Autocrine Gastrins in Colon Cancer Cells Up-regulate Cytochrome c Oxidase Vb and Down-regulate Efflux of Cytochrome c and Activation of Caspase-3*

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Suppressions of the gastrin gene in human colon cancer cells by stably expressing antisense (AS) gastrin RNA results in significant growth suppression of AS cells. To understand mechanisms mediating the growth effects of autocrine gastrins, differential expression of transcripts by AS and control (C) clones of a representative cell line (HCT-116) was analyzed to identify target genes of autocrine gastrins. Six differentially expressed transcripts were confirmed and sequenced. Of these, the RNA and protein levels of cytochrome c oxidase (COX) Vb were significantly higher in C versus AS cells. The expression of COX Vb by colon cancer cells was proportional to the expression of gastrin. Higher levels of COX Vb coprecipitated with cytochrome c in the mitochondria of C versus AS cells. Treatment of mitochondria with digitonin resulted in a 2-fold higher release of cytochrome c from AS versus C mitochondria. As a corollary, the cytosolic levels of cytochrome c were significantly higher in AS versus C cells, which correlated with ~2- and ~3-fold higher activation of caspase-9 and -3, respectively, in AS versus C cells in response to camptothecin. Thus, autocrine gastrins may support growth/survival of cells by up-regulating COX Vb, which may decrease the sensitivity of the cancer cells to apoptotic stimuli by increasing retention of cytochrome c in mitochondria.

Gastrins are peptide hormones that play an important role in acid secretion from the stomach (1). Circulating gastrins play an equally important role in the growth of gastrointestinal mucosa (2). Recent studies with gastrin gene knockout mice confirmed an important role of circulating gastrins in the growth of colonic mucosa (3). Growth-promoting effects of amidated gastrins on mouse and human colon cancer cells have also been demonstrated in vitro and in vivo (4–8). Colon cancer cells additionally express the gastrin gene (9–11). We and others demonstrated that down-regulation of gastrin gene expression significantly reduces the proliferative and tumorigenic potential of human colon cancer cell lines (12) and a mouse colonic cell line (13), indicating a significant growth-promoting role of autocrine gastrins in colon cancer cells. Although amidated gastrins are the major forms of gastrins present in circulation, non-amidated gastrins (progastrin and Gly-extended gastrin) are the predominant forms of gastrins expressed by colon cancer cells (14–16). We now know that non-amidated gastrins are biologically active and exert significant growth-promoting effects on several cell types, including human and mouse colon cancer cells (13, 14, 17, 18). In experiments with transgenic mice that overexpress progastrin or Gly-extended gastrin, a growth-promoting role of non-amidated gastrins for colonic mucosa was reported (19, 20). Thus, studies with colon cancer cells and transgenic mice confirmed that non-amidated gastrins exert significant growth effects on both normal and cancerous colonic mucosas. Our recent studies indicated that non-amidated gastrins can also function as co-carcinogens in chemical colon carcinogenesis; amidated gastrins were less effective as co-carcinogens for unknown reasons (21–23).

Several receptor subtypes and intracellular mechanisms are believed to mediate the growth-promoting effects of endocrine (circulating) gastrins (reviewed in Ref. 23). However, intracellular mechanisms mediating the growth-promoting effects of autocrine gastrins (which are largely composed of non-amidated gastrins) are as yet unknown (23). The goal of this study was to identify target genes/pathways that may mediating the growth-promoting effects of autocrine gastrins on human colon cancer cells. To achieve this goal, we used a representative human colon cancer cell line (HCT-116) that is dependent on autocrine gastrins for maintaining its growth in vitro and in vivo (12, 24). In a previous study, we demonstrated that overexpression of antisense (AS) gastrin significantly suppresses growth of AS-HCT-116 cells compared with that of control (C) HCT-116 cells (12). We postulated that differential expression of transcripts by AS versus C clones of HCT-116 cells will allow us to identify target genes/pathways that are perhaps regulated directly or indirectly by autocrine gastrins. Using the method of differential display, we identified at least 35 transcripts that were differentially expressed; six were confirmed by reverse slot blot analysis. Sequence analysis confirmed one of the transcripts to be a well known gene, mitochondrial (MT) cytochrome c oxidase (COX) Vb. In this study, we have investigated the significance of this rather unexpected finding. Our results suggest that COX Vb may be one of the target genes of autocrine gastrins in human colon cancer cells, the RNA levels of which correlate with gastrin expression. A novel observation made was that the suppression of endogenous gastrin caused

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†The abbreviations used are: AS, antisense; C, control; MT, mitochondrial/mitochondria; COX, cytochrome c oxidase; cyt c, cytochrome c; Ab, antibody; PCR, polymerase chain reaction.
down-regulation of COX Vb, resulting in efflux of cytochrome c (cyt c) from MT and activation of caspase-9 and -3. Our findings suggest that endogenous growth factors such as autocrine gastrins can enhance cancer cell growth via up-regulation of COX Vb, which may not only support the higher energy requirements of cancer cells, but may also indirectly play a role in the survival of the cancer cells due to its association with cyt c.

