Precursor peptide progastrin$_{1-80}$ reduces apoptosis of intestinal epithelial cells and upregulates cytochrome $c$ oxidase Vb levels and synthesis of ATP

Hai Wu, Azarmidokht Owlia, and Pomila Singh

Department of Anatomy and Neurosciences, University of Texas Medical Branch, Galveston, Texas 77555-1043

Submitted 9 May 2003; accepted in final form 17 July 2003

Wu, Hai, Azarmidokht Owlia, and Pomila Singh. Precursor peptide progastrin$_{1-80}$ reduces apoptosis of intestinal epithelial cells and upregulates cytochrome $c$ oxidase Vb levels and synthesis of ATP. Am J Physiol Gastrointest Liver Physiol 285: G1097–G1110, 2003. First published July 24, 2003; 10.1152/ajpgi.00216.2003.—We recently reported that downregulation of gastrin gene expression in colon cancer cells significantly suppresses relative levels of mitochondrial cytochrome $c$ (cyt $c$) oxidase Vb (Cox Vb) RNA and protein. These unexpected findings suggested the possibility that gastrin gene products (mainly progastrin (PG)) may be directly or indirectly mediating the observed effects in colon cancer cells. Because colon cancer cells do not respond to exogenous PG, we examined the possibility of whether PG regulates Cox Vb expression in gastrin-responsive intestinal epithelial cells (IECs) in vitro. Levels of Cox Vb RNA and protein were significantly increased in a dose-dependent manner in response to PG. Mitochondrial synthesis of ATP was also increased by approximately three- to fivefold in response to optimal concentrations (0.1–1.0 nm) of PG. Possible antiapoptotic effects of PG were additionally examined, because activation of caspases 9 and 3 had been noted in colon cancer cells downregulated for gastrin gene expression. We measured a significant loss in the levels of cyt $c$ in the cytosol of PG-treated vs. control IEC cells, which correlated with a significant loss in the activation of caspases 9 and 3, resulting in a significant loss in DNA fragmentation on PG treatment of the cells. Our results thus suggest the novel possibility that the precursor PG peptide exerts direct antiapoptotic effects on IECs, which may contribute to the observed growth effects of PG on these cells. Additionally, Cox Vb gene appears to be an important intracellular target of PG, resulting in an increase in ATP levels, which may also contribute to the observed increase in the growth of target cells in response to PG.

gastrins; growth; apoptosis; colon cancer cells; adenosine 5′-triphosphatase

UNDER PHYSIOLOGICAL conditions, the completely processed amidated forms of gastrins (G17, G34) are present as the major circulating forms of gastrin and play an important role in acid secretion from the parietal cells (61). Circulating gastrins also play an important role in the growth of gastrointestinal mucosa (19, 20, 29, 32). In the late 1980s, we learned that COOH-terminal amidation was not crucial for measuring biological effects of gastrins (50). Glycine-extended gastrin was reported to be mitogenic for rat intestinal epithelial cells (IECs) (53), Swiss 3T3 fibroblasts (53), pancreatic cancer cells (44), colon cancer cells (53, 57), and gastric epithelial cells (17) and to promote invasiveness of human colon cancer cells (24). In recent years, we have learned that the full-length precursor molecule progastrin (PG) is also biologically active. Transgenic mice overexpressing PG, either in the liver (hGAS) or in the intestinal mucosa (FABP-hPG) demonstrated hyperproliferation of colonic crypt cells (12, 56, 63), and PG-expressing mice were at a high risk for developing preneoplastic and neoplastic lesions in the colonic mucosa in response to azoxymethane (12, 55, 56). Factors contributing to growth and cocarcinogenic effects of PG on the colonic mucosal cells in vivo may be due to indirect or direct effects of the precursor peptides on the large IECs. We recently reported significant growth-promoting effects of the full-length recombinant human PG (rhPG$_{1-80}$) peptide on IEC-18 and IEC-6 cells in vitro (7, 51). Others have similarly reported growth-promoting effects of PG molecules on a transformed gastric epithelial cell line in vitro (4, 38). The direct growth-promoting effects of PG on IEC cells in vitro may reflect either a proliferative and/or antiapoptotic effect of PG.

Our recent studies suggested that downregulation of gastrin gene expression results in the activation of caspases 9 and 3 in gastrin-dependent human colon cancer cells (65). Our studies with human colon cancer cells further suggested the novel possibility that endogenous gastrins (mainly PG) may support the growth/survival of the cells by upregulating mitochondrial (Mt) cytochrome $c$ (cyt $c$) oxidase Vb (Cox Vb) levels (65). It is, however, not known whether these surprising findings were a direct result of the downregulation of gastrin gene expression (and hence endogenous PG), or if the effects were due to downstream effects of the loss of gastrin gene expression. The present studies were therefore undertaken to examine the novel possibility of whether PG can directly regulate Cox Vb expression and perhaps reduce the apoptotic response.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
of the target cells to proapoptotic agents. To fulfill these objectives, we measured relative levels of Cox Vb RNA and protein in response to increasing doses of recombinant PG₁₈₀ peptide and examined possible activation of the Cox Vb promoter in PG-expressing cells. In addition, relative levels of cyt c and activated caspases 9 and 3 were measured in the cytosol of PG-stimulated vs. control IEC cells. ATP levels were measured as a functional readout of changes in Cox Vb levels, whereas DNA fragmentation was measured as a readout of changes in the activation of caspases 9 and 3.

MATERIALS AND METHODS

Materials. Leupeptin, aprotinin, benzamidine, PMSF, sodium orthovanadate, EDTA, HEPES, EGTA, and camptothecin were obtained from Sigma (St. Louis, MO). Monoclonal anticytotoxicshrome Vb antibodies (Abs) (A6456) were purchased from Molecular Probes (Eugene, OR). Monoclonal anti-cyt c Abs (65981A) were purchased from Pharmingen (San Diego, CA). Polyclonal anti-caspase 3 (H277) and anti-caspase 9 (H83) Abs were procured from Santa Cruz Biotechnology (Santa Cruz, CA). [α-³²P]dCTP (3,000 Ci/mmol) was from ICN (Costa Mesa, CA). TRI reagent was obtained from Molecular Research Center (Cincinnati, OH). Avian myeloblastosis virus reverse transcriptase, oligo(dT) primer, RNAase inhibitor, and random-primer DNA labeling kit were from GIBCO-BRL (Grand Island, NY). The PCR-II plasmid and TA cloning kit were obtained from Invitrogen (Carlsbad, CA). The full-length rhPG₁₈₀ was generated as recently described (7, 51).

