stoughton, MA).

Proliferation and cell survival assays. To analyze cell proliferation, cells (30–40% confluent) were cultured in 24-well plates and maintained for 16 h in serum-free medium (DMEM for Hs 766T and PL 45 or Iscove's medium for CFPAC-1 and CAPAN-1). Fresh medium was added in the presence or absence of 5 or 50 nmol/l Ex-4, 10 μmol/l forskolin, or 10% fetal bovine serum (FBS) and incubated at 37°C for the indicated period of time. 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 assessed by measuring the bioreduction of a MTS tetrazolium salt at 490 nm using the Cell-Titer 96 aqueous cell proliferation assay kit according to the manufacturer's suggestions (Promega, Madison, WI). To measure the extent of cell death/survival, cells (at a confluency of 60–70%) were maintained for 16 h in medium lacking serum before treatment with LY294002 (50 μmol/l final), cycloheximide (80 μmol/l final), indomethacin (600 μmol/l final), or cyclopamine (10 μmol/l) in the same medium in the presence or absence of 5 or 50 nmol/l Ex-4, 10 μmol/l forskolin, or 10% FBS for 48 h (LY294002, cycloheximide, and indomethacin) or 72 h (cyclopamine). Control cultures were subjected to the same manipulations as treated cells but in the absence of drugs. At the end of the incubation period, the number of viable cells in each condition was determined.

Animals. All animal studies were approved by the Animal Care Committee of the University Health Network, Toronto General Hospital. Experiments were performed on 10-week-old athymic nude male mice (Harlan Labs, Indianapolis, IN). All mice were housed in plastic-bottomed, wired-lid cages, maintained in temperature- and humidity-controlled rooms with a 12-h light/dark cycle, and given water and chow ad libitum throughout the course of the experiment.

Tumor implantation experiments. Cell suspensions of CFPAC-1 and PL 45 cells (5×10^5 cells each) were implanted by subcutaneous injection into opposite flanks of nude mice. Six days after cell injection, mice were given daily intraperitoneal injections with either 100 μl PBS solution ($n = 8$ mice) or 24 nmol/kg Ex-4 ($n = 10$ mice) for 4 weeks. On the final day of the experiment, mice were injected with 100 mg/kg BrdU and killed by anesthesia 4.5 h later. Blood samples were obtained by cardiac puncture and mixed with 10% (vol/vol) trasylol EDTA (500,000 kIU/ml trasylol, 1.2 mg/ml EDTA, and 0.1 mmol/l diprotin A). Plasma was collected by centrifugation at 4°C and stored at −80°C. The pancreas and tumors were removed and weighed, and small portions were quickly frozen on dry ice for RNA and protein analysis. Just before mice were killed, blood was drawn from a tail vein, and blood glucose

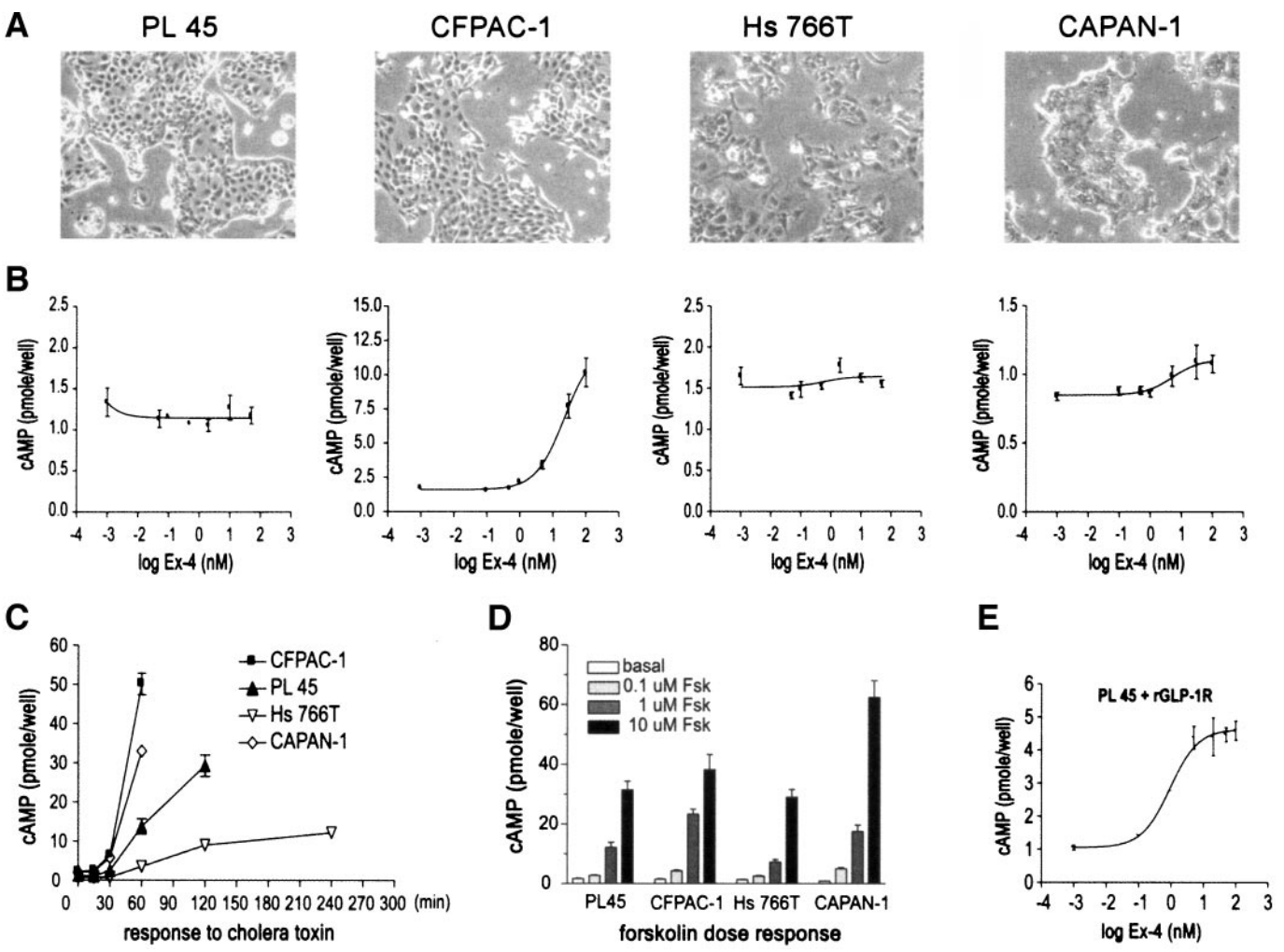

FIG. 2. cAMP accumulation after Ex-4 treatment is only observed in CFPAC-1 cells. *A*: Morphology of pancreatic cancer cell lines. Magnification ×100. *B*: GLP-1R–expressing pancreatic cancer cell lines were treated with the indicated concentrations of Ex-4 for 10 min. cAMP content was measured by a radioimmunoassay. Data represent means ± SE of four independent experiments performed in triplicate. cAMP levels in pancreatic cancer cell lines after treatment with 1 μg/ml cholera toxin (*C*), forskolin (Fsk) for 10 min (*D*), or PL 45 (CRL2558) cells transiently transfected with the rat GLP-1R treated with the indicated concentrations of Ex-4 for 10 min (*E*). cAMP content was measured by a radioimmunoassay. Data represent means ± SE of three (*D* and *E*) or two (*C*) independent experiments performed in triplicate.

levels were measured by the glucose oxidase method using an Ascensia Elite blood glucose meter (Bayer, Toronto, ON, Canada).