**EXPERIMENTAL PROCEDURES**

Materials—Aprotinin, benazemidine, digitonin, camptothecin, mercaptoethanol, phenylmethylsulfonyl fluoride, sodium orthovanadate, EDTA, HEPES, and TTA were obtained from Sigma. Cyt c was obtained from Pharmingen (San Diego, CA). Polyclonal anti-cyt c antisera (Abs) (Santa Cruz Biotechnology, Santa Cruz, CA) and UV-cross-linked at 254 nm using a UCV-515 were obtained from BIORAD Research Labs Inc. (Plymouth Meeting, PA). Monoclonal anti-cyt c Abs (65981A) were obtained from Pharmingen (San Diego, CA). Polycononal anti-cyt c Abs (sc-7159) and protein A/G Plus-agarose beads were procured from Santa Cruz Biotechnology (Santa Cruz, CA). Anchored oligo(dT) primers and arbitrary primers were obtained from GenHunter Corp. (Nashville, TN). [α-32P]dATP (3000 Ci/mmol) and [α-32P]dCTP (3000 Ci/mmol) were obtained from ICN (Costa Mesa, CA). The random primer DNA labeling kit was obtained from Life Technologies, Inc. The PCR 2.1-Topo plasmid and TA cloning kit were obtained from Invitrogen (Carlsbad, CA). Colonies of the human colon carcinoma cell lines Colo-205, Colo-320, HCT-116, HT-29, DLD-1, and CaCo-2 were obtained and maintained in culture as described previously (12, 25). AS and C clones of HCT-116 cells, which overexpress either the antisense gastrin RNA or the control vector, respectively, were generated as described previously (12). We have previously reported the growth and gastrin expression characteristics of various AS and C clones of HCT-116 cells (12). For the present studies, we chose a representative antisense clone (AS-2) that demonstrated significant suppression of endogenous gastrin expression and negligible secretion of gastrin gene products in the conditioned medium of the cells (12). A representative control clone (C-2) that demonstrated gastrin expression and growth characteristics very similar to those of the wild-type, non-transfected cells was used. The antisense and control clones were maintained in hygromycin-containing growth medium as described previously (12).

**Identification of Differentially Expressed Transcripts by AS-HCT-116 and C-HCT-116 Cells**—Total cellular RNA was isolated from AS-HCT-116 and C-HCT-116 cells using the TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) following the manufacturer’s instructions. RNA was cleared from DNA using a Message Clean kit (GenHunter Corp.). The RNA was reverse-transcribed using different 1-base-anchored H-T7-M primers (where M may be A, G, or C; provided by GenHunter Corp.) at 65 °C for 5 min, at 37 °C for 60 min, and at 75 °C for 5 min. Ten min after incubation at 37 °C, Moloney murine leukemia virus reverse transcriptase (100 units) was added, and incubation was continued for another 50 min. The reverse-transcribed cDNA samples were PCR-amplified using H-T7-M primers in conjunction with eight different primers (Ap1–Ap8; GenHunter Corp.) and [α-32P]dATP following the manufacturers’ instructions. The PCR products were separated by electrophoresis on 6% denaturing polyacrylamide gels. The bands that were found to be differentially expressed in AS-HCT-116 and C-HCT-116 cells were cut out of the gel, eluted, and reamplified by PCR using the above H-T7-M and Ap1–Ap8 primers, and subjected to reverse transcription—slot blot analysis.

**Reverse Blot Analysis**—Total cellular RNA was prepared from AS and C cells as described above. Ten μg of RNA from AS and C cells were reverse-transcribed into 32P-labeled cDNA using 5 μl of [α-32P]dCTP (50 μCi) in the reaction buffer. Reverse transcription was carried out as described previously (26), and a quick-spin column was used to remove the unincorporated [32P]dCTP. Thirty μl of the PCR products were purified and prepared for blotting. The PCR products were boiled for 5 min with 5 μl of 2 N NaOH to denature the DNA, followed by neutralization with 5 μl of 3 N sodium acetate (pH 5.0) in a total volume of 105 μl of distilled H2O. Fifty μl of each cDNA sample thus prepared were slot-blotted in duplicate on nylon membranes (Micron Separations, Westborough, MA) and UV-cross-linked at 254 nm using a UVC-515 ultraviolet multi-linker, and the membrane was rinsed in 6× SSC buffer buffer (pH 6.0) for 15 min, followed by a 5-min wash in 2× SSC buffer, then incubation with the radiolabeled cDNA probes, prepared from the RNA samples (as described above), for 2 h using Rapid-Hyb buffer (Amerham Pharmacia Biotech) following the manufacturer’s instructions.