Cell culture. Rat intestinal cell lines IEC-6 and IEC-18 (American Type Culture Collection, Rockville, MD), which are pluripotent and can be induced to differentiate into either large or small IECs (40), were grown as monolayer cultures that demonstrated gastrin expression and growth characteristics. After seeding the cultures, restriction-digested plasmid was purified from the cultures, and the plasmid was then used as a probe for Northern blot analysis by our published methods (14). The primers used for amplifying rat Cox Vb cDNA were designed based on the published rat Cox Vb cDNA sequence in GeneBank accession no. X 14208 (14). The primers used for amplifying rat Cox Vb cDNA were sense: 5'AGT CGC TTG GCT AGT CTT TA-3' and antisense: 5'AGT CGC TTG GCT AGT CTT TA-3'. This resulted in the amplification of 494 bp of Cox Vb cDNA. The cDNA fragment was subcloned into PCR-II vector and amplified in the top 10 Escherichia coli cells (Invitrogen). The plasmid was purified from the cultures, restriction-digested with EcoRI enzyme, and the DNA fragment confirmed. The purified and confirmed rat Cox Vb cDNA fragment was then used as a probe for Northern blot analysis by our published methods (49, 54, 65). Briefly, the cDNA fragment was labeled with [α-³²P]dCTP by using the random primer DNA labeling kit and hybridized with RNA blots by using rapid-Hyb buffer (Amersham Life Sciences) according to the supplier’s instructions. After developing the blot, the membrane was stripped and rehybridized with labeled 18S probe (as the control) by our published methods (49, 65).

Fractionation of mitochondria and cytosol from IEC and HCT-116 cells. Cells treated as described in Treatment of IEC-116-C and HCT-116-AS cells with camptothecin for measuring apoptotic end points. Camptothecin (an extract of the Chinese tree Camptotheca acuminata) is a potent inhibitor of topoisomerase II, a molecule required for DNA synthesis (42). Camptothecin has been shown to induce apoptosis in a dose-dependent manner in vitro (21) and is generally used for inducing apoptosis (38a). Subconfluent HCT-116-AS and HCT-116-C cells, growing logarithmically in 60-mm cell culture plates in complete growth medium containing 10% FCS were treated with 5 μM camptothecin in 0.1 mM DMSO, for 5 h at 37°C in 5% CO₂ incubator. Control cells were treated with equivalent concentrations of DMSO. At the end of the treatment, cells were washed twice with ice-cold PBS followed by analysis with either the DNA ladder assay kit or the DNA ladder assay kit and hybridized with RNA blots by using rapid-Hyb buffer (Amersham Life Sciences) according to the supplier’s instructions. After developing the blot, the membrane was stripped and rehybridized with labeled 18S probe (as the control) by our published methods (49, 65).

AJP-Gastrointest Liver Physiol • VOL 285 • DECEMBER 2003 • www.ajpgi.org
tion of either mitochondria or cytosol as recently described (65). Briefly, the treated and control cells were washed twice with cold PBS and lysed in buffer A (in mM: 20 HEPEs, pH 8.0, 10 KCl, 1.5 MgCl₂, 1 EDTA, 1 EGTA, 250 sucrose, 1 DTT, 1 PMSF, 10 benzamidine, 0.2 sodium orthovanadate, and 10 μg/ml leupeptin and 10 μg/ml aprotinin) for 30 min on ice at a concentration of 10⁶ cells/ml lysis buffer. The cell lysates were collected in homogenization tubes and subjected to homogenization by using Dounce homogenizer B pestle. The cell lysates were cleared by centrifugation twice at 2,500 rpm for 5 min at 4°C, and the supernatant was subjected to another centrifugation at 13,000 rpm for 30 min at 4°C. The supernatant and pellets were collected separately. The supernatant was further centrifuged at 40,000 rpm for 1 h at 4°C. The resulting supernatant was labeled cytosol fraction. The pellet from the 13,000 rpm centrifugation was resuspended in buffer A and centrifuged at 13,000 rpm for 30 min at 4°C. The resulting supernatant was discarded, and the pellet containing the Mt fraction was used for measuring either ATP levels or lysed further for protein extraction as described below.

Preparation of protein extracts from Mt fractions. For Western blotting experiments, the Mt fractions were lysed for preparation of protein extracts by suspending the Mt pellets in buffer B (in mM: 10 Tris-Cl, pH 7.4, 150 NaCl, 5 EDTA, 1 PMSF, 1 sodium orthovanadate, and 0.5% Triton X-100 and 10 μg/ml aprotinin) for 30 min on ice; Mt from 10⁶ cells/100 μl of lysis buffer were used. The lysate was centrifuged at 4°C at 13,000 rpm, and the supernatant was labeled as Mt protein extract. Protein concentrations in the cytosolic and Mt fractions were determined by using BCA Protein Assay Kit (Pierce), and aliquots were stored at −70°C.

Western blot analysis of Mt protein extracts and cytosolic fractions from control and treated IEC cells. Cytosolic fractions and Mt extracts were prepared from control and treated IEC cells as described in Preparation of protein extracts from Mt fractions and were used for Western immunoblotting with specific Abs as follows. Equal amounts of protein (50 μg) from the cytosolic and Mt extracts were separated by electrophoresis on 0.1% SDS/15% polyacrylamide gels by our published procedures (49, 65). The protein was transferred electro-photorectically to a nitrocellulose membrane (Hybond; Amer- sham Pharmacia Biotech) and blocked in 10 μg/ml blocking buffer B (20 HEPES, pH 8.0, containing 150 μM NaCl, 0.1% Tween 20, and 5% (wt/vol) nonfat dry milk, as described previously (49, 65). Complete transfer of proteins was confirmed by Coomasie blue staining of the gels (49, 65). The membranes were processed by Western immunoblot analysis by using one or more of the following specific primary Abs (1 μg/ml anti-cyt c Ab; 3 μg/ml anti-Cox Vb Ab; 1:2,000 dilution of anti-caspases 9 and 3 Abs) followed by incubation with the appropriate peroxidase-conjugated Ab by our published procedures (49, 65). The antigen-Ab complexes were detected by using chemiluminescence reagent kit (Amersham Pharmacia Biotech). The cytosolic samples were additionally processed for West- ern immunoblot analysis with anti-β-actin Abs (Santa Cruz) as loading controls, whereas the Mt samples were addition- ally processed with Mt Cox 1 Abs (Molecular Probes) as loading controls. The relative density of the bands was dens- itometrically analyzed with the Documentation Analysis System (model Alphalager 2000; Alpha Innotech, San Le- andro, CA). The ratio of the densitometric readings for the protein of interest vs. the appropriate control protein was arbitrarily assigned a value of 1.0 for all control (nonpeptide treated) samples. The ratio for the peptide treated samples is expressed as a factor of the control samples.