Histology and immunocytochemistry. The pancreas and tumors were fixed overnight in 5% glacial acetic acid/25% formaldehyde (vol/vol) or in 10% formalin, respectively, and embedded in paraffin. Paraffin sections were immunostained for insulin (rabbit anti-insulin; 1:30 dilution; Dako Diagnostics, Mississauga, ON, Canada) or Pdx-1 (1/200; a gift of Dr. H. Edlund, Umea, Sweden) as primary antibody and biotinylated goat anti-rabbit (1:200 dilution; Vector Labs, Burlington, ON, Canada) as secondary antibody. The sections were then washed with PBS and incubated with horseradish peroxidase–conjugated Ultra Streptavidin (ID Labs, London, ON, Canada) for 30 min, and color was developed with freshly prepared 3,3′-diaminobenzidine tetrahydrochloride (Dako Diagnostics) solution.

Serial sections were treated with 2 N HCl for 30 min, blocked with 10% normal horse serum, and then immunostained for BrdU using mouse anti-BrdU (1:1,000 dilution; Caltag Laboratories, Burlingame, CA) as primary antibody. Sections were then exposed to biotinylated horse anti-mouse IgG (1:200 dilution; Vector Labs) and subsequently stained with horseradish peroxidase–conjugated Ultra-Streptavidin (ID Labs), and color was developed with freshly made 3,3-diaminobenzidine (Vector Labs) solution. Sections were lightly counterstained with Mayer's hematoxylin, dehydrated through graded alcohol, cleared in xylene, and mounted in Permount (Fisher, Hampton, NH). Nonspecific immunopositivity was assessed using preimmune serum and omission of primary antiserum.

SDS-PAGE and Western blot analysis. Cells were maintained in serum-free medium for 16 h followed by treatment in the presence or absence of Ex-4, 10 μmol/l forskolin, 25 ng/ml EGF, or 100 nmol/l PMA as indicated. 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 mmol/l Tris, pH 8.0, and 150 mmol/l NaCl) supplemented with protease (1:40 dilution) and phosphatase (1:100 dilution) inhibitor cocktails and 500 μmol/l sodium orthovanadate. Thirty micrograms of cell lysate was then used for Western blot analysis as described (24,25) and exposed to Kodak BioMax MR film. Extracts from a small sample of each tumor were prepared by disrupting the tissue using a 1.5-ml pellet pestle (Nalge Nunc International; Kontes Glass Company, Vineland, NJ) in radioimmunoprecipitation assay buffer (50 mmol/l Tris, pH 8.0, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 150 mmol/l NaCl) containing protease and phosphatase inhibitors, and 45 μg tumor lysate was used for Western blot analysis.

Antibodies to p90Rsk phosphorylated on Ser 380, Akt phosphorylated on Ser 473, cAMP-responsive element–binding protein (CREB) phosphorylated on Ser 133, and p44/42 MAPK phosphorylated on Thr 202 and Tyr 204 were from Cell Signaling Technology (Beverly, MA) and were used at 1:1,000 dilutions to detect catalytically active forms of the kinases. Procaspase 3 and cleaved caspase 3 were also from Cell Signaling Technology and were used at 1:1,000 dilutions. Poly(ADP-ribose) polymerase (PARP) was from PharMingen and used at a 1:2,500 dilution. A primary antibody against heat shock protein 90 (Hsp90) (Transduction Laboratories, San Diego, CA) was used as a loading control at a 1:2,000 dilution. Densitometry was performed on blots exposed to Biomax MR film (Eastman Kodak) using a Hewlett-Packard ScanJet 3p scanner and NIH Image Software.

Statistical analysis. Statistical significance was assessed by one-way ANOVA using Bonferroni's multiple comparison post test and, where appropriate, by unpaired Student's *t* test using GraphPad Prism 3 (GraphPad