The confirmed cDNAs were cloned into PCR-II plasmids using the TA cloning kit and subjected to nucleotide sequencing using an automated DNA Sequencer (Applied Biosystems, Foster City, CA) as described previously (26). The sequences were screened against the cDNA database using the GeneTool software program (Life Science Software Resources, Long Lake, MN).

**Northern Blot Analysis**—Total cellular RNA was isolated from CaCo-2, Colo-205, Colo-320, DLD-1, AS-HCT-116, and HT-29 cells using TRI reagent. Equal amounts of RNA (20 μg/lane) were separated on 2.2× formaldehyde-containing 1.2% agarose gel and transferred to nylon membranes as described previously (12, 25, 26). The confirmed cDNA fragments were subcloned into pcRII vectors, restriction-digested, purified, and used as probes for Northern blot analysis following our published procedures (25). Briefly, the cDNA fragments were labeled with [α-32P]dCTP using the random primer DNA labeling kit and hybridized with the RNA blots using Rapid-Hyb buffer according to the supplier’s instructions.

**Fractionation of MT and Cytosolic Fractions from AS and C Cells and Treatment with Digitonin—**Subconfluent AS-HCT-116 and C-HCT-116 cells cultured in complete growth medium containing 10% fetal calf serum (12) were washed twice with cold phosphate-buffered saline and lysed in buffer A (20 mM HEPES (pH 8.0), 1.0 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 mM benzamidine, and 0.2 mM sodium orthovanadate) for 30 min on ice at a concentration of 107 cells/ml of lysis buffer A. The cell lysates were cleared by centrifugation and subjected to digitonin dehydration using a Dounce homogenizer B pestle. The cell lysates were centrifuged at 8000 rpm for 30 min at 4 °C in a Beckman CS-15R centrifuge. The cell pellets were discarded, and the supernatant was subjected to another centrifugation at 20,000 rpm for 30 min at 4 °C. The supernatant and pellets were collected. At this stage, the supernatant was further centrifuged at 40,000 rpm for 1 h at 4 °C. The resulting supernatant was labeled the cytosolic fraction and used as such. The pellet from the 13,000 rpm centrifugation was resuspended in buffer A and recentrifuged at 13,000 rpm for 30 min at 4 °C. The resulting supernatant was discarded, and the pellet containing the MT fraction was either treated with digitonin or lysed further for protein extraction as described below. Protein concentrations in the cytosolic and MT fractions were determined using BCA reagent (Pierce), and aliquots containing 50 μg/tube were stored at −70 °C.

**Preparation of Protein Extracts from MT Fractions—**For Western blot experiments, the MT fractions were lysed for preparation of protein extracts by suspending the MT pellets (prepared as described above) in buffer B (10 mM Tris–Cl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 10 μg/ml aprotenin, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM EDTA, and 0.1% SDS) for 30 min on ice. To both 10 μg of proteins from AS-HCT-116 and C-HCT-116 cells/100 μl of lysis buffer B were used. The lysate was centrifuged at 4 °C for 30 min at 13,000 rpm in a microcentrifuge, and the supernatant was labeled as the MT protein extract. The protein concentrations in the MT extracts were measured as described above, and aliquots were stored at −70 °C.

**Western Blot Analysis of MT Protein Extracts and Cytosolic Fractions**—Cytosolic fractions and MT extracts prepared as described above were used in immunoprecipitation experiments and for Western immunoblotting with specific Abs. Equal amounts of protein (50 μg) from the cytosolic and MT extracts were separated by electrophoresis on 10% SDS and 15% polyacrylamide gels following our published procedures (25). The protein was transferred electrophoretically to a nitrocellulose membrane (Hybond, Amersham Pharmacia Biotech) and blocked in 10 mM Tris–Cl (pH 8.0) containing 150 μM NaCl, 0.1% Tween 20, and 5% (w/v) nonfat dry milk as described previously (25). Complete transfer of proteins was confirmed by Coomassie Blue staining of the gels following our published procedures (25). To confirm equal loading of the proteins, duplicate sets of gels were stained with Coomassie Blue, and the relative concentration of proteins, across the lanes, was analyzed densitometrically (as described below). In all cases, the majority of the protein bands, separated by 15% polyacrylamide gel electrophoresis, were present in equal amounts in the AS and C samples (data not shown). The membranes were subjected to Western immunoblot analysis using the specific primary Abs (anti-cyt c Ab, 1 μg/ml; and anti-COX Vb Ab, 3 μg/ml), followed by incubation with the appropriate peroxidase-conjugated Ab (1:2000 dilution) according to our published procedures (25). The ABC peroxidase-conjugated Ab (Dako) was used for COX Vb (30, 31). All the Abs used in these studies have been reported. The antigen-Ab complexes were detected using a chemiluminescence reagent kit (Amerham Pharmacia Biotech). The relative density of the bands was densitometrically analyzed with the Documentation @ Analysis system (Model AlphalImager™ 2000, Alpha Innotech Corp., San Leandro, CA). In a few experiments, the MT and cytosolic protein extracts were subjected to immu
noprecipitation with specific Abs as described below. Immunoprecipitation—Equal amounts of protein (100 μg; from either cytosolic or MT extracts) were incubated with anti-cyt c Ab (2 μg/ml) overnight at 4 °C with gentle rocking using the Red Rocker (Hoefer Scientific Instruments, San Francisco, CA). Protein A/G Plus-agarose beads (40 μl) were added to each tube, and incubation was continued for another 2 h at 4 °C with gentle rocking. The beads were pelleted by centrifugation at 4000 rpm for 5 min and washed four times with buffer A and once with phosphate-buffered saline. The pellets were then boiled in 40 μl of SDS-polyacrylamide gel electrophoresis samples for 5 min, followed by centrifugation at 4000 rpm. The samples were loaded onto SDS-polyacrylamide gel and processed for gel electrophoresis and Western blot analysis as described above.