DNA ladder (fragmentation) assay. The DNA Ladder Iso- lation Kit (Oncogene Research Products) was used for visual- izing DNA fragmentation in apoptotic cells. The control and peptide-stimulated IEC cells, treated with camptothecin as described in Treatment of HCT-116-C and HCT-116-AS cells with camptothecin for measuring apoptotic end points, were used for analysis of DNA fragmentation by the DNA Ladder Isolation Kit. Subconfluent HCT-116-AS and HCT- 116-C cells growing in normal growth medium containing 1% FCS and treated with camptothecin as described in Treat- ment of HCT-116-C and HCT-116-AS cells with camptothecin for measuring apoptotic end points were also used in these assays. A significant percentage of camptothecin treated HCT-116 cells became detached from the cell culture dishes and were observed as floating cell populations in the cell culture medium of these dishes. IEC cells, on the other hand, were less susceptible to detachment after camptothecin treatment, which may be due to the fact that IEC cells express insulin-like growth factors (36, 47) that may delay complete detachment of the cells. Therefore, at the end of the treatment, both floating and attached IEC cells were har- vested separately, and aliquots were counted by using a coulter counter (Beckman Coulter, Miami, FL). Fragmented DNA and high molecular weight DNA were recovered from the attached and floating cell samples, as per the protocols provided with the kit. The DNA fragments were resuspended in the buffer provided with the kit and separated by standard agarose gel electrophoresis and stained with ethidium bromide for visualization of the DNA ladders. DNA markers of the appropriate size range (provided with the kit) were also run along with the samples. The gels were photographed at the end of the run, and the extent of fragmentation analyzed visually.

ELISA assay for measuring relative levels of apoptotic death in different cellular samples. The control and peptide-stimulated IEC cells that were additionally treated with camptothecin (as described in Treatment of HCT-116-C and HCT-116-AS cells with camptothecin for measuring apoptotic end points) were processed for quantitative measurement of cytoplasmic histone-associated DNA-fragments (mono- and oligonucleosomes) by using the cell death detection ELISA®. LUX kit (Roche Laboratories) as per the protocols provided with the kit. HCT-116-AS and HCT-116-C cells growing logarithmically were treated with camptothecin (as described in Treatment of HCT-116-C and HCT-116-AS cells with camptothecin for measuring apoptotic end points), and processed as per the protocols provided with the ELISA kit. The assay is based on the quantitative sandwich/enzyme-immu- noassay-principle and uses mouse monoclonal Abs against DNA and histones, respectively. The assay thus allows for the specific determination of mononucleosomes in the cell lysates, which was photometrically measured at 405 nm with the help of an ELISA reader (μQuant; Bio-Tek Instruments, Winooski, VT). Reagent blanks were subtracted from the cell sample readings, and the optical density readings in the peptide-stimulated vs. control cells are presented as a quantitative measure of apoptotic death in the various samples.

Relative levels ATP synthesis by oxidative phosphorylation in the mitochondria of cellular samples. The relative levels of ATP were measured in control and peptide-stimulated IEC cells with the help of a mitochondrial ATP assay (Roche Diagnostics) by using a luminous reagent kit. HCT-116-AS and HCT-116-C cells growing in normal growth medium containing 1% FCS and treated with camptothecin as described in Treatment of HCT-116-C and HCT-116-AS cells with camptothecin for measuring apoptotic end points were also used in these assays. A significant percentage of camptothecin treated HCT-116 cells became detached from the cell culture dishes and were observed as floating cell populations in the cell culture medium of these dishes. IEC cells, on the other hand, were less susceptible to detachment after camptothecin treatment, which may be due to the fact that IEC cells express insulin-like growth factors (36, 47) that may delay complete detachment of the cells. Therefore, at the end of the treatment, both floating and attached IEC cells were har- vested separately, and aliquots were counted by using a coulter counter (Beckman Coulter, Miami, FL). Fragmented DNA and high molecular weight DNA were recovered from the attached and floating cell samples, as per the protocols provided with the kit. The DNA fragments were resuspended in the buffer provided with the kit and separated by standard agarose gel electrophoresis and stained with ethidium bromide for visualization of the DNA ladders. DNA markers of the appropriate size range (provided with the kit) were also run along with the samples. The gels were photographed at the end of the run, and the extent of fragmentation analyzed visually.

ELISA assay for measuring relative levels of apoptotic death in different cellular samples. The control and peptide-stimulated IEC cells that were additionally treated with camptothecin (as described in Treatment of HCT-116-C and HCT-116-AS cells with camptothecin for measuring apoptotic end points) were processed for quantitative measurement of cytoplasmic histone-associated DNA-fragments (mono- and oligonucleosomes) by using the cell death detection ELISA®. LUX kit (Roche Laboratories) as per the protocols provided with the kit. HCT-116-AS and HCT-116-C cells growing logarithmically were treated with camptothecin (as described in Treatment of HCT-116-C and HCT-116-AS cells with camptothecin for measuring apoptotic end points), and processed as per the protocols provided with the ELISA kit. The assay is based on the quantitative sandwich/enzyme-immu- noassay-principle and uses mouse monoclonal Abs against DNA and histones, respectively. The assay thus allows for the specific determination of mononucleosomes in the cell lysates, which was photometrically measured at 405 nm with the help of an ELISA reader (μQuant; Bio-Tek Instruments, Winooski, VT). Reagent blanks were subtracted from the cell sample readings, and the optical density readings in the peptide-stimulated vs. control cells are presented as a quantitative measure of apoptotic death in the various samples.

Relative levels ATP synthesis by oxidative phosphorylation in the mitochondria of cellular samples. The relative levels of ATP were measured in control and peptide-stimulated IEC cells with the help of a mitochondrial ATP assay (Roche Diagnostics) by using a luminous reagent kit. HCT-116-AS and HCT-116-C cells growing in normal growth medium containing 1% FCS and treated with camptothecin as described in Treatment of HCT-116-C and HCT-116-AS cells with camptothecin for measuring apoptotic end points were also used in these assays. A significant percentage of camptothecin treated HCT-116 cells became detached from the cell culture dishes and were observed as floating cell populations in the cell culture medium of these dishes. IEC cells, on the other hand, were less susceptible to detachment after camptothecin treatment, which may be due to the fact that IEC cells express insulin-like growth factors (36, 47) that may delay complete detachment of the cells. Therefore, at the end of the treatment, both floating and attached IEC cells were har- vested separately, and aliquots were counted by using a coulter counter (Beckman Coulter, Miami, FL). Fragmented DNA and high molecular weight DNA were recovered from the attached and floating cell samples, as per the protocols provided with the kit. The DNA fragments were resuspended in the buffer provided with the kit and separated by standard agarose gel electrophoresis and stained with ethidium bromide for visualization of the DNA ladders. DNA markers of the appropriate size range (provided with the kit) were also run along with the samples. The gels were photographed at the end of the run, and the extent of fragmentation analyzed visually.
were washed three times with HEPES-buffered Hank’s balanced salt solution containing 0.1% dextrose (wt/vol). The cells were pelleted in centrifuge tubes at 200 g for 10 min and washed with five volumes of 10% cold tricloroacetic acid (wt/vol) followed by sonication on ice for 5 min by using Fisher Sonic Dismembrator (model 300). The sonicate was centrifuged at 4°C at 10,000 g, and the extract was neutralized by 4 M KOH. The mixture was then again centrifuged for 30 min at 15,000 g at 4°C and the supernatant lyophilized and stored at −70°C for measuring ATP. On the day of the assay, the lyophilized supernatant was dissolved in 2 ml sterile water (pH 7.8), and the adenosine 5′-triphosphate bioluminescent kit (Cat. no. FL-AA; Sigma) was used for measuring relative levels of ATP in the various samples.