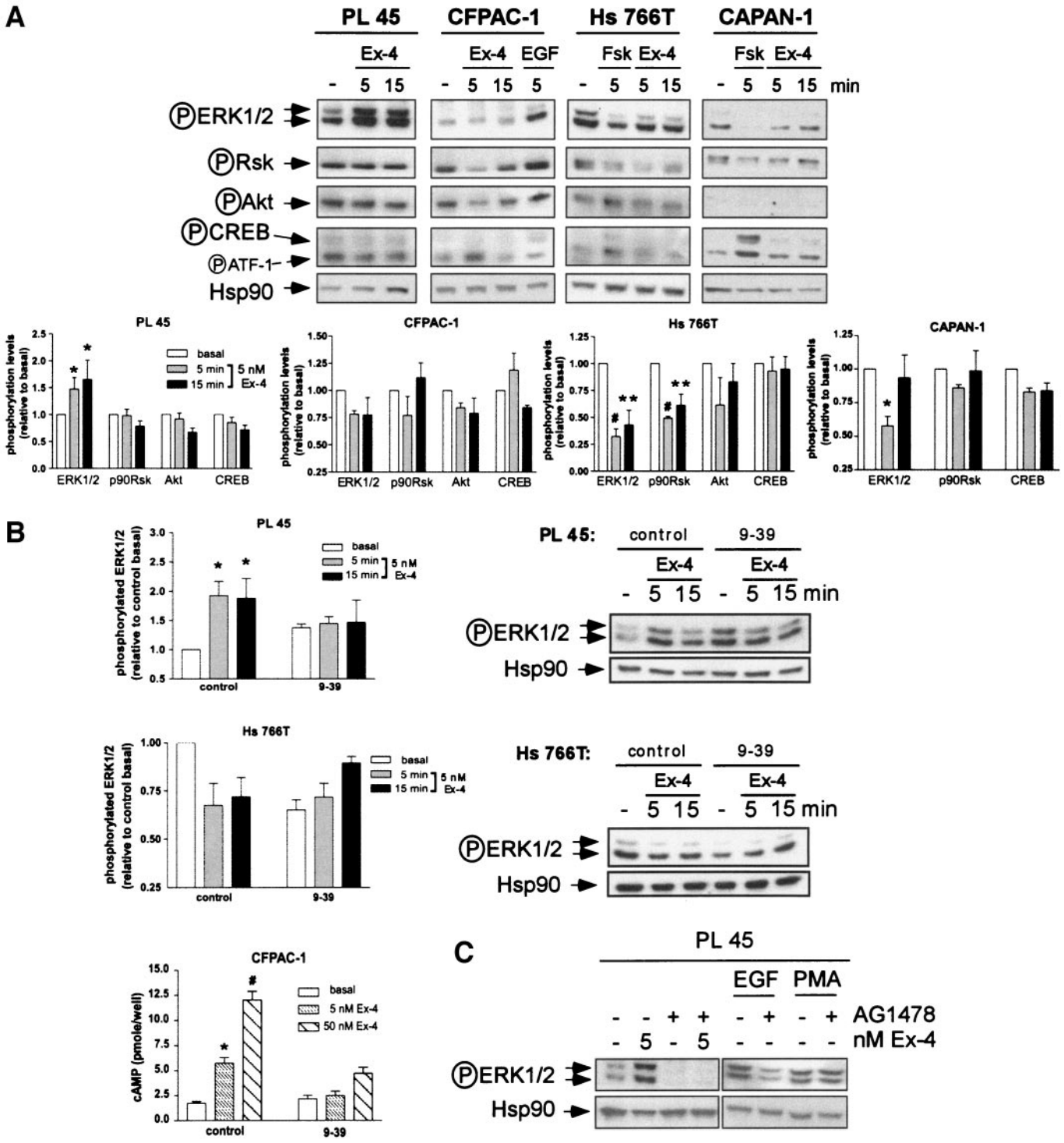

FIG. 3. GLP-1R–mediated effects on ERK1/2 phosphorylation in GLP-1R–expressing pancreatic cancer cell lines. *A*: Cell lines were serum-starved overnight (−) and stimulated for 5 or 15 min with 5 nmol/l Ex-4, 10 μmol/l forskolin (Fsk), or 25 ng/ml EGF as indicated. After treatments, equal amounts of whole-cell lysate were immunoblotted using antisera against phosphorylated/activated ERK1/2 (Thr202/Tyr204), phosphorylated p90Rsk (Ser 380), phosphorylated Akt (Ser 473), phosphorylated CREB (Ser 133), or Hsp90 as a loading control. Representative blots from three independent experiments with similar results are shown. Densitometric quantification of the data, corrected for the intensity of Hsp90 expression, from three independent experiments is shown in the *bottom panels*. Data represent means ± SD. *$P < 0.05$, **$P < 0.01$, #$P < 0.001$, Ex-4–treated vs. basal levels. *B*: PL 45 and Hs 766T cells were serum-starved overnight (−) before treatment with 5 nmol/l Ex-4 for 5 or 15 min in the presence or absence of the GLP-1R antagonist exendin-(9-39) (1 μmol/l) added to cells for 20 min before and during stimulation with 5 nmol/l Ex-4 as indicated. After treatments, equal amounts of whole-cell lysate were immunoblotted using antisera against phosphorylated/activated ERK1/2 (Thr202/Tyr204) or Hsp90 as a loading control. Representative blots from two independent experiments with similar results are shown on the *right*. Quantifications of the level of ERK1/2 phosphorylation from two independent experiments are shown on the *left*. The intensity of the signal was quantified by densitometry and corrected for the intensity of the Hsp90 signal. Data are means ± SD. The effect of exendin-(9-39) on cAMP levels in CFPAC-1 cells after treatment with 5 or 50 nmol/l Ex-4 is shown in the *bottom panel*. Data represent means ± SD. *$P < 0.05$, **$P < 0.01$, #$P < 0.001$, Ex-4–treated vs. basal levels. *C*: PL 45 cells were serum-starved overnight (*first lane*) before treatment with 5 nmol/l Ex-4 for 5 min or pretreated for 1 h with 1 μmol/l AG1478 before stimulation with Ex-4. As a control for EGFR activation, cells were pretreated with or without AG1478 before stimulation with 25 ng/ml EGF for 5 min. Alternatively, cells were stimulated with 100 nmol/l PMA as a control to confirm the specificity of the EGFR inhibitor (AG1478). Equal amounts of whole-cell lysates were then immunoblotted against phosphorylated ERK1/2 or Hsp90. Shown are representative blots from two independent experiments with similar results.

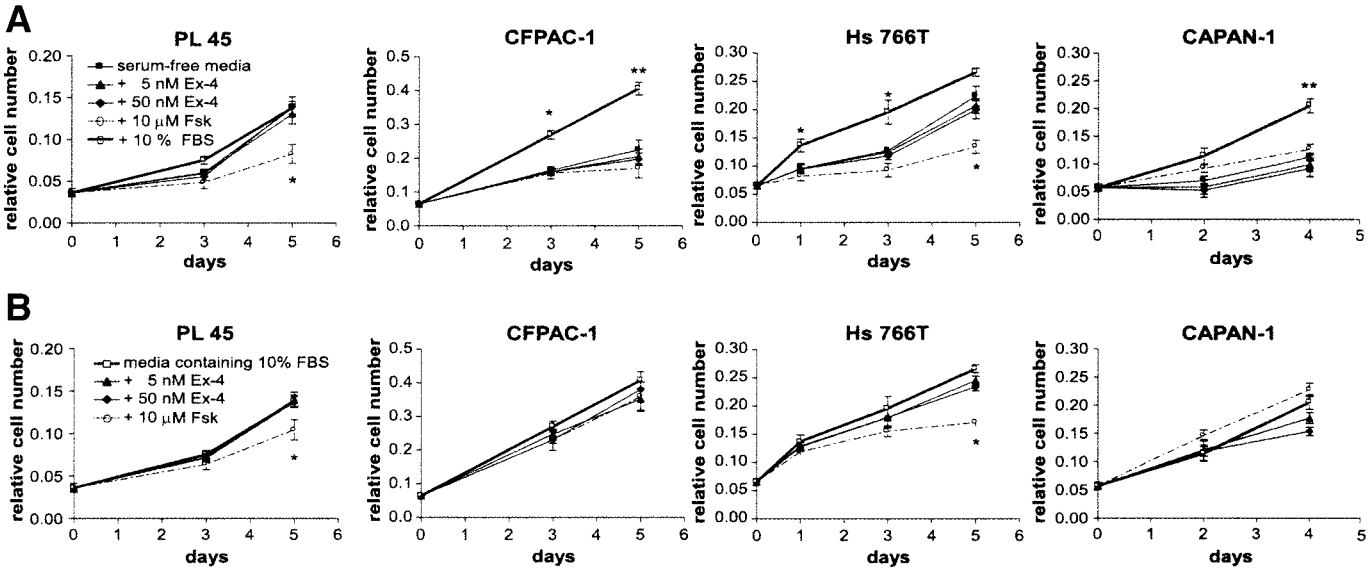

FIG. 4. Ex-4 does not modulate cell proliferation of pancreatic cancer cell lines expressing the GLP-1R. Cells maintained in serum-free medium (*A*) or growth medium (containing 10% FBS) (*B*) were treated in the presence or absence of 5 or 50 nmol/l Ex-4, 10 μmol/l forskolin, or 10% FBS; and fresh medium and treatments (Ex-4 or forskolin) were replaced every 24 h. At the indicated time points, cell viability was measured using a tetrazolium salt bio-reduction assay (MTS assay). *$P < 0.05$, **$P < 0.01$, alone vs. treatment. Data are the means ± SE from four independent experiments, each performed in triplicate.

Software, San Diego, CA). A P value of <0.05 was considered to be statistically significant.

RESULTS

Human pancreatic cancer cells express the GLP-1R. To identify pancreatic carcinoma cell lines that express the GLP-1R, we used RT-PCR and Southern blot analysis to examine RNA from six human pancreatic cancer cell lines obtained from the American Type Culture Collection. RT-PCR was performed using internal primers directed against the human GLP-1R that resulted in the amplification of an 890-bp cDNA product. GLP-1R cDNAs were amplified from RNA from three human pancreatic ductal adenocarcinoma (CAPAN-1, CFPAC-1, and PL 45) and two carcinoma (PANC-1 and Hs 766T) cell lines but not from HPAC cells (Fig. 1). Sequence analysis of GLP-1R cDNAs from the different pancreatic cell lines did not reveal any mutations in the region encompassing intracellular loops 2 and 3, which are reported to be essential for G-protein coupling selectivity (data not shown). Because the effects of GLP-1 on differentiation of PANC-1 cells transfected with Pdx-1 have previously been reported (15), all subsequent studies were carried out using CFPAC-1, Hs 766T, PL 45, and CAPAN-1 cells (Fig. 2*A*)

Activation of downstream signaling pathways in GLP-1R–expressing pancreatic cancer cell lines. Stimulation of the GLP-1R leads to the activation of pleiotropic signaling pathways in human islet cells involving adenylyl cyclase, phospholipase C, phosphatidylinositol 3-kinase (PI 3-kinase), and extracellular regulated kinases 1 and 2 (ERK1/2) (26,27). Accordingly, we assessed whether GLP-1R stimulation in human pancreatic cancer cell lines was coupled to activation of similar signal transduction pathways. Surprisingly, Ex-4 induced a robust increase in levels of intracellular cAMP only in CFPAC-1 cells (Fig. 2*B*). The lack of a significant GLP-1–stimulated cAMP response in PL 45, Hs 766T, and CAPAN-1 cells was not attributable to a generalized defect in cAMP generation or deficient coupling of Gαs to adenylate cyclase, because cholera toxin (which activates Gαs through ADP-ribosylation) and forskolin (an activator of adenylate cyclase) markedly increased the levels of cAMP in all four cell lines (Fig. 2*C* and *D*). However, Ex-4 stimulated cAMP accumulation in PL 45 cells transfected with the rat GLP-1R (Fig. 2*E*). Taken together, these results demonstrate that all of the signaling components required for coupling to adenylate cyclase are present in these cell lines.