Treatment of AS-HCT-116 and C-HCT-116 Cells with Pro-apoptotic Agents (Camptothecin, Ceramide-2, and Menadione) and Analysis of Caspase-3 and -9 Activities—Camptothecin (an extract of the Chinese tree Camptotheca acuminata) is a potent inhibitor of topoisomerase-1, a molecule required for DNA synthesis (32). Camptothecin has been shown to induce apoptosis in a dose-dependent manner in vitro (33) and is used generally for inducing apoptosis (34). Ceramide functions as a second messenger in the induction of apoptosis by tumor necrosis factor-α, Fas ligand, and other agents (35). The specific mechanisms mediating the apoptotic effects of ceramides appear to involve ceramide-activated serine/threonine protein kinase and a cytosolic ceramide-activating sphosphoprotein phosphatase (36). Menadione is a known potent oxidant and causes depolarization of mitochondrial membranes, which is believed to result in the release of cytochrome c and the initiation of apoptosis. Subconfluent AS-HCT-116 and C-HCT-116 cells growing logarithmically in 60-mm cell culture plates in complete growth medium containing 10% fetal calf serum were treated with 5 μM camptothecin in <0.1 mM MeSO, with 50 μM C-2-ceramide, or with 50 μM menadione for 5 h at 37 °C in 5% CO2 incubators. Control cells were treated with an equivalent concentration of MeSO. At the end of the treatment, cells were washed twice with ice-cold phosphate-buffered saline, collected by scraping with a rubber policeman as described previously (25), and subjected to fractionation for cytosolic preparation as described above. Equal amounts of cytosolic protein (50 μg) from the MT fraction from the soluble fraction at 13,000 rpm for 30 min at 4 °C. The supernatant, representing extramitochondrial membrane, was collected and subjected to Western blot analysis with specific Abs against cyt c as described above.

RESULTS

To identify differences in RNA expression by colon cancer cells that were expressing high versus low levels of gastrin, we chose C and AS clones of HCT-116 cells for reasons described under the Introduction. Differential expression of RNA transcripts by a representative antisense clone (AS-2) and a representative control clone (C-2) was determined as described under “Experimental Procedures.” Thirty-five transcripts were identified as being differentially expressed in the C and AS clones. All 35 cDNA bands were further amplified by PCR, subcloned, and subjected to reverse slot blot analysis. Confirmation of six bands was confirmed by reverse slot blot analysis; the differential display of the six positive bands is shown in Fig. 1. The cDNAs thus positively confirmed were subjected to sequencing as described under “Experimental Procedures,” and the nucleotide sequences were screened against the cDNA database from GenBank™ using the BLAST program and GeneTool software. Four cDNAs were for known genes, Bruton's tyrosine kinase (BTK), high mobility group 2 protein (HMG-2), Y12F7F12, and COX Vb; and two were for unknown genes (Fig. 1). BTK, HMG-2, COX Vb, and an unknown gene were down-regulated in antisense compared with control cells. In contrast, Y12F7F12 and an unknown gene were significantly up-regulated in AS versus C cells (Fig. 1).

Of the four known genes, the biological functions of MT COX are well described. COX is the terminal complex of the respiratory chain and plays an important role in the biosynthesis of ATP (31, 38). It consists of 13 subunits, the larger components (I–III) of which are encoded by the MT DNA, and the rest are smaller in size and are encoded by the genome in the nucleus (38, 40). Although the larger subunits (I–III) are involved in the catalytic activity of COX, the smaller subunits are important in its regulation (38). COX Vb, one of the smaller subunits of the COX complex, has been reported to be important in the regulation of COX activity (41). In view of its importance in energy metabolism, we chose to focus on this unexpected finding and examined the biological significance of COX Vb expression in relation to gastrin expression by colon cancer cells.