Analysis of Cox Vb promoter activity in HCT-116 cells by measuring relative levels of chloramphenicol acetyl transferase enzyme. The ML221–475bp human Cox Vb functional promoter fragment (3, 27) in chloramphenicol acetyl transferase (pCAT)-enhancer vector (Promega) (obtained from Dr. M. Lomax, University of Michigan) was transformed in DH5α cells and purified by using the QIAprep Maxprep Kit (QIAgen). HCT-116 and HCT-116-C cells were seeded into 60-mm tissue culture plates (2 × 10⁶ cells/plate) in normal growth medium containing 10% FCS. Logarithmically growing cells were transfected with 2 μg of Cox Vb promoter-CAT vector (ML221) by using lipofectin reagent (Life Technologies, Carlsland, CA) as per the protocols provided by the company. Control cells were transfected with the pCAT vector alone that did not contain the Cox Vb promoter fragment. Transiently transfected cells were assayed for relative levels of the CAT enzyme, 48 h after transfection as described below. The cells in culture to be assayed were washed three times with PBS followed by the addition of 400 μl reporter lysis buffer (Promega) at RT for 15 min. Lysed cells were scraped and the total contents of the cell culture dish transferred to a microcentrifuge tube, and the lysates heated at 60°C for 10 min to inactivate endogenous deacetylases. Heated lysates were centrifuged at 13,000 rpm for 5 min, and the supernatant containing the cellular extracts was transferred to a fresh tube. Relative levels of the CAT enzyme in the cellular extracts were then measured in a microcentrifuge tube containing 100 μl of the cell extract, 3 μl of the [14C]chloramphenicol (0.05 mCi/ml) (New England Nuclear Life Science Products), 5 μl of n-butyl-CoA (5 mg/ml), and distilled water to a final volume of 125 μl. Chloramphenicol acetyltransferase (Promega) was used as a positive control. Reaction tubes containing no cellular extract or chloramphenicol acetyltransferase were used as negative controls. Reaction tubes were incubated at 37°C for 3 h followed by centrifugation of the tubes at 13,000 rpm for 10 min at RT. Xylene (300 μl) was added to each tube and vortexed for 30 s. Tubes were once again centrifuged at 13,000 rpm for 5 min to separate the xylene phase from the aqueous phase. The upper xylene phase was carefully removed to a fresh tube and 100 μl of 0.25 M Tris·HCl (pH 8.0) added to the xylene extract. The xylene extract was once again separated from the aqueous phase and transferred to a scintillation vial. Scintillation fluid (5 ml) (Scintisafe Econo; Fisher Scientific) was added to the xylene extract in the scintillation vial, and radioactivity in the samples was measured with the help of a liquid scintillation counter (Tri Analytic, Brandon, FL). Cox Vb measured in each sample was converted to cpm (counts per minute) (based on the efficiency of the counter for 14C) and represented the levels of butyrylated chloramphenicol products in the samples. Counts in the negative control samples were deducted from all other samples, and the relative levels of n-butyrylated chloramphenicol products were determined for each sample. Relative levels of chloramphenicol thus measured represented the relative activity of Cox Vb promoter in the cells.

RESULTS

Effect of PG and G17 on relative levels of Cox Vb RNA and protein in IEC cells. In initial experiments, we examined the effects of 0.1–1.0 nM PG on the levels of Cox Vb RNA, because 0.1–1.0 nM rhPG was maximally effective in stimulating the growth of IEC cells in vitro (7, 51). In a few experiments, we additionally examined the relative potency of the completely processed gastrin peptide G17 at optimally effective doses of 0.1–1.0 nM, because G17 was less than half as effective as the full-length precursor molecule (PG) in stimulating the growth of IEC cells in vitro (7, 51). PG was maximally effective at 0.1 nM, resulting in a three- to fourfold increase in the steady-state levels of Cox Vb RNA in IEC cells. At high doses, PG was less effective but still caused a significant increase in the relative levels of Cox Vb RNA in IEC cells (Fig. 1A). G17 treatment also resulted in a significant increase in the steady-state levels of Cox Vb RNA in IEC cells by approximately two- to threefold at concentrations of 0.1–0.5 nM (Fig. 1B). The data in Fig. 1, A and B, confirmed that the completely processed form of gastrin (G17) was approximately one-half as effective as the full-length precursor molecule (PG) in stimulating an increase in the relative levels of Cox Vb RNA in IEC cells, which correlated with the relative potency of the two peptides on the growth of IEC cells in culture (7, 51).

Previously, we (65) reported that anti-Cox Vb-Abs (Abs) used in our studies were specific for the Cox Vb protein and detected Cox Vb in the Mt fraction but not in the cytosolic fractions, as expected. In the present studies, we therefore examined the relative levels of Cox Vb only in the Mt fraction of IEC cells in response to direct stimulation with the indicated gastrin-like peptides. In preliminary studies, we confirmed that the relative levels of Mt Cox 1 subunit are not changed at either the RNA or protein levels in response to PG or G17 stimulation in IEC cells (data not shown). We therefore used the Cox 1 protein as a loading control and corrected the Cox Vb densitometric readings with Cox 1 units in each sample. The Cox Vb readings for the control (nontreated) samples, after correction with the loading control (Cox 1), were arbitrarily assigned a value of 1.0 (Fig. 2). All other data for treated samples are presented as a factor of the control values (Fig. 2). As can be seen from Fig. 2A, PG was once again maximally effective at the low concentrations of 0.1–0.5 nM and increased the relative levels of Cox Vb protein in the Mt fraction of IEC-6 cells by approximately two- to threefold. In previous studies (51), we reported that both PG and G17 demonstrated biphasic dose-dependent effects on the growth of IEC cells in vitro in which the peptides were ineffective in stimulating the growth of IEC cells at pharmacological concentrations of >10 nM. In the present studies, we similarly observed that the peptides were ineffective in stimulating an increase in the levels of Cox Vb protein.
at concentrations higher than 10 nM. Representative data demonstrating negligible effects of 100 nM PG are presented in Fig. 2A. The molecular mechanisms responsible for the observed biphasic effects of the peptides are largely unknown, but several mechanisms have been postulated as discussed previously (51). G17 was once again 30–50% less effective than PG in stimulating an increase in the relative levels of Cox Vb protein in the Mt fraction of IEC cells at equivalent concentrations (Table 1). The relative potency of G17...
Table 1. Dose-dependent effects of PG and G17 on relative levels of mitochondrial Cox Vb protein and cytosolic cyt c levels in IEC-18 cells