To determine whether the endogenous GLP-1R expressed in these cell lines is coupled to signaling pathways other than adenylate cyclase, we assessed the phosphorylation of p44/p42 MAPK (ERK1/2), p90Rsk (a downstream effector of ERK1/2), and Akt (a downstream effector of PI-3 kinase) using antibodies that recognize specific phosphorylated residues required for their activation. In contrast to the lack of effect of Ex-4 on cAMP accumulation, stimulation of the GLP-1R in PL 45 cells induced ERK1/2 phosphorylation/activation (Fig. 3*A*). Nevertheless, ERK1/2 phosphorylation was not coupled to p90Rsk phosphorylation in the same experiments (Fig. 3*A*). Furthermore, no change in the levels of Akt or CREB phosphorylation were observed in PL 45 cells after Ex-4 treatment (Fig. 3*A*), suggesting that neither the PI 3-kinase nor the cAMP/PKA pathway is activated by the GLP-1R in these cells.

Ex-4 produced a modest reduction in levels of phosphorylated ERK1/2, p90Rsk, and Akt in CFPAC-1 cells (Fig. 3*A*), suggesting that both MAPK and PI 3-kinase signaling pathways are inhibited after GLP-1R stimulation. In contrast, Ex-4 and, to a greater extent, forskolin significantly inhibited ERK1/2 phosphorylation in Hs 766T and CAPAN-1 cells, with a concomitant decrease in p90Rsk phosphorylation. Although forskolin promoted CREB phosphorylation in CAPAN-1 cells, Ex-4 failed to produce a robust increase in CREB phosphorylation in any of these cell lines (Fig. 3*A*). Nevertheless, despite the lack of a cAMP response observed in PL 45, Hs 766T, and CAPAN-1 cells after GLP-1R stimulation, our data indicate that the endogenous GLP-1R expressed in each cell line is func-

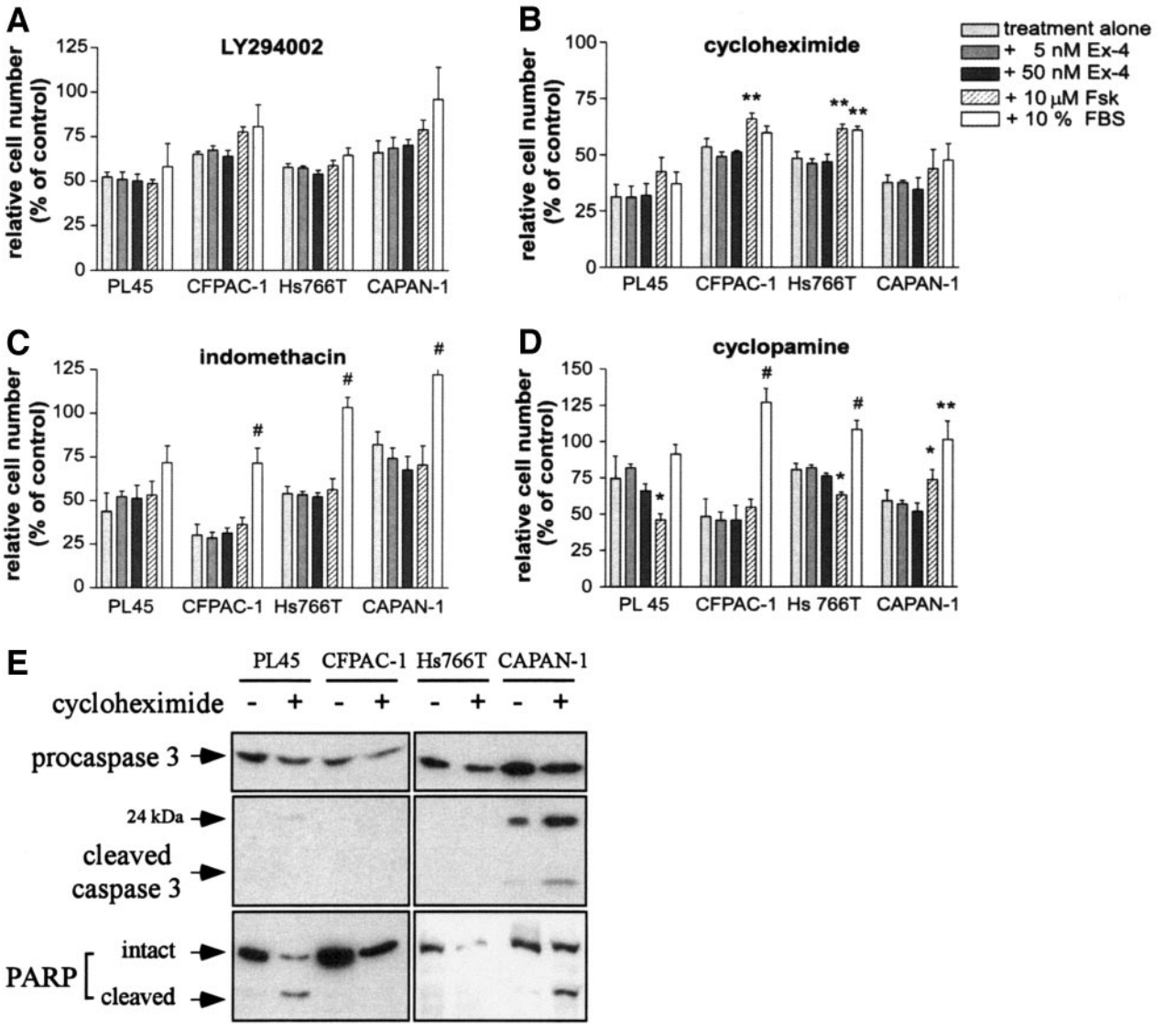

FIG. 5. Ex-4 does not protect GLP-1R–expressing pancreatic cancer cell lines from drug-induced cell death. *A–D*: Cells were maintained for 16 h in medium lacking serum before treatment with LY294002 (50 μmol/l final), cycloheximide (80 μmol/l final), indomethacin (600 μmol/l final), or cyclopamine (10 μmol/l) in the same medium in the presence or absence of 5 or 50 nmol/l Ex-4, 10 μmol/l forskolin, or 10% FBS for 48 h (LY294002, cycloheximide, and indomethacin) or 72 h (cyclopamine), at which point cell viability was measured using a tetrazolium salt bio-reduction assay (MTS assay) and is expressed as a percentage relative to vehicle-treated cells. Fresh medium and treatments were replaced every 24 h. *$P < 0.05$, **$P < 0.01$, #$P < 0.001$, treatment alone vs. treatment + Ex-4, forskolin, or FBS. Data are the means ± SE from four (LY294002, cycloheximide, and indomethacin) or three (cyclopamine) independent experiments, each performed in triplicate. Cell number obtained for cells cultured in vehicle alone was designated a relative value of 100%, whereas cell viability after exposure to either LY294002, cycloheximide, indomethacin, or cyclopamine (treatment alone) or treatment plus Ex-4, forskolin, or FBS was expressed as a percentage relative to the cell number obtained in the absence of drug. *E*: Serum-starved cells were treated with ethanol alone (−) or 80 μmol/l cycloheximide (+) for 48 h, after which attached and floating cells were combined, lysed, and immunoblotted against procaspase 3, cleaved caspase 3, or PARP. Data are representative of two independent experiments.

tional, even though the signaling pathways modulated by the GLP-1R varied from cell line to cell line.