Differential Expression of COX Vb RNA by Human Colon Cancer Cells in Relation to Gastrin Expression—To determine if the steady-state levels of COX Vb RNA correlate with endogenous gastrin levels, total cellular RNA was isolated from several human colon cancer cells that are known to express different levels of endogenous gastrin RNA and was analyzed by Northern blotting for COX Vb transcripts. Northern blot data from a representative experiment (one of a total of three experiments) are shown in Fig. 2A. Colon cancer cells expressing high levels of gastrin RNA (more than three to five copies of gastrin RNA/cell) (11, 12) are labeled H, and colon cancer cells expressing relatively lower levels of gastrin RNA (less than one to two copies of gastrin RNA/cell) (11, 12) are labeled L. The ratio of COX Vb RNA to 18 S RNA was determined by densitometric analysis of the Northern blot data, and the results obtained from three separate experiments are shown in a bar graph in Fig. 2B. As shown in Fig. 2B, the relative levels of COX Vb RNA were ~4-fold higher in C-HCT-116 cells than in AS-HCT-116 cells, and the difference was statistically significant (p < 0.05). Similarly, all cell lines expressing relatively low (L) levels of gastrin RNA (HT-29, Colo-205, and CaCo-2) expressed significantly lower levels of COX Vb RNA compared with colon cancer cells expressing high (H) levels of gastrin (Colo-205, DLD-1, and C-HCT-116) (Fig. 2B).

Relative Levels of COX Vb Protein in the MT of AS-HCT-116 Versus C-HCT-116 Cells—To confirm the RNA results at the protein level, cytosolic and MT protein extracts were prepared from AS-HCT-116 and C-HCT-116 cells, and equal amounts of protein were analyzed by Western immunoblotting using anti-
COX Vb Abs as described under “Experimental Procedures.” Western blot data from a representative experiment of a total of several experiments is shown in Fig. 3A. The relative levels of COX Vb protein were determined by densitometric analysis of three separate blots from three experiments, and the resulting data are presented in a bar graph in Fig. 3B. The densitometric units measured for C samples were arbitrarily assigned a 100% value, and the densitometric units for the AS samples are presented as a percent of those measured for the C samples in Fig. 3B. As expected, COX Vb was present only in the MT samples, and no band was seen in the cytosolic samples (Fig. 3A), confirming the specificity of the anti-COX Vb Abs used in these studies. Consistent with Northern blot results, COX Vb protein levels were also found to be significantly lower (5-fold less) in AS versus C cells.

Association of COX Vb with Cytochrome c in the MT of AS-HCT-116 and C-HCT-116 Cells—Cytochrome c is an important substrate for the MT COX enzymes, but it is not known if cyt c exists as a bound complex with the COX Vb subunit. Cytochrome c not only plays an important role in the oxidative respiratory pathway as a substrate for the COX enzymes, it also plays an important role in the apoptotic pathway for most epithelial cells (28, 41, 43). The association of cyt c with higher levels of COX Vb in gastrin-expressing colon cancer cells could then potentially make it less available for initiating apoptotic events in the cytosol. It was therefore important to first establish if cyt c existed as a bound complex with the COX Vb subunit in MT. MT and cytosolic lysates were prepared from AS and C cells as described under “Experimental Procedures,” and equal amounts of MT and cytosolic proteins were immunoprecipitated with anti-cyt c Ab. The anti-cyt c immunoprecipitates were analyzed by sequential immunoblotting with specific Abs against COX Vb and cyt c. Immunoblot data from a representative experiment of two separate experiments are shown in Fig. 4A. As shown in Fig. 4A, the anti-cyt c immunoprecipitates of the MT proteins from both AS and C cells clearly coprecipitated with COX Vb, indicating an association of cyt c with the COX Vb subunit in the MT of the cells. More importantly, the levels of COX Vb associated with cyt c were significantly higher (>3 times) in the MT of C versus AS cells. Once again, as expected, no COX Vb was immunoprecipitated with cyt c using cytosolic samples, confirming the specificity of the Ab and the immunoprecipitation reaction (Fig. 4A).

The relative levels of cyt c protein were significantly higher (by ~2-fold) in the cytosol of AS versus C cells (Fig. 4A). We hypothesized that higher levels of cyt c in the cytosol of AS cells reflected lower levels of COX Vb in the MT of AS versus C cells and hence lower retention of cyt c in the MT of AS cells. To test this possibility, we conducted the following experiments with digitonin.

MT pellets were prepared from AS and C cells as described under “Experimental Procedures” and treated with an optimal concentration of digitonin (100 μg/ml). The release of cyt c from MT into the extramitochondrial medium was analyzed by immunoblot analysis using anti-cyt c Ab. The results from a representative experiment of two separate experiments are shown in Fig. 4B. The immunoblot data from Fig. 4B were densitometrically analyzed, and the densitometric units measured for the AS samples was arbitrarily assigned a 100% value. The densitometric units measured for the C samples are presented as a percent of those measured for the AS samples. These results confirm the possibility that high levels of COX Vb in the gastrin-expressing C cells may have been more effective in retaining cyt c within the MT, whereas lower levels of COX Vb in AS cells may have allowed for a significantly higher release of cyt c from MT into the medium by AS samples. To confirm if cyt c levels were also...
that higher levels of COX Vb in the MT of C cells may have retained higher levels of cyt c in the MT of C cells.