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>0.1 nM</th>
<th>0.5 nM</th>
<th>1.0 nM</th>
<th>10.0 nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cox Vb</td>
<td>100.0</td>
<td>243 ± 45</td>
<td>160 ± 20</td>
<td>ND</td>
<td>194 ± 41</td>
</tr>
<tr>
<td>cyt c</td>
<td>100.0</td>
<td>29 ± 13</td>
<td>56 ± 18</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values for each parameter measured in control (nonpeptide treated) cells, were arbitrarily assigned a 100% value. All other values in PG- and G17-treated samples are presented as a percentage of the control values. Each value represents the mean ± SE of 4–12 observations from 2–6 separate experiments. The relative levels of mitochondrial Cox Vb protein and cytochrome c (cyt c) levels were measured by Western immunoblot analysis as described in MATERIALS AND METHODS. † In a few cases, only 1 experiment was conducted to confirm the dose-dependent effects, and in these cases, the average of 2–3 observations from a single experiment is presented, in which the intraexperimental variation was < 10–20%. PG, progastrin; Cox Vb, cytochrome c oxidase Vb; IEC, intestinal epithelial cells; ND = not done. *P < 0.05 vs control values.

and PG at the optimally effective dose of 0.1 nM from 3 separate experiments with IEC-18 cells is presented in Fig. 2B.

Effect of PG and G17 peptides on the relative levels of cyt c protein in the cytosol of IEC cells. Representative Western blot data from one of three experiments with IEC-6 cells demonstrating dose-dependent effects of rhPG on cytosolic cyt c levels are presented in Fig. 3A. The corresponding β-actin levels in these samples are also shown. Data from all the experiments are presented as bar graphs in which the densitometric readings for the cyt c bands in control (nontreated) samples were corrected for the corresponding β-actin readings and arbitrarily assigned a value of 1.0. Cyt c values from treated samples (after correction for loading) are presented as a fraction of the control values. At the optimally effective doses (0.1–1.0 nM), PG treatment significantly reduced the relative levels of cyt c protein in the cytosolic fraction of IEC cells by >50%. At the extremely low concentrations of 0.01 nM, PG was ineffective; PG was similarly ineffective at 0.01 nM concentrations in stimulating the growth of IEC cells (51). G17 was once again less effective than PG in reducing the relative levels of cyt c in the cytosolic fraction of IEC cells, especially at the dose of 1.0 nM (Fig. 3B, Table 1).

Assay for measuring relative levels of apoptotic death in different cellular samples. There were, however, some differences in the dose-dependent effects of PG on specific end points measured in the present study. Whereas PG was maximally effective at the low concentration of 0.1 nM in increasing the steady-state levels of Cox Vb RNA and protein (Figs. 1 and 2), PG was more effective at the higher concentration of 1 nM in reducing the relative levels of cyt c in the cytosolic fraction of IEC cells (Fig. 3). These differences in the dose-dependent effects of PG on the various end points suggest that PG at different concentrations may stimulate different intracellular pathways that may be mediating the changes in the observed end points; the latter possibility is being currently examined in our laboratory.
Effect of PG and G17 on the activation of caspases 9 and 3 in IEC cells. The relative levels of procaspase 9 and activated caspase 9 in the cytosolic fraction of camptothecin-treated IEC cells were measured as described in MATERIALS AND METHODS. rhPG treatment of IEC-6 cells resulted in a significant loss in the activation of caspase 9 in a dose-dependent manner in which PG was maximally effective at a dose of 1.0 nM PG (Fig. 4A). A shift in the dose-dependent efficacy of rhPG to the right of the curve was once again evident when examining the activation of caspase 9 compared with an increase in the levels of Cox Vb RNA and protein (Figs. 1, 2, and 4); the dose-dependent activation of caspases 9 and 3, however, resembled the dose-dependent increase in cytosolic cyt c levels (Figs. 3 and 4).

G17 was once again less effective than PG in reducing the activation of caspases 9 at equivalent concentrations (Fig. 4B). A significant effect of 1.0 nM PG on the loss of activation of caspase 9 was confirmed in IEC-18 cells in three separate experiments (Fig. 4C). At the maximally effective dose of 1.0 nM, PG significantly reduced the activation of caspase 3 by >50% in both IEC-6 and IEC-18 cells. Representative data from three experiments with IEC-18 cells are presented in Fig. 4D.

Fig. 4. Effect of rhPG and G17 on the activation of procaspases 9 and 3 in IEC cells. IEC-6 (A and B) and IEC-18 (C and D) cells were treated with the indicated doses of rhPG or G17 in the presence of camptothecin as described in MATERIALS AND METHODS. The control (0.0) and peptide-stimulated cells were processed for measuring relative levels of procaspases and activated caspases by Western blot analysis as described in MATERIALS AND METHODS. The autoradiographic data from representative experiments of several experiments are presented in A, top–D, top. The relative levels of procaspase 9 (P-C-9) and activated caspase 9 (A-C-9) in response to peptide stimulation are presented in A–C. The relative levels of procaspase 3 (P-C-3) and activated caspase 3 (A-C-3) in response to an optimally effective dose of PG are presented in D. The molecular mass of the eletrophoretically separated pro- and activated caspases (in terms of kDa) is indicated to the left of the autoradiographs. Data from 3 separate experiments are presented as bar graphs in A–D. The ratio of densitometric readings for the activated caspases/β-actin bands were determined, and the relative levels of activated caspases in the peptide-stimulated samples are presented in relation to the control values as described in the legend of Fig. 3. Each bar graph represents means ± SE of data from 3 separate experiments A–D. *P < 0.05 vs. control (Con) (0.0 peptide) values.
Effect of endogenous and exogenous PG on the apoptotic death of colon cancer and IEC cells in response to camptothecin. We previously reported a significant decrease in the surrogate markers of apoptosis in a representative PG-expressing human colon cancer cell line (HCT-116-C) compared with that in cells downregulated for the expression of gastrin gene (HCT-116-AS) (65). In the present studies, we have demonstrated for the first time a loss in the surrogate markers of apoptosis in IEC cells in a dose-dependent manner in response to stimulation with increasing concentrations of PG and G17. To examine if a loss in surrogate markers of apoptosis results in reducing the apoptotic death of colon cancer and IECs, we measured relative levels of apoptotic death qualitatively (DNA Ladder Assay) and quantitatively (ELISA assay), as described in MATERIALS AND METHODS.