The diverse number of signaling pathways activated by Ex-4 in these cell lines prompted us to determine whether Ex-4 was acting through the known GLP-1R. Accordingly, we repeated key experiments in the presence or absence of exendin-(9-39), a well-characterized GLP-1R antagonist (28). Exendin-(9-39) completely abrogated the Ex-4–dependent induction of ERK1/2 in PL 45 cells and reduced the basal levels of ERK1/2 in Hs 766T cells (Fig. 3*B*), consistent with its ability to function as an inverse agonist at the GLP-1R (29). Furthermore, the Ex-4–mediated induction of cyclic AMP in CFPAC-1 cells was completely attenuated in the presence of exendin-(9-39) (Fig. 3*B*). Taken together, these findings are consistent with Ex-4 exerting its actions on pancreatic cancer cells through the known GLP-1R.

To further explore the mechanism by which GLP-1R activation mediates ERK1/2 phosphorylation in PL 45 cells, we examined the effects of different kinase inhibitors on ERK1/2 activation. Phosphorylation of ERK1/2 by Ex-4 was completely blocked by the specific EGF receptor (EGFR) inhibitor, AG1478, demonstrating that EGFR activation is essential for GLP-1R–mediated ERK1/2 phosphorylation (Fig. 3*C*). Similarly, pretreatment with AG1478 abolished ERK1/2 phosphorylation induced by EGF but not PMA, illustrating the specificity of this inhibitor (Fig. 3*C*). These results demonstrate that GLP-1R activation in

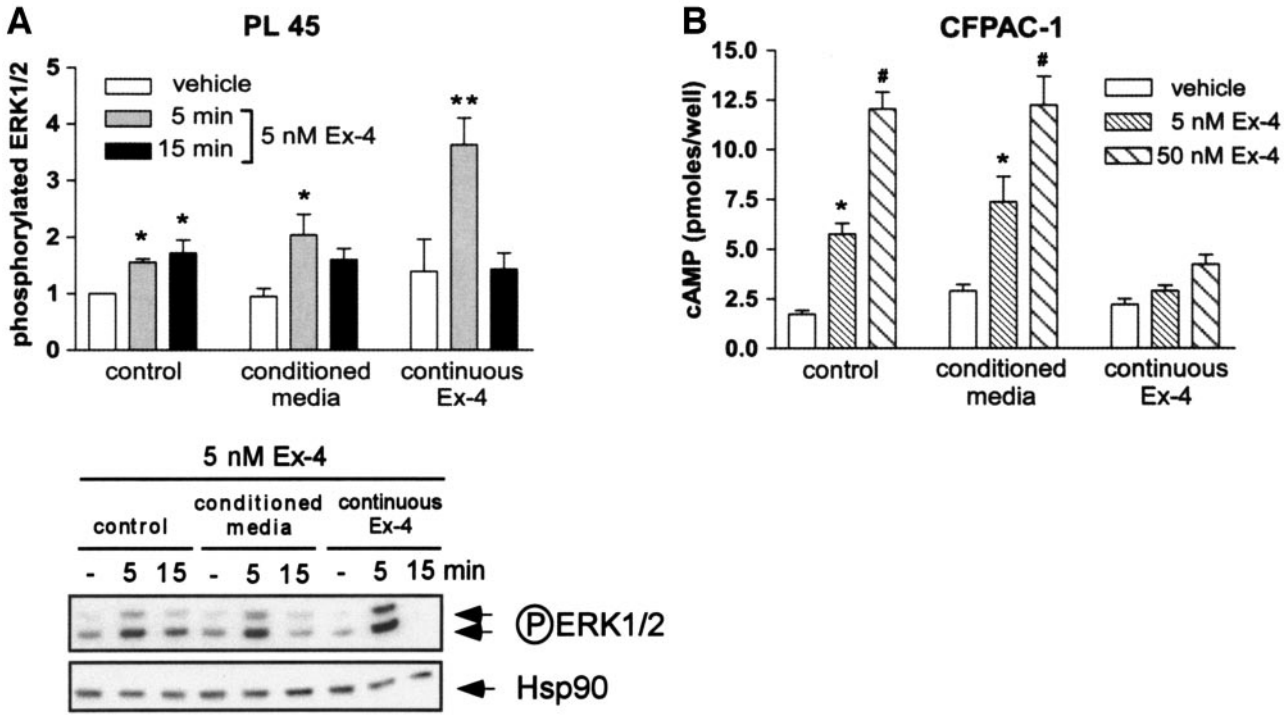

FIG. 6. Ex-4–mediated signaling is diminished in CFPAC-1 but not in PL 45 cells after continuous exposure to Ex-4. Cells were cultured in the presence or absence of 5 or 50 nmol/l Ex-4 for 4 days. Fresh medium and Ex-4 were replaced every 24 h. On day 4, cells were serum-starved for 24 h in the presence or absence of 5 or 50 nmol/l Ex-4. The following day, conditioned medium was collected from cells grown in the presence or absence of Ex-4 and used to stimulate new groups of cells not previously exposed to Ex-4. In separate experiments, serum-free medium containing the indicated concentrations of fresh Ex-4 was used to stimulate previously unexposed cells (control) or to rechallenge cells that were previously exposed to Ex-4 for 4 days (continuous Ex-4). For cells continuously exposed to Ex-4 for 4 days, cells were then restimulated with the same concentration of Ex-4 used in the 4-day continuous exposure experiment. *A*: After treatment for the indicated time periods, equal amounts of whole-cell lysate were immunoblotted using antisera against phosphorylated/activated ERK1/2 (Thr202/Tyr204) or Hsp90 as a loading control. Representative blots from two independent experiments with similar results are shown. Densitometric quantification of the level of ERK1/2 phosphorylation from two independent experiments, corrected for the intensity of the Hsp90 signal, is shown. Data are means ± SD. *$P < 0.05$, **$P < 0.01$, vehicle vs. treatment. *B*: cAMP levels in CFPAC-1 cells after a 10-min treatment period were measured by a radioimmunoassay. Data represent means ± SE of three independent experiments performed in triplicate. $P < 0.05$; $P < 0.001$ vehicle vs. treatment.

PL 45 cells promotes EGFR transactivation, which mediates GLP-1R–induced activation of ERK1/2, consistent with previous observations in islet cell lines (11,30).