It is now known that cyt c is released from MT in response to apoptotic stimuli and is one of the several factors required for activation of caspase-3, which is believed to be an important initiating event in the pathway to apoptosis (28, 41, 43). We therefore hypothesized that less availability of cyt c in the cytosol of gastrin-expressing C cells (due to higher retention of cyt c in the MT of C cells, as suggested by the results presented in Figs. 4, 5B and C) may result in lower activation of caspase-3 in response to apoptotic stimuli. To investigate this possibility, the following experiment was conducted.

**Effect of Camptothecin Treatment on Activation of Caspase-3 and -9 in AS-HCT-116 Versus C-HCT-116 Cells**—AS and C cells were treated with an optimal dose of camptothecin as described under “Experimental Procedures.” Twenty-four h post-treatment, the cells were washed, and cytosolic protein was prepared. Equal amounts of cytosolic protein were analyzed by Western immunoblotting with anti-caspase-9 and -3 Abs. Immunoblot data from a representative experiment (of a total of three experiments) are presented in Fig. 6 (A and C). Six immunoblots of each from the three experiments were densitometrically analyzed, and the densitometric units for the AS samples were arbitrarily assigned a 100% value. The densitometric units for the MT fractions. *p < 0.05 versus AS samples, and the data are shown as a percent of those measured for the AS samples in the MT fractions. **p < 0.05 versus the corresponding cyt c values in the MT fractions.

**Fig. 5.** Relative levels of cyt c in the cytosolic and MT fractions of AS-HCT-116 and C-HCT-116 cells. MT (MIT) and cytosolic (CYT) extracts were prepared from AS and C cells as described under “Experimental Procedures.” Equal amounts of protein (50 μg) from the MT and cytosol extracts were processed for Western immunoblotting with anti-cyt c Abs as described under “Experimental Procedures.” A representative autoradiograph of a Western immunoblot is shown in A. The arrows indicate the 15.3-kDa cyt c protein band in the samples. The bands in A were densitometrically analyzed, and the relative density of cyt c bands in the cytosol of the AS samples was arbitrarily assigned a 100% value. The densitometric readings for all other bands are represented as a percent of those measured for the cytosol of the AS samples in B. Black bars represent MT values, and gray bars represent cyt c in cytosolic fractions.

Suppression of Autocrine Gastrins Down-regulates COX Vb

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116 cells to apoptotic stimuli, we conducted additional studies with two other known pro-apoptotic stimuli, C$_2$-ceramide and menadione, as described under “Experimental Procedures.” The experiment was carried out as described above for camptothecin, and the data were analyzed similarly. The data from three separate experiments are presented in Table I. Treatments of the cells with camptothecin once again resulted in a significantly higher activation of caspase-3 (−3-fold higher) in AS versus C samples. Menadione was slightly less effective than camptothecin, but similarly caused a significant increase in the levels of activated caspase-3 (−2-fold higher) in AS versus C samples. C$_2$-ceramide was the least effective of the three pro-apoptotic agents, but the difference in the levels of activated caspase-3 in the AS versus C samples was once again statistically significant. The pro-apoptotic efficacy of C$_2$-ceramide is critically dependent on the reciprocal influence of sphingoid bases and diglycerides on protein kinase C activity (44). In other words, the efficacy of ceramide-driven apoptosis depends upon the balance between relative levels of ceramides and diglycerides (which oppose ceramide action) (44). It is thus possible that HCT-116 cells have a high concentration of the Va isoform, which is expressed under aerobic conditions (49). The Va isoform is expressed under aerobic (O$_2$ > 0.5 m) conditions, and the Vb isoform is expressed under anaerobic (O$_2$ < 0.5 m) conditions (49), wherein the Vb isoform has a higher turnover rate and a higher intramolecular transfer rate than the Va isoform. Isomerization of COX V significantly affects the binuclear reaction center around the catalytic subunits I and II and alter the kinetics of interaction with the isoforms of cyt c (49). The results of the present study demonstrate 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. Although specific binding sites for the COX II subunit are located on cyt c (38), it is not known if COX Vb has specific binding sites for cyt c.

The fact that COX Vb is specifically up-regulated under anaerobic conditions of low oxygen tension is especially relevant to tumorigenesis, wherein hypoxia within the tumors may provide the necessary feedback for specific elevation of COX Vb protein in cancer cells. Our gene expression studies further suggest that up-regulation of autocrine growth factors such as gastrin gene products may provide yet another mechanism for elevating COX Vb levels and thus support the increasing energy demands of rapidly growing cancer cells.