DNA fragmentation (as detected by ethidium bromide staining of the DNA fragments), in response to the proapoptotic agent camptothecin, was surprisingly negligible in PG-expressing HCT-116-C cells, but was clearly visible in HCT-116-AS cells (downregulated for PG expression), in response to camptothecin treatment (Fig. 5A). This difference, however, was only observed in the floating cell populations and not in cells that remained attached to the plastic cell culture dishes, suggesting that colon cancer cells undergoing apoptotic death lose their ability to remain attached to the plastic surface of the cell culture dishes. A significant increase in the apoptotic death of HCT-116-AS cells was reflected by a significant increase in the total number of floating cells/culture dish for AS cells compared with that for HCT-116-C cells (Table 2). A significant increase in the apoptotic death of floating HCT-116-AS cells in response to camptothecin was further confirmed quantitatively by an ELISA assay (Table 3).

Unlike the HCT-116 cells, IEC cells for the most part (>90%) remained attached to the cell culture dishes even after camptothecin treatment. We therefore analyzed the total cell populations (attached and floating) of control and PG-stimulated IEC cells. An optimally effective dose of 1 nM PG was used in experiments with IEC-18 cells based on the results presented in Figs. 3 and 4. PG treatment resulted in significantly reducing apoptotic death of camptothecin-treated IEC cells when measured either by DNA ladder assay (Fig. 5B) or ELISA assay (Table 3). Because in PG-expressing colon cancer cells we only analyzed the floating cell populations, the inhibition of proapoptotic effects of camptothecin appear to be almost 100% (Fig. 5A). In the case of IEC cells, on the other hand, we had to examine both the attached and floating cells (for reasons described above), and hence PG treatment appeared to be less effective in reducing apoptotic death in response to camptothecin (Fig. 5B, Table 3). Thus the results obtained with the two cell lines are not strictly comparable but provide strong evidence that PG expression/treatment significantly reduces apoptotic response of cells treated with proapoptotic agents. In the absence of camptothecin treatment, we did not measure significant levels of apoptosis in both the HCT-116 and IEC cells under the subconfluent cell culture conditions used in the present studies.

Effect of PG on ATP levels in the mitochondria of target cells. Our studies so far have confirmed that endogenous PG (65) and exogenous PG at physiological concentrations of 0.1–1.0 nM (present studies), can significantly increase the steady-state levels of Cox Vb RNA and protein. The primary role of Cox Vb subunit is in increasing the efficiency of the Cox holoenzyme in oxidative phosphorylation and generation of ATP (9). We therefore examined whether the observed increase in Cox Vb levels in colon cancer cells and IEC cells in response to endogenous and exogenous PG, respectively, results in an increase in the levels of ATP in
Table 2. Percent distribution of cells that are either attached or floating in the medium in response to camptothecin treatment in HCT-116-C and HCT-116-AS cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>PG*</th>
<th>Total No. of Cells (×10^6)</th>
<th>Attached Distribution</th>
<th>Floating Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT-116-C</td>
<td>+</td>
<td>1.0 ± 0.025</td>
<td>0.75 ± 0.02</td>
<td>0.25 ± 0.004</td>
</tr>
<tr>
<td>HCT-116-AS</td>
<td>–</td>
<td>0.75 ± 0.052</td>
<td>0.25 ± 0.06</td>
<td>0.50 ± 0.04</td>
</tr>
</tbody>
</table>

Each data point represents the mean ± SE value from 6 separate 35-mm cell culture dishes from 2 separate experiments. *HCT-116-C cells express PG while HCT-116-AS cells are downregulated for the expression of endogenous PG (54).

Table 3. Relative levels of DNA fragmentation in the floating cell populations of HCT-116 cells and in total cell populations of IEC cells, in response to camptothecin, in the presence or absence of PG expression/treatment (as measured by an ELISA assay using the Cell Death Detection Assay Kit)

<table>
<thead>
<tr>
<th>Cells</th>
<th>PG</th>
<th>OD Readings at 405 nm/5 × 10^4 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT-116-C</td>
<td>+</td>
<td>0.08 ± 0.001</td>
</tr>
<tr>
<td>HCT-116-AS</td>
<td>–</td>
<td>0.68 ± 0.009</td>
</tr>
<tr>
<td>IEC-18</td>
<td>+</td>
<td>0.21 ± 0.003</td>
</tr>
<tr>
<td>IEC-18</td>
<td>–</td>
<td>0.53 ± 0.004</td>
</tr>
</tbody>
</table>

Each data point represents the means ± SE value from 6 separate 35-mm cell culture dishes from 2 experiments. In the absence of camptothecin, DNA fragmentation was minimal in HCT-116 and IEC cells under the conditions of the current study (in which cells were examined at subconfluent densities). IEC cells at subconfluent densities are less responsive to PG (as previously reported (7, 51)), and hence all experiments were conducted at subconfluent cellular densities in the current studies. *HCT-116-C cells express PG while HCT-116-AS cells are downregulated for the expression of endogenous PG (54). IEC-18 cells were treated with (+) or without (−) an optimal concentration of PG (1.0 nM) as described in MATERIALS AND METHODS. OD, optical density.

DISCUSSION

In the present studies, we report for the first time that the full-length precursor PG1–80 peptide (rhPG) directly and significantly upregulates the steady-state levels of Cox Vb at the RNA and protein levels, which correlated with the number of molecules of ATP in the mitochondria of IECs in vitro. We further observed that the number of ATP molecules in a representative human colon cancer cell line (HCT-116-C, which express significant levels of endogenous PG (54)) was approximately four- to fivefold higher compared with that in cells downregulated for PG expression (HCT-116-AS).