Ex-4 does not modulate the growth rate of pancreatic cancer cell lines expressing the GLP-1R. GLP-1R activation promotes β-cell proliferation in cell lines in vitro and in the rodent pancreas in vivo. To determine whether GLP-1R activation stimulates proliferation of pancreatic cancer cell lines, we monitored the growth rate of cells maintained in media with or without Ex-4 or forskolin for up to 5 days. The number of PL 45 cells increased similarly when maintained in either serum-free medium (Fig. 4*A*) or medium containing 10% FBS (Fig. 4*B*), suggesting that these cells proliferate equally well independent of serum. In the presence of forskolin, however, the number of viable PL 45 cells was somewhat reduced after 5 days, suggesting that a chronic rise in intracellular cAMP levels either inhibits the growth rate of these cells or is detrimental to their survival. In contrast, Ex-4 did not affect the number of viable cells grown under either serum-free or serum-supplemented conditions (Fig. 4*A* and *B*). Forskolin also decreased the growth rate of Hs 766T cells but not CFPAC-1 or CAPAN-1 cells (Fig. 4*A* and *B*). Furthermore, Ex-4 did not alter the growth rate of any of these cell lines under serum-free or serum-supplemented conditions. Taken together, these results demonstrate that despite the activation of distinct signal transduction pathways in specific cells, GLP-1R activation did not modulate the growth rate of any of the GLP-1R–expressing pancreatic cancer cells examined here.

Ex-4 does not rescue GLP-1R–expressing pancreatic cancer cells from drug-induced cell death. Because GLP-1R activation reduces apoptosis in rat (9) and human (6,12) β-cells and cell lines (7), we examined whether GLP-1R stimulation could rescue pancreatic cancer cells from drug-induced cell death. Cells were treated with cycloheximide (a protein synthesis inhibitor), indomethacin (a nonsteroidal anti-inflammatory drug), LY294002 (an inhibitor of PI 3-kinase), or cyclopamine (an inhibitor of the hedgehog signaling pathway) in the presence or absence of Ex-4, and cell viability was assessed after 2 or 3 days of drug exposure. Ex-4 had no effect on survival of pancreatic cancer cell lines after exposure to cytotoxic agents (Fig. 5). In contrast, serum attenuated cycloheximide-induced cell death in Hs 766T cells (Fig. 5) and increased the number of viable CFPAC-1, Hs 766T, and CAPAN-1 cells after exposure to indomethacin or cyclopamine (Fig. 5). Similarly, forskolin increased survival in CFPAC-1 and Hs 766T cells after treatment with cycloheximide (Fig. 5) and in CAPAN-1 cells exposed to cyclopamine. In contrast, cell viability was further reduced in cyclopamine-treated PL 45 and Hs 766T cells in the presence of forskolin (Fig. 5). Furthermore, pretreatment with 0.5, 5, or 50 nmol/l Ex-4 for 24 h did not increase cell survival after exposure of all four cell lines to cycloheximide, indomethacin, or LY294002 (data not shown), sug-

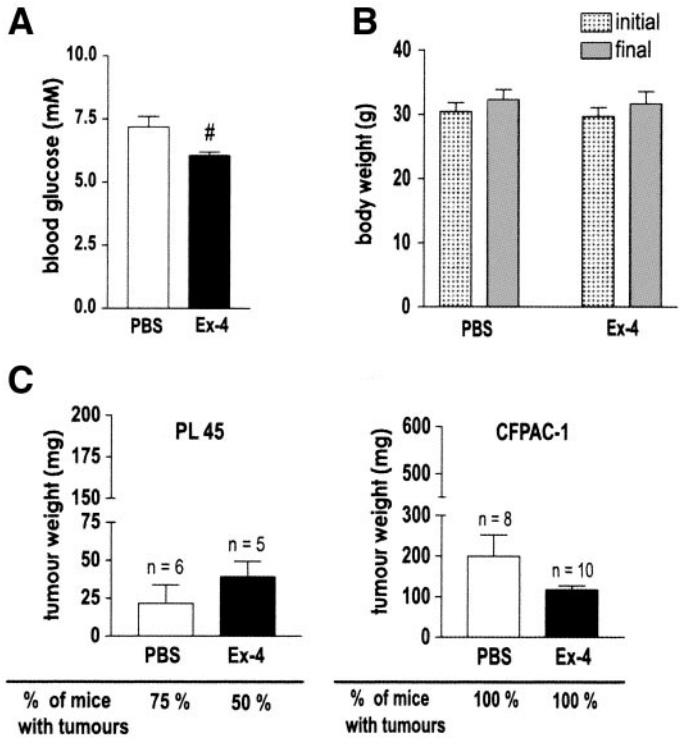

FIG. 7. Ex-4 treatment does not modulate tumor formation after implantation into nude mice. *A*: Blood glucose levels of nude mice after 4 weeks of PBS or Ex-4 treatment (measured 4.5 h after final intraperitoneal injection with PBS or Ex-4, as indicated). Data are the means ± SD from $n = 8$ (PBS) or $n = 10$ (Ex-4) mice. #$P < 0.001$, PBS vs. Ex-4 treatment. *B*: Body weight of mice at the beginning of the experiment, just before tumor implantation (initial), and at the end of the 4-week treatment period (final) with PBS or Ex-4. Data are the means ± SD from $n = 8$ (PBS) or $n = 10$ (Ex-4) mice. *C*: Tumor weights of implanted CFPAC-1 (*right*) or PL 45 cells (*left*) at the end of the 4-week treatment period with PBS or Ex-4, as indicated. Data are the means ± SD. The number of mice (n) is indicated above each bar, and the percentage of mice that developed tumors is indicated underneath the graph.

gesting that neither concomitant nor pretreatment with GLP-1R agonists prevents pancreatic cancer cell death induced by these agents.

To determine whether the reduction in cell survival after exposure to these agents was associated with activation of apoptotic pathways and not simply necrosis, we examined the levels of procaspase 3, cleaved caspase 3, and intact PARP. Treatment of pancreatic cancer cells with cycloheximide reduced the levels of procaspase 3 and intact uncleaved PARP (a downstream substrate of active executioner caspases) (Fig. 5*E*). Furthermore, an increase in the levels of cleaved caspase 3 and cleaved PARP were also observed in PL 45 and CAPAN-1 cells (Fig. 5*E*), demonstrating that cycloheximide induced apoptosis in these cells. Similar results were also observed after treatment with LY294002 and indomethacin (data not shown). Taken together, these results demonstrate that Ex-4 does not rescue or protect GLP-1R–expressing pancreatic cancer cell lines from a variety of apoptosis-inducing agents.

Because Ex-4 did not significantly perturb cell growth or survival in vitro, we examined whether prolonged exposure of cultured cells to Ex-4 was associated with loss of biological activity as a result of peptide degradation or receptor downregulation. We first examined whether conditioned medium from cells incubated with Ex-4 was capable of activating ERK1/2 phosphorylation and cAMP accumulation in cells previously grown in the absence of Ex-4. Conditioned medium significantly increased ERK1/2 phosphorylation in PL 45 cells and cyclic AMP accumulation in CFPAC-1 cells not previously exposed to Ex-4 (Fig. 6*A* and *B*). These findings strongly suggest that Ex-4 was not completely degraded in the medium under these experimental conditions.

We next ascertained whether the ERK1/2 or adenylate cyclase pathway was downregulated after continuous exposure to Ex-4. A robust increase in levels of phosphorylated ERK1/2 was observed after Ex-4 treatment of PL 45 cells continuously exposed to Ex-4 for 4 days, with levels of phosphorylated ERK1/2 returning to baseline control values after 15 min (Fig. 6*A*). In contrast, CFPAC-1 cells continuously exposed to 5 or 50 nmol/l Ex-4 for 4 days exhibited markedly reduced cAMP accumulation upon rechallenge with Ex-4 (Fig. 6*B*), without reducing levels of GLP-1R mRNA transcripts in the same experiments (data not shown). These findings demonstrate that chronic exposure of CFPAC-1 cells to Ex-4 may result in desensitization of GLP-1R signaling coupled to cyclic AMP accumulation, consistent with previous observations in studies of Ex-4 and islet cells (31).