The majority of the colon cancer cell lines, including HCT-116 cells, which express significant concentrations of autocrine gastrins in human colon cancer cells. Two of these (Y12FF112 and an unknown gene) were negatively regulated, whereas four (BTK, HMG-2, COX Vb, and an unknown gene) were positively regulated in response to autocrine gastrins. At the present time, we do not know if this regulation is at the transcriptional or post-transcriptional level. Since the biological function of the COX enzyme is well described, we examined the significance of this rather unexpected finding. COX is the terminal enzyme complex of the electron transfer chain (38) and oxidizes cyt c with transfer of electrons to generate ATP by oxidative phosphorylation (38). Mammalian COX is composed of three major catalytic subunits (COX I–III), which are encoded by the MT genome (38). In addition, at least 10 smaller regulatory subunits, which are encoded by the nuclear genome, form part of the complex eukaryotic COX enzyme (45, 46). The nuclear subunits are synthesized as precursor proteins, transported into the MT, and processed, and the complex is assembled (36, 46). Although 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 (46, 47). There are significant species and tissue differences in the relative expression of the nuclear subunits (31, 41). The nuclear subunits are differentially regulated by environmental and developmental signals, which allows the tissues to adjust to different energy demands (reviewed in Refs. 38 and 47).

The gene for COX Vb (COX5B) is located on chromosome 2 (48), and oxygen regulates expression of the V isoforms in yeast (49). The Vb isoform is expressed under aerobic (O$_2$ > 0.5 m) conditions, and the Vb isoform is expressed under anaerobic (O$_2$ < 0.5 m) conditions (49), wherein the Vb isoform has a higher turnover rate and a higher intramolecular transfer rate than the Vb isoform. Isomerization of COX V significantly affects the binuclear reaction center around the catalytic subunits I and II and alter the kinetics of interaction with the isoforms of cyt c (49). The results of the present study demonstrate 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. Although specific binding sites for the COX II subunit are located on cyt c (38), it is not known if COX Vb has specific binding sites for cyt c.

The fact that COX Vb is specifically up-regulated under anaerobic conditions of low oxygen tension is especially relevant to tumorigenesis, wherein hypoxia within the tumors may provide the necessary feedback for specific elevation of COX Vb protein in cancer cells. Our gene expression studies further suggest that up-regulation of autocrine growth factors such as gastrin gene products may provide yet another mechanism for elevating COX Vb levels and thus support the increasing energy demands of rapidly growing cancer cells.
gastrointestinal tumors, are largely unresponsive to exogenous gastrins (23). To confirm a role of gastrins in regulating COX Vb levels, we have therefore been using intestinal epithelial cell lines (IEC-6 and IEC-18) that are known to be responsive to exogenous gastrins (50). In preliminary studies, we examined the activation of the COX Vb promoter using transient transfection assays with the COX Vb promoter-chloramphenicol acetyltransferase plasmid (obtained from Dr. Margaret Lomax). We measured a significant increase in the COX Vb promoter activity in response to 1 nM gastrin.2

Significant changes in the expression of specific subunits of the MT COX holoenzyme in cancer versus benign normal cells from various tissues have been reported using the method of differential display (51–53). The COX VIc subunit was significantly up-regulated in human prostate carcinoma versus normal human prostate tissues (51). Other authors have reported overexpression of either COX II (52) or COX Va (53) in breast carcinoma specimens versus normal breast tissue, suggesting that tumors originating from different tissues may up-regulate specific subunits of the COX holoenzyme, resulting in increased COX catalytic activity. Mechanisms mediating up-regulation of COX subunits are as yet unknown, but are likely to be cancer cell-specific. In the case of gastrin-dependent human colon cancers, our present studies suggest that up-regulation of autocrine gastrins results in overexpression of COX Vb. Recently, COX VIIa was identified as an estrogen-responsive gene using the genomic binding site cloning method (54), suggesting that estrogens directly regulate expression of specific subunits of the COX holoenzyme. It remains to be seen if autocrine gastrins can similarly, directly or indirectly, regulate COX Vb gene expression. The intracellular pathways that mediate the growth/survival effects of autocrine gastrins may potentially regulate expression of COX Vb RNA. Raf-1, a cytoplasmic Ser/Thr protein kinase that plays an important role in mitogen-activated protein kinase/extracellular signal-regulated kinase kinase and that is up-regulated in many cancers, was recently shown to up-regulate expression of the COX II subunit (55). Up-regulation of the COX II gene in head and neck squamous carcinoma cells contributes to resistance of the cells to platinum-derived cytotoxic drugs (56). Somatic mutations in MT COX genes are commonly present in human colorectal tumors (57). The specific functional effect of these mutations has yet to be identified, however. It is likely that the combined effect of mutations in MT COX genes and the up-regulation of specific nuclear subunits in response to cancer-specific growth factors results in the increased catalytic activity of the COX holoenzyme.