Cox is the terminal enzyme complex of the electron transfer chain and oxidizes cyt c with transfer of electrons to generate ATP by oxidative phosphorylation (9). Mammalian Cox is composed of three major catalytic subunits (Cox I–III), which are encoded by the Mt genome (9). In addition, at least 10 smaller regulatory subunits, which are encoded by the nuclear genome, form part of the complex eukaryotic Cox enzyme (10, 39). The nuclear subunits are synthesized as precursor proteins, transported into the mitochondria and processed, and the complex is assembled (10, 39). All these cells. The relative levels of ATP were measured in the cells as described in MATERIALS AND METHODS. The total number of ATP molecules/cell were four- to fivefold higher in HCT-116-C cells compared with that in HCT-116-AS cells (Fig. 6A). Similarly, the total number of ATP molecules in an equal number of IEC-18 cells was significantly increased by two- to fourfold in response to 0.1–1 nM PG (Fig. 6B). At a dose of 0.1 nM, PG was most effective in increasing the levels of ATP in the IEC-18 cells. Importantly, the dose-dependent effects of PG in increasing the relative levels of Cox Vb RNA and protein (Figs. 1 and 2) correlated closely with the possibility that PG expression by HCT-116-C cells may have directly or indirectly resulted in significantly increasing the promoter activity of the Cox Vb gene; downregulation of PG expression in HCT-116-AS cells may have resulted in the loss of transcriptional activity of the Cox Vb promoter in the AS cells. The rat Cox Vb promoter sequence is significantly different from that of the human promoter (18). At the present time, we do not have a promoter-reporter construct for the rat Cox Vb gene and have not examined transcriptional activation of Cox Vb promoter in IEC cells in response to exogenous PG, as yet.
though the specific functional role for many of the nuclear subunits has yet to be established, biochemical analysis of the Cox mutants in yeast indicated that the nuclear subunits are critically required for Cox function or assembly (10, 39). There are significant species and tissue differences in the relative expression of the nuclear subunits (5, 60). The nuclear subunits are differentially regulated by environmental and developmental signals, which allows the tissues to adjust to different energy demands (8; reviewed in Ref. 39).

The gene for Cox Vb (Cox5B) is located on chromosome 2 (3), and oxygen regulates expression of the V isoforms in yeast (2). The Va isoform is expressed under aerobic (O$_2$ > 0.5 μM) conditions, and the Vb isoform is expressed under anaerobic (O$_2$ < 0.5 μM) conditions (2) in which the Vb isoform has a higher turnover rate and a higher intramolecular transfer rate than the Va isoform. Isoforms of Cox V significantly affect the binuclear reaction center around the catalytic subunits I and II and alter the kinetics of interaction with the isoforms of cyt c (2). The fact that Cox Vb is specifically upregulated under anaerobic conditions of low oxygen tension is especially relevant to tumorigenesis in which hypoxia within the tumors may provide the necessary feedback for specific elevation of Cox Vb protein in cancer cells. Endogenous or exogenous growth factors such as PG may provide yet another important mechanism for elevating Cox Vb levels resulting in increasing the catalytic efficiency of the holoenzyme and increased generation of ATP. Our present studies strongly support the latter possibility, because ATP levels were significantly upregulated in

![Fig. 6](http://ajpgi.physiology.org/)

**Fig. 6.** A: generation of ATP in the mitochondria of HCT-116-C (C) and HCT-116-AS (AS) cells as measured in a bioassay. The 50 × 10⁶ cells in culture were processed for measuring relative levels of ATP in a bioassay by using the adenosine triphosphate bioluminescent assay kit (Sigma) as described in MATERIALS AND METHODS. Data in each bar graph represent observations from 3 separate experiments. *P < 0.05 vs. AS values. B: generation of ATP in the mitochondria of control and PG-treated IEC-18 cells as measured in a bioassay. Cells in culture were treated with or without PG and processed for measuring ATP concentrations in a bioassay, as described in MATERIALS AND METHODS. Concentration of ATP measured in mitochondria of control (non-PG-treated) cells was arbitrarily assigned a value of 1.0. Relative levels of ATP in PG-treated cells are shown in relation to that measured in control (0.0) cells. Data in each bar graph represent means ± SE of 3 observations from 3 separate experiments. *P < 0.05 vs. control values.

![Fig. 7](http://ajpgi.physiology.org/)

**Fig. 7.** CoxVb promoter activity in HCT-116-C and HCT-116-AS cells. Cells were transiently transfected with either the control or the hCoxVb promoter-chloramphenicol acetyl transferase (CAT)-expressing vector and CAT activity measured in these cells after 48 h by using the CAT Enzyme Assay System Z-1000 (Promega), as described in MATERIALS AND METHODS. The relative levels of CAT enzyme measured in the HCT-116-C and HCT-116-AS cells are shown as a measure of Cox Vb promoter activity in A. Cells transfected with the control vector demonstrated negligible CAT enzyme activity. Data in A are presented as a percent in B, whereas the CAT activity measured in HCT-116-C cells was arbitrarily assigned a 100% value. Data represent mean values from 3 separate experiments. *P < 0.05 vs. HCT-116-C values.
response to endogenous and exogenous PG in human colon cancer cells and nontransformed IECs, respectively. A significant increase in ATP levels in response to PG, in the target cells, is likely to play an important role in increasing the energy demands of the target cells induced to grow more rapidly in response to endogenous (54) and exogenous PG (51). The latter concept is further supported by the finding that the hormone melatonin significantly increases the activity of several Mt respiratory enzymes including Cox, resulting in an increased production of ATP in cells (31) known to be responsive to growth effects of the hormone.

Significant changes in the expression of specific subunits of the Mt Cox holoenzyme in cancer vs. benign/normal cells from various tissues have been reported by using the method of differential display (6, 45, 62). The Cox Vlc subunit was specifically upregulated in human prostate carcinoma (62), whereas Cox II (45) and Cox Va (6) was reported to be upregulated in breast carcinoma specimens, suggesting that tumors originating from different tissues may upregulate specific subunits of the Cox holoenzyme, resulting in increased Cox catalytic activity. By using the method of differential display, we discovered that Cox Vb subunit was specifically and significantly upregulated in gastrin-expressing human colon cancer cells, compared with that in cells downregulated for the gastrin gene expression (65). Importantly, upregulation of Cox Vb RNA and protein correlated with the level of gastrin gene expression by human colon cancer cells (65). Our present studies further suggest that the upregulation in Cox Vb RNA and protein may be a specific response to PG stimulation in normal and cancerous target cells. Cox II expression similarly appears to be regulated by herregulin β1 in mammary epithelial cells via ErbB2 (59). Raf-1, a cytoplasmic Ser/Thr protein kinase that plays an important role in mitogen-activated response of several hormones, was shown to upregulate Cox II expression (37), providing a mechanistic pathway for the observed effects.