Ex-4 does not modulate the propagation of GLP-1R–expressing pancreatic cancer cell lines in vivo. To ascertain whether sustained GLP-1R activation modulates pancreatic cancer cell growth in vivo, we implanted CFPAC-1 and PL 45 cells subcutaneously into separate flanks of nude mice followed by daily injections with either PBS or Ex-4 for 4 weeks. CFPAC-1 and PL 45 cells were chosen for this experiment because these cells displayed an increase in intracellular cAMP levels or ERK1/2 activation in response to Ex-4 treatment, respectively. Ex-4 treatment significantly decreased blood glucose levels in mice (Fig. 7*A*), however, there was no significant difference in body weight (Fig. 7*B*) or the weight of the tumors after 4 weeks of treatment with either PBS or Ex-4 (Fig. 7*C*), demonstrating that Ex-4 does not modulate tumor growth in vivo. Somewhat surprisingly, however, the cell line that proliferated equally well in either serum-free or growth conditions in vitro (PL 45 cells; Fig. 4*A*) and also contains an oncogenic K-*ras* mutation (32) developed much smaller tumors compared with tumors arising after implantation of CFPAC-1 cells. Despite the comparatively reduced weight of PL 45 tumors, the number of $BrdU^+$ cells (Fig. 8) was higher (~ 15%) than that observed with CFPAC-1 cells (~10%) (compare Fig. 8*C* with *D*). Furthermore, although Ex-4 treatment had no effect on the number of $BrdU^+$ cells in CFPAC-1 tumors (Fig. 8*D*), BrdU incorporation into PL 45 tumor cells was significantly reduced after Ex-4 treatment (Fig. 8*C*). Taken together, these results demonstrate that chronic treatment with Ex-4 does not promote the progression of pancreatic tumor development or growth in vivo.

Effect of Ex-4 treatment on signaling molecules in tumors of implanted CFPAC-1 cells. To ascertain the consequences of GLP-1R activation on downstream signaling pathways in implanted tumor cells, we examined the levels of phosphorylated proteins in tumor extracts from CFPAC-1 tumors. The small size of PL 45-derived tumors precluded our ability to carry out a similar analysis with these tumors. CFPAC-1 tumors from Ex-4–treated mice displayed a modest but nonsignificant increase in levels of ERK1/2 phosphorylation (Fig. 9). Similarly, no significant differences in the phosphorylation state of p90Rsk or CREB were observed in CFPAC-1 tumors from PBS- versus Ex-4–treated mice. However, Ex-4 treatment did

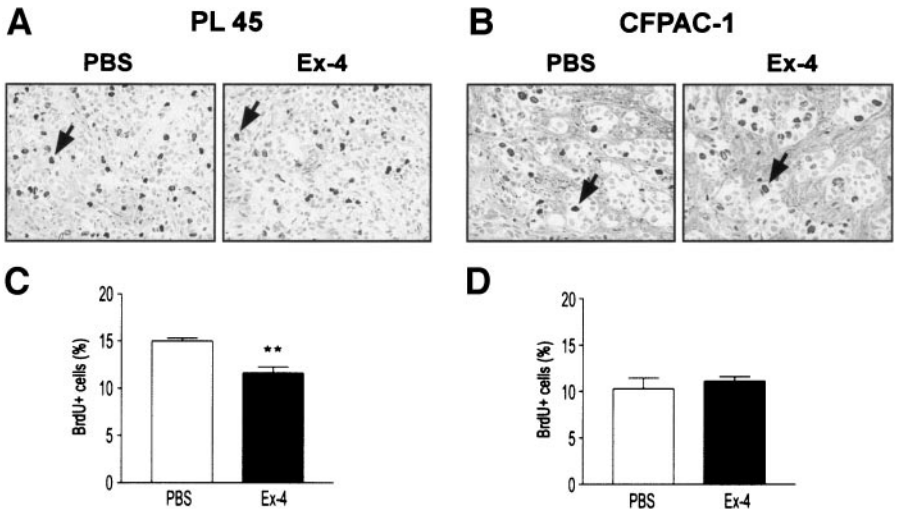

FIG. 8. BrdU incorporation in tumors propagated in nude mice. Histology and BrdU immunostaining of tumors from implanted GLP-1R–expressing pancreatic cancer cell lines after a 4-week treatment with PBS (*left*) or Ex-4 (*right*), as indicated. Shown are representative photomicrographs of tumors from implanted PL 45 (*A*) and CFPAC-1 (*B*). Representative BrdU$^+$ cells are indicated by an arrow. Magnification ×200. The number of BrdU$^+$ cells after treatment were quantified and shown as a percentage of the total number of cells counted. For each tumor, 800–900 cells were counted and assessed for BrdU incorporation, and a total of four (PL 45) or six (CFPAC-1) tumors were assessed for each treatment. Data are the means ± SD. **$P <$ 0.01, PBS vs. Ex-4.

result in a small but significant increase in levels of phosphorylated Akt (Fig. 9). Thus, similar to findings derived from studies of the identical cell lines cultured in vitro, a 4-week exposure to Ex-4 produced only modest effects on known molecular targets of GLP-1R signaling in human pancreatic cancer cells propagated in vivo.

Because GLP-1 has been shown to induce differentiation of pancreatic exocrine (AR42J) or ductal (CAPAN-1, rat ARIP, and PANC-1) cells into insulin-producing endocrine cells (13–15), we next examined whether Ex-4 treatment induced the expression of β-cell markers in tumor cells. Immunohistochemical analysis of CFPAC-1 or PL 45 tumors derived from our implantation experiments did not reveal expression of either insulin or PDX-1 (data not shown). Hence, differentiation of pancreatic exocrine cells toward a more endocrine phenotype is not an invariable consequence after sustained treatment with GLP-1R agonists.

DISCUSSION

The observation that GLP-1R agonists lower levels of blood glucose in diabetic subjects together with the expansion of β-cell mass by these agents via proliferative and anti-apoptotic actions (18) has generated substantial interest in the development of GLP-1R agonists for the treatment of type 2 diabetes. Nevertheless, the proliferative and anti-apoptotic actions of these agents may have theoretical unintended consequences on tumor formation in either the endocrine or the exocrine pancreas. Several human subjects have recently presented with hyperinsulinemic hypoglycemia and pancreatic nesidioblastosis after gastric bypass surgery (33). Because plasma levels of gut hormones, including GLP-1, may be significantly increased in some patients after gastric bypass (34,35), it seems reasonable to consider the possible contribution of hormones such as GLP-1 to the pathophysiology of nesidioblastosis in affected subjects. Our data demonstrate that although human pancreatic cancer cell lines may express a functional GLP-1R, activation of GLP-1R signaling is not coupled to stimulation of cell proliferation or resistance to cell death in short-term studies.