Augenlicht and co-workers (58) hypothesized that MT play a pivotal role in coordinating proliferation and apoptosis in rapidly renewing tissues such as colonic mucosa. 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 (47), the importance of up-regulation of COX Vb in gastrin-dependent human colon cancers (as shown in the present studies) highlights a possible role of COX Vb in the survival of human colon cancer cells. Recent reports confirmed that COX activity may be related to apoptotic potential of cells (59). For example, cell death was induced in hematopoietic cells by down-regulating MT respiratory enzymes (59). Similarly, treatment of human leukemia cells with adriamycin resulted in loss of expression of COX II and IV genes and promotion of apoptosis (60). Virulent Mycobacterium tuberculosis caused apoptotic death of macrophages by apparently down-regulating the COX VIIa subunit (61). These reports thus suggest that down-regulation of one or more specific COX subunits somehow translates into initiation of apoptotic events. Our present studies indicate that increased release of cyt c in AS cells, expressing suppressed levels of the gastrin gene and COX Vb, represents one such mechanism that significantly enhances the sensitivity of the cells to apoptotic stimuli, resulting in increased activation of the caspase enzyme. Recent reports from other laboratories support our current findings (62, 63). In one such report, Bax-induced growth 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 to the cytosol (62). Treatment of Chinese hamster ovary cells with cAMP-elevating agents significantly inhibited COX activity with a concomitant release of cyt c into the cytosol (63), almost mimicking the results of the present studies with gastrin-dependent human colon cancer cells. 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 elevated cAMP (63). Thus, COX Vb may play a critical role in specific cancer cell types in regulating COX activity, which can then result in the differential release of cyt c into the cytosol, as discussed above.

The release of cyt c is a requirement for initiating apoptosis (28, 45). Microinjection experiments with cyt c have confirmed the important role of this MT protein in the initiation of the apoptotic pathway via activation of the caspase enzymes (64). The sequence of activation of the caspases is now believed to include the release of cyt c from MT, followed by its binding to apoptotic protease-activating factor-1, which triggers the activation of caspase-9, followed by the activation of caspase-3, which is then followed by the activation of at least four other caspases (caspase-2, -6, -8, and -10) (65). Thus, both caspase-9 and -3 represent critical and penultimate molecules that are required for initiation of apoptosis in response to cyt c release. However, several other pathways impinge upon this activation process, wherein cyt c, released into the cytosol, plays an important permissive role that sensitzes the cells to potent apoptotic stimuli (Refs. 66–68 and this study). Several potent apoptotic stimuli have been described in the literature in recent years (34), including topoisomerase-I inhibitors (such as camptothecin) (32, 33) and p53 (39, 66). The differential display method was used to identify target genes of overexpression of wild-type p53 in a leukemia cell line (66). One gene identified was cyclin G1, which co-immunoprecipitated with the COX II subunit and which apparently resulted in activation of the caspase-3 enzyme (66). Overexpression of wild-type p53 in SAOS-2 cells resulted in bax expression and cyt c release accompanied by activation of caspase-3 (39). In our present studies, we observed that suppression of gastrin gene regulation resulted in down-regulation of COX Vb expression, which resulted in an increased release of cyt c, triggering an increased activation of caspase-9 and -3 in response to camptothecin and other apoptotic stimuli. It remains to be seen if the p53-mediated pathway is somehow connected to the observed effects in response to down-regulation of the gastrin gene in human colon cancer cells.

Just as we observed a significant increase in the sensitivity of the colon cancer cells overexpressing antisense gastrin RNA to pro-apoptotic stimuli, overexpression of c-myc was similarly shown to sensitize the cells to pro-apoptotic stimuli via release of MT cyt c into the cytosol, which was blocked by the survival factor insulin-like growth factor I (42). The c-Myc-initiated apoptosis was not mediated via the p53 or CD95/Fas signaling pathway (42). It was concluded that although c-Myc promotes apoptosis by releasing cyt c, its ability to activate apoptosis was critically dependent upon other signals (42). We similarly

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2 H. Wu and P. Singh, unpublished data.
measured a significant increase in the activation of caspase-9 and -3 in AS cells only in response to pro-apoptotic stimuli, confirming the notion that pathways mediating the increased release of cyt c in response to either loss of gastrin gene expression in colon cancer cells (this study) or overexpression of c-Myc in growth factor-starved fibroblasts (42) result only in sensitizing the cells to more potent pro-apoptotic stimuli, thus making the cells less able to survive under hostile conditions. Our present studies thus for the first time demonstrate an important link between gastrin gene expression, expression of the COX Vb subunit, cyt c release, and regulation of sensitivity to pro-apoptotic stimuli.

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Autocrine Gastrins in Colon Cancer Cells Up-regulate Cytochrome Oxidase Vb and Down-regulate Efflux of Cytochrome c and Activation of Caspase-3

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