Because upregulation in Cox Vb RNA levels correlated with an upregulation in Cox Vb protein levels in response to endogenous and exogenous PG (65, present studies), it appeared likely that PG stimulation of target cells may result in directly or indirectly upregulating the activation of Cox Vb expression at the transcriptional level. To examine this possibility, we examined the activation of the human Cox Vb promoter by using transient transfection assays with the Cox Vb promoter-CAT plasmid and measured a two- to threefold difference in the hCox Vb promoter activity in PG-expressing HCT-116-C cells compared with that in HCT-116-AS cells (that were downregulated for gastrin gene expression). The possibility that growth-promoting hormones/signaling molecules can directly or indirectly regulate the expression of specific subunits of the Cox holoenzyme at the transcriptional level, receives support from several recent studies (37, 59, 64). Cox VIIa was identified as an estrogen-responsive gene by using the genomic binding site cloning method (64), suggesting that estrogens directly regulate expression of specific subunits of the Cox holoenzyme. Herregulin β1 (59) and the signaling molecule Raf-1 (37) were reported to regulate specific subunits of the Cox holoenzyme, whereas our studies suggest that gastrins specifically upregulate Cox Vb gene expression.

Augenlicht and coworkers (16) hypothesized that mitochondria play a pivotal role in coordinating proliferation and apoptosis in rapidly renewing tissues such as colonic mucosa. We recently reported that Cox Vb in colon cancer cells can be immunoprecipitated with anti-cyt c Ab, suggesting that the Cox Vb subunit is physically associated with cyt c within the holoenzyme (65). Specific binding sites for the Cox II subunit are located on cyt c (8, 9). It is however, not known if Cox Vb has specific binding sites for cyt c. By virtue of the fact that Cox Vb is involved in regulating the binding affinity of cyt c for the catalytic subunits I and II (9), and the possibility that Cox Vb is physically associated with cyt c (65), the importance of upregulation of Cox Vb in response to PG highlights a possible role of Cox Vb in the survival of gastrin-expressing gastrin-responsive cells.

Recent reports further confirm that Cox activity may be related to the apoptotic potential of cells (11, 28, 33–35, 41, 46, 67). Cell death was induced in hematopoietic cells by downregulating Mt respiratory enzymes (46). Treatment of human leukemia cells with adriamycin resulted in loss of expression of Cox II and IV genes and promotion of apoptosis (35). Virulent Mycobacterium tuberculosis caused apoptotic death of macrophages by apparently downregulating the Cox VIIc subunit (41). These reports thus suggest that downregulation of one or more specific Cox subunits somehow translates into initiation of apoptotic events and vice versa.

Our studies provide further support for the concept that upregulation of one or more Cox subunits can somehow translate into protection of the cells from apoptotic effects of proapoptotic agents. In response to optimal concentrations of exogenous PG (0.1–1.0 nM), we measured a significant increase in the levels of Cox Vb, associated with a significant decrease in the release of cyt c from the mitochondria into the cytosol of the cells, which apparently resulted in significantly reducing activation of caspases 9 and 3 in response to proapoptotic agents such as camptothecin.

The possibility that upregulation of Cox subunits play an antia apoptotic role in many cells receives further support from the findings that Bax-induced apoptotic effects on yeast cells were believed to be directly related to a decrease in the amount of Cox holoenzyme and a dramatic increase in the release of cyt c (30). Treatment of Chinese hamster ovary cells with cAMP-elevating agents significantly inhibited Cox activity with a concomitant release of cyt c into the cytosol (66), almost mimicking the results of the our studies. Importantly, the regulatory subunit of protein kinase A interacted with Cox Vb in regulating Cox activity and cyt c release in Chinese hamster ovary cells with ele-
vated cAMP (66). Thus Cox Vb may play a critical role in specific cell types in response to endogenous/exogenous PG in regulating Cox activity, which can then result in the differential release of cyt c into the cytosol.

Release of cyt c is a requirement for initiating apoptosis in response to many, but not all, apoptotic stimuli (25, 26). Both caspase 9 and 3 represent critical and penultimate molecules that are required for initiation of apoptosis in response to cyt c release (58). However, several other pathways impinge on this activation process in which cyt c released into the cytosol plays an important permissive role that sensitizes the cells to potent apoptotic stimuli (22, 43, 65). Several potent apoptotic stimuli have been described in the literature in recent years (38a), including topoisomerase-1 inhibitors (such as camptothecin) (21, 42) and p53 (23, 43). In our studies, we observed that absence of endogenous/exogenous PG caused downregulation of Cox Vb expression, which perhaps resulted in significantly increasing the release of cyt c, triggering an increased activation of caspases 9 and 3 in response to camptothecin. It remains to be seen whether the p53-mediated pathway is somehow connected to the observed effects of PG on human colon cancer and IEC cells.

Just as we observed a significant increase in the sensitivity of the normal and cancerous IECs to proapoptotic stimuli in the absence of PG, overexpression of c-Myc similarly sensitized the cells to proapoptotic stimuli via release of Mt cyt c into the cytosol, which was blocked by the survival factor insulin-like growth factor I (22). The c-Myc-initiated apoptosis was not mediated via the p53 or CD95/Fas signaling pathway (22). It was concluded that although c-Myc promotes apoptosis by releasing cyt c, its ability to activate apoptosis was critically dependent on other signals (22). We similarly measured a significant increase in the activation of caspases 9 and 3 in nongastrin-stimulated IEC cells in response to proapoptotic stimuli, confirming the notion that pathways mediating the increased release of cyt c in response to either loss of gastrin stimulation (65, present studies) or overexpression of c-Myc in growth factor-starved fibroblasts (22), results only in sensitizing the cells to more potent proapoptotic stimuli, thus making the cells less able to survive under hostile conditions.

Because activation of caspase 3 is known to cause fragmentation of the nuclear DNA that results in the apoptotic death of the cells (23, 26, 43, 58), we also examined whether nuclear DNA fragmentation was significantly reduced in colon cancer and IEC cells in response to endogenous and exogenous PG, respectively. Our results confirmed that histone-associated DNA fragments were significantly increased in HCT-116-AS cells compared with that in the gastrin-expressing HCT-116-C cells, in response to camptothecin. PG stimulation of IEC cells similarly resulted in significantly reducing the DNA fragmentation of the cells in response to camptothecin, compared with non-PG-stimulated IEC cells, once again confirming antiapoptotic effects of exogenous PG. In previous studies, we and others (reviewed in Refs. 48 and 52) have demonstrated significant mitogenic effects of gastrin-like peptides on IEC cells, suggesting that several pathways, including mitogenic effects, antiapoptotic effects, and an upregulation of ATP may contribute to the reported growth effects of PG and gastrin-like peptides on normal and cancerous intestinal epithelial cells.

DISCLOSURES

This work was supported by National Cancer Institute CA-97959 and CA-72992 to Pamina Singh.

REFERENCES


22. Hollande F, Choquet A, Blanc EM, Lee DJ, Bali JP, and Baldwin GS. Involvement of phosphatidylinositol 3-kinase and...