Although the pancreatic cancer cell lines studied here were selected for expression of the GLP-1R, we observed cell-specific differences in GLP-1R–dependent activation of ERK1/2 and cAMP. One possible explanation for this differential responsivity to GLP-1R agonists is that the downstream consequences of GLP-1R activation may be dependent in part on the level of GLP-1R expression in each cell type (36). For example, we observed that Ex-4 activated ERK1/2 but not cyclic AMP formation in PL 45 cells expressing an endogenous GLP-1R, however, transfection of PL45 cells with a GLP-1R cDNA was associated with dose-dependent increases in cyclic AMP formation in response to Ex-4. Hence the relative level of GLP-1R expression may be an important determinant of signal transduction pathway activation in pancreatic cancer cells. Furthermore, it seems quite likely that the relative level of GLP-1R expression in different human pancreatic tumors may also vary considerably, hence further analysis of GLP-1R expression in larger numbers of human pancreatic tumors seems warranted.

The differential effects of Ex-4 on downstream signaling molecules and cell proliferation and/or the different degrees of sensitivity to drug-induced cell death exhibited by these cell lines may also reflect differences in specific mutations and/or overexpression of signaling molecules inherent to these pancreatic cancer cells. It is known that pancreatic cancers exhibit numerous genetic and functional alterations, such as overexpression of mitogenic growth factors and their receptors (37), as well as mutations in the proto-oncogene K-*ras* and the tumor suppressor genes p53, p16, and Smad4 (rev. in 38). However, all four cell lines used in this study contain p53 mutations and do not express p16 either because of homozygous deletion of the p16 gene or because p16 is transcriptionally silenced, and all but Hs 766T cells contain oncogenic K-*ras* mutations. Hence it seems likely that multiple inherent genetic alterations may also contribute to the differences observed in the biological properties and the response to GLP-1R activation exhibited by these cell lines.

The levels and duration of Ex-4 exposure either in cell culture experiments in vitro or in mice harboring implanted tumors in vivo are considerably different than levels of exposure to GLP-1 or Ex-4 that might be expected in human subjects. Levels of circulating bioactive intact endogenous GLP-1 in human subjects range from 5 to 50 pmol/l (39) and may exceed 300 pmol/l in subjects after gastric bypass surgery (35). Similarly, human subjects treated with exenatide (synthetic Ex-4) for type 2 diabetes receive 10-μg injections twice daily, resulting in circulating levels of exenatide that generally peak at <75 pmol/l (40,41). In contrast, pancreatic cancer cells were exposed

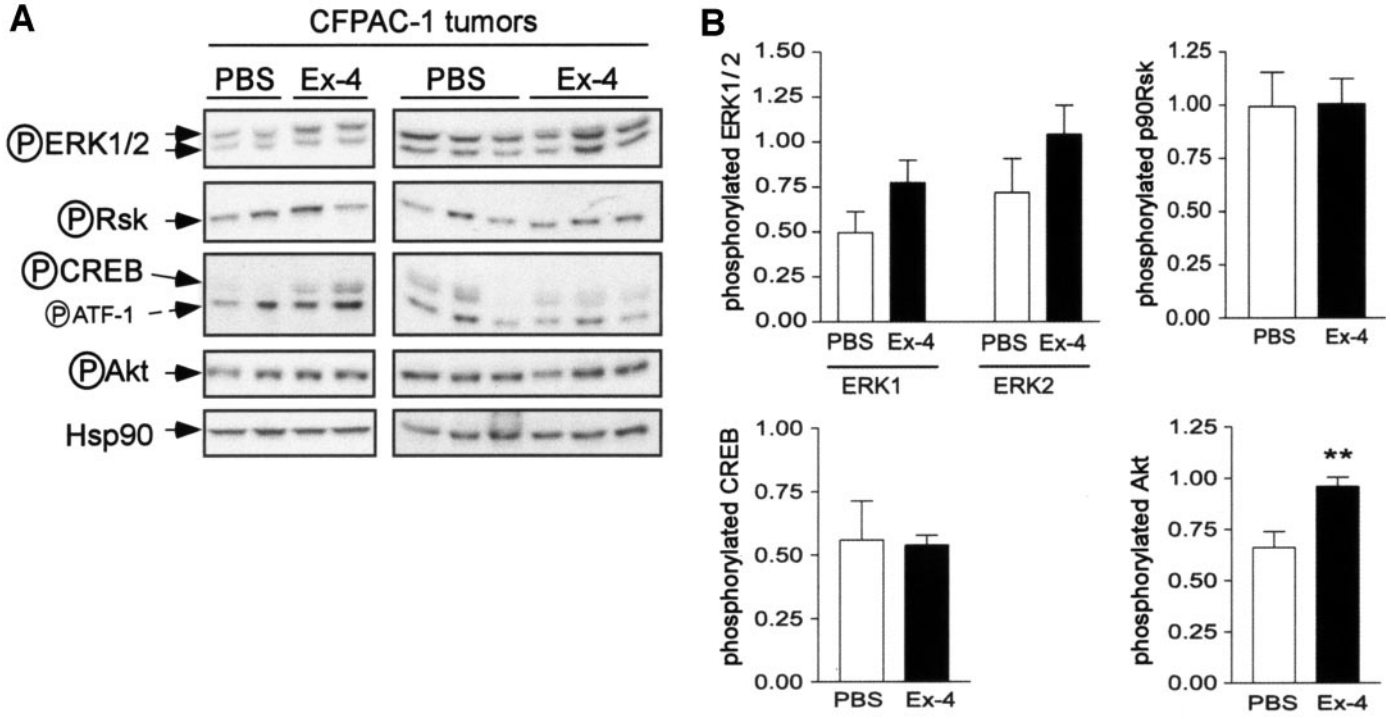

FIG. 9. Levels of phosphorylated signaling molecules in tumor extracts from implanted CFPAC-1 cells. *A*: Tumors from mice treated with or without Ex-4, as indicated, were excised, and a portion was lysed in radioimmunoprecipitation assay buffer. Equal amounts of whole-cell lysate were then immunoblotted against phosphorylated/activated ERK1/2, phosphorylated Akt (Ser 473), phosphorylated p90Rsk (Ser 380), phosphorylated CREB (Ser 133), or Hsp90 as a loading control. *B*: Quantification of the level of phosphorylation of the indicated signaling molecules in each tumor. The intensity of the signal was quantified by densitometry and corrected for the intensity of the Hsp90 signal. Data are means ± SD. **$P < 0.01$, PBS vs. Ex-4.

to nanomolar concentrations of Ex-4, and mice treated with Ex-4 received daily injections of 24 nmol/kg, or ~2.5 μg/25 g mouse. Although we were not able to determine the plasma levels of Ex-4 in tumor-bearing mice, we suspect that the circulating levels of Ex-4 in these mice are likely to be considerably higher than levels of GLP-1 or Ex-4 observed in human subjects.

In conclusion, although treatment with GLP-1R agonists expands β-cell mass in normal and diabetic rodents and promotes survival of human islet cells (6,12), our data demonstrate that acute or prolonged treatment with Ex-4 does not enhance the propagation of human pancreatic cancer cell lines in vitro or in vivo. Nevertheless, our studies do not address the question of whether prolonged continuous GLP-1R activation may promote the development or growth of normal human pancreatic exocrine, ductal, or islet cells or pancreatic cancer. The GLP-1R agonist exenatide (Ex-4) has recently been approved as a new agent for the treatment of type 2 diabetes in the U.S., and there is ongoing interest in developing longer-acting GLP-1R agonists with extended circulating pharmacokinetic profiles (42). Moreover, several reports have linked the development of islet nesidioblastosis and pancreatic endocrine tumors in patients after gastric bypass (33) with markedly increased levels of circulating GLP-1 (35). Hence, a more detailed understanding of how sustained GLP-1R activation regulates cell proliferation in the normal and neoplastic pancreas seems prudent.

